Separated leaves and stems of lucerne were extracted stepwise with ethanol-water (4 : 1) which removed colouring matter and soluble sugars and with cold water which extracted a complex mixture of acidic polysaccharides which were contaminated with inorganic material and protein, and subsequent extraction of the lucerne stem with ethylendiaminetetra acetic acid yielded a pectic acid which was purified via the calcium salt and regenerated as ammonium pectate. Lucerne stem ammonium pectate on partial acid hydrolysis furnished the following acidic oligosaccharides:

4-\(\alpha\)-(\(\alpha\)-galactopyranosyluronic acid)-\(\alpha\)-galacturonic acid,

\(\alpha\)-galactopyranosyluronic acid-(1 \(\rightarrow\) 4)-\(\alpha\)-galactopyranosyluronic acid -(1 \(\rightarrow\) 4)-\(\alpha\)-galacturonic acid,

2-\(\alpha\)-(\(\alpha\)-galactopyranosyluronic acid)-L-rhamnose,

\(\alpha\)-galactopyranosyluronic acid-(1 \(\rightarrow\) 2)-\(\alpha\)-L-rhamnopyranosyl-(1 \(\rightarrow\) 4)-\(\alpha\)-galactopyranosyluronic acid-(1 \(\rightarrow\) 2)-L-rhamnose,

6-\(\alpha\)-(\(\beta\)-glucopyranosyluronic acid)-L-galactose,

4-\(\beta\)-(\(\beta\)-glucopyranosyluronic acid)-L-fucose.

The significance of these results is discussed in relation to the structure of lucerne pectic acid.

The water-extracted polysaccharides from the separated leaves and stems were passed through columns of Sulphoethyl-Sephadex C-50 to remove cations and some of the protein, the resulting solutions were further deproteinised, and the polysaccharides were fractionated on diethylaminoethyl-Sephadex A-50 by elution with water containing increasing proportions of formic acid to yield various polysaccharide fractions and evidence was obtained for the presence of three distinct polysaccharide types, a neutral arabinogalactan, acidic arabinogalactan and highly esterified pectinic acids.

Partial acid hydrolysis of the neutral and acidic arabinogalactans gave the three galactobioses, 3-, 4-, and 6-\(\beta\)-galactopyranosyl-L-galactose. Methylation studies of both the neutral and acidic arabinogalactans indicated the presence of the following structural units in the methylated polysaccharides, 2,3,4- and 2,3,5-tri-, 2,3-, 2,5- and 3,5-di-\(\alpha\)-methyl arabinose, 2,3,4,6-tetra-, 2,3,4-, 2,3,6- and 2,4,6-tri and 2,4-di-\(\alpha\)-methyl galactose together with a trace of 2,3,4-tri-\(\alpha\)-methyl rhamnose. In addition the oligosaccharide 3-\(\beta\)-arabinopyranosyl-\(\Lambda\)arabinose was detected on mild hydrolysis of an acidic arabinogalactan, while the acidic methyl glycosides from a methylated acidic arabinogalactan were separated and remethanolysed to afford the methyl glycosides of the following sugars, 3,4-di- and 3-\(\alpha\)-methylrhamnose, 2,3,4-tri-\(\alpha\)-methyl glucuronic acid.
2,3-di-0-methyl galacturonic acid. The structural significance of these results is discussed.

The pectinic acid fractions of highest degree of esterification from both leaves and stems were partially hydrolysed and in both cases the same acidic oligosaccharides were identified as had previously been characterised as partial hydrolysis products of the pectic acid extracted from lucerne with ethylenediaminetetra-acetic acid. The only discernable difference between the pectic acid and the water-extracted pectins was that the latter polysaccharides had higher degrees of esterification. Graded precipitation with sodium acetate of the de-esterified water-extracted pectins afforded five polysaccharide fractions which were analysed and examined electrophoretically and again the results are discussed.

The extracellular polysaccharides from sycamore cambial cells were divided into water-soluble and water-insoluble fractions. The water-soluble polysaccharides contained an acidic component which was precipitated as its copper salt. The neutral polysaccharides were fractionated on a cellulose column to yield an arabinogalactan and a xyloglucan.

Partial acid hydrolysis of the arabinogalactan afforded the two galactobioses, 3- and 6-0-β-D-galactopyranosyl-D-galactose. A methylation study indicated the presence of the following structural units in the methylated polysaccharide, 2,3,5-tri- and 2,3-di-0-methyl arabinose, 2,3,4-tetra-, 2,4,6- and 2,3,4-tri- and 2,4-di-0-methyl galactose. A methylation study of the xyloglucan indicated the presence in the polysaccharide of the following structural units 2,3,4-tri-, 3,4- and 2,3-di-0-methyl xylose, 2,3,4-tri-0-methyl fucose, 2,3,4,6-tetra-, 2,3,6-tri- and 2,3-di-0-methyl glucose. Enzymic degradation of the xyloglucan afforded two oligosaccharides which were characterised as 4-0-β-D-xylopyranosyl-D-xylene and 4-0-β-D-glucopyranosyl-D-glucopyranose and a minor component which contained glucose, xylose and fucose. The significance of these results are discussed in relation to the structures of the two neutral polysaccharides.

 Araucaria bidwillii gum on partial hydrolysis furnished oligosaccharides which were characterised as 6-0-β-D-galactopyranosyl-D-galactose and the trisaccharide, 0-β-D-galactopyranosyl-(1 → 6)-0-β-D-galactopyranosyl-(1 → 6)-D-galactose. The characterisation of the two oligosaccharides together with the results of a previous study confirmed the occurrence of acid-catalysed anomerisation at the glycosidic bond during acetolysis of the carboxyl-reduced gum, affecting a high proportion of 1,6 linkages.
STRUCTURAL STUDIES ON
PECTIC SUBSTANCES WITH A SPECIAL
REFERENCE TO THE POLYSACCHARIDES
OF LUCERNE AND SYCAMORE

by

JOHN A. MOLLOY, M.Sc.

A thesis presented for the degree of Doctor of Philosophy
University of Edinburgh

July, 1968
To my parents and to Cait
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INTRODUCTION

General

Previous investigations of lucerne had indicated the presence of various polysaccharide components. Cold water extraction yielded a complex mixture of acidic polysaccharides which were contaminated with inorganic material and protein and subsequent extraction with ammonium oxalate or ethylenediaminetetra-acetic acid (disodium salt) yielded a pure pectic acid. A large part of the present investigation has been concerned with the pectic acid and with the water-extracted polysaccharides, which were shown to consist of a mixture of highly esterified pectins and associated arabinogalactans. A study of these arabinogalactans provided evidence for the presence of two distinct polysaccharide types, acidic arabinogalactans containing appreciable proportions of uronic acid and a neutral arabinogalactan devoid of uronic acid. Subsequent to this work an investigation was carried out on a related topic, on the extracellular polysaccharides from sycamore cambial cells. A previous investigation of this polysaccharide mixture had indicated the presence of a pectinic acid and an arabinogalactan and attention was at first focussed on the arabinogalactan. However, in the course of this study a xyloglucan was isolated from the extracellular polysaccharide mixture and the xyloglucan was examined in some detail.
The wood arabinogalactans have basic structural features in common with the acidic galactan-based polysaccharides from the exudate gums. Araucaria bidwillii gum acid is a representative of the latter group. Accordingly a study of A. bidwillii gum was included in the present investigation of arabinogalactans with a view to elucidating the problem of acid-catalysed anomeration of certain glycosidic bonds which was suspected to occur during acetolysis of the gum.
PART I
THE PECTIC SUBSTANCES

A large section of the present investigation was concerned with the pectic and pectinic acids present in lucerne together with the arabinogalactans which occur in association with them. Therefore, a brief introduction to the chemistry of pectic substances is here given. Pectins occur in plants in both the primary and to a lesser extent in the secondary cell wall and in the intercellular layer. Pectins are most abundant in soft tissues composed mainly of primary cell walls. The pectins occur in fruit juices and are associated with hemicelluloses and celluloses in the cell wall. In hard tissue such as wood they are present in negligible amount. Pectins are important commercially because of their gel-forming properties. Of the two types of gel which are formed, the first type is a pectin-sugar-acid-water gel which is formed through hydrogen bonding and is the most important industrially.

The other type of gel is an ion-bonded gel formed by cross-linking through carboxyl groups and multivalent cations especially calcium.

Definition of the terms used in pectin chemistry.

A committee of the American Chemical Society recommended a nomenclature for the terms used in pectin chemistry which were subsequently revised and can be stated as follows:

Pectic substance. "Pectic substance is a group designated
for the complex colloidal carbohydrate derivatives, which occur in or are prepared from plants and contain a large proportion of galacturonic acid units which are thought to exist in a chain-like combination. The carboxyl groups of the polygalacturonic acid may be partly esterified by methoxyl groups and partly or completely neutralised by one or more bases.

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

**Pectin.** "Pectin or pectins are those water-soluble pectinic acids of varying methoxyl content and degree of neutralisation which are capable of forming gels with sugar and acid under suitable conditions."

The function of pectic substances in plants has not yet been fully elucidated. It is considered that the insoluble pectic substances in the intercellular layer are responsible for its rigidity, whereas in the cell wall the pectic substances, because of their hydrophilic property of being able to hold quantities of excess water, provide for the hydration of young growing cell walls. This theory can be related to their presence in most abundance in the primary cell wall.

Molecular weights of pectins have been reported as varying considerably from about 10,000 to 400,000 for materials isolated from different sources and also from the same source by different extraction procedures. It is
probable, however, that this wide variation can be related to the large pectin molecules undergoing a partial degradation during extraction procedures. Direct evidence for the occurrence of degradation was provided by Schneider and co-workers.\textsuperscript{5,6} The nitration of alcohol extracted and dried slices of sugar beet tissue gave nitrated pectin of molecular weight 50,000 to 100,000, whereas pectins obtained by hot-water extraction of the same material had after nitration molecular weights of 30,000 to 50,000. Also it was reported that the molecular weight of nitrated apple pomace pectin obtained from a first extract was substantially higher than that obtained from subsequent extracts. However, in view of the susceptibility of pectic substances to degradation under various conditions, which will be discussed later, the whole question of the molecular weights of pectins would need to be re-examined.

Pectic substances are composed mainly of galacturonic acid residues but they also contain varying proportions of galactose and arabinose residues. Early work in pectin chemistry suggested that there were three distinct polysaccharides present, a galacturonan, a galactan and an arabinan. It is interesting to note, however, that all three homopolymers have never been isolated from a single source. It now appears likely that, although in some cases separate galactan or arabinan may occur in association with pectic material, galactose and arabinose are also linked to
the galacturonic acid residues. It is probable that cer-
in both cases, the 1,4-lactone of 2,3-di-6-methyl-β-galact-
tain homopolysaccharide preparations may have arisen from
degradation of pectin molecules during the severe conditions
of extraction and fractionation earlier employed.
Nevertheless, the work on the 'homopolysaccharides' helped
to establish some of the important features of the structu-
ral chemistry of pectins. The major components of pectins
are now known to be complex heteropolysaccharides as these
materials retain galactose and arabinose units, even after
exhaustive fractionation. 7

Pectin galacturonans. In the early investigations, the
pure galacturonans obtained were degradation products, since
acid hydrolysis was used to remove the accompanying galactose
and arabinose residues. One of the first investigations of
a pectic galacturonan was carried out by Levene and Kneider, 8
who carried out periodate oxidation studies and showed that
the galacturonan chains consist of galacturonic acid residues
linked through either 1 and 4 positions or the 1 and 5
positions dependent on whether the uronic acid units were
in the pyranose or furanose forms respectively. Luckett
and Smith 9 isolated a galacturonan from citrus pectin with
hot mineral acid and Beaven and Jones 10 isolated a galact-
uronan from strawberry pectin by methanolysis with hot
methanolic hydrogen chloride. These galacturonans were
about eight units in length indicating appreciable hydrolysis
of the uronic acid as well as of the neutral residues. The
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In both cases, the 1,4-lactone of 2,3-di-O-methyl-D-galactaric acid was isolated as the sole oxidation product. The authors on the basis of these observations suggested that the galacturonans consisted of chains of 1,4 linked α-D-galactopyranosyluronic acid residues (Structure I).

Jones and Reid \cite{11,12} isolated galacturonobiosate and galacturonotriose by the enzymic degradation of apple pectic acid using an enzyme from *Aspergillus foetidus*. The galacturonobiose was shown to be 4-O-(α-D-galactopyranosyluronic acid)-D-galacturonic acid (II) by reduction to 4-O-α-D-galactopyranosyl-D-galactose.

Thus these results confirmed the earlier proposed structure.

The more recent extraction procedures, which do not involve the use of acid, yield acidic polysaccharides which contain neutral sugars, and pure galacturonans are considered to be of relatively infrequent occurrence. However,
Bishop \textsuperscript{13} isolated a galacturonan by the extraction of sunflower heads with hot ammonium oxalate-oxalic acid solution. Recently Zitko and Bishop \textsuperscript{14} fractionated by precipitation with sodium acetate, the pectic acids obtained by saponification of pectins from sunflower heads, sugar beet, apple and citrus fruits. In all cases (with the possible exception of sugar beet pectic acid) it was shown that the pectic acid contained two acidic components, one thought to be a galacturonan free of neutral sugars, the other a pectic acid which contained neutral sugars as well, although the pure galacturonan was isolated only in the case of sunflower heads pectic acid. Timell and Bhattacharjee \textsuperscript{16} further fractionated the ammonium pectate from the bark of amabilis fir by acidification and ultracentrifugation to obtain a homogeneous galacturonan. Both galacturonans, from sunflower heads \textsuperscript{15} and amabilis fir,\textsuperscript{16} were reduced to the corresponding galactan, and methylation studies again indicated that galacturonans were linear molecules of α-D-galacturonic acid residues linked through the 1 and 4 positions. (Structure I).

Pectic galactans. The first pectic galactan was isolated from white lupin seeds by hot alkaline extraction by Hirst and co-workers.\textsuperscript{17} The purification procedure involved the use of dilute oxalic acid to hydrolyse the accompanying arabinose residues. From the results of methylation and from the observed specific rotations of the polysaccharide
and its methylated derivative, it was concluded that the galactan consisted of a linear chain of β-D-galactose residues linked through positions 1 and 4. (Structure III).

\[
\begin{align*}
\text{CH}_2\text{OH} & \quad \text{CH}_2\text{OH} \\
\text{O} & \quad \text{CH}_2\text{OH} \\
\text{OH} & \quad \text{O} \\
\text{OH} & \quad \text{OH} \\
\end{align*}
\]

The possibility cannot be excluded, however, that the white lupin seed galactan may have arisen from the base-catalysed degradation of pectin during the initial alkaline extraction. Moreover the white lupin seed galactan may have arisen by the removal of arabinose residues from an arabinogalactan. There is recent evidence which indicates the presence of an arabinogalactan in lupin seeds. Hot water extraction of blue lupin seeds yielded an arabinogalactan and methylation studies indicated the presence of chains of β-D-galactopyranose residues linked through positions 1 and 4, to which were attached occasional branches of one or two arabinose residues probably attached at C3 of the galactopyranose residues. There were anomalies in respect of the occurrence of branch points in the polysaccharide, but the results nevertheless indicated the presence in lupin seeds of an authentic arabinogalactan. There is evidence elsewhere to show that separate galactans
can exist in pectic substances. A galactan of similar structure to that of white lupin seeds has been isolated in small proportion from commercial citrus pectin after precipitation of the acidic polysaccharide as its copper salt \(^{19}\) and another galactan of similar structure has been isolated from the seeds of *Strychnos nux-vomica*. \(^{20}\) Recently Timell and co-workers have isolated a galactan from the compression wood of red spruce. \(^{21}\) This galactan is significant as from its method of extraction it must be an authentic component of the wood. Analysis showed that the polysaccharide contained a framework of at least 300 1,4 linked \(\beta-D\)-galactopyranose residues, five to eight branches occur per average molecule, but their mode of attachment to the framework was not ascertained. There was evidence to indicate the presence of a small proportion of uronic acid residues and that the galactan is located in the outer regions of the cell wall. A related galactan to that from red spruce compression wood was isolated by Bouveng and Meier \(^{22}\) from delignified Norwegian spruce compression wood, and shown to contain chains of 1,4 linked \(\beta-D\)-galactopyranose residues. The Norwegian spruce galactan also contained 13% uronic acid, present as both galacturonic acid and glucuronic acid. Another interesting wood galactan was isolated by Meier \(^{23}\) from beech tension wood. This polysaccharide contained both \(\beta-1,4\) as well as \(\beta-1,6\) linked \(D\)-galactose residues and was the first natural polysaccharide in which this combination was demonstrated.
Besides pure galactans, arabinogalactans have also been shown to be associated with pectic substances. An arabinogalactan has been isolated from defatted soybean flour.\textsuperscript{24} This polysaccharide consists of chains of 1,4 linked β-D-galactopyranose residues with side chains of L-arabinofuranose residues linked through 1 and 5 positions to the galactan framework at position 3. Arabinogalactans were considered to fall into two main classes, those normally associated with pectins consisting of 1,4 linked galactose residues in the β-configuration such as the arabinogalactan from soybeans,\textsuperscript{24,25} and the highly branched arabinogalactans from coniferous woods containing 1,3 and 1,6 linked galactose residues also in the β-configuration.\textsuperscript{26} But recent investigations have shown that arabinogalactans associated with pectins do not necessarily contain galactose residues which are mutually linked by 1,4 linkages only. This problem emerged from an investigation of lemon peel pectin and the extracellular polysaccharides from sycamore cambial cells\textsuperscript{27} and also emerged during the present investigation. The problem of the intergalactose linkages in the arabinogalactans which occur in association with pectins will be dealt with in detail in the discussion.

**Pectic arabinans.** Pectic arabinans have been isolated from various sources by Hirst and Jones, from peanuts,\textsuperscript{28,29,30} from sugar beet,\textsuperscript{31} from citrus fruits\textsuperscript{32} and from apple.\textsuperscript{33} In a typical case,\textsuperscript{31} methylation and hydro-
lysis of the alkali extracted sugar beet arabinan gave equimolecular proportions of 2,3,5-tri-, 2,3-di- and 2-O-methyl-L-arabinose. The high negative specific rotation and the acid lability of the pentose units led to the suggestion that all the arabinose residues are in the furanose form and that the configuration is α. A branched structure was suggested for the arabinan (Structure IV).

\[
\begin{align*}
\alpha-\text{L-Araf} \\
\downarrow & 1 \\
\rightarrow 5\alpha-\text{L-Araf} & 1 \\
\end{align*}
\]

\[
\text{IV}
\]

This structure has been supported by further investigations, by Barry degradation studies, \(^{34}\) by methylation and periodate oxidation studies \(^{35}\) and by the isolation of the oligosaccharides 5-O-\text{L-arabinofuranosyl-} \text{L-arabinofuranose} and 3-O-\text{L-arabinofuranosyl-} \text{L-arabinose} by partial acid hydrolysis of the arabinan. \(^{36}\) However, in the light of present knowledge of the susceptibility of pectins to degradation by alkali doubt must be cast on the authenticity of the 'arabinan' as an undegraded constituent of sugar beet pectin. In the light of recent evidence this arabinan
would appear to be in fact a heteropolysaccharide containing proportions of galactose, rhamnose and galacturonic acid, of a type most likely to arise from degradation of pectic material.

More recently an arabinan of the pectic type was isolated from mustard seeds, which is in all likelihood an authentic polysaccharide component of the seeds as it was isolated under conditions designed to minimise degradation of the polysaccharide. Methylation of the arabinan followed by hydrolysis yielded four major products, \( \text{L-arabinose, } 2-\text{Q-methyl-L-arabinose, } 2,3-\text{di-Q-methyl-L-arabinose and } 2,3,5-\text{tri-Q-methyl-L-arabinose} \) in the molar proportions 2 : 11 : 6 : 12. The ease of hydrolysis of the arabinan suggested that the arabinose units were in the furanose form, while an \( \alpha \)-linkage between the arabinofuranose residues was indicated by the high negative specific rotation of the polysaccharide. The structure indicated by these results is typical of that normally associated with pectic arabinans, the arabinose units being joined through 1,5 and 1,3 linkages, but it is more highly branched and contains a proportion of 1,2 linkages. A similar type of arabinan has been isolated from the aqueous extracts of maritime pine, but this polysaccharide contains a small proportion of galactose.

**Pectins as heteropolysaccharides.** Recent structural investigations of pectic materials have led to the view that
some neutral sugars are integral constituents of the acidic polysaccharides. Pectic substances extracted under non-degradative conditions and fractionated to remove neutral polysaccharides, still contain neutral sugar residues. McCready and Gee found that purified pectinic acids from several fruits and vegetables gave on hydrolysis galacturonic acid together with varying amounts of galactose, arabinose, rhamnose and xylose. The same four sugars were present in a purified pectic acid isolated from the fresh-water alga, *Nitella translucens*, and they were also obtained along with 2-2-methyl-2-xylose and 2-2-methyl-
L-fucose from the hydrolysis of sisal flash pectin. Pectic substances present in lucerne, soybeans and lemon peel, despite exhaustive fractionation contained all the above-mentioned neutral sugars together with L-fucose. Neukom and co-workers, as mentioned previously, attempted to fractionate sugar beet pectin on diethylaminoethyl-cellulose and found that all the fractions contained neutral sugars.

L-rhamnose was the first neutral sugar conclusively shown to be an integral constituent of acidic polysaccharide components of pectic material. It is linked directly to galacturonic acid as is shown by the isolation of the aldobiouronic acid, 2-2-(\(\alpha\)-D-galactopyranosyluronic acid)-L-rhamnose, (Structure V),...
from partial hydrolysis of the pectic acid from lucerne, the pectic acid from the bark of amabilis fir, the acidic polysaccharides of soybeans and lemon peel pectin. Higher oligosaccharides containing rhamnose and galacturonic acid, \( \alpha-D\)-galactopyranosyluronic acid-(1 \rightarrow 2)-\( \beta-L\)-rhamnopyranosyl-(1 \rightarrow 4)-\( \alpha-D\)-galactopyranosyluronic acid-(1 \rightarrow 2)-\( \alpha-L\)-rhamnose (Structure VI), and the trisaccharides \( \alpha-D\)-galactopyranosyluronic acid-(1 \rightarrow 4)-\( \alpha-D\)-galactopyranosyluronic acid-(1 \rightarrow 2)-\( \alpha-L\)-rhamnose (Structure VII) and \( \alpha-D\)-galactopyranosyluronic acid-(1 \rightarrow 2)-\( \beta-L\)-rhamnopyranosyl-(1 \rightarrow 2)-\( \alpha-L\)-rhamnose (Structure VIII) (evidence for the structure of oligosaccharides VI-VIII is chromatographic, the conformation of the sugars are based on other evidence, and the configurations of the rhamnosyl linkages are based on specific rotations), have been obtained by the partial degradation of pectins from various sources, from the acidic polysaccharides of soybeans and from lemon peel pectin.
The isolation of the rhamnose containing oligosaccharides (V-VIII), shows that in the main chain of pectins occasional rhamnose residues occur. It is apparent that the rhamnose units may alternate with galacturonic acid units, or that the rhamnose units are adjacent in certain parts of the molecule. Further information on the role of the rhamnose residues has been obtained from methylation studies, which indicate that in the main chain of pectins some of the rhamnose residues may constitute branch points.

Small amounts of two glucuronic acid-containing aldobiouronic acids, 6-O-(β-D-glucopyranosyluronic acid)-D-galactose (Structure IX) and 4-O-(β-D-glucopyranosyluronic acid)-L-fucose (Structure X), have recently been isolated from some pectins, from lemon peel pectin and from soybean acidic polysaccharides. The exact structural significance of these oligosaccharides has not
In recent investigations of lucerne pectic acids all of the above-mentioned oligosaccharides (Structures V–X) have been isolated. The structural significance of these oligosaccharides in relation to lucerne pectic acid will be discussed later in conjunction with the results of the present investigation.

As mentioned earlier galactose and arabinose residues must also be integral constituents of the acid polysaccharide components of pectic materials, as these sugars are not removed even by exhaustive fractionation of the polysaccharide. Methylation studies have shown that a large proportion of the arabinose and galactose residues exist as non-reducing end groups, but they may be also attached in structural units similar to those in the respective pectic homopolysaccharides. In lemon peel pectin galactose residues are mainly non-reducing end groups while arabinose residues are linked in the branched structure of arabinans of the pectic type. In sisal pectic acid however, the arabinose is present predominantly as end groups. In contrast, \(4-O-\beta-D\)-galacto-
pyranosyl-\(\beta\)-galactose and its higher homologues have been isolated as partial hydrolysis products from soybean acidic polysaccharides \(^{44,45}\) and from commercial citrus pectin,\(^{19}\) showing the presence of chains of 1,4 linked galactose residues in these pectins. An enzymic degradation of pectic material from soybeans gave an oligosaccharide, not fully characterised, in which chains of 1,4 linked galactopyranose residues are linked either directly or through xylose or rhamnose to a single unit of galacturonic acid (Structure XI).

\[
\begin{align*}
\beta-\text{D-Galp} &\xrightarrow{4\beta-\text{D-Galp}} 4\beta-\text{D-Galp} &\xrightarrow{\text{D-Galp}} \text{L-Rhap} \\
\text{D-Xylp} &\quad & & \quad \\
\end{align*}
\]

Recently enzymic degradation of acidic polysaccharides from soybeans \(^{44}\) and also from lemon peel pectin \(^{19}\) yielded an oligosaccharide, 3-\(\text{O-}\beta-\text{D-xylopyranosyl-}\beta\)-galacturonic acid, which shows that xylose is an integral constituent sugar of these pectins. Recently from the degradation of lucerne pectic acid with a pectinase preparation,\(^{46}\) an interesting disaccharide was characterised which contained arabinose linked directly to galacturonic acid through position 3. This was the first direct evidence for the linkage of arabinose to the galacturonan chain, and it will be discussed later in connection with the degradation of lucerne pectic acids.
Degradation of pectic substances. Pectic substances are very susceptible to degradation under a variety of conditions. The subject of degradation can be considered in four separate categories, degradation in acid solution, in alkaline solution, degradation in neutral solution and degradation by enzymes.

The arabinofuranose units in pectic substances are extremely labile to acid and thus only the weakest of acidic solutions can be used in the extraction and fractionation of pectins without degradation occurring. On the other hand, the partial degradation of pectins with acid can be used to isolate various acidic oligosaccharides and thus be used to positive advantage in structural investigations. Polysaccharides, in oxygen-free solution, undergo alkaline degradation which begins at the reducing end of the molecule and proceeds in a step-wise manner along the sugar chain. In chains with sugar residues linked through 1 and 3 or 1 and 4 positions, this 'peeling process' proceeds with the release of the corresponding saccharinic acids, but chains with sugar residues linked through the 1 and 2 positions are resistant to alkali. Pectic substances contain 1,4 linked galacturonic acid residues in chains and they degrade under alkaline conditions to saccharinic acids. However, the extent of degradation in this type of process is small and the decrease in molecular weight is
not readily detected by methods such as viscosity measurements.

It has been shown by Vollmert \(^{48}\) that scission of some of the glycosidic linkages within the pectin chain occurs during the alkaline saponification of pectin at 20°C. This produces a rapid decrease in molecular weight. Moreover Vollmert has shown that pectic acid is not depolymerised under the same conditions and it was concluded that the extent of depolymerisation is related to the degree of esterification of the pectin. Launer and Tomimatsu \(^{49}\) have established that for approximately eighty de-esterifications which occur one glycosidic linkage is split during alkaline saponification of pectin at 25°C. Neukom and Deuel \(^{50}\) have studied the effect of temperature on the degradation of citrus pectin during alkaline saponification and they found that the viscosity of the sodium pectate solution produced is dependent on the saponification temperature selected. Similar results were obtained with the glycol esters of the pectic acid, but in both cases the de-esterified sodium pectate formed was not depolymerised by alkali under these conditions, but at higher temperatures was further degraded by the usual step-wise alkaline degradation from the reducing end. More recently the effects of temperature and pH in the alkaline saponification of pectins have been investigated \(^{51}\) and suitable conditions have been deter-
mined for saponification of pectins without any appreciable degradation of the molecule. The instability of the esters of pectic acids to alkali is explained by assuming that chain scission occurs via a \( \beta \)-dealkoxylation mechanism (Structure XII).

As depicted the glycosidic linkage in the \( \beta \)-position to the ester carbonyl group is cleaved following the removal of the activated hydrogen at \( C_5 \) and the formation of a double bond between \( C_4 \) and \( C_5 \). In the case of pectic acids the non-esterified carboxyl group at \( C_6 \) is not sufficient to activate the hydrogen at \( C_5 \) and therefore
degradation does not proceed.

Pectin is susceptible to degradation in hot solutions of phosphate buffer.\(^5\) Again this is due to the presence of carboalkoxy groups as pectic acid is quite stable when similarly treated. Albersheim, Neukom and Deuel\(^5\) investigated the mechanism of the degradation of citrus pectin in phosphate buffer at pH 6.8, and they found that, even under these neutral conditions, the characteristics of the degradation are the same as in alkaline solution. The degradation is a function of the degree of esterification, \(\beta\)-elimination and the concomitant cleavage of glycosidic bonds occur. The presence of a \(\beta\)-elimination mechanism was indicated by an increasing absorption at 235 nm characteristic of \(\alpha,\beta\) unsaturation and also by the formation, by ozonolysis, of oxalic acid which is only produced from unsaturation in the \(C_4, C_5\) position of uronic acids.\(^5\)

Barrett and Northcote\(^5\) degraded purified apple pectinic acid by heating with hot phosphate buffer at pH 6.8 and obtained two polysaccharide components separable by zone electrophoresis and Sephadex gel filtration. The high molecular weight component was composed mainly of neutral sugars (69\%), whereas the low-molecular weight component contained a very high proportion of galacturonic acid (98\%). The authors concluded that the pectinic acid molecules contained long chains of
galacturonosyl residues but also contained more neutral sections with large arabinofuranose 'blocks' covalently linked to the main chains, and that during the degradation the molecule was split into two types of fragment, one containing uronic acid predominately and the other mainly neutral sugars.

Pectic substances can also be degraded by enzymes of which there are three major types. The first group contains the pectinesterases which catalyse the de-esterification of the natural and synthetic esters of polygalacturonans.

The second group includes the polygalacturonases which catalyse the hydrolysis of glycosidic linkages. Among these are the fungal polygalacturonases. These enzymes are probably mixtures of three or more components, one of which hydrolyses pectin to galacturonobiose and galacturonotriose while another hydrolyses these to galacturonic acid.\(^{57,58}\) The action of yeast polygalacturonase on pectins has been examined by Phaff and co-workers.\(^{59,60,61,62,63}\) This enzyme is an endopolygalacturonase which catalyses a random hydrolysis of pectic acid to galacturonic acid and galacturonobiose via a series of oligogalacturonides. Evidence has been presented to show that yeast endopolygalacturonase is a single enzyme and an interesting feature is that the galacturonic acid end group in the oligo- or polyuronide chain inhibits the hydrolysis of neighbouring glycosidic linkages. This enzyme is unable to
attack galacturonobiase for example. A series of oxidised and reduced derivatives of oligouronides were hydrolysed using this enzyme \(^6\) and it was shown that the bond at the non-reducing end of the chain is protected from attack by yeast endo-polygalacturonase.

Acidic oligosaccharides containing neutral sugars have been isolated by enzymic degradation of pectic substances. Aspinall and co-workers have isolated \(\beta-\text{D}-\text{xylopyranosyl-D-galacturonic acid}\) from soybean \(^4\) and lemon peel pectins\(^1\) while the oligosaccharide \(\alpha-(\text{galactopyranosyluronic acid})-(1 \rightarrow 2)-\text{D}-\text{rhamnopyranosyl-(1 \rightarrow 4)}-\text{galacturonic acid}\) has been isolated by enzymolysis of lemon peel pectin.\(^\text{19}\) A third type of pectic enzyme \(^6\) catalyses the degradation of pectins by way of a trans-elimination mechanism similar to that previously observed for the neutral and alkaline degradation of pectins.

(Structure XIII).

\[
\begin{align*}
\text{COOCH}_3 & \quad \text{COOCH}_3 \\
\text{OH} & \quad \text{OH} \\
\text{OH} & \quad \text{OH} \\
\text{OH} & \quad \text{OH} \\
\text{OH} & \quad \text{OH} \\
\end{align*}
\]

A product is obtained containing 4,5 unsaturated hexuronic acid residues. The enzyme is designated as pectin trans-eliminase and it only attacks pectins and not pectic acid. Unsaturated products have also been obtained from pectic
materials by the use of the pectolytic enzyme from *Bacillus polymixae.* This enzyme in contrast is a polygalacturonic acid transeliminase which is more active on pectic acid than on pectin and which attacks the molecule from the non-reducing end. Hasegawa and Nagel have characterised 4-O-(4-deoxy-β-L-threo-hex-4-enosyluronic acid)-D-galacturonic acid from the action of this enzyme on pectic acid.

**Fractionation and purification of pectins.** In the extraction of pectic substances care must be taken to ensure that degradation is minimised. Before commencing extraction any enzymes present must be inactivated by treating the freshly cut material with 90% ethanol. The only methods of extraction not liable to cause degradation are cold water extraction, from which the yield of pectin is not high, or extraction in hot solutions of such reagents as ammonium oxalate or ethylene-diaminetetra acetic acid (disodium salt) buffered between pH 4 and 5. It is clear that to guard against degradation some efficiency must be sacrificed.

The problems encountered in the fractionation of pectins include the separation of neutral polysaccharides from acidic polysaccharides and the further fractionation of acidic polysaccharides of varying uronic acid contents. Acidic polysaccharides can be precipitated as their insoluble calcium or copper salts. This precipitation procedure does not always yield a pure acidic component.
as some neutral material may be co-precipitated. Recently Neukom and co-workers \(^7\) chromatographed sugar-beet pectin on diethylaminoethylcellulose (phosphate form). All the fractions on hydrolysis were shown to contain galactose and arabinose as well as galacturonic acid which shows, as was indicated earlier, that these neutral sugars are integral constituents of pectin. Similarly apple, citrus fruit and sugar-beet pectins \(^68\) were fractionated to yield acidic polysaccharides and in addition neutral components which were either an arabinogalactan or a mixture of galactan and arabinan. Diethylaminoethylcellulose as ion-exchange medium is limited by its low capacity but recently an ion-exchange resin of high capacity, diethylaminoethyl-Sephadex A-50 has been made available. This ion-exchange resin was used to fractionate lemon peel pectin. \(^19\) Because of its large capacity it can be used to fractionate pectins on a preparative scale. Zitko and Bishop \(^14\) have applied the technique of fractionation by precipitation with different concentrations of sodium acetate of the pectic acids obtained by saponification of pectins from sunflower heads, sugar-beet, apple and citrus fruits, to isolate a galacturonan from sunflower and acidic polysaccharides containing various proportions of neutral sugars.

A useful criterion of the homogeneity of pectic acids can be supplied by electrophoresis techniques, by moving
boundary electrophoresis $^{14, 16, 56}$ or by zone electrophoresis.$^{56}$

THE ARABINOGLACTANS

Cell walls in plants contain cellulose as the major polysaccharide component. The cellulose exists as long thread-like macromolecules which consist of chains of $1, 4$ linked $\beta-D$-glucopyranose residues. Interspersed with these macromolecules are areas rich in lignin and the other major polysaccharide component 'the hemicelluloses'. There is also some pectin present in the cell wall.

The function of the hemicelluloses has not been completely elucidated. They undoubtedly contribute to the rigidity of the cell wall while many also seem to be concerned with metabolic processes. The extraction of hemicelluloses often requires strong alkali which can result in degradation of these polymers. The insolubility of the hemicelluloses in situ may be due to a chemical combination with lignin or the physical restraint which polymers impose on the solution of each other.

Hemicellulose polysaccharides can be classified by the presence of similar arrangements of sugars in the main chains.$^{69}$ For example the xylan, mannanglucomannan and arabinogalactan groups comprise three well-known families of structurally related polysaccharides. In regard to the arabinogalactan it should be noted that
they occur largely outside the cell wall. The xylan group is based on chains of 1,4 linked β-D-xylopyranose residues which accommodate the two most commonly encountered side chains in this group, 4-O-methyl-D-glucuronic acid or 4-O-methyl-D-glucuronic acid and L-arabinose. 69

The characteristic of the glucomannan family is that the main chains of the polysaccharides contain 1,4 linked β-D-glucopyranose and β-D-mannopyranose residues. A related so-called 'galactoglucomannan' is included in this family. This polysaccharide contains in addition D-galactose residues attached to the main chain. 69

The arabinogalactan group is characterised by the presence of 1,3 and 1,6 linked D-galactopyranose residues. The presence of such water-soluble, highly branched arabinogalactans in the wood of conifers appears to be a general characteristic of these trees. Most species of conifer, however, contain only a small proportion of this type of polysaccharide, but among members of the genus Larix, sometimes as much as 25% of the wood may consist of an arabinogalactan. 69

Arabinogalactans have in general been investigated by techniques of (a) hydrolysis and partial hydrolysis, (b) methylation studies together with hydrolysis of the methylated polysaccharides and (c) periodate oxidation studies generally in conjunction with Barry degradation.
or Smith degradation.

The classic investigation of an arabinogalactan was carried out by White 70-73 on the arabinogalactan from Western Larch (Larix occidentalis). He found the ratio of D-galactose to L-arabinose to be 6 : 1. Methylation of the arabinogalactan followed by partial methanolysis gave methyl 6-O-(2,3,4,6-tetra-O-methyl-β-D-galactopyranosyl)-2,3,4-tri-O-methyl-D-galactopyranoside, together with the corresponding compound lacking the methoxyl group on C3 of the D-galactose moiety, which indicated the presence in the polysaccharide of structural units (XIV) and (XV).

\[
\begin{align*}
\text{XIV} & \quad \beta-D-Galp(1 \rightarrow 6)-D-Galp 1 \\
\text{XV} & \quad \beta-D-Galp(1 \rightarrow 6)-D-Galp 3
\end{align*}
\]

In a separate experiment the L-arabinose residues were removed by mild hydrolysis and the degraded polysaccharide was methylated and hydrolysed to give 2,4-di-, 2,3,4-tri-, 2,4,6-tri- and 2,3,4,6-tetra-O-methyl-D-galactose in the ratio of 2 : 1 : 1 : 2 as compared to a ratio of 3 : 1 : 0 : 2 in the original polysaccharide. Thus the removal of the arabinose residues resulted in the formation of one residue of 2,4,6-tri-O-methyl-D-galactose in place of one residue of 2,4,6-di-O-methyl-D-galactose. The arabinose residue must therefore have been attached to C6 of a 1,3 linked galactose unit. The only methylated L-arabinose residue was 2,3,5-tri-O-methyl-L-arabinose
indicating that all the arabinose residues were present as terminal furanose units. On the basis of these results White suggested the following structure for Western Larch arabinogalactan. (Structure XVI).

\[
\beta-D-Galp_1
\]

\[
\beta-D-Galp-(1 \rightarrow 3)-D-Galp-(1 \rightarrow 3)-D-Galp-(1 \rightarrow 3)-D-Galp-(1 \rightarrow 6)-D-Galp_6
\]

\[
\text{XVI} \quad L-Araf_1 \quad D-Galp_1 \quad \rightarrow 3-D-Galp_1
\]

The structure with minor alterations is still today the accepted structure for Western Larch arabinogalactan and it shows the structural features common to all the coniferous arabinogalactans, that is the backbone of 1,3 linked \(\beta-D\)-galactopyranose residues with 1,6 linked \(\beta-D\)-galactopyranose side chains. The proportion of arabinose units varies but such units are always found situated around the periphery of the molecule.

A very detailed examination of Western Larch arabinogalactan was carried out recently by Bouveng and Lindberg 74-78. These authors found that the arabinogalactan could be resolved into two fractions A and B, molecular weights 100,000 and 16,000 respectively, by electrophoresis on glass fiber sheets in borate buffer. Both fractions were shown to have similar structures with the type of galactan core proposed by White (XVI). The arabinose was shown to be present as terminal \(L\)-arabinose, as \(3-O-\beta-L\)-arabinopyranosyl-\(L\)-arabinofuranose and perhaps also as units of
three residues. Another related arabinogalactan from European Larch has also been studied, by Hirst and coworkers 79 and more recently by Aspinall, 80 while the arabinogalactan from Scots pine has been investigated in detail by Aspinall and Wood. 81

It is now considered that many, if not all of the coniferous wood arabinogalactans, contain small proportions of \(\alpha\)-glucuronic acid. Such residues have, for example, been detected in the arabinogalactans from tamarack larch (2.5%), 82-84 mountain larch (6.8%) 85 and maritime pine (2.9%). 86 These acidic arabinogalactans have been shown to be of the same structural type as Western and European Larch arabinogalactans. The \(\alpha\)-glucuronic acid is always present as non-reducing end groups and are linked to galactose residues by 1,6 linkages, the only aldobiouronic acid detected on partial hydrolysis being 6-\(\beta\)-(\(\alpha\)-\(\alpha\)-glucopyranosyluronic acid)-\(\alpha\)-galactose (IX p. 17).

It has become increasingly apparent that there is no clear line of demarkation between the wood arabinogalactans and the acidic galactan based polysaccharides found in several plant gum exudates. These polysaccharides also contain \(\alpha\)-galactose and \(L\)-arabinose as their main structural units and they possess an underlying framework of 1,3 and 1,6 linked galactopyranose units. Of these exudate gums detailed study has been
made of those from *Acacia senegal*, Acacia pyonantha, *Araucaria bidwillii*, *Khaya senegalensis* and *Asafoetida* gums. Attention will be here confined to the polysaccharides in *Araucaria bidwillii*, the chemistry of which will be discussed in a separate section.

The similarity between the wood arabinogalactans and the gum polysaccharides is significant and it has been suggested that the arabinogalactans in wood might act as precursors of exudate gums. Timell has pointed out that larch arabinogalactans are located largely outside the cell walls in greatest concentration in the heartwood, near the heartwood-sapwood boundary. He has therefore suggested that the arabinogalactans can be looked upon as a mucilage which is deposited in the non-functioning part of the xylem. A brief discussion will now be given of some less well known hemicelluloses.

**THE XYLOGLUCANS**

The combination of xylose with glucose residues in hemicellulose polysaccharides is generally not well known but recently a number of xyloglucans have been reported. One such polysaccharide is the so-called 'amyloid' extracted from the cotyledons of *Tamarindus indica* by Kooiman. This polysaccharide is representative of a group of xyloglucans which give a characteristic colour reaction with potassium tri-iodide in the presence of sodium sulphate, a reaction designated 'the amyloid
Tamarindus-amyloid was investigated by partial hydrolysis and enzymic degradation studies and shown to consist of a main chain of 1,4 linked β-D-glucopyranose residues. To this main chain D-xylose residues are attached by α-1,6 linkages to three glucose units out of every four. The polysaccharide also contained a proportion of D-galactose residues which are attached to the xylose residues by a β-1,2 linkage. Thus the following structure was assigned to Tamarindus-amyloid. (Structure XVII).

\[ \beta-D-Galp_1 \rightarrow 4\beta-D-Glp_1 \rightarrow 4\beta-D-Glp_1 \rightarrow 4\beta-D-Glp_1 \rightarrow 4\beta-D-Glp_1 \rightarrow \]

\[ \alpha-D-Xylp_1 \rightarrow \alpha-D-Xylp_1 \rightarrow \alpha-D-Xylp_1 \rightarrow \]

\[ \beta-D-Galp_1 \rightarrow \beta-D-Galp_1 \]

Kooiman recently reported an investigation of an amyloid extracted with alkali from the seeds of Annona muricata L, which also contains glucose, xylose and galactose and has a structure very similar to that of Tamarindus-amyloid. 101

An alkali soluble xyloglucan from mustard seeds has recently been examined. 102 This xyloglucan is fucose-containing, as that sugar is present in addition to glucose, xylose and a trace of galactose. The polysaccharide gave the distinctive amyloid reaction, it is structurally related to the Tamarindus-amyloid and was classed as a
cotyledon amyloid. There are, however, a number of important structural differences between the two polysaccharides.

Recently xyloglucans from the roots of sugar maple were reported. Alkaline extraction of the roots, followed by differential precipitation with Cetavlon yielded two acidic xyloglucans which were examined by methylation studies. Both of the xyloglucans consisted of a chain of β-1,4 linked D-xylose and D-glucose units, in one polysaccharide some of each of the glucose and xylose residues were branched through C3, in the other polysaccharide branching occurred at C3 of the xylose residues only. The terminal non-reducing units were D-xylose or acidic sugar residues.

Another polysaccharide intermediate between glucan and xylan was recently isolated by Timell from the bark of Engelmann Spruce. This xyloglucan contains galactose, glucose and xylose and was homogeneous by the criterion of its constancy of composition on repeated fractionation. The polysaccharide contained sequences of 1,4 linked β-D-glucopyranose residues most of which carried branches linked through C6. The majority of the galactose units occurred as non-reducing end groups, although many occurred as a 1,6 linked galactan chain. The xylose occurred as a sequence of units linked through positions 1 and 4, but it was not established whether this xylan was linked to the glucan. Another similar
galactoxyloglucan has been reported from the compression wood of red spruce. These are the first reports of the presence of polysaccharides of the xyloglucan type in wood.

It is apparent that as a general class xyloglucans may be as variable in structure as are for example the xylans.
Part II

Polysaccharide Components of Lucerne

Lucerne or alfalfa (*Medicago sativa*) is a major legume crop belonging to the same family as beans, peas and clover. It is an excellent fodder plant as it is a rich source of protein and calcium.

Plant polysaccharides can be divided into two groups, (a) reserve polysaccharides and (b) cell wall polysaccharides. Lucerne contains small amounts of reserve polysaccharides and a proportion of sucrose and raffinose. The cell wall polysaccharides of lucerne include cellulose, hemicellulose and pectins.

An investigation of the polysaccharides of lucerne was first carried out by Myhre and Smith. These authors extracted lucerne with aqueous sodium hydroxide and isolated a polysaccharide fraction which contained the residues of L-arabinose (12.0%), D-xylose (67.3%), D-galactose (8.1%), D-glucose (8.1%), L-rhamnose (4.5%), D-galacturonic acid and 4-O-methyl-D-glucuronic acid.

Partial acid hydrolysis of the polysaccharide gave five acidic oligosaccharides:

1. 2-O-(4-O-methyl-α-D-glucopyranosyluronic acid)-D-xylose.
2. 2-O-(4-O-methyl-α-D-glucopyranosyluronic acid)-(1→2)-O-β-D-xylopyranosyl-(1→4)-β-D-xylose.
3. 2-O-(α-D-galactopyranosyluronic acid)-L-rhamnose.
4. 6-O-(β-D-glucopyranosyluronic acid)-D-galactose.
5. 2-O-(α-D-glucopyranosyluronic acid)-D-xylose.

However, this so-called 'hemicellulose' was undoubtedly a mixture of pectic materials and a polysaccharide of the xylan group. The presence of both these polysaccharides in lucerne had earlier been indicated, and this has been confirmed by further investigations. It is evident that oligosaccharides 1 and 2 arise from a xylan polysaccharide and oligosaccharide 3 (V, p. 15) arises from pectic acid. Oligosaccharide 4 (IX, p. 17) has recently been isolated from various pectins.

Aspinall and Fanshawe carried out a step-wise extraction of lucerne and obtained a number of polysaccharide fractions.

Diagram 1.

Lucerne (freshly cut)

\[ \xrightarrow{\text{Boiling Ethanol-Water (4 : 1)}} \text{Ethanol Soluble Sugars} \]

Residue A

\[ \xrightarrow{\text{Cold or Hot Water Extraction}} \text{Water-Soluble Polysaccharides} \]

Residue B

\[ \xrightarrow{\text{Hot Ammonium Oxalate or Ethylenediaminetetra-acetate}} \text{Ammonium Pectate} \]

Residue C

The extraction scheme devised is shown in Diagram 1.
This scheme with minor modifications was continued to be used in later investigations. Ex

Extraction with hot-ethanol-water (4 : 1) removed colouring matter and soluble sugars among which D-glucose, D-fructose, sucrose and raffinose were identified. Cold and hot water extractions yielded complex mixtures of acidic polysaccharides which were contaminated with inorganic material and protein. Extraction with hot ammonium oxalate solution gave ammonium pectate which was purified via the calcium salt and found to be homogeneous by diethylaminoethylcellulose chromatography. Extraction of the residue with lime-water gave an arabinan rich polysaccharide.

The ammonium pectate from the ammonium oxalate extraction contained residues of D-galacturonic acid (50%), D-galactose, L-arabinose, L-rhamnose and traces of L-fucose, 2-O-methyl-L-fucose and 2-O-methyl-D-xylose. Partial acid hydrolysis of the polysaccharide furnished oligomers of galacturonic acid, 4-O-(α-D-galactopyranosyluronic acid)-D-galacturonic acid (II, p. 7) and the trisaccharide 2-O-α-D-galactopyranosyluronic acid-(1 → 4)-2-O-α-D-galactopyranosyluronic acid-(1 → 4)-D-galacturonic acid (Structure XVIII).

![Diagram](XVIII)
Also identified was \(2{-}\text{O-} (\alpha{-}\text{D-galactopyranosyluronic acid}){-}\text{L-rhamnose} \) (V, p. 15), which was the same as that isolated by Myhre and Smith from their so-called hemicellulose preparation, and tentatively higher oligosaccharides containing galacturonic acid and rhamnose residues, and two disaccharides thought to contain galacturonic acid and fucose and galacturonic acid and galactose respectively. The structures of the last three oligosaccharides were not fully established and subsequently the last two were shown in fact to be glucuronic acid containing oligosaccharides, but it was conclusively shown that rhamnose was an integral constituent of lucerne pectic acid.

The present investigation of the pectic substances of lucerne was part of a larger project supervised by Dr. Aspinall, and was carried out simultaneously with investigations of lucerne pectic acids by Dr. Uddin and Dr. Gestetner. The results of these latter investigations will be described and discussed in conjunction with the discussion of the results of the present investigation.

In another series of step-wise extractions of polysaccharides from the separated leaves and stems of lucerne, Aspinall and McGrath have isolated a \(4{-}\text{O-methylglucuronoxylan} \). This hemicellulose polysaccharide was extracted with dilute alkali from lucerne residue C, which had been previously extracted with ethanol : water (4 : 1), with water and with solutions of ethylenediaminetetra-acetic acid
disodium salt. Partial acid hydrolysis of the xylan yielded oligosaccharides 1 and 2 obtained by Myhre and Smith as well as their higher homologues. In addition the following neutral oligosaccharides were obtained:

6. $\beta-D-Xylp\_1 \rightarrow (\rightarrow 4\beta-D-Xylp\_1)n \rightarrow 4\ Xylp$

7. $Xylp\_1 \rightarrow 3\ Rha$

8. $Xylp\_1 \rightarrow 4\ Xylp\_1 \rightarrow 3\ Rha$

On the basis of the partial hydrolysis results together with methylation results it was concluded that lucerne leaf and stem xylans contain linear chains of $1,4$ linked $\beta-D$-xylopyranose residues with approximately every ninth residue carrying through C$_2$ a single unit side chain of $4-O$-methyl-$\alpha-D$-glucuronic acid and the main chain contains some rhamnose residues (Structure XIX).

$$\text{XIX} \quad 4-O-Me-\alpha-D-GpA\_1$$

The seeds of lucerne contain a substantial amount of galactomannans. Andrews, Hough and Jones extracted from lucerne seeds with hot water a galactomannan having similar composition to galactomannans from other leguminous plants. Hirst and Jones had earlier reported an unusual type of galactomannan obtained by extracting lucerne seeds with hot potassium hydroxide solution.
THE EXTRACELLULAR POLYSACCHARIDES FROM SYCAMORE CAMBIAL CELLS.

There are three types of cells in wood tissues, phloem, cambial and xylem cells. These are named depending on their situation in the tree, the cambial cells being the growth cells. Thornber and Northcote have made a quantitative determination of the changes that occur in the composition of a cambial cell during its differentiation into xylem and phloem tissue in four species of tree including sycamore \((Acer pseudoplatanus)\). These authors report that the composition of the cambial cell was very similar in each of the species examined and that secondary thickening of the cambial cell wall involved the deposition of \(\alpha\)-cellulose, hemicellulose and lignin with little change in the amount of pectic substances per cell. The carbohydrate composition of the different polysaccharide fractions from the various regions in the tree were determined and in the case of sycamore secondary thickening was accompanied by the formation of glucans, xylans, uronic anhydride and mannans but little change occurred in the amount of arabinans and galactans decreased. More recently, Northcote and co-workers have examined and compared the pectic polysaccharides of sycamore cambial and sycamore callus tissue and these authors report that the pectins of callus and cambium are very similar and are
comparable to apple fruit pectin. It was concluded that any differences were not of species difference but were more characteristic of the growth conditions of the cells.

It was discovered by Albersheim and co-workers that sycamore cambial cells grow rapidly in complex yeast extract media and that the cells secrete polysaccharides into the culture medium. The investigation of the extracellular polysaccharides from the culture medium showed these polysaccharides to be similar in composition to the noncellulosic wall polysaccharides of cambial cells and the authors concluded that the extracellular polysaccharides are synthesised in a normal manner within the wall and then excreted into the culture medium. Moreover, these authors report that the extracellular polysaccharides are rapidly labeled if the cells are supplied with radioactive glucose. Recently Northcote and co-workers also reported on the extracellular polysaccharides in the media of sycamore suspension-callus tissues during an investigation of the metabolism of the pectic substances of actively growing sycamore cells. These authors reported that the extracellular polysaccharides were rich in glucose and xylose and poor in arabinose. They reported, moreover, that pectic polysaccharides in the medium are associated with old cultures, where cell necrosis is likely, and also they noted the presence of a polygalacturronase in the filtered medium of old cultures.
The metabolism of inositol by sycamore cell cultures was investigated recently. The authors concluded that inositol, when contained in the growth medium, is largely directed towards the synthesis of cell wall pentose and uronic acid units in the sycamore cells. It was also reported that in the cultures of sycamore cambial cells, the biosynthetic processes in the primary cell wall continued to produce pectic substances, whereas the processes involved in the biosynthesis of secondary cell wall polysaccharides were not activated. An examination of the extracellular polysaccharides grown in complex yeast medium by Albersheim was carried out by Aspinall and Craig. Despite the presence of a mannan which was a contaminant and a constituent of the yeast used as a nutrient for the sycamore cambial cells, it proved possible to fractionate the extracellular polysaccharides and isolate a pectinic acid, an arabinogalactan and a xylan-glucan mixture, which was not examined. It seemed quite certain that the pectinic acid and the arabinogalactan were genuine products elaborated by the sycamore cambial cells. The arabinogalactan was shown to contain galactose residues which were linked through 1 and 3 and 1 and 6 positions and thus to be similar to the water-soluble arabinogalactans from coniferous woods. No other arabinogalactan of this type had previously been isolated from deciduous wood.
ARAUCARIA BIDWILLII GUM

Araucaria bidwillii gum represents the first carbohydrate exudate reported for any conifer being an exudate from the Australian bunya pine. In the original investigation by Aspinall and Fairweather, the polysaccharide from the gum exudate was subjected to hydrolysis, partial hydrolysis and methylation studies and also to periodate oxidation and Smith degradation to yield degraded polysaccharides which were in turn subjected to hydrolysis, methylation and periodate oxidation studies, and the main features of the gum acid were established. The polysaccharide was shown to possess a highly branched structure with a core of D-galactose residues mutually linked by 1,3 and 1,6 linkages and with outer chains terminated by end groups of L-arabinofuranose, L-rhamnopyranose, D-galactopyranose, D-glucuronic acid and 4-O-methyl-D-glucuronic acid. In addition, the position of attachment of the end groups of D-glucuronic acid (and its 4-O-methyl ether) were pinpointed by the characterisation, as products of partial acid hydrolysis, of the two aldobiouronic acids, 6-O-(β-D-glucopyranosyluronic acid)-D-galactose (a) and 6-O-(4-O-methyl-β-D-glucopyranosyluronic acid)-D-galactose (b)

(a) β-D-GpA 1 → 6 D-Gal

(b) 4-O-Me β-D-GpA 1 → 6 D-Gal

A subsequent investigation of a freshly isolated sample of the gum carbohydrate was directed towards
assessing the homogeneity of the polysaccharide and to ascertain the points of attachment of the rhamnose end groups.

By ion exchange chromatography of the gum carbohydrates an arabinogalactan was obtained in 3% yield (i.e. in quantities insufficient to cause serious contamination of the acidic polysaccharide). This arabinogalactan designated *A. bidwillii* arabinogalactan, was homogeneous by the criteria of ultra-centrifugation and free-boundary electrophoresis and contained arabinose and galactose in the molar ratio of 4 to 1. Methylation and Smith degradation studies seemed to indicate that the polysaccharide is essentially an arabinan containing linear chains of arabinofuranose residues which are joined by 1,2 and 1,3 linkages. There are occasional branch points which accommodate end groups of arabinofuranose and galactopyranose residues. However, there were some anomalies in the results obtained, including the impossibility of obtaining a fully methylated polysaccharide, and further investigation will be needed to establish a definite structure for the arabinogalactan.

The major acidic component of the gum carbohydrates, obtained in 97% yield from ion exchange chromatography, was designated *A. bidwillii* gum. The acidic polysaccharide contained as constituent sugars, glucuronic acid (in part as its 4-O-methyl ether), galactose, arabinose and rham-
nose but these sugars were present in different proportions (molar percentages 18, 56, 17 and 9) from the same sugars in the gum sample originally examined (10, 69, 16 and 5). The gum was examined by methylation studies and the carboxyl reduced gum was examined by methylation and acetolysis studies. The results obtained indicated that, despite quantitative differences in the proportions of the constituent units, the nature of the sugar residues and of the linkages between them were the same in the sample under investigation as those in the sample previously examined. The results from both investigations can be incorporated in the following partial structure of *A. bidwillii* gum (Structure XX) -

\[
\begin{align*}
\cdots & \text{Galp}_1 \rightarrow 3 \text{Galp}_1 \rightarrow 3 \text{Galp}_1 \rightarrow 3 \text{Galp}_1 \rightarrow 3 \text{Galp}_1 \rightarrow 3 \text{Galp}_1 \\
& 6 \uparrow \quad 6 \uparrow \quad 6 \uparrow \quad 6 \uparrow \quad 6 \uparrow \\
& 1 \quad 1 \quad 1 \quad 1 \\
& \text{(Galp)}_n \quad R \quad \text{(Galp)}_n \quad \text{(Galp)}_n \quad \text{(Galp)}_n \\
& 6 \uparrow \quad 6 \uparrow \quad 6 \uparrow \quad 6 \uparrow \quad 6 \uparrow \\
& 1 \quad 1 \quad 1 \quad 1 \\
& \text{Galp} \quad 4\text{-Me-GpA} \quad \text{RgA} \quad \text{Rhap} \\
\end{align*}
\]

\[
R = \text{Araf}_1 \rightarrow 3 \text{Araf}_1 \rightarrow 3 \text{Araf}_1 \rightarrow (\text{tr})
\]

\[
n = 0, 1, 2
\]

Acetolysis of the carboxyl-reduced polysaccharide
gave a mixture of sugars which were isolated and identified as \(4-O-L\)-rhamnopyranosyl-\(\beta\)-glucose (c), \(O-L\)-rhamnopyranosyl-(1 → 4)-\(O-D\)-glucopyranosyl-(1 → 6)-\(D\)-galactose (d), \(6-O-D\)-glucopyranosyl-\(D\)-galactose (e), \(6-O-(4-O-methyl-D\)-glucopyranosyl-\(D\)-galactose (f), \(3-O-\beta-D\)-galactopyranosyl-\(D\)-galactose (g), the polymer homologous tri- (h) and tetrasaccharides (i), \(6-O-D\)-galactopyranosyl-\(D\)-galactose (j), the polymer-homologous trisaccharide (k) and the trisaccharide \(O-D\)-galactopyranosyl-(1 → 6)-\(O-D\)-galactopyranosyl-(1 → 3)-\(D\)-galactose (l).

- L-Rhap 1 → 4 \(D\)-G (c).
- L-Rhap 1 → 4 \(D\)-Gp 1 → 6 \(D\)-Gal (d).
- \(D\)-Gp 1 → 6 \(D\)-Gal (e).
- 4-O-Me \(D\)-Gp 1 → 6 \(D\)-Gal (f).
- \(\beta-D\)-Galp 1 → 3 \(D\)-Gal (g).
- \(\beta-D\)-Galp 1 → 3 \(\beta-D\)-Galp 1 → 3 \(D\)-Gal (h).
- \(\beta-D\)-Galp 1 → 3 \(\beta-D\)-Galp 1 → 3 \(\beta-D\)-Galp 1 → 3 \(D\)-Gal (i).
- \(D\)-Galp 1 → 6 \(D\)-Gal (j).
- \(D\)-Galp 1 → 6 \(D\)-Galp 1 → 6 \(D\)-Gal (k).
- \(D\)-Galp 1 → 6 \(D\)-Galp 1 → 3 \(D\)-Gal (l).

The formation from the carboxyl-reduced polysaccharide of the oligosaccharide (g-l) is fully consistent with a structure (XX) consisting of a chain of 1,3 linked \(D\)-galactose residues carrying side chains of 1,6 linked \(D\)-galactose residues attached at \(C_6\) of main chain units. The branch points were pinpointed by the isolation of oligosaccharide (l) containing both 1,3 and 1,6 linkages.

Oligosaccharides (e) and (f) clearly have their origin in units of the two aldobiouronic acids (a) and
(b) present in the parent polysaccharide. The isolation of the two rhamnose containing oligosaccharides (c) and (d) provides clear evidence for the attachment by 1,4 linkages of L-rhamnopyranose residues to residues of D-glucuronic acid.

In both series of investigations of A. bidwillii gum 95,98 the low specific rotations of the polysaccharides and their methylated derivatives have suggested that the D-glucuronic acid and D-galactose residues have the β-configuration. Moreover, the two aldobiouronic acids (a and b) formed on partial hydrolysis of the earlier polysaccharide sample were assigned β-D-glucopyranosyluronic acid linkages on the basis of specific rotations.

However, examination of the oligosaccharides formed on acetolysis showed that all those containing 1,6 linkages had high positive specific rotations indicating the presence of α-D-glucopyranosyl or α-D-galactopyranosyl linkages. In the case of the oligosaccharide (e), 6-D-glucopyranosyl-D-galactose, the presence of the α linkage was confirmed by n.m.r. spectroscopy and periodate oxidation studies. Since the disaccharide (e) originated from the same structural unit in the parent polysaccharide as the aldobiouronic acid (a), it was therefore suspected that acid-catalysed anomerisation had taken place during acetolysis and that all the other 1,6 linkages were similarly affected. Lindberg 119 has shown that acid-catalysed anomerisation at the glycosidic bond does take
place during acetolysis and he has used this in preparing 6-\(\alpha\)-\(D\)-glucopyranosyl-\(D\)-glucose from gentiobiose, 6-\(\alpha\)-\(D\)-glucopyranosyl-\(D\)-glucose. Aspinall and co-workers have also found evidence for anomerisation during acetolysis of various mannans in which oligosaccharides containing 1,4 linked \(\alpha\)-\(D\)-mannopyranose residues were isolated as minor products.\textsuperscript{120-122} However, the presence in \textit{A. bidwillii} gum of \(\beta\) linkages only needed to be conclusively demonstrated especially in respect of 1,6 linked galactose residues.
SECTION I

PECTIC SUBSTANCES OF LUCERNE

DISCUSSION
DISCUSSION

LUCERNE STEM PECTIC ACID

The lucerne crop was cut at the beginning of October, 1965, and was separated into leaves and stems. The stem pectic acid was isolated as follows: the freshly cut stem was sliced into portions, extracted with ethanol-water (4:1) to inactive enzymes, dried, milled and extracted with cold water to remove water-soluble polysaccharides. The water extracted residue was extracted with a 2% solution of ethylenediaminetetra-acetic acid disodium salt at 70°. The polysaccharide was precipitated with acetone and purified via the calcium salt. The calcium pectate was converted to the ammonium pectate by heating with an 0.5% solution of ammonium oxalate and finally isolated as such and freeze-dried. Lucerne stem ammonium pectate was examined 46 by chromatography on diethylaminoethyl cellulose and more than 95% of the polysaccharide was eluted in a single band with sodium hydroxide solution. The uronic acid content was estimated at 73%, a low proportion of the galacturonic acid residues (ca. 1 in 6) were esterified, the specific rotation was [α]D +200°, and hydrolysis yielded the usual sugars associated with pectins, galactose, arabinose and rhamnose as the major components together with traces of glucose, xylose, fucose, 2-O-methylxylose and 2-O-methylfucose.
Partial Acid Hydrolysis of Lucerne Stem Pectic Acid.

The lucerne stem pectic acid was subjected to partial acid hydrolysis with a view to isolating acidic oligosaccharides, to obtaining information about the fine structure and to making a structural comparison with the corresponding pectic acid from lucerne leaf.

Stem pectic acid was partially hydrolysed in $\text{H}_2\text{SO}_4$ to give a mixture of mono- and oligosaccharides and degraded galacturonans which contained ca. 97% of uronic acid residues and were devoid of neutral sugars. The mixture of neutral and acidic sugars was fractionated on a column of diethylaminoethyl-Sephadex A-25 (formate form). Elution with water gave neutral sugars identified chromatographically as galactose, arabinose, rhamnose together with traces of fucose, xylose, 2-0-methylfucose and 2-0-methyl-xylose. Elution with water containing increasing amounts of formic acid gave large amounts of galacturonic acid together with various acidic oligosaccharides which were further purified by paper partition chromatography.

The two oligosaccharides isolated in largest amounts contained galacturonic acid only. The first of these was identified as $\beta_4$-$\text{D}$-galactopyranosyluronic acid-$\text{D}$-galacturonic acid (1, Table I) while the other was identified as the polymer homologous trisaccharide $\alpha_0$-$\text{D}$-galactopyranosyluronic acid-$\alpha_0$-$\text{D}$-galactopyranosyluronic acid-$\alpha_0$-$\text{D}$-galacturonic acid (2, Table I). The two oligosaccharides were characterised by isolation
of their calcium salts. The linkages in the two acidic oligosaccharides (1 and 2, Table I) were confirmed by methylation analysis of the corresponding galactobiose and and galactotriose derived from galacturonobiose and galacturonontriose, respectively.

The two oligouronides, as their respective methyl esters methyl glycosides, were dissolved in pyridine and subjected to trimethylsilylation and the trimethylsilyl derivatives were reduced with lithium aluminium hydride in ethereal solution. The resulting methyl glycosides of the neutral oligosaccharides were methylated, the methylated products were methanolyzed and by examination of the cleavage products by gas-liquid partition chromatography the methyl glycosides of 2,3,4,6-tetra- and 2,3,6-tri-O-methyl galactose were identified.

Two oligosaccharides containing rhamnose and galacturonic acid were also isolated. The first of these was characterised as 2-α-D-galactopyranosyluronic acid)-L-rhamnose (3, Table I) by conversion into the crystalline methyl glycoside pentamethyl ether dihydrate. The other rhamnose containing oligosaccharide was identified as the tetrasaccharide 2-α-D-galactopyranosyluronic acid-(1 → 2)-β-D-galactopyranosyl-(1 → 4)-α-D-galactopyranosyluronic acid-(1 → 2)-L-rhamnose, 19,44,45 on the basis of the following (4, Table I). The oligosaccharide contained galacturonic acid and rhamnose in the molar ratio of
1.05 : 1 whereas the derived glycitol contained these sugars in the molar ratio 1.9 : 1. The oligosaccharide on partial hydrolysis gave predominately 2-\(\beta\)-\((\text{galactopyranosyluronic acid})\)-rhamnose, whereas partial hydrolysis of the derived glycitol gave the same aldobiouronic acid together with 2-\(\beta\)-\((\text{galactopyranosyluronic acid})\)-rhamnitol. Gas-liquid chromatography of methanolysis products of the methylated tetrasaccharide glycitol gave peaks with the retention times of 1,3,4,5-tetra-\(\beta\)-methyl rhamnitol and of the methyl glycosides of 3,4-di-\(\beta\)-methyl rhamnose, 2,3-di-\(\beta\)-methyl galacturonic acid and 2,3,4-tri-\(\beta\)-methyl galacturonic acid.

Finally two glucuronic acid containing oligosaccharides were isolated. One of these was chromatographically indistinguishable from 4-\(\beta\)-\((\beta\-D-glucopyranosyluronic acid)\)\(-\text{D-fucose}\) (7, Table I).\(^{19,44,45}\) Hydrolysis of the oligosaccharide offered fucose, glucuronic acid and glucurone whereas the carboxyl-reduced disaccharide gave glucose and fucose on hydrolysis. Gas-liquid chromatography of the methanolysis products from the methylated sugar gave peaks with the retention times of methyl glycosides of 2,3-di-\(\beta\)-methyl fucose and 2,3,4-tri-\(\beta\)-methyl glucuronic acid. The specific rotation suggested a \(\beta\)-configuration for the linkage. The other oligosaccharide containing glucuronic acid was chromatographically indistinguishable from 6-\(\beta\)-\((\beta\-D-glucopyranosyl-
Hydrolysis of the oligosaccharide afforded galactose, glucuronic acid and glucurone. Hydrolysis of the carboxyl-reduced oligosaccharide gave glucose and galactose. Gas-liquid chromatography of the methanolysis products from the methylated aldobiouronic acid gave peaks with the retention times of the methyl glycosides of \(2,3,4\)-tri-O-methyl glucuronic acid and \(2,3,4\)- and \(2,3,5\)-tri-O-methyl galactose. These results suggest structure (8, Table I) for this aldobiouronic acid.

**Evaluation of Results from Partial Degradation of Lucerne Pectic Acid.**

The oligosaccharides isolated from lucerne stem pectic acid in the course of the present investigation are listed in Table I. The present investigation was part of a larger group study of lucerne pectic acids. Lucerne leaf pectic acid was examined by partial hydrolysis and both leaf and stem pectic acids were examined by acetolysis studies by Dr. Uddin and the oligosaccharides isolated in these studies are also included in Table I. From the combined study there was no apparent difference between lucerne leaf and stem pectic acids, and therefore, the subsequent enzymic degradation of lucerne pectic acid by Dr. Gestetner was carried out on the pectic acid extracted directly from the whole lucerne crop. The results of this study are also included in Table I.
Table I.

Acidic Oligosaccharides from Partial Degradation of Lucerne Pectic Acids.

<table>
<thead>
<tr>
<th>Oligosaccharides</th>
<th>Stem</th>
<th>Leaf</th>
<th>Combined Leaf + Stem</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a</td>
<td>b</td>
<td></td>
</tr>
<tr>
<td>1. α-D-GalpA 1 → 4 D-GalpA</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>2. α-D-GalpA 1 → 4 α-D-GalpA</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 → 4 D-GalpA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. α-D-GalpA 1 → 2 L-Rha</td>
<td>+</td>
<td>+</td>
<td>+*</td>
</tr>
<tr>
<td>4. α-D-GalpA 1 → 2 L-Rhap 1 → 4</td>
<td>+</td>
<td>+</td>
<td>+*</td>
</tr>
<tr>
<td></td>
<td>α-D-GalpA 1 → 2 L-Rha</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. α-D-GalpA 1 → 4 α-D-GalpA</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 → 2 L-Rha</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6. α-D-GalpA 1 → 2 L-Rhap 1 → 2</td>
<td>+</td>
<td>+*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>L-Rha</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7. β-D-Gpa 1 → 4 L-FucP</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>8. β-D-Gpa 1 → 6 D-Gal</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>9. β-D-Xylp 1 → 3 D-GalpA</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10. L-Araf 1 → 3 D-GalpA</td>
<td>+</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Detected by paper chromatography only.

a = Partial acid hydrolysis, b = Acetolysis, c = Enzymic degradation.

It is apparent that the same series of acidic oligosaccharides has been isolated from lucerne stem pectic acid by partial acid hydrolysis during the present investigation (1-4, 7 and 8, Table I) as has been isolated from lucerne leaf pectic acid by partial acid hydrolysis by Dr. Uddin. Partial acid hydrolysis of lucerne leaf
pectic acid yielded another oligosaccharide (5, Table I) which was characterised as \(\beta-\alpha-D\)-galactopyranosyluronic acid-(1 \rightarrow 4)-\(\beta-\alpha-D\)-galactopyranosyluronic acid-(1 \rightarrow 2)-L-rhamnose. However, this same oligosaccharide has been isolated from lucerne stem pectic acid by partial acetolysis by Dr. Uddin (5, Table I). Another rhamnose containing oligosaccharide (6, Table I) has been identified among the acetolysis products of lucerne stem pectic acid, and tentatively from lucerne leaf pectic acid, and it has been characterised as \(\beta-\alpha\)-galactopyranosyluronic acid)-(1 \rightarrow 2)-\(\beta-\alpha\)-rhamnopyranosyl-(1 \rightarrow 2)-rhamnose. Both leaf and stem pectic acids yielded degraded galacturonans on partial hydrolysis. Thus it is apparent from these results that there are no discernable structural differences between the pectic acids from lucerne leaves and stems as the same type of acidic oligosaccharides have been isolated from both samples. Thus the results of the group study are combined and discussed in relation to the structure of 'lucerne pectic acid'.

The Basal Chain of Pectic Acid.

The isolation of galacturononobiose (1, Table I) and galacturononotriose (2, Table I) and the formation of degraded galacturonans during partial acid hydrolysis show that lucerne pectic acid has a typical pectic structure with basal chains of 1,4 linked \(\alpha-D\)-galactopyranosyluronic acid residues. The isolation of the aldobiouronic acid,
2-O-(α-D-galactopyranosyluronic acid)-L-rhamnose (3, Table I) shows unambiguously that L-rhamnose is a constituent sugar of the basal chain. The presence of rhamnose in the basal chain is confirmed by the isolation of the tetrasaccharide (4, Table I) in which the galacturonic acid and rhamnose residues alternate. The isolation of the oligosaccharide (6, Table I) from acetolysis shows that at least two rhamnose units may be adjacent in parts of the structure. It is apparent that blocks of uninterrupted galacturonic acid residues occur in the galacturonorhamman chain, that these uninterrupted regions are interspersed with rhamnose residues which are unevenly distributed and concentrated in certain areas of the chain. Thus the basal chain of lucerne pectic acid can be represented as follows (Structure XXI).

\[ \rightarrow (4_{\text{D}}\text{-GalpA})_n \rightarrow 4_{\text{D}}\text{-GalpA} \rightarrow 2_{\text{L}}\text{-Rhap} \rightarrow 4_{\text{D}}\text{-GalpA} \rightarrow \]

Further information about the role of rhamnose residues has been obtained by methylation of the carboxyl reduced pectic acid. From the methanolysis products of the methylated polysaccharide, 3,4-di-O-methyl rhamnose was identified indicating the presence of 2-O-substituted rhamnose residues in the polysaccharide, while the identification of 2,3,4-tri-O-methyl rhamnose and 3-O-methyl rhamnose indicated that some rhamnose residues are present as non-reducing end groups, while certain of the rhamnose
residues in the galacturonorhamnan chain act as branch points through position 4, as had been indicated earlier.\textsuperscript{43} The same type of galacturonorhamnan basal structure has been shown to be present in lemon peel pectin\textsuperscript{19} and in the acidic polysaccharides from soybean cotyledons\textsuperscript{44} and hulls,\textsuperscript{45} and is a structural feature of a wide range of plant polysaccharides containing galacturonic acid and rhamnose residues. The relative proportion of galacturonic acid to rhamnose in the naturally occurring galacturonorhamnans varies widely however. At one end of the scale, tragacanthic acid contains rhamnose residues in its basal chain as indicated by the isolation from this polysaccharide of 2-O-(\(\alpha\)-D-galactopyranosyluronic acid)-L-rhamnose (3, Table I),\textsuperscript{123} but the rhamnose is a minor component and the basal chain of tragacanthic acid approximates closely to a galacturonan (Structure XXII).

\[
XXII \rightarrow 4\alpha-D-GalpA 1 \rightarrow 4\alpha-D-GalpA 1 \rightarrow 4\alpha-D-GalpA 1 \rightarrow 3 \rightarrow 3 \\
\beta-D-Xylp 1 \quad \beta-D-Xylp 1 \quad R = L-Fucp or D-Galp
\]

On the other hand, the polysaccharide components of the exudate gums of the \textit{Sterculia}\textsuperscript{124,125} and \textit{Khaya} genera,\textsuperscript{126,127} contain interior chains in which L-rhamnose (and D-galacturonic acid) are major constituents. In the case of the \textit{Khaya} genera, the galacturonorhamnan basal chains are similar to those in pectins (XXI), while in the case
of *Sterculia urens* gum, for example, the existing evidence indicates that the basic structure of the molecule can be represented by the two partial formulae (Structure XXIII, A and B), the exact molecular arrangement of which has not yet been defined.

XXIII  A. \(\rightarrow 4 \ \text{GalA} \ 1 \rightarrow 2 \ \text{Rha} \ 1 \rightarrow 4 \ \text{GalA} \ 1 \rightarrow 2 \ \text{Rha} \rightarrow\)

\[
\begin{array}{cccc}
3 & 4 & 3 & 4 \\
1 & * & 1 & *
\end{array}
\]

\[\text{GA} \quad * \quad \text{GA} \quad *\]

B. \(\rightarrow 4 \ \text{GalA} \ 1 \rightarrow 4 \ \text{Gal} \ 1 \rightarrow\)

\[
\begin{array}{c}
2 \\
1 \\
\text{Gal}
\end{array}
\]

The portion (XXIII, B) of the molecule represents a main chain containing galacturonic and galactose residues to which are attached galactose residues as non-reducing end groups linked to the galacturonic acid residues at \(C_2\). But the partial formula (XXIII, A) represents a portion of the molecule in which galacturonic acid and rhamnose residues alternate in a basal galacturonorhamman chain. The rhamnose residues in the main chain are substituted at \(C_4\) and the galacturonic acid residues are substituted at \(C_3\) by glucuronic acid residues present as non-reducing end groups.
Other Residues Present in Pectic Acid.

The two neutral residues which occur in pectic acids in greater proportion are galactose and arabinose, but nevertheless, conclusive evidence for the attachment of these sugars to the galacturonorhamman chains of pectins had not been provided. From the degradation of lucerne pectic acid with a pectinase preparation, recently carried out by Dr. Gestetner, an oligosaccharide was isolated (10, Table I) which was characterised as 3-O-arabinofuranosyl-galacturonic acid. This provides definite evidence that arabinose is an integral constituent of lucerne pectic acid and represents the first oligosaccharide of its type to have been isolated from a pectic substance. In the course of the same study, another oligosaccharide was isolated containing galactose and galacturonic acid only, in which the uronic acid was present as the reducing unit. This oligosaccharide was not fully characterised, but its isolation indicates that galactose is also an integral constituent of lucerne pectic acid. Further information on the nature of the galactose and arabinose residues in lucerne pectic acid was provided by a methylation study of the carboxyl-reduced pectic acid. The results of the methylation study confirmed that the basal chain of pectic acid is in fact a chain of 1,4 linked α-D-galactopyranosyluronic acid residues some of which act as branch points at 3 or 2 position. It was also indicated that
the D-galactose residues in the outer chains of the polysaccharide occur as single non-reducing end groups (XXIV), while the arabinose residues are present as multiple units (XXV-XXVIIa)

\[
\text{D-Galp} 1 \rightarrow \text{L-Araf} 1 \rightarrow \text{5-L-Araf} 1 \rightarrow \text{5-L-Araf} 1 \rightarrow \text{3}
\]

Thus it is apparent in respect of the modes of linkage of the galactose and arabinose residues that lucerne pectic acid most closely resembles lemon peel pectin as similar structural features (XXIV-XXVIIa) have been found in the latter polysaccharide. In contrast to this situation, galactose in the acidic polysaccharides of soybeans and commercial citrus pectin occurs in multiple unit side chains of 1,4 linked β-D-galactopyranose residues. On the other hand, pectins from the bark of amabilis fir and from sisal flash contain the arabinose residues present as single non-reducing end groups.

In the course of the enzymic degradation of lucerne pectic acid another oligosaccharide was isolated in which xylose was linked directly to galacturonic acid through C₃ position, (9, Table I). This oligosaccharide was characterised as 3-Ω-xylopyranosyl-galacturonic acid and its isolation conclusively proves that xylose is a constituent sugar of lucerne pectic acid. This is the
same oligosaccharide as had earlier been isolated from soybean \(^4\) and lemon peel pectic acids.\(^{19}\) Further evidence for the presence of xylose (and some fucose residues) in the outer chains of lucerne pectic acid, both present as non-reducing end groups, was provided by the methylation study mentioned above.\(^{109}\) The two glucuronic acid-containing oligosaccharides \(^4-O-(\beta-D\text{-glucopyranosyluronic acid})-\alpha\text{-fucose (7, Table I) and 6-O-(\beta-D-glucopyranosyluronic acid})-\beta\text{-galactose (8, Table I) were isolated from lucerne pectic acid in small amounts by partial hydrolysis and there is no direct evidence yet for their location in the pectic acid. However, they provide evidence for the presence of glucuronic acid as a minor constituent sugar of the pectic acid. It is likely that these two aldobiouronic acids arise from side chains in lucerne pectic acid and they appear to be authentic components of typical pectins \(^4,19,45\) and also of tragacanthic acid.\(^{123}\)
Isolation and Fractionation of Water-extracted Alfalfa Polysaccharides.

Lucerne leaves and stems were separated and each was extracted with boiling ethanol-water (4:1) to remove soluble sugars and inactive enzymes, dried and then milled. The milled leaf was extracted with cold water (three times) and the extract was passed through a bed of sulfoethyl-Sephadex C-50 to remove cations and adsorb some of the protein present. The resulting solution was emulsified with phenol and set aside at 0°C, crystalline phenol was separated, and the protein content of the aqueous solution was seen to have been substantially reduced. The solution was concentrated and adsorbed on a column of diethylaminoethyl-Sephadex A-50 (formate form) and polysaccharide fractions were eluted with water containing increasing proportions of formic acid. In all, 9 fractions were obtained, eluted with 0, 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, 10.0 and 15.0% of formic acid respectively. Details of the 9 fractions isolated are recorded in Table II.
Table II.

WATER-SOLUBLE LUCERNE LEAF POLYSACCHARIDE FRACTIONS

<table>
<thead>
<tr>
<th>Polysaccharide fraction</th>
<th>Formic acid in eluant (%)</th>
<th>Wt (g) isolated</th>
<th>Polysaccharide (g, as galactan)</th>
<th>Protein (%)</th>
<th>Volume of eluant (ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>0</td>
<td>0.50</td>
<td>0.30</td>
<td>1.0</td>
<td>5,500</td>
</tr>
<tr>
<td>2.</td>
<td>0.5</td>
<td>1.40</td>
<td>0.90</td>
<td>3.6</td>
<td>13,500</td>
</tr>
<tr>
<td>3.</td>
<td>1</td>
<td>1.70</td>
<td>1.00</td>
<td>4.0</td>
<td>10,000</td>
</tr>
<tr>
<td>4.</td>
<td>2</td>
<td>1.75</td>
<td>1.11</td>
<td>4.5</td>
<td>10,000</td>
</tr>
<tr>
<td>5.</td>
<td>4</td>
<td>2.20</td>
<td>1.60</td>
<td>6.0</td>
<td>12,000</td>
</tr>
<tr>
<td>6.</td>
<td>6</td>
<td>0.91</td>
<td>0.45</td>
<td>9.8</td>
<td>10,000</td>
</tr>
<tr>
<td>7.</td>
<td>8</td>
<td>0.50</td>
<td>0.37</td>
<td>8.5</td>
<td>10,000</td>
</tr>
<tr>
<td>8.</td>
<td>10</td>
<td>0.45</td>
<td>0.37</td>
<td>12.5</td>
<td>10,000</td>
</tr>
<tr>
<td>9.</td>
<td>15</td>
<td>0.36</td>
<td>0.28</td>
<td>15.1</td>
<td>10,000</td>
</tr>
</tbody>
</table>

The milled lucerne stem was extracted with cold water (three times), the extract was shaken three times with chloroform-pentan-1-ol (35:10), the aqueous phase being separated at each stage, the protein content of which was reduced. The solution was concentrated and passed through a column of sulphoethyl-Sephadex C-50 to remove cations and effect a further substantial reduction in the amount of protein present. Finally, the solution was adsorbed on a column of diethylaminoethyl-Sephadex A-50 (formate form) and polysaccharide fractions were eluted with water containing increasing proportions of formic acid. In all, 8 fractions were obtained, eluted with 0, 0.5, 1.0, 2.0, 5.0, 8.0, 10.0 and 15% of formic acid respectively.
Details of the 8 fractions isolated are recorded in Table III.

Table III

WATER-SOLUBLE LUCERNE STEM POLYSACCHARIDE FRACTIONS

<table>
<thead>
<tr>
<th>Polysaccharide fraction</th>
<th>Formic acid in eluant (%)</th>
<th>Wt (g) Isolated</th>
<th>Polysaccharide (g. as galactan)</th>
<th>Protein (%)</th>
<th>Volume of eluant (ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>0</td>
<td>0.28</td>
<td>0.07</td>
<td>5.0</td>
<td>4,000</td>
</tr>
<tr>
<td>2.</td>
<td>0.5</td>
<td>0.30</td>
<td>0.14</td>
<td>4.3</td>
<td>4,000</td>
</tr>
<tr>
<td>3.</td>
<td>1.0</td>
<td>0.13</td>
<td>0.05</td>
<td>3.1</td>
<td>2,000</td>
</tr>
<tr>
<td>4.</td>
<td>2.0</td>
<td>0.44</td>
<td>0.22</td>
<td>1.7</td>
<td>4,000</td>
</tr>
<tr>
<td>5.</td>
<td>5.0</td>
<td>1.10</td>
<td>0.45</td>
<td>2.7</td>
<td>6,000</td>
</tr>
<tr>
<td>6.</td>
<td>8.0</td>
<td>0.70</td>
<td>0.35</td>
<td>2.1</td>
<td>5,000</td>
</tr>
<tr>
<td>7.</td>
<td>10.0</td>
<td>0.40</td>
<td>0.12</td>
<td>4.2</td>
<td>3,000</td>
</tr>
<tr>
<td>8.</td>
<td>15.0</td>
<td>0.18</td>
<td>0.08</td>
<td>5.0</td>
<td>3,000</td>
</tr>
</tbody>
</table>

Samples of all leaf and stem fractions were analysed for uronic acid contents and methoxyl contents (both corrected for the small amounts of protein still present) and the hydrolysis products were examined by paper chromatography. The results are summarised in Table IV.
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Lucerne Stem</td>
<td>1.</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-13.2</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>+++</td>
<td>tr</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.5</td>
<td>20.5</td>
<td>3.1</td>
<td>+15.1</td>
<td>+</td>
<td>+++</td>
<td>tr</td>
<td>+++</td>
<td>tr</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.0</td>
<td>28.9</td>
<td>3.0</td>
<td>+34.0</td>
<td>+</td>
<td>+++</td>
<td>tr</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>2.0</td>
<td>39.4</td>
<td>5.35</td>
<td>+71.5</td>
<td>+++</td>
<td>+++</td>
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<td>++</td>
<td>+</td>
<td>+</td>
<td>tr</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>5.0</td>
<td>72.0</td>
<td>8.5</td>
<td>+192</td>
<td>++++</td>
<td>++++</td>
<td>tr</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>tr</td>
</tr>
<tr>
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<td>6</td>
<td>8.0</td>
<td>71.3</td>
<td>8.0</td>
<td>+190.5</td>
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<td>++</td>
<td>+</td>
<td>+</td>
<td>tr</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>10.0</td>
<td>69.4</td>
<td>4.1</td>
<td>+215</td>
<td>++++</td>
<td>++++</td>
<td>tr</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>tr</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>15.0</td>
<td>70.9</td>
<td>3.5</td>
<td>+212.5</td>
<td>++++</td>
<td>++++</td>
<td>tr</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>tr</td>
</tr>
<tr>
<td>Lucerne Leaf</td>
<td>1.</td>
<td>0</td>
<td>12.0</td>
<td>2.1</td>
<td>+20.5</td>
<td>tr</td>
<td>+++</td>
<td>-</td>
<td>+++</td>
<td>tr</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.5</td>
<td>18.0</td>
<td>2.2</td>
<td>+32.0</td>
<td>+</td>
<td>+++</td>
<td>tr</td>
<td>+++</td>
<td>tr</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.0</td>
<td>31.1</td>
<td>4.5</td>
<td>+66.7</td>
<td>+</td>
<td>+++</td>
<td>tr</td>
<td>+++</td>
<td>tr</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>2.0</td>
<td>41.5</td>
<td>6.1</td>
<td>+92.5</td>
<td>+++</td>
<td>+++</td>
<td>tr</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>tr</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>4.0</td>
<td>80.3</td>
<td>9.0</td>
<td>+185</td>
<td>++++</td>
<td>++++</td>
<td>tr</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>tr</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>6.0</td>
<td>79.4</td>
<td>8.8</td>
<td>+200</td>
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<td>++++</td>
<td>tr</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>tr</td>
</tr>
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<td></td>
<td>7</td>
<td>8.0</td>
<td>76.0</td>
<td>6.9</td>
<td>+195</td>
<td>++++</td>
<td>++++</td>
<td>tr</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>tr</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>10.0</td>
<td>79.5</td>
<td>3.4</td>
<td>+206.5</td>
<td>++++</td>
<td>++++</td>
<td>tr</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>tr</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>15.0</td>
<td>75.9</td>
<td>3.0</td>
<td>+215</td>
<td>++++</td>
<td>++++</td>
<td>tr</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>tr</td>
</tr>
</tbody>
</table>
The small amounts of polysaccharide samples which were obtained (especially from stems) did not permit a detailed examination in all cases. However, three distinct polysaccharide types were recognised, a neutral arabinogalactan, acidic arabinogalactans and pectinic acid.

The Neutral Arabinogalactan

Lucerne leaf polysaccharide fraction 1 (eluted from diethylaminoethyl-Sephadex A-50 with water) still contained 12% of uronic acid and partial acid hydrolysis yielded trace quantities of acidic oligosaccharides. Therefore, leaf polysaccharide fraction 1 was de-esterified under the mildest possible conditions and further fractionated by chromatography on diethylaminoethyl-cellulose to give a neutral arabinogalactan. The arabinogalactan was partially hydrolysed and gave three galactobiose which were chromatographically indistinguishable from 3-, 4-, and 6-α-β-D-galactopyranosyl-D-galactose. The highly branched nature of the polysaccharide was established by methylation and by detection by gas chromatography of (a) the methyl glycosides formed on methanolysis and of (b) the aldonolactones formed by hydrolysis of the methyl glycosides followed by oxidation with aqueous bromine.

The presence of the following methylated sugars as structural units in the methylated polysaccharide was
indicated. The presence of 2,4-di-O-methyl galactose was also confirmed by paper chromatography.

**Sugars**

<table>
<thead>
<tr>
<th>Sugars</th>
<th>Relative Amounts</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3,4-tri-O-methyl rhamnose</td>
<td>tr</td>
</tr>
<tr>
<td>2,3,4-tri-O-methyl arabinose</td>
<td>+++</td>
</tr>
<tr>
<td>2,3,5-tri-O-methyl arabinose</td>
<td>++</td>
</tr>
<tr>
<td>2,3-di-O-methyl arabinose</td>
<td>++</td>
</tr>
<tr>
<td>2,5-di-O-methyl arabinose</td>
<td>+++</td>
</tr>
<tr>
<td>3,5-di-O-methyl arabinose</td>
<td>++</td>
</tr>
<tr>
<td>2,3,4,6-tetra-O-methyl galactose</td>
<td>++</td>
</tr>
<tr>
<td>2,3,4-tri-O-methyl galactose</td>
<td>+++</td>
</tr>
<tr>
<td>2,3,6-tri-O-methyl galactose</td>
<td>++</td>
</tr>
<tr>
<td>2,4,6-tri-O-methyl galactose</td>
<td>+</td>
</tr>
<tr>
<td>2,4-di-O-methyl galactose</td>
<td>+++</td>
</tr>
</tbody>
</table>

The neutral arabinogalactan contained galactose and arabinose residues in a highly branched structure. There was an absence of branching through arabinose residues, so this sugar must be a constituent of a heteropolysaccharide. The presence of structural units (XXIV-XXXII) has been established.

\[
\text{Araf}_1 \rightarrow \rightarrow \text{5Araf}_1 \rightarrow \rightarrow \text{2Araf}_1 \rightarrow
\]

\[
\text{XXV} \quad \text{XXVI} \quad \text{XXVII}
\]

\[
\rightarrow \text{3Araf}_1 \rightarrow \rightarrow \text{Galp}_1 \rightarrow \rightarrow \text{3Galp}_1 \rightarrow
\]

\[
\text{XXVIII} \quad \text{XXIV} \quad \text{XXIX}
\]

\[
\rightarrow \text{4Galp}_1 \rightarrow \rightarrow \text{6Galp}_1 \rightarrow \rightarrow \text{3Galp}_1 \rightarrow
\]

\[
\text{XXX} \quad \text{XXXI} \quad \text{XXXII}
\]
The galactose residues are present as structural units XXIV, XXIX to XXXII and are mutually joined by 1,3, 1,4 and 1,6 linkages. There is no evidence that the polysaccharide is a single molecular species nor can the way in which the linkages are mutually distributed be yet indicated. But the results do indicate that there is present in lucerne either a single polysaccharide containing all three intergalactose linkages or a mixture of two polysaccharides, one containing 1,4 linked β-D-galactopyranose residues and the other containing 1,3 and 1,6 linked β-D-galactopyranose residues. Galactans and arabinogalactans of plant origin which have been characterised in reasonable detail fall into two main classes, those associated with pectins containing 1,4 linked β-D-galactopyranose residues (i.e. the galactan from lupin seeds 17 and the arabinogalactan from soybeans24,25) and the highly branched arabinogalactans from coniferous woods 26 which contain 1,3 and 1,6 linked β-D-galactopyranose residues. But the present results indicate the presence in lucerne of one or more polysaccharides containing galactose residues linked by all three intergalactose linkages. The same complex situation is encountered in the arabinogalactan from gum tragacanth 128 and recently an arabinogalactan of similar complexity has been found in association with lemon peel pectin.27 In neither of these cases has rigorous evidence been obtained
to show that the three types of linkage are present in a single molecular species. Recently an arabinogalactan found in association with pectin acid in the extracellular polysaccharide mixture from sycamore cambial cells, and re-examined in the course of the present investigation, has been shown to contain galactose residues linked by 1,3 and 1,6 linkages only.

It is clear that arabinogalactans associated with pectins do not necessarily contain $\beta$-D-galactopyranose residues which are mutually linked by 1,4 linkages only and that more detailed investigations of these polysaccharides is necessary. It is interesting to note that an arabinogalactan of the structural type here discussed was recently isolated by Unrau from *Centrosema* seed. The polysaccharide was shown to contain a complex branched structure and to contain a preponderance of 1,4 linked galactose residues, but evidence also indicated the presence in the polysaccharide of galactose residues mutually linked by 1,3 and 1,6 linkages.

**The Acidic Arabinogalactans.**

Lucerne leaf polysaccharide fractions 2 and 3 (eluted from diethylaminoethyl-Sephadex A-50 with 0.5% and 1.0% formic acid respectively) were partially hydrolysed, and both gave the three galactobioses, identified chromatographically as 3-, 4- and 6-$\alpha$-$\beta$-D-galactopyranosyl-\(\beta\)-galactose. In addition from polysaccharide fraction
on mild acid hydrolysis, the arabinobiose, 3-O-β-L-arabinofuranosyl-β-l-arabinose was chromatographically identified. Leaf fractions 2 and 3 were moreover methylated, and the methyl glycosides and aldonolactones derived from the methylated polysaccharides were analysed by gas chromatography. Components with the retention times of the following sugars were recognized in both cases, 2,3,4-, and 2,3,5-tri, 2,3-, 2,5- and 3,5-di-O-methyl arabinose, 2,3,4,6-tetra, 2,3,4-, 2,3,6- and 2,4,6-tri- and 2,4-di-O-methyl galactose, 2,3,4-tri-O-methyl glucuronic acid, 3-O-methyl rhamnose and in trace amounts 2,3,4-tri-O-methyl rhamnose or 2,3,4-tri-O-methyl xylose.

In order to obtain information on the nature of the acidic sugar constituents of leaf polysaccharide fraction 3, the neutral and acidic methyl glycosides formed on methanolysis of the methylated polysaccharide were separated. The acidic methyl glycosides must have consisted of mixtures of mono- and oligosaccharide derivatives, since further methanolysis gave the methyl glycosides of the following sugars:

<table>
<thead>
<tr>
<th>Sugars</th>
<th>Relative Amounts</th>
</tr>
</thead>
<tbody>
<tr>
<td>3,4-di-O-methyl rhamnose</td>
<td>minor</td>
</tr>
<tr>
<td>3-O-methyl rhamnose</td>
<td>major</td>
</tr>
<tr>
<td>2,3,4-tri-O-methyl glucuronic acid</td>
<td>minor</td>
</tr>
<tr>
<td>2,3-di-O-methyl galacturonic acid</td>
<td>major</td>
</tr>
</tbody>
</table>

The acidic arabinogalactans (leaf fractions 2 and 3)
contain galactose and arabinose in a highly branched structure and from partial hydrolysis and methylation results the same structural units XXXIV-XXXII occur in both polysaccharides as in the neutral arabinogalactan.

Moreover, from the identification of the disaccharide \(3-O-\beta-L\)-arabinopyranosyl-\(L\)-arabinose from polysaccharide fraction 2, the following structural unit is indicated:

\[
\text{Arap} \quad 1 \rightarrow 3 \quad \text{Araf} \quad 1 \rightarrow
\]

XXXIII

The same structural problem is present in both cases as in the case of the neutral arabinogalactan and a polysaccharide or polysaccharides are present containing galactose residues which are mutually linked by 1,3, 1,4 and 1,6 linkages. Polysaccharide fractions 2 and 3 contain 31.5% and 41.5% of uronic acid respectively and from their method of preparation fractions 2 and 3 must contain arabinogalactans with integral hexuronic acid residues. Leaf fraction 3 may be a homogeneous acidic arabinogalactan or (in the absence of evidence for homogeneity) an acidic arabinogalactan associated with a highly esterified pectin. The identification of the methyl glycosides from the acidic fraction of leaf fraction 3 (above) shows that the major component of the acidic glycoside mixture was a partially methylated aldobiouronic acid derived from structural unit XXXIV, in the parent polysaccharide, which also must contain terminal glucuronic
acid residues XXXV and also structural unit XXXVI at least in minor proportions:

\[
\begin{align*}
&\rightarrow \text{4GalpA} \quad 1 \rightarrow 2 \text{Rhap} \quad 1 \rightarrow & \\
&\quad \quad \downarrow & \\
&\text{XXXIV} \quad 1 & \\
&\rightarrow \text{4GalpA} \quad 1 \rightarrow 2 \text{Rhap} \quad 1 \rightarrow & \\
&\quad \quad & \\
&\text{XXXVI} \quad 1
\end{align*}
\]

Structural units XXXIV and XXXVI represent characteristic portions of pectins \(^{19,45,46}\) but as indicated, hard evidence on the homogeneity of leaf fraction 3 is lacking.

**The Pectinic Acid.**

Leaf polysaccharide fraction 5 and stem polysaccharide fraction 5, the pectin samples with the highest degree of esterification, were subjected to partial acid hydrolysis, and the chromatographic patterns of the products were examined with a view to comparing these fractions with the lucerne pectic acids extracted from leaf and stem with ethylenediamine-tetra-acetic acid di-sodium salt.

Leaf and stem fractions 5 on hydrolysis in \(\bar{\text{N}}\)-sulphuric acid, both yielded degraded galacturonans and mixtures of mono- and oligosaccharides which were partially fractionated on diethylaminoethyl-Sephadex A-25. Elution with water gave neutral sugars and then the columns were eluted with water containing increasing amounts of formic acid to give large amounts of galacturonic acid and
various acidic oligosaccharides which were further examined by paper chromatography, and by comparison with authentic samples the following sugars were tentatively identified in each case, $2-O-(\alpha-D$-galactopyranosyluronic acid)$-\text{l-rhamnose}$ (3, Table I), $4-O-(\beta-D$-glucopyranosyluronic acid)$-\text{L-fucose}$ (7, Table I), $6-O-(\beta-D$-glucopyranosyluronic acid)$-\text{D-galactose}$ (8, Table I), $4-O-(\alpha-D$-galactopyranosyluronic acid)$-\text{D-galacturonic acid}$ (1, Table I) and the trisaccharide $\text{Q-}\alpha-D$-galactopyranosyluronic acid$-(1 \rightarrow 4)-\text{Q-}\alpha-D$-galactopyranosyluronic acid$-(1 \rightarrow 4)-\text{D-galacturonic acid}$ (2, Table I), and in addition from leaf polysaccharide fraction 5 the oligosaccharide $\text{Q-}\alpha-D$-galactopyranosyluronic acid$-(1 \rightarrow 2)-\text{Q-}\text{l-rhamnopyranosyl}$-(1 $\rightarrow 4)-\text{Q-}\alpha-D$-galactopyranosyluronic acid$-(1 \rightarrow 2)$-\text{l-rhamnose}$ (4, Table I) was identified. Moreover, oligosaccharide (3, Table I) from leaf fraction 5 was methylated and methanolyzed and its identity was confirmed by gas chromatographic detection of the methyl glycosides of the following sugars, 3,4-di-$Q$-methylrhamnose and 2,3,4-tri-$Q$-methylgalacturonic acid.

Leaf polysaccharide fractions 5 to 9 and stem polysaccharide fractions 5 to 8, eluted from diethylaminoethyl-Sephadex with 4-15% formic acid, all contained a high proportion of hexuronic acid residues (69-80%) and gave on hydrolysis the sugars associated with typical pectins, galactose, arabinose, rhamnose with traces of fucose, xylose, glucose, 2-$Q$-methylfucose and 2-$Q$-methylxylose.
Since the polysaccharide fractions had progressively lower methoxyl contents it is quite clear that the fractionation of these higher fractions was based on differences in the degree of esterification, the more highly esterified pectins being least strongly adsorbed on the Sephadex column and the least highly esterified pectins being most strongly adsorbed. All of the pectin fractions were more highly esterified than the pectic acids extracted from lucerne leaf or stem with ethylenediamine-tetra-acetic acid di-sodium salt.\textsuperscript{43,46}

In respect of the partial hydrolysis studies on leaf and stem fractions 5, the fractions with the highest degree of esterification, it is clear that the acidic oligosaccharides recognized had all been characterised as partial hydrolysis products from lucerne leaf and stem pectic acids in the course of the present joint study.\textsuperscript{46} These results indicate that there are no apparent differences in the nature and mode of linkage of the sugar residues in the pectin fractions examined here and the pectic acid extracted from lucerne with disodium ethylenediamine-tetra-acetate. The only discernable difference is that the water extracted pectin fractions from both leaf and stem have higher degrees of esterification. Further information in respect of the water-extracted pectinic acids was provided by carrying out a sodium acetate fractionation of the de-esterified polysaccharides.
Sodium Acetate Fractionation of De-esterified Pectinic Acid.

Bishop and Zitko \(^{14}\) have fractionated the pectic acids obtained from sunflower heads, sugar-beet, apple and citrus pectins, by graded precipitation with sodium acetate. The pectic acid gave a number of acidic polysaccharide fractions of varying uronic acid content including a galacturronan free of neutral sugars (from sunflower heads only). This technique was now applied to lucerne pectinic acid.

Lucerne leaf fractions 5 to 9, and stem fractions 5 to 8, were combined and de-esterified under the mildest possible conditions \(^{51}\) to give pectic acid. Fractionation of the pectic acid by Bishop’s method gave four fractions precipitated by increasing concentrations of sodium acetate in the solution and a fifth and final fraction was obtained by precipitation of the residual polysaccharide with ethanol. Each fraction was examined for uronic acid content, specific rotation, possible heterogeneity by electrophoresis and the presence of neutral sugars. The results are tabulated in Table V.
Table V.

<table>
<thead>
<tr>
<th>Sodium Acetate Fraction of the Pectic Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pectic Acid Fraction No.</td>
</tr>
<tr>
<td>-------------------------</td>
</tr>
<tr>
<td>Pectic Acid</td>
</tr>
<tr>
<td>I</td>
</tr>
<tr>
<td>II</td>
</tr>
<tr>
<td>III</td>
</tr>
<tr>
<td>IV</td>
</tr>
<tr>
<td>V Ethanol</td>
</tr>
</tbody>
</table>

The results show that the pectic acid contained a number of molecular components and that a partial fractionation of these components was achieved. All of the fractions were shown to still contain neutral sugars, even the first fraction precipitated at lowest sodium acetate concentration, which fraction approximated most closely to a galacturonan. The galacturonic acid contents of the fractions decreased progressively with increasing sodium acetate concentration, and that of the ethanol-precipitated fraction was lowest. The specific rotations of the fractions decreased with decreasing uronic acid content. The electrophoretic pattern of the pectic acid contained two peaks indicating the presence of at least two components, one of which was preferentially precipitated with sodium acetate to yield fractions I and III in which this component predominated. Fraction I gave a single symmetrical
peak on electrophoresis and thus appeared to be electrophoretically homogeneous. There was no apparent discontinuity on electrophoresis between fraction I and fraction III which latter fraction showed, however, a tailing edge and contained a lower uronic acid content. So it appears that the component of greater electrophoretic mobility of the pectic acid contained a broad molecular weight distribution. The ethanol precipitated material was correspondingly enriched in the second, electrophoretically slower component of the pectic acid but a third distinct peak was here observable. Thus by means of graded precipitation with sodium acetate a partial fractionation was achieved between the component of the pectic acid showing greatest electrophoretic mobility and the components of lower electrophoretic mobility. There was no indication in water-extracted pectinic acid of a pure galacturonan, as the fraction precipitated at lowest sodium acetate concentration still contained neutral sugars. Bishop and Zitko examined by electrophoresis the fractions obtained by graded precipitation with sodium acetate of the pectic acids from sunflower heads, sugar-beet, apple and citrus fruit. All four pectic acids contained two components excepting perhaps the citrus pectic acid where the component of lower electrophoretic mobility was detected only in one minor fraction. In all cases the component of the pectic acids of greater electrophoretic
mobility was preferentially precipitated by sodium acetate, and the fraction precipitated at lowest sodium acetate concentration from the sunflower heads pectic acid did not contain neutral sugars and was in fact a galacturonan. The fraction precipitated from sugar-beet pectin at lowest sodium acetate concentration still contained appreciable quantities of neutral sugars, and it was, therefore, considered an exception in respect to the presence of a galacturonan, but in the case of the other pectic acids the authors suggested that the two components were a linear galacturonan free of neutral sugars and an acidic polysaccharide containing neutral sugars as well. The failure to resolve the galacturonan completely in the case of apple pectic acid, for example, was considered to be caused by a broad molecular weight distribution. In the present study of lucerne water-extracted pectic acid, it was observed that the component of higher electrophoretic mobility was preferentially precipitated with sodium acetate, as in the investigations of Zitko and Bishop, but there was no evidence to indicate the presence in the pectic acid of a pure galacturonan.

Also, the results of the present study differ from those obtained by a sodium acetate fractionation of lucerne pectic acid extracted with ethyldiamine-tetra-acetic acid disodium salt. In this case the original pectic acid and the various fractions had similar
electrophoretic mobilities. The peaks were not completely symmetrical but in no case was a distinct second peak observable. Again all fractions contained neutral sugars, and it was concluded that the acidic polysaccharide fractions formed a series of molecular species with no discontinuities in the composition.
EXPERIMENTAL
General Methods

Paper Partition Chromatography.

Paper chromatography was carried out on Whatman No. 1 and No. 4 filter papers. The following solvent systems were employed (v/v):-

(A) Ethyl acetate : pyridine : water (10 : 4 : 3).
(C) Butan-1-ol : pyridine : water (3 : 2 : 2).
(E) Ethylacetate : acetic acid : formic acid : water (18 : 8 : 3 : 9).
(F) Methylethyl ketone : acetic acid : water (9 : 1 : 1, saturated with boric acid).
(G) Ethyl acetate : pyridine : acetic acid : water (5 : 5 : 1 : 3).
(H) Butan-1-ol-ethanol-water (4 : 1 : 5, upper layer).
(I) Butan-2-on-water-ammonia (200 : 17 : 1).

The following abbreviations have been used to describe the chromatographic mobilities of sugars:

The \( R_{\text{Gal}} \) value of a sugar refers to the ratio of its movement relative to \( \alpha \)-galactose.

The \( R_{\text{GalA}} \) value refers to the rate of movement of a sugar relative to \( \alpha \)-galacturonic acid.
Thick paper chromatographic separations were carried out on Whatman 3MM filter sheets, which had been previously washed with water in a Soxhlet extractor. The positions of the sugar bands were located by cutting thin strips and developing them with the appropriate spray reagent. The appropriate parts of the filter sheets were then cut out and the sugars were eluted with water.

**Chromatographic Spray Reagents**:

1. **Aniline oxalate.**

   Unless otherwise stated, the reducing sugars were detected by spraying the dried chromatogram with a saturated solution of aniline oxalate in ethanol and developed at 100° for 5 mins.

2. **Periodate permanganate reagent** was used to detect sugar alcohols.

3. **Alkaline silver nitrate** was used to detect reducing and non-reducing sugars in minute quantities.

**Paper ionophoresis**:

Ionophoresis was carried out on Whatman No. 1 paper in borate buffer (pH 10) at a potential of 350 volts for 4-6 hrs. The sugars were located by spraying the oven-dried electrophoretogram with p-anisidine hydrochloride 1% in a solution of glacial acetic acid in butan-1-ol (10% v/v).

**Moving boundary electrophoresis** of polysaccharides was carried out in 0.05M sodium tetra-borate buffer (pH 9.25)
using a Tiselius type, Spinco Model H apparatus.
(Performed by Mr. I. W. Cottrell).

Evaporations were carried out under reduced pressure at or below 40°.

Specific rotations were measured at about 18° in aqueous solution (unless otherwise stated) using the sodium D-line as light source.

**Column Chromatography:**

**D.E.A.E.-Cellulose Columns.** The ion-exchange cellulose was washed alternately with 0.5M-hydrochloric acid and 0.5M-sodium hydroxide and the washings were repeated a second time. The cellulose was then washed with water until free from base and packed as a slurry in water. Diethylaminoethyl-cellulose was then generated in the phosphate form by elution with 0.5M-sodium dihydrogen phosphate buffer (pH 6). Equilibration of the column was by 0.005M-sodium dihydrogen phosphate solution of the same pH. The polysaccharide was dissolved in a little water and allowed to soak into the column overnight prior to elution.

**Cellulose Columns.** The cellulose was washed with N-sodium hydroxide followed by water until free from alkali and was then added to the column as a slurry in water and thoroughly washed with water.

**Charcoal-Celite Columns.** The celite was boiled with hydrochloric acid : water (1 : 1) twice, filtered and washed with water until free from acid. The charcoal was
washed three times in boiling water, fine material being
decanted off between washings. The mixture of charcoal : celite (1 : 1) was packed in the column as a water slurry
and washed with water prior to use.

**D.E.A.E.-Sephadex Columns.** Diethylaminoethyl-Sephadex (A-25 or A-50) was allowed to swell in water. It was
then washed with 0.5 M-hydrochloric acid, followed by water
until free from chloride ions and then with 0.5 M-sodium
hydroxide followed by water until free from base. Finally,
the resin was generated in the formate form by stirring
three times with 15% formic acid for 15 mins. The column
was then packed and eluted with water until free from
formic acid.

**S.E.-Sephadex C-50.** Sulphoethyl-Sephadex C-50 was allowed
to swell in water and was then washed repeatedly with 0.5 M-
hydrochloric acid on a Buchner funnel. The excess acid was
removed by washing with water. The resin was poured as a
slurry in water into a column and washed with water until
the bed had equilibrated.

**Gas-liquid Partition Chromatography.** Qualitative
separations of methyl glycosides and aldonolactones of
methylated sugars were carried out on a "Pye Argon
Chromatograph," (Section I) or on "Hewlett Packard Model
5750B" (Sections II and III) using the following columns:

(a) 5% by weight of neopentylglycol adipate polyester on
dichlorodimethylsilane treated Celite, 80-100 mesh at
150° or 165° C.
(b) 15% by weight of polyethyleneglycol adipate on dichlorodimethylsilane treated Celite at 175°C.

(c) 10% by weight of polyphenylether \([n\text{-bis(m-phenoxyphe-noxy) benzene}]\) on dichlorodimethylsilane treated Celite at 200°C.

(d) 10% by weight of Carbowax 20M on Chromosorb W, 80-100 mesh.

The retention times \((T)\) of the methylated sugars are relative to that of methyl 2,3,4,6-tetra-\(\text{O}\)-methyl-\(\beta\)-\(\text{D}\)-glucopyranoside.

**Small scale hydrolyses** were carried out by heating the oligo- or polysaccharides (1-5 mg.) in \(\text{H}_{2}\text{SO}_{4}\) (0.5-2 ml.) in a sealed tube at 100°C. The duration of the hydrolysis varied from four hours for neutral oligosaccharides to 12-18 hrs. for acidic polysaccharides. The solutions were neutralized with barium carbonate and filtered. After de-ionization with Amberlite resin IR-120[H], the solutions were concentrated and examined by paper chromatography.

**Methanolyses** were carried out by heating the methylated polysaccharides (5-10 mgs.) or oligosaccharides (1-2 mgs.) at 100°C with methanolic 4% hydrogen chloride in sealed tubes for the desired length of time. The cooled solution was neutralized with silver carbonate, centrifuged, and the solution carefully concentrated in the presence of chloroform.

**Small-scale methylations** were carried out by the Kuhn
The oligosaccharide (1-5 mg.) was dissolved in N,N dimethyl formamide (0.2-0.5 ml.) and methyl iodide (0.5-1.0 ml.) and silver oxide (1-5 mg.) were added. The mixture was shaken in the dark for eighteen hours, centrifuged, and the supernatant concentrated. The N,N dimethyl formamide was removed azeotropically with redistilled toluene and the resultant syrup was dried in vacuo prior to methanolysis.

Methylation: The procedure normally used was the sodium hydride – methyl sulfoxide method. A typical case is described.

Sodium hydride (1.5 grams, 50% in oil) was washed three times with n-pentane and then stirred in methyl sulfoxide (15 ml.) at 50° under an atmosphere of nitrogen for 45 min. A sample of polysaccharide (500 mg.) was dissolved in methyl sulfoxide (50 ml.) by heating at 60° for 20 min. After cooling the methylsulphinyl carbanion solution (7 ml.) was added (30% in excess of theoretical). A gel formed but disappeared after stirring at room temperature for 4 hr. Methyl iodide was then added drop-wise, the temperature was not allowed to rise above 25°C, and stirring was continued at room temperature overnight. The resulting solution was dialyzed for 24 hr. The methylated polysaccharide was extracted repeatedly with chloroform, and the chloroform extracts were combined and concentrated. Purification of the methylated polysac-
Charide was effected by precipitation (twice) from chloroform with petrol ether (B.P. 60-80°).

**Borohydride reductions** were carried out by the addition of sodium borohydride to an equal amount of sugar dissolved in water. The solution was allowed to stand overnight, excess borohydride was destroyed with Amberlite resin IR-120[H], and the solution was concentrated to a syrup. Finally boric acid was removed by exhaustive evaporation with methanol.

**Demethylations** were carried out in dichloromethane and boron trichloride.

**Methoxyl determinations** were carried out by the semi-micro Zeisel method.

**Phenol-sulphuric acid reagent** was used to determine total sugar content. D-Galactose was used as the reference sugar.

**Uronic acid contents** were determined by:

(a) The carbazole colorimetric reagent.
(b) A modified carbazole procedure.
(c) Potentiometric titration.

\(L\)-Rhamnose was estimated by the \(L\)-cysteine method.

**Aldonolactones:** The methylated sugar (10 mg) was dissolved in water (2 ml) bromine (5 drops) was added and the mixture was kept in the dark at room temperature for three days. Excess bromine was removed by aeration. The solution was neutralized with silver carbonate and centrifuged. Silver salts were precipitated from the supernatant with hydrogen sulphide and the solution was again centrifuged. Finally the solution was concentrated
to dryness. The residue was extracted with acetone and again dried.

Protein estimations were carried out using the Folin-phenol reagent,\textsuperscript{145} using Bovine Plasma Albumin (Fraction 5) as reference material.

Melting points were determined using a Kofler hot-stage microscope.

Organic solvents were dried and purified by methods quoted by Vogel.\textsuperscript{146}
**LUCERNE PECTIC ACID**

Extraