STRUCTURAL INVESTIGATIONS ON PLANT POLYSACCHARIDES, WITH SPECIAL REFERENCE TO THE PECTIC GROUP OF SUBSTANCES

by

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TO MY PARENTS AND EDITH

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SECTION 2

THE EXTRACELLULAR POLYSACCHARIDES FROM SYCAMORE CAMBIAL CELLS

INTRODUCTION

Plants contain a wide range of polysaccharides. The occurrence of these throughout the plant is as diverse as their nature. They constitute cell walls, appear in the intercellular layer and can be exuded by the plant. This thesis will be concerned mainly with pectins, which occur in both primary and secondary cell walls, in the intercellular layer and in fruit juices, and with hemicelluloses which occur in cell walls together with cellulose.

SECTION 1

Pectins

Definition of the terms used in Pectin Chemistry.

Pectic Substances

Pectic substances is a group designation for those complex, colloidal carbohydrate derivatives which occur in, or are prepared from, plants and contain a large proportion of anhydrogalacturonic acid units existing in a chain-like combination. The carboxyl groups of the polygalacturonic acids may be partly esterified by methyl groups and partly or completely neutralised by one or more bases (1).

Pectinic Acids

The term pectinic acid is used for colloidal polygalacturonic acids containing more than a negligible proportion of methyl ester groups.

Pectin

The general term pectin designates those water-soluble pectinic acids of varying methyl ester content and degree of neutralisation which are

capable of forming gels with sugar and acid under suitable conditions.

Pectic Acid

Pectic acids are those pectic substances which are essentially free of methyl ester groups.

The pectic substances occur as both water-soluble and insoluble components of plant tissue and differ in proportions of individual sugars both with the variety and maturity of the plant and with the part of the tissue or cell included in the sample (2). They occur mainly in the primary cell walls and in the intercellular layer but also occur, to a lesser extent, in the secondary cell walls. Some plant juices and saps are also quite rich in these substances. Thus they are most abundant in soft tissues which are composed mainly of primary cell walls (i.e. fruit and roots) (3)(4) and much less so in wood where the volume of the primary layer is small.

On the basis of solubilities, and staining reactions of pectinic acids, the pectic substances in the cell wall are believed to differ from those in the intercellular layer and the soluble pectins present in the intercellular layer of ripe fruits are believed to differ from the insoluble pectins.

The functions of pectic substances in plants are varied and have not yet been fully elucidated. It would appear that the insoluble pectic substances in the intercellular layer contribute to its rigidity but the function of the pectic substances in cell walls is not so obvious. However, from their hydrophilic nature, they may be necessary for the

proper hydration of young growing cell walls. This hypothesis is supported by their presence in largest proportions in primary cell walls (5).

Pectic substances are important commercially because of the ease with which they form gels. Of the two types of gel which is formed, the pectin-sucrose-acid type is the most important industrially. This is formed as a result of the tendency of aggregations of pectin molecules to crystallise. The other type is formed by cross linking chains through carboxyl groups by divalent calcium.

Molecular weights of pectins have been reported to vary from around 10,000 to around 400,000. It would seem, however, that this wide range of values can be explained by partial degradation of large molecules with molecular weights of several hundred thousand to smaller molecules with molecular weights between ten and fifty thousand. Henglein and Schneider (6) obtained evidence of this by nitrating pectins in situ. Direct nitration of alcohol extracted and dried slices of sugar-beet tissue gave nitrated pectins of molecular weights of 50,000 - 100,000. Pectins obtained by hot-water extraction of the same material had, after nitration, molecular weights of 30,000 - 50,000. Further evidence in support of this was obtained by Schneider and Bock (7) who reported that the molecular weight of nitrated pectinic acids form apple pomace was much higher from the first extract than from subsequent extracts.

Pectic substances although composed mainly of galacturonic acid residues contain varying amounts of arabinose and galactose residues. The early work on these substances suggested that there were three separate

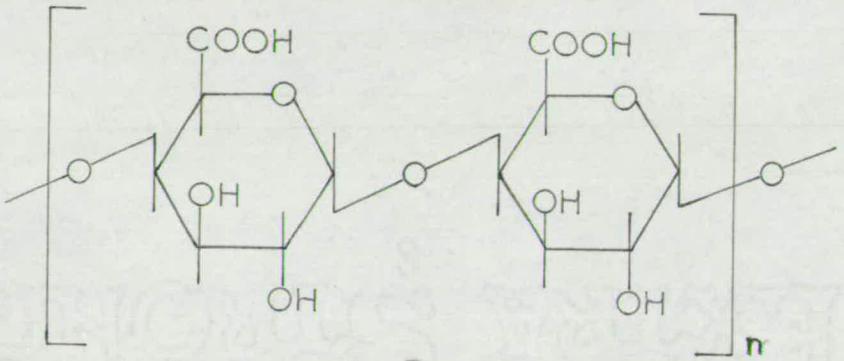
polysaccharides present: a galacturonan, a galactan, and an arabinan. It would now appear that, although there may be some separate galactan and arabinan, galactose and arabinose residues are also linked to the galacturonic acid residues and that the isolation of three homopolysaccharides, the Pectic Triad, was due to degradation during extraction of a heteropolysaccharide containing all three sugars. There is as yet no direct evidence for a linkage between arabinose or galactose units and galacturonic acid units and the theory of a heteropolysaccharide is based on the retention of the neutral sugars after exhaustive fractionations. The early investigations on the 'homopolysaccharides', however, give an insight into the basic structural features of pectic substances.

Pectic Galacturonans

In the early investigations, a homopolysaccharide, containing galacturonic acid residues only, was obtained by acid hydrolysis of associated galactose and arabinose residues. Hot mineral acid was used (8) and, in the case of strawberry pectin (9), hot methanolic hydrogen chloride which yielded a galacturonan of about eight units indicating appreciable hydrolysis of the galacturonic acid residues as well as the neutral residues. In some instances, the pectic material was extracted with alkali which could lead to a β-elimination type of degradation which will be discussed more fully later.

One of the first examinations of a pectic galacturonan was made by Levene and Kreider (10) who showed from oxidation studies that the galacturonic acid residues were linked through either the 1 and 4 or 1 and 5 positions dependent on the galacturonic acid units being in the pyranose or furanose forms respectively.

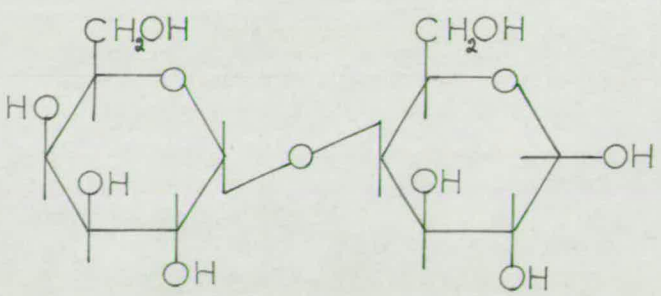
The linear structure was confirmed from methylation studies by Luckett and Smith on the galacturonan from citrus pectin (8) and by Beaven and Jones on the galacturonan from strawberry pectin (9). In both cases, a structure composed of galacturonic acid units, in the pyranose form, linked through the 1 and 4 positions (I) was favoured due to the high



I

positive rotation of the galacturonans and their stability towards hydrolytic agents.

Further structural features of pectic galacturonans have been elucidated from studies on galacturonobiose and galacturonotriose obtained from the action of the enzymes from Aspergillus foetidus on apple pectic acid (11)(12). The reduction of galacturonobiose to 4-O-α-D galactopyranosyl - D - galactose showed that it consisted of two D - galactopyranosiduronic acid residues connected by an α1→4 linkage (II)



II

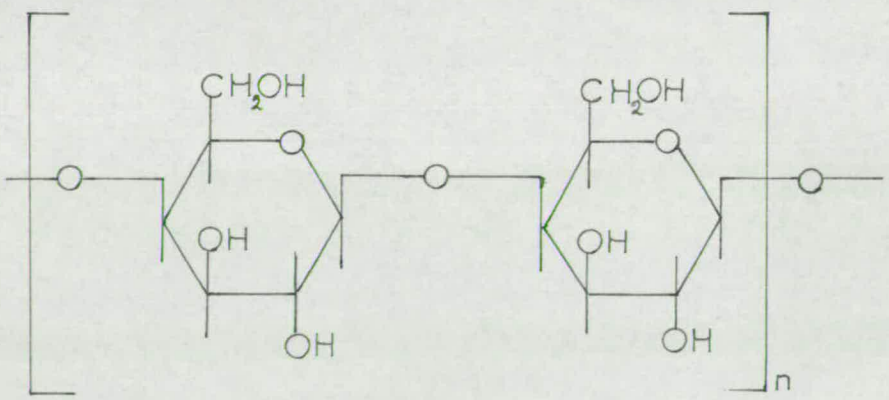
but the reduction of galacturonotriose to the corresponding galactotriose, which had a high positive rotation indicative of α linkages, and subsequent methylation was less conclusive in that the possibility of a branched structure was not resolved. However, the results supported earlier conclusions that the galacturonic acid residues in pectin existed in the pyranose form and that the linkage was α.

More recently, the extraction procedures which have not involved the use of acid have yielded acidic polysaccharides, containing neutral sugar residues, and galacturonans, as strictly homopolysaccharides, are now recognised to be of relatively infrequent occurrence. However, the extraction of sunflower heads with hot ammonium oxalate-oxalic acid solution has yielded a galacturonan (13) and galacturonans have also, in some instances, been obtained from the further fractionation of the acidic polysaccharides. Zitko and Bishop (14), by further fractionating the acidic polysaccharides in sunflower, sugar-beet, apple, and citrus pectins, isolated a pure galacturonan in each case and Timell and Bhattacharjee (15), on further fractionation of the pectin present in the bark of amabilis fir, obtained a pure galacturonan. On reduction of the galacturonan to the corresponding galactan and subsequent methylation of the galactan, they concluded that the galacturonan was a linear molecule with the galacturonic acid units in the pyranose form linked through the 1 and 4 positions in the α configuration.

Pectic Galactans

Hirst, Jones and Walder (16) first isolated a pectic galactan from the seeds of white lupin. Hot alkaline extraction of the seeds yielded a

pectic material which contained a high percentage of D - galactose residues. A pure galactan containing a small amount of uronic acid was obtained after the removal of the arabinan by extraction with 70% methanol followed by hydrolysis with mild oxalic acid of the remaining arabinose residues, and after the removal of the acidic polysaccharide by precipitation as the calcium salt. In view of the utilisation of oxalic acid to hydrolyse the last of the arabinose residues, the authenticity of the galactan is questionable in that it may have arisen from the removal of arabinose residues from an arabinogalactan, and in light of recent investigations into base catalysed degradation of pectic substances, the degradation of a large complex molecule, during alkaline extraction, resulting in the mixture of polysaccharides extracted, is a possiblity which cannot be overlooked. Methylation studies on the galactan showed that it was a linear polymer built up of D - galactopyranose residues linked through the 1 and 4 positions (III) and from optical rotatory power, the authors concluded



III

that the galactose residues were linked in the β configuration.

Although there is doubt as to the authenticity of the galactan from Lupinus albus seeds, there is evidence that separate galactans do exist in

pectic substances as a structurally similar galactan has been isolated as a minor component from a commercial citrus pectin by precipitation of the acidic polysaccharide as the insoluble copper salt (17). Andrews, Hough and Jones (18) have also isolated a galactan of this type from the seeds of Strychnos nux-vomica using an alkaline extraction procedure.

In addition to these homopolysaccharides, arabinogalactans with β - D - galactopyranose residues linked through the 1 and 4 positions constituting the main chain and arabinose residues attached as side chains have also been isolated. Such a polysaccharide, containing L - arabinose to D - galactose residues in the approximate proportion of 1:2, has been shown to be the main component of a polysaccharide mixture extracted from defatted soyabean flour with hot water after the removal of proteins with very dilute alkali (19). Methylation studies indicated that the main chain of the polysaccharide was composed of D - galactopyranose residues linked through the 1 and 4 positions while some branching at the 3 position accommodated side chains of L arabinofuranose residues linked through the 1 and 5 positions.

An arabinogalactan, with the predominant linkage of galactose residues through the 1 and 4 positions but, on the whole, of a more complex structure, has been isolated from Centrosema plumari seeds (20).

Acidic polysaccharides containing chains of (1 → 4) - linked β - D - galactopyranose residues as the main structural feature have also been isolated from wood. Bouveng and Meier (21) isolated a polysaccharide of this type in the solution from the delignification of Norwegian spruce compression wood with chlorite which contained 13% uronic acid residues in

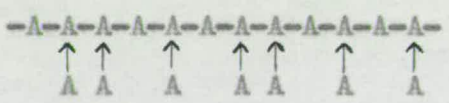
the form of both galacturonic and glucuronic acid residues. The neutral part of the polysaccharide was shown to consist of a chain of (1 → 4)-linked β- D - galactopyranose residues. Alkaline extraction of beech tension wood has yielded a still more complex polysaccharide containing as one of its structural features chains of D - galactose residues linked through the 1 and 4 positions (22).

Pectic Arabinans

Polysaccharides of this type were first isolated from various sources by Hirst and Jones (23)(24)(25)(26) who carried out structural investigations which have been supplemented by further studies on sugar-beet arabinan (27)(28). However, in light of more recent evidence (29), it would appear that the so-called sugar-beet arabinan is a heteropolysaccharide containing arabinose, galactose, rhamnose, and galacturonic acid residues and, in addition, is not an alkali stable polysaccharide as was previously believed (30) in that it can be degraded by further treatment under the conditions of extraction. However, in light of present knowledge of the susceptibility of pectins, containing a high proportion of esterified galacturonic acid residues, to degradation by alkali, doubt must be cast on the authenticity of the "arabinan" as an undegraded constituent of sugar-beet pectin.

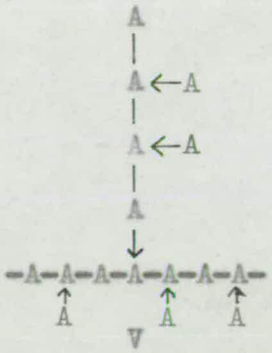
In two instances, arabinans of the pectic type have been isolated under non-degradative conditions. Studies on the arabinan isolated from mustard seed (31)(32), in particular, have yielded the most complete structure so far obtained for pectic arabinans. Methylation of the mustard seed arabinan and subsequent hydrolysis gave L - arabinose,

2 - O - methyl - L - arabinose, 2, 3 - di - O - methyl L - arabinose and 2, 3, 5 - tri - O - methyl - L - arabinose in the molar proportions 2:11:6:12. It would appear from the non-reducing end groups that the arabinose residues are present in the furanose form, the units being joined by 1 → 5 linkages with a considerable proportion of 1 → 3 branch linkages. Furthermore, the isolation of a small amount of arabinose from the methylated polysaccharide would indicate branching through the C_2 position of a relatively small proportion of units that are also linked through the C_3 and C_5 positions. An $\propto$ linkage between the arabinofuranose residues was deduced from the high negative rotation of the polysaccharide. An insight into the fine structure of the arabinan was obtained from Smith degradation studies which yielded a series of oligosaccharides containing 1 → 5 linkages. These oligosaccharides were identified by methylation followed by quantitative gas-liquid chromatography and indicated that the arabinan must have one of the three structures (IV), (V) or (VI) or have characteristics of any one of these present to a greater or lesser extent in the molecule.

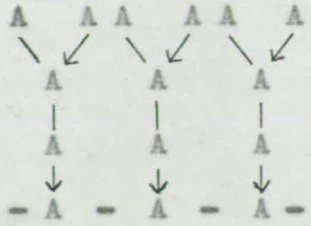


-, (1 → 5) linkage
→, (1 → 3) linkage

IV



V



VI

The arabinan isolated from the aqueous extracts of maritime pine (33) would appear to have a similar structure although, in this case, a small amount of galactose residues were shown to be present as non-reducing end groups.

The Complex Acidic Polysaccharides in Pectins

Pectic substances, extracted under mild conditions which are unlikely to cause degradation and fractionated to remove any neutral polysaccharides, give acidic polysaccharides which still contain neutral sugar residues.

The table below, compiled by McCready and Gee (34) illustrates the sugars liberated by partial hydrolysis of purified pectinic acids.

Source	Anhydrouronic Acid*	Arabinose	Galactose	Rhamnose	Xylose
Orange	92.1	+	+	+	0
Grapefruit	91.7	+	+	+	0
Lemon	90.4	+	+	+	0
Fig	87.1	+	+	+	0
Carrot	76.7	+	+	+	0
Apple	88.0	+	+	+	+
Peach	86.8	+	+	+	+
Pea Pod	84.8	+	+	+	+
Apricot	83.1	+	+	+	+
Pear	82.6	+	+	+	+
Sugar Beet	82.3	+	+	+	+
Avocado	79.0	+	+	+	+

*Content corrected for water, ash, and methylene impurity from carbomethoxyl.

In addition to these neutral constituents, 2 - O - methyl - D - xylose and 2 - O - methyl - L - fucose, which were first isolated from plum leaf hemicellulose (35), have been isolated from sisal pectic acid (36) and subsequently from other pectic acids along with L - fucose which was first isolated in pectins from lucerne pectic acid and which is present in a relatively high proportion in the acidic polysaccharides from soyabeans (37).

Although the previously mentioned neutral sugars would appear to be constituent sugars of the complex acidic polysaccharides in pectins, until recently, however, only rhamnose residues had been shown to be directly linked to galacturonic acid residues from the isolation of the aldobiouronic acid, 2 - O - (α - D - galactopyranosyluronic acid) - L - rhamnose, first from the pectic type acidic polysaccharide from grapes (38) and later from lucerne pectic acid (39), the pectic acid from the bark of amabilis fir (15), and the acidic polysaccharides from soyabeans (40), (41). A larger oligosaccharide (VII) containing both galacturonic acid and rhamnose residues has also been isolated from partial hydrolysis studies on lucerne pectic acid (42)and the acidic polysaccharides from soyabeans (40)(41) which have also yielded the trisaccharide (VIII) and from acetolysis studies, the trisaccharide (IX) and the tetrasaccharide (X).

GalpA $1\overset{\alpha}{\rightarrow}2$ Rhap $1\overset{\beta}{\rightarrow}4$ GalpA $1\overset{\alpha}{\rightarrow}2$ Rhap

VII

GalpA $1\overset{\alpha}{\rightarrow}4$ GalpA $1\overset{\alpha}{\rightarrow}2$ Rhap

VIII

GalpA $1\overset{\alpha}{\rightarrow}2$ Rhap $1\overset{\beta}{\rightarrow}2$ Rhap

IX

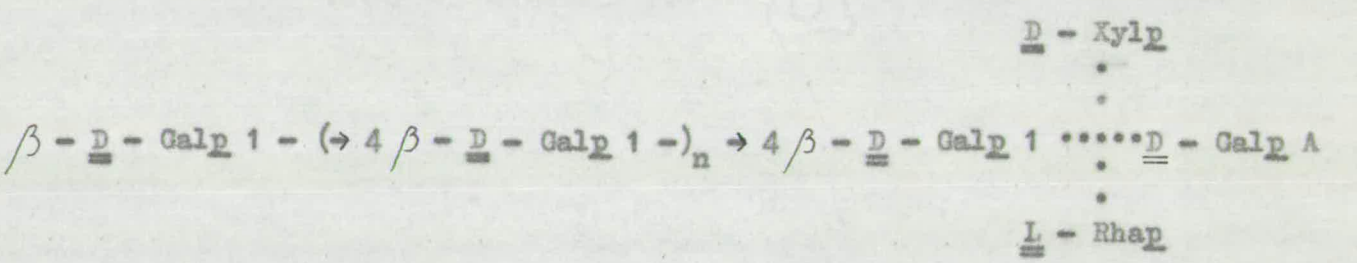
GalpA $1\overset{\alpha}{\rightarrow}4$ GalpA $1\overset{\alpha}{\rightarrow}2$ Rhap $1\overset{\beta}{\rightarrow}2$ Rhap

X

Further information on the structural significance of the rhamnose residues has been obtained from methylation studies on pectic acids from different sources by Aspinall and Cañas-Rodriguez (36), Aspinall and Fanshawe (39), and Timell and Bhattacharjee (15) which, when taken with the results from partial degradation studies, show that the main chain of the polysaccharide is composed of (1 → 4) - linked D - galacturonic acid residues, some of which provide branch points with a small number of L - rhamnose residues, some of which also provide branch points.

Galactose and arabinose are the predominant neutral sugar residues found in the complex acidic polysaccharides in pectins but as yet no direct evidence has been obtained from structural studies for linkages between these sugar residues and those of galacturonic acid although indirect evidence from the failure to remove them on fractionation and, in certain cases, from the relatively large proportions of residues of these sugars which occur as non-reducing end groups, as shown by methylation studies. Examination of the methylated complex acidic polysaccharides from various sources has shown that although the residues of galactose and arabinose may frequently be attached to the basal chains as non-reducing end groups, they may also be attached in structural units with linkages similar to those in the respective pectic homopolysaccharides. In the case of sisal pectic acid (36), a predominance of arabinose end groups was indicated along with (1 → 4) - linked chains of galactose residues, lucerne pectic acid (39), on the other hand, yielded a predominance of galactose end groups along with arabinose residues linked in a branched structure as in pectic arabinans, whereas the pectic acid from the bark of

amabilis fir (15) yielded both neutral sugars mainly in the form of end groups. The methylation evidence which indicates that galactose and arabinose residues in the complex acidic polysaccharides may occur in structural units similar to those in pectic galactans and arabinans respectively is further substantiated by the isolation of 4 - O - β - D - galactopyranosyl - D - galactose and the corresponding galactotriose from commercial citrus pectin (17) and from the acidic polysaccharides from soyabeans (40)(41), and by the isolation, from enzymic degradation of the acidic polysaccharides from soyabeans (40) of the acidic oligosaccharide (XI), not yet fully characterised, in which (1 → 4) - linked linear chains of



XI

D - galactopyranose residues are linked either directly or through xylose or rhamnose residues to a single galacturonic acid reducing unit.

Although the existence of a linkage between arabinose or galactose residues and those of galacturonic acid is still mainly hypothetical, direct evidence has been obtained for a linkage between arabinose and galactose residues by Mullar and Smith (29) from Smith degradation studies on an acidic polysaccharide from sugar beet, the so-called sugar beet arabinan, which has afforded O - α - L - arabinofuranosyl - (1 → 3) - O - β - D - galactopyranosyl - (1 → 1) - glyceritol indicating that, in this instance at least, the arabinofuranose units are linked to the

galactopyranose units. They suggest that the arabinan portion of the polysaccharide is joined to the core of galactose, rhamnose and galacturonic acid residues by linkage to the galactose residues.

Of the other neutral sugars present in the complex acidic polysaccharides, D - xylose residues which are generally present in lower proportions than those of galactose or arabinose, have been shown to be directly linked to galacturonic acid residues. Aspinall et al (40) have now isolated from enzymic degradation of the acidic polysaccharides from soyabeans the dissaccharide, 3 - O - β - D - xylopyranosyl - D - galacturonic acid which has also been obtained from tragacanthic acid (43).

Degradation of Pectic Substances

The subject of degradation of pectic substances can be divided into four separate sections: degradation in acidic solution, degradation in alkaline solution, degradation in neutral solution and degradation by enzymes.

The acid labile glycosidic bonds between arabinofuranose units in pectic substances allow only the weakest of acidic solutions to be used without degradation occurring. The increasing stability through galactopyranose bonds to galacturonic acid bonds allows the isolation of fragments such as 2 - O -(α - D galactopyranosyluronic acid) - L - rhamnose from partial hydrolysis experiments. (Acetolysis is a different type of acidic degradation resulting in markedly different "cracking patterns", the reason for this is not yet understood.)

Polysaccharides, in oxygen free solution, undergo alkaline degradation which begins at the reducing end of the molecule and proceeds

in a stepwise manner along the sugar chain. The rate of degradation depends on the mode of linkage of the sugar residues which compose the chain. Chains with sugar residues linked through the 1 and 3 or 1 and 4 positions are labile to alkali and degradation proceeds in a peeling process with the release of saccharinic acids but chains with sugar residues linked through the 1 and 2 positions are resistant to alkali.

Pectic substances contain galacturonic acid residues linked through the 1 and 4 positions and consequently they degrade by this peeling process yielding saccharinic acids (44). This type of degradation, however, does not produce such a rapid decrease in molecular weight and hence is not so readily detected by viscosity measurements as is scission of the same number of glycosidic linkages within the chain which Vollmert (45) has shown to take place during alkaline saponification of pectin at 20°C. He also showed that pectic acid is not depolymerised under the same conditions. Thus depolymerisation of pectic substances in this manner is only possible if there is some esterification of the galacturonic acid residues and furthermore the degree of depolymerisation is a function of the extent of esterification.

Neukom and Deuel (46) have shown that the viscosity of the sodium pectate produced by saponification of citrus pectin can be varied at will by selecting the proper saponification temperature. This is illustrated in the following table.

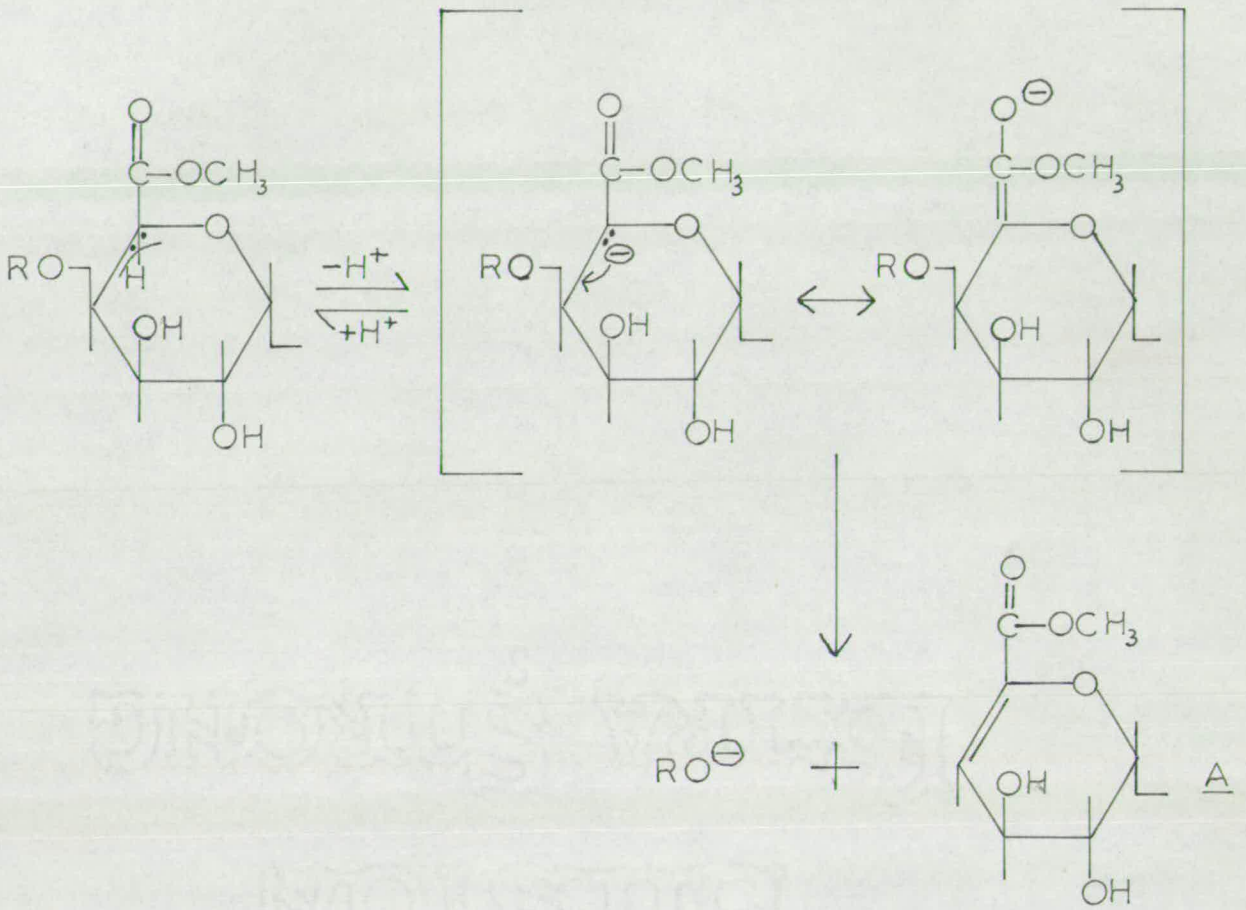
Saponification		Viscosity
Temp. °C	Time	η sp./c
-10+	3 days	1.132
5	24 hours	0.858
20	2 "	0.571
35	1 "	0.354
50	15 mins.	0.215
100	5 "	0.062
Original	Pectin *	1.322

+Saponified in the presence of glycerol.

*Degree of Esterification = 65%

They also observed that similar results were obtained with the glycol esters of pectic acid. In both cases the sodium pectate formed was no longer depolymerised by alkali in this manner but at the higher temperatures was further degraded by the usual stepwise alkaline degradation from the reducing end groups.

The instability of the esters of pectic acid can be explained by assuming a "β dealkoxylation" mechanism as suggested by Whistler and BeMiller (47) (XII).



R = the remaining portion of the polysaccharide molecule.

