

THE MOLECULAR STRUCTURE OF PECTIC SUBSTANCES

by

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TO MY PARENTS

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INTRODUCTION

Definition of the Terms Used in Pectin Chemistry.

Pectin or Pectic Substance.

Pectic substance is a group designation for those mostly colloidal carbohydrate derivatives which occur in, or are prepared from, plants and contain a large percentage of galacturonic acid residues existing in chain like combination. The carboxyl groups of the polygalacturonic acids may be partially esterified by methyl groups and partly or completely neutralized by one or more bases (1).

Pectic Acids.

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

INTRODUCTION

Polyuronides, which are components of almost all living organisms are most abundant in the higher orders of land plants and in seaweeds. The group known as the pectic substances is plentiful and widespread, being found in all higher plants and in wood. (2).

Pectic substances are found mainly in the primary cell walls and in the intercellular layer. They occur also in the secondary walls and in the walls of fruit cells either in solution or in colloidal suspension. They are, consequently most abundant in soft tissues which are composed mainly of

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primary walls (i.e. fruit and roots) (3)(4) and much less so in wood, where the volume of the primary layer is small.

Pectic substances occurring in the cell wall differ from these in the intercellular layer. The former consist mainly of the methyl ester of the acid, which is water soluble and readily extractable, whilst the latter exist as insoluble salts (usually of Ca, Mg, or Fe) or in combination with cellulose or some other polysaccharide of high molecular weight.

The role of pectic substances in plant physiology is not yet fully understood. Their location in the plant indicates that they are structural constituents. They may also assist in cell adhesion or provide important food reserves for the plant. It is possible that the hydrophillic nature of the pectin gel helps to maintain the turgidity of cells which gives soft tissues their necessary rigidity. It is obvious that different types of pectic substance can perform exceedingly varied biological functions.

Interest was first aroused in pectic substances because of their gelling power and their ability to act as emulsifying agents. Of the two types of gel which can be formed, the most important industrially is the jelly obtained using pectin, sugar and acid. The gel is formed as a result of the tendency of aggregations of pectin molecules to crystallise. It consists of a giant framework of partially associated, partially

hydrated micella.

A gel of a different type is formed when calcium, or some other divalent cation, is added to an aqueous dispersion of pectin. Cross links are formed through ionic linkages between carboxyl groups.

The molecular weights of pectins which have been determined vary from 10,000 to several hundreds of thousands. The first values for the molecular size of the native pectins from apple and sugar beet were obtained by Schneider and co-workers (5). These workers prepared the nitro and acetyl derivatives in order to avoid the anomalous behaviour due to charge effects which occurred using the unsubstituted polysaccharides. From osmotic and viscometric measurements on the nitrate, values for the molecular weight of 30,000 - 100,000 were found.

From measurements of the sedimentation and diffusion constants of pectins isolated directly from fruit juices, Svedberg and Gralén (6) reported values for the molecular weight of 25,000 - 35,000 for apple, pear and plum pectins and a higher value of 40,000 - 50,000 for orange pectin. Using the same methods, Sæverborn (7) obtained values of 50,000 - 100,000 for pectins from apple and citrus fruit albedo. The same author has reported (8) that the pectins isolated from plant juices have a considerably higher value for the molecular weight ($200 - 400$) $\cdot 10^3$ than those extracted

even with mild reagents, which had molecular weights of about 60,000.

MacLay and co-workers (9) obtained molecular weights of 23,000 - 71,000 for citrus and apple pectins. These values were obtained from viscosity measurements.

Speiser and Eddy (10) obtained values of 32,000 - 213,000 for the molecular weight of nitrated apple pectin. Owens, Miers and MacLay (11) obtained the molecular weight distribution curves for apple and lemon pectins in a similar way and found the molecular weights ranging from 20,000 to 150,000.

It appears that the molecular size of the pectic substances depends to a large extent on their method of isolation. The free substances would appear to possess a high molecular weight of the order of 200,000.

Extraction of Pectin.

Laboratory methods of extracting pectic materials must involve the minimum amount of degradation for chemical and physical characterisation. Before commencing extraction, however, it is best to inactivate enzymes by treating the freshly cut material with boiling 90% ethanol. This also removes colouring matter, organic acids and sugars. At this point, to make further extraction procedures more efficient the pectin should be ground. Cold water extraction removes water soluble pectin, while hot water (above 60°C) removes still greater amounts. Mineral acids have also been used, but these involve

extensive degradation (i.e. hydrolysis of associated araban) (12).

Alkaline reagents which have also been used, cause de-esterification of the pectic acids. It has been shown that the alkaline de-esterification of pectin is accompanied by depolymerisation even in an oxygen free atmosphere (13), (14), (15), (16).

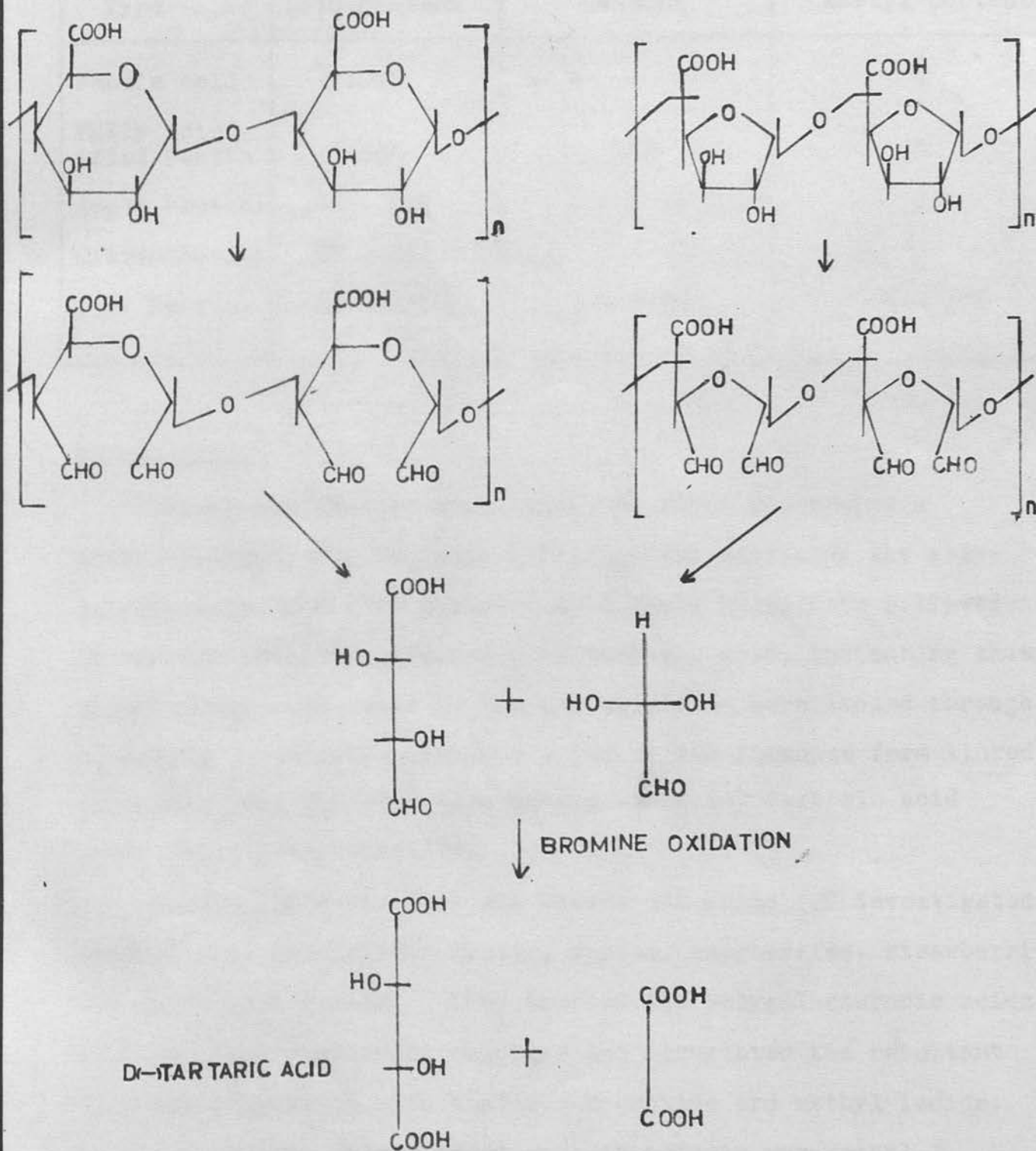
Certain salt solutions also effect total extraction of pectic materials. Ammonium oxalate, which is the most commonly used, has been reported as extracting 90% of the pectic substance in cotton fibre in two successive eight hour periods at 90°. (17). Other calcium removing agents such as ammonium citrate, fluoride, arsenate or phosphate can also be used.

The Pectic Triad.

Pectic polysaccharides exist in plants in close physical union with araban and galactan. So firm is this association that on extraction a triad containing a galacturonan, a galactan and an araban is usually obtained. It is difficult to say whether pectins are physical mixtures of polysaccharides or whether they consist of highly complex polysaccharides. The table below illustrates some typical galacturonic acid contents of some pectic substances.

Fig.1

LEVENE AND KREIDER (18) TREATMENT OF POLYGALACTURONIC ACID.



Type	Galacturonic Acid Content	Methoxyl Content	Acetyl Content
Pectic Acid	100%	0	0
Fully Esterified Pectin	84%	16%	0
Apple Pectins	70 - 80%	6 - 9%	0
Citrus Pectins	75 - 85%	7 - 10%	0
Beet Pectins	60 - 65%	5 - 7%	5 - 10%

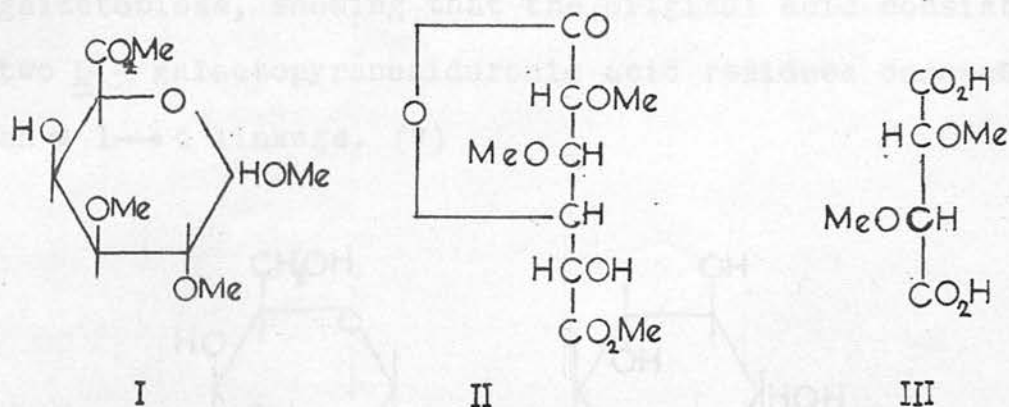
Pectic Acids.

Levene and Kreider were among the first to examine a pectic polygalacturonic acid (18). After oxidising the polygalacturonic acid with periodic acid, acid hydrolysis followed by bromine oxidation yielded D(-) tartaric acid, indicating that galacturonic acid units in the pyranose form were linked through C₁ and C₄. Galacturonic acid units in the furanose form linked through C₁ and C₅ would also have given D (-) tartaric acid under those conditions. (Fig. I)

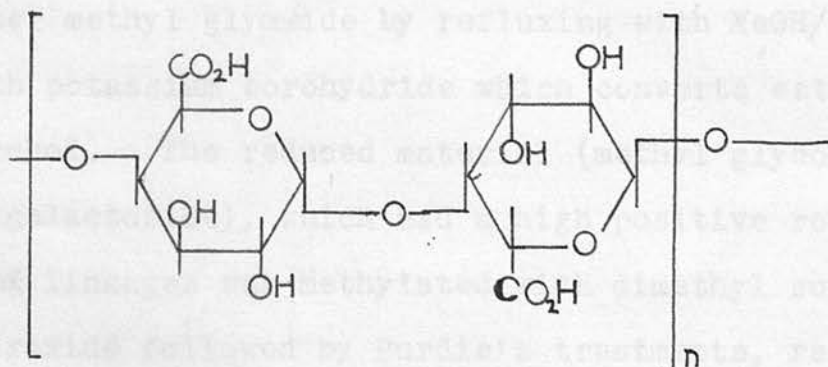
Hirst (19), Jones (21) and Beaven and Jones (20) investigated pectic acids from citrus fruits, apples, raspberries, strawberries and white lupin seeds. They treated the polygalacturonic acids with methanolic hydrogen chloride and methylated the resultant degraded polyesters with thallos hydroxide and methyl iodide. In all cases the only product on methanolysis was methyl 2, 3-di-O-methyl-D-galacturonoside (I), the structure of which was

HYDROLYSIS,

proved by Δ oxidation with bromine water followed by esterification to give the 1 : 4 lactone of 2,3 - dimethyl mucic ester (II), which with periodic acid gave I - dimethoxy - succinic acid (III) after oxidation of the aldehyde with bromine water.



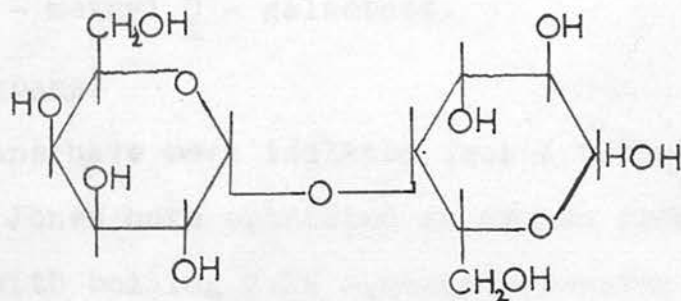
This result in conjunction with the stability of pectic acid to hydrolysis and its high positive rotation lead them to suggest that the pectic acid molecule consisted of a series of pyranose α - D - galacturonic acid residues linked through positions one and four. (IV)



IV

Luckett and Smith (22) obtained results in agreement with this by the direct methylation of pectic acid obtained from citrus pectin.

Jones and Reid (23), (24) investigated the di- and tri-galacturonic acids obtained by treating apple pectic acid with an enzyme from Aspergillus foetidus. The digalacturonic acid was reduced to the corresponding α 1 \rightarrow 4 linked galactobiose, showing that the original acid consisted of two D - galactopyranosiduronic acid residues connected by an α 1 \rightarrow 4 linkage. (V)



V

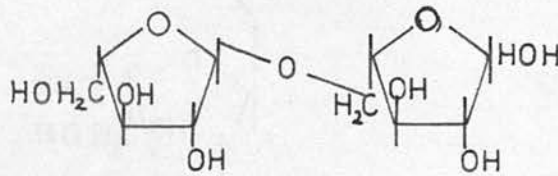
The trigalacturonic acid was converted to the methyl ester methyl glycoside by refluxing with MeOH/HCl and reduced with potassium borohydride which converts ester groups to alcohol. The reduced material (methyl glycoside of a trigalactoside), which had a high positive rotation indicative of α linkages was methylated with dimethyl sulphate and sodium hydroxide followed by Purdie's treatments, resulting in a fully methylated trisaccharide which was hydrolysed. The products were separated on a cellulose column and characterised by obtaining crystalline derivatives. The sugars obtained were

2, 3, 4, 6 - tetra - Q - methyl - D - galactose, 2, 3, 6 - tri - Q - methyl - D - galactose and 2, 6 - di - Q - methyl - D - galactose in approximately equal proportions. The isolation of these sugars suggested that the trisaccharide was either an unbranched chain, which should furnish one mole of tetra - Q - methyl - D - galactose and two moles of tri - Q - methyl - D - galactose or a branched chain, which should furnish two moles of tetra - Q - methyl - D - galactose and one mole of di - Q - methyl D - galactose.

Pectic Arabans.

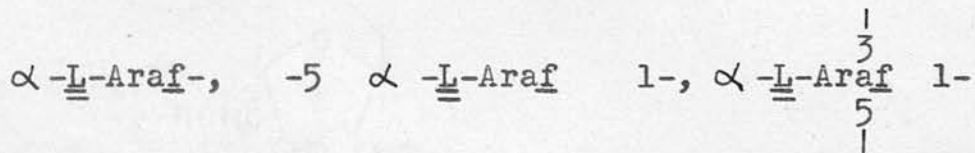
Arabans have been isolated from a variety of sources. Hirst and Jones have extracted an araban from peanuts by stirring with boiling 0.2% aqueous potassium hydroxide for four hours, the araban-pectic acid complex being separated after methylation (25). They also extracted arabans from peanuts, apple pomace and citrus fruits using hot 70% ethanol (26), (27). Another araban was extracted from sugar beet chips using hot lime water (28). In all cases methylation and hydrolysis gave equimolar portions of 2, 3, 5 - tri - Q - methyl - L - arabinose, 2, 3 - di - Q - methyl - L - arabinose, and 2 - mono - Q - methyl - L - arabinose. The ease of hydrolysis of these arabans and their strong negative rotation indicated that all the arabinose was present in the furanose form and that all the linkages were α . Hough and Powell (29) by graded hydrolysis of sugar beet araban, were able to isolate

5 - 0 - L - arabinofuranosyl - L - arabinofuranose (VI) and
 3 - 0 - L - arabinofuranosyl - L - arabinose.

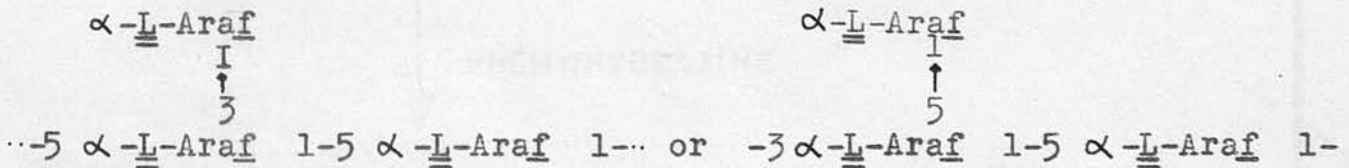


VI

On the basis of the evidence obtained so far several structures may be assigned to the araban. The polysaccharide must contain



but the repeating unit may be either



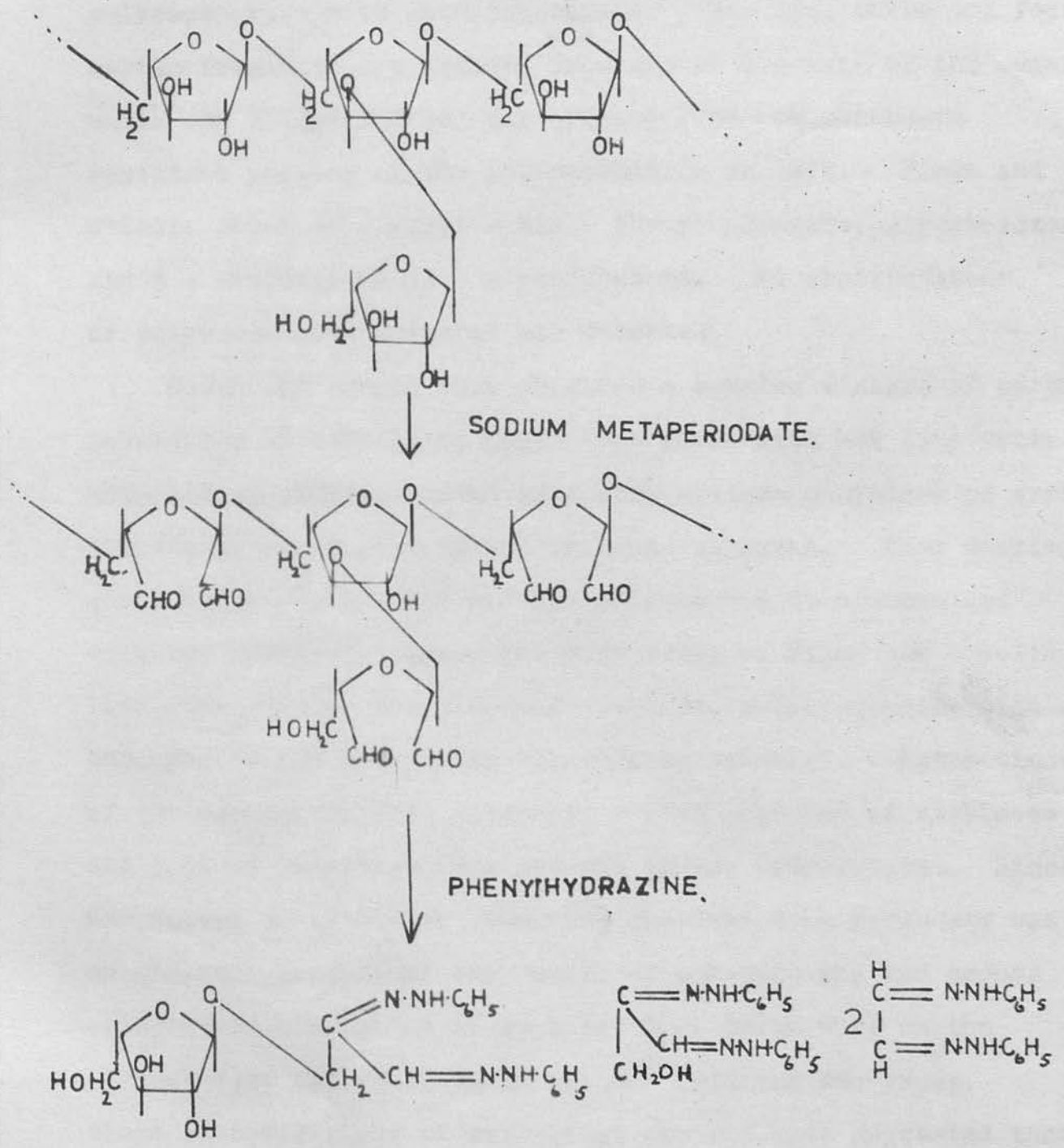
VII

VIII

or a simple variation of one of these. By application of the Barry degradation to sugar beet araban, Finan and O'Colla (30) were able to show that the structure had to be either VII or VIII

Fig II.

BARRY DEGRADATION OF ARABAN



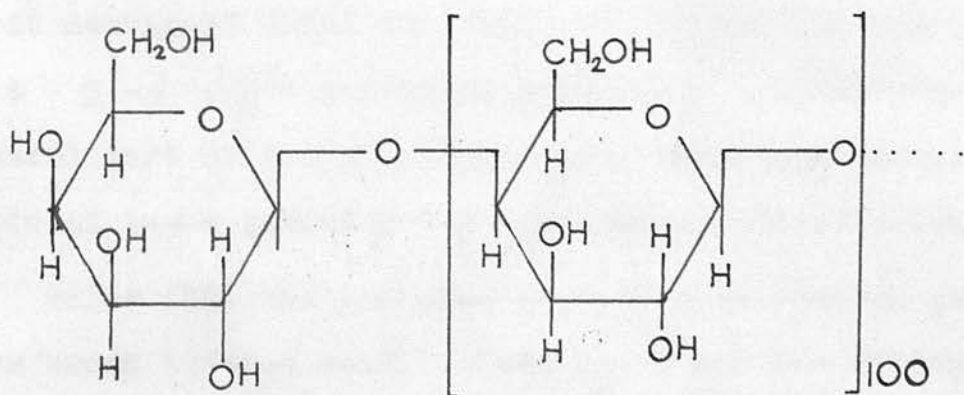
or a ramified modification of one of these. The Barry (Fig. II) degradation involves the treatment of the periodate degraded polysaccharide with phenylhydrazine. The two, three and four carbon fragments are removed from glycol cleavage of the sugar units and a degraded polymer arising from the periodate resistant portion of the polysaccharide is left. Finan and O'Colla obtained glyoxal - bis - phenylhydrazone, glycerosazone and 3 - arabinofuranosyl - glycerosazone. No arabinosazone or polysaccharide material was detected.

Hough and Powell (31) obtained a complex mixture of pectic substances by extracting sugar beet chips with hot lime water. Methylation studies showed that this mixture contained an araban intimately associated with a branched galactan. They carried out a Barry degradation on this polysaccharide mixture and obtained results in agreement with those of Finan and O'Colla. They also reduced the periodate oxidised polysaccharide with sodium borohydride and hydrolysed the reduced material. Estimations of the monosaccharides liberated showed that 14% of arabinose and 3.5% of galactose were present in the hydrolysates. Since the amount of arabinose remaining unoxidised by periodate was an indirect measure of the number of non-reducing end groups attached to the araban, it appeared that there were on the average five arabinose units per non-reducing end group. Since investigations of methylated arabans have suggested that one in every three arabinofuranosyl units represents a branching

point the estimate obtained by periodate oxidation studies must be held in reserve until further evidence is available.

Pectic Galactans.

Hirst, Jones, and Walder first isolated a pectic galactan from white lupin seeds (32). Advantage was taken of the fact that the carbohydrate fraction extracted with boiling aqueous sodium hydroxide (0.2%) contained a pectic material in which the percentage of uronic acid component and of araban was unusually low thus making it relatively easy to obtain a sample of galactan. Contaminating araban was removed by extraction with 70% alcohol and pectic acid removed by precipitation as the calcium salt. On methylation and hydrolysis they obtained 2, 3, 6 - tri - O - methyl - D - galactose and 2, 3, 4, 6 - tetra - O - methyl - D - galactose in the molecular ratio 100 - 1. Since the rotation of the methylated galactan was low and negative, they suggested that it consisted of a chain of 1 - 4 linked β - D - galactose units. (IX)



IX

Andrews, Hough and Jones (33) isolated a galactan from the seeds of Strychnos nux-vomica by extracting the freshly precipitated water extract of the seeds with cold Fehling's solution. Methylation and periodate oxidation studies showed that it consisted of chains of β - D - galactopyranose units, the majority of which were linked 1 \rightarrow 4, but with a small number of branching points arising from units linked through C₍₁₎, C₍₂₎, and C₍₆₎. The complex mixture of pectic substances isolated by extraction of sugar beet chips with lime water appears to consist of an araban and a branched galactan similar to the one from Strychnos nux-vomica (31).

Bouveng and Meier (34) isolated a galactan from Norwegian Spruce compression wood which contained 13% of uronic acid residues. Both glucuronic acid and galacturonic acid were present. Attempts to fractionate the polysaccharide were unsuccessful. Partial hydrolysis yielded a number of neutral oligosaccharides belonging to the same homologous series, the first member of which was obtained crystalline and identified as 4 - O - β - D - galactopyranosyl - D - galactose. The neutral part of the polysaccharide, therefore consisted of a chain of 1 \rightarrow 4 linked β - D - galactopyranose residues.

Meier (35) has isolated a still more complex galactan from beech tension wood. Both 1 \rightarrow 6 and 1 \rightarrow 4 linkages were present. Hydrolysis gave galacturonic acid, 4 - O - methyl - D - glucuronic acid and a number of other neutral sugars.

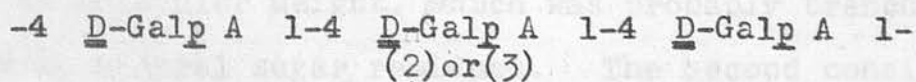
Sugar Residues other than Galacturonic Acid found in Pectin.

The view that the pectic triad consists of a mixture of three homopolysaccharides requires some modification in view of recent work. Attempts to isolate a pure galacturonan have usually been unsuccessful except where the methods of extraction have been harsh enough to effect possible hydrolysis of glycosidic linkages and degradation of one or more of the carbohydrate polymers present. In this way, Hirst and Jones isolated a pectic acid from apples which had a uronic acid content of 96% (36). The only recent case of the isolation of a pure galacturonan has been reported by Bishop (37), who obtained a pectic acid from sunflower seed heads, which contained 96% of uronic acid residues and gave only galacturonic acid on hydrolysis.

All other recent workers have reported the presence of neutral sugars in pectic acids. Aspinall and Cañas-Rodriguez (38) extracted a pectic acid from sisal flesh which contained rhamnose, arabinose, galactose and traces of 2 - O - methyl - D - xylose and 2 - O - methyl - L - fucose, as well as galacturonic acid. Since attempts to fractionate the polysaccharide were unsuccessful, they suggested that the neutral sugars were constituents of the acidic polysaccharide. Neukom et al (39) subjected a pectic acid from sugar beet to chromatography on diethylaminoethyl cellulose. They found that hydrolysis of the fractions obtained gave arabinose and galactose as well as galacturonic acid. Since they successfully separated an

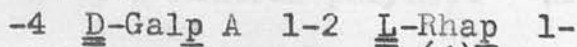
artificial mixture of an araban and a pectic acid in this way, these results strongly suggested that the arabinose and galactose were bound to the pectic acid through glycosidic linkages. Similar results were obtained for citrus pectin, apple pectin and beet pectin samples (both prepared in the laboratory and obtained commercially) by Zitko, Rosik and Vašátko (40). These workers, however, did manage to isolate pure arabinogalactans from all their samples. In the case of the commercial apple pectin sample, a pure galactan was also obtained. McCready and Gee (41) were able to separate the pectic substances from several fruits into two fractions by precipitation from aqueous solution as the copper complexes. Both fractions, however, contained the same neutral sugars as well as galacturonic acid i.e. arabinose, xylose, galactose, and rhamnose. Aspinall and Fanshawe (42) isolated a pectic acid from lucerne by extraction with ammonium oxalate. Paper chromatography showed that the hydrolysate contained substantial amounts of arabinose, galactose, and rhamnose together with traces of fucose, xylose, 2 - Q - methylxylose, and 2 - Q - methylfucose. All attempts to fractionate the polysaccharide were unsuccessful and it travelled as a single peak on chromatography on diethylaminoethyl cellulose. Methanolysis of the methylated pectic acid, followed by reduction and hydrolysis gave 2, 3, 5 - tri- and 2, 3 - di - Q - methyl, and 2 - Q - methyl - L - arabinose, 2, 3, 4, 6 - tetra -, 2, 3, 4 - tri -, and 2, 3 - di - Q - methyl, and 2 - and 3 - Q - methyl - D - galactose,

and β , 4 - di - O - methyl - and β - O - methyl - L - rhamnose, From these results they concluded that the main chain of the polysaccharide was composed of 1 \rightarrow 4 - linked α - D - galacturonic acid residues (X), some of which provided branching points. Since they had isolated 2 - O - (α - D - galactopyranosyluronic acid) - L - rhamnose as a partial hydrolysis product, this provided definite evidence that L - rhamnose residues were components of the pectic acid. The isolation of β - O - methyl - L - rhamnose suggested that some of the rhamnose residues acted as branching points (XI). The methyl ethers of L - arabinose isolated showed that some L - arabinofuranose end-groups were present in the polysaccharide, although the ring size of non-terminal residues were not defined. If all the L - arabinose residues were present in the furanose form, then the modes of linkage of this sugar would be the same (XII - XIV) as those in the arbins from pectic materials. The high proportion of the D - galactose residues which were present as end group (XV), showed that these were probably linked in some way to the framework of D - galacturonic acid residues.



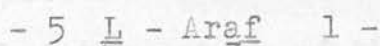
(2) or (3)

X

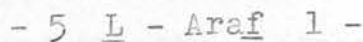


(4)

XI



XII



XIII



XIV



XV

The aldobiouronic acid 2 - $\underline{\underline{O}}$ - (α - $\underline{\underline{D}}$ - galactopyranosyluronic acid) - $\underline{\underline{L}}$ - rhamnose has been isolated by Deuel and Büchi (43) from a soluble galacturonic acid containing polysaccharide isolated from grapes. Anderson and King (44) in a study of the pectic complex from the fresh water green algae Nittella translucens obtained galactose, arabinose, xylose, and rhamnose on hydrolysis. In a study of the pectin present in the inner bark of white birch Timell and Jabbar Mian (45) isolated a pectic material containing $\underline{\underline{D}}$ - galacturonic acid, galactose, and $\underline{\underline{L}}$ - arabinose residues in the ratio 66 : 7 : 27 and traces of glucose, xylose, and rhamnose units. Attempts to resolve the material by fractionation were unsuccessful, but free boundary electrophoresis indicated the presence of three different polysaccharides. One was an $\alpha 1 \rightarrow 4$ linked polygalacturonic acid of moderate molecular weight, which was probably branched and contained no neutral sugar residues. The second consisted of galacturonic acid and neutral sugar residues, while the third appeared to be a neutral polymer. Arabinose residues were

present as non-reducing end groups in the furanose form but the nature of the galactose and the three other sugar residues could not be determined.

Enzymic Hydrolysis of Pectin.

Pectic enzymes fall into two main classes (46), (47); the pectinesterases which hydrolyse methyl ester groups and the polygalacturonases which cleave glycosidic linkages.

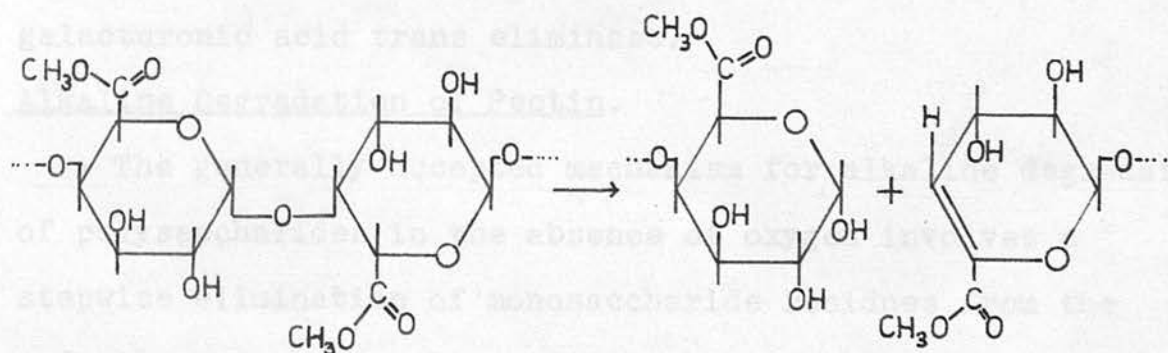
Yeast polygalacturonase causes the incomplete hydrolysis of pectic acid to a mixture of mono-, di-, and trigalacturonic acids (48), (49). The enzyme requires at least two neighbouring free carboxyl groups to permit hydrolysis of the corresponding linkage. One of the end groups in an oligo- or polyuronic chain hinders hydrolysis when the two galacturonic acid units in question are close to this end group. The further the site of the attack of the enzyme is from the interfering end group, the greater the velocity of the hydrolysis becomes until at a distance at which hindrance becomes negligible maximum velocity is attained. Thus tetragalacturonic acid gives trigalacturonic acid and galacturonic acid, trigalacturonic acid gives digalacturonic acid and galacturonic acid, while digalacturonic acid is not attacked (50).

Fungal polygalacturonases catalyse the complete breakdown of pectic acid to galacturonic acid. Di-, tri-, and tetragalacturonic acids are produced only in the initial stages of hydrolysis (51). It has been suggested that fungal polygalacturonases are complexes of three or more enzymes, one of

which hydrolyses pectin to di- and trigalacturonic acids, while another hydrolyses these to galacturonic acid (52), (53).

As yet, no case of an acidic oligosaccharide which contains a neutral sugar residue has been reported as a product of enzymic hydrolysis. Reid (54) has shown that the majority of fungal polygalacturonases cause the breakdown of pectin to galacturonic acid, arabinose, and galactose.

A third type of pectin splitting enzyme is the pectin trans eliminase described by Albersheim *et al* (55). The hydrolysis products obtained using this enzyme contained not only the usual lower uronides, but also a product possessing at the non-reducing end a double bond between C₄ and C₅ of the galacturonic acid residue. They suggested the following mechanism. (XVI)



XVI

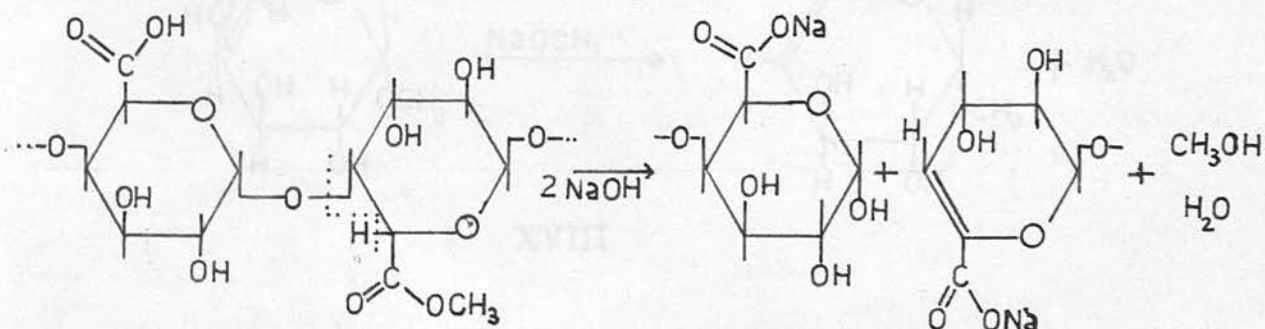
In contrast to most polygalacturonases, pectin trans eliminase will only catalyse the breakdown of pectin and

not of pectic acid. Different samples of pectin from an identical source vary greatly in their susceptibility to degradation by pectin trans eliminase (56). The ability of the enzyme to attack pectin depends not only on the degree of esterification of the polygalacturonic acid chain, but also on the length of the chain and the structural variations within the chain due to the presence of attached neutral sugars. Similar unsaturated products have been reported by Nagel and Vaughn (57) and by Starr and Moran (58). Using the Bacillus polymyxa enzyme, they studied its effect on the lower uronides as well as on the polygalacturonan. They showed that the attack was from the non-reducing end. This enzyme was more active on pectic acid than on pectin and was therefore a polygalacturonic acid trans eliminase.

Alkaline Degradation of Pectin.

The generally accepted mechanism for alkaline degradation of polysaccharides in the absence of oxygen involves a stepwise elimination of monosaccharide residues from the reducing end. The glycosidic linkages within the chain are usually extremely alkali resistant. Vollmert (13) has shown, however, that alkaline saponification of pectin at 20°C effects the splitting of a few glycosidic linkages within the chain. Launer and Tomimatsu (16) have shown

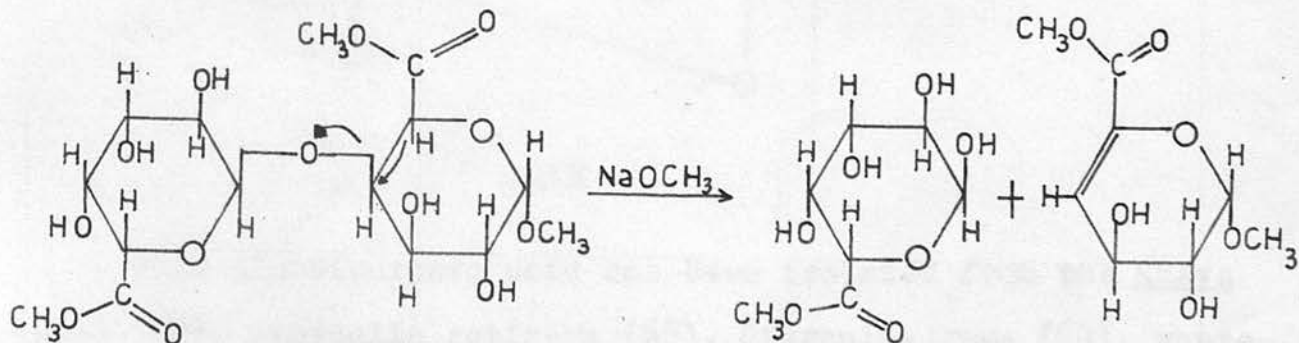
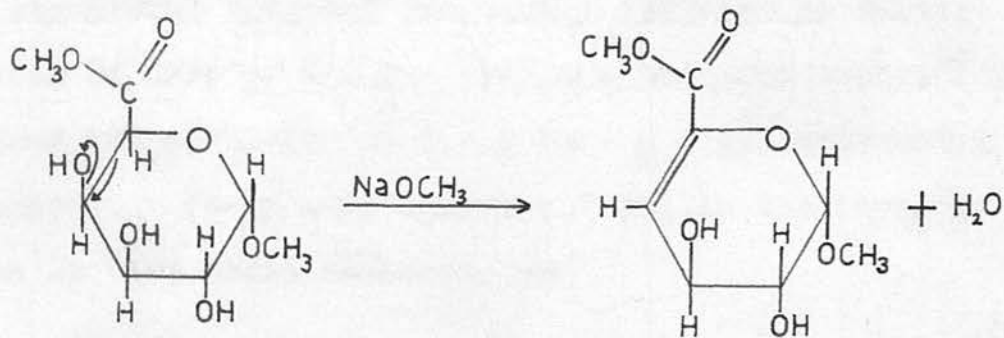
that during saponification, for approximately every eighty de-esterifications which occur, one glycosidic linkage is split. Neukom and Deuel (15) have also found this splitting of glycosidic linkages during alkaline saponification and have suggested that a " β dealkoxylation" mechanism offered the best explanation. According to this mechanism the glycosidic linkage in the β position to the ester carboxyl group of pectin is cleaved following the removal of the activated hydrogen at C_5 and the formation of a double bond between C_4 and C_5 as shown. (XVII)



XVII

The non-esterified carboxyl group at C_6 is not sufficient to activate the hydrogen at C_5 in alkali, therefore the above splitting does not occur with sodium pectate. A similar mechanism accounts for the ready breakdown of pectin on heating with buffer at pH 6.8 (59).

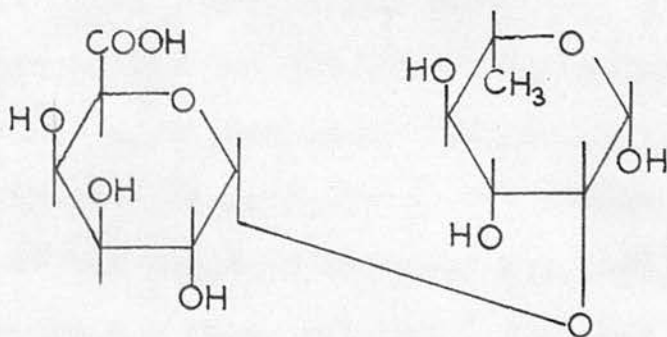
A β elimination mechanism of the type described has been used by Heim and Neukom (60), (61) to account for Δ 4, 5 unsaturated products they obtained by treatment of the methyl ester methyl glycoside of galacturonic acid and the methyl ester methyl glycoside of digalacturonic acid with sodium methoxide. (XVIII, XIX)



Other Galacturonic Acid Containing Polysaccharides.

D - Galacturonic acid frequently occurs as a component of plant gums. It has been found in gums from species of Sterculia (62), Astragalus (gum tragacanth) (63), Opuntia (64), Cochlospermum (65) and Khaya (66), where it occurs together with 4 - O - methyl - D - glucuronic acid. Other sugar residues associated with these gums are D - galactose and L - rhamnose. In addition to these, however, D - xylose, L - arabinose, and L - fucose are often found (67).

A structural fragment frequently isolated on partial hydrolysis of many of the D - galacturonic acid containing plant gums and mucilages is 2 - O - α - D - galacturonosyl - L - rhamnose. It is most commonly found in the backbone with one or both units branched. (XX)



XX

This aldobiouronic acid has been isolated from the Khaya gums (66), Sterculia setigera (68), Sterculia urens (69), where it constitutes the backbone of the gum, Cochlospermum gossypium

The polysaccharide consists of an essentially linear chain of 1→4 linked α - D - galacturonic acid residues, the majority of which carry xylose containing side chains through C₃.

GENERAL METHODS OF INVESTIGATION

Paper Chromatography

Descending chromatography was carried out on Whatman No. 1 filter paper except where otherwise stated. The following solvent systems (v/v) were used:

- (a) Ethyl acetate : pyridine : water, (20 : 6 : 3)
- (b) Ethyl acetate : acetic acid : formic acid : water, (18 : 3 : 1 : 4)
- (c) Ethyl acetate : acetic acid : formic acid : water, (18 : 3 : 5 : 9)
- (d) Butan-1-ol : ethanol : water, (4 : 1 : 5, upper layer).
- (e) Ethyl acetate : acetic acid : water, (10 : 5 : 6)
- (f) Ethyl acetate : acetic acid : water, (2 : 1 : 2, upper layer).
- (g) Benzene : ethyl acetate : water, (160 : 47 : 15, upper layer).
- (h) Methyl-ethyl ketone half saturated with water.

EXPERIMENTAL

Except where otherwise stated, chromatograms were run with standard reference sugars, air dried, and sprayed with the appropriate reagent.

Chromatographic Spray Reagents

Reducing sugars were detected by spraying with a 1.5% solution of p-aniline HCl in butan-1-ol and heating at 100° for 2 - 3 minutes.

Sugar alcohols and small amounts of reducing sugars were detected with silver nitrate reagent (73).

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(18 : 8 : 3 : 9)
- (d) Butan-1-ol : ethanol : water, (4 : 1 : 5, upper layer).
- (e) Ethyl acetate : acetic acid : water, (10 : 5 : 6)
- (f) Ethyl acetate : acetic acid : water, (2 : 1 : 2, upper layer).
- (g) Benzene : ethanol : water, (169 : 47 : 15, upper layer).
- (h) Methyl ethyl ketone half saturated with water.

Except where otherwise stated, chromatograms were run with standard reference sugars, air dried, and sprayed with the appropriate reagent.

Chromatographic Spray Reagents

Reducing sugars were detected by spraying with a 1.5% solution of p-anisidine HCl in butan-1-ol and heating at 140° for 2 - 3 minutes.

Sugar alcohols and small amounts of reducing sugars were detected with silver nitrate reagent (73).

The value R_G refers to the distance travelled by a methylated sugar relative to the distance travelled by 2, 3, 4, 6 - tetra - O - methyl - D - glucose. R_{Gal} and R_{GalA} , similarly, refer to distances travelled by sugars relative to D - galactose and D - galacturonic acid respectively. The abbreviation M_G quoted in ionophoretic examinations, refers to the distance travelled by a sugar relative to the distance travelled by D - glucose, correction being made for electro-endosmotic flow by incorporating a standard of 2, 3, 4, 6-tetra - O - methyl - D - glucopyranose.

Paper Ionophoresis (74, 75) was carried out on Whatman No.1 paper in 0.1 M borate buffer (pH10), applying a potential of 350 volts over a period of five hours. The dried papers were sprayed with p-anisidine HCl and developed at 140°.

Whatman 3 MM paper sheets (a thick paper with a medium flow rate used for chromatographic fractionation of sugar mixtures) were exhaustively extracted with water in a Soxhlet extractor. The position of the sugars was determined by cutting off side strips, which were sprayed with the appropriate chromatographic reagent. The appropriate parts of the filter sheet were then cut out and extracted with cold water, until the extract gave a blank reading with the phenol-sulphuric acid reagent.

Column Chromatography(a) Charcoal - Celite Columns.

Activated charcoal was washed several times with boiling distilled water, which was decanted. Celite (grade 545) was washed with concentrated hydrochloric acid : water (1 : 1), left overnight, filtered and washed till free of chloride. Equal weights of charcoal and Celite were mixed and packed into suitable columns as a water slurry, which was allowed to settle under gravity and thoroughly washed with distilled water. Columns of this type are used to separate mixtures of oligosaccharides (76) or mixtures of methylated sugars (77). The sugar mixtures were applied to the top of the column, allowed to soak into the absorbent and eluted with gradients of water : water-ethanol mixtures of increasing concentration. The eluate was collected in an automatic fraction collector. Aliquot portions were withdrawn from every fifth tube and examined chromatographically. Fractions containing the same sugars or mixtures of sugars were combined and evaporated to dryness. It was found that fractions were always contaminated with Celite. Methylated sugars were dissolved in A.R. acetone and the Celite filtered off. Oligosaccharides were taken up in water and evaporated to dryness several times. Celite was left on the walls of the flask.

(b) Diethylaminoethylcellulose Columns (39)

Diethylaminoethylcellulose was washed alternately with 0.1 N HCl and 0.1 N NaOH (500 ml per washing). The suspension was stirred for 10 minutes, centrifuged, and the turbid solution decanted. It was then washed three times with distilled water. A perforated disc was placed in the bottom of a column, then a layer of glass wool, followed by a layer (2 cm.) of silver sand, and finally a layer (1 cm.) of acid washed Celite. The cellulose was added as a water slurry and packed under air pressure. A layer of glass wool was placed on top of the column. The initial generation and subsequent regenerations of the cellulose to the phosphate form was done by eluting the column with two litres of 0.5 M sodium dihydrogen phosphate (adjusted to pH6.1 with sodium hydroxide), followed by equilibration with one litre of 0.005 M sodium dihydrogen phosphate solution (pH6.1). The polysaccharide mixture in a small volume of water was absorbed onto the top of the column and left overnight.

(c) Sephadex Columns.

Dry sephadex (G - 25) was stirred in a beaker with a dilute salt solution. After sedimentation and decantation to remove the finest gel particles, the suspension was transferred to a suitable column through a funnel. The column was filled with water and the suspension was added with stirring,

the gel grains sedimenting slowly to the bottom of the tube. When a layer (2 - 5 cm.) had formed, the stopcock was carefully opened to allow a slow flow of water. When all the gel grains had settled, a circular paper was placed on the top of the bed and the column thoroughly washed with water. Polysaccharide mixtures in a small volume of water were applied to the top of the column and left overnight.

Squat Charcoal Columns.

A filter paper was placed in a Buchner funnel of appropriate size and covered with a layer of paper pulp. Purified charcoal was added as a water slurry and allowed to settle under gravity. Oligosaccharides were eluted from the resultant squat column in a stepwise manner by increasing concentrations of ethanol-water mixtures.

Ion Exchange Resins.

In general, solutions were deionised by passing them in turn through columns of Amberlite resins IR - 120 (H) and IR - 4B (OH) of appropriate size. Columns of anion exchange resin were used to separate acidic sugars from neutral sugars, and to fractionate mixtures of acidic sugars.

Amberlite ion exchange resin (CG - 45) was suspended in distilled water and packed into a column. The column was eluted with 4% sodium hydroxide solution (6 bed volumes) and

washed with carbon dioxide free water. The resin was converted to the formate form with 10% formic acid and washed with carbon dioxide free water. Sugar solutions were applied to the column and left overnight. Neutral sugars were eluted with water, and acidic sugars were fractionated by gradient elution with formic acid.

Gas-Liquid Partition Chromatography (78), (79).

This was effected using a "Pye Argon Chromatograph" according to the procedure of Bishop and Cooper. Separations were made on the following columns (120 x 0.5 cms.) at gas flow rates of 80 - 100 ml/min. ; (a) 15% by weight of butan - 1, 4 - diol succinate polyester on acid washed Celite (80 - 100 mesh.) at 175°, (b) 10% by weight of polyphenyl ether (M - bis - (M - phenoxy phenoxy benzene) on acid washed Celite at 200°.

Evaporations were carried out under reduced pressure at 40°.

Small Scale Hydrolysis were effected in the following ways;

(a) N-sulphuric acid hydrolysis : samples 5 mg. for polysaccharides, 1 - 3 mg. for oligosaccharides were heated with N-sulphuric acid (2 ml.) at 100° in a sealed tube for an appropriate time (3hr. for oligosaccharides, and up to 16 hr. for polysaccharides). The solutions were neutralised with barium carbonate, filtered, the barium ions were removed with

Amberlite resin IR - 120 (H) and the solution was concentrated.

(b) Formic acid hydrolysis : samples (10 - 20 mg.) were heated with acid (2 ml.) at 100° for 12 hr. in a sealed tube, the formic acid was removed by repeated distillation with water under reduced pressure and the resulting formyl esters were hydrolysed with N sulphuric acid at 100° for 2 hr.

Methanolysis of methylated polysaccharides was done by refluxing with methanolic hydrogen chloride (4%) for 18 hr. Methyl glycoside formation of sugars was achieved by refluxing for 6 hr. Acid was neutralised with silver carbonate, which was filtered off, extracted with chloroform and the extract and filtrate combined and concentrated. It was essential not to evaporate to dryness samples which were to be examined by vapour phase chromatography, because of the extreme volatility of the methyl glycosides of some methylated sugars.

Reductions of aldobiouronic acids were achieved by formation of the methyl ester methyl glycoside, which was dissolved in water and an equal weight of sodium borohydride added.

After about 18 hours excess hydride was destroyed and sodium ions were removed by shaking the solution with Amberlite resin IR - 120 (H). The filtrate was evaporated to dryness. Borate ions were removed by repeated evaporation with methanol.

Melting points were determined either by using a Kofler hot stage microscope or in a capillary tube in a Gallenkamp melting point apparatus.

Methoxyl determinations were carried out by the semi-micro Zeisel method (80).

Estimation of sugars was carried out by the phenol - sulphuric acid method (81).

Sugar solution (1 ml.) containing 10 - 70 mg. sugar was pipetted into a tube (15 x 2 cm.). Phenol (1 ml., 5% solution of A.R. phenol in water) reagent was added, followed by analar concentrated sulphuric acid (5 ml.) from a fast delivery pipette. The solution was mixed, and left at room temperature for 45 minutes. Absorbance was measured at 490 m μ for hexoses and 480 m μ for pentoses and uronic acids on a "Unicam" S.P. 500 spectrophotometer.

Uronic acid determinations were carried out on small quantities of material (ca. 5 mg.) using carbazole-sulphuric acid reagent (82). Where more material was available (50 - 100 mg.) or a more accurate result required the estimation was done by decarboxylation and titrimetric determination of the carbon dioxide evolved (83).

Nitrogen determinations were carried out by the micro-Kjeldahl method.

Optical rotations were observed at $18^{\circ} \pm 2^{\circ}$ in the stated solvent.

Acetyl determinations. The acetyl content of an acetylated polysaccharide was estimated by saponifying with sodium hydroxide, acidifying and distilling off the acetic acid, which was titrated with standard alkali (84).

Periodate Uptake was determined spectrophotometrically by the method of Aspinall and Ferrier (85).

Polysaccharide (ca. 10 mg.) was dissolved in 0.03 M sodium metaperiodate and incubated in the dark at 35°. Aliquots (1 ml.) were withdrawn at suitable intervals, diluted 250 times with distilled water and the optical densities of the resulting solutions measured in the "Unicam" S.P.500 spectrophotometer at 22.5 μ against a distilled water blank and compared with these obtained for the original solution of periodate (diluted 250 times) and of an equimolecular solution of iodate.

Periodate oxidation of methylated sugars (86). The sugar (1 - 2 mg.) was dissolved in 0.5 M sodium metaperiodate solution (0.2 ml.) and left at 0° for one hour. Excess periodate was destroyed by the addition of ethylene glycol (1 drop) and the solution made alkaline to phenolphthalein with 0.5 N sodium hydroxide solution. The solution was evaporated to dryness, extracted with acetone, concentrated and examined chromatographically in solvent D.

Methylations with Silver Oxide and Methyl Iodide (87)

Methyl iodide was purified by refluxing with silver oxide and distilling in a dry system.

Silver oxide was prepared by the addition of sodium hydroxide (40 g. in 500 ml.) to a solution of silver nitrate (170 g. in 500 ml.) and washing the product with cold water (4 L.) and hot water (1 L.). The silver oxide was filtered off, ground with acetone (1 L.), filtered, and washed with ether. The silver oxide was dried in a vacuum desiccator and stored in the dark.

Partially methylated polysaccharide was dissolved in methyl iodide and silver oxide added in tenths to the refluxing mixture over 6 hours. Refluxing was continued a further 12 hours and the suspension cooled and filtered. The silver residues were extracted continuously with chloroform. The methyl iodide and chloroform solutions were combined, concentrated and poured into light petroleum (60 - 80°, 20 : 1). The methylated material which separated was centrifuged off.

The methylation was repeated until no further increase in methoxyl content was observed.

Small Scale Methylations of Oligosaccharides (88).

Oligosaccharides (1 - 2 mg.) were dissolved in dimethylformamide (0.2 ml.), methyl iodide (0.2 ml.) and silver oxide

(200 mg.) added and the flask shaken in the dark for 18 hours. Silver oxide was filtered off, extracted with chloroform, and the filtrate and extracts combined and concentrated. Dimethylformamide was removed with a high vacuum pump at room temperature. The methylated oligosaccharide was methanolised and examined by gas chromatography.

Aniline Derivatives of methylated sugars were prepared by refluxing the sugars (10 mg.) with equimolar quantities of freshly distilled aniline in dry ethanol (5 ml.) for 30 minutes in an atmosphere of CO_2 . Removal of the solvent left a syrup, which was crystallised and recrystallised from the given solvent.

Aldonolactones were prepared by dissolving the sugar (10 - 100 mg) in water (5 ml.), adding bromine (10 - 20 drops), and leaving the mixture in the dark at room temperature for 3 days. Excess bromine was removed by aeration and the solution neutralised with silver carbonate. Silver ions were removed by treatment with hydrogen sulphide and the filtrate evaporated to dryness. The lactone was crystallised by slow evaporation of an ethanolic solution.

Purification of organic solvents for column chromatography and for reactions where specified was effected by the methods described in the literature (89).

Commercial Pectin

The pectin of the orange is a commercial grade of pectin. It is a natural product of the orange fruit and is used in the food industry as a thickening agent. The pectin is obtained from the orange peel and is purified by various methods. The pectin is a natural product of the orange fruit and is used in the food industry as a thickening agent. The pectin is obtained from the orange peel and is purified by various methods. The pectin is a natural product of the orange fruit and is used in the food industry as a thickening agent. The pectin is obtained from the orange peel and is purified by various methods.

SECTION I

CITRUS PECTIN

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Commercial Citrus Pectin.

The citrus pectin examined was a commercial sample obtained from Eastman Organic Chemicals. Examination of the sample by paper chromatography without previous hydrolysis showed that free glucose was present. This was removed by dialysis against running tap water. The solution was then deionised with Amberlite resins IR-120 (H) and IR-4B (OH), concentrated and freeze dried. The citrus pectin obtained had $[\alpha]_D + 178^\circ \pm 5^\circ$ (C. 0.7 in water) [Found : uronic anhydride (by decarboxylation), 44.2%; OMe, 3.9%; N, 0.42; ash, 4.1%]. Chromatography of the hydrolysate in solvent systems (A), (B) and (D) showed the presence of galactose, arabinose, glucose, rhamnose, and acidic oligosaccharides.

Since the presence of glucose was unexpected, an iodine staining reaction was carried out to test for the presence of starch. A solution of 0.2% iodine in 2% potassium iodide acidified with hydrochloric acid, was added to a solution of the freeze dried polysaccharide in water. As no blue colour was observed in the solution, starch was absent. The glucose in the hydrolysate, therefore, must have arisen some other way.

Attempted Fractionations of Citrus Pectin.(a) Extraction with Aqueous Ethanol.

Citrus pectin was extracted with boiling ethanol-water

(7 : 3) for two days, cooled and centrifuged. The polysaccharide, which was precipitated from the supernatant by the addition of the concentrated solution to 95% aqueous ethanol (4 vol.), was removed at the centrifuge, dissolved in water, concentrated to remove ethanol and isolated by freeze drying. It contained only 0.5% by weight of the original polysaccharide and had $[\alpha]_D + 3^\circ$ (C.0.8 in water) [Found : uronic anhydride (carbazole - sulphuric acid method), 4.8%]. Chromatography of the hydrolysate in solvent system (A) showed galactose and arabinose as major constituents in approximately equal quantities together with traces of galacturonic acid and rhamnose.

(b) Extraction with Saturated Lime Water.

Freeze dried polysaccharide (10 g.) was dissolved in water (300 ml.) at 65° on a water bath. Calcium hydroxide (7.5 g.) was added with stirring. The suspension, which became yellow after a few minutes, was stirred for two hours, cooled and centrifuged. Soluble material (100mg), isolated after treatment of the supernatant with Amberlite resins IR-120 (H) and IR -4B (OH) and freeze drying, had $[\alpha]_D -7^\circ$ (C. 0.5 in water) [Found : uronic anhydride (carbazole-sulphuric acid method), 3.9%]. Chromatography of the hydrolysate in solvent system (A) gave galactose and arabinose

in approximately equal quantities and traces of rhamnose and galacturonic acid.

(c) Precipitation of Calcium Pectate.

Calcium chloride solution (5% w/v in water) was added dropwise with stirring to a solution of citrus pectin (5% w.r.t. polysaccharide) until precipitation of calcium pectate was complete. The isolated calcium pectate was identical to the original polysaccharide having $[\alpha]_D + 182^\circ$ (C. 0.8 in water) [Found : uronic anhydride (decarboxylation), 44.0%]. The supernatant did not contain a significant amount of polysaccharide material (estimated using phenol-sulphuric acid reagent).

Fractionation on Diethylaminoethylcellulose (39).

Citrus pectin (340 mg.) was dissolved in water (20 ml.) and an aliquot portion removed and assayed for total carbohydrate (estimated as galactose) by the phenol-sulphuric acid method. This indicated the presence of 336 mg. of polysaccharide in the original solution. The polysaccharide solution was then applied to a column of diethylaminoethylcellulose (50 g. 36 x 4 cm.) in the phosphate form. Elution was effected with 0.025 M-(600 ml.), 0.05 M-(500 ml.), 0.10 M-(500 ml.), and 0.25 M sodium dihydrogen phosphate (NaH_2PO_4) (pH 6, 1 l.), followed by a gradient of sodium

Fig. III

FRACTIONATION OF CITRUS PECTIN ON
DEAE-CELLULOSE.

Polysaccharide conc.

in mg. per 20ml.

5

10

0.0025M

50

0.005M

Fraction no.

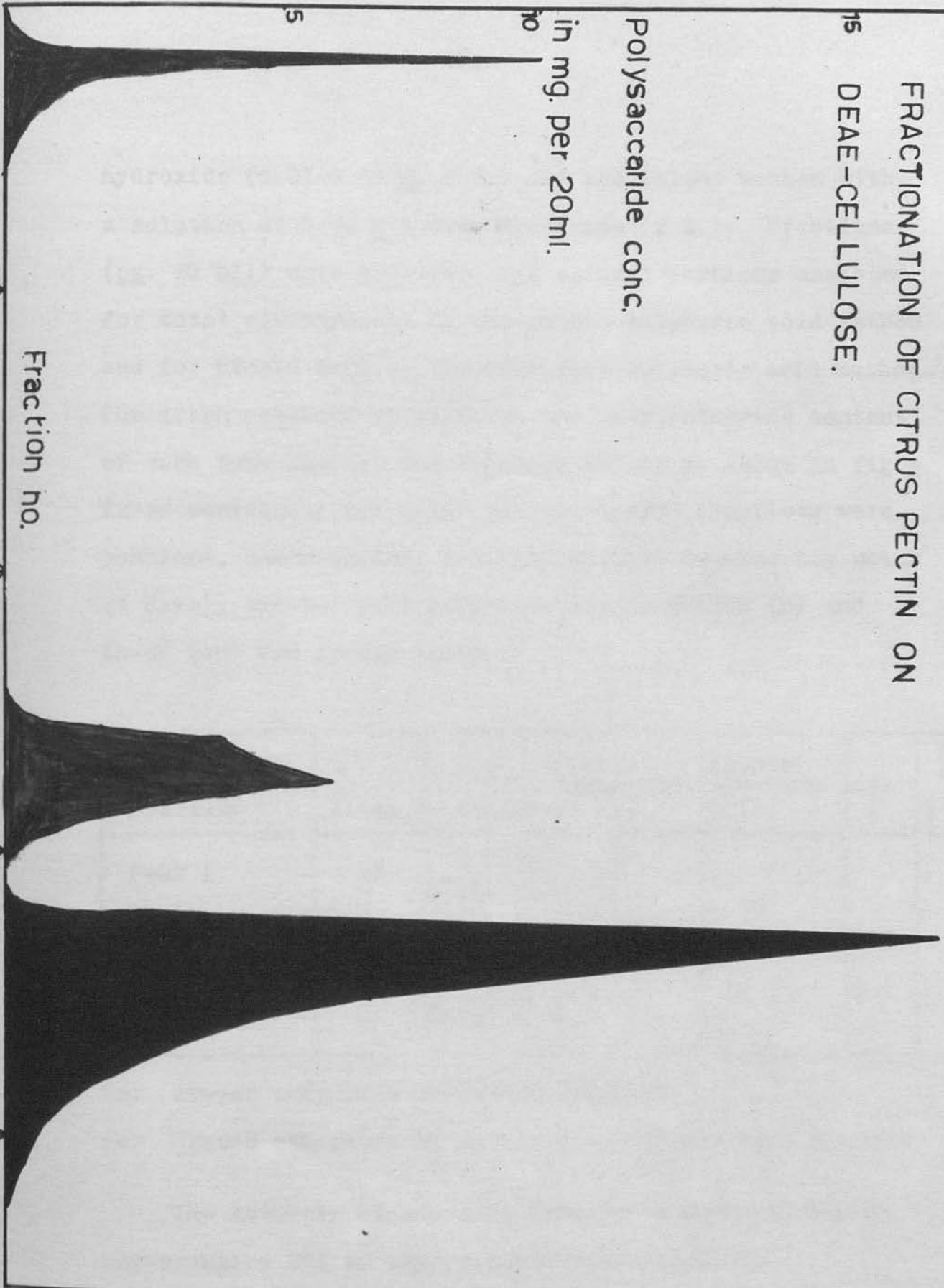
100

0.010M

150

0.025M

200



hydroxide (0.01-0.30 M, 2 ℓ.) and the column washed with a solution of 0.30 M sodium hydroxide (2 ℓ.). Fractions (ca. 20 ml.) were collected and aliquot portions analysed for total carbohydrate by the phenol-sulphuric acid method and for uronic acid by the carbazole-sulphuric acid method. The graph obtained by plotting the polysaccharide content of each tube against the fraction number is shown in fig. III. Tubes containing the major polysaccharide fractions were combined, concentrated, dialysed against running tap water (4 days), treated with Amberlite resins IR-120 (H) and IR-4B (OH) and freeze dried.

Polysaccharide Fraction	Wt(mg.)	Eluant	Uronic Anhydride % (a)	Uronic Anhydride % (b)	OMe %	$[\alpha]_D$ $\pm 5^\circ$
Peak I	18	0.025M NaH ₂ PO ₄	< 5			+ 3°
Peak II	46	0.10M NaH ₂ PO ₄	44.5	44.0	8.5	+ 181°
Peak III	218	0.25M NaH ₂ PO ₄	45.7	48.5	1.1	+ 179°

(a) Uronic anhydride by decarboxylation.

(b) Uronic anhydride by carbazole-sulphuric acid reagent.

The recovery of material from the column was 282 mg. representing 85% of the polysaccharide applied.

Samples (5 mg.) of each peak were hydrolysed and examined in solvent systems (A) and (B).

Fraction	Glucose	Galactose	Arabinose	Rhamnose	Galacturonic Acid
I	+	+	+		tr.
II	+	+	+	+	+
III	+	+	+	+	+

tr. = trace.

The polysaccharides from peaks II and III which were eluted close together (fig.III) contained the same sugars present in the same relative proportions (by visual estimation from the chromatograms). With the exception of the methyl ester contents, the physical constants of the two polysaccharides were similar. It has been shown that the degree of esterification is an important factor in determining at what stage a polysaccharide is eluted from a diethylaminoethylcellulose column (90).

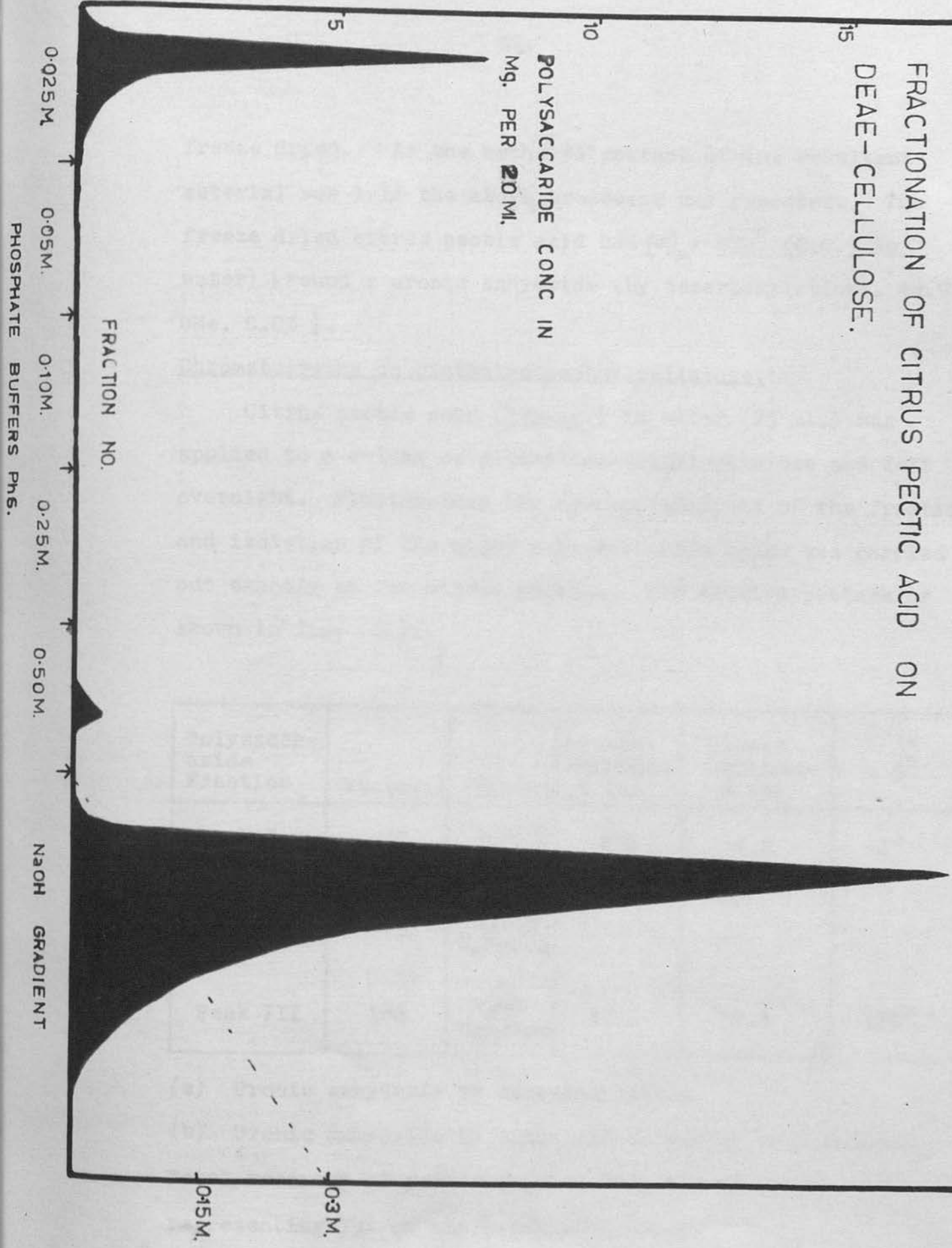
Saponification of Citrus pectin.

Citrus pectin (0.1%) in sodium hydroxide solution (0.05N) was saponified at 25° for 90 minutes. Cations were removed with Amberlite resin IR-120 (H) and the solution

Fig. IV

FRACTIONATION OF CITRUS PECTIC ACID ON
DEAE-CELLULOSE.

POLYSACCARIDE CONC IN
MG. PER 20 ML.



freeze dried. As the methoxyl content of the resultant material was 1.1% the above treatment was repeated. The freeze dried citrus pectic acid had $[\alpha]_D + 179^\circ$ (C.O.7 in water) [Found : uronic anhydride (by decarboxylation), 44.9%; OMe, 0.0%].

Chromatography on Diethylaminoethylcellulose.

Citrus pectic acid (320 mg.) in water (25 ml.) was applied to a column of diethylaminoethylcellulose and left overnight. Elution from the column, analysis of the fractions, and isolation of the major polysaccharide peaks was carried out exactly as for citrus pectin. The elution pattern is shown in fig. (IV).

Polysaccharide Fraction	Wt. (mg.)	Eluant	Uronic Anhydride % (a)	Uronic Anhydride % (b)	$[\alpha]_D \pm 5^\circ$
Peak I	40	0.025M NaH ₂ PO ₄	< 5	4.2	-1°
Peak II	3	0.25M NaH ₂ PO ₄			
Peak III	186	NaOH Gradient	45.2	50.1	175°

(a) Uronic anhydride by decarboxylation.

(b) Uronic anhydride by carbazole-sulphuric acid reagent.

Total recovery of polysaccharide from the column was 239 mg. representing 75% of the material applied.

Samples (5 mg.) of each peak were hydrolysed and examined in solvent systems (A) and (B).

Fraction	Glucose	Galactose	Arabinose	Rhamnose	Galacturonic Acid
I	tr.	+	+		tr.
II	tr.	+	+		tr.
III	+	+	+	+	+

tr. = trace

Fractionation of Citrus Pectin as the Copper Salt.

A variety of pectic substances have been fractionated by precipitation from aqueous solution with cupric ion and thus freed from copper soluble polysaccharides (41).

Citrus pectin (100 g.) was dissolved in water (10 l.) and 15% copper sulphate solution (500 ml.) was added slowly, dropwise and with stirring. The resulting precipitate was removed by centrifugation, dissolved in water and the pH was adjusted to 2 with dilute hydrochloric acid. Polysaccharide was then precipitated by the addition of a solution of 90% ethanol-water (2 vol.). The gelatinous precipitate was strained with muslin, pressed dry and copper ions were removed by treating it several times with a solution of 70% ethanol-water containing 1% of hydrochloric acid.

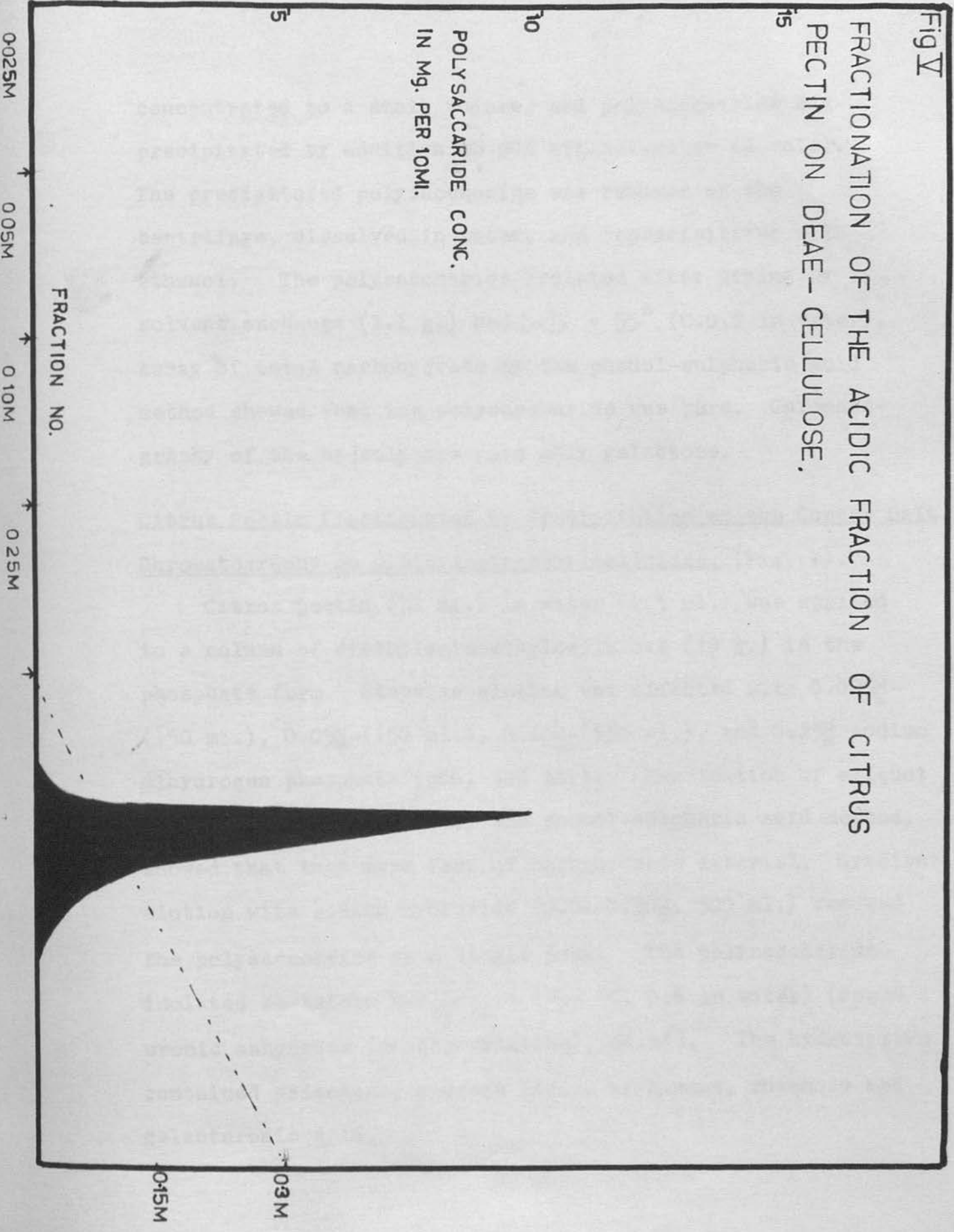
Finally, the precipitate was washed three times with 70% ethanol-water, dissolved in water and freeze dried. The freeze dried polysaccharide had $[\alpha]_D^{20} + 181^\circ$ (C.0.8 in water) [Found : uronic anhydride (decarboxylation), 43.8%]. Chromatography of the hydrolysate in solvent system (A) showed galactose, glucose (tr.), arabinose, rhamnose and galacturonic acid.

The supernatant left after removal of the polysaccharide precipitated as the copper salt, was adjusted to pH 7 with sodium hydroxide (350 ml. 2N) and a blue precipitate which separated was removed at the centrifuge. Hydrolysis and chromatographic examination of this material, showed that it did not contain any polysaccharide. The supernatant was treated with cation and anion exchangers, concentrated and polysaccharide was precipitated by the addition of ethanol (3 vol.). The precipitate (5.2 g.) was removed by centrifugation and dried by solvent exchange with ethanol-ether. Estimation of total carbohydrate (as D - galactose) by the phenol-sulphuric acid method showed that the fraction was heavily contaminated with ionic material. Chromatography of the hydrolysate in solvent system (A) showed that galactose was present as the major constituent with traces of arabinose and glucose. An aqueous solution of the polysaccharide was treated with ion exchange resins,

Fig V

FRACTIONATION OF THE ACIDIC FRACTION OF CITRUS
PECTIN ON DEAE-CELLULOSE.

POLYSACCHARIDE CONC.
IN MG. PER 10ML.



concentrated to a small volume, and polysaccharide was precipitated by addition to 90% ethanol-water (4 vol.). The precipitated polysaccharide was removed at the centrifuge, dissolved in water, and reprecipitated with ethanol. The polysaccharide isolated after drying by solvent exchange (1.1 g.) had $[\alpha]_D + 55^\circ$ (C.0.2 in water). Assay of total carbohydrate by the phenol-sulphuric acid method showed that the polysaccharide was pure. Chromatography of the hydrolysate gave only galactose.

Citrus Pectin Fractionated by Precipitation as the Copper Salt. Chromatography on Diethylaminoethylcellulose. (Fig. V).

Citrus pectin (50 mg.) in water (2.5 ml.) was applied to a column of diethylaminoethylcellulose (10 g.) in the phosphate form. Stepwise elution was effected with 0.025M- (150 ml.), 0.05M- (150 ml.), 0.10M- (150 ml.), and 0.25M sodium dihydrogen phosphate (pH6, 300 ml.). Examination of aliquot portions of the eluants by the phenol-sulphuric acid method, showed that they were free of carbohydrate material. Gradient elution with sodium hydroxide (0.01-0.30M, 300 ml.) removed the polysaccharide as a single peak. The polysaccharide isolated as before had $[\alpha]_D + 179^\circ$ (C. 0.6 in water) [Found : uronic anhydride (decarboxylation), 44.0%]. The hydrolysate contained galactose, glucose (tr.), arabinose, rhamnose and galacturonic acid.

Partial Hydrolysis of Citrus Pectin.(a) With N Sulphuric Acid.

Citrus pectin (25 g. in 500 ml. water), purified by precipitation as the copper salt, was heated to 100° in a water bath. 2 N Sulphuric acid (500 ml.) at 100° was added and the solutions mixed. After two hours a degraded polysaccharide separated from the solution. When the hydrolysis was complete (7 hr.), the degraded polysaccharide was removed by centrifugation and the supernatant was added to acetone (1 vol.). As no further degraded material separated, the solution was concentrated to remove acetone, and the sulphuric acid was neutralised with methyl di-n-octylamine (5% v/v in chloroform). Treatment with Amberlite resin IR-120 (H) removed cations from the solution, which was then concentrated to a syrup (7.3 g). A further 0.8 g. of syrup was obtained by repeating the hydrolysis on the degraded polysaccharide.

The degraded polysaccharide (13 g.) had $[\alpha]_D^{25} + 216^\circ$ (as the ammonium salt, C.0.95 in water). Hydrolysis with formic acid and chromatography in solvent systems (A) and (B) showed that only galacturonic acid was present.

The syrup (8.1 g.) was dissolved in water and applied to a column of Amberlite resin CG-45 (10 x 45 cm) in the formate form. Elution with water gave a mixture of neutral

sugars (3.9 g.) which was not examined further.

Acidic sugars were eluted with gradients of (i) 0.0-0.25 N - (1 l.), (ii) 0.25 - 0.50 N - (1.5 l.), (iii) 0.50 - 0.75 N - (1.5 l.), (iv) 0.75 - 1.50 N - (1.5 l.), (v) 1.50 - 2.00 N formic acid (1.5 l.), followed by 3 N formic acid (5 l.).

The following fractions were obtained:

Fractions	Wt(mg.)	R _{Gal} in Solvent System (B).						Major Hydrolysis
		1.0	0.75	0.61	0.51, 0.48	0.21	<0.20	Products
1.	130	+	+			+		Gal A, Rha.
2.	172	+	+			+		Gal A, Rha.
3.	1,330	+	+			+		Gal A, Rha.
4.	214	+	+	+		+	+	Gal A, Rha, Gal, Gluc.
5.	123	+	+	+	+	+	+	Gal A, Rha, Gal, Ara.
6.	224	+		+	+	+	+	Gal A, Glu, Ara, Gal.
7.	377	+		+	+	+	+	Gal A, Glu, Ara, Gal.
8.	133	+		+	+	+	+	Gal A, Glu, Ara, Gal.
9.	61	+		+	+	+	+	Gal A.
10.	260	+			+	+	+	Gal A.

The above mixtures were further fractionated by filter

sheet chromatography using Whatman No. 3MM paper in solvent system B. Fractions with $R_{\text{GalA}} > 0.2$ were successfully separated in this way. Oligosaccharides with $R_{\text{GalA}} < 0.2$ were eluted from the paper, concentrated and separated by filter sheet chromatography on Whatman No. 3MM paper in solvent system (C).

Sugar I.

This sugar (1.36 g.), R_{GalA} 1.0 in solvent systems (B), (C), and (E) had $[\alpha]_D + 58^\circ$ (C.0.8 in water) and was characterised as D - galacturonic acid by conversion into the 2, 5 - dichloro-phenylhydrazone, m.p. and mixed m.p. 180-182°.

Sugar II.

This sugar (161 mg.), R_{GalA} 0.75 in solvent system B, M_G 0.60 (borate buffer pH 10), had $[\alpha]_D + 84^\circ$ (C.0.51 in water) and gave on hydrolysis galacturonic acid and rhamnose.

A sample (5 mg.) was methanolysed, reduced with sodium borohydride and hydrolysed. Chromatography of the hydrolysate gave galactose and rhamnose.

A sample (2 mg.) was dissolved in dimethyl formamide (0.2 ml.), methyl iodide (0.2 ml.) and silver oxide (200 mg.) added and the flask shaken for 20 hours. Methanolysis of the methylated disaccharide and examination of the products by gas chromatography showed that the major components had the retention times of the methyl glycosides of 3, 4 - di - O - methyl - L - rhamnose and

2, 3, 4 - tri - Q - methyl - D - galacturonic acid.

Dimethyl sulphate (1 ml.) and 30% aqueous sodium hydroxide (1 ml.) were added dropwise with vigorous stirring during two hours to the sugar (45 mg.) in water (2 ml.). Further additions of dimethyl sulphate (5 ml.) and sodium hydroxide (10 ml.) were made over eight hours. Additions of dimethyl sulphate (6 ml.) and sodium hydroxide (12 ml.) were made on four successive days. The reaction mixture was heated on a boiling water bath (30 min.), cooled and adjusted to pH 9 with dilute sulphuric acid. Sodium sulphate was precipitated with methylated spirits and removed by filtration. The sodium sulphate was thoroughly washed with methylated spirits and the filtrate and washings combined. The solution was made just alkaline, concentrated to a small volume, acidified with sulphuric acid and extracted with chloroform. The dried extract (anhydrous sodium sulphate) furnished a syrup (22 mg.) which crystallised from chloroform-light petroleum (100 - 120°) to give 2 - Q - (2, 3, 4 - tri - Q - methyl - D - galactopyranosyluronic acid) - 3, 4 - di - Q - methyl - L - rhamnopyranoside as the crystalline dihydrate, m.p. and mixed m.p. 68° (hot stage), m.p. and mixed m.p. 114 - 116° (capillary), $[\alpha]_D^{20} + 91^\circ$ (C.1.1 in chloroform). The X-ray powder photograph was identical to that of an authentic sample.

Sugar III

This sugar (15 mg.), R_{Gala} 0.61 in solvent system (B) stained red with aniline oxalate both in the visible and in ultra violet light. Hydrolysis showed galacturonic acid, arabinose, xylose and glucose. Attempted small scale methylations were unsuccessful.

Sugars IV and V

These sugars (35 mg.) ran together and had R_{Gala} 0.46 and 0.51 in solvent system (B). The faster sugar stained red with aniline oxalate, while the slower one stained yellow. They did not separate in any of a variety of solvent systems tried, nor on electrophoresis. A number of experiments were carried out using diethylaminoethylcellulose on thin layer plates in solvent systems involving, methanol : acetic acid : water, methanol : formic acid : water, and methanol : hydrochloric acid : water, in differing proportions. No separation was achieved.

Hydrolysis on a boiling water bath for 4 hours showed galacturonic acid, galactose, arabinose, xylose, and glucose. Methanolysis, reduction with sodium borohydride and hydrolysis gave galactose and smaller amounts of glucose, arabinose, and xylose. Attempted small scale methylations were unsuccessful.

Sugar VI

This sugar (310 mg.), R_{Gala} 0.2 in solvent system (B)

was chromatographically indistinguishable from digalacturonic acid in solvent systems (C), (E), and (F), in which it had R_{GalA} values of 0.59, 0.50, and 0.66 respectively and on electrophoresis in borate buffer in which it had M_{G} 1.0 and M_{GalA} 0.8. On hydrolysis only galacturonic acid was obtained.

An aqueous solution of the sugar was neutralised with calcium carbonate and filtered. Ethanol (1.5 vol) was added to the solution and the precipitated salt was removed at the centrifuge and dried by solvent exchange with ethanol-ether. It had $[\alpha]_D^{20} + 113^\circ$ (C.1.3 in N hydrochloric acid).

Attempts to methylate the sugar were unsuccessful.

Sugar VII

This sugar (50 mg.), R_{GalA} 0.05 in solvent system (B) gave only galacturonic acid on hydrolysis and was chromatographically indistinguishable from trigalacturonic acid in solvent systems (C), (E), and (F) in which it had R_{GalA} values of 0.21, 0.27, and 0.48 respectively and on electrophoresis in borate buffer (pH 10) in which it had M_{G} 0.98. It gave a calcium salt which had $[\alpha]_D^{20} + 142^\circ$ (C.0.36 in N hydrochloric acid).

Partial hydrolysis with N sulphuric acid gave a syrup which contained sugars chromatographically identical to digalacturonic acid and galacturonic acid.



Sugar VIII

This sugar (15 mg.) gave only galacturonic acid on hydrolysis and had $R_{\text{GalA}} = 0.33$ in solvent system (F). (49) Partial hydrolysis with N sulphuric acid gave a syrup containing sugars chromatographically identical to trigalacturonic acid, digalacturonic acid, and galacturonic acid. It formed a calcium salt which had $[\alpha]_D +159^\circ$.

(b) Hydrolysis with 0.5 N Sulphuric Acid.

Citrus pectin purified by fractionation as the copper salt was partially hydrolysed with 0.5 N sulphuric acid and the hydrolysate examined for neutral oligosaccharides in solvent system (A). These were present in the greatest quantities after 45 minutes.

Citrus pectin (40 g.) was heated in 0.5 N sulphuric acid (2 L.) on a boiling water bath for 45 minutes. Degraded polysaccharide was removed by centrifugation and the supernatant was added to acetone (1 vol). A further quantity of degraded polysaccharide which separated from the solution was removed at the centrifuge. After concentration of the supernatant to remove acetone, the sulphuric acid was neutralised with methyl di-n-octylamine (5% v/v in chloroform). The solution was treated with cation and anion exchangers and evaporated to dryness to give a clear syrup (260 mg.) which contained xylose, arabinose, glucose, galactose, and a series of oligosaccharides.

The syrup was absorbed on charcoal-Celite (1 : 1, 20 x 2.5 cm.); elution with water (1 L.) gave a mixture of monosaccharides (210 mg.) which was not further examined. Gradient elution was carried out with 0.0 - 5.0% - (2 L.), 5.0% - (2 L.), 5.0 - 10.0% - (2 L.), 10.0% - (2 L.), and 10 - 30.0% aqueous ethanol (2 L.). Fractions (10 ml.) were collected and every fifth tube examined chromatographically in solvent system (A). Tubes containing the same sugars were bulked giving the following fractions:

Fraction No.	Wt.(mg.)	R _{Gal} (in solvent system (A))	M _G (borate buffer pH 10)
I	15	0.58	0.48
II	12	0.28	0.40
III	4	0.15	0.36

Oligosaccharide I.

This sugar gave only galactose on hydrolysis and crystallised on seeding with 4 - Q - β - D - galactopyranosyl - D - galactose. On recrystallisation from ethanol-water it had m.p. and mixed m.p. 203°, $[\alpha]_D + 68^\circ$ (equil. value), (C. 2.5 in water). The X-ray powder photograph was identical to that of an authentic sample. Examination of the methanolysis products of the methylated disaccharide by gas chromatography

showed that the main components had the retention times of the methyl glycosides of 2, 3, 4, 6 - tetra -, and 2, 3, 6 - tri - Q - methyl - D - galactose.

Oligosaccharide II

This sugar (12 mg.), which was a clear syrup, had $[\alpha]_D + 56^\circ$ (C. 1.8 in water). Hydrolysis gave only galactose. Examination of the methanolysis products of the methylated trisaccharide showed that the main components had the retention times of the methyl glycosides of 2, 3, 4, 6 - tetra -, and 2, 3, 6 - tri - Q - methyl - D - galactose.

Chromatographic examination of the products formed on partial hydrolysis with 0.5 N sulphuric acid showed galactose and galactobiose.

Oligosaccharide III

This sugar (4 mg.), which was a clear syrup, had $[\alpha]_D + 51^\circ$ (C. 0.4 in water) and gave only galactose on hydrolysis. Examination of the methanolysis products of the methylated sugar showed that the main components had the retention times of the methyl glycosides of 2, 3, 4, 6 - tetra - and 2, 3, 6 - tri - Q - methyl - D - galactose.

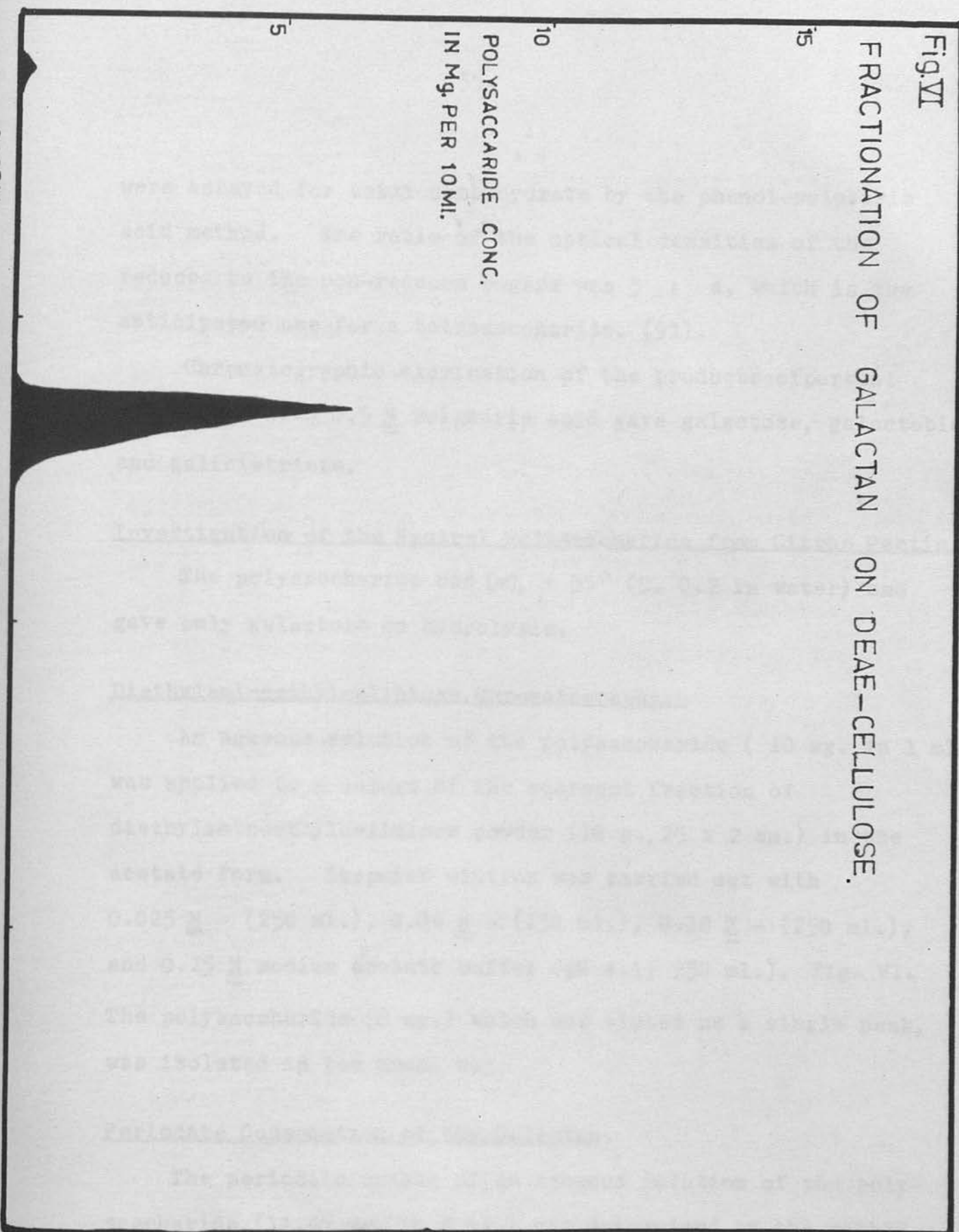
An aliquot portion (ca. 0.2 mg.) was dissolved in water (2 ml.) and the resulting solution was divided into two 1 ml. portions. To one of these, sodium borohydride (ca. 1 mg.) was added and the solutions were left overnight. The solutions

Fig. VI

FRACTIONATION OF GALACTAN ON DEAE-CELLULOSE.

15
10
5
POLYSACCARIDE CONC.
IN Mg. PER 10ML.

0025M
0.04M
SODIUM ACETATE BUFFER Ph 4.1.
0.10M
0.25M



were assayed for total carbohydrate by the phenol-sulphuric acid method. The ratio of the optical densities of the reduced to the non-reduced sugars was 3 : 4, which is the anticipated one for a tetrasaccharide. (91).

Chromatographic examination of the products of partial hydrolysis with 0.5 N sulphuric acid gave galactose, galactobiose, and galactotriose.

Investigation of the Neutral Polysaccharide from Citrus Pectin.

The polysaccharide had $[\alpha]_D + 55^\circ$ (C. 0.2 in water) and gave only galactose on hydrolysis.

Diethylaminoethylcellulose Chromatography.

An aqueous solution of the polysaccharide (10 mg. in 1 ml.) was applied to a column of the coarsest fraction of diethylaminoethylcellulose powder (10 g., 25 x 2 cm.) in the acetate form. Stepwise elution was carried out with 0.025 M - (250 ml.), 0.04 M - (250 ml.), 0.10 M - (250 ml.), and 0.25 M sodium acetate buffer (pH 4.1, 250 ml.). Fig. VI. The polysaccharide (8 mg.) which was eluted as a single peak, was isolated in the usual way.

Periodate Consumption of the Galactan.

The periodate uptake of an aqueous solution of the polysaccharide (11.46 mg. in 5 ml.) was determined by the method of Aspinall and Ferrier (85). 0.92 Moles of periodate were

consumed per sugar unit and periodate uptake was complete after 18 hours.

Small Scale Smith Degradation.

Polysaccharide (10 mg.) was oxidised with 0.015 M sodium metaperiodate for 18 hours, the excess of reagent was destroyed with ethylene glycol, and iodic acid was neutralised with barium hydroxide and barium carbonate. The filtered solution was treated with sodium borohydride (5 mg.) for 18 hours. The resulting solution was treated alternately with cation and anion exchangers and the remaining boric acid was removed as methyl borate by evaporation with methanol. Acetal linkages were cleaved by the addition of N sulphuric acid. After 3.5 hours the solution was neutralised with barium carbonate, filtered, deionised, and concentrated to a syrup. Chromatographic examination of the syrup in solvent system (A) showed that the major product was chromatographically indistinguishable from threitol. It was found, however, that threitol and erythritol did not separate in this solvent system or in any of a variety which were tried. Paper electrophoresis (hydrated sodium molybdate (25 g. in 1200 ml. water), pH 5, 2 - 8 v/cm. across 10 cm. wide lengths of Whatman No. 3 MM. filter paper for 2 hours.) separated a mixture of threitol and erythritol (92). The respective M_S values of erythritol

and threitol were 1.0 and 0.7, where M_S refers to the true distance of migration of the alcohol, relative to the true distance of migration of sorbitol. In this way it was shown that the product from the periodate oxidised polysaccharide was threitol.

Methylation of the Galactan.

Galactan (600 mg.) in water (10 ml.) was methylated by the addition of dimethylsulphate (12.5 ml.) and 30% aqueous sodium hydroxide (25 ml) in the usual way. Further additions of Haworth's reagents were made on four successive days. The reaction mixture was then heated on a boiling water bath (30 min.) and cooled. Sodium sulphate was precipitated by the addition of methylated spirits (1 vol.) and removed at the centrifuge. The sodium sulphate was washed with methylated spirits and the filtrate and washings were combined. The neutral solution was extracted with chloroform in a liquid-liquid extractor. The dried extracts (anhydrous sodium sulphate) yielded, on removal of the solvent, a crisp brown solid. Methylation was completed by refluxing with methyl iodide and silver oxide. The fully methylated galactan (310 mg.) had $[\alpha]_D^{20} - 8^\circ$ (C.0.9 in chloroform) [Found : OMe, 42.1%].

Methylated galactan (310 mg.) was heated in a sealed tube with 4% methanolic hydrogen chloride (10 ml.) at 100° for 5 hours. After neutralisation with silver carbonate and removal of the

solvent, an aliquot portion of the resulting syrup was examined by gas chromatography. The major peaks had the retention times of the methyl glycosides of 2, 3, 6 - tri - and 2, 3, 4, 6 - tetra - O - methyl - D - galactose. The major portion of the methanolysed, methylated galactan was hydrolysed with N sulphuric acid (100 ml.) for 5 hours on a boiling water bath, and after neutralisation and treatment with ion exchange resins, was applied to a charcoal-Celite column (30 x 3 cm.). Sugars were eluted from the column with a gradient of ethanol - water (1.5 - 6.0%, 4 l.). Fractions (10 ml.) were collected and every fifth tube examined by paper chromatography in solvent systems (C) and (G). Tubes containing the same sugars or mixtures of sugars were bulked and evaporated to dryness to give the following fractions:

Fraction	Wt. (mg.)	Probable Identity
I	3	A mixture of dimethyl galactoses.
II	87	Trimethyl galactose and a trace of the dimethyl galactoses.
III	125	2, 3, 6-tri- <u>O</u> -methyl- <u>D</u> -galactose.
IV	8	2, 3, 4, 6-tetra- <u>O</u> -methyl- <u>D</u> -galactose.

Fraction I

Chromatography in solvent system (D) showed that two dimethyl sugars were present, one staining red with p-anisidine HCl and the other yellow (visible and u/v light). Paper electrophoresis in borate buffer (pH.10) gave two spots, one having an M glucose value of 0.28 identical to that of an authentic sample of 2, 6 - di - O - methyl - D - galactose. The two sugars were chromatographically indistinguishable from a mixture of methylated sugars obtained by Mr. T. Dolan of this department, one of which has been positively identified as 2, 6 - di - O - methyl - D - galactose and the other tentatively identified as 3, 6 - di - O - methyl - D - galactose.

Periodate oxidation of the mixture according to the method of Lemieux and Bauer (86) and subsequent chromatographic examination of the products gave material staining a brilliant yellow with aniline oxalate with an R_F 0.15 in solvent system (C). This is the characteristic product of a dimethyl hexose.

Fraction II

Chromatography of the syrup showed 2, 3, 6 - tri - O - methyl - D - galactose and traces of the dimethyl sugars in fraction I.

Fraction III

The syrup R_{Gal} 0.80 in solvent system (D), had $[\alpha]_D^{20} + 82^\circ$

(C. 0.90 in chloroform) and was chromatographically indistinguishable from 2, 3, 6 - tri - Q - methyl - D - galactose. The sugar was characterised by conversion into 2, 3, 6 - tri - Q - methyl - D - galactonolactone, which on recrystallisation from ethanol had $[\alpha]_D - 31^\circ$ (C. 1.0 in chloroform) and m.p. and mixed m.p. 98° .

Fraction IV.

The syrup (8 mg.) had $[\alpha]_D + 98^\circ$ (C. 0.7 in chloroform) and was chromatographically indistinguishable from 2, 3, 4, 6 - tetra - Q - methyl - D - galactose in solvent systems (D), (G), and (H).

It was characterised as the crystalline aniline derivative which had m.p. and mixed m.p. $197 - 198^\circ$.

Partial Hydrolysis of the Galactan.

Galactan (30 mg.) was partially hydrolysed in N sulphuric acid. Chromatographic examination of the products showed that the optimum time for the formation of oligosaccharides was 30 minutes.

Galactan (170 mg. in 20 ml. N sulphuric acid) was hydrolysed at 100° on a water bath for 30 minutes and the hydrolysate was added to acetone (1 vol.). No degraded polysaccharide precipitated from the solution. Acetone was removed by concentration and the sulphuric acid was

neutralised with Amberlite - 2 B.D.H. liquid ion exchange resin (5% v/v in CHCl_3). The solution was concentrated and applied to a charcoal-Celite column (2 x 10 cm.). Gradient elution with aqueous ethanol 1 - 10% (2 L.), followed by 10 - 35% (4 L.) gave the following fractions:

Fraction No.	Wt. (mg.)	R _{Gal.}	M _G (Borate buffer pH 10)	Probable Identity
I	35	1	0.95	<u>D</u> -galactose
II	21	0.58	0.48	Galactobiose
III	16	0.28	0.40	Galactotriose
IV	11	0.14	0.35	Galactotetraose
V	5	0.07, 0.14	0.32 0.35	A mixture of galactotetraose and galactopentaose.

Sugar I

This sugar crystallised and had $[\alpha]_D + 81^\circ$ (C. 1.2 in water), m.p. and mixed m.p. 164 - 167°, confirming that it was D-galactose.

Sugar II

This sugar crystallised on seeding with 4 - 0 - β - D - galactopyranosyl - D - galactose. On recrystallisation from ethanol - water it had $[\alpha]_D + 68^\circ$ (equil. C. 2.3 in water), m.p. and mixed m.p. 203°. The X-ray powder photograph was identical to that of an authentic sample. Examination of

the methanolysis products of the methylated sugar showed that the main components had the retention times of the methyl glycosides of 2, 3, 4, 6 - tetra -, and 2, 3, 6 - tri - Q - methyl - D - galactose.

Sugar III

This sugar (16 mg.) had $[\alpha]_D + 55^\circ$ (C.1.3 in water) and gave only galactose on hydrolysis. Examination of the methanolysis products of the methylated trisaccharide showed that the main components had the retention times of the methyl glycosides of 2, 3, 4, 6 - tetra -, and 2, 3, 6 - tri - Q - methyl - D - galactose. Partial hydrolysis with 0.5 N sulphuric acid gave galactose and galactobiose. A sample (ca. 0.3 mg.) was dissolved in water (2 ml.) and divided into two 1 ml. portions. One of these was treated with sodium borohydride (1 mg.) and left 18 hours. Assay of the two solutions for total carbohydrate by the phenol-sulphuric acid method showed that the ratio of the optical densities of the reduced sugar to the original was 2 : 3 as expected for a trisaccharide. (91).

Sugar IV

This sugar (11 mg.) had $[\alpha]_D + 50^\circ$ and gave only galactose on hydrolysis. Partial hydrolysis with 0.5 N sulphuric acid gave galactose, galactobiose and galactotriose. Examination

of the methanolysis products of the methylated sugar showed that the main components had the retention times of the methyl glycosides of 2, 3, 4, 6 - tetra -, and 2, 3, 6 - tri - Q - methyl - D - galactose. The ratio of the optical densities for a sample which had been reduced with sodium borohydride to that of the original solution as estimated by the phenol-sulphuric acid method was 3 : 4, the value expected for a tetrasaccharide.

Sugar V.

This sugar (5 mg.) was contaminated with Sugar IV. Partial hydrolysis with 0.5N sulphuric acid gave galactose, galactobiose, galactotriose, and galactotetraose. Examination of the methanolysis products of the methylated sugar showed that the main components had the retention times of the methyl glycosides of 2, 3, 4, 6-tetra, and 2, 3, 6-tri - Q - methyl - D - galactose. The M_G value in borate buffer compares favourably with that quoted by Bouveng and Meier (34) for galactopentaose.

DISCUSSION

Citrus pectin had a characteristically high positive rotation ($[\alpha]_D^{25} + 176^\circ$) and a uronic acid content of 4-5%. Paper chromatography showed that the hydrolyzate contained galactose, glucose, arabinose, and rhamnose in addition to galacturonic acid. Attempts were made to fractionate the polysaccharide by precipitation as the calcium salt and extraction of neutral polysaccharides with boiling ethanol-water (7:3) and with saturated lime water. In each case, however, the regenerated polysaccharide was virtually unchanged in optical rotation and uronic anhydride content. The extractions with ethanol-water and with lime water removed small quantities (ca. 0.2%) of polysaccharide which gave galactose and arabinose on hydrolysis.

DISCUSSION

Citrus pectin was chromatographed on diethylaminoethyl-cellulose by the procedure of Seakins et al. (39). Stepwise elution with increasing concentrations of sodium phosphate buffer at pH 6.5 gave three polysaccharide fractions. Fraction I, eluted with 0.025M buffer, contained residues of galactose and arabinose, with traces of galacturonic acid and glucose. Fractions II and III, eluted with 0.10M and 0.25M buffers respectively, gave galactose, glucose, arabinose, rhamnose, and galacturonic acid on hydrolysis. They were identical

DISCUSSION.

Citrus pectin had a characteristically high positive rotation ($[\alpha]_D + 178^\circ$) and a uronic acid content of 44%. Paper chromatography showed that the hydrolysate contained galactose, glucose, arabinose, and rhamnose in addition to galacturonic acid. Attempts were made to fractionate the polysaccharide by precipitation as the calcium salt and extraction of neutral polysaccharides with boiling ethanol-water (7 : 3) and with saturated lime water. In each case, however, the regenerated polysaccharide was virtually unchanged in optical rotation and uronic anhydride content. The extractions with ethanol-water and with lime water removed small quantities (ca. 0.5%) of polysaccharide which gave galactose and arabinose on hydrolysis.

Citrus pectin was chromatographed on diethylaminoethyl-cellulose by the procedure of Neukom et al. (39). Stepwise elution with increasing concentrations of sodium phosphate buffer at pH 6.5 gave three polysaccharide fractions. Fraction I, eluted with 0.025M buffer, contained residues of galactose and arabinose, with traces of galacturonic acid and glucose. Fractions II and III, eluted with 0.10M and 0.25M buffers respectively, gave galactose, glucose, arabinose, rhamnose, and galacturonic acid on hydrolysis. They were identical

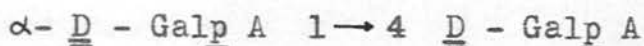
in optical rotation and uronic acid content and differed only in methyl ester content. In view of this, it seemed likely that these two fractions were eluted from the column with different buffers because of their differing degrees of esterification and not because of any fundamental variation in structure, since it is known that the extent to which an acidic polysaccharide is esterified is an important factor in determining how strongly it is retained by diethylaminoethylcellulose (90). That this was the case was confirmed by saponification of citrus pectin with aqueous sodium hydroxide and chromatography on diethylaminoethylcellulose. Two fractions were obtained; a neutral polysaccharide eluted with 0.025M buffer and an acidic polysaccharide eluted as a single broad band with increasing concentrations of sodium hydroxide. To obtain samples of these two polysaccharides on a preparative scale, citrus pectin was fractionated by the addition of copper sulphate solution to an aqueous solution of the polysaccharide. The acidic material was precipitated as the insoluble copper salt, leaving the neutral fraction in solution. The regenerated acidic polysaccharide had $[\alpha]_D + 181^\circ$, a uronic acid content of 44%, and contained residues of galactose, glucose, arabinose, rhamnose, and galacturonic acid. On chromatographing on diethylaminoethylcellulose it was eluted as a single broad band with

increasing concentrations of sodium hydroxide.

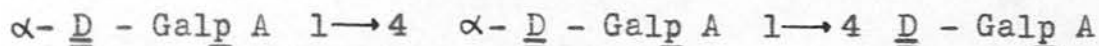
Acid hydrolysis of the acidic polysaccharide under conditions favouring the formation of aldobiouronic acids afforded neutral monosaccharides, a mixture of D-galacturonic acid and acidic oligosaccharides, and a degraded polysaccharide. The degraded polysaccharide had a high positive rotation ($[\alpha]_D + 214^\circ$) and gave only galacturonic acid on hydrolysis. The mixture of acidic sugars was fractionated by elution with increasing concentrations of formic acid from anion exchange resin followed, where necessary, by partition chromatography on filter sheets. Eight acidic sugars were obtained. Sugar I was characterised as D-galacturonic acid by preparation of a crystalline derivative, D-galacturonic acid, 2, 5-dichlorophenylhydrazone. Sugar II was characterised as 2 - Q - (α - D - galactopyranosyluronic acid) - L - rhamnose. (XXII). Methylation with Haworth's reagents gave a crystalline methylated sugar which had the specific rotation, melting point, and X-ray powder photograph characteristic of methyl 2 - Q - (α - D - galactopyranosyluronic acid) - L - rhamnoside pentamethyl ether dihydrate.



Sugars VI and VII gave only galacturonic acid on hydrolysis and were chromatographically indistinguishable from authentic samples of di- and trigalacturonic acids respectively in a number of different solvent systems. They both formed calcium salts which had rotations identical to those of authentic samples confirming that sugar VI was digalacturonic acid (XXIII) and that sugar VII was trigalacturonic acid (XXIV).

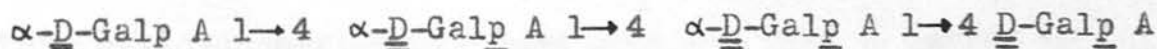


XXIII



XXIV

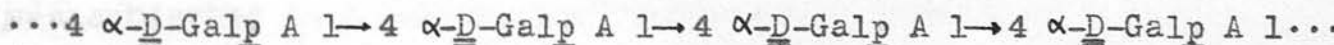
Sugar VIII gave only galacturonic acid on hydrolysis and yielded galacturonic acid, digalacturonic acid, and trigalacturonic acid on partial hydrolysis. The R_{Gala} value in solvent system (F) was similar to that reported for tetragalacturonic acid. (49) (XXV).



XXV

Small quantities of three other sugars were also isolated. Sugar III was chromatographically pure and stained red with aniline oxalate. The hydrolysate contained galacturonic acid, glucose, arabinose, and xylose. Sugars IV and V could not be separated. Sugar IV stained red with aniline oxalate and sugar V stained yellow. The hydrolysate contained galacturonic acid, galactose, glucose, and xylose.

The isolation of a degraded polysaccharide containing only galacturonic acid and of di-, tri-, and tetragalacturonic acids as products of the partial hydrolysis, indicated that the acidic polysaccharide from citrus pectin was a typical pectic acid, consisting of chains of 1→4 - linked α -D-galacturonic acid residues (XXVI).



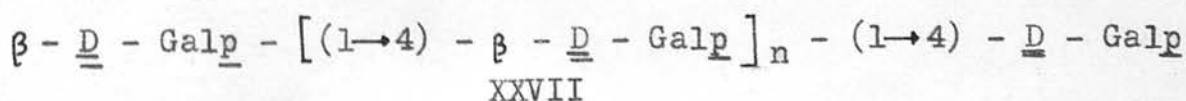
XXVI

The isolation of the aldobiouronic acid 2 - 0 - (α - D - galactopyranosyluronic acid) - L - rhamnose (XXII) confirmed that L - rhamnose residues were components of this polysaccharide. This oligosaccharide has been obtained from a number of

polysaccharides containing both galacturonic acid and rhamnose; lucerne pectic acid, (42) the pectic acid from soybean hulls, (93) the pectic complex from soybean meal, (94) and from gums of the Khaya and Sterculia genera, (66), (69), (70) and from Cochlospermum gossypium (100). It may be that the L - rhamnose residues in citrus pectin are interposed between blocks of 1→4 - linked α -D - galacturonic acid residues as in the Khaya and Sterculia gums.

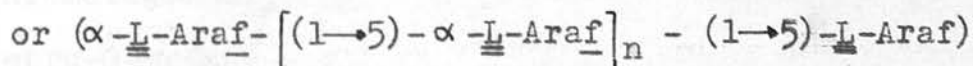
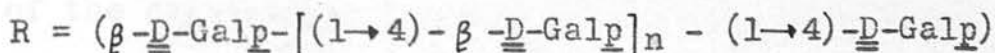
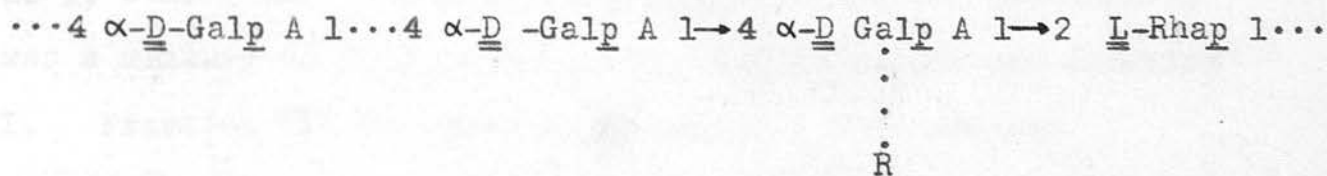
Galactose and arabinose have been shown to be components of the acidic polysaccharide. Recent work has shown that these sugars are integral constituents of pectic polysaccharides. (38 - 45), (93), (94).

In order to obtain more information about the location of these neutral sugar residues, the acidic polysaccharide was subjected to a partial hydrolysis under conditions favouring the formation of neutral oligosaccharides. A degraded polysaccharide and a water soluble mixture of neutral sugars and oligosaccharides were obtained. The mixture of sugars was fractionated on a charcoal - Celite column by successive elution with gradients of aqueous ethanol. A homologous series of galactose containing oligosaccharides was obtained. (XXVII).



The first member of the series, 4 - Q - β - D - galactopyranosyl - D - galactose (XXVII, n = 0) was characterised as the crystalline sugar by comparison (m.p. and mixed m.p., specific rotation, and X-ray powder photograph) with an authentic specimen. The second member of the series, 0 - β - D - galactopyranosyl - (1 \rightarrow 4) - Q - β - D - galactopyranosyl - (1 \rightarrow 4) - D - galactose (XXVII), n = 1) gave galactose and 4 - Q - β - D - galactopyranosyl - D - galactose on partial hydrolysis. Methanolysis of the methylated sugar yielded the methyl glycosides of 2, 3, 6 - tri -, and 2,3, 4, 6 - tetra - Q - methyl - D - galactose. Similarly the third member of the series was shown to be 0 - β - D - galactopyranosyl - (1 \rightarrow 4) - 0 - β - D - galactopyranosyl - (1 \rightarrow 4) - 0 - β - D - galactopyranosyl - (1 \rightarrow 4) - D - galactose. (XXVII), n = 2). The isolation of this homologous series of oligosaccharides showed that side chains of polygalactose units were attached in some way to the pectic acid main chain. A similar series of galactose containing oligosaccharides has recently been isolated from a pectic acid complex obtained from soybean meal (94). Partial hydrolysis of this complex yielded a variety of acidic oligosaccharides including 2 - Q - (α - D - galactopyranosyluronic acid) - L - rhamnose and di -

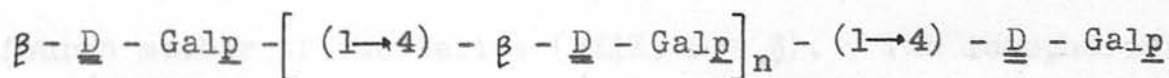
and trigalacturonic acids. Work on lucerne pectic acid had indicated that the galactose units in that polysaccharide were present as single unit end groups (42). In the acidic polysaccharide from citrus pectin, however, galactose is present as chains of 1→4 - linked β - D - galactose residues. Further proof of the presence of side chains of polygalactose units has recently been obtained from a methylation study of the polysaccharide, (95) which indicated the presence of relatively large amounts of 2, 3, 6 - tri - Q - methyl - D - galactose and smaller amounts of 2, 3, 4, 6 - tetra - Q - methyl - D - galactose. The presence of 2, 3, 5 - tri -, and 2, 3 - di - Q - methyl - L - arabinose was also indicated. On the basis of the results obtained, the following partial structure summarises the main structural features of the acidic polysaccharide from citrus pectin. (XXVIII).



The neutral polysaccharide isolated from the supernatant after removal of the insoluble acidic fraction had $[\alpha]_D + 55^\circ$ and gave galactose on hydrolysis. On chromatographing on diethylaminoethylcellulose according to the procedure of Jermyn (96) the polysaccharide was eluted as a single broad band. A sample of the galactan was methylated with Haworth's reagents and the methylation was completed with methyl iodide and silver oxide. The fully methylated polysaccharide was refluxed with methanolic hydrogen chloride and the resulting methyl glycosides hydrolysed with sulphuric acid. The mixture of methylated sugars was fractionated on a charcoal-Celite column using gradients of ethanol-water. Four fractions were obtained. Fraction I contained small quantities of two sugars which were tentatively identified as 2, 6-di-, and 3, 6-di-Q-methyl-D-galactose. Fraction II was a mixture of 2, 3, 6-tri-Q-methyl-D-galactose and fraction I. Fraction III was chromatographically pure and was characterised as 2, 3, 6-tri-Q-methyl-D-galactose by formation of the crystalline lactone derivative. Fraction IV was also chromatographically pure and was characterised as 2, 3, 4, 6-tetra-Q-methyl-D-galactose by formation of the crystalline aniline derivative. The major component, 2, 3, 6-tri-Q-

methyl-D-galactose must have arisen from the main chain of the galactan and the 2, 3, 4, 6-tetra-O-methyl-D-galactose must have originated from non-reducing D-galactose end group. It was not possible at this point to say whether the small quantities (ca. 1%) of the di-O-methyl galactoses were structurally significant, or arose from incomplete methylation of the polysaccharide or from demethylation during hydrolysis. Essentially, however, the polysaccharide consisted of linear chains of 1→4-linked D-galactose residues.

Partial hydrolysis of a sample of the galactan afforded a mixture of mono- and oligosaccharides which was fractionated on a charcoal-Celite column by gradient elution with aqueous ethanol, to give a homologous series of galactose containing oligosaccharides (XXIX).



XXIX

The first member of the series was a disaccharide which crystallised on seeding and was identified by comparison (m.p. and mixed m.p., specific rotation, and X-ray powder

photograph) with an authentic specimen of 4 - $\underline{0}$ - β - \underline{D} - galactopyranosyl- \underline{D} - galactose. (XXIX, n = 0). The second member of the series was a trisaccharide which gave, on partial hydrolysis, galactose and 4 - $\underline{0}$ - β - \underline{D} - galactopyranosyl - \underline{D} - galactose. Methanolysis of the methylated sugar yielded the methyl glycosides of 2, 3, 4, 6 - tetra and 2, 3, 6 - tri - $\underline{0}$ - methyl - \underline{D} - galactose. Estimation of the DP by Timell's modification (91) of the method of Peat, Whelan and Roberts (97) showed that it was a trisaccharide. The structure was, therefore, 0 - β - \underline{D} - galactopyranosyl - (1 \rightarrow 4) - $\underline{0}$ - β - \underline{D} - galactopyranosyl - (1 \rightarrow 4) - \underline{D} - galactose. (XXIX, n = 1). Similarly the third member of the series (XXIX, n = 2) was shown to be 0 - β - \underline{D} - galactopyranosyl - (1 \rightarrow 4) - $\underline{0}$ - β - \underline{D} - galactopyranosyl - (1 \rightarrow 4) - $\underline{0}$ - β - \underline{D} - galactopyranosyl - (1 \rightarrow 4) - \underline{D} - galactose. Chromatographic and ionophoretic evidence was obtained for the fourth member of the series (XXIX, n = 3). The ionophoretic values of all these oligosaccharides were similar to those quoted by Bouveng and Meier (34) for the same oligosaccharides isolated by partial hydrolysis of the galactan from Norwegian spruce compression wood.

A sample of citrus pectin galactan was oxidised with sodium metaperiodate. Reduction with sodium borohydride and hydrolysis

of acetal linkages with sulphuric acid in the cold, according to the method of Smith and his collaborators (98) gave a product which was chromatographically and ionophoretically indistinguishable from threitol. Electrophoresis with molybdate buffer (92) confirmed that the product was threitol and not erythritol. The isolation of threitol provided additional evidence that the polysaccharide consisted of 1→4 - linked galactose residues. Hydrolysis of the syrup and subsequent chromatographic examination showed that galactose was not present in the hydrolysate. The absence of periodate resistant galactose residues confirmed that the dimethyl sugars isolated on methylation of the galactan arose from incomplete methylation. From these results it was concluded that the galactan isolated from citrus pectin was of the type classically associated with pectic acids (105) and consisted of linear chains of 1→4 - linked β - D - galactopyranose residues.

In each case the main chain is composed of 1→4 - linked β - D - galacturonic acid residues and the polysaccharide contains residues of β - rhamnose, β - galactose, and β - arabinose.

The pectic group of polysaccharides contains three recognised structural species, pectic acid, araban, and galactan. Hitherto, however, when one of these species has been required the source has been chosen appropriately.

and soybean (93) pectic acids. Notably it is known about the

The galactan obtained from Lupinus albus, for example, was isolated from a pectin which contained much less pectic acid and araban than usual. Furthermore, many polysaccharides have been extracted using alkaline solutions which decompose pectin (25), (31). In the work described here, however, a galactan has been isolated from citrus pectin under conditions which make extensive degradation of any of the polysaccharide components unlikely and from a source not notably rich in galactan. It seems probable that many other pectic acids have associated with them small quantities of araban or galactan, since chromatography of a number of pectins on diethylaminoethylcellulose (39), (40) gives, in addition to the main acidic fractions, small neutral fractions containing residues of galactose or of galactose and arabinose.

The acidic polysaccharide isolated from citrus pectin has a number of structural features in common with the pectic acids from lucerne, (42) sisal, (38) and soybeans (93). In each case the main chain is composed of 1→4 - linked α -D - galacturonic acid residues and the polysaccharide contains residues of L - rhamnose, D - galactose, and L - arabinose as constituent sugars. The aldobiouronic acid 2 - O - (α - D - galactopyranosyluronic acid) - L - rhamnose, which confirms that L - rhamnose residues are components of the acidic polysaccharide has also been isolated from lucerne (42) and soybean (93) pectic acids. Nothing is known about the

location of the galactose and arabinose residues in these polysaccharides, although the results of a methylation study of lucerne indicated that galactose was present as single unit side chains and that arabinose probably existed as chains of 1→5 - linked α -L - arabinofuranose residues. The isolation of a homologous series of galactose containing oligosaccharides from the acidic fraction of citrus pectin, provides conclusive evidence that, in this polysaccharide, the galactose exists as chains of 1→4 - linked β -D - galactose units. In containing chains of polygalactose units attached to the uronic acid backbone the acidic polysaccharide from citrus pectin resembles a pectic acid type complex which has been isolated from soybean meal. This complex also yields as products of partial hydrolysis, 2 - O - (α - D - galactopyranosyluronic acid) - L - rhamnose and di-, and trigalacturonic acids. The results of a methylation study of the acidic polysaccharide from citrus pectin (95) confirms the presence of chains of polygalactose units and indicates that arabinose is present as chains of 1→5 linked α -L - arabinofuranose units.

Citrus pectin, therefore, contains an acidic polysaccharide composed of chains of 1→4 linked α -D - galacturonic acid

residues to which are attached residues of $\underline{\underline{L}}$ - rhamnose. Also present in the polysaccharide are chains of 1→4-linked β - $\underline{\underline{D}}$ - galactose residues and probably chains of 1→5-linked α - $\underline{\underline{L}}$ - arabinofuranose residues. Associated with this acidic polysaccharide is a galactan of the classical pectic type i.e. composed of linear chains of 1→4-linked β - $\underline{\underline{D}}$ - galactose residues. It is of interest that recent work in these laboratories (99) has revealed the existence of an analagous situation in mustard seed pectin. An acidic polysaccharide has been separated, methylation of which shows that it is composed of chains of 1→4 - linked α - $\underline{\underline{D}}$ - galacturonic acid units to which are attached residues of $\underline{\underline{L}}$ - rhamnose and which contains chains of 1→5-linked α - $\underline{\underline{L}}$ - arabinofuranose residues. Associated with this polysaccharide is a neutral araban of the classical pectic type.

The isolation of such an araban makes it more likely that the 2, 3 - di - $\underline{\underline{O}}$ - methyl - $\underline{\underline{L}}$ - arabinose obtained on methylation of lucerne pectic acid (42) and of the acidic polysaccharide from citrus pectin (95) arises from non-terminal 1→5 - linked α - $\underline{\underline{L}}$ - arabinofuranose residues. An approximation to an araban of this type was isolated from lucerne pectic acid by extraction with lime water. In

view of the harsh nature of the extraction procedure, it seems likely that this araban was a product of degradation.

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With the exception of a pectic acid isolated from sunflower seed heads (37) all recent workers have reported the presence of neutral sugars, especially rhamnose, galactose, and arabinose in pectic acids. The acidic fraction from citrus pectin is a typical polysaccharide of this type. It appears that pectic substances are similar to the hemicelluloses particularly the xylan group, in that all polysaccharides of the pectic type belong to the same general family in being built up from chains of (1→4) linked α -D - galactopyranosyluronic acid residues. The majority of pectins carry other sugar residues (most commonly L - rhamnose, L - arabinose, and D - galactose) attached to the uronic acid main chain. Most of the polysaccharides are probably mixtures of closely related molecular species in which variations in detailed structure are superimposed on the variations in molecular size commonly found in natural polymers. At least one type of variation, namely that due to differing methyl ester contents has been demonstrated in the case of citrus pectin.

The pectic complex from soybean meal (94) is also thought to consist of several acidic polysaccharides of a similar type since a number of peaks were obtained on diethylaminoethylcellulose chromatography, between which no differences could be detected. It is also possible that citrus pectin contains not only a mixture of acidic polysaccharides containing neutral sugar residues, but also a polysaccharide composed solely of D - galacturonic acid residues of the type isolated by Bishop (37). The existence of a situation of this kind has been demonstrated by Timell and Jabbar Mian (45) who obtained three polysaccharide components from the inner bark of white birch. One of these consisted of galacturonic acid residues, another contained galacturonic acid, galactose and arabinose, and the third was largely neutral. A more detailed investigation into problems of this type, must await the development of methods for the separation of closely related molecular species.

Lucerne, 1912

Extraction of the Carbohydrates from Lucerne.

Lucerne (1.0 kg.) was extracted with 50% ethanol, cold water, hot water, ammonia oxalate and hot water in that order.

Ammonium Oxalate Extraction.

The residue from the hot water extraction was extracted twice with 0.5% ammonium oxalate solution at 60 - 90° for 3 hours. The residue was filtered off and washed. Calcium chloride solution (10%) was added to the extract until precipitation of calcium oxalate was complete. The calcium oxalate was washed, suspended in 0.5% ammonium oxalate and heated on a water bath for 30 minutes. Calcium

SECTION II

LUCERNE PECTIC ACID

oxalate was reprecipitated by the addition of acetone to the solution. The pectic acid was reprecipitated from 50% acetone (1 part) and dissolved in water. The solution was concentrated to remove acetone and freeze dried. The freeze dried ammonium oxalate had $[\eta] = 2.5 \text{ dl/g}$ (0.5% in water). Found: organic anhydride (decarboxylation), 30.0%; N, 0.2; ash, 1.5%.

Chromatography of the hydrolyzed pectic acid gave galactose, glucose, arabinose, rhamnose, small amounts of xylose, fucose, 2-O-methyl arabinose, 2-O-methyl galactose, galacturonic acid and a small amount of uronic acid.

Lucerne Pectic AcidExtraction of the Carbohydrates from Lucerne.

Lucerne (1.1 Kg.) was extracted with 80% ethanol, cold water, hot water, ammonium oxalate, and hot lime water in that order.

Ammonium Oxalate Extraction.

The residue from the hot water extraction was extracted twice with 0.5% ammonium oxalate solution at 80 - 90° for 3 hours. The residue was filtered off and washed. Calcium chloride solution (10%) was added to the extract until precipitation of calcium pectate was complete. The calcium pectate was washed, suspended in 0.3% ammonium oxalate and heated on a water bath at 90° for 30 minutes. Calcium oxalate was removed at the centrifuge and ammonium pectate was precipitated by the addition of acetone to the solution. The polysaccharide was reprecipitated from 50% acetone (4 vol.) and dissolved in water. The solution was concentrated to remove acetone and freeze dried. The freeze dried ammonium pectate had $[\alpha]_D + 205^\circ \pm 5^\circ$ (C.0.5 in water). [Found : uronic anhydride (decarboxylation), 50.0% ; N, 0.2 ; ash, 4.8%].

Chromatography of the hydrolysate gave galactose, glucose, arabinose, rhamnose, small amounts of xylose, fucose, 2 - O - methyl xylose, 2 - O - methyl fucose, galacturonic acid and a complex mixture of acidic oligosaccharides.

Esterification of Pectic Acid with Ethylene Oxide.

Ammonium pectate (35 g.) was dissolved in water and the solution was deionised with Amberlite resins, IR - 120(H) and IR - 4B(OH) and freeze dried. Ethylene oxide (400 ml.) was added to a suspension of the pectic acid (32 g.) in water (3 l.) and the mixture was shaken at room temperature for fourteen days. At the end of this period, the esterification was complete with the formation of a viscous solution and the change of pH from 2 to 7. The glycol ester was precipitated by the addition of acetone (2 vol.), and the gelatinous precipitate was washed with acetone-alcohol. Finally it was dissolved in water and the solution was freeze dried. The 2-hydroxyethyl pectate (39 g.) had $[\alpha]_D^{25} + 175^\circ$ (C.0.4 in water) [Found : uronic anhydride (decarboxylation), 42.0% (equivalent to 51% in the free acid); glycol released on saponification, 44% (equivalent to 52% of acidic groups in the native polysaccharide)]. The ethylene glycol content was estimated by saponification with sodium hydroxide, periodate oxidation of the released ethylene glycol and estimation of the resultant formaldehyde with chromotropic acid reagent. (10).

Reduction of 2 - Hydroxyethyl Pectate with Sodium Borohydride.

2 - Hydroxyethyl pectate (39 g.) was dissolved in water (1 l.) containing glycerol (15 g.), the solution was cooled to 0° , sodium borohydride (9 g.) in water (35 ml.) was added

with stirring and boric acid (0.5 g.) was added to maintain the pH of the solution at 8. The solution was kept at 0° for 3 days, excess of borohydride was destroyed and sodium ions were removed with Amberlite resin IR - 120(H), boric acid was removed with Amberlite resin IR - 4B(OH), and polysaccharide was precipitated by the addition of acetone, redissolved in water, and isolated by freeze-drying. The partially reduced pectic acid (28 g.) had $[\alpha]_D^{20} + 208^\circ \pm 5^\circ$ (C. 0.3 in water) [Found : uronic anhydride (by decarboxylation), 26.0%]. Repetition of this sequence of operations resulted in a further loss of material without significantly reducing the acidic content.

Acylation of the Partially Reduced Pectic Acid.

Partially reduced pectic acid (28 g.) was dissolved in formamide (1.2 l. dried with anhydrous calcium sulphate and distilled under reduced pressure) by adding it batchwise to the solvent with vigorous stirring at 50° during 6 hours. Pyridine (340 g.) was added during 4 hours to the viscous solution, vigorous stirring being maintained to prevent gelling. The solution was stirred overnight and cooled to 30°, propionic anhydride (190 g.) was added dropwise during 19 hours and the solution was again stirred overnight. Two further additions of propionic anhydride (15 g. each) were made during subsequent days and the final solution was

cooled and poured into ice (400 g.) and water (4 ℓ.) containing 2% of hydrochloric acid. The resulting suspension was stirred for 1 hour and the precipitate was removed at the centrifuge, washed four times with cold 0.5% hydrochloric acid and four times with water, suspended in water overnight, washed again with water, removed by centrifugation and dried at 30° in a vacuum oven. The propionate was dissolved in chloroform, the solution was treated with cation - and anion - exchangers and the polysaccharide ester (30 g.), $[\alpha]_D + 98^\circ$ (C. 0.45 in chloroform), was precipitated by the addition of light petroleum (b.p. 60 - 80°) (4 vol.).

Diborane Reduction of the Propionated Polysaccharide.

A series of trial experiments were carried out to determine the best conditions for the reduction. Generation of the diborane in situ caused the formation of artefacts due to the reduction of some propionyl groups to propyl groups. To avoid this, diborane was generated externally, when the formation of such artefacts was negligible.

The system used consisted of a diborane generator, a trap containing diglyme and sodium borohydride and an empty trap. The generator consisted of a round bottomed flask (1 ℓ.) containing sodium borohydride (50 g.) and diglyme (diethyleneglycol dimethyl ether, 300 ml.). Diborane

was generated by the addition to the flask of boron trifluoride diethyl etherate through a dropping funnel, the top of which was connected to a nitrogen supply. A solution of the propionated polysaccharide (25 g.) in diglyme (1 l.) was periodically saturated with diborane during a reaction time of four weeks, the mixture being shaken from time to time when gel formation occurred. The final reaction mixture was poured into methanol-water (9 : 1) and the precipitated polysaccharide derivative was removed by centrifugation and heated in aqueous 5% ammonia at 65° for 45 minutes. The resulting solution was concentrated, and the carboxyl-reduced polysaccharide (10 g.) $[\alpha]_D^{25} + 210^\circ$ (C. 0.37 in water), was precipitated by the addition of ethanol and dried [Found : uronic anhydride, 3.8%]. Estimated by decarboxylation with HI (g. 1.7) and estimation of the resulting CO₂ by a vapour phase infra red method]. (102)

Methylation of Carboxyl-reduced Pectic Acid.

Carboxyl-reduced pectic acid (1 g.) in water (36 ml.) was methylated by the addition of sodium hydroxide (25 ml.) and dimethyl sulphate (125 ml.) in the usual way. Three further additions of Haworth's reagents were made over three successive days. The solution was then adjusted to pH 8

with acetic acid, heated on a boiling water bath (1 hr.), cooled, dialysed, concentrated, and freeze dried. The partially methylated polysaccharide was methylated twice with methyl iodide and silver oxide to give methylated carboxyl-reduced pectic acid (0.52 g), $[\alpha]_D^{25} + 154^\circ$ (C. 0.5 in chloroform) [Found : OMe, 43.3%, not raised on further methylation]. A sample of the methylated polysaccharide was heated with methanolic hydrogen chloride and the resulting mixture of methyl glycosides was examined by gas chromatography on columns a and b. Components were detected which had the retention times of methyl glycosides of 2, 3, 6 - tri - O - methylgalactose (major component), 2, 3, 4, 6 - tetra - O - methylgalactose, 2, 3, 5 - tri and 2, 3 - di - O - methylarabinose, and 3, 4 - di - and 3 - O - methylrhamnose.

Periodate Oxidation of Carboxyl-reduced Pectic Acid.

Carboxyl-reduced polysaccharide (12.2 mg.) was dissolved in water (5 ml.) and the periodate consumption was determined by the method of Aspinall and Ferrier (85). Periodate oxidation was complete after 40 hours when the consumption was 0.85 mole. per sugar residue. The periodate consumption of Lucerne pectic acid was 0.87 mole. per sugar residue.

Large Scale Periodate Oxidation of Carboxyl-reduced Pectic Acid and Subsequent Degradation.

Carboxyl-reduced polysaccharide (5 g.) was oxidised with 0.15 M - sodium metaperiodate (15 g.) for 40 hours (uptake of reagent was constant and corresponded to the consumption of 0.85 mole of reagent per sugar residue) and the excess of reagent was destroyed by the addition of ethylene glycol. The solution was shaken with Amberlite resin IR - 120(H) to remove sodium ions; iodic acid was removed by neutralisation with barium hydroxide and barium carbonate, and the filtered solution was treated with sodium borohydride (1.5 g.) for 48 hours. Excess of hydride was destroyed and sodium ions were removed with Amberlite resin IR - 120(H) and the solution was treated again with cation - and anion - exchangers, and concentrated. The residue was hydrolysed with N - sulphuric acid (300 ml.) at room temperature for 4 hours and the solution was neutralised with barium hydroxide and barium carbonate, concentrated and poured into ethanol. No degraded polysaccharide was precipitated and concentration afforded a syrup (3.4 g.) which was shown by paper chromatography to contain glycollaldehyde, glycerol, threitol and other components having $R_{Galactose}$ 1.48, 1.05, 0.57 and 0.21 in solvent system A.

Methylation of Degraded Polysaccharide.

A sample of the syrup (50 mg.) was methylated in dimethyl formamide with methyl iodide and silver oxide, methanolysed, and examined by gas chromatography. Large peaks caused by threitol and other breakdown products interfered with the examination.

A sample (100 mg.) of the syrup was applied to a charcoal-Celite column (1 : 1, 20 x 3 cm.) and left overnight. Elution with water removed threitol and low molecular weight products. The other sugars were removed with 40% ethanol-water. Concentration yielded a syrup which was methylated as before by the method of Kuhn et al (88), methanolysed, and examined by gas chromatography. Components were observed which had the retention times of the methyl glycosides of 2, 3, 6-tri-, 2, 3, 4, 6-tetra-o-methylgalactose and 2, 3, 5-tri-o-methyl-arabinose. Several peaks were obtained with relatively low retention times which were attributed to 1, 3, 4-tri-o-methyl-threitol.

Fractionation of the Syrup on a Charcoal Column.

The syrup (3.3 g.) was applied to a charcoal-Celite column (1 : 1, 30 x 6 cm.); elution with water (4 l.) gave a fraction which contained glycollaldehyde, glycerol, and threitol. Gradient elution was carried out with 0.0 - 5.0% -

(2 l.), 5.0% - (2 l.), 5.0 - 10.0% - (2 l.), 10.0% - (2 l.), and 10 - 30.0% aqueous ethanol (2 l.). Every tenth tube was examined chromatographically in solvent system (A) and tubes containing the same mixtures of sugars were bulked. The fractionation achieved was extremely poor, further separation by filter sheet chromatography in solvent system (A) being required in every case.

Fraction I

This fraction contained threitol and several faster travelling products. Separation by filter sheet chromatography gave a non-reducing sugar $[\alpha]_D + 4^\circ$ (c.l.0 in water) which was chromatographically indistinguishable from threitol. Electrophoresis in molybdate buffer (92) confirmed this. Hydrolysis of the syrup isolated from the leading edge of the threitol band on the filter sheet chromatogram showed that rhamnose was present as well as threitol, although no pure rhamnose containing material could be separated from the threitol.

Fraction II.

This non-reducing sugar had $R_{\text{Galactose}} 1.5$ in solvent system (A). Hydrolysis with Nsulphuric acid gave arabinose and threitol. Methanolysis of the methylated sugar and examination by gas chromatography indicated the presence of

the methyl glycosides of 2, 3, 5 - tri - 0 - methlarabinose and other peaks of low retention time.

Fraction III.

This non-reducing sugar (12 mg.) $[\alpha]_D + 35^\circ$ (c.1.5 in water) had $R_{Galactose}$ 0.6 in solvent system (A). Hydrolysis with Nsulphuric acid for 8 hours yielded galactose and threitol. Examination of the methanolysis products of the methylated sugar showed that the main components had the retention times of the methyl glycosides of 2, 3, 4, 6-tetra - 0 - methyl-galactose and of the peaks attributed to 1, 3, 4 - tri - 0 - methyl - threitol. The periodate consumption of the sugar, determined spectrophotometrically by the method of Aspinall and Ferrier (85) was three moles of oxidant per sugar mole. Aliquot portions of the reaction solution used for this determination of the periodate consumption were tested with chromotropic acid reagent (101). During the periodate oxidation, one mole of formaldehyde was formed per sugar mole.

Fraction IV.

This non-reducing sugar, R_{Gal} 0.36, gave glucose (major component), arabinose, and threitol on hydrolysis.

Fraction V.

This non-reducing sugar (13 mg.) had $[\alpha]_D + 138^\circ$ (c. 1.4 in water) and $R_{Galactose} 0.2$ in solvent system (A). Hydrolysis with Nsulphuric acid for 8 hours gave galactose and threitol. Examination of the methanolysis products of the methylated sugar showed that the main components had the retention times of 2, 3, 6 - tri -, and 2, 3, 4, 6 - tetra - O - methylgalactose and of peaks attributed to 1, 3, 4 - tri - O - methyl threitol. Each sugar mole consumed four moles of periodate and released one mole of formaldehyde.

DISCUSSION

DISCUSSION

The results of a methylation study on lucerne pectic acid carried out by Aspinall and Fanshawe (42) had indicated that some of the 1→4 - linked α -D - galacturonic acid residues provided branching points in the structure. There were indications that some of the arabinose and rhamnose residues also acted as branching points. It was decided, therefore, to subject lucerne pectic acid to a Smith degradation (98) in order to obtain more detailed structural information by the isolation of these units in the polysaccharide which were resistant to oxidative cleavage by periodate. When the Smith degradation is applied to acidic polysaccharides, difficulties are encountered, since the oxidised fragments which contain uronic acid groups are less readily removed by mild acid hydrolysis than the other acetal systems (106). Investigations by Smith and Stephen (103) and Aspinall and Fanshawe (42) into the use of diborane for the reduction of carboxyl groups in hexuronic acid derivatives had indicated that a high proportion of hexuronic acid residues in suitably substituted polysaccharides could be reduced with this reagent to the corresponding hexose residues. If such a reduction was effected on lucerne pectic acid, the resulting carboxyl-

reduced polysaccharide could be subjected to periodate oxidation, reduction, and cold hydrolysis. Furthermore, the investigation of Aspinall and Fanshawe (42) had shown the absence of branched galactose residues in the pectic acid, so that it could be assumed that any periodate resistant galactose residues isolated on Smith degradation of the carboxyl-reduced polysaccharide arose from branched galacturonic acid residues in the parent material.

Combined alfalfa leaves and stem were extracted successively with ethanol-water (4 : 1), cold water, and hot water as described by Aspinall and Fanshawe (42). Further extraction with 0.5% ammonium oxalate solution at ca. 90° for 3 hours, followed by precipitation of the pectic acid as the insoluble calcium salt, regeneration with ammonium oxalate solution and precipitation with acetone, furnished ammonium pectate which had a characteristically high positive rotation $[\alpha]_D + 205^\circ$ and a uronic anhydride content of 50%. Hydrolysis of a sample of the polysaccharide gave galactose, glucose (of unknown origin), arabinose, rhamnose, small amounts of xylose, fucose, 2 - O - methylxylose and 2 - O - methylfucose, galacturonic acid, and a complex mixture of acidic oligosaccharides. An aqueous solution of ammonium pectate was treated with cation - and anion - exchangers and freeze dried to give pectic acid. An aqueous suspension of pectic acid was esterified by shaking

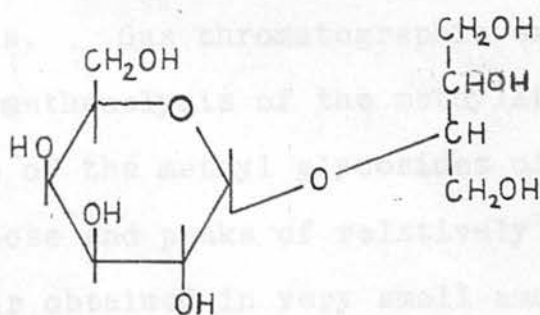
with ethylene oxide for 14 days. The 2-hydroxyethyl pectate so formed was reduced by treatment in aqueous solution with sodium borohydride at pH8. The partially reduced pectic acid obtained after treatment with ion exchange resins and freeze drying had $[\alpha]_D + 208^\circ$ and a uronic acid content of 26%. Repetition of this sequence of operations failed to reduce the acidic content significantly. The polysaccharide was propionated in formamide solution with propionic anhydride and pyridine by Carson and Maclay's method (104). The propionate was dissolved in chloroform, deionised with cation and anion exchangers and precipitated with light-petroleum (60 - 80°). The purified propionated polysaccharide, which had $[\alpha]_D + 98^\circ$, was dissolved in diethylene glycol dimethyl ether (diglyme) and reduced by periodic saturation with diborane during an interval of four weeks, the mixture being shaken from time to time to break up the gel which formed. Acyl groups were removed by heating in aqueous 5% ammonia at 65° for 45 minutes. The polysaccharide isolated had $[\alpha]_D + 210^\circ$ and a uronic anhydride content of 4%. Hydrolysis of a sample gave galactose, glucose (?), arabinose, rhamnose, small amounts of xylose, and traces of methylated sugars. To ensure that the reduced polysaccharide was structurally representative of the parent pectic acid, a sample was methylated, methanolysed, and examined by gas chromatography. Components having the

retention times of the methyl glycosides of 2, 3, 6 - tri - O - methylgalactose (major component), 2, 3, 4, 6 - tetra - O - methylgalactose, 2, 3, 5 - tri -, and 2, 3 - di - O - methyl arabinose, and 3, 4 - di -, and 3 - O - methyl rhamnose were present. This confirmed that all the major sugars present in the parent pectic acid were present in the carboxyl-reduced polysaccharide.

A trial periodate oxidation of the carboxyl reduced material showed that oxidation was complete when 0.85 moles of oxidant had been consumed per sugar unit.

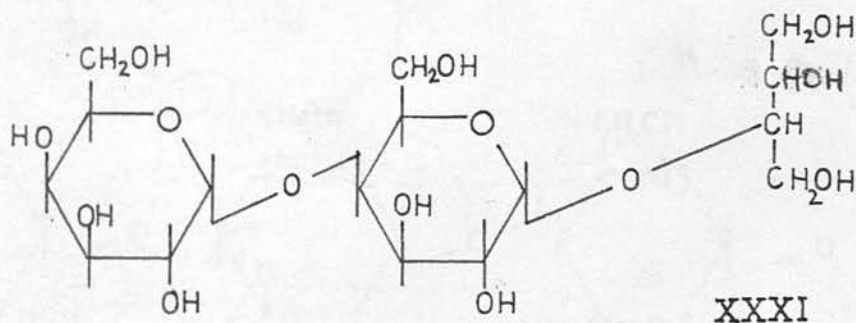
A large quantity of the reduced polysaccharide was oxidised with sodium metaperiodate until no more reagent was consumed (0.85 moles). Polyaldehyde was reduced to the polyalcohol with sodium borohydride and inorganic ions were removed with ion exchange resins. Acetal linkages were cleaved by treatment with sulphuric acid at room temperature for 4 hours and the neutralised solution was concentrated and poured into ethanol (2 vol.). No degraded polysaccharide precipitated from solution. Concentration of the solution and chromatographic examination of the syrup obtained showed the presence of glycoll-aldehyde, glycerol, threitol (major component), and four non-reducing sugars. The syrup was applied to a charcoal-Celite column and fractionated by gradient elution with water containing increasing concentrations of ethanol. The fractionation

achieved was extremely poor, further separations by chromatography on filter sheets being required. In all, five fractions were obtained. The major fraction, as anticipated for the degradation of a 1→4 linked galactan, was threitol. Another fraction gave only galactose and threitol on hydrolysis. Methanolysis of the methylated sugar yielded the methyl glycosides of 2, 3, 4, 6 - tetra - Q - methylgalactose and a number of peaks of relatively low retention time attributed to 1, 3, 4 - tri - Q - methyl - threitol. The sugar had a periodate consumption corresponding to 3 moles of oxidant per mole of sugar and formed 1 mole of formaldehyde during the oxidation. From these results the sugar must be O - α - D - galactopyranosyl - (1→2) - D - threitol(XXX).



Another fraction, present in almost the same amount as galactosyl threitol, also gave galactose and threitol on hydrolysis. Methanolysis of the methylated sugar yielded the methyl glycosides of 2, 3, 4, 6 - tetra -, and 2, 3, 6 - tri -

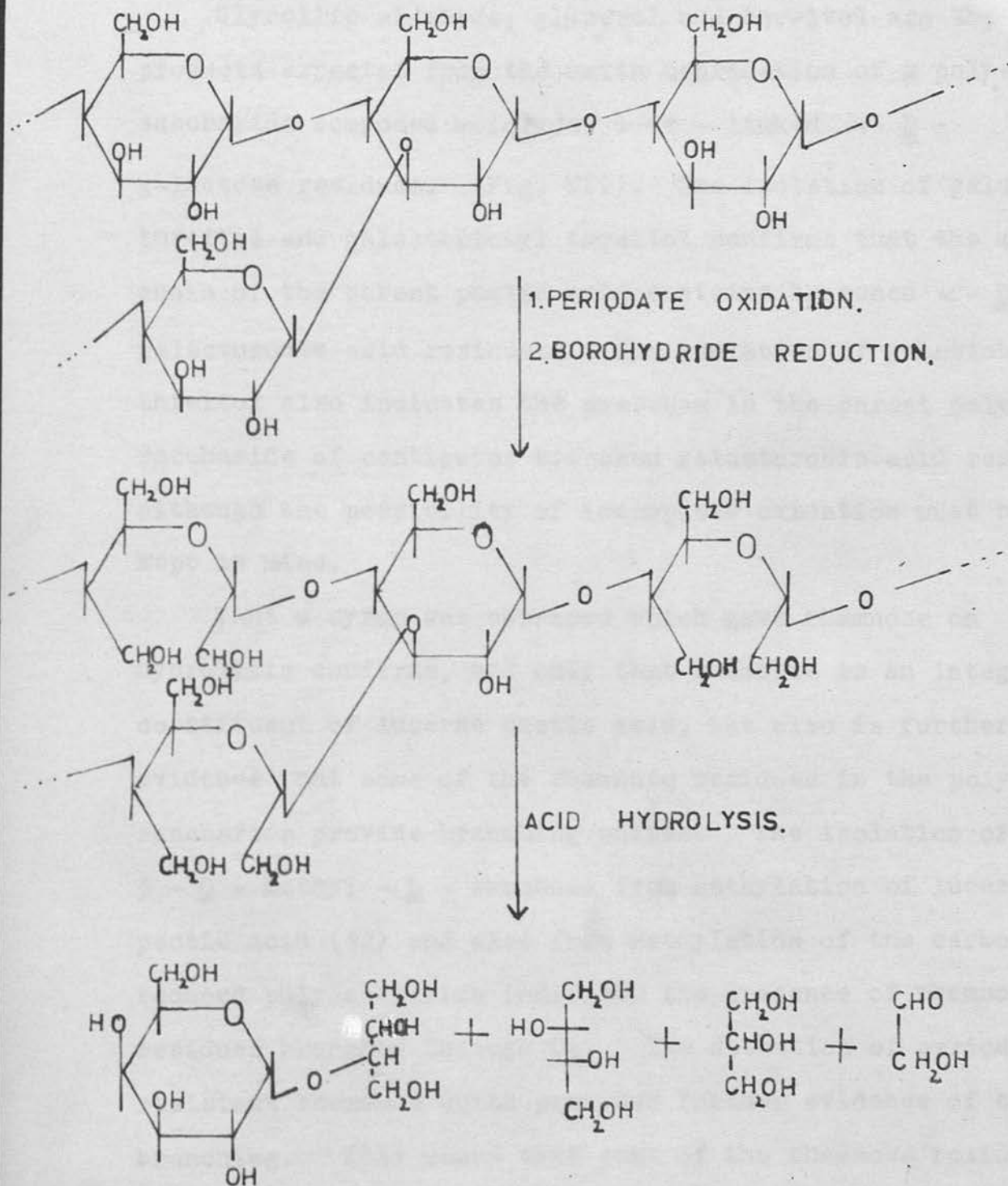
Q - methylgalactose and a number of peaks of relatively low retention time attributed to 1, 3, 4 - tri - Q - methyl - threitol. The sugar had a periodate consumption, corresponding to 4 moles of oxidant per mole of sugar and formed 1 mole of formaldehyde during the oxidation. This sugar was $O - \alpha - \underline{D}$ - galactopyranosyl - (1 \rightarrow 4) - $O - \alpha - \underline{D}$ - galactopyranosyl - (1 \rightarrow 2) - D - threitol. (XXXI).



A third fraction obtained gave arabinose and threitol on hydrolysis. Gas chromatographic examination of the products of methanolysis of the methylated sugar indicated the presence of the methyl glycosides of 2, 3, 5 - tri - Q - methylarabinose and peaks of relatively low retention time. Another sugar obtained in very small amount gave glucose, arabinose and threitol on hydrolysis. Hydrolysis of a fraction which contained mainly threitol yielded rhamnose, although it was not possible to separate threitol and this rhamnose containing sugar.

Fig. VII.

SMITH DEGRADATION.



Glycollic aldehyde, glycerol and threitol are the products expected from the Smith degradation of a polysaccharide composed mainly of 1→4 - linked α -D-galactose residues. (Fig. VII). The isolation of galactosyl threitol and galactobiosyl threitol confirms that the main chain of the parent pectic acid contains branched α -D-galacturonic acid residues. The isolation of galactobiosyl threitol also indicates the presence in the parent polysaccharide of contiguous branched galacturonic acid residues, although the possibility of incomplete oxidation must be kept in mind.

That a syrup was obtained which gave rhamnose on hydrolysis confirms, not only that rhamnose is an integral constituent of lucerne pectic acid, but also is further evidence that some of the rhamnose residues in the polysaccharide provide branching points. The isolation of 3-O-methyl-L-rhamnose from methylation of lucerne pectic acid (42) and also from methylation of the carboxyl-reduced polysaccharide indicated the presence of rhamnose residues branched through C₄. The detection of periodate resistant rhamnose units provides further evidence of this branching. This means that some of the rhamnose residues in lucerne pectic acid are similar to those obtained in the gums of the Khaya and Sterculia genera (66), (69) in that

they are linked through positions 1 and 2 in the main chain and that side chains are attached through position 4.

Similarly the isolation of a product giving arabinose on hydrolysis confirms that arabinose residues are integral constituents of lucerne pectic acid and that some of these residues provide branching points. Aspinall and Fanshawe (42) obtained 2 - O - methyl - L - arabinose on methylation of lucerne pectic acid which suggested the presence of arabinose branched at C₃. This is borne out by the Smith degradation. The fact that no higher oligosaccharides composed of arabinose units were detected indicates that citrus pectic acid does not contain concentrations of ramified arabinose residues.

Making due allowance for incomplete oxidation, it seems likely that the sugars obtained as products of the Smith degradation of carboxyl-reduced lucerne pectic acid represent branching points in the parent polysaccharide. This agrees with evidence obtained on methylation which had indicated the presence of branched residues of rhamnose and arabinose as well as residues^{of} galacturonic acid. Since the quantities of sugars isolated from the Smith degradation was small the number of branching points in lucerne pectic acid is not great.

The treatment of Laccaria Peptic acid with Ferric Chloride and Hydrogen Peroxide. (Fisher's Research), (197)

Ferric acetate (1 g.) was added to an aqueous solution of Laccaria peptic acid (100 mg. in 100 ml.) and the flask shaken for four hours. Insoluble ferric acetate was removed by centrifugation and 3% Hydrogen peroxide (1 g.) was added. Aliquot portions (10 ml.) were removed after 3 hours, 6 hours and then every 12 hours up to 54 hours, pipetted directly into an equal volume of alcohol and the precipitated polysaccharide was removed at the centrifuge. The supernatant liquids were treated with Amberlite resin 12 - SECTION III evaporated to dryness. Paper chromatographic examination of the products in solvent systems (A) and (B) indicated the presence of arabinose, galacturonic acid, and acidic oligosaccharides.

Large Molecule Degradation.

Laccaria peptic acid (2 g.) was dissolved in water (1 l.) and the solution concentrated to 100 ml. Ferric acetate (1 g.) was added, the reaction vessel was shaken for four hours, and insoluble ferric acetate was removed at the centrifuge. 3% Hydrogen peroxide (3 ml.) was added and the solution shaken continuously for 48 hours. After 12 hours a considerable decrease in viscosity was observed.

The Treatment of Lucerne Pectic Acid with Ferric Chloride and Hydrogen Peroxide. (Fenton's Reagent). (107)

Ferric acetate (1 g.) was added to an aqueous solution of lucerne pectic acid (200 mg. in 100 ml.) and the flask shaken for four hours. Insoluble ferric acetate was removed by centrifugation and 30% hydrogen peroxide (1 g.) was added. Aliquot portions (10 ml.) were removed after 3 hour, 6 hours and then every 12 hours up to 51 hours, pipetted directly into an equal volume of alcohol and the precipitated polysaccharide was removed at the centrifuge. The supernatant liquids were treated with Amberlite resin IR - 120 (H) and evaporated to dryness. Paper chromatographic examination of the products in solvent systems (A) and (B) indicated the presence of arabinose, galacturonic acid, and acidic oligosaccharides.

Large Scale Degradation.

Lucerne pectic acid (2 g.) was dissolved in water (1 l.) and the solution concentrated to 100 ml. Ferric acetate (1 g.) was added, the reaction vessel was shaken for four hours, and insoluble ferric acetate was removed at the centrifuge. 30% Hydrogen peroxide (3 ml.) was added and the solution shaken continuously for 48 hours. After $1\frac{1}{2}$ hours a considerable decrease in viscosity was observed.

After 48 hours the solution was added to an equal volume of ethanol and the polysaccharide which precipitated was removed at the centrifuge and treated again with ferric chloride and hydrogen peroxide. The combined supernatants were concentrated to remove ethanol, treated with Amberlite resin IR - 120 (H), concentrated and applied to a column of anion exchange resin. Neutral sugars were eluted with water and acidic sugars with 2N formic acid. The total yield of acidic sugars was 62 mg. Chromatographic examination indicated the presence of a number of acidic oligosaccharides.

For purposes of comparison and to provide suitable reference sugars, lucerne pectic acid (1 g.) was partially hydrolysed with N sulphuric acid (25 ml.) on a boiling water bath for 3.5 hours. Degraded polysaccharide was precipitated by the addition of acetone (1 vol.) and removed at the centrifuge. The supernatant solution was concentrated to remove acetone, neutralised with barium carbonate, filtered, and treated with Amberlite resin IR - 120(H) to remove barium ions. The solution, which contained acidic and neutral sugars, was applied to a column of Amberlite resin CG-45 (formate form). Neutral sugars were eluted with water, and acidic sugars were removed with 2N formic acid. The mixture of acidic oligosaccharides obtained was examined in

solvent system (B) and compared with the mixture of acidic sugars obtained by treatment with ferric acetate and hydrogen peroxide.

R _{Gala} Values of Acidic Sugars obtained with Fenton's Reagent	R _{Gala} Values of Sugars obtained by partial hydrolysis.	Coloured with p-Anisidine HCl.	Probable Identity
0.04	0.04	Brown	Trigalacturonic Acid
0.20	0.10 0.20	Red	Digalacturonic Acid
0.45	0.45	Red - Brown	
0.63(tr.)	0.60	Red - Brown	Galacturonosyl rhamnose.
0.85	0.85	Orange-Red	

Alkaline Degradation of Polysaccharides.Degradation of the 2-hydroxyethyl ester of tragacanthic acid.

The 2 - hydroxyethyl ester of tragacanthic acid (630 mg.) was dispersed in dry methanol (40 ml.) and a solution of sodium (0.25 g.) in dry methanol (25 ml.) added. Air was displaced from the flask with dry nitrogen and the stoppered flask shaken for 14 days. Insoluble material was removed at the centrifuge and the solution was treated with Amberlite resin IR - 120(H). The major portion of the solution was then concentrated to a syrup and examined by paper chromatography in solvent systems (A) and (B). This showed the presence of galactose, xylose, a trace of fucose and two oligosaccharides with R_G values of 1.4 and 0.95 in solvent system (A) and 1.0 and 0.6 respectively in solvent system (B). These oligosaccharides were chromatographically indistinguishable from 2 - \underline{O} - α - \underline{L} - fucosyl - \underline{D} - xylose and 2 - \underline{O} - β - \underline{D} - galactosyl - \underline{D} - xylose. Several faster moving sugars were also detected.

The methanol insoluble residue was dissolved in water, decolourised with charcoal, deionised with Amberlite resin IR - 120 (H) and aliquot portions of the aqueous and methanolic solutions were analysed for carbohydrate by the phenol-sulphuric acid method and for the presence of unsaturated products with

thiobarbituric acid (108). The reaction with thiobarbituric acid provided evidence for the presence of unsaturated degradation products in both the aqueous and methanolic solutions, although the reaction with the aqueous solution was considerably weaker.

The experiment was repeated using a commercial sample of a fully esterified citrus pectin. The methanolic solution contained arabinose and galactose with slower moving material with R_{Gal} 0.7. The reaction with thiobarbituric acid again provided evidence for the presence of unsaturated products.

Alkaline Degradation of Polysaccharides.Degradation of the 2-hydroxyethyl ester of tragacanthic acid.

The 2 - hydroxyethyl ester of tragacanthic acid (630 mg.) was dispersed in dry methanol (40 ml.) and a solution of sodium (0.25 g.) in dry methanol (25 ml.) added. Air was displaced from the flask with dry nitrogen and the stoppered flask shaken for 14 days. Insoluble material was removed at the centrifuge and the solution was treated with Amberlite resin IR - 120(H). The major portion of the solution was then concentrated to a syrup and examined by paper chromatography in solvent systems (A) and (B). This showed the presence of galactose, xylose, a trace of fucose and two oligosaccharides with R_G values of 1.4 and 0.95 in solvent system (A) and 1.0 and 0.6 respectively in solvent system (B). These oligosaccharides were chromatographically indistinguishable from 2 - \underline{O} - α - \underline{L} - fucosyl - \underline{D} - xylose and 2 - \underline{O} - β - \underline{D} - galactosyl - \underline{D} - xylose. Several faster moving sugars were also detected.

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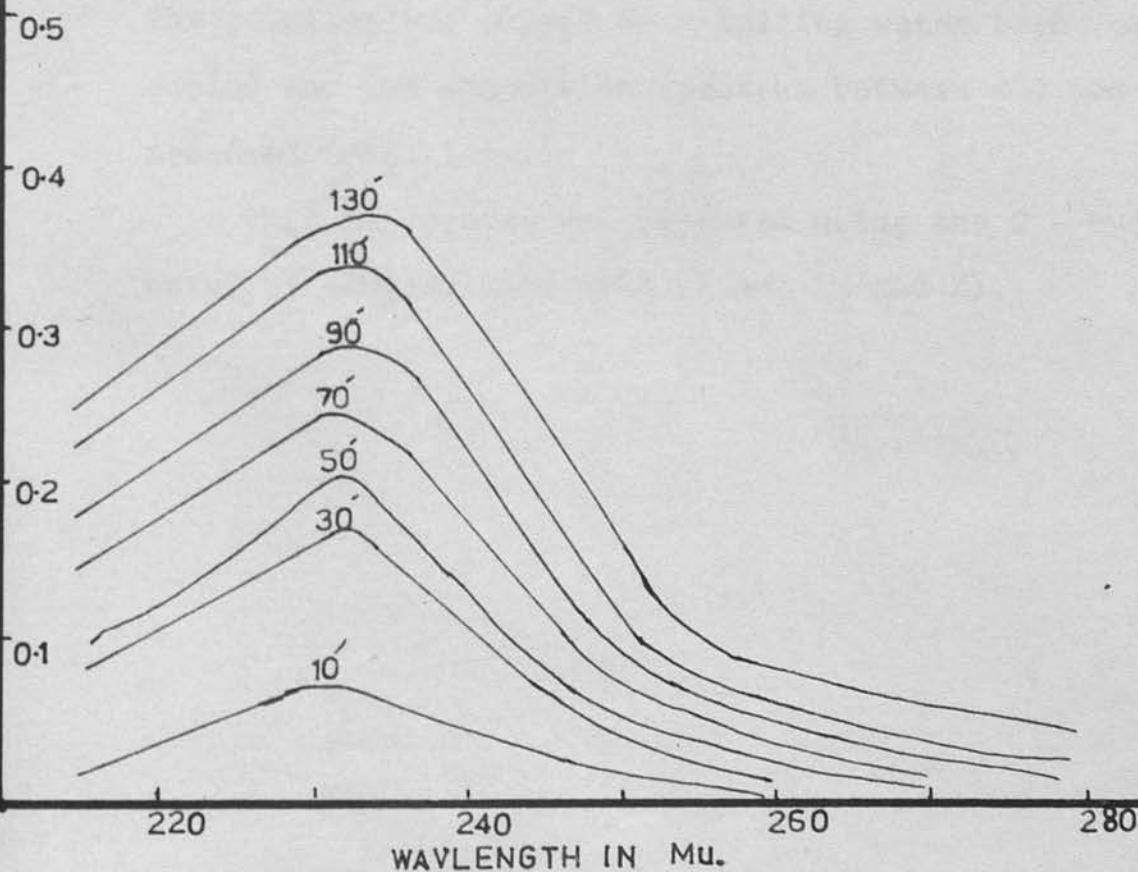
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Fig.X

INCREASE IN ULTRA VIOLET ABSORPTION OF A SOLUTION OF THE ETHYLENE GLYCOL ESTER OF GUM TRAGACANTH DURING A 2HR. BUFFER TREATMENT

OPTICAL DENSITY



Degradation of Polysaccharides in Neutral Solution.Degradation of a commercial sample of 100% esterified citrus pectin.

Citrus pectin (400 mg.) was dissolved in water (20 ml.) and heated on a water bath at 95° . 0.1M Sodium phosphate buffer (20 ml.), pH 6.8 at 95° was added, the solution mixed, and the heating continued for 130 minutes. Aliquot portions (1 ml.) were removed at 10 minute intervals, diluted ten times and the ultra-violet absorption measured on a Unicam spectrophotometer. (Fig. VIII). At the end of 130 minutes an aliquot portion 1 ml. was taken and 5 ml. of 0.5M hydrochloric acid and 10 ml. of 0.01M thiobarbituric acid were added. The solution was placed on a boiling water bath for 30 minutes, cooled and the absorption spectrum between 480 and 580 μ measured (Fig. IX).

This experiment was repeated using the 2 - hydroxyethyl ester of tragacanthic acid (Figs. IX and X).

DISCUSSION

The generally accepted mechanism for the alkaline degradation of polysaccharides in the absence of oxygen involves a stepwise elimination of anomeric residues from the reducing end. The glycosidic linkages within the chain are usually extremely alkali resistant. In the case of pectin(methyl ester of pectic acid) however, it has been found that glycosidic linkages within the chain are readily split by alkali producing fragments of lower molecular weight (15). This lability of pectin in alkaline solutions has been attributed to the presence of unusual glycosidic linkages by a β -D-galacturonic acid. This type of reaction, which results in the formation of Δ -4,5 unsaturated galacturonic acid end groups, has been shown to occur when pectins are either heated in neutral solution (12) or degraded with certain pectinases (59). It was hoped to achieve a controlled degradation of this type so that only these glycosidic linkages in the β position to ester carboxyl groups would be cleaved. Unfortunately it was impossible to find an anhydrous solvent which was polar enough to dissolve the polysaccharide. The investigation was, therefore, carried out on a heterogeneous reaction mixture. The isolation and

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The generally accepted mechanism for the alkaline degradation of polysaccharides in the absence of oxygen involves a stepwise elimination of monosaccharide residues from the reducing end. The glycosidic linkages within the chain are usually extremely alkali resistant. In the case of pectin (methyl ester of pectic acid) however, it has been found that glycosidic linkages within the chain are readily split by alkali producing fragments of lower molecular weight (15). This lability of pectin in alkaline solutions has been attributed to the scission of internal glycosidic linkages by a β - elimination mechanism. This type of reaction, which results in the formation of Δ - 4,5 unsaturated galacturonic acid end groups, has been shown to occur when pectins are either heated in neutral solution (12) or degraded with certain pectinases (55). It was hoped to achieve a controlled degradation of this type so that only these glycosidic linkages in the β position to ester carboxyl groups would be cleaved. Unfortunately it was impossible to find an anhydrous solvent which was polar enough to dissolve the polysaccharide. The investigation was, therefore, carried out on a heterogeneous reaction mixture. The isolation and

chromatographic identification of xylose, fucosyl-xylose, and galactosyl-xylose as products of the degradation (Fig.XI) of the 2 - hydroxyethyl ester of tragacanthic acid together with the detection of unsaturated reaction products with thiobarbituric acid showed that the required elimination reaction had proceeded to some extent. The sugar fragments isolated must have been attached to modified galacturonic acid residues at some intermediate stage. Since a similar β elimination occurs in neutral solution, it was decided to investigate the degradations of the 2 - hydroxyethyl ester of gum tragacanth and of a fully esterified commercial citrus pectin under these conditions. It was hoped that under the milder conditions, products attached to modified galacturonic acid residues would be obtained. The preliminary investigations, however, were not encouraging and did not indicate the formation of unsaturated products to any great extent. Using the value of the molar extinction coefficient quoted by Suzuki (109) it was calculated that the final concentration of unsaturated material was only 3%.

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