XII

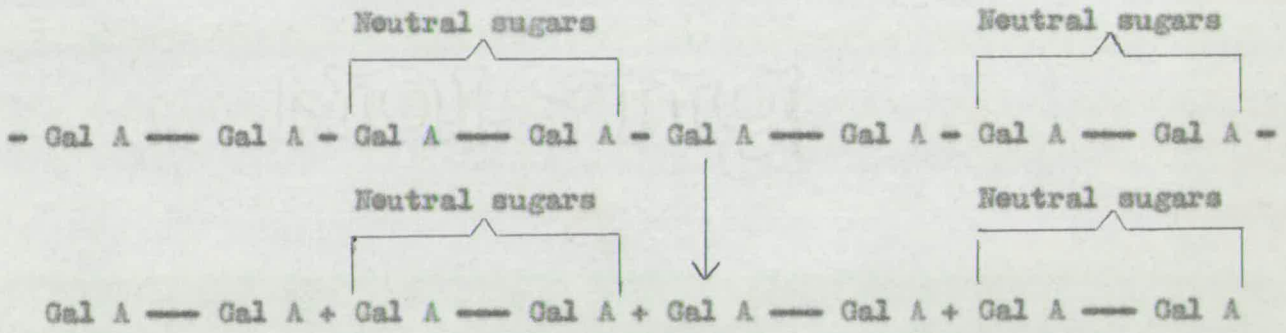
The esters of the α,β unsaturated acids (A), which are formed, are more slowly hydrolysed than the ester of the galacturonic acid (48).

Depolymerisation and de-esterification are competitive reactions which take place at the same time, the amount of each product depending on the relative rates of reaction. Launer and Tominatsu (49) have concluded that for every eighty de-esterifications which occur, one glycosidic bond is cleaved.

The instability of pectin in hot solutions of phosphate buffer is an unusual phenomenon, its susceptibility to degradation being due to the presence of methyl esterified carboxyl groups (50). As in alkaline solution, pectic acid is quite stable when similarly treated.

Investigations into the mechanism of the neutral degradation of pectin have been carried out by Albersheim, Neukom and Deuel (51) to prove the cleavage of glycosidic bonds and the formation of double bonds by buffer treatment at pH 6.8. They showed that degradation, as measured by reduction in relative viscosity, depends both on the length of heating and the temperature. Verification that degradation is the result of splitting of glycosidic linkages was obtained by correlating the reduction in viscosity with the increase in free aldehyde groups. The extent of breakdown was found to be approximately proportional to the degree of esterification of pectin and de-esterification, which would account for the reduction in the rate of glycosidic breakdown with increased lengths of degradation, was also shown to take place as heating proceeded. The presence of a β-elimination mechanism was indicated by an increasing absorption at 235 mμ, characteristic of α, β unsaturation, and also by the formation, by ozonation, of oxalic acid which is only produced from unsaturation in the C_4, C_5 position of uronic acids (52). Thus, not only are the characteristics of degradation in neutral solutions the same as those in alkaline solutions but it would also seem that even at pH 6.8 the α-hydrogen atom is sufficiently activated to be removed and that the mechanisms are identical.

Further investigations into the effect of hot phosphate buffers on pectic substances have been carried out by Barrett and Northcote (53) who obtained two polysaccharide components after heating a purified pectinic acid from apple pectin at pH 6.8. These components were separated by gel filtration and it was found that the high-molecular weight component contained a large proportion of the total neutral sugar residues of the pectinic acid whereas the low-molecular weight component contained a very high percentage of galacturonic acid. They suggest that the original molecule could be considered to contain large arabinofuranose 'blocks' covalently attached to the galacturonosyl chains and that degradation divides the molecule into two different types of fragments: one containing principally galacturonosyl residues and the other mainly neutral residues (XIII).



XIII

In addition to the conclusions drawn by the authors, it can be argued from gel filtration evidence, assuming that the galacturonic acid chains of both types of degraded fragments are approximately the same length, that these chains must contain between 20 and 50 galacturonic acid units. From a β-elimination type of degradation, the galacturonic

acid units at the non-reducing end of each chain will be α, β unsaturated.

Pectic enzymes can be divided into three major groups. The first group contains the pectinesterases which catalyse the de-esterification of the natural and synthetic methyl esters of polygalacturonic acids. The second group contains the polygalacturonases which catalyse the hydrolysis of glycosidic linkages and the third group contains the pectin transeliminases which would seem to degrade pectin by a β-elimination type of mechanism.

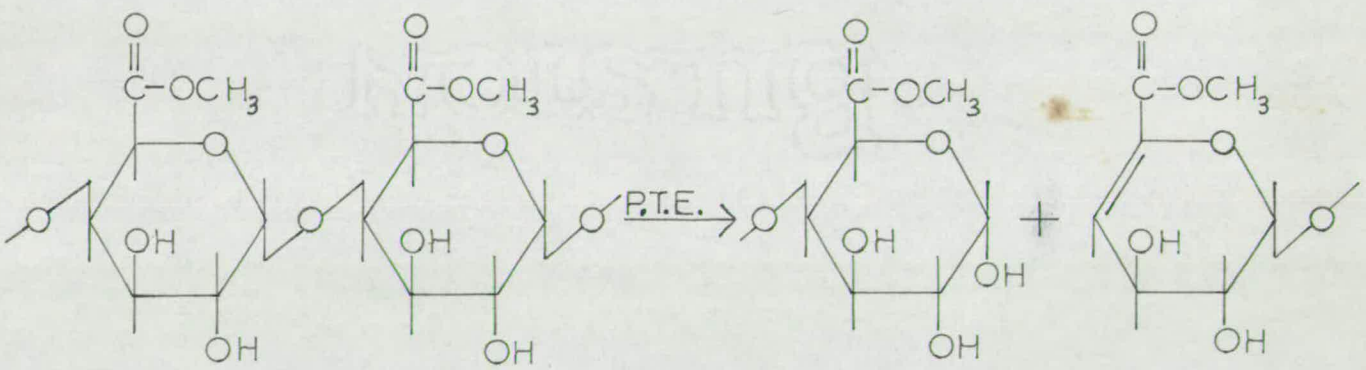
Fungal polygalacturonases only produce galacturonobiose, galacturonotriose and galacturonotetraose in the initial stages of hydrolysis (54) and eventually give complete breakdown to galacturonic acid, arabinose and galactose (55). It has been suggested that fungal polygalacturonases are mixtures of three or more enzymes, one of which hydrolyses pectin to galacturonobiose and galacturonotriose, while another hydrolyses these to galacturonic acid (56)(57).

Yeast polygalacturonase, however, catalyses a random hydrolysis of pectic acid to a mixture of galacturonobiose and galacturonic acid through a series of oligogalacturonides (58)(59). Demain and Phaff (60) studied the action of yeast polygalacturonase on galacturonobiose, galacturonotriose and galacturonotetrose and concluded that galacturonic acid end group inhibits hydrolysis of neighbouring glycosidic linkages. Thus galacturonobiose is not attacked by the enzyme and the rate of hydrolysis increases as the oligouronide chain lengthens. Patel and Phaff (61) have examined the action of the enzyme on oligomers of galacturonic acid which had been modified at the reducing end and have shown that the

bond at the non-reducing end of the chain is protected from attack by yeast polygalacturonase.

Although L - rhamnose is contained in the main chain of pectins, no rhamnose containing oligosaccharides have so far been isolated from enzymic hydrolysis. 3 - O - β - D xylopyranosyl - D - galacturonic acid, however, has been isolated from the enzymic degradation products of the pectic material from soyabean cotyledons (40).

Enzymic degradation can also give unsaturated products. Pectin transeliminase (62) gives not only the usual lower uronides but also a product possessing a galacturonic acid residue with a double bond between C_4 and C_5 at the non-reducing end which would indicate that some type of transelimination reaction was taking place, similar to that described earlier, with the enzyme only attacking pectin and not pectic acid (XIV).



XIV

The efficiency of the enzymic attack, however, depends not only on the degree of esterification but also on the length of the polygalacturonic acid chain and structural variations within the chain due to attached neutral sugars. The pectolytic enzyme from Bacillus polymyxa gives

similar products (63)(64), attacking from the non-reducing end, but this enzyme is more active on pectic acid than on pectin and is therefore a polygalacturonic acid transeliminase. Hasegawa and Nagel (65) have characterised 4 - O - α - D - (4,5 - dehydrogalacturonosyl) - D - galacturonic acid, isolated by the action of this enzyme on pectin.

Extraction of Pectin

It is generally recognised now that the rather severe methods of extraction used in the early work which produced discrete arabinans, galactans, and galacturonans, may have been instrumental to their formation by the degradation of a larger molecule containing all three sugars.

Pure galacturonans were obtained by deliberate acid hydrolysis (8)(9) of neutral 'blocks'. This procedure would also degrade the galacturonan to some extent.

Alkaline extraction was frequently used and this would inevitably cause degradation by a β-elimination type of mechanism.

The studies of Hullar and Smith (29) on sugar-beet arabinan suggest that it is a degradation product of a larger pectic acid molecule. This is supported by the isolation of an arabinan from lucerne pectic acid with hot lime water (39). This arabinan could not be extracted with water but after its isolation was found to be readily soluble in water. Thus on the basis of solubilities it would seem to be a degradation product. These results are in accordance with the conclusions of Barrett and Northcote (53) from which one would expect a mixture of polysaccharides if sugar-beet and lucerne pectinic acids are degraded in the same way as that of apple.

Before commencing extraction, however, any enzymes present must be inactivated by treating the freshly cut material with 90% ethanol. This also removes colouring matter, acids, and sugars.

Cold water extraction is the least likely to produce degradation but the yield of pectin is not high. Higher yields of pectin are obtained by the use of hot water (above 60°), mineral acids, alkaline reagents, and certain salt solutions of which ammonium oxalate is the most commonly used, 90% extraction of pectic substance being reported by Whistler, Harris, and Martin (66) after two successive eight hour periods at 90°. However, the use of these more efficient methods is likely to promote one of the previously discussed types of degradation.

Thus it would seem that cold water extraction and extraction in hot solution, buffered between pH 4 and 5, are the only methods for extracting pectin that are not liable to cause degradation and in order to safeguard against degradation, efficiency must be sacrificed.

Fractionation of Pectic Substances

Two types of problem are encountered in the fractionation of pectic substances, the separation of acidic polysaccharides from neutral polysaccharides and the further fractionation of acidic polysaccharides of varying acid content. Frequently, non-solvent precipitation effects a degree of fractionation of a mixture of acidic and neutral polysaccharides, the acidic polysaccharides being precipitated more readily. Furthermore, selective precipitation of the acidic polysaccharides can be achieved by formation of the insoluble salt of the acid (copper, calcium or quaternary ammonium) but acidic polysaccharides are not always obtained in a pure form

due to co-precipitation of some of the neutral polysaccharides. However, in some cases neutral polysaccharides have been isolated in a pure form from the supernatant liquid by this technique.

Chromatography on diethylaminoethylcellulose provides an analytical procedure for distinguishing between polysaccharides on the basis of the acidic sugars present. Neukom et al (67) subjected a pectic acid from sugar-beet to this chromatographic technique and found that the fractions, on hydrolysis, gave galactose and arabinose residues as well as those of galacturonic acid which supports the hypothesis that arabinose and galactose units are bound to the galacturonic acid units in the polysaccharide through glycosidic linkages. Similar results were obtained from citrus pectin, apple pectin, and sugar-beet pectin by Zitko, Rosík and Vašátko (68) but in addition they isolated a neutral component, which was either an arabinogalactan or an arabinan and galactan, from each sample. This procedure, however, cannot be conveniently employed to separate larger quantities of polysaccharide because of the low capacity of the ion-exchange medium and the large amounts of buffer solution needed to desorb the acidic polysaccharides. During the present investigations, large scale separation of acidic and neutral polysaccharides by ion-exchange chromatography has been achieved using diethylaminoethyl Sephadex A - 50 which has a much larger capacity than diethylaminoethylcellulose.

Moving boundary electrophoresis has been used to determine the homogeneity of pectic acid samples (14)(15)(53) as has zone electrophoresis (53) but the sensitivity of such methods may not be great enough to detect small quantities of neutral polysaccharide.

Acidic polysaccharides of varying acid content and varying methyl ester content have been separated on diethylaminoethylcellulose (67) and moving boundary electrophoresis (14)(15).

The subfractionation of related acidic polysaccharides on a preparative scale is described in two separate papers. Timell and Bhattacharjee (15) further fractionated the ammonium pectate from the bark of amabilis fir by acidifying the solution and removing the precipitated galacturonan by ultracentrifugation. The soluble material contained both galacturonic acid residues and those of arabinose and galactose. Zitko and Bishop (14) fractionated pectic acids from sunflower heads, sugar-beet, apples and citrus fruits by precipitation with sodium acetate to give in each case a galacturonan, free of neutral sugars, and a series of acidic polysaccharides containing both galacturonic acid residues and neutral sugars. The results would seem to justify the earlier conclusion by Aspinall and Cañas - Rodriguez (36) that pectin might consist of "a mixture of acidic polysaccharides, one composed solely of D - galacturonic acid residues and the other or others containing both neutral sugars and D - galacturonic acid residues".

However, in both cases, conditions were such that there may have been degradation of the type described by Barrett and Northcote (53) although the authors think it unlikely. Timell and Bhattacharjee extracted the pectic acid using hot potassium acetate and hot ammonium oxalate solutions and Zitko and Bishop de-esterified the samples with alkali before fractionation.

Thus in pectin chemistry one of the main difficulties is in obtaining an undegraded sample on which to carry out structural investigations.

SECTION 2

This section concerns a study of some extracellular polysaccharides obtained from the incubation of sycamore cambial cells in a solution of sucrose by Dr. P. Albersheim. The investigations were carried out in order to compare the structure of the extracellular polysaccharides with those extracted from cell walls in woody tissue.

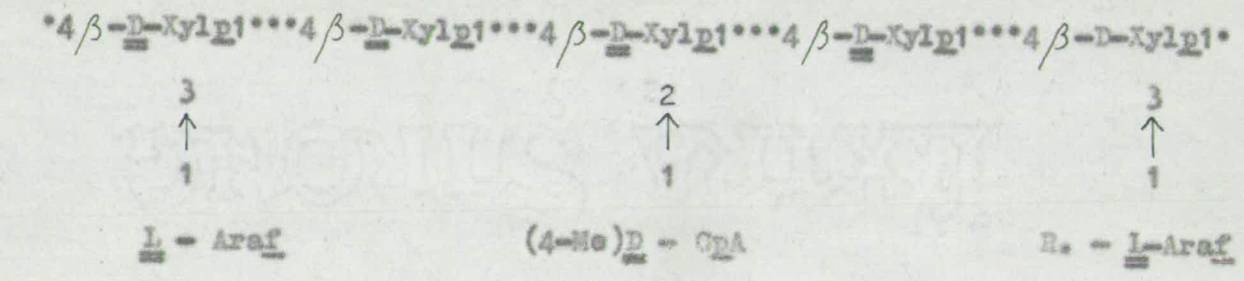
Cell-wall Polysaccharides

Cellulose is the major constituent of plant cell-walls. It exists in long thread like macromolecules, arranged in bundles which are so well organised in certain areas due to hydrogen bonding between free hydroxyl groups in the (1 → 4) - linked β - D - glucopyranose residues of adjacent molecules that crystallinity is observed (69). Interspersed between these regions are amorphous regions which appear to be rich in lignin and other polysaccharides known as 'hemicelluloses', and some pectin. The exact function of the hemicelluloses in the cell wall is not known, for while they certainly contribute to the rigidity of the cell wall, many seem to be concerned with metabolic processes.

The extraction of hemicelluloses often requires strong alkali, which can result in degradation and chemical modification of the polymers, and it has been suggested that the difference in solubility of these polysaccharides before and after extraction may be due to a chemical combination with lignin or a physical restraint which individual polymers impose on the solution of each other and especially the restraint which lignin imposes on the polysaccharides by interpenetration.

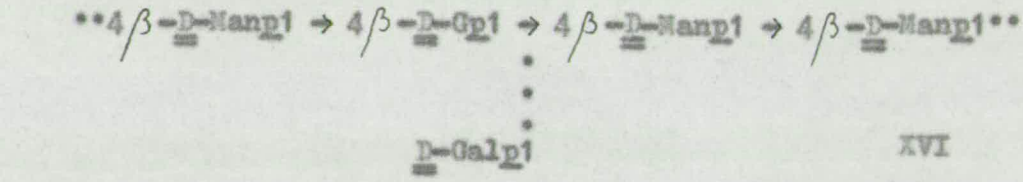
The hemicellular extracts contain a small number of structurally distinct families of polysaccharides which can be classified by the presence of similar arrangements of sugars in the main chains.

The xylan family has (1 → 4) - linked β - D - xylopyranose residues in the main chain and contains a homopolysaccharide of xylose, and xylans substituted by 4 - O - methyl-D - glucuronic acid residues or by both 4 - O - methyl-D-glucuronic acid and L arabinose residues (70) (XV).



XV

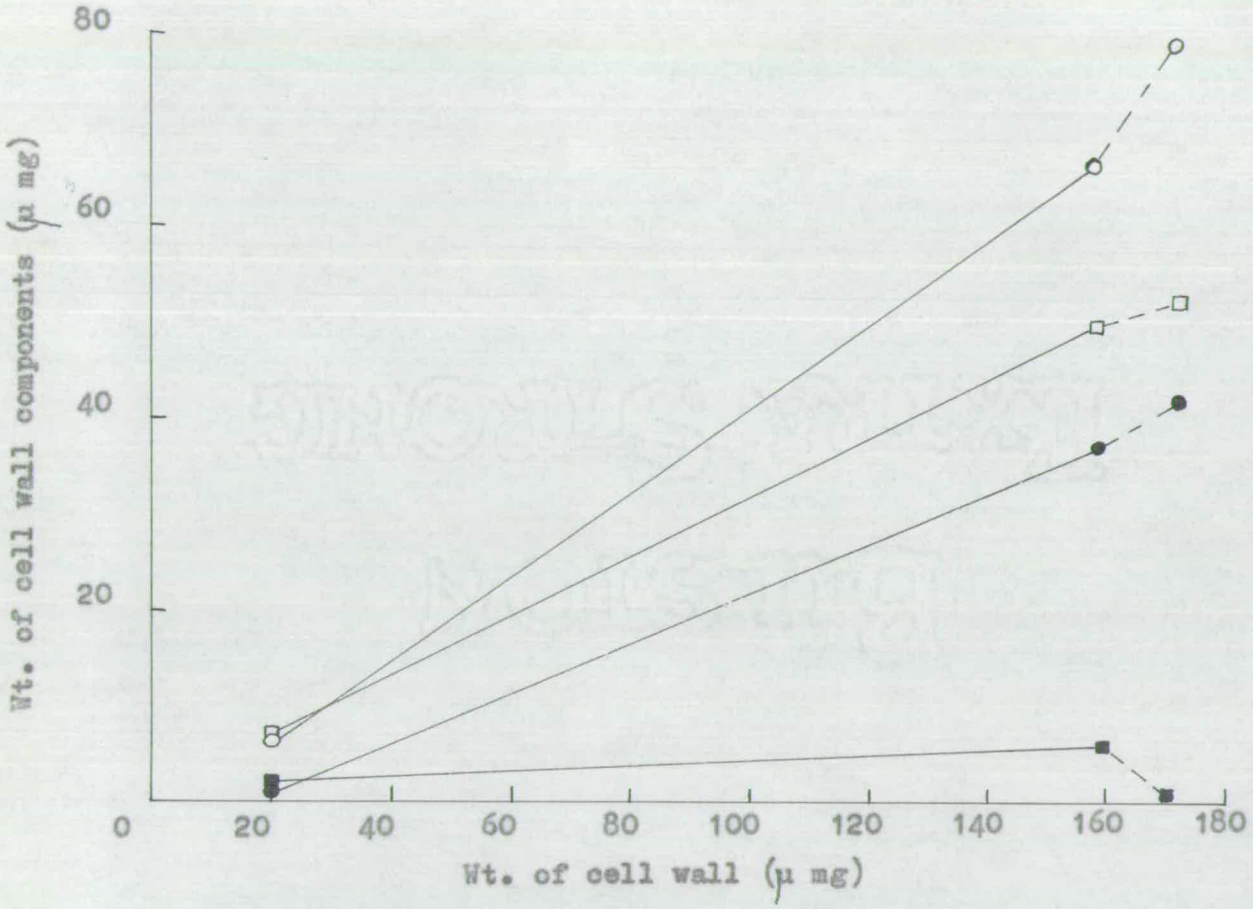
The characteristic main chain linkage of the glucomannan family is (1 → 4), β - D - glucopyranose and β - D - mannopyranose residues both being contained in the main chain. The second polysaccharide in this family contains D - galactose residues attached to the main chain. (70) (XVI)



XVI

A third family of hemicellulose polysaccharides is the arabinogalactan group which contains (1 → 3) and (1 → 6) - linked D - galactopyranose residues. The L - arabinose and D - galactose structure may also be substituted with D - glucuronic acid. (70)

Fig. 1



■ , Pectic substances; ● , lignin; ○ , α- cellulose; □ , hemicellulose

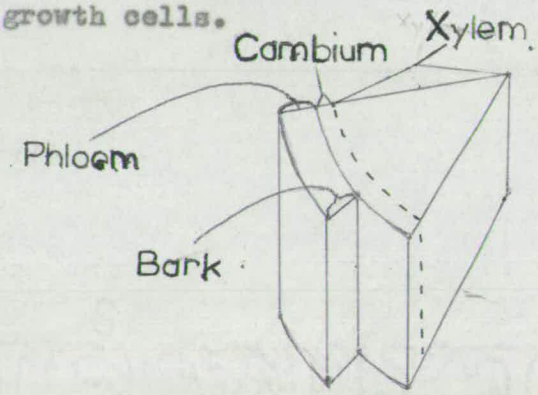
——— , Cambial cell to sapwood development

— — — , sapwood to heartwood development.

The fractionation and purification of these polysaccharides is often difficult and only recently have satisfactory methods been developed in this field.

Sycamore Cambial Cells

Woody tissue contains three types of cells which are named phloem, cambial and xylem depending on their situation in the tree (XVII), the cambial cells being the growth cells.



XVII

Thornber and Northcote (71)(72)(73) have investigated the changes in chemical composition of a cambial cell during its differentiation into xylem and phloem tissue in the sycamore. The results showed that secondary thickening of the cambial cell wall occurred by the formation of ∝- cellulose, hemicellulose and lignin with little change in weight of the pectic substances, and that the secondary thickening was accompanied by the formation of glucans 56 - 67%; xylans 20 - 30%; uronic anhydride 8 - 11%; and mannans 2 - 6% but little change occurred per cell of the amount of arabinans, and galactans decreased. (Fig 1.) gives a diagramatic increase in the weight of components per cell.

Extracellular Polysaccharides

Although many extracellular polysaccharides have been isolated from micro-organisms, grown in a sugar medium, and their structures studied (74)(75)(76)(77), the extracellular polysaccharides from sycamore cambial cells are the only extracellular polysaccharides from plant cells that has so far been reported in the literature.

Albersheim et al (78) found that sycamore (Acer pseudoplatanus) cells grew rapidly in complex yeast extract medium to secrete the polysaccharides, which have been studied in this thesis, into the culture medium. These polysaccharides are reported to be similar in composition to the noncellulosic wall polysaccharides of the cells and differ slightly from the composition of sycamore cambial cells from the intact tree (71)(72)(73).

SECTION 1

CITRUS PECTIN

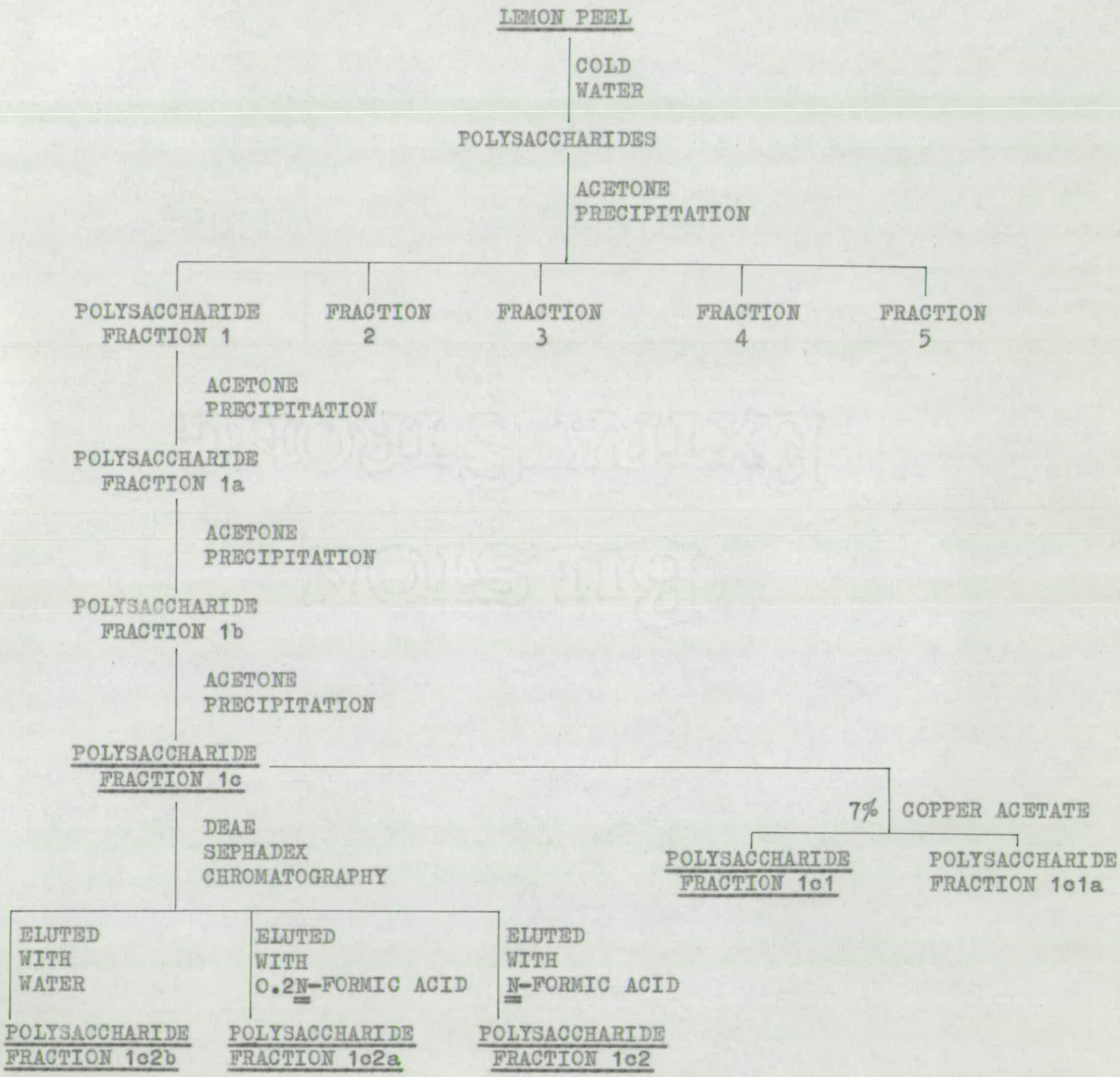
DISCUSSION

Powdered lemon peel was kindly supplied by Mr. W. C. Platt of the Ventura Coastal Lemon Corporation, Ventura, California, U.S.A. The dried peel had been previously extracted with propan-2-ol to remove lemon oil and gave on hydrolysis galacturonic acid, galactose, glucose, arabinose, xylose and rhamnose together with a small amount of mannose.

The extraction and fractionation procedures are summarised in the flow sheet on the following page.

During extraction and subsequent fractionation, strict precautions were taken to avoid any possible degradation of the pectin, whether by acid or by a β - elimination type of mechanism, by keeping the pH of the pectin solutions between 4 and 5. The amount of pectin solubilised by extraction within this pH range in cold solution amounted to about 10% by weight of the peel which was extracted. It would appear that only part of the pectin is extracted under these conditions as extraction with hot acidic solution at pH 2 has yielded more pectin (101).

The pectin extracted with cold water was subjected to fractionation by non-solvent precipitation with acetone. After repeated precipitations, polysaccharide 1c, $[\alpha]_D$ + 216°, with uronic acid content 75% and methoxyl content 10.7% indicating that approximately 80% of the carboxyl groups are esterified, was obtained which contained mainly galacturonic acid with galactose and arabinose and small amounts of glucose, xylose and rhamnose. Ion-exchange chromatography of this fraction on diethylaminoethylcellulose showed that neutral polysaccharides, containing galactose and arabinose as


LEMON PEEL
COLD WATER
POLYSACCHARIDES
ACETONE PRECIPITATION
POLYSACCHARIDE FRACTION 1
FRACTION 2
FRACTION 3
FRACTION 4
FRACTION 5
ACETONE PRECIPITATION
POLYSACCHARIDE FRACTION 1a
ACETONE PRECIPITATION
POLYSACCHARIDE FRACTION 1b
ACETONE PRECIPITATION
POLYSACCHARIDE FRACTION 1c
7% COPPER ACETATE
POLYSACCHARIDE FRACTION 1c1
POLYSACCHARIDE FRACTION 1c1a
DEAE SEPHADEX CHROMATOGRAPHY
ELUTED WITH WATER
ELUTED WITH 0.2N-FORMIC ACID
ELUTED WITH N-FORMIC ACID
POLYSACCHARIDE FRACTION 1c2b
POLYSACCHARIDE FRACTION 1c2a
POLYSACCHARIDE FRACTION 1c2

constituent sugars, were present. These polysaccharides amounted to about 5% of the pectin. The acidic polysaccharides were eluted in two major peaks and a minor peak probably due to varying acid and ester content within the pectin.

The amount of neutral polysaccharides was reduced to 4% when a portion of polysaccharide fraction 1c was fractionated via its insoluble copper salt (92) to yield polysaccharide fraction 1c1, $[\alpha]_D$ + 221°, with uronic acid content 76% and methoxyl content 10.2%.

The neutral polysaccharides were finally removed by chromatography on diethylaminoethyl Sephadex A-50. This ion-exchange medium was found to have a much greater capacity than diethylaminoethylcellulose, being able to absorb at least a tenth of its own weight of citrus pectin, and was thus much more suitable for large scale separations.

Citrus pectin (polysaccharide fraction 1c) was absorbed on diethylaminoethyl Sephadex A-50 which was eluted first with water followed by 0.2$\underline{\underline{N}}$ - formic acid and $\underline{\underline{N}}$ - formic acid. The formic acid was removed by dialysis and the pH of the solution adjusted to 4.5 with potassium acetate which is soluble in ethanol and therefore does not precipitate with the polysaccharides. The pH was held at 4.5 during concentration to minimise the risk of any kind of degradation.

Citrus pectinic acid (polysaccharide fraction 1c2), $[\alpha]_D$ + 217°, with uronic acid content 76% and methoxyl content 10.2% showing that no de-esterification had taken place, was desorbed from the diethylaminoethyl Sephadex A-50 as the major fraction with $\underline{\underline{N}}$ - formic acid and contained mainly galacturonic acid with galactose and arabinose and a small amount of rhamnose.

Ion-exchange chromatography of the citrus pectinic acid on diethylaminoethylcellulose gave an elution pattern similar to that obtained from diethylaminoethylcellulose chromatography of polysaccharide fraction 1c except for the absence of a peak corresponding to neutral polysaccharides confirming that a pure pectinic acid free of neutral polysaccharides had been obtained. It is probable, however, that the citrus pectinic acid contains a limited spectrum of polymers of varying acid content and varying methyl ester content from its resolution into three peaks on diethylaminoethyl-cellulose.

The material eluted with 0.2N - formic acid contained polysaccharide fraction 1c2a, $[\alpha]_D$ + 78°, uronic acid content 27%, which contained mainly galactose and arabinose with a small amount of galacturonic acid.

The water eluates contained polysaccharide fraction 1c2b, $[\alpha]_D$ + 38°, with uronic acid content 10%, which gave mainly galactose, arabinose, and glucose on hydrolysis together with lesser amounts of xylose, fucose, and rhamnose, and a trace of galacturonic acid.

It is possible that acidic degradation may have taken place during desorption of the citrus pectinic acid with formic acid but this is thought unlikely since no carbohydrate material was present in the supernatant solution after precipitation nor was any other fraction present on rechromatography of the pectinic acid on diethylaminoethyl Sephadex A-50.

Thus it would seem likely that the pectinic acid isolated by chromatography on diethylaminoethyl Sephadex A-50 is a genuine example of citrus pectinic acid and that the neutral sugars detected are component sugars of the pectin molecule.

Citrus pectin was examined with a view to obtaining evidence of the linkages between the various sugar components. Polysaccharide fraction 1c1 was subjected to partial hydrolysis studies designed to release acidic oligosaccharides and polysaccharide fraction 1c was degraded by a commercial pectinase preparation in order to obtain further evidence of the linkages between neutral sugar residues and galacturonic acid. The presence of a small amount of neutral polysaccharides in these fractions is immaterial as only oligosaccharides containing acidic units were considered. Citrus pectinic acid was subjected to graded acetolysis to obtain information on the linkages between neutral sugar residues themselves as well as between neutral sugars and galacturonic acid. Further structural information was obtained from methylation studies on carboxyl reduced citrus pectinic acid. Information on the structures of the polysaccharides present in polysaccharide fractions 1c2a and 1c2b was also obtained from methylation studies.

Partial Degradation of Citrus Pectin

Treatment of citrus pectin (polysaccharide fraction 1c1) with N - sulphuric acid resulted in the formation of a mixture of acidic oligosaccharides, galacturonic acid, and neutral monosaccharides together with an insoluble degraded polysaccharide which contained mainly galacturonic acid with very small amounts of glucose, mannose, and xylose.

Chromatography of the mixture of sugars on charcoal gave a number of fractions when eluted with water followed by water containing 35% ethanol. The first of the fractions eluted with water contained galacturonic acid

and oligomers of galacturonic acid only. The later fractions eluted with water and the one eluted with water containing 35% ethanol contained acidic oligosaccharides, galacturonic acid and neutral monosaccharides and were suitably combined on the basis of the acidic oligosaccharides which they contained to give two further fractions. This separation was unexpected as the purpose of the charcoal column had been to separate the neutral monosaccharides and some galacturonic acid, which should have been eluted with water, from the acidic oligosaccharides, which should have been eluted with water containing 35% ethanol, and was attributed to the charcoal not having been washed with acid before use.

The three fractions obtained from the charcoal column were each separated into acidic and neutral components on diethylaminoethyl Sephadex A-25. The neutral components contained only monosaccharides in the form of galactose, arabinose, xylose, fucose and rhamnose together with traces of 2 - O - methyl fucose and 2- O - methyl xylose. The acidic components were further separated on diethylaminoethyl Sephadex A-25 and, where necessary, on filter sheets to give six acidic oligosaccharides and some material which was immobile on paper chromatography and contained galacturonic acid only.

The acidic oligosaccharides which were present in the largest amounts were 4 - O - (α - D - galactopyranosyluronic acid) - D - galacturonic acid (XVIII) and the polymer homologous trisaccharide, O - α - D - galacto-pyranosyluronic acid - (1 → 4) - O - α - D - galactopyranosyluronic acid - (1 → 4) - O - α - D - galacturonic acid (XIX) which were characterised by

Galp A 1 $\overset{\alpha}{\rightarrow}$ 4 Galp A

XVIII

Galp A 1 $\overset{\alpha}{\rightarrow}$ 4 Galp A 1 $\overset{\alpha}{\rightarrow}$ 4 Galp A

XIX

isolation of the calcium salts. The linkages in these oligouronides were confirmed by the reduction of the methyl glycoside of each to the methyl glycosides of the corresponding galactobiose, 4 - O - α - D - galactopyranosyl - D - galactose (XX), and galactotriose, O - α - D - galactopyranosyl - (1 → 4) - O - α - D - galactopyranosyl - (1 → 4) - O - α - D - galactose (XXI), followed by methylation and identification of the cleavage

Galp 1 $\overset{\alpha}{\rightarrow}$ 4 Galp

XX

Galp 1 $\overset{\alpha}{\rightarrow}$ 4 Galp 1 $\overset{\alpha}{\rightarrow}$ 4 Galp

XXI

products of the methylated oligosaccharides. This was achieved by the formation of the respective methyl ester methyl glycosides to solubilise the oligouronides in pyridine followed by trimethylsilylation and reduction with lithium aluminium hydride of the ether soluble products.

The methyl glycosides of the neutral oligosaccharides, (XX) and (XXI), were then methylated. The methylated disaccharide obtained from sequential reduction and methylation of (XVIII) gave on methanolysis the methyl glycosides of 2, 3, 4, 6 tetra - O - methyl - D - galactose and 2, 3, 6 tri - O - methyl - D - galactose which were identified by gas-liquid partition chromatography indicating that the linkage in structure (XVIII) is correct. The methylated trisaccharide obtained from sequential reduction and methylation of (XIX) gave on hydrolysis 2, 3, 4, 6 tetra - O - methyl - D - galactose, characterised as the crystalline aniline derivative, and 2, 3, 6 tri - O -

methyl - D - galactose, characterised by conversion into the crystalline 2, 3, 6 tri - O - methyl - D - galactonolactone, in the ratio of 1:1.7 together with a small amount of 2, 6 di - O - methyl - D - galactose which if assumed to be due to undermethylation of (residues otherwise giving 2, 3, 6 tri - O - methyl - D - galactose would raise the above ratio to 1:2 and confirm the linkages in structure (XIX). Undermethylation of the same galactotriose yielding 2, 6 di - O - methyl - D - galactose has also been reported by Jones and Reid (11).

Two oligosaccharides containing rhamnose and galacturonic acid were also isolated. The first of these gave the methyl glycosides of 3, 4 di - O - methyl - L - rhamnose and 2, 3, 4 tri - O - methyl - D - galacturonic acid after methylation and methanolysis and was characterised as 2 - O - (α-D - galactopyranosyluronic acid) - L - rhamnose (XXII) by conversion into the crystalline methyl glycoside pentamethyl ether. The second contained galacturonic acid and rhamnose residues in the molar ratio of 1.2:1 (87)(89) while the derived glycitol contained these residues in the ratio of 2.2:1 showing that rhamnose was the reducing group. Mild acid hydrolysis of the glycitol afforded 2 - O - (α - D - galactopyranosyluronic acid) - L - rhamnose and 2 - O - (α - D - galactopyranosyluronic acid) - L - rhamnitol while methylation of the tetrasaccharide glycitol followed by methanolysis afforded 1, 3, 4, 5 tetra - O - methyl - L - rhamnitol and the methyl glycosides of 3, 4 di - O - methyl - L - rhamnose, 2, 3, 4 tri - O - methyl - D - galacturonic acid, and 2, 3 di - O - methyl - D - galacturonic acid. These results suggest that the sugar has the structure (XXIII).

Assuming that the configuration of the linkage from galacturonic acid to

Galp A 1 $\overset{\alpha}{\rightarrow}$ 2 Rhap

XXII

Galp A 1 $\overset{\alpha}{\rightarrow}$ 2 Rhap 1 $\overset{\beta}{\rightarrow}$ 4 Galp A 1 $\overset{\alpha}{\rightarrow}$ 2 Rhap

XXIII

rhamnose is α as in the aldobiouronic acid then it is probable from the ~~optical~~ specific rotation of +92° that the configuration of the linkage from rhamnose to galacturonic acid is β.

In addition to these oligosaccharides containing galacturonic acid, two aldobicuronic acids were obtained which contained glucuronic acid. The first of these gave glucuronic acid, glucurone and galactose on hydrolysis and on methylation and subsequent methanolysis gave the methyl glycosides of 2, 3, 4 tri - O - methyl - D - galactose and 2, 3, 5 tri - O - methyl - D - galactose. On the basis of this and the specific rotation of +6° which was similar to that obtained for the same disaccharide isolated from soyabeans (40)(41), it may be assigned the tentative structure, 6 - O - (β - D - glucopyranosyluronic acid) - D - galactose (XXIV). The second gave glucuronic acid, glucurone, and fucose on hydrolysis and the methyl glycosides of 2, 3, 4 tri - O - methyl - D - glucuronic acid and 2, 3 di - O - methyl - L - fucose after methylation and subsequent methanolysis. On the basis of these cleavage products together with the ~~optical~~ specific rotation of -70° which was similar to that obtained for this disaccharide isolated from soyabeans (40)(41), the structure, 4 - O - (β - D - glucopyranosyluronic acid) - L - fucose (XXV) may be assigned to the disaccharide.

Gp A 1 $\overset{\beta}{\rightarrow}$ 6 Galp

XXIV

Gp A 1 $\overset{\beta}{\rightarrow}$ 4 Fucp

XXV

A further sample of citrus pectin (polysaccharide fraction 1c2) was subjected to graded acetolysis which furnished an insoluble degraded polysaccharide, containing galacturonic acid only, and a mixture of mono- and oligosaccharides. This mixture was separated into neutral and acidic components on diethylaminoethyl Sephadex A-25. The neutral components were composed mainly of galactose and arabinose with lesser amounts of xylose and rhamnose but there were no detectable neutral oligosaccharides. The acidic components were further fractionated on diethylaminoethyl Sephadex A-25 and, where necessary, on filter sheets to yield oligomers of galacturonic acid and three rhamnose and galacturonic acid containing oligosaccharides.

The first of these was characterised as 2 - O - (α - D - galactopyranosyluronic acid) - L - rhamnose by conversion into the crystalline methyl glycoside pentamethyl ether as before. The second oligosaccharide gave galacturonic acid and rhamnose on hydrolysis and 2 - O - (α - D - galactopyranosyluronic acid) - L - rhamnose and rhamnose on partial hydrolysis while partial hydrolysis of the glycitol gave the same aldobiouronic acid and rhamnitol. Sequential methylation and methanolysis of the trisaccharide glycitol gave 1, 3, 4, 5 tetra - O - methyl - L - rhamnitol and the methyl glycosides of 3, 4 di - O - methyl - L rhamnose and 2, 3, 4 tri - O - methyl - D - galacturonic acid. These results together with the ~~optical~~ specific rotation suggest that this fragment has the probable structure (XXVI). The third oligosaccharide, when subjected to

GalpA 1 $\xrightarrow{\alpha}$ 2 Rhap1 $\xrightarrow{\beta}$ 2 Rhap

XXVI

hydrolysis, partial hydrolysis of the glycitol and methylation of the glycitol, gave results which indicated that it was identical to the acidic tetrasaccharide (XXIII).

Citrus pectin (polysaccharide fraction 1c) was also subjected to enzymic degradation using a commercial pectinase preparation. The degradation products were separated into neutral and acidic components on diethylaminoethyl Sephadex A-25, the acidic components being further fractionated on diethylaminoethyl Sephadex A-25 and, where necessary, on filter sheets to give four oligosaccharide components.

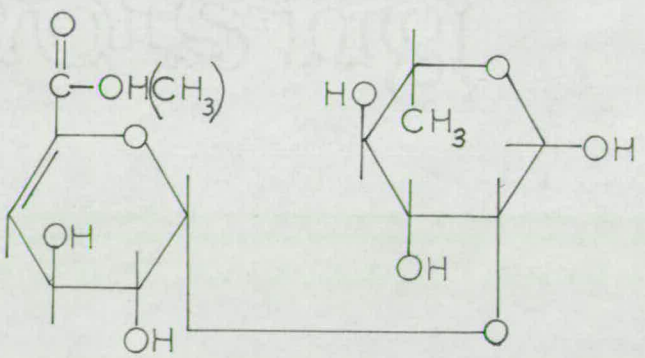
The first of these gave galacturonic acid and xylose on hydrolysis while the derived glycitol gave xylose only. Methylation of the glycitol followed by methanolysis gave γ - 2, 3, 5, 6 tetra - O - methyl - D - galactonolactone and the methyl glycosides of 2, 3, 4 tri - O - methyl - D - xylose. On the basis of these results together with the ~~optical~~ specific rotation and chromatographic mobility it is possible to assign the tentative structure (XXVII) to the disaccharide.

Xylp 1 $\xrightarrow{\beta}$ 3 Galp A

XXVII

The second oligosaccharide had twice the mobility of galacturonic acid in Solvent B and contained 46% rhamnose (89) but after hydrolysis of the oligosaccharide and the glycitol only rhamnose and rhamnitol respectively could be detected. Methylation of the oligosaccharide followed by methanolysis gave on gas-liquid partition chromatography the methyl glycoside of 3, 4 di - O - methyl - L - rhamnose and a number of peaks

which could not be identified. Examination of the absorption spectra of the oligosaccharide showed that it had an absorption maximum at 231 mμ and when reacted with thiobarbituric acid (51) gave a red coloured solution which had an absorption maximum at 547 m μ. On the basis of the chromatographic mobility of the oligosaccharide and the proportion of rhamnose it contains, it would appear that it is a disaccharide. The absorption maximum of 231 m μ of the disaccharide suggests that it contains an α,β unsaturated acid. This is confirmed by the absorption maximum at 547 m μ obtained after the disaccharide had been reacted with thiobarbituric acid. In light of this and the distinctive orange colour given by the disaccharide with aniline oxalate spray reagent and also from the isolation of 4 - C - (4 - deoxy - β - L - threo - hex - 4 - enosyluronic acid) - D galacturonic acid from other enzymic degradation studies (65), the structure (XXVIII) is suggested for the disaccharide. The α configuration is proposed



XXVIII

from the ~~optical~~ specific rotation of +87°. From the isolation of this unsaturated disaccharide it would appear that the pectinase preparation contains some

pectin transeliminase activity. The possible instability of the α,β unsaturated acid to acid could explain its absence on paper chromatography and also the unknown peaks on the gas chromatogram which could have arisen from its degradation product or products.

The third oligosaccharide contained galacturonic acid and rhamnose residues in the molar ratio of 1.9:1 while the glycitol contained these residues in the ratio of 1:1. Alkaline degradation of the oligosaccharide with triethylamine (99) gave 2 - O - (α - D - galactopyranosyluronic acid) - L - rhamnose. Sequential methylation, methanolysis and gas-liquid partition chromatography of the glycitol gave the methyl glycosides of 3, 4 di-O - methyl - L - rhamnose and 2, 3, 4 tri - O - methyl - D - galacturonic acid together with a peak which was not identified but was suspected to have arisen from the methyl ester or lactone of 2, 4, 5, 6 - tetra - O - methyl - D - galactonic acid. In an effort to confirm this, 4 - O - (α - D - galactopyranosyluronic acid) - D - galactonic acid was methylated as was 3 - O - α - D - galactopyranosyl - D - galactonolactone (3 - O - α - D - galactopyranosyl - D - galactose was kindly supplied by Dr. T. C. S. Dolan) and on methanolysis and gas-liquid partition chromatography both gave a peak which had the same relative retention times on different columns as had the unidentified peak in the methanolysis products of the methylated oligosaccharides. This evidence, however, is not conclusive as the gas chromatograms contained other peaks which could not be accounted for.

As the ratio of galacturonic acid to rhamnose is 2:1, the oligosaccharide must be a trisaccharide or a hexasaccharide or so on. From its

mobility, however, it would appear to be a trisaccharide. On the basis of the formation of 2 - O - (α - D - galactopyranosyluronic acid) - L - rhamnose from alkaline degradation, the structure (XXIX) is suggested with

Galp A 1 → 2 Rhap 1 → ? Gal A

XXIX

the linkage between the rhamnose unit and the galacturonic acid unit at the reducing end through either the 3 or 4 positions. This structure is substantiated from methylation evidence both from the products obtained, 3, 4 di - O - methyl - L - rhamnose and 2, 3, 4 tri - O - methyl - D - galacturonic acid, and from the absence of any 1, 3, 4, 5 tetra - O - methyl - L - rhamnitol and any 2, 3 di - O - methyl - D - galacturonic acid. Although the evidence obtained from the methylation of 4 - O - (α - D - galactopyranosyluronic acid) - D - galactonic acid and 3 - O - α - D - galactopyranosyl - D - galactonolactone is not conclusive, it would suggest, along with the absence of any 2, 3, 5, 6 tetra - O - methyl - D - galactonolactone or any 2, 3, 4, 6 tetra - O - methyl - D - galactonolactone, that the linkage is through the 4 position and that the trisaccharide has the structure (XXX). The suggested configurations for the two linkages are

Galp A 1 $\overset{\alpha}{\rightarrow}$ 2 Rhap 1 $\overset{\beta}{\rightarrow}$ 4 Gal A

XXX

based on an ~~optical~~ specific rotation of +85°.

The fourth oligosaccharide contained mainly galactose together with glucose, arabinose, xylose, rhamnose, galacturonic acid and probably a small amount of glucuronic acid. No change in the hydrolysis pattern was observed

after formation of the glycitol or after alkaline degradation with triethylamine (99). Sequential methylation, methanolysis and gas-liquid partition chromatography gave a complicated chromatogram from which the methyl glycosides of the following sugars were identified.

2, 3, 4 tri - O - methyl - L - rhamnose
2, 3, 5 tri - O - methyl - L - arabinose
3, 4 di - O - methyl - L - rhamnose
2, 3, 4, 6 tetra - O - methyl - D - galactose
2, 3, 6 tri - O - methyl - D - galactose
2, 3, 4 tri - O - methyl - D - galacturonic acid

This would appear to be a high molecular weight fragment containing a predominance of neutral sugar residues. The linkages between the sugar residues seem to be of the usual type found in pectins but from the complicated nature of the gas chromatograms it is impossible to draw any firm conclusions as to the structure of the oligosaccharide.

Methylation of Carboxyl Reduced Citrus Pectinic Acid

This methylation was carried out in order to obtain more information on the linkages present in the polysaccharide.

Citrus pectinic acid was reduced by the method of Rees and Samuel (100). This involved the formation of the 2-hydroxyethyl esters of the free acid groups in the pectin with ethylene oxide, acetylation, and finally reduction with lithium borohydride of the acetylated, esterified pectin suspended in tetrahydrofuran. The reduced polysaccharide gave galactose as the only detectable sugar after hydrolysis. This was due to the relatively large amount of galactose present after reduction in relation to the other monosaccharides.

Methylation of the reduced polysaccharide and subsequent examination of the methanolysed products by gas-liquid partition chromatography yielded the methyl glycosides of the sugars in table XXIV.

TABLE XXIV

Sugar	Relative Amounts
2, 3, 4 tri - O - methyl - L - rhamnose	tr.
2, 3, 5 tri - O - methyl - L - arabinose	++
3, 4 di - O - methyl - L - rhamnose	+
3 - O - methyl - L - rhamnose	+
2, 3 di - O - methyl - L - arabinose	+
2 - O - methyl - L - arabinose	tr.
2, 3, 4, 6 tetra - O - methyl - D - galactose	++
2, 3, 6 tri - O - methyl - D - galactose	++++
2, 6 di - O - methyl - D - galactose	++

1, 3, 4, 5 tetra - O - methyl - L - rhamnitol was also detected in approximately the same amount as 3, 4 di - O - methyl - L - rhamnose.

The detection of a large amount of 2, 3, 6 tri - O - methyl - D - galactose indicates the essentially linear nature of the polymer.

Methylation of the Two Minor Fractions eluted from Diethylaminoethyl Sephadex A-50

The fraction eluted from diethylaminoethyl Sephadex A-50 with 0.2N - formic acid (polysaccharide fraction 1c2a) was methylated, the fully methylated polysaccharide was methanolysed and the methyl glycosides of the sugars obtained together with their relative amounts are given in table XXV.

TABLE XXV

Sugar	Relative Amounts
2, 3, 4 tri - O - methyl - L - rhamnose	tr.
2, 3, 5 tri - O - methyl - L - arabinose	++++
2, 3, 4 tri - O - methyl - L - arabinose	tr.
3, 4 di - O - methyl - L - rhamnose	tr.
2, 3 di - O - methyl - L - arabinose	+++
2, 5 di - O - methyl - L - arabinose	+
2, 3, 4, 6 tetra - O - methyl - D - galactose	+
2, 3, 6 tri - O - methyl - D - galactose	++
2, 4, 6 tri - O - methyl - D - galactose	+
2, 3, 4 tri - O - methyl - D - galactose	++
2, 6 di - O - methyl - D - galactose	tr.
2, 4 di - O - methyl - D - galactose	++

Hydrolysis of the methylated polysaccharide and examination of the methylated sugars by paper chromatography showed that small amounts of L - arabinose and 2 - O - methyl - L - arabinose were present along with the sugars above.

The fraction which was not absorbed on diethylaminoethyl Sephadex A-50 (polysaccharide fraction 1c2b) was methylated and the methyl glycosides of the sugars obtained after methanolysis are given in table XXVI along with their relative amounts.

TABLE XXVI

Sugar	Relative Amounts
2, 3, 4 tri - O - methyl - D - xylose	tr.
2, 3, 5 tri - O - methyl - L - arabinose	++++
2, 3, 4 tri - O - methyl - L - arabinose	tr.
3, 4 di - O - methyl - L - rhamnose	tr.
2, 3, 4, 6 tetra - O - methyl - D - glucose	tr.
2, 3 di - O - methyl - L - arabinose	+++
2, 3, 4, 6 tetra - O - methyl - D - galactose	+
2, 3, 6 tri - O - methyl - D - galactose	++
2, 3, 6 tri - O - methyl - D - glucose	++
2, 6 di - O - methyl - D - galactose	tr.

L - arabinose and 2 - O - methyl - L - arabinose were identified in this case also along with the methylated sugars above by paper chromatography after hydrolysis of the fully methylated polysaccharide.

The failure to detect any methylated galacturonic acid residues in both methylated fractions and the change in ~~optical~~ specific rotation from +80° to -95° in the first case and from +38° to -71° in the second case could be indicative of fractionation taking place during the methylation procedure or of alkaline degradation which would seem more likely with the fraction absorbed on diethylaminoethyl Sephadex A-50 as absorption indicates that only acidic polysaccharides are present.

EVALUATION OF THE STRUCTURAL INVESTIGATIONS

The structural significance of the results obtained for citrus pectinic acid can be discussed in relation to their bearing on the galacturonan chain, on the rhamnose portion of that chain and on the sites of attachment and structure of side chains.

The isolation of galacturonobiose (XVIII), galacturonotriose (XIX),

Gal$\underline{p}$ A 1 $\overset{\alpha}{\rightarrow}$ 4 Gal$\underline{p}$ A — XVIII

Gal$\underline{p}$ A 1 $\overset{\alpha}{\rightarrow}$ 4 Gal$\underline{p}$ A 1 $\overset{\alpha}{\rightarrow}$ 4 Gal$\underline{p}$ A — XIX

the indication of larger fragments containing galacturonic acid only, and the formation of a degraded polysaccharide containing predominantly galacturonic acid residues, as products of partial hydrolysis shows that citrus pectin has a typical pectic structure with basal chains of (1 - 4) - linked α- $\underline{D}$ - galactopyranosyluronic acid residues (XXXI). This is

$$\cdots 4\alpha\text{-}\underline{D}\text{-Gal}\underline{p}\text{A1}\rightarrow 4\alpha\text{-D-Gal}\underline{p}\text{A1}\rightarrow 4\alpha\text{-}\underline{D}\text{-Gal}\underline{p}\text{A1}\rightarrow 4\alpha\text{-}\underline{D}\text{-Gal}\underline{p}\text{A1}\cdots$$

XXXI

confirmed from methylation studies on carboxyl reduced citrus pectin which yielded a high proportion of 2, 3, 6 tri - $\underline{O}$ - methyl - $\underline{D}$ - galactose indicating linear chains of the above type.

The isolation of a number of oligosaccharides from partial hydrolysis (XXII & XXIII), acetolysis (XXII, XXIII & XXVI), and enzymolysis (XXX) containing both galacturonic acid and rhamnose residues would suggest that

α-$\underline{D}$-Gal$\underline{p}$A1$\rightarrow$2$\underline{L}$-Rha — XXII

α-$\underline{D}$-Gal$\underline{p}$A1$\rightarrow$2β-$\underline{L}$-Rha$\underline{p}$1$\rightarrow$4α-$\underline{D}$-Gal$\underline{p}$A1-2$\underline{L}$-Rha — XXIII

α-$\underline{D}$-Gal$\underline{p}$A1$\rightarrow$2β-$\underline{L}$-Rha$\underline{p}$1$\rightarrow$2$\underline{L}$-Rha — XXVI

α-$\underline{D}$-Gal$\underline{p}$A1$\rightarrow$2β-$\underline{L}$-Rha$\underline{p}$1$\rightarrow$4$\underline{D}$-GalA — XXX

rhamnose is also a constituent of the basal chains of citrus pectin. The detection of contiguous rhamnose units, no more than two have been found together, trisaccharide (XXVI), together with the presence of alternating galacturonic acid and rhamnose units, tetrasaccharide (XXIII), when considered with the high proportion of galacturonic acid to rhamnose units would suggest that the rhamnose units are present in groups in the basal chain rather than being randomly distributed.

Oligosaccharides containing galacturonic acid and rhamnose residues have also been obtained from lucerne pectic acid (39)(42), from the pectic acid from the bark of amabilis fir (15), and from the acidic polysaccharides from soyabeans (40)(41).

If all the rhamnose units are assumed to be contained in the basal chain, then the detection of 2, 3, 4 tri - O - methyl - L - rhamnose and 1, 3, 4, 5 tetra - O - methyl - L - rhamnitol from methylation studies on carboxyl reduced citrus pectin would suggest that a number of these residues are present at the reducing and non-reducing ends of the chain, 1, 3, 4, 5 tetra - O - methyl - L - rhamnitol being assumed to have arisen from the reduction of the reducing end group during reduction of the carboxyl groups. Only limited evidence is available regarding the glycosidic configuration of the rhamnosidic residues. However, from the ~~optical~~ specific rotations of certain oligosaccharides containing both galacturonic acid and rhamnose, some are assigned a β configuration.

The failure to remove all the neutral sugar residues on repeated fractionation can be taken as evidence that these residues are present in side chains in citrus pectin and some indication of the sites of attachment

of these side chains is obtained from the detection of 3 - O - methyl - L - rhamnose and 2, 6 di - O - methyl - D - galactose on methylation of carboxyl reduced citrus pectinic acid. Due to the difficulty in getting complete methylation 2, 6 di - O - methyl - D - galactose could possibly arise from undermethylation of 2, 3, 6 tri - O - methyl - D - galactose as it was detected as an undermethylation product from O - α - D - galactopyranosyl - (1 → 4) - O - α - D - galactopyranosyl - (1 → 4) - D - galactose. However, some of it at least must arise from the attachment of xylose residues to the basal chain as the pseudo-aldobiouronic acid, 3 - O - β - D - xylopyranosyl - D - galacturonic acid has been isolated from enzymic degradation studies. These partially methylated sugars, then, suggest that branching takes place through the 4 position in some of the rhamnose residues (XXXII) and the 3 position in some of the galacturonic acid residues (XXXIII). This would also appear to be the case in sisal pectic

acid (36), lucerne pectic acid (39), the pectic acid from the bark of amabilis fir (15), and the acidic polysaccharides from soyabeans (41), although, in these instances, apparent branching through the 2 position and both the 2 and 3 positions in some of the galacturonic acid residues was also detected.

As regards the side chains themselves, the arabinose residues would appear to be present in a highly branched structure similar to that found

in pectic arabinans as methylation studies on carboxyl reduced citrus pectinic acid have yielded 2, 3, 5 tri - O - methyl - L - arabinose, 2, 3 di - O - methyl - L - arabinose and 2 - O - methyl - L - arabinose. This type of arabinose side chain is also found in lucerne pectic acid (39). The galactose residues, on the other hand, appear to be present only as single non-reducing end groups as no evidence has been obtained from acetolysis studies for (1 → 4) - linked chains of galactose residues. This type of single unit side chain has also been found in lucerne pectic acid (39) whereas chains of (1 → 4) - linked β - D - galactopyranose residues have been found in sisal pectic acid (36), commercial citrus pectin (17), and the acidic polysaccharides from soyabeans (40), (41).

Xylose, fucose, 2 - O - methyl fucose and 2 - O - methyl xylose have also been detected in small amounts in citrus pectin but of these, only xylose has been located in the molecule from the isolation of the pseudo-aldobiouronic acid 3 - O - β - D - xylopyranosyl - D-galacturonic acid which indicates that the xylose residues are linked to the basal chain through the 3 position in a few of the galacturonic acid residues. This pseudo-aldobiouronic acid has also been isolated from enzymic degradation of tragacanthic acid (43) and the acidic polysaccharides from soyabeans (40).

Acidic oligosaccharides (XXIV) and (XXV) containing glucuronic acid have also been isolated from partial hydrolysis studies. Although there

β - D - Gp A 1 → 6D-Galp — XXIV

β - D - Gp A 1 → 4L-Fucp — XXV

is no evidence for the location of glucuronic acid units in citrus pectin,

it is almost certain that glucuronic acid is a constituent sugar as these oligosaccharides have also been isolated from lucerne pectic acid (42) and from the acidic polysaccharides from soyabeans (40)(41). Similar oligosaccharides have been isolated from tragacanthic acid in which the glucuronic acid residues are present as end groups on side chains (94).

The small amount of polysaccharides of low uronic acid content (27%) eluted from diethylaminoethyl Sephadex A-50 with 0.2N - formic acid were methylated in order to gain some insight into their structure. The detection of 2, 3, 6 tri -, and a small amount of 2, 6 di - O - methyl - D - galactose would indicate that some of the galactose residues are linked through the 1 and 4 positions whereas the detection of 2, 3, 4 tri -, 2, 4, 6 tri -, and 2, 4 di - O - methyl - D - galactose would indicate that others are linked through the 1 and 6, and 1 and 3 positions. The arabinose residues would appear to be linked mainly through the 1 and 5 positions from the detection of 2, 3 di - O - methyl - L - arabinose although there is evidence from the presence of some 2, 5 di - O - methyl - L - arabinose that some arabinose residues may be linked through the 1 and 3 positions. Branching is also evident in the arabinose structure from the detection of 2 - O - methyl - L - arabinose and L - arabinose which is not thought to be due to undermethylation, from the high methoxyl content and also from the absence of any unmethylated galactose.

As this fraction was absorbed on diethylaminoethyl Sephadex A-50 it would appear to contain genuine acidic polysaccharides. The absence of any methylated galacturonic acid residues is thus more likely to be due to alkaline degradation during methylation rather than to inadvertent

fractionation during methylation. From the diverse nature of the linkages between the galactose residues it would appear that this fraction contains either an extremely complex acidic polysaccharide or a mixture of acidic polysaccharides.

The small amount of polysaccharides (uronic acid content 10%) which were not absorbed on diethylaminoethyl Sephadex A-50 were also methylated and yielded 2, 3, 6 tri - O - methyl - D - galactose and a small amount of 2, 6 di - O - methyl - D galactose as the only methylated galactose residues indicating that the galactose residues are linked through the 1 and 4 positions with some branching through the 3 position. The arabinose residues seemed to be present in a pectic arabinan type of structure as 2, 3, 5 tri - O - methyl - L - arabinose, 2, 3 di - O - methyl - L arabinose, and small amounts of 2 - O - methyl - L - arabinose and L - arabinose were detected. The L - arabinose is again assumed to be an authentic product and not due to undermethylation from the high methoxyl content and the absence of any unmethylated galactose.

From these methylation results it would appear that this fraction is composed of a pectic type arabinogalactan or a pectic arabinan and galactan together with some acidic polysaccharide which may have been removed by inadvertent fractionation during methylation or have been subjected to alkaline degradation during methylation. The detection of 2, 3, 6 tri - O - methyl - D - glucose would suggest that a contaminating glucan of the starch type is also present.

SUMMARY

The basal chains of citrus pectinic acid, extracted from lemon peel with cold water, are composed of (1 → 4) - linked α - D - galacto-pyranosyluronic acid units interspersed with rhamnose units which are not randomly dispersed but are located in groups. To the basal chains are attached side chains of arabinose units in a pectic arabinan type of structure and galactose units probably as single unit end groups. Xylose units are also attached to the basal chains through the 3 position in the galacturonic acid units.

On the basis of the galactose residues, this citrus pectinic acid is significantly different from commercial citrus pectin as, in that case, side chains of (1 → 4) - linked β - D - galactopyranose residues were detected (17). This could mean that the extraction procedure with cold water solubilises a pectinic acid of high ester content and single unit galactose side chains and more drastic extraction procedures such as used in the commercial process are needed to solubilise pectinic acids of lower ester content which may have larger side chains of galactose residues. Hot acidic solution has been shown to extract more pectin from lemon peel (101) but no structural investigations have been carried out on it as yet.

EXPERIMENTAL

GENERAL METHODS OF INVESTIGATION

Paper Chromatography

Qualitative chromatographic separations were carried out on Whatman No. 1 paper. Quantitative chromatographic separations were carried out on Whatman No. 3 MM filter sheets which had previously been extracted for 24 hours with boiling water.

The following solvent systems (v/v) were employed to develop the chromatograms.

A. ethyl acetate : pyridine : water. (10 : 4 : 3)

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

C. ethyl acetate : acetic acid : formic acid : water (18 : 8: 3: 9)

D. ethyl acetate : pyridine : acetic acid: water (5 : 5: 1: 3)

E. butan - 1 - ol : ethanol : water (4 : 1 : 5 upper layer)

F. butan - 2 - one : water : ammonia (200 : 17 : 1)

The R_X value of a sugar refers to its mobility relative to sugar X.

The R_G value of a methylated sugar refers to its mobility relative to 2, 3, 4, 6 tetra - O - methyl - D - glucose.

Paper Ionophoresis

Electrophoresis was carried out in borate buffer at pH 10 (79) for 4 hours, applying a potential voltage of 350 v. The M_G value of the sugar refers to its mobility relative to D - glucose, a correction being made for electroendosmotic flow by incorporating 2, 3, 4, 6 tetra - O - methyl - D- glucose.

Chromatographic Spray Reagents

Reducing sugars were detected by spraying with a saturated solution of aniline oxalate in methylated spirits.

Sugar alcohols and small amounts of reducing sugars were detected with silver nitrate reagent (80).

Sugar alcohols were also detected with periodate-permanganate reagent (81).

Column Chromatography

Cellulose Columns

Cellulose was washed with N sodium hydroxide followed by distilled water until free from alkali. The cellulose was then added to the column, plugged with a layer of silver sand on glass wool, as a slurry and washed thoroughly with water.

Charcoal Columns

Activated charcoal was washed several times with distilled water, which was decanted. The charcoal was then packed into a suitable column in a slurry, which was allowed to settle under gravity and was thoroughly washed with water.

Charcoal-Celite Columns (82)

Charcoal was prepared as above. Celite was washed first with 1 : 1 hydrochloric acid followed by water until free from chloride ions. A slurry composed of equal weights of charcoal and Celite in water was added to a column, plugged with a layer of silver sand on glass wool, and

allowed to settle under gravity. The column was then washed thoroughly with water.

DEAE Cellulose Columns (67)

Diethylaminoethylcellulose was washed alternately with 0.1 N - hydrochloric acid and 0.1 N - sodium hydroxide. After washing free from alkali with water, the column was packet as a slurry. The column was then generated in the phosphate form by elution with 0.5 M - sodium dihydrogen phosphate buffer (pH 6, 2000 ml) and finally equilibrated with 0.005 M - sodium dihydrogen phosphate buffer (pH 6, 1000 ml). The polysaccharide mixture, in a small volume of water, was absorbed on to the column and eluted with the desired buffers.

DEAE Sephadex Columns

Diethylaminoethyl Sephadex (A - 25 and A - 50) was initially swollen in water and the finest gel particles removed by decantation. The gel was then washed alternately with 0.5 N - sodium hydroxide and 0.5 N - hydrochloric acid and washed free of chloride ions with water. The Sephadex was then generated in the formate form by washing with 3 N - formic acid and, after packing in a slurry, free acid was removed by washing with water.

Gas-Liquid Partition Chromatography

Qualitative separations were carried out on the "Pye Argon" instrument using the following columns:-

(a) 15% by weight of butan - 1, 4 - diol succinate polyester on dichloro-dimethylsilane treated Celite (80 - 100 mesh) at 175°.
(b) 10% by weight of polyphenyl ether [m - bis - (m - phenoxy - phenoxy) benzene] on dichloro-dimethylsilane treated Celite at 200°.
(c) [illegible] by weight of neopentylglycol adipate polyester on dichloro-dimethysilane treated Celite at 125°, 150° or 175°.
(d) 11% by weight of diethylene glycol succinate polyester on dichloro-dimethylsilane treated Celite at 150°.
(e) 5% by weight of cyano ethylmethyl silicone on dichloro-dimethylsilane treated Celite at 125°.

Retention times (T) of methyl glycosides are given relative to the mobility of methyl 2, 3, 4, 6 tetra - O - methyl - β - D - glucopyranoside.

Evaporations were carried out under reduced pressure below 40°.

Hydrolysis

(A) Hydrolysis with sulphuric acid. Samples of polysaccharide (5 - 10 mg) or oligosaccharides (1 - 2 mg) were heated at 100° with a given normality of sulphuric acid for the desired length of time. The cooled solutions were neutralised with barium carbonate and the precipitate was removed at the centrifuge. Any residual barium ions were removed with Amberlite IR 120(H) resin before concentrating.
(B) Hydrolysis with hydrochloric acid. Samples of methylated polysaccharides (5 - 10 mg) and oligosaccharides (1 - 2 mg) were hydrolysed with N hydrochloric acid at 100° for the desired length of time. The

cooled solution was neutralised with silver carbonate and the precipitate was removed at the centrifuge. Any residual silver ions were removed with Amberlite IR 120(H) resin before concentrating.

(C) Methanolysis. Samples of methylated polysaccharides (5 - 10 mg.) and oligosaccharides (0.5 - 2 mg.) were heated at 100° in methanolic 4% hydrogen chloride for the desired length of time. The cooled solution was neutralised with silver carbonate and the precipitate was removed at the centrifuge before concentrating the solution to a small volume in the presence of chloroform.

Small Scale Methylations (83) were carried out by dissolving the oligosaccharide (0.5 - 2 mg.) in N, N dimethylformamide (0.5 ml.) and adding methyl iodide (1 ml.) and silver oxide (0.2 g.). The mixture was then shaken for 24 hrs. at room temperature in the dark. Excess chloroform was added and the precipitate removed at the centrifuge before evaporating to dryness under reduced pressure. The N, N dimethylformamide was removed by azeotropic distillation under reduced pressure with toluene.

Demethylations were carried out by the method of Bonner, Bourne and McNally (84).

Methoxyl determinations were carried out by the semi-micro Zeisel method (85).

Estimation of Sugars by Phenol-Sulphuric Acid Method (86)

To the sugar solution (2 ml.) containing (20 - 150 μg.) sugar

was added 5% aqueous phenol solution (1 ml.) and concentrated sulphuric acid (5 ml.). The solution was mixed, set aside for 10 mins. and kept at 30° for 10 mins. Absorbance was measured at 490 m μ for hexoses and 480 m μ for pentoses and uronic acids.

Estimation of Uronic Acid Anhydride by Carbazole Method (87)

To the sugar solution (1 ml.) containing (5 - 100 μ.g.) of uronic acid at 0° was added concentrated sulphuric acid (12 ml.) and the solution was heated to 100° for 20 mins. After cooling, carbazole reagent (1 ml.) was added and the tubes left for exactly 25 mins. before measuring the absorbance at 520 m μ.

Estimation of Uronic Acid Anhydride by Decarboxylation was carried out using the decarboxylation method of Anderson (88).

Estimation of Rhamnose by Cysteine Hydrochloride was carried out according to Dische (89).

Thiobarbituric Acid Reaction for α,β unsaturated acids was carried out using the method of Albersheim, Neukom, and Deuel (51).

Optical Rotations were observed in aqueous solutions, at $20\pm2°$, unless otherwise stated.

Melting Points were determined either by using a Kofler hot-stage microscope or in capillary tube.

Borohydride Reduction

The oligosaccharide (2 - 3 mg.) was dissolved in water and left overnight with excess potassium borohydride. Any remaining borohydride was destroyed with Amberlite IR 120(H) resin, and the solution was evaporated to dryness, boric acid being removed by repeated evaporation with methanol as the volatile methyl borate.

Acetylation (90)

The polysaccharide (1 g.) was dissolved in formamide (25 ml.). The solution was then heated to 40° and anhydrous pyridine (7 ml.) added dropwise with vigorous stirring. Acetic anhydride (18 ml.) and anhydrous pyridine (20 ml.) were then added dropwise with vigorous stirring at 40° over a period of 2 hours. The acetylation was completed by vigorous stirring for 3 days at room temperature.

The solution was poured into ice-cold hydrochloric acid (2 N, 200 ml.) and the acetylated polysaccharide was removed at the centrifuge. It was then washed with cold water and dissolved in chloroform, the remaining traces of water being removed by repeated distillation with chloroform. The solution was then dried over anhydrous sodium sulphate, concentrated and precipitated from light petroleum (B.P. 60-80°). After washing with light petroleum, the polysaccharide acetate was dried over phosphorous pentoxide and paraffin wax.

Aniline Derivatives were prepared by refluxing equimolar portions of the sugar and freshly distilled aniline in ethanol for the appropriate time. The reaction was carried out under nitrogen in the dark and,

after evaporating off excess solvent, the derivative was recrystallised from an appropriate solvent.

Aldonolactones were prepared by dissolving the sugar (10 - 100 mg.) in water (5 ml.) and adding bromine (10 - 30 drops). The mixture was left in the dark for 3 days and then excess bromine was removed by aeration. The solution was neutralised with silver carbonate, centrifuged, silver salts precipitated with hydrogen sulphide, centrifuged and concentrated. The syrup was extracted with acetone and the derivative was crystallised from the appropriate solvent.

Organic Solvents were purified and dried by methods quoted by Vogel (91).

EXTRACTION AND FRACTIONATION

Powdered lemon peel was kindly supplied by Mr. W. C. Platt of the Ventura Coastal Lemon Corporation, Ventura, California, U.S.A. The dried peel had been previously extracted with propan - 2 - ol to remove lemon oil and gave on hydrolysis the following sugars; galacturonic acid (+++++), galactose (++), glucose (+), mannose (tr.), arabinose (+++), xylose (+), and rhamnose (+).

Trial Extraction and Preliminary Investigations

The powdered peel (50 g.) was stirred in water (1.5 l.) at room temperature for 24 hrs., the solution was centrifuged and the supernatant solution was poured into ethanol (4 vol.) containing 1% hydrochloric acid. The precipitated polysaccharide fraction A was washed free of acid with ethanol, washed with ether and dried *in vacuo*. The residual peel was stirred in water (1.5 l.) for a further 24 hrs. and the solution treated as above to give polysaccharide fraction B. The supernatant solutions from the ethanol precipitations were neutralised with silver carbonate, centrifuged and concentrated to syrups, fractions C and D, which were dried over phosphorus pentoxide *in vacuo*.

The residue from the cold water extractions was then stirred in water (1.5 l.) at 55° for 8 hrs. and the solution treated as before to yield polysaccharide fraction E.

Polysaccharide fractions A, B and E were hydrolysed in N - sulphuric acid and the hydrolysates were chromatographed in solvents A and B. Fractions C and D were chromatographed directly in solvents A and B.

TABLE I

Fraction	Gal.A	Gal.	G.	Man.	Ara.	Xyl.	Rha.	Oligo(a)	Wt.(g)
A.	++++	++	++	tr.	+++	+	+	/	3.486
B.	++++	++	++	tr.	+++	+	+	/	1.457
C.	/	+	+++	+	tr.	/	/	++	0.066
D.	/	+	+++	+	tr.	/	/	++	0.016
E.	++++	++	+	+	+++	+	+	/	1.028

(a) Oligosaccharides

tr. trace

Polysaccharide fractions A and B were combined and a sample of these (140 mg.) was chromatographed on diethylaminoethyl cellulose (30 g; phosphate form). The column was eluted successively with 0.05 M, 0.10 M, 0.25 M and 0.50 M - sodium dihydrogen phosphate buffers at pH 6 and finally with 0.50 M - potassium chloride solution. Fractions (25 ml.) were collected and analysed by the phenol-sulphuric acid method for total sugars to give a plot of polysaccharide content against eluate (diagram 1.). Details of the five polysaccharide fractions eluted are given in table II.

DIAGRAM 1.

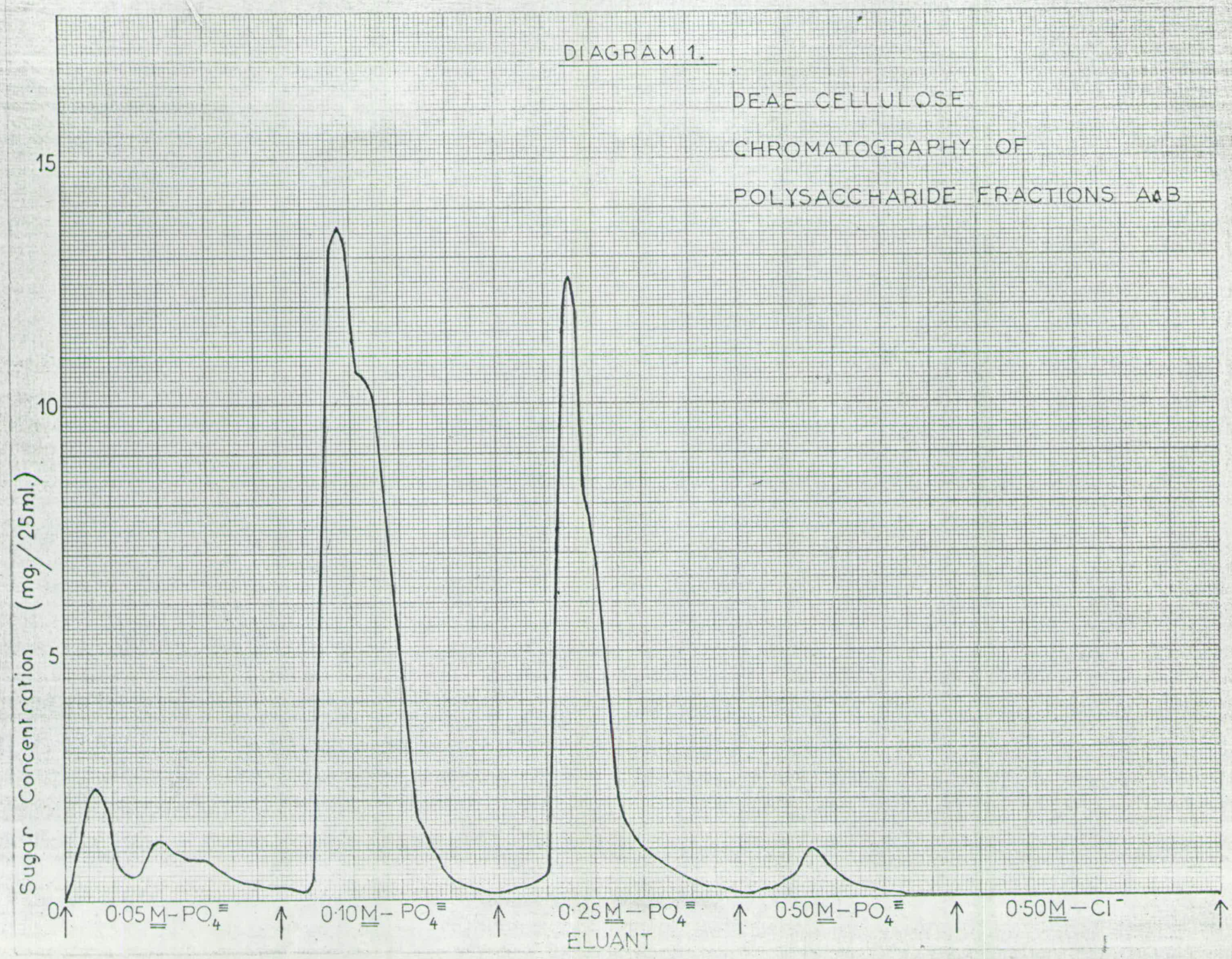

DEAE CELLULOSE
CHROMATOGRAPHY OF
POLYSACCHARIDE FRACTIONS A & B
Sugar Concentration (mg./25ml.)
15
10
5
0
0·05 M – PO4
0·10 M – PO4
0·25 M – PO4
0·50 M – PO4
0·50 M – Cl⁻
ELUANT

TABLE II

Eluant	Wt. (mg.)	Sugars on hydrolysis							
		GalA.	G.A.	Gal.	G.	Man.	Ara.	Xyl.	Rha.
0.05M-$PO_4^{\equiv}$	11.7	/	/	+	tr.	/	+++	/	/
	9.1	++	+	+	tr.	+	+++	++	tr.
0.10M-$PO_4^{\equiv}$	98.5	++++	/	+	tr.	/	+	/	tr.
0.25M-$PO_4^{\equiv}$	41.7	++++	/	+	tr.	/	+	/	tr.
0.50M-$PO_4^{\equiv}$	3.7	++++	/	+	tr.	/	+	/	tr.

Trial Fractionation by Non-solvent Precipitation

The powdered peel (50 g.) was stirred in water (1.5 l.) at room temperature for 24 hrs. at pH 4.8. The insoluble material was removed at the centrifuge and extracted for a further 24 hrs. with water (1.5 l.). The insoluble material was again removed at the centrifuge and the supernatant solutions from both extractions were combined. Acetone was then added to the solution with vigorous stirring until appreciable precipitation took place (1.5 vol.). The precipitate was removed at the centrifuge, redissolved in water, and freeze dried. The supernatant solution was concentrated, the pH being held between 4 and 5, and also freeze dried. Details of both fractions are given in table III.

TABLE III

Fraction	Wt. (g.)	$[\alpha]_D$	c	Sugars on hydrolysis						
				GalA.	Gal.	G.	Man.	Ara.	Xyl.	Rha.
Precipitate	9.006	+189°	0.206	++++	++	tr.	tr.	++	tr.	tr.
Supernatant	1.061	+144°	0.314	++	+	+	tr.	+++	+	tr.

Chromatography of Acetone Precipitate on Diethylaminoethylcellulose

A sample of the acetone precipitate (56 mg.) was chromatographed on diethylaminoethylcellulose (30 g.; phosphate form) as above. A plot of polysaccharide content against eluate is given in diagram 2. and details of the fractions are given in table IV.

TABLE IV

Eluant	Wt. (mg.)	Sugars on hydrolysis						
		GalA.	Gal.	G.	Man.	Ara.	Xyl.	Rha.
0.05M-PO_4 ≡	2.9	/	+	tr.	tr.	+++	+	tr.
0.10M-PO_4 ≡	29.8	++++	+	tr.	/	+	/	tr.
0.25M-PO_4 ≡	11.3	++++	+	tr.	/	+	/	tr.
0.50M-PO_4 ≡	1.3	++++	+	tr.	/	+	/	tr.

Large Scale Extraction, and Fractionation by Non-solvent Precipitation

The powdered peel (1000 g.) was stirred in water (30 l.) at pH 4.8 for 24 hrs. at room temperature. The insoluble material was removed at the centrifuge and extracted for a further 24 hrs. at room temperature with water (30 l.) at pH 4.8. The insoluble material was again removed at the centrifuge and the supernatant solutions from both extractions were combined and poured into acetone (1.5 vol.) to give a gelatinous precipitate which was washed with 70% aqueous acetone. The precipitate was then dissolved in water and freeze dried to give polysaccharide fraction 1.

Polysaccharide fraction 1 was dissolved in water (20 l.) and reprecipitated with acetone (1.5 vol.). The precipitate was then redissolved in water and freeze dried to give polysaccharide fraction1a.

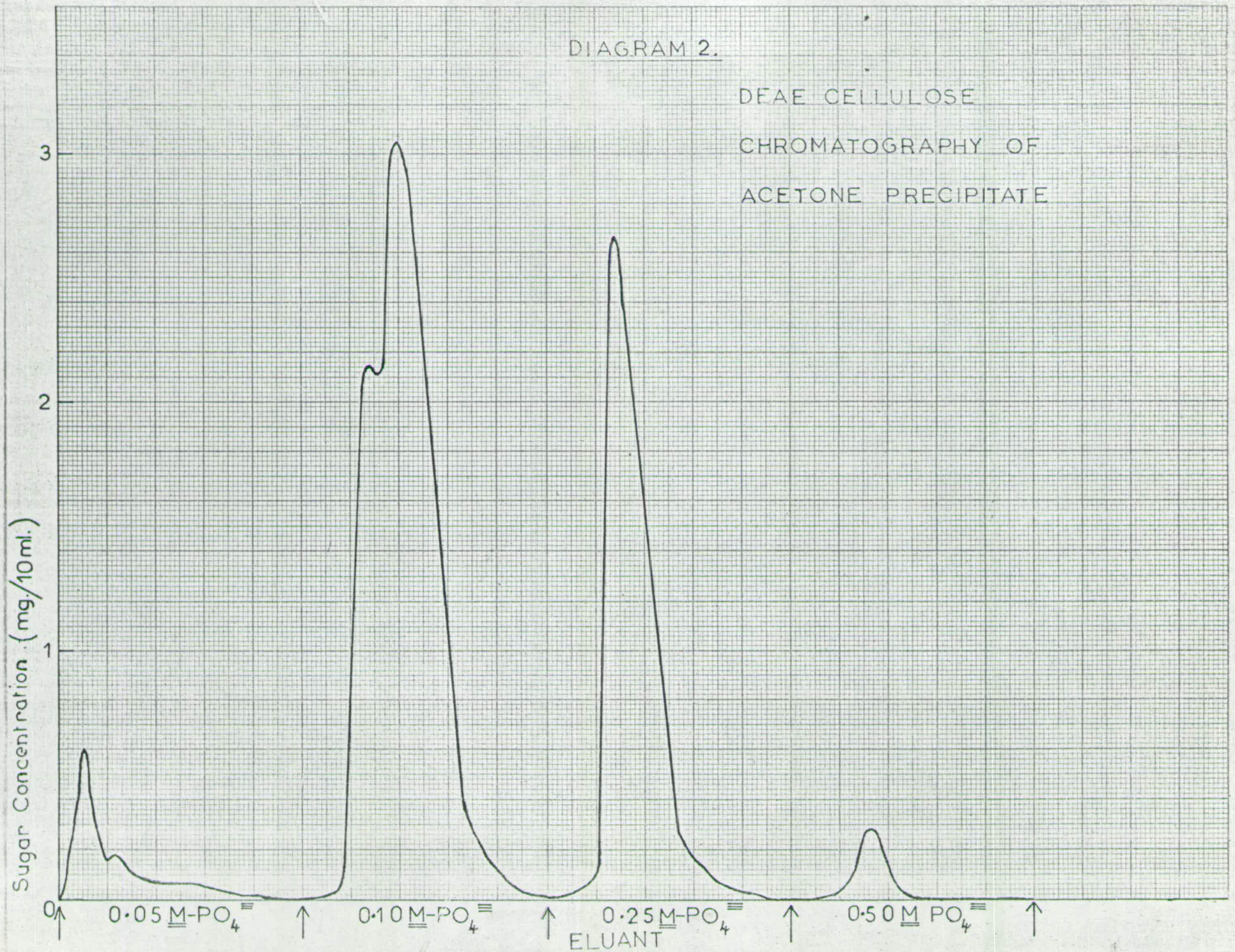


DIAGRAM 2.
DEAE CELLULOSE
CHROMATOGRAPHY OF
ACETONE PRECIPITATE
Sugar Concentration (mg./10 ml.)
3
2
1
0
0·05 M-PO4
0·10 M-PO4
0·25 M-PO4
0·50 M PO4
ELUANT

Reprecipitation with acetone was repeated a further two times to give polysaccharide fractions 1b and 1c.

The supernatant solutions from the acetone precipitations were combined and concentrated, the pH being held between 4 and 5. During concentration a fine precipitate appeared which was removed at the centrifuge and dried after washing with ether to give fraction 2. The supernatant solution was then poured into acetone (9 vol.) and the precipitate was removed at the centrifuge, redissolved in water and freeze dried to give polysaccharide fraction 3. The supernatant solution was again concentrated, poured into acetone (25 vol.) and the precipitate freeze dried as before to give polysaccharide fraction 4. The supernatant solution was then concentrated to a syrup which was dried over phosphorus pentoxide *in vacuo* to give fraction 5.

Details of each fraction are given in table V.

TABLE V

Fraction	Wt. (g.)	$[\alpha]_D$	c	Sugars on hydrolysis						
				GalA.	Gal.	G.	Man.	Ara.	Xyl.	Rha.
1	80	+193°	0.321	++++	+	tr.	/	++	+	tr.
1a	75	+200°	0.301	++++	+	tr.	/	++	tr.	tr.
1b	71	+216°	0.236	++++	++	tr.	/	+++	tr.	tr.
1c	68	+218°	0.310	++++	++	tr.	/	+++	tr.	tr.
2	1.6	n.d.	/	tr.	tr.	++	/	tr.	tr.	+
3	8	+174°	0.357	++++	++	tr.	/	+++	tr.	tr.
4	3.2	+122°	0.334	+	++	+	+	+++	+	tr.
5	1.1	n.d.	/	/	/	+	/	tr.	/	+

n.d. = not determined

Chromatography of Polysaccharide Fraction 1c. on Diethylaminoethylcellulose

A sample of polysaccharide fraction 1c (65 mg.) was chromatographed on diethylaminoethylcellulose (30 g.; phosphate form) as above. A plot of polysaccharide content against eluate is given in diagram 3. and details of the fractions eluted are given in table VI.

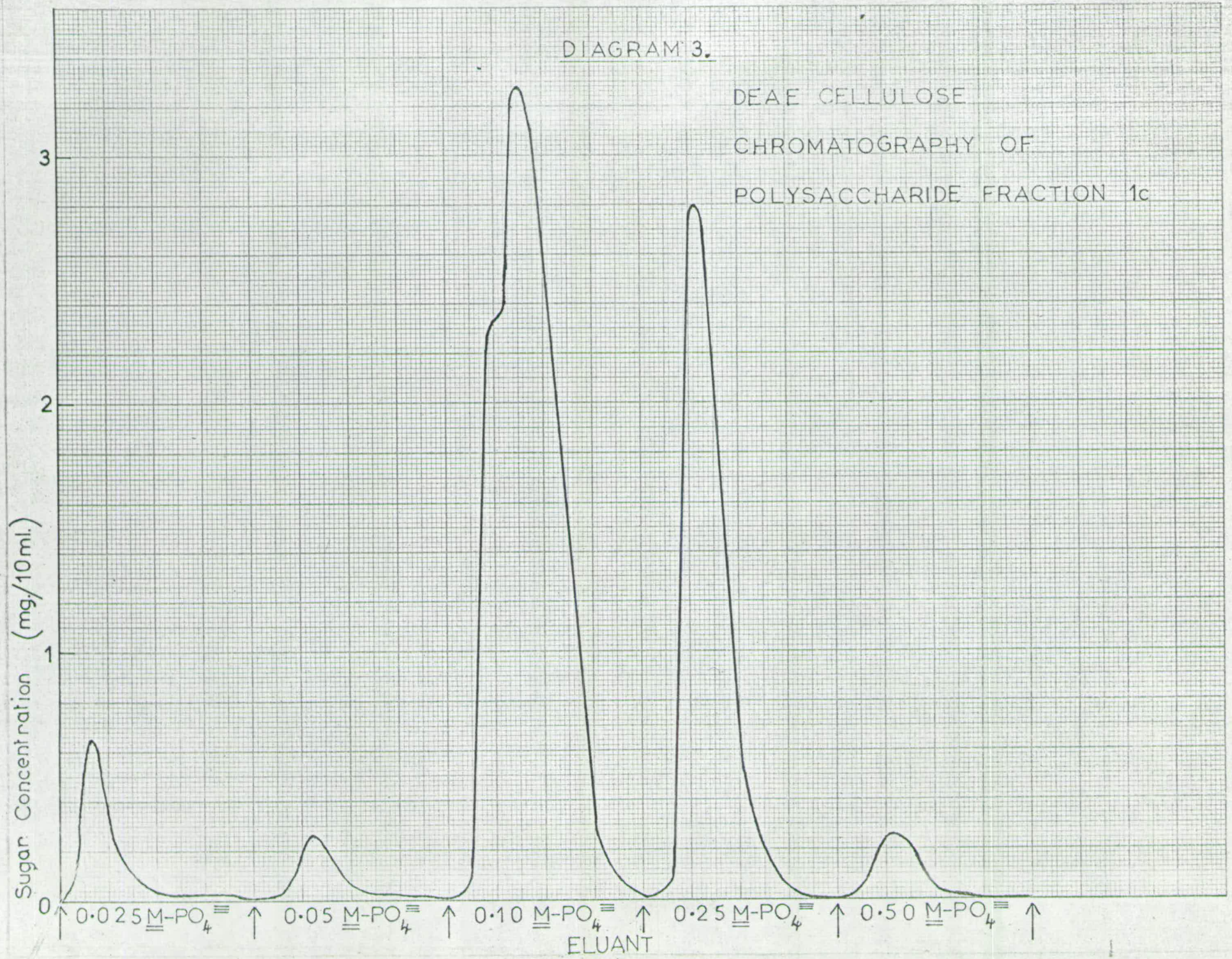
TABLE VI

Eluant	Wt. (mg.)	Sugars on hydrolysis						
		GalA.	Gal.	G.	Man.	Ara.	Xyl.	Rha.
0.025M-PO_4	3.4	/	+	+	/	+++	/	/
0.05M-PO_4	1.7	+++	+	+	+	+	tr.	tr.
0.10M-PO_4	30.2	++++	++	tr.	/	+++	tr.	+
0.25M-PO_4	16.1	++++	++	tr.	/	+++	tr.	+
0.50M-PO_4	1.8	++++	+	tr.	/	+	/	+

Fractionation with Copper Acetate

A sample of polysaccharide fraction 1c. (20 g.) [Found: uronic anhydride (by decarboxylation), 75%; OMe, = 10.7%] was dissolved in water (4 l.) and 7% copper acetate solution (200 ml.) was added dropwise with vigorous stirring. The copper salt was removed at the centrifuge and decomposed by washing with acetone containing 1% hydrogen chloride. The precipitate was washed free of hydrogen chloride with ethanol, dissolved in water and the solution was freeze dried to give polysaccharide fraction 1c1 [Found: uronic anhydride (by decarboxylation), 76%; OMe, = 10.2%]. The supernatant solution was dialysed against running tap water until the blue colour disappeared, concentrated, and poured

DIAGRAM 3.


DEAE CELLULOSE
CHROMATOGRAPHY OF
POLYSACCHARIDE FRACTION 1c
Sugar Concentration (mg./10ml.)
0
1
2
3
0·025 M-PO4
0·05 M-PO4
0·10 M-PO4
0·25 M-PO4
0·50 M-PO4
ELUANT

into acetone (25 vol.) to give polysaccharide fraction 1c1a.

Details of both fractions are given in table VII.

TABLE VII

Fraction	Wt. (g.)	$[\alpha]_D$	c	Sugars on hydrolysis						
				GalA.	Gal.	G.	Man.	Ara.	Xyl.	Rha.
1c1	18.2	+221°	0.285	++++	++	tr.	/	+++	tr.	+
1c1a	1.2	+142°	0.336	++	++	+	tr.	+++	+	tr.

Chromatography of Polysaccharide Fraction 1c1 on Diethylaminoethyl-cellulose

A sample of polysaccharide fraction 1c1 (60 mg.) was chromatographed on diethylaminoethylcellulose (30 g.; phosphate form) as above. A plot of polysaccharide content against eluate is given in diagram 4. and details of the fractions eluted are given in table VIII.

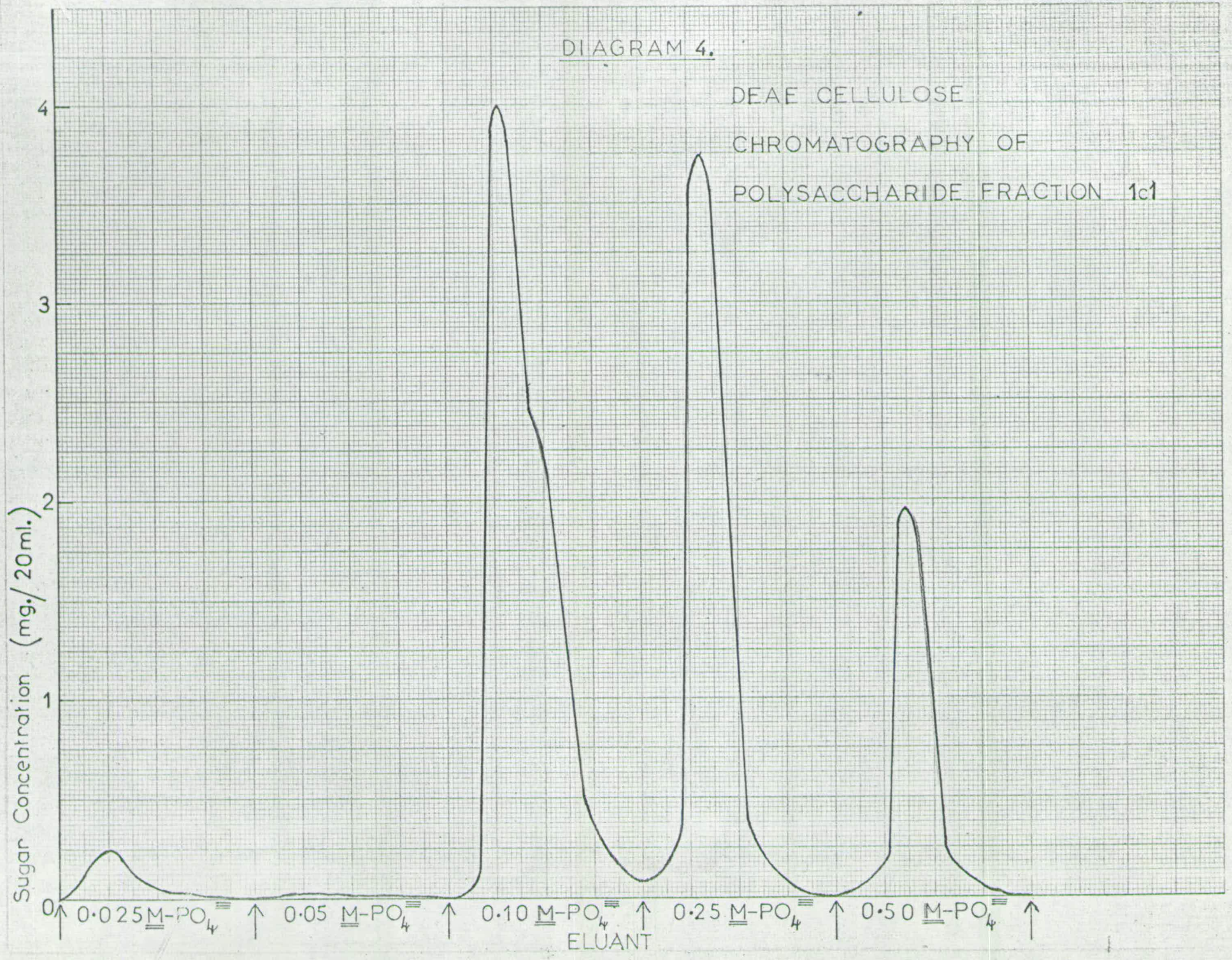
TABLE VIII

Eluant	Wt. (mg.)	Sugars on hydrolysis						
		GalA.	Gal.	G.	Man.	Ara.	Xyl.	Rha.
0.025M-$PO_4^{\equiv}$	2.8	/	+	+	/	+	+	/
0.10M-$PO_4^{\equiv}$	28.8	++++	+	tr.	/	++	tr.	tr.
0.25M-$PO_4^{\equiv}$	18.0	++++	+	tr.	/	++	tr.	tr.
0.50M-$PO_4^{\equiv}$	8.6	++++	+	tr.	/	++	tr.	tr.

Trial Fractionations by Chromatography on Diethylaminoethyl Sephadex A - 50 eluting with Formic Acid

A sample of polysaccharide fraction 1c (500 mg.) was dissolved in the minimum volume of water (20 ml.) and chromatographed on diethylaminoethyl

DIAGRAM 4.


DEAE CELLULOSE
CHROMATOGRAPHY OF
POLYSACCHARIDE FRACTION 1c1
Sugar Concentration (mg./20 ml.)
0
1
2
3
4
0·025 M-PO4
0·05 M-PO4
0·10 M-PO4
0·25 M-PO4
0·50 M-PO4
ELUANT

Sephadex A-50 (5g; formate form). The column was eluted successively with water, 0.1N, 0.2N, 0.4N, 0.6N, N and 2N formic acid and fractions (20 ml.) were collected and analysed by the phenol-sulphuric acid method for total sugars. A plot of polysaccharide content against eluate is given in diagram 5. Each fraction was stirred with Amberlite 4(B) resin to bring the pH to 4.5. During this treatment the fractions eluted with 0.6N, and 2N formic acid were absorbed on the resin and could only be recovered by washing the resin with 0.5N - sodium hydroxide. These fractions were disregarded. The other fractions were concentrated, precipitated with ethanol (2 vol.) and freeze dried.

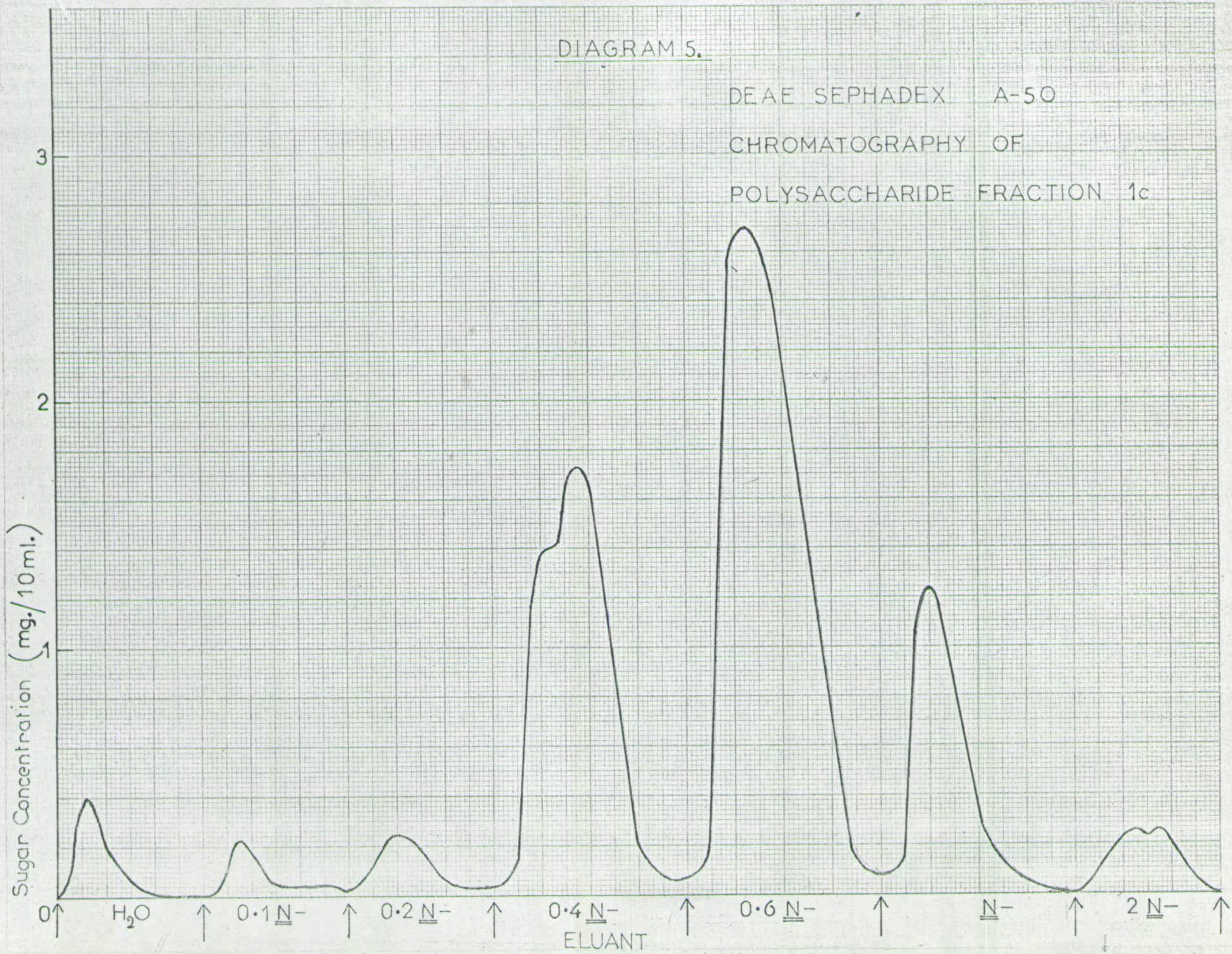
Details of each fraction are given in table IX.

TABLE IX

Eluant	Wt.	Sugars on hydrolysis						
	(mg.)	GalA.	Gal.	G.	Man.	Ara.	Xyl.	Rha.
H_2O	6.0	/	+	++	tr.	++	tr.	/
0.1N-acid	3.8	/	+	/	/	+	/	/
0.2N-acid	11.1	+	+	+	/	++	/	/
0.4N-acid	79.5	++++	+	tr.	/	++	tr.	tr.
0.6N-acid	172.1	——	——	——	n.d.	——	——	——
N-acid	65.1	++++	+	tr.	/	++	/	tr.
2N-acid	22.8	——	——	——	n.d.	——	——	——

A further sample of polysaccharide fraction 1c (500 mg.) was chromatographed on diethylaminoethyl Sephadex A-50 (5 g.; formate form).

DIAGRAM 5.


DEAE SEPHADEX A-50
CHROMATOGRAPHY OF
POLYSACCHARIDE FRACTION 1c
Sugar Concentration (mg./10 ml.)
3
2
1
0
H2O
0·1 N-
0·2 N-
0·4 N-
0·6 N-
N-
2 N-
ELUANT

The column was eluted successively with water, 0.2N and N - formic acid, the eluates being collected in bulk. The formic acid solutions were dialysed for 36 hrs., potassium acetate was added to the solutions to bring the pH to 4.5 and the solutions were concentrated to small volumes keeping the pH at 4.5 by addition of acetic acid. The polysaccharide fractions were then precipitated from ethanol (2 vol.) containing 4% acetic acid and the precipitates were removed at the centrifuge. Each precipitate was washed free of acetic acid with ethanol, redissolved in water and freeze dried.

Details of the fractions are given in table X.

TABLE X

Eluant	Wt. (mg.)	OMe	Sugars on hydrolysis						
			GalA.	Gal.	G.	Man.	Ara.	Xyl.	Rha.
water	16.5	n.d.	/	+	+	/	+	tr.	/
0.2N-acid	30.4	n.d.	+	++	/	/	+++	tr.	tr.
N-acid	265	9.5%	++++	+	tr.	/	++	tr.	tr.

When the N - formic acid fraction was rechromatographed in this way, all the polysaccharide was eluted with N - formic acid.

Large Scale Fractionation of Polysaccharide Fraction 1c on Diethylaminoethyl Sephadex A-50

A sample of polysaccharide fraction 1c (12 g.) was chromatographed on diethylaminoethyl Sephadex A-50 (100 g.; formate form). A 2%

solution of the polysaccharide was applied to the column (I) which was eluted successively with water (12 l.), 0.2N - formic acid (12 l.) and N - formic acid (20 l.), the eluate being tested periodically for polysaccharide content by the phenol-sulphuric acid method. The formic acid solutions were dialysed for 36 hrs., the pH of the solutions was then adjusted to 4.5 by the addition of potassium acetate and the solutions were concentrated, the pH being held at 4.5 by the addition of acetic acid. The polysaccharide fractions were then precipitated from ethanol containing 4% acetic acid and the precipitates were removed at the centrifuge. Each precipitate was washed free of acetic acid with ethanol, redissolved in water and freeze dried.

The column was regenerated by elution with 3N - formic acid (5 l.) followed by elution with water until the pH of the eluate was above 4.

A further sample of polysaccharide fraction 1c (12 g.) was then fractionated as above (column II).

A new column of diethylaminoethyl Sephadex A-50 was prepared before fractionation of the last sample of polysaccharide fraction 1c (10 g.) (column III).

Details of each column are given in table XI.

TABLE XI

Column	Eluant	Wt. (g)	$[\alpha]_D$	c	Sugars on hydrolysis						
					GalA.	Gal.	G.	Ara.	Xyl.	Fuc.	Rha.
I	water	0.180	+ 37°	0.248	tr.	++	+	+++	tr.	tr.	tr.
	0.2N-acid	0.360	+ 76°	0.184	+	++	/	+++	/	/	tr.
	N-acid	6.842	+216°	0.562	++++	++	/	+++	tr.	/	tr.
II	water	0.196	+ 38°	0.204	tr.	++	+	+++	tr.	tr.	tr.
	0.2N-acid	0.381	+ 78°	0.204	+	++	/	+++	/	/	tr.
	N-acid	7.292	+218°	0.504	++++	++	/	+++	tr.	/	tr.
III	water	0.146	+ 38°	0.218	tr.	++	+	+++	tr.	tr.	tr.
	0.2N-acid	0.301	+ 80°	0.196	+	++	/	+++	/	/	tr.
	N-acid	5.869	+217°	0.602	++++	++	/	+++	tr.	/	tr.

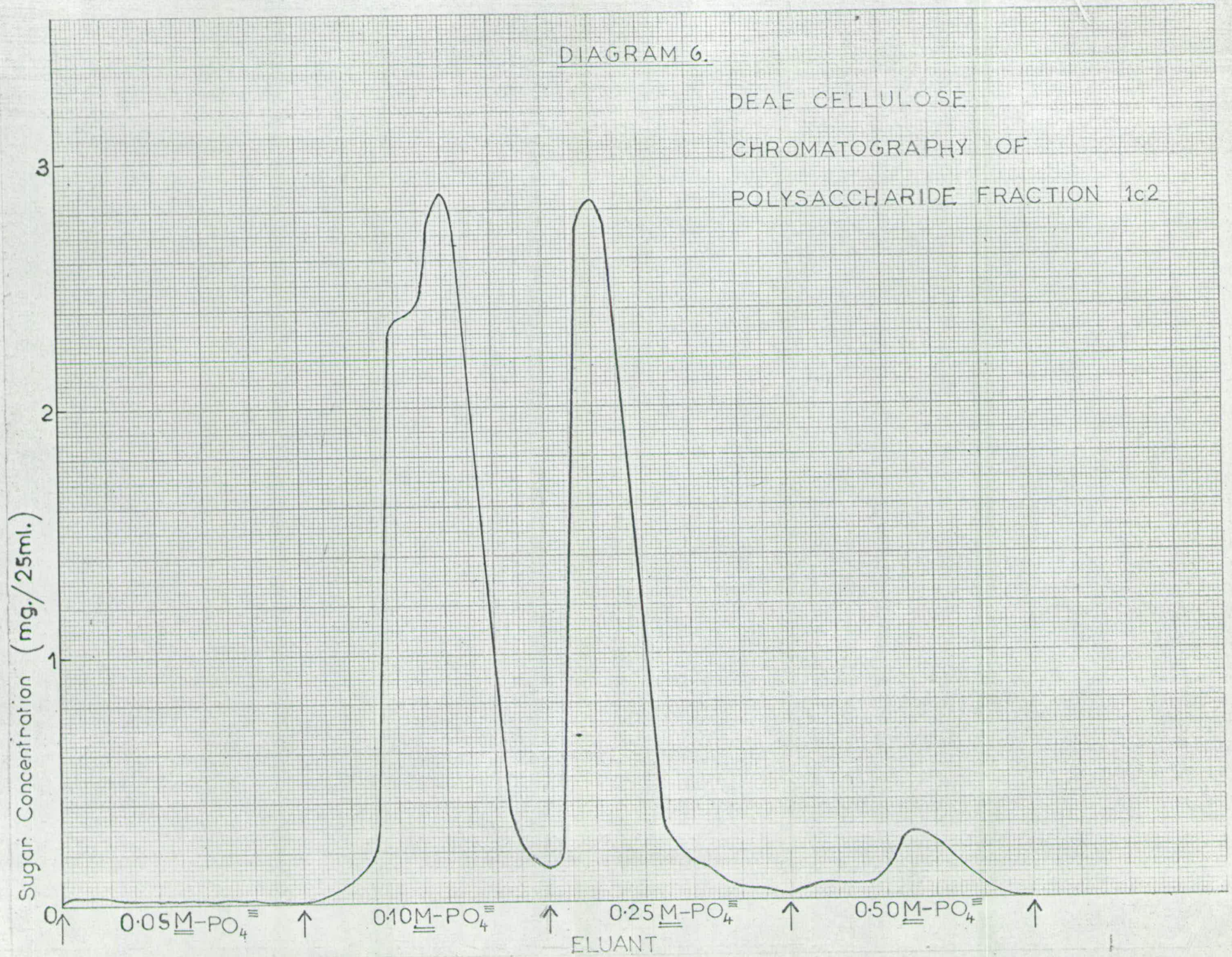
The polysaccharide fractions eluted with N - formic acid were combined to form polysaccharide fraction 1c2. [Found: uronic anhydride (by decarboxylation), 76%; OMe, = 10.2%].

The polysaccharide fractions eluted with 0.2N - formic acid were also combined to form polysaccharide fraction 1c2a. [Found: uronic anhydride (by decarboxylation), 27%] as were the fractions eluted with water to give polysaccharide fraction 1c2b. [Found: uronic anhydride (by decarboxylation), 10%]

Chromatography of Polysaccharide Fraction 1c2 on Diethylaminoethylcellulose

A sample of polysaccharide fraction 1c2 (50 mg.) was chromatographed on diethylaminoethylcellulose (30 g., phosphate form) as above. A plot of polysaccharide content against eluate is given in diagram 6. and

DIAGRAM 6.


DEAE CELLULOSE
CHROMATOGRAPHY OF
POLYSACCHARIDE FRACTION 1c2
Sugar Concentration (mg./25ml.)
3
2
1
0
0·05 M-PO4
0·10 M-PO4
0·25 M-PO4
0·50 M-PO4
ELUANT

details of the fractions eluted are given in table XII.

TABLE XII

Eluant	Wt. (mg.)	Sugars on hydrolysis						
		GalA.	Gal.	G.	Man.	Ara.	Xyl.	Rha.
0.10M-$PO_4^{\equiv}$	29.6	++++	++	tr.	/	+++	tr.	tr.
0.25M-$PO_4^{\equiv}$	15.0	++++	++	tr.	/	+++	tr.	tr.
0.50M-$PO_4^{\equiv}$	1.7	++++	++	tr.	/	+++	tr.	tr.

PARTIAL HYDROLYSIS OF CITRUS PECTIN

Citrus pectin (polysaccharide fraction 1c1) (17g.) was heated at 100° in $\underline{\underline{N}}$ - sulphuric acid (750 ml.) for 4 hrs. After 1½ hrs. heating, degraded polysaccharide separated out and remained insoluble throughout the reaction. The degraded polysaccharide was removed at the centrifuge and after the addition of acetone (1 vol.) to the supernatant solution a further precipitate of degraded polysaccharide was removed. The supernatant liquid was concentrated to remove acetone and the solution was neutralised, partly with a saturated solution of barium hydroxide and finally with barium carbonate. The precipitated barium sulphate was removed at the centrifuge and the solution was concentrated, passed through a column of Amberlite IR 120 (H) resin and finally concentrated to a syrup. The residual barium sulphate was washed twice with a very dilute solution of sulphuric acid (pH 4), the combined washings were neutralised by shaking with Amberlite LA - 1 resin (5% v/v in chloroform) and then passed through a column of Amberlite IR 120(H) resin before concentration to a syrup.

The two precipitates of degraded polysaccharide were combined and heated in $\underline{\underline{N}}$ - sulphuric acid (500 ml.) at 100° for a further ¼ hrs. The insoluble material was again removed at the centrifuge washed free of acid with water, washed with ethanol followed by ether and dried to give a degraded polysaccharide (5.94 g.) which on further hydrolysis in $\underline{\underline{N}}$ - sulphuric acid at 100° for 8 hrs. gave predominantly galacturonic acid with traces of glucose, mannose and xylose. The supernatant solution was treated as above to give two further syrups.

The syrups were redissolved in water, combined, concentrated and dried over phosphorus pentoxide in vacuo for 24 hrs.

The combined syrups (8.25 g.) were absorbed on charcoal (500 g.) and the squat column eluted with water (12 l.) at a rate of 60 ml./hr. Fractions (1 l.) were collected and suitably combined to give fractions A to F. The column was then eluted with water containing 35% ethanol to give fraction G. Details of these fractions are given in table XIII.

TABLE XIII

Fraction	Wt(g.)	Sugars, $R_{GalA.}$ (Solvent B) *
A	3.231	1.00(brown), 0.22(brown), 0.06(brown)
B	0.859	1.00(brown), 0.74(red), 0.22(brown), 0.06(brown)
C	0.702	1.00(brown), 0.84(orange), 0.21(brown), 0.06(brown)
D	0.635	1.00(brown), 0.84(orange), 0.21(brown), 0.06(brown)
E	0.283	1.00(brown), 0.42(red), < 0.1(brown)
F	0.605	0.42(red), < 0.1(brown)
G	1.330	1.00(brown), 0.74(red), 0.42(red), < 0.1(brown)

* Colour when chromatogram developed with aniline oxalate.

The fractions were thus combined as follows:-

A to give I	3.231 g.
C & D to give II	1.337 g.
B, E, F & G to give III	3.077 g.

Fractions I, II and III were, in turn, chromatographed on diethylaminoethyl Sephadex A - 25 (30 g; formate form) and neutral sugars were recovered by eluting the columns with water. The water

eluates were combined, concentrated and dried over phosphorus pentoxide *in vacuo* to give a syrup (1.47 g.) which contained galactose, arabinose, xylose, fucose and rhamnose together with traces of 2 - O - methyl fucose and 2 - O - methyl xylose. Fragments containing one or two acid residues were desorbed by gradient elution with water containing 0 → 0.4N - formic acid. Further fragments containing two acid residues were desorbed by elution with 0.4N - formic acid and fragments containing three or more acid residues were desorbed by N, and 3N - formic acid. The oligosaccharides were further separated, where necessary, by filter sheet chromatography. The formic acid elution patterns of columns I, II and III are given in tables XIV, XV and XVI respectively.

TABLE XIV

Eluant	$R_{GalA.}$ (Solvent B)	Colour *	Wt. (g.)
Water ↓ 0.4N-formic acid	1.00	brown	0.601
	0.20	brown (O_5)	0.307
0.4N-formic acid	0.20	brown (O_5)	0.253
N-formic acid	0		1.021
	0.06	brown (O_6)	
3N-formic acid	0		0.485
	0.06	brown (O_6)	

* Colour when chromatogram developed with aniline oxalate.

O_n refers to oligosaccharide n.

TABLE XV

Eluant	$R_{GalA.}$ (Solvent B)	Colour *	Wt. (g.)
Water ↓ 0.4N-formic acid	0.36	pink	0.063
	0.84	orange (O_1)	
	1.00	brown	0.106
0.4N-formic acid	0.21	brown (O_5)	0.061
N-formic acid	0	brown	0.274
	0.06	brown (O_6)	
3N-formic acid	0	brown	0.423
	0.06	brown (O_6)	

TABLE XVI

Eluant	$R_{GalA.}$ (Solvent B)	Colour *	Wt. (g.)
water			
	0.33	yellow	0.067
	0.74	red	
	0.09	red	0.205
	0.22	yellow (O_2)	
	0.34	yellow	
	0.42	red	
	0.73	yellow	
	1.00	brown	
	0.22	yellow (O_2)	0.189
	0.37	red	
	0.60	yellow (O_3)	
	1.00	brown	
	0.12	orange (O_4)	0.195
	0.23	brown (O_5)	
	1.00	brown	
0.4N-formic acid			
0.4N-formic acid	0.23	brown (O_5)	0.042
N-formic acid	0	brown	1.357
	0.06	brown (O_6)	
3N-formic acid	0	brown	0.533
	0.06	brown (O_6)	

Hydrolysis of the material from each column which was immobile in Solvent B gave galacturonic acid only.

Oligosaccharide 1. (47 mg.) $R_{GalA.}$ = 0.83 (Solvent B), M_G = 0.56, $[\alpha]_D$ + 96° (c 0.47) stained orange with aniline oxalate, gave galacturonic acid and rhamnose on hydrolysis and was chromatographically and ionophoretically indistinguishable from 2 - O - (α- D - galacto-pyranosyluronic acid) - L - rhamnose.

A sample of the sugar (2 mg.) was methylated by the Kuhn procedure and methanolysis and subsequent gas-liquid partition chromatography gave peaks with the retention times of the methyl glycosides of the following sugars.

Sugar	T (Column a)
3, 4 di - O - methyl - L - rhamnose	0.97
2, 3, 4 tri - O - methyl - D - galacturonic acid	6.9

A further sample of the sugar (24 mg.) was methylated by the Haworth procedure. Methyl sulphate (1 ml.) and 30% sodium hydroxide (2 ml.) were added simultaneously to the sugar in water (2 ml.) with constant stirring over a period of 2 hrs. Nitrogen was passed through the solution. Five further additions of methyl sulphate (2 ml.) and 30% sodium hydroxide (4 ml.) were made on consecutive days. The resulting solution was heated at 100° for 1 hr., allowed to cool and made acid to pH 4 before being poured into methylated spirits (8 vol.). The precipitated sodium sulphate was removed at the centrifuge, the pH

adjusted to 8 and the solution concentrated to a small volume. The pH was again adjusted to 4, the solution was extracted with chloroform (4 x 25 ml.) and the combined chloroform solutions concentrated to give the methylated disaccharide which was crystallised and recrystallised from a chloroform-light petroleum (B.P. 100 - 120°) mixture. The crystals $[\alpha]_D$ + 91° (c 0.63 in chloroform) had a m.p. and mixed m.p. 69° (Kofler hot-stage microscope), m.p. and mixed m.p. 119° (capillary tube) and gave an x-ray powder photograph and an infra-red spectra identical to that of methyl 2 - O - (2, 3, 4 tri - O - methyl-α-D - galacto-pyranosyluronic acid) - 3, 4 di - O - methyl - L - rhamnoside dihydrate.

Oligosaccharide 2. (8 mg.) R_{GalA} = 0.26 (Solvent B) M_G = 1.04, $[\alpha]_D$ + 6° (c 0.4) stained yellow with aniline oxalate, gave galactose, glucuronic acid, and glucurone on hydrolysis and was chromatographically and ionophoretically indistinguishable from 6 - O - (β - D gluco-pyranosyluronic acid) - D - galactose.

The disaccharide was methylated by the Kuhn procedure and methanolysis and subsequent gas-liquid partition chromatography gave peaks with the retention times of the methyl glycosides of the following sugars.

Sugar	T (Column a)
2, 3, 4 tri - O - methyl - D - glucuronic acid	2.41, 3.12
2, 3, 4 tri - O - methyl - D - galactose	7.11
2, 3, 5 tri - O - methyl - D - galactose	4.34

Oligosaccharide 3. (15 mg.) R_{GalA} = 0.60 (Solvent B), M_G = 0.63, $[\alpha]_D$ - 70° (c 0.8) stained yellow with aniline oxalate, gave fucose, glucuronic acid, and glucurone on hydrolysis and was chromatographically and ionophoretically indistinguishable from 4 - O - (β - D - glucopyranosyl-uronic acid) - L - fucose.

The sugar was methylated by the Kuhn procedure and methanolysis and subsequent gas-liquid partition chromatography gave peaks with the retention times of the methyl glycosides of the following sugars.

Sugar	T (Column a)
2, 3 di - O - methyl - L - fucose	1.06, 1.51, 2.07
2, 3, 4 tri - O - methyl - D - glucuronic acid	2.44, 3.18

Oligosaccharide 4. (8 mg.) R_{GalA} = 0.12 (Solvent B), = 0.56 (Solvent C), M_G = 0.67, $[\alpha]_D$ + 92° (c 0.4) contained galacturonic acid and rhamnose residues in the molar ratio of 1.2:1, while the derived glycitol contained these sugar residues in the ratio of 2.2:1. Mild acid hydrolysis of the glycitol, obtained by borohydride reduction, gave 2 - O - (α - D - galactopyranosyluronic acid) - L - rhamnose and 2 - O - (α - D - galactopyranosyluronic acid) - L - rhamnitol. The derived glycitol was also methylated by the Kuhn procedure and methanolysis and subsequent gas-liquid partition chromatography indicated that 1, 3, 4, 5 tetra - O - methyl - L - rhamnitol [T = 1.10 (column c)] and the methyl glycosides of the following sugars were present.

Sugar	T (Column c)
3, 4 di - O - methyl - L - rhamnose	0.86
2, 3 di - O - methyl - D - galacturonic acid	4.49
2, 3, 4 tri - O - methyl - D - galacturonic acid	6.50, 6.83

Oligosaccharide 5. (508 mg.) R_{GalA} = 0.21 (Solvent B), = 0.50 (Solvent C) M_G = 0.95 stained brown with aniline oxalate, gave on hydrolysis galacturonic acid only and was chromatographically and ionophoretically indistinguishable from 4 - O - (α - D - galactopyranosyluronic acid) - D - galacturonic acid.

The acid (30 mg.) in water (10 ml.) was neutralised with calcium carbonate, excess solid being removed by filtration. Acetone (2 vol.) was then added and the precipitated salt was filtered off, washed with acetone and ether, and dried to give a white solid $[\alpha]_D$ + 115° (c 0.68 in 0.5N - hydrochloric acid).

The acid (30 mg.) was treated overnight with methanolic 1% hydrogen chloride (5 ml.). The solution was neutralised with silver carbonate and the silver residue was removed by centrifugation before the solution was concentrated to a syrup (31 mg.). The syrup, dried over phosphorus pentoxide in vacuo for 24 hrs., was dissolved in anhydrous pyridine (3 ml.), hexamethyldisilazane (0.6 ml.) and chlorotrimethylsilane (0.3 ml.) were added and the mixture was shaken vigorously for 30 sec. After standing at room temperature for 1 hr., the solution was evaporated to dryness and the residue extracted with ether (2 x 10 ml.)

The combined ether extractions were then evaporated to dryness to give a syrup (52 mg.) which was redissolved in ether (5 ml.) and refluxed with lithium aluminium hydride (100 mg.) for 2 hrs. with vigorous stirring. The solution was cooled and ethyl acetate was added dropwise until evolution of hydrogen ceased indicating that all the remaining lithium aluminium hydride had been destroyed. The precipitate was removed at the centrifuge and dissolved in 0.5N - sulphuric acid (3 ml.). The solution was neutralised with barium carbonate, deionised with Amberlite IR 120(H) resin and concentrated to a syrup (21 mg.) which was methylated by the Kuhn procedure. Methanolysis of the methylated disaccharide and subsequent gas-liquid partition chromatography gave peaks with the retention times of the methyl glycosides of the following sugars.

Sugar	T (Column c)
2, 3, 4, 6 tetra - O - methyl - D - galactose	1.47, 1.80
2, 3, 6 tri - O - methyl - D - galactose	2.88, 3.74, 4.14

Oligosaccharide 6. (923 mg.) R_{GalA} = 0.24 (Solvent C) M_G = 0.95 stained brown with aniline oxalate, gave on hydrolysis galacturonic acid only and was chromatographically and ionophoretically indistinguishable from O - α - D - galactopyranosyluronic acid - (1 → 4) - O - α - D - galactopyranosyluronic acid - (1 → 4) - O - α - D - galacturonic acid.

The acid (30 mg.) in water (10 ml.) was neutralised with calcium carbonate, excess solid being removed by filtration. Acetone (2 vol.)

was then added and the precipitated salt was filtered off, washed with acetone and ether, and dried to give a white solid $[\alpha]_D$ + 135° (c 0.72 in 0.5N - hydrochloric acid).

The acid (110 mg.) was treated overnight with methanolic 1% hydrogen chloride (20 ml.). The solution was neutralised with silver carbonate and silver residues were removed at the centrifuge before concentration to a syrup (111 mg.). The syrup, dried over phosphorus pentoxide in vacuo for 24 hrs., was dissolved in anhydrous pyridine (12 ml.) and hexamethyldisilazane (2.4 ml.) and chlorotrimethylsilane (1.2 ml.) were added. The mixture was shaken vigorously for 30 secs. and allowed to stand at room temperature for 1 hr. before evaporation to dryness. The residue was extracted with ether (2×25 ml.) and the combined extracts were concentrated to a syrup (180 mg.) which was redissolved in ether (20 ml.) and refluxed with lithium aluminium hydride (350 mg.) with vigorous stirring for 2 hrs. The solution was cooled and the excess lithium aluminium hydride destroyed with ethyl acetate as above. The precipitate was removed at the centrifuge and dissolved in 0.5N - sulphuric acid (12 ml.). The solution was neutralised with barium carbonate, deionised with Amberlite IR 120(H) resin and concentrated to a syrup (87 mg.).

The syrup was dissolved in water (2 ml.), and methyl sulphate (4 ml.) and 30% sodium hydroxide (8 ml.) were added dropwise with vigorous stirring over a period of 2 hrs. to the solution under an atmosphere of nitrogen. The additions were repeated each day for a further four days and on the sixth day the solution was heated on a boiling water bath for

1 hr., cooled, centrifuged, and the pH of the supernatant solution adjusted to 4 before extraction with chloroform (4 x 60 ml.). The combined chloroform extracts were dried over anhydrous sodium sulphate and concentrated to a syrup (73 mg.) which was dissolved in methyl iodide (10 ml.) and refluxed with silver oxide (100 mg.) for 16 hrs. The mixture was filtered and the residue was extracted in a Soxhlet extractor with chloroform for 8 hrs. The filtrate and extractions were combined and concentrated to a syrup (66 mg.). A sample of this (6 mg.) was methanolysed and subsequent gas-liquid partition chromatography gave peaks with the retention times of the methyl glycosides of the following sugars.

Sugar	Relative Proportions	T (Column c)
2, 3, 4, 6 tetra-O-methyl-D-galactose	+	1.50, 1.85
2, 3, 6 tri - O - methyl-D-galactose	++	2.94, 3.82, 4.25
2, 6 di-O-methyl-D-galactose	tr.	8.40, 9.64, 11.3

The methylated trisaccharide (60 mg.) was hydrolysed in N - hydrochloric acid (3 ml.) at 100° for 8 hrs. The solution was neutralised with silver carbonate, centrifuged and deionised with Amberlite IR 120(H) resin before concentrating to a syrup (57 mg.). The syrup was absorbed on a charcoal-Celite column (20 g. 1:1) which was eluted with water containing increasing quantities of ethanol to give three fractions.

Fraction 1. (4 mg.) R_G = 0.56 (Solvent E), = 0.19 (Solvent F), gave on methanolysis and subsequent gas-liquid partition chromatography peaks with the retention times of the methyl glycosides of 2, 6 di - O - methyl - D - galactose only.

Fraction 2. (29 mg.) R_G = 0.76 (Solvent E), = 0.51 (Solvent F), $[\alpha]_D$ + 82° (c 0.58 in chloroform) gave on methanolysis and subsequent gas-liquid partition chromatography peaks with the retention times of the methyl glycosides of 2, 3, 6 tri - O - methyl - D - galactose. The sugar was characterised by conversion into 2, 3, 6 tri - O - methyl - D - galactonolactone, which on recrystallisation from ethanol had m.p. and mixed m.p. 97 - 98°.

Fraction 3. (17 mg.) R_G = 0.89 (Solvent E), = 0.86 (Solvent F) $[\alpha]_D$ + 97° (c 0.34 in chloroform) gave on methanolysis and subsequent gas-liquid partition chromatography peaks with the retention times of the methyl glycosides of 2, 3, 4, 6 tetra - O - methyl - D - galactose. The sugar was characterised as the crystalline aniline derivative which had m.p. and mixed m.p. 196 - 197°.

ACETOLYSIS OF CITRUS PECTIN

Citrus pectin (polysaccharide fraction 1c2) (12 g.) was acetylated as described in "General Methods" giving an acetylated polysaccharide (12.3 g.). This material was dispersed thoroughly in acetic acid (320 ml.) and acetic anhydride (320 ml.) was added dropwise with constant stirring. Concentrated sulphuric acid (32 ml.) was then added over a period of two hours with vigorous stirring which was continued throughout the reaction. After about 30 hrs., a scum appeared which increased in quantity. After 75 hrs., the solution was centrifuged and hydrolysis of the residue gave galacturonic acid only. The supernatant solution was poured into ice-water (1 l.) and the precipitated acetylated products were removed at the centrifuge. The precipitate was extracted with chloroform and the aqueous supernatant solution was brought to pH 3 with sodium bicarbonate before extracting it, also with chloroform (4 x 150 ml.). The combined chloroform extracts were dried over anhydrous sodium sulphate and concentrated to a syrup (6.5 g.). The syrup was dissolved in chloroform (15 ml.) and methanol (30 ml.) and deacetylated with 0.5N - barium methoxide (30 ml.). After leaving at 0° for 24 hrs., the solution was poured into water, neutralised with dilute sulphuric acid, and filtered, barium ions were removed with Amberlite IR 120(H) resin, and the solution was filtered and concentrated to a syrup (2.0 g.).

The syrup was absorbed on a column of diethylaminoethyl Sephadex A - 25 (25 g., formate form) and elution with water, followed by concentration furnished a syrup of neutral sugars (181 mg.). This syrup was applied to a charcoal-Celite column (20 g. 1:1) and

monosaccharides (124 mg.), including galactose, arabinose, xylose and rhamnose were recovered by elution with water. No oligosaccharides were detected when the column was eluted with water containing increasing quantities of ethanol. Elution of the diethylaminoethyl Sephadex A-25 column with 0.05N - formic acid yielded fragments containing one acid residue and gradient elution with 0.05N - formic acid containing increasing amounts of 0.4N - formic acid yielded fragments containing two acid residues. Further fragments containing two acid residues were eluted with 0.4N - formic acid and fragments containing three or more acid residues were eluted with N and 3N - formic acid. The oligosaccharides were further separated, where necessary, by filter sheet chromatography. The formic acid elution pattern of the column is given in table XVII.

TABLE XVII

Eluant	$R_{GalA.}$ (Solvent B)	Colour *	Wt. (g.)
0.05N-formic acid	0.49	orange (O_2)	0.019
	0.77	orange (O_1)	0.042
	1.00	brown	0.711
0.05N-formic acid ↓ 0.4N-formic acid	0.12	orange (O_3)	0.082
	0.22	brown	
0.4N-formic acid	0.21	brown	0.103
N-formic acid	0	brown	0.230
	0.06	brown	
3N-formic acid	0	brown	0.344
	0.06	brown	

* Colour when chromatogram developed with aniline oxalate.

O_n refers to oligosaccharide n.

Hydrolysis of the fractions eluted with 0.4N, N, & 3N - formic acid gave galacturonic acid only.

Oligosaccharide 1. (20 mg.; eluted with 0.05N - formic acid) R_{GalA} = 0.77 (Solvent B), M_G = 0.55, $[\alpha]_D$ = + 90° (c 0.4) gave galacturonic acid and rhamnose on hydrolysis and was chromatographically and ionophoretically indistinguishable from 2 - O - (α - D - galactopyranosyluronic acid) - L - rhamnose. Methylation of the sugar by the Kuhn method and subsequent gas-liquid partition chromatography of the methanolysed products gave peaks with the retention times of the methyl glycosides of 3, 4 di - O - methyl - L - rhamnose and 2, 3, 4 tri - O - methyl - D - galacturonic acid. Methylation of the sugar by the Haworth procedure and crystallisation and recrystallisation from chloroform - light petroleum (B.P. 100 - 120°) yielded a methylated disaccharide, with m.p and mixed m.p 68 - 69° (Kofler hot-stage microscope), $[\alpha]_D$ + 92° (c 0.3 in $CHCl_3$), which gave an x-ray powder photograph and infra-red spectra identical to that from methyl 2 - O - (2, 3, 4 tri - O - methyl - α - D - galactopyranosyluronic acid) - 3, 4 di - O - methyl - L - rhamnoside dihydrate.

Oligosaccharide 2. (11 mg.; eluted with 0.05N - formic acid) R_{GalA} = 0.49 (Solvent B) $[\alpha]_D$ + 84° (c = 0.2) gave galacturonic acid and rhamnose on hydrolysis. Partial acid hydrolysis gave rhamnose and 2 - O - (α - D - galactopyranosyluronic acid) - L - rhamnose while similar hydrolysis of the glycitol gave rhamnitol and the same aldobiouronic acid. Methylation of the derived glycitol by the Kuhn method and subsequent gas-liquid partition chromatography of the methanolysed products gave

peaks with the retention times of 1, 3, 4, 5 tetra - O - methyl - L - rhamnitol [T, 1.12 (Column c)] and the methyl glycosides of the following sugars.

Sugar	T (Column c)
3, 4 di - O - methyl - L - rhamnose	0.86
2, 3, 4 tri - O - methyl - D - galacturonic acid	6.46, 6.80

Oligosaccharide 3. (8 mg.; eluted with 0.05 - 0.4N - formic acid) R_{GalA} = 0.12 (Solvent B) = 0.59 (Solvent C) $[\alpha]_D$ + 91° (c 0.3) gave galacturonic acid and rhamnose on hydrolysis. The derived glycitol, on partial hydrolysis, gave 2 - O - (α - D - galactopyranosyluronic acid) - L - rhamnose and 2 - O - (α - D - galactopyranosyluronic acid) - L - rhamnitol, and on sequential methylation, methanolysis and gas-liquid partition chromatography gave peaks with retention times of 1, 3, 4, 5 tetra - O - methyl - L - rhamnitol [T, 0.81 (Column b), 1.12 (Column c)] and the methyl glycosides of the following sugars.

Sugar	T.	
	Column b	Column c
3, 4 di - O - methyl - L - rhamnose	0.62	0.86
2, 3, 4 tri - O - methyl - D - galacturonic acid	3.78, 4.17	6.78
2, 3 di - O - methyl - D - galacturonic acid	1.90, 2.18	4.44

ENZYMIC DEGRADATION OF CITRUS PECTIN

A trial enzymolysis was carried out using a commercial pectinase preparation which had been dialysed for 3 days at 4° to remove the large amount of glucose that was present, and freeze dried. A sample of pectinic acid (100 mg.) (polysaccharide fraction 1c) and a sample of pectic acid (100 mg.), which was obtained by saponifying the pectinic acid by the method of Zitko and Bishop (14), were each dissolved in water (50 ml.) and pectinase (10 mg.) was added to each. Samples (2 ml.) were removed at hourly intervals and the enzyme in these was destroyed by heating at 100° for 7 mins. Paper chromatography of the samples showed that the enzyme degraded the pectinic acid as well as the pectic acid but at a reduced rate. No further degradation of the pectinic acid seemed to take place after 24 hrs. whereas this state was reached in only 3 hrs. with the pectic acid. Small amounts of oligosaccharides were detectable on paper chromatography of the hydrolysates.

Citrus pectin (20 g.) (Polysaccharide fraction 1c) was dissolved in water (10 l.) and a solution of pectinase (2 g.) in water (100 ml.) was added with vigorous stirring at room temperature. The pectinase had previously been dialysed for 3 days and freeze dried. The stirring was continued for 24 hrs. and the solution was then added to an equal volume of acetone. The precipitate of degraded polysaccharide and deactivated enzyme was removed at the centrifuge and gave on hydrolysis galacturonic acid, galactose, glucose and arabinose together with lesser amounts of xylose and rhamnose. The supernatant solution was then concentrated and poured into acetone (4 vol.) and allowed to stand at 4° for 2 days. The

precipitate, which appeared to contain an appreciable amount of crystalline galacturonic acid was also removed at the centrifuge, redissolved in water and concentrated to a syrup (S_1) (9 g.) keeping the pH at 7. The supernatant solution was also concentrated to a syrup (S_2) (11 g.) keeping the pH at 7. Direct chromatography of syrups S_1 and S_2 yielded the monosaccharides in table XXI.

TABLE XXI

Fraction	Sugars							
	GalA.	Gal.	G.	Man.	Ara.	Xyl.	Fuc.	Rha.
S_1	++++	+	tr.	/	++	tr.	/	tr.
S_2	++++	+	tr.	/	++	tr.	/	tr.

In view of the detection of oligosaccharides in the trial enzymolysis, fractions S_1 and S_2 were chromatographed separately on diethylaminoethyl Sephadex A-25 (50 g.) (formate form). The columns were washed with water to remove neutral sugars (1.2 g. - S_1) (3.9 g. - S_2), which included galactose and arabinose with small amounts of glucose, xylose and rhamnose in both cases, and then with 0.05$\underline{\underline{N}}$ - formic acid to remove fragments containing one acid unit followed by gradient elution with 0.05$\underline{\underline{N}}$ - formic acid containing increasing amounts of 0.4$\underline{\underline{N}}$ - formic acid to remove fragments with two acid units. The formic acid elution patterns of the diethylaminoethyl Sephadex A-25 chromatography of fractions S_1 and S_2 are given in tables XXII and XXIII respectively.

TABLE XXII

Eluant	R_{GalA} (Solvent B)	Colour *	Wt. (g.)
0.05N- formic acid	1.00	brown	7.861
0.05N- formic acid ↓ 0.4N- formic acid	0.12	brown (O_3)	0.051

TABLE XXIII

Eluant	R_{GalA} (Solvent B)	Colour *	Wt. (g.)
0.05N- formic acid	0.53	red (O_1)	0.293
	1.00	brown	
	1.00	brown	5.865
	2.02	orange (O_2)	0.042
0.05N- formic acid ↓ 0.4N- formic acid	0		0.031

* Colour when chromatogram developed with aniline oxalate.

O_n refers to oligosaccharide n.

The fractions were further separated, where necessary, by filter sheet chromatography.

Oligosaccharide 1. (12 mg.) R_{GalA} = 0.53 (Solvent B), $[\alpha]_D$ + 28° (c 0.24) gave on hydrolysis galacturonic acid and xylose only. The derived glycitol gave on hydrolysis xylose only. Methylation of the derived glycitol by the Kuhn method followed by methanolysis and subsequent

gas-liquid partition chromatography gave peaks corresponding to γ - 2, 3, 5, 6 tetra - O - methyl - D - galactonolactone [T = 4.38 (Column c)] and the methyl glycosides of 2, 3, 4 tri - O - methyl - D - xylose [T = 0.41, 0.52 (column c)].

Oligosaccharide 2. (19 mg.) R_{GalA} = 2.02 (Solvent B), $[\alpha]_D$ + 87° (c 0.30) gave on hydrolysis rhamnose only but was shown to contain only 46% rhamnose. The derived glycitol gave rhamnitol only on hydrolysis. Methylation of the oligosaccharide by the Kuhn procedure followed by gas-liquid partition chromatography gave a number of peaks, one of which was identified as the methyl glycoside of 3, 4 di - O - methyl - L - rhamnose [T = 0.83 (Column c)]. The oligosaccharide was shown to have an absorption maximum at 231 m μ and when reacted with thiobarbituric acid yielded a red coloured solution with an absorption maximum at 547 m μ.

Oligosaccharide 3. (29 mg.) R_{GalA} 0.12 (Solvent B), 0.50 (Solvent C) $[\alpha]_D$ + 85°. (c 0.40) contained galacturonic acid and rhamnose residues in the molar ratio of 1.9:1, while the derived glycitol, obtained from reduction with sodium borohydride, contained these sugar residues in the ratio of 1:1. A sample of the oligosaccharide (2 mg.) was heated at 100° for 16 hrs. in 50% aqueous methanol (1 ml.) containing 2.5% triethylamine. The solution was then evaporated to dryness, the residue was redissolved in water and the solution was deionised with Amberlite IR 120(H) resin before paper chromatography which gave 2 - O - (α - D - galactopyranosyluronic acid) - L - rhamnose only. Methylation of the derived glycitol of oligosaccharide 3 by the Kuhn procedure followed by methanolysis and gas-liquid partition chromatography gave an unknown peak and the methyl

glycosides of the following sugars.

Sugars	T	
	Column b	Column c
3, 4 di - O - methyl - L - rhamnose	0.63	0.87
2, 3, 4 tri - O - methyl - D - galacturonic acid	4.24	6.54 6.86
Unknown peak	3.70	9.95

The glycitol of the disaccharide, 4 - O - (∝ - D - galactopyranosyluronic acid) - D - galacturonic acid, (5 mg.) was formed and methylated by the Kuhn procedure. The disaccharide, 3 - O - ∝ - D - galactopyranosyl - D - galactose, (25 mg.) was oxidised with bromine (0.25 ml.) in water (2.5 ml.) in the presence of barium carbonate (25 mg.). The mixture was shaken vigorously for 15 mins. and stored in the dark for 72 hrs. at room temperature with occasional shaking. Excess bromine was removed from the resulting mixture by aeration, barium ions were removed from the filtered solution with Amberlite IR 120(H) resin, hydrobromic acid was neutralised by shaking the solution with Amberlite LA-1 resin (5% v/v in light petroleum), and the aqueous solution was concentrated. The resulting syrup was methylated by the Kuhn procedure which was repeated to yield a methylated aldobionic acid. The methylated galacturonobiitol and the methylated aldobionic acid were methanolysed and subsequent gas-liquid partition chromatography on columns b and c showed that one of the peaks in the chromatograms of each methylated product had a similar relative retention time to the unknown peak in the chromatograms of the oligosaccharide (O_3).

Degraded Polysaccharide
~~Oligosaccharide 4.~~ (71 mg.) R_{GalA} 0.0 (Solvents B and C) gave galacturonic acid (+), galactose (+++), glucose (tr.), arabinose (+), xylose (tr.), and rhamnose (+) as did the derived glycitol. A sample of the material (5 mg.) was heated at 100° for 16 hrs. in 50% aqueous methanol (2 ml.) containing 2.5% triethylamine. The solution was then concentrated, the residue was redissolved in water and the resulting solution was deionised with Amberlite IR 120(H) resin before hydrolysis followed by paper chromatography which gave the same hydrolysis products as above and in similar amounts. A further sample (10 mg.) was methylated, first by the Haworth procedure, five additions of methyl sulphate (1 ml.) and 30% aqueous sodium hydroxide (2 ml.) being made, and then by the Kuhn procedure. Methanolysis and subsequent gas-liquid partition chromatography gave a number of peaks, some of which were identified as the methyl glycosides of the following methylated sugars.

Sugars	T		
	Column a	Column b	Column c
2, 3, 4 tri - O - methyl - L - rhamnose	0.45	(0.47)	0.44
2, 3, 5 tri - O - methyl - L - arabinose	0.53	(0.47)	0.50
	0.72	(0.62)	0.66
3, 4 di - O - methyl - L - rhamnose	0.98	(0.62)	0.87
2, 3, 4, 6 tetra - O - methyl - D - galactose	1.68	(1.57)	1.87
2, 3, 6 tri - O - methyl - D - galactose	3.11	(1.57)	2.88
	4.12	1.90	3.80
	4.43	2.08	4.36
		2.29	
2, 3, 4 tri - O - methyl-D-galacturonic acid	6.73	4.30	6.64

PREPARATION AND METHYLATION OF CARBOXYL-REDUCED CITRUS PECTINIC ACID

Citrus pectinic acid (polysaccharide fraction 1c2) (1g.) was dissolved in water (100 ml.) and passed through a column of Amberlite IR 120(H) resin before treating with ethylene oxide (25 ml.) at room temperature for 5 weeks when esterification was complete (pH 6.8). The esterified polysaccharide was precipitated from ethanol (3 vol.), redissolved in water and freeze dried. The 2-hydroxyethyl ester (1 g.) was then acetylated as described in "General Methods" giving the acetate of the esterified polysaccharide (1.1 g.) which was suspended in tetrahydrofuran (25 ml.) and a mixture of tetrahydrofuran (25 ml.) and lithium borohydride (1.1 g.) was added. The mixture was refluxed with vigorous stirring for 20 hrs., cooled, and the residual lithium borohydride was destroyed by the dropwise addition of water. A further volume of water (50 ml.) was added and the remaining tetrahydrofuran was removed under reduced pressure. The pH of the solution was then adjusted to 7 with dilute sulphuric acid and the precipitate (1.) which appeared was removed at the centrifuge. The supernatant solution was then dialysed for 3 days and a precipitate (2.) was also removed at the centrifuge. The supernatant solution was concentrated and poured into ethanol (6 vol.) to yield a precipitate (3.) which was redissolved in water and freeze dried to give a white solid (54 mg.). The three precipitates gave galactose only on hydrolysis. Precipitate 1. was then dissolved in 0.5N - sodium hydroxide, the solution was poured into ethanol (3 vol.), the precipitate was washed thoroughly with ethanol and ether and dried to give precipitate 1a

(322 mg.) $[\alpha]_D$ + 175° (c 0.5 in 0.5N - NaOH) which was shown by the phenol/sulphuric acid method to contain at least 93% carbohydrate. Precipitate 2. was insoluble in 0.5N - sodium hydroxide and after repeated washing with water, ethanol, and finally ether gave, after drying, a pale brown solid (123 mg.).

A sample of precipitate 1a (220 mg.) was dissolved in 0.5 - sodium hydroxide (4 ml.) and methyl sulphate (4 ml.) and 30% sodium hydroxide (8 ml.) were added dropwise over a period of 4 hrs. with vigorous stirring. Nitrogen was passed through the solution. Four similar additions were made on consecutive days and on the sixth day the solution was heated at 100° for 1 hr. After cooling, the pH was adjusted to 4 with dilute sulphuric acid and a precipitate was removed at the centrifuge. The precipitate was dissolved in chloroform which was dried over anhydrous sodium sulphate before the solution was concentrated to a syrup (136 mg.). The supernatant solution was then extracted with chloroform (4 x 100 ml.) and the extracts were combined, dried, and concentrated to a syrup (108 mg.). A sample of each syrup was methanolysed and gas-liquid partition chromatography of the methanolysed products gave identical chromatograms.

The combined syrups were dissolved in methyl iodide (25 ml.) and silver oxide (400 mg.) added in four portions at hourly intervals with constant stirring and refluxing which was continued overnight. The silver residues were removed by filtration and were extracted with hot chloroform in a Soxhlet extractor for 8 hrs. The supernatant solution and the extracts were combined, dried, and concentrated to a syrup

(222 mg.) which was redissolved in methyl iodide and the methylation procedure repeated to give a syrup (216 mg.). Gas-liquid partition chromatography of a methanolysed sample showed that substantial amounts of methyl glycosides of 2, 6 di - O - methyl - D - galactose were present which was attributed to undermethylation.

The partially methylated polysaccharide was then dissolved in methyl iodide (25 ml.), and N, N - dimethylformamide (5 ml.) and silver oxide (300 mg.) was added and the mixture shaken vigorously in the dark for 3 days. The silver residues were then removed at the centrifuge and extracted with hot chloroform in a Soxhlet extractor for 8 hrs. The supernatant solution and extracts were combined and concentrated to remove methyl iodide and chloroform. Water (50 ml.) was then added, the solution was dialysed against running tap water for 3 days and concentrated to dryness. The residue was then extracted with chloroform and the chloroform extractions were concentrated to a syrup (195 mg.). The syrup was redissolved in methyl iodide (25 ml.) and N, N - dimethylformamide (5 ml.) and the above procedure repeated to give a syrup (184 mg.) which was redissolved in a small volume of chloroform and precipitated by the addition of light petroleum. (B.P. 60 - 80°) (20 vol.) The precipitate was then dried at 60° over phosphorus pentoxide in vacuo to give a fully methylated polysaccharide (148 mg.) [Found: OMe, 42.8%] $[\alpha]_D$ + 177° (c 0.3 in $CHCl_3$).

A sample (20 mg.) of the methylated polysaccharide was methanolysed with methanolic 4% hydrogen chloride. The products were examined by

gas-liquid partition chromatography and gave peaks with the retention times of the methyl glycoside of the following sugars in table XVIII.

TABLE XVIII

Sugar	Relative Proportions	T				
		Column a	Column b	Column c	Column c*	Column d
2,3,4 Me_3-Rha	tr.	0.47	n.d.	n.d.	0.40	0.45
2,3,5 Me_3-Ara	++	0.56 0.72	0.48	0.49 0.64	0.45 0.65	0.61 0.78
3,4 Me_2-Rha	+	1.03	(0.66)	0.84	0.83	1.07
3 Me -Rha	+	n.d.	1.01	n.d.	n.d.	n.d.
1,3,4,5Me_4-Rha-ol	+	1.10	(0.84)	1.05	1.10	1.13
2,3 Me_2-Ara	+	1.52 (1.76) 2.03	(0.66) (0.84)	1.16	1.21 1.63	(1.88) 2.30
2 Me -Ara	tr.	n.d.	1.24	n.d.	n.d.	
2,3,4,6 Me_4-Gal	++	(1.76)	n.d.	1.48 1.78	1.91 2.05	(1.88)
2,3,6 Me_3-Gal	++++	3.21 3.93 4.30 4.70	1.61 2.07 2.23 (2.51)	2.83 3.67 4.12		3.66 4.40 4.98 5.43
2,6 Me_2-Gal	++	10.3 11.2 15.8	(2.51) 3.03 3.58	8.32 11.0 12.6		

* Column temperature 125°.

n.d. - not detected.

METHYLATION OF THE POLYSACCHARIDES ELUTED FROM THE DIETHYLAMINOETHYL SEPHADEX A-50 COLUMNS WITH 0.2N - FORMIC ACID

Sodium hydride (1.5 g) was washed three times with light petroleum (B.P.30-40°) and then stirred in dimethyl sulphoxide (15 ml.) at 50° under an atmosphere of nitrogen for 45 mins. A sample of the polysaccharides eluted from the diethylaminoethyl Sephadex A-50 columns with 0.2N - formic acid (polysaccharide fraction 1c2a)(500 mg.) was dissolved in dimethyl sulphoxide (50 ml.) by heating at 60° for 15 mins. and the required amount (35% excess of theoretical) of the methylsulphinyl carbanion (6 ml.) was added. A gel formed but disappeared after stirring at room temperature for 4 hrs. (the time required for alkoxide formation). Methyl iodide (3 ml.) was then added to the stirred solution at a rate so that the temperature did not rise above 25° and stirring was continued at room temperature overnight. The clear solution was then concentrated to remove any remaining methyl iodide and the solution was dialysed for 24 hrs. The non-diffusible material was concentrated to a syrup, dissolved in chloroform and the chloroform solution filtered before pouring into a large excess of light petroleum (B.P.60-80°). The precipitate was removed at the centrifuge and dried over phosphorus pentoxide *in vacuo* at 60° to give a methylated polysaccharide (292 mg.) [Found: OMe = 40.2%], $[\alpha]_D$ - 95° (c 0.484 in $CHCl_3$).

A sample of the methylated polysaccharide (30 mg.) was methanolysed with methanolic 4% hydrogen chloride and examination of the products with gas-liquid partition chromatography gave peaks with the retention times of

the methyl glycosides of the following sugars in table XIX.

TABLE XIX

Sugar	Relative Amounts	T					
		Column a	Column b	Column c	Column e*	Column d	Column e
2,3,4Me_3-Rha.	tr.	0.46	n.d.	n.d.	0.40	0.47	0.43
2,3,5Me_3-Ara.	++++	0.54 0.73	0.47 (0.64)	0.49 0.65	0.45 0.62	0.59 0.78	0.50 0.71
2,3,4Me_3-Ara.	tr.	(1.05)	n.d.	(0.87)	0.90	(1.17)	n.d.
3,4Me_2-Rha.	tr.	(1.05)	n.d.	(0.87)	0.82	(1.17)	n.d.
2,3Me_2-Ara.	+++	1.57 1.91	(0.64) 0.82 0.95	1.18 (1.50)	1.20 1.61	1.89 2.34	1.04 1.43
2,5Me_2-Ara.	+	n.d.	n.d.	n.d.	1.50	2.18	1.30 2.53
2,3,4,6Me_4-Gal.	+	n.d.	n.d.	(1.50) 1.76	1.86 2.02	n.d.	1.85
2,3,6Me_3-Gal.	++	3.23 (4.15) 4.66	1.63 (2.09) 2.32 (2.51)	2.80 3.57 4.08		3.64 4.38 4.83 5.43	3.01 (3.79)
2,4,6Me_3-Gal.	+	(4.15) 4.66	(2.09) 2.32	n.d.		n.d.	3.47 (3.79)
2,3,4Me_3-Gal.	++	7.32	(2.51) 2.89	6.67		8.69	6.45 7.07
2,6Me_2-Gal.	tr.	n.d.	n.d.	8.61		n.d.	
2,4Me_2-Gal.	++	n.d.	3.57 4.17	14.5 16.7		n.d.	12.45 13.4

* Column temperature 125°.

A further sample of the methylated polysaccharide (20 mg.) was heated in methanolic 4% hydrogen chloride at 100° overnight and the methyl glycosides were hydrolysed with N - sulphuric acid (2 ml.) at 100° for 4 hrs. Small amounts of L - arabinose and 2 - O - methyl L - arabinose along with the sugars above were detected on paper chromatography in Solvent F.

METHYLATION OF THE POLYSACCHARIDES ELUTED FROM THE DIETHYLAMINOETHYL SEPHADEX A-50 COLUMNS WITH WATER

The methylsulphinyl carbanion was prepared as above. A sample of the polysaccharides eluted from the diethylaminoethyl Sephadex A-50 columns with water (polysaccharide fraction 1c2b) (250 mg.) was dissolved in dimethyl sulphoxide (50 ml.) by heating at 60° for 15 mins. and the required amount (35% excess of theoretical) of the methylsulphinyl carbanion (3 ml.) was added. After alkoxide formation was complete (4 hrs.), methyl iodide (1.5 ml.) was added to the stirred solution at a rate so that the temperature did not rise above 25° and the reaction was continued as above to yield a precipitate of methylated polysaccharide (175 mg.) [Found: OMe = 39.7%], $[\alpha]_D$ - 71° (c 0.406 in $CHCl_3$).

A sample of the methylated polysaccharide (30 mg.) was methanolysed with methanolic 4% hydrogen chloride and gas-liquid partition chromatography of the methanolysis products gave peaks with the retention times of the methyl glycosides of the following sugars in table XX.

TABLE XX

Sugars	Relative Amounts	T				
		Column a	Column b	Column c	Column c*	Column e
$2,3,4Me_3$-Xyl.	tr.	0.44 (0.54)	n.d.	0.40 (0.47)	0.36 (0.46)	0.37 (0.46)
$2,3,5Me_3$-Ara.	++++	(0.54) 0.69	0.47 (0.65)	(0.47) 0.62	(0.46) 0.64	(0.46) 0.71
$2,3,4Me_3$-Ara.	tr.	n.d.	n.d.	0.89	0.96	n.d.
$3,4Me_2$-Rha.	tr.	n.d.	n.d.	0.85	0.83	n.d.
$2,3,4,6Me_4$-G.	tr.	n.d.	n.d.	1.03 (1.49)	1.03 (1.65)	n.d.
$2,3Me_2$-Ara.	+++	1.53 (1.84)	(0.65) 0.83 0.95	1.18 (1.49)	1.21 (1.65)	1.04 1.45
$2,3,4,6Me_4$-Gal.	+	n.d.	n.d.	1.81	1.86 2.03	1.83
$2,3,6Me_3$-Gal.	++	3.06 4.10 (4.51)	(1.59) (2.20) 2.38	2.85 3.70 4.17		2.92 3.76
$2,3,6Me_3$-G.	++	3.31 (4.51)	(1.59) (2.20)	3.20 4.44		4.16
$2,6Me_2$-Gal.	tr.	n.d.	n.d.	8.62 9.75 12.0		6.95

* Column temperature 125°

A further sample of the methylated polysaccharide (20 mg.) was methanolysed with methanolic 4% hydrogen chloride at 100° overnight and the methyl glycosides were hydrolysed with N - sulphuric acid (2 ml.) at 100° for 4 hrs. Small amounts of L - arabinose and 2 - O - methyl - L - arabinose together with the methylated sugars above were detected on paper chromatography of the hydrolysate in Solvent F.

SECTION 2

THE EXTRACELLULAR POLYSACCHARIDES FROM SYCAMORE CAMBIAL CELLS

DISCUSSION

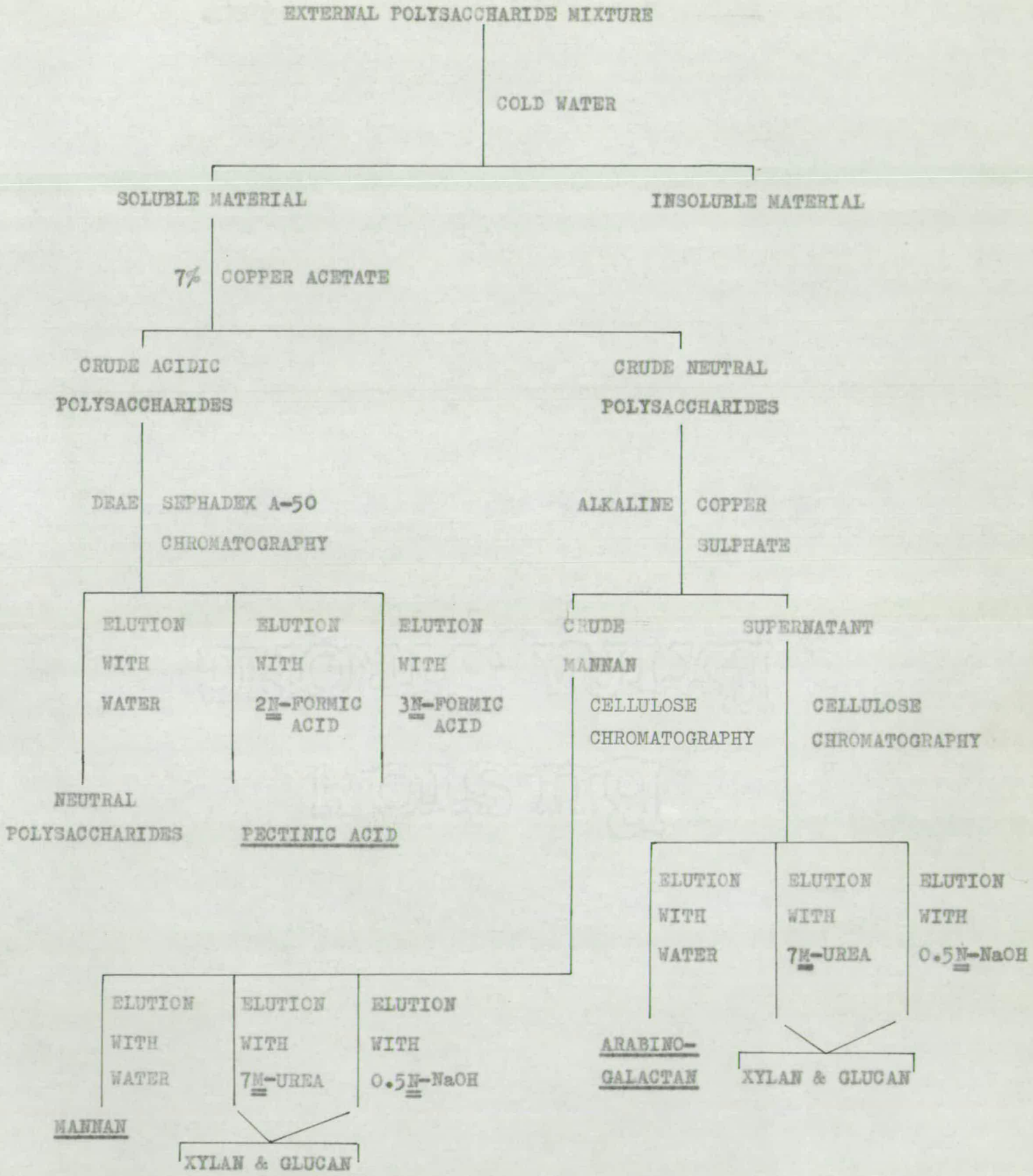
THE EXTRACELLULAR POLYSACCHARIDE MIXTURE

The external polysaccharide mixture was obtained as a pale brown powder from Dr. P. Albersheim. The polysaccharides had been secreted into the culture medium by the sycamore cambial cells when the cells were mildly rotated at 23° at pH 5.5 under conditions of interrupted darkness in a medium consisting mainly of sucrose and yeast extract with small amounts of 2, 4 dichlorophenoxyacetic acid and various minerals. After twelve days, the cells were removed by filtration, the polysaccharides were precipitated from ethanol and the precipitate was washed with acetone and dried by dessication.

The insolubility in water of half the polysaccharide mixture when received would suggest that some of the mixture had undergone a change in physical state during the drying process. Both soluble and insoluble fractions gave on hydrolysis similar mixtures of galacturonic acid, galactose, glucose, mannose, arabinose, xylose, fucose and rhamnose and contained similar amounts of uronic acid. The sugar composition of the external polysaccharide mixture seemed to be similar to that of the wall polysaccharides obtained from the cambial region of the sycamore tree (71)(72)(73) except for a somewhat greater amount of mannose.

Ion-exchange chromatography of the polysaccharide mixture on diethylaminoethylcellulose indicated that it was composed of neutral polysaccharides and a range of acidic polysaccharides of the pectic type, with varying amounts of galacturonic acid and varying degrees of esterification.

The fractionation scheme carried out on the polysaccharide mixture is summarised on the following page. The separation of acidic and neutral polysaccharides in the water-soluble fraction was achieved by precipitation of the acidic polysaccharides as their copper salts (92). The acidic polysaccharides were further purified by chromatography on diethylaminoethyl Sephadex A-50 giving two acidic fractions of similar composition, optical rotation, and uronic acid content, which were combined, constituting a pectinic acid, $[\alpha]_D$ + 178°, with uronic acid content of 67% and methoxyl content of 3.4%. Two of the neutral polysaccharides were also obtained in a pure form. A crude separation of the neutral polysaccharides was achieved by the ability of some mannose containing polysaccharides to form a complex with copper in alkaline solution (93). Cellulose was found to absorb all the neutral polysaccharides present except a mannan and an arabinogalactan . On the basis of this, a pure mannan, $[\alpha]_D$ + 75°, was obtained by chromatography of the crude mannan on cellulose and a pure arabinogalactan, $[\alpha]_D$ - 49°, was also obtained from chromatography on cellulose of the polysaccharides which did not complex with copper. In addition to these polysaccharides which have been isolated in a pure state, it is probable from the monosaccharides detected on hydrolysis that a xylan and glucan are also present.


EXTERNAL POLYSACCHARIDE MIXTURE
COLD WATER
SOLUBLE MATERIAL
INSOLUBLE MATERIAL
7% COPPER ACETATE
CRUDE ACIDIC POLYSACCHARIDES
CRUDE NEUTRAL POLYSACCHARIDES
DEAE SEPHADEX A-50 CHROMATOGRAPHY
ALKALINE COPPER SULPHATE
ELUTION WITH WATER
ELUTION WITH 2N-FORMIC ACID
ELUTION WITH 3N-FORMIC ACID
CRUDE MANNAN
SUPERNATANT
NEUTRAL POLYSACCHARIDES
PECTINIC ACID
CELLULOSE CHROMATOGRAPHY
CELLULOSE CHROMATOGRAPHY
ELUTION WITH WATER
ELUTION WITH 7M-UREA
ELUTION WITH 0.5N-NaOH
ELUTION WITH WATER
ELUTION WITH 7M-UREA
ELUTION WITH 0.5N-NaOH
MANNAN
XYLAN & GLUCAN
ARABINO-GALACTAN
XYLAN & GLUCAN

THE PECTINIC ACID

The pectinic acid was subjected to partial hydrolysis involving N- sulphuric acid. The hydrolysate was separated into neutral and acidic fractions on diethylaminoethyl Sephadex A-25. Examination of the neutral fraction showed that it contained galactose, arabinose and rhamnose together with small amounts of xylose, glucose and fucose. The acidic fraction was further fractionated on diethylaminoethyl Sephadex A-25 and, where necessary, on filter sheets to yield five acidic oligosaccharides.

The first of these gave galacturonic acid and rhamnose on hydrolysis and the methanolysis products of the methylated oligosaccharide contained the methyl glycosides of 3, 4 di - O - methyl - L - rhamnose and 2, 3, 4 tri - O - methyl - D - galacturonic acid. These results when taken with the chromatographic and ionophoretic mobilities of the oligosaccharide confirmed that it was 2 - O - (α - D galactopyranosyluronic acid) - L - rhamnose.

The second oligosaccharide was tentatively identified as 4 - O - (β - D - glucopyranosyluronic acid) - L - fucose from its chromatographic and ionophoretic mobilities and from the release of glucuronic acid, glucurone and fucose on hydrolysis.

The third oligosaccharide was tenatively identified as 6 - O - (β - D - glucopyranosyluronic acid) - D - galactose from its chromatographic and ionophoretic mobilities and from the release of glucuronic acid, glucurone and galactose on hydrolysis.

The fourth and fifth oligosaccharides gave galacturonic acid only on hydrolysis and from their chromatographic and ionophoretic mobilities were tentatively identified as 4 - O - (∝ - D - galactopyranosyluronic acid) - D - galacturonic acid and O - ∝ - D - galactopyranosyluronic acid - (1 → 4) - O - ∝ - D - galactopyranosyluronic acid - (1 → 4) - D - galacturonic acid respectively.

The main sugars, galactose, arabinose, rhamnose and galacturonic acid, released on hydrolysis of the polysaccharide, its high uronic acid content and high positive rotation together with the isolation of these five oligosaccharides, which were also obtained from citrus pectin, verify that it is a typical pectinic acid. The isolation of the galacturonobiose, the galacturonotriose and 2 - O - (∝ - D - galactopyranosyluronic acid) - L - rhamnose indicate that the main chain is built up of contiguous galacturonic acid residues linked through the 1 and 4 positions interspersed with occasional rhamnose residues. No definite structural interpretation, however, can be placed on the two glucuronic acid containing aldobiouronic acids which have also been isolated from citrus pectin as described previously in this thesis, from lucerne pectic acid (42), and from the acidic polysaccharides in soyabeans (40)(41). Similar glucuronic acid containing aldobiouronic acids have been isolated from tragacanthic acid (94) where the glucuronic acid residues would appear to be present as end groups.

THE ARABINOGALACTAN

Partial hydrolysis of the arabinogalactan with N - sulphuric acid yielded two oligosaccharide components which were tentatively identified as 3 - O - β - D - galactopyranosyl - D - galactose and 6 - O - β - D - galactopyranosyl - D - galactose on the basis of their paper chromatographic mobilities.

When the arabinogalactan was methylated with methyl sulphate and sodium hydroxide, fractionation took place which resulted in two partially methylated products being isolated, M.1. being water soluble and M.2. being water insoluble. These two fractions were further methylated in parallel by methyl iodide and silver oxide to yield fully methylated fractions M.1. and M.2. with $[\alpha]_D - 35^\circ$ and $[\alpha]_D - 80^\circ$ respectively, M.1. being the minor fraction. Methanolysis of samples of these fractions yielded the methyl glycosides of the following sugars which were identified by gas-liquid partition chromatography.

Sugar	Relative Amounts	
	M.1.	M.2.
2, 3, 4 tri - O - methyl - D - xylose	tr.	tr.
or 2, 3, 4 tri - O - methyl - L - rhamnose		
2, 3, 5 tri - O - methyl - L - arabinose	++++	++++
2, 3, 4 tri - O - methyl - L - arabinose	tr.	tr.
2, 3 di - O - methyl - L - arabinose	+	+
2, 3, 4, 6 tetra - O - methyl - D - mannose	+	/
2, 3, 4, 6 tetra - O - methyl - D - galactose	tr.	tr.
3, 4, 6 tri - O - methyl - D - mannose	tr.	/
2, 4, 6 tri - O - methyl - D - galactose	+	+
2, 3, 4 tri - O - methyl - D - galactose	+	+
2, 4 di - O - methyl - D - galactose	++	++

tr. - trace

It is evident from the methylated derivatives of mannose obtained in M.1. and from its less negative rotation that the arabinogalactan must have been contaminated by a small amount of mannan and that partial fractionation had taken place during methylation to yield as the major fraction a pure partially methylated arabinogalactan (M.2.) and as the minor fraction a partially methylated arabinogalactan containing some partially methylated mannan (M.1).

From the detection of 3 - O - β - D - galactopyranosyl - D - galactose and 6 - O - β - D - galactopyranosyl - D - galactose from partial hydrolysis and also 2, 4, 6 tri - O - methyl - D - galactose, 2, 3, 4 tri - O - methyl -

D - galactose and 2, 4 - di - O - methyl - D - galactose from methylation studies, it would appear that the galactan portion of the polysaccharide is of a branched nature. In view of the relatively high proportion of 2, 3, 5 tri - O - methyl - L - arabinose obtained from the methylated polysaccharide, most of the arabinose residues must be present as end group which, together with the absence of 2 - O - methyl - L - arabinose, confirms that the arabinose residues are constituents of an arabinogalactan and not a discrete arabinan.

On the basis of the galactose residues being linked through the 1 and 3, and 1 and 6 positions, this arabinogalactan is similar to the water-soluble arabinogalactans from coniferous woods (95) and differs from the pectic type of arabinogalactan, isolated from soyabeans (19), which contains galactose residues linked through the 1 and 4 positions only. From its dissimilarity to the pectic type arabinogalactans it would appear that this arabinogalactan is not associated with the pectinic acid. No other arabinogalactan of this type has so far been isolated from deciduous wood.

THE MANNAN

Methylation of the mannan by the method of Srivastava (96) yielded a partially methylated polysaccharide which was completely methylated by silver oxide and methyl iodide. Methanolysis and subsequent gas-liquid partition chromatography of the methyl glycosides indicated the presence of the following methylated sugars.

Sugar	Relative Amounts
2, 3, 4, 6 tetra - O - methyl - D - mannose	++
3, 4, 6 and/or 2, 3, 4 tri - O - methyl - D - mannose	+
2, 4, 6 tri - O - methyl - D - mannose	+
3, 4 di - O - methyl - D - mannose	+

The detection of the above methylated sugars together with its high positive rotation (+ 75°), indicative of α linkages, suggested that this was a yeast mannan (97) and not the usual linear type of mannan which is found in plants (98) and contains β linkages.

The isolation of such a mannan cast doubts as to its authenticity as an extracellular polysaccharide produced by sycamore cambial cells and it was suspected to be a contaminant. Careful examination of the growth conditions of the sycamore cambial cells showed that it could not have been produced by micro-organisms but after further investigations, Albersheim found that the mannan was a constituent of the yeast which was used as a nutriant for the sycamore cambial cells and was being released into the culture medium.

The confirmation that the mannan was not being produced by the sycamore cambial cells explains the relatively high proportion of mannose in the external polysaccharide mixture when compared with the wall polysaccharides from the cambial region of the sycamore tree and suggests that the suspected glucan may have arisen from the same source.

Thus, until conditions, which do not involve the use of yeast and consequently avoid this type of contamination, can be evolved for the incubation of the cells, comparisons between the polysaccharides produced and those in the cell walls must be somewhat restricted. However, there seems little doubt that the pectinic acid and the arabinogalactan are genuine products of the sycamore cambial cells and the formation of this type of arabinogalactan, similar to those found in coniferous woods, is of interest as arabinogalactans have not been reported in deciduous woods up till now.

EXPERIMENTAL

PRELIMINARY EXPERIMENTS ON THE POLYSACCHARIDE MIXTURE

The polysaccharide mixture (12.4 g.) was stirred in cold water (4 l.) for 24 hours, and the insoluble material was removed at the centrifuge and dried by solvent exchange to give polysaccharide A (6.3 g.). Further extraction of polysaccharide A with boiling water, N - ammonium hydroxide, and N - sodium hydroxide failed to solubilise any more material. The supernatant solution was concentrated, poured into ethanol (4 vol.), and the precipitate was removed at the centrifuge, redissolved in water and any remaining traces of ethanol were removed under reduced pressure before freeze drying to give polysaccharide B. (5.7 g.). The hydrolysis pattern of the two fractions is shown in table I.

TABLE I

Extract	Uronic Acid Anhydride % a	Sugars on Hydrolysis							
		GalA.	Gal.	G.	Man.	Ara.	Xyl.	Fuc.	Rha.
A	18.6	+	+	+	+	+	+	tr.	tr.
B	17.3	+	+	+	+	+	+	tr.	tr.

a - by decarboxylation tr. - trace

A sample of polysaccharide B. (110 mg.) was chromatographed on diethylaminoethylcellulose (30 g.), generated in the phosphate form. The column was eluted successively with 0.05 M, 0.10 M, 0.25 M and 0.50 M - sodium dihydrogen phosphate buffers at pH 6 and finally with

0.50M - potassium chloride solution. Fractions (10 ml.) were collected and analysed by the phenol-sulphuric acid method for total sugars to give a plot of polysaccharide content against eluate (diagram 1.). Polysaccharide peaks were analysed for uronic acids by the carbazole method. Details of the four polysaccharide fractions eluted are given in table II.

TABLE II

Eluant	Wt. (mg.)	U.A.* %	Sugars on Hydrolysis							
			GalA.	Gal.	G.	Man.	Ara.	Xyl.	Fuc.	Rha.
0.05M-$PO_4^{\equiv}$	49	1	/	+	+	++	+	++	/	/
0.10M-$PO_4^{\equiv}$	11	33	+++	+	+	tr.	+	tr.	tr.	tr.
0.25M-$PO_4^{\equiv}$	13	21	+++	+	tr.	/	+	tr.	tr.	tr.
0.50M-$PO_4^{\equiv}$	14	54	+++	+	/	/	+	tr.	tr.	tr.
0.50M-Cl^{-}	6	n.d.	+++	+	+	tr.	+	tr.	tr.	tr.

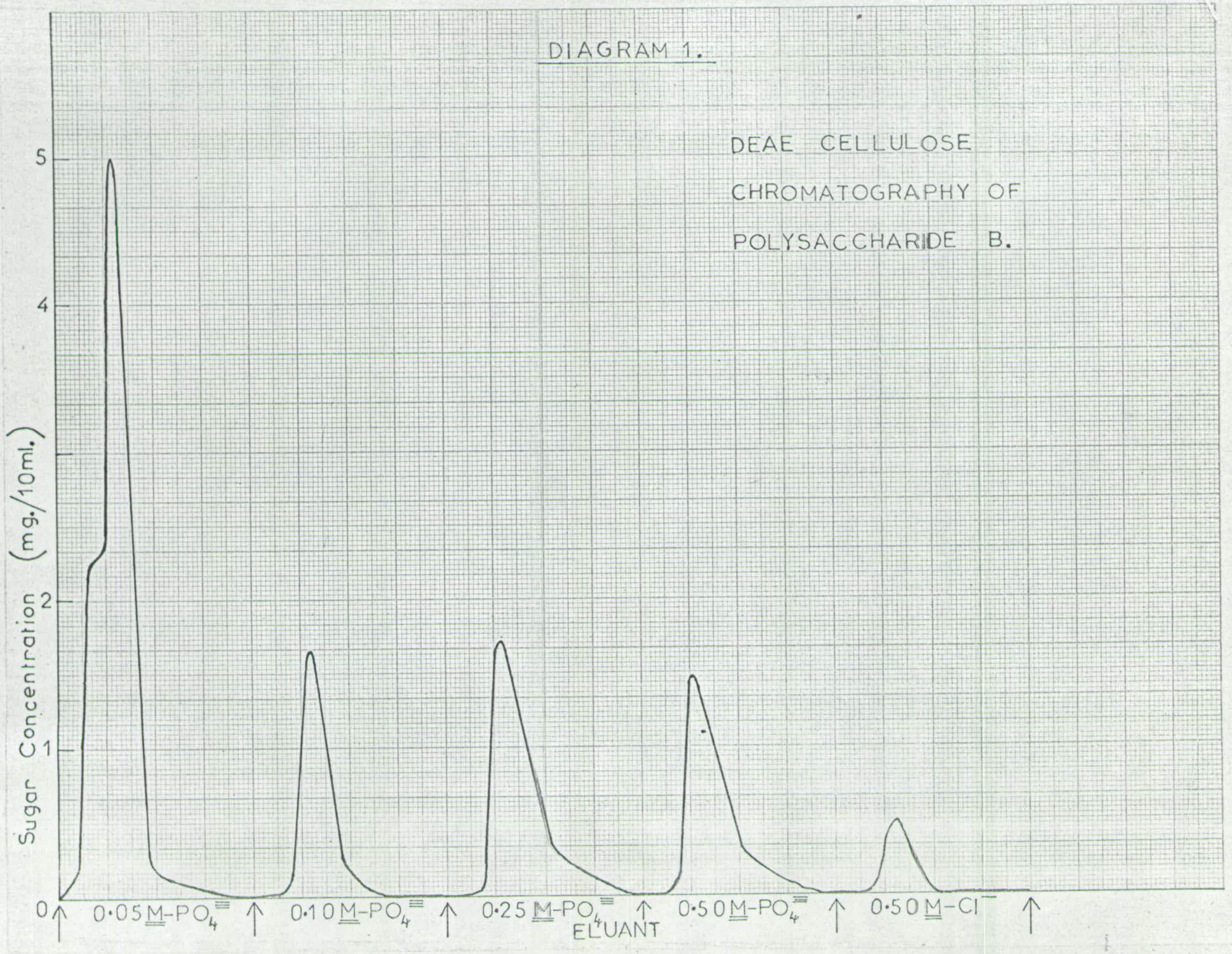
* Uronic Acid Anhydride by carbazole method.

n.d. = not determined

FRACTIONATION OF THE POLYSACCHARIDES

The polysaccharide mixture B. (5.6 g.) was dissolved in water (1.5 l.) and copper acetate solution (100 ml.) was added dropwise with vigorous stirring. The acidic polysaccharide was precipitated as its copper salt which was decomposed with acetone containing 1% hydrogen chloride. The

DIAGRAM 1.


DEAE CELLULOSE
CHROMATOGRAPHY OF
POLYSACCHARIDE B.
Sugar Concentration (mg./10ml.)
0
1
2
4
5
0·05 M-PO4
0·10 M-PO4
0·25 M-PO4
0·50 M-PO4
0·50 M-Cl
ELUANT

precipitate was washed free of hydrogen chloride with ethanol, dissolved in water and the solution was freeze dried to give polysaccharide fraction 1. which was further purified by chromatography on diethylamino-ethyl Sephadex - A50 (15 g.; formate form) to give polysaccharide fractions 1a., 1b., and 1c. eluted with water, 2 N - formic acid, and 3 N - formic acid respectively. The formic acid eluants were dialysed for 24 hrs., the pH of the solutions was adjusted to 4.5 with potassium acetate and the solutions were concentrated before precipitating the polysaccharides in ethanol containing 4% acetic acid. The precipitates were washed free of acetic acid with ethanol, dissolved in water and the solutions were freeze dried.

The supernatant solution from the copper acetate precipitation was concentrated to 500 ml. and Fehling's solution (20 ml.) was added dropwise with vigorous stirring. The precipitated material was removed at the centrifuge and the copper complex was destroyed in the same way as the copper salt to give polysaccharide fraction 2. Polysaccharide fraction 2. was then dissolved in the minimum amount of water (ca. 30 ml.) and applied to a cellulose column (60 g.) to give polysaccharide fractions 2a., 2b., and 2c., eluted with water, 7M - urea, and 0.5M - sodium hydroxide respectively. The sodium hydroxide eluate was neutralised with N - hydrochloric acid and was then dialysed against running tap water for two days, as was the urea eluate. The three fractions were each precipitated from ethanol (4 vol.) and redissolved in water before freeze drying.

The supernatant solution from the Fehling's solution precipitation was neutralised with $\underline{\underline{N}}$ - hydrochloric acid and dialysed against running tap water until no blue colour remained before precipitation from ethanol (4 vol.) and freeze drying to give polysaccharide fraction 3. Polysaccharide fraction 3. was then chromatographed on cellulose (60 g.) to give polysaccharide fractions 3a., 3b., and 3c., eluted with water, $7\underline{\underline{M}}$ - urea and $0.5\underline{\underline{M}}$ - sodium hydroxide respectively. The sodium hydroxide solution was neutralised with $\underline{\underline{N}}$ - hydrochloric acid and was then dialysed against running tap water for two days, as was the urea eluate. The three fractions were each precipitated from ethanol (4 vol.) and redissolved in water before freeze drying.

Details of each fraction are given in table III.

TABLE III

Fraction	Wt. (g)	$[\alpha]_D$	C	Sugars on Hydrolysis							
				GalA.	Gal.	G.	Man.	Ara.	Xyl.	Fuc.	Rha.
1	1.555			++++	++	+	+	+	+	/	tr.
1a	0.625			/	++	+	+	++	++	/	/
1b	0.308	+175°	0.452	++++	++	/	/	+	tr.	/	tr.
1c	0.396	+180°	0.452	++++	++	/	/	+	tr.	/	tr.
2	1.858			/	+	+	++	tr.	++	tr.	tr.
2a	0.803	+ 75°	0.320	/	tr.	tr.	++++	/	tr.	/	/
2b	0.577			/	+	++	+	tr.	++	tr.	/
2c	0.296			/	+	++	tr.	tr.	+++	tr.	/
3	1.998			+	+++	++	tr.	+++	+++	+	+
3a	1.010	- 49°	0.510	/	++	tr.	tr.	++	/	/	tr.
3b	0.370				++	+++	tr.	+	+++	+	/
3c	0.356			/	tr.	++	/	/	+++	tr.	/

Polysaccharide fractions 1b. /Found: U.A. (by decarboxylation) = 65%/ and 1c. /Found: U.A. (by decarboxylation) = 68%/ were combined constituting a pectinic acid /Found: U.A. (by decarboxylation) = 67%/ /Found: OMe = 3.4%/. Polysaccharide fractions 2a. and 3a. constitute the mannan and the arabinogalactan respectively

THE PECTINIC ACID

Partial Hydrolysis

A sample of the pectinic acid (450 mg.) was dissolved in N - sulphuric acid (30 ml.) and heated at 100° in a sealed tube for 4 hrs. The insoluble material, which was removed at the centrifuge, was shown by hydrolysis for a further 12 hrs. in N - sulphuric acid to contain D - galacturonic acid only. The supernatant solution was neutralised by stirring with barium carbonate, treated with Amberlite IR 120 (H) resin and concentrated to a syrup (248 mg.).

The syrup was then dissolved in the minimum volume of water and applied to a diethylaminoethyl Sephadex A - 25 column (5 g.; formate form) which was eluted with water (1.5 l.), to give neutral sugars (118 mg.), followed by water containing increasing quantities of formic acid to give four acidic oligosaccharides which were further separated, where necessary, by chromatography on Whatmann 3 MM paper.

Oligosaccharide 1. (3 mg.; eluted with 0.05 N - formic acid), $R_{GalA.}$ = 0.86 (Solvent B.), M_G = 0.56 gave galacturonic acid and rhamnose only on hydrolysis and was chromatographically and ionophoretically indistinguishable from 2 - O - (α - D - galactopyranosyluronic acid) - L - rhamnose. Methylation of the sugar by the Kuhn method and subsequent gas-liquid partition chromatography of the methanolysis products gave peaks with the retention times of the following sugars.

Sugar	T. (Column c)
3, 4 di - O - methyl - L - rhamnose	0.85
2, 3, 4 tri - O - methyl - D - galacturonic acid	6.83, 6.50

Oligosaccharide 2. (2 mg.; eluted with 0.05 N - formic acid) $R_{GalA.}$ = 0.59 (Solvent B) M_G = 0.62 was chromatographically and ionophoretically indistinguishable from 4 - O - (β - D - glucopyranosyluronic acid) - L- fucose and gave glucuronic acid, fucose and glucurone on hydrolysis.

Oligosaccharide 3. (2 mg.; eluted with 0.05 N - formic acid) R_{Gal} = 0.23 (Solvent B) M_G = 1.08 gave glucuronic acid, glucurone and galactose only on hydrolysis and was chromatographically and ionophoretically indistinguishable from 6 - O - (β - D - glucopyranosyluronic acid) - D - galactose.

Oligosaccharide 4. (8 mg.; eluted with 0.4 N - formic acid) $R_{GalA.}$ = 0.25 (Solvent B), = 0.50 (Solvent C) M_G = 0.96 gave galacturonic acid only on hydrolysis and was chromatographically and ionophoretically indistinguishable from 4 - O - (α - D - galactopyranosyluronic acid) - D - galacturonic acid.

Oligosaccharide 5. (8 mg.; eluted with 0.5 N - formic acid) $R_{GalA.}$ = 0.25 (Solvent C) M_G = 0.95 gave galacturonic acid only on hydrolysis and was ionophoretically and chromatographically indistinguishable from

D - ∝ - D - galactopyranosyluronic acid - (1 → 4) - D - ∝ - D -
galactopyranosyluronic acid - (1 → 4) - D - galactopyranosyluronic acid.

THE ARABINOGALACTAN

Partial Hydrolysis

A sample of the polysaccharide (10 mg.) was heated in N - sulphuric acid (1 ml.) in a sealed tube at 100° for 30 mins. and, after cooling, the solution was neutralised with barium carbonate, centrifuged and any barium ions were removed with Amberlite IR 120 (H) resin before concentrating to a small volume. The hydrolysate was chromatographed in solvents A and B and the following table IV compares the oligosaccharides O_1 and O_2, which were obtained, with authentic galactose oligosaccharides and shows that they are chromatographically indistinguishable from 3 - O - β - D - galactopyranosyl - D - galactose and 6 - O - β - D - galactopyranosyl - D - galactose respectively.

TABLE IV

Oligosaccharides	R_{Gal} Solvent A	R_{Gal} Solvent B
Galp1 $\xrightarrow{\beta}$ 4 Galp	0.56	0.40
Galp1 $\xrightarrow{\beta}$ 3 Galp	0.49	0.32
Galp1 $\xrightarrow{\beta}$ 6 Galp	0.32	0.22
Galp 1 $\xrightarrow{\beta}$ 4 Galp 1 $\xrightarrow{\beta}$ 4 Galp	0.28	0.15
O_1	0.48 tr.	0.33 tr.
O_2	0.31	0.24

Methylation

The polysaccharide (850 mg.) was dissolved in water (20 ml.) through which a continuous stream of nitrogen was passed. Methyl sulphate (10 ml.)

and 30% sodium hydroxide solution (20 ml.) were added dropwise over a period of four hours to the solution in an ice bath with vigorous stirring. The additions were repeated each day for a further four days and on the sixth day the solution was heated in a boiling water bath for 1 hr., cooled, centrifuged and the pH of the supernatant adjusted to 4 with dilute sulphuric acid. The solution was then extracted with chloroform (4 x 200 ml.), the chloroform solution was dried over anhydrous sodium sulphate and concentrated under reduced pressure to give a syrup M.1. (305 mg.).

The residue from centrifugation was extracted with acetone, the solution was concentrated to a syrup which was then extracted with chloroform and the chloroform extract was dried over anhydrous sodium sulphate before concentration to a syrup M.2. (853 mg.)

The two fractions, M.1 and M.2., were further methylated separately.

Each fraction was dissolved in methyl iodide (25 ml. for M.1.) (50 ml. for M.2.) and silver oxide (0.700 g. for M.1.) (1.5 g. for M.2.) was added portionwise each hour for four hours with constant refluxing and stirring. The reaction was continued overnight, the mixture was then cooled, filtered and the silver residues were washed with chloroform and then extracted with chloroform in a Soxhlet extractor for 8 hrs. The chloroform extracts were combined and concentrated to a syrup (250 mg. - M.1.) (805 mg. - M.2.)

The silver oxide/methyl iodide methylation was repeated and the syrups obtained were dissolved in a small volume of chloroform and

precipitated from a large excess of light petroleum (B.P. 60°/80°) to give white precipitates which were dried over phosphorous pentoxide in vacuo for 24 hrs. before the measurements in table V were obtained.

TABLE V

Fraction	OMe%	$[\alpha]_D$	c in $CH Cl_3$	Wt.(g.)
M.1.	39.7	-35°	0.520	0.153
M.2.	39.5	-80°	0.510	0.444

Methanolysis of samples (20 mg.) of each fraction and subsequent gas-liquid partition chromatography gave peaks corresponding to the methyl glycosides of the methylated sugars in table VI.

TABLE VI

Methylated Sugars	Relative Proportions		T.		
			M.1.	M.2.	
	M.1.	M.2.	Column.a.	Column.a.	Column.c.
2,3, 4 Me_3-Xyl. or 2, 3, 4 Me_3-Rha.	tr.	tr.	0.46	0.47	0.44
2, 3, 5 Me_3-Ara.	++++	++++	0.55 0.70	0.54 0.71	0.48 0.61
2, 3, 4 Me_3-Ara.	tr.	tr.	1.03	1.03	0.86
2, 3 Me_2-Ara.	+	+	1.54	1.59	1.19 1.50
2, 3, 4,6 Me_4-Man.	+	/	1.40	/	/
2, 3, 4,6 Me_4-Gal.	tr.	tr.	1.76	1.80	1.81
3, 4, 6 Me_3-Man.	tr.	/	3.13	/	/
2, 4, 6 Me_3-Gal.	+	+	4.16 4.75	4.10 4.65	3.58 4.19
2, 3, 4 Me_3-Gal.	+	+	7.25	7.30	6.82
2, 4 Me_2-Gal.	++	++	17.1 19.4	17.2 19.5	14.2 16.2

THE MANNAN

Methylation

The polysaccharide (200 mg.), which had been previously dried at 60° in vacuo over phosphorus pentoxide, was dissolved in the minimum volume of dimethyl sulphoxide (4 ml.) and barium oxide (1 g.) and methyl iodide (4 ml.) were added. The mixture was stirred at room temperature for 48 hrs., centrifuged and the residue was washed with chloroform. The supernatant solution and the chloroform extracts were combined and more chloroform was added until the precipitation of organic salts was complete. The salts were removed by centrifugation and the red-brown solution was decolourised by washing with a 15% solution of sodium thiosulphate and then washed with a small volume of water to remove any residual dimethyl sulphoxide before concentrating under reduced pressure to give a syrup (161 mg.).

The syrup was dissolved in methyl iodide (5 ml.) to which was added silver oxide (50 mg.) in four portions at hourly intervals with constant stirring and refluxing. The reaction was continued overnight and the residual silver salts were removed at the centrifuge. The residue was extracted for 8 hrs. in a Soxhlet extractor with chloroform and the extracts were combined with the supernatant solution before evaporation to dryness to give a syrup (143 mg.)

The silver oxide/methyl iodide methylation was repeated twice and the final syrup was dissolved in a small volume of chloroform and the fully methylated mannan (124 mg.), $[\alpha]_D = +73°$ $[c = 0.510]$

[Found: OMe, 43.2%] was precipitated from a large excess of light petroleum (B.P. 60°-80°) to give a white powder which was dried over phosphorus pentoxide in vacuo.

A sample of the methylated mannan (10 mg.) was methanolysed and the resultant methyl glycosides on gas-liquid partition chromatography gave peaks with the retention times of the methyl glycosides of the sugars in table VII.

TABLE VII

Methylated Sugars	T		Relative Proportions
	Column a	Column b	
2, 3, 4, 6 Me_4-Man.	1.43	1.32	++
3, 4, 6 Me_3-Man. and/or 2,3,4 Me_3 Man.	3.09	1.70	+
2, 4, 6 Me_3-Man.	3.83	1.98	+
3, 4 Me_2-Man.	7.35	2.26	+

A further sample of the methylated mannan (4 mg.) was heated in methanolic 4% hydrogen chloride at 100° overnight and the methyl glycosides were hydrolysed with $\underline{\underline{N}}$ - sulphuric acid (1 ml.) at 100° for 4 hrs. Demethylation of the hydrolysate gave mannose only on paper chromatography in solvents A and B.

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BIBLIOGRAPHY

BIBLIOGRAPHY

1. G. L. Baker, G. H. Joseph, Z. I. Kertesz, H. H. Mottern, and A. G. Olsen, Chem. Eng. News., 1944, 22, 105.

2. F. W. Norris, and C. E. Resch, Biochem J., 1937, 19, 1945.

3. E. Roberts, Botan. Gaz., 1916, 62, 497.

4. C. L. Hinton, "Fruit Pectins, their Chemical Behaviour and Jellying Properties", Dept. of Scientific and Industrial Research, 1939.

5. Z. I. Kertesz, "The Pectic Substances" p. 278.

6. F. A. Henglein and G. Schneider, Ber. deut. chem. Ges., 1936, 69B, 309.

7. G. G. Schneider and H. Bock, Ber. deut. chem. Ges., 1937, 70, 1617.

8. S. Luckett and F. Smith, J., 1940, 1106 and 1506.

9. G. H. Beaven, and J. K. N. Jones, J., 1947, 1218.

10. F. A. Levene and L. C. Kreider, J. Biol. Chem., 1937, 120, 591.

11. J. K. N. Jones and W. W. Reid, J., 1954, 1361.

12. J. K. N. Jones and W. W. Reid, ibid, 1955, 1890.

13. C. T. Bishop, Canad. J. Chem., 1955, 33, 1521.

14. V. Zitko and C. T. Bishop, Canad. J. Chem., 1965, 43, 3206.

15. S. S. Bhattacharjee and T. E. Timell, Canad. J. Chem., 1965, 43, 758.

16. E. L. Hirst, J. K. N. Jones, and (Mrs.) W. O. Walder, J., 1947, 1225.

17. J. L. Whyte, Ph.D. Thesis, Edinburgh University, 1964.

18. P. Andrews, L. Hough, and J. K. N. Jones, J., 1954, 806.

19. M. Morita, Agric. and Biol. Chem. (Japan) 1965, 29, 564.

20. A. M. Unrau, Canad. J. Chem., 1964, 42, 916.

21. H. O. Bouveng and H. Meier, Acta. Chem. Scand., 1959, 13, 1884.

22. H. Meier, Acta Chem. Scand., 1962, 16, 2275.

23. E. L. Hirst and J. K. N. Jones, J., 1938, 496.

24. E. L. Hirst and J. K. N. Jones, J., 1939, 452.

25. G. H. Beaven, E. L. Hirst and J. K. N. Jones, J., 1939, 1865.

26. E. L. Hirst and J. K. N. Jones, J., 1948, 2311.

27. P. A. Finan and P. S. O'Colla, Chem. and Ind., 1958, 493.

28. L. Hough and D. B. Powell, J., 1960, 16.

29. T. L. Hullar and F. Smith, Unpublished Results.

30. P. Andrews, L. Hough, D. B. Powell, and B. M. Woods, J., 1959, 774.

31. E. L. Hirst, D. A. Rees, and N. G. Richardson, Biochem J., 1965, 95, 453.

32. N. G. Richardson, Ph.D. Thesis, Edinburgh University, 1965.

33. J. Roudier and L. Eberhard, Bull. Soc. chim. France, 1965, 460.

34. R. M. McCready and M. Gee, J. Agric. and Food Chem., 1960, 8, 510.

35. P. Andrews and L. Hough, J., 1958, 4476.

36. G. O. Aspinall and A. Cañas-Rodriguez, J., 1958, 4020.

37. G. O. Aspinall, R. Begbie, A. Hamilton, and J. N. C. Whyte, Unpublished Results.

38. W. Büchi, H. Deuel, Helv. Chim. Acta., 1954, 37, 1392.

39. G. O. Aspinall and R. S. Fanshawe, J., 1961, 4215.

40. G. O. Aspinall, I. W. Cottrell, (Mrs.) S. V. Egan, I. M. Morrison, and J. N. C. Whyte, Unpublished Results.

41. G. O. Aspinall, K. Hunt, and I. M. Morrison, Unpublished Results.

42. G. O. Aspinall and M. Uddin, Unpublished Results.

43. G. O. Aspinall and J. Baillie, J., 1963, 1702.

44. H. Neukom, P. Heim, and P. Albersheim, 144th Meeting Amer. Chem. Soc., 1963, p. 10c.

45. B. Vollmert, Makromol. Chemie, 1950, 5, 110.

46. H. Neukom and H. Deuel, Chem. and Ind., 1958, 683.

47. R. L. Whistler and J. N. BeMiller, Advances in Carbohydrate Chemistry, 1958, 13, 289.

48. J. Hine, Physical Organic Chemistry, (McGraw-Hill Book Co. 1962) p.285.

49. H. F. Launer and Y. Tomimatsu, J. Org. Chem., 1961, 26, 541.

50. P. Albersheim, Biochem. Biophys. Research Communs., 1959, 1, 253.

51. P. Albersheim, H. Neukom and H. Deuel, Arch. Biochem. Biophys., 1960, 60, 46.

52. A. Linker, K. Meyer, and P. Hoffman, J. Biol. Chem., 1956, 219, 13

53. A. J. Barrett and D. H. Northcote, Biochem. J., 1965, 94, 617.

54. R. M. McCready and E. A. McComb, J. Agric. and Food Chem., 1953, 1, 1165.

55. W. W. Reid, J. Science Food Agric., 1950, 1, 234.

56. J. Dingle, W. W. Reid, and G. L. Solomons, J. Science Food Agric., 1953, 4, 149.

57. A. Ayres, J. Dingle, A. Phipps, W. W. Reid, and G. L. Solomons, Nature, 1952, 170, 834.

58. B. S. Luh and H. J. Phaff, Arch. Biochem. Biophys., 1951, 33, 212.

59. A. L. Demain and H. J. Phaff, Arch. Biochem Biophys., 1954, 51, 114.

60. A. L. Demain and H. J. Phaff, J. Biol. Chem., 1954, 210, 381.
A. L. Demain and H. J. Phaff, ibid, 1956, 218, 875.

61. D. S. Patel and H. J. Phaff, ibid, 1959, 234, 237.

62. R. Albersheim, H. Neukom and H. Deuel, Helv. Chim. Acta., 1960, 43, 1422.

63. C. W. Nagel and R. H. Vaughn, Arch. Biochem. Biophys., 1961, 94, 328.

64. M. P. Starr and F. Moran, Bacteriol. Proc., 1961, 169.

65. S. Hasegawa and C. W. Nagel, J. Biol. Chem., 1962, 237, 619.

66. R. L. Whistler, A. R. Martin, and M. Harris, J. Research National Bur. Standards, 1940, 24, 555.

67. H. Neukom, H. Deuel, W. J. Heri and W. Kündig, Helv. Chim. Acta., 1960, 43, 64.

68. J. Rosík, V. Zitko, and J. Vašátko, Coll. Czech. Chem. Commun., 1962, 27, 1346.

69. E. L. Hirst, J., 1955, 2974.

70. G. O. Aspinall, Chim. Biochim. Lignine Cellulose Hemicelluloses, Actes Symp. Intem., p 421, Les Imprimeries Réunies de Chambéry, France (1964).

71. J. P. Thornber and D. H. Northcote, Biochem. J. 1961, 81, 449.

72. J. P. Thornber and D. H. Northcote, ibid., 1961, 81, 455.

73. J. P. Thornber and D. H. Northcote, ibid., 1962, 82, 340.

74. W. N. Haworth, H. Riastrick, and M. Stacey, Biochem. J., 1937, 31, 640.

75. I. R. Siddiqui and G. A. Adams, Canad. J. Chem., 1961, 39, 1683.

76. H. Bender, J. Lehmann, and K. Wallenfels, Biochem. Biophys. Acta., 1959, 36, 309.

77. G. A. Adams and S. M. Martin, Canad. J. Biochem., 1964, 42, 1403.

78. G. E. Becker, Paul A. Hui, and P. Albersheim, Plant Physiology, 1964, 39, 913.

79. A. B. Foster, Chem. and Ind, 1952, 828.

80. W. E. Trevelyan, D. P. Proctor, and J. S. Harrison, Nature, 1950, 166, 444.

81. R. U. Lemieux and H. F. Bauer, Analyt. Chem., 1954, 26, 920.

82. W. J. Whelan, J. M. Bailey, and P. J. P. Roberts, J., 1953, 1293.

83. R. Kuhn, H. Trischmann, and I. Löw, Angew. Chem., 1955, 67, 32.

84. T. G. Bonner, E. J. Bourne, and S. McNally, J., 1960, 2929.

85. R. Belcher and A. L. Godbert, Semi-micro Quant. Org. Anal., (Longmans, Green & Co., 1954)

86. M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, Analyt. Chem., 1956, 28, 350.

87. E. A. McComb and R. M. McCready, Analyt. Chem., 1952, 24, 1630.

88. D. M. W. Anderson, Talanta, 1959, 2, 73.

89. Z. Dische and L. B. Shettles, J. Biol. Chem., 1948, 175, 595.

90. J. F. Carson and W. D. Maclay, J. Amer. Chem. Soc., 1946, 68, 1015.

91. A. I. Vogel, Practical Org. Chem. (Longmans, Green & Co. 1956).

92. A. J. Erskine and J. K. N. Jones, Canad. J. Chem., 1956, 34, 821.

93. G. A. Adams, Canad. J. Chem., 1957, 35, 556.

94. G. O. Aspinall, D. B. Davies, and R. N. Fraser, Unpublished Results.

95. G. O. Aspinall, Advances in Carbohydrate Chemistry, 1959, 14, 429.

96. H. C. Srivastava, S. N. Harshe, and Prem Pal Singh, Tetrahedron Letters, 1963, 27, 1869.

97. J. A. Cifonelli and F. Smith, J. Amer. Chem. Soc., 1955, 77, 5682.

98. M. L. Wolfrom, M. L. Laver, and D. L. Patin, J. Org. Chem., 1961, 26, 4533.

99. (Mrs.) V. P. Rege, T. J. Painter, W. M. Watkins, and W. T. J. Morgan, Nature, 1964, 203, 360.

100. D. A. Rees and J. W. B. Samuel, Chem. and Ind., 1965, 2008.

101. G. O. Aspinall and I. W. Cottrell, Unpublished Results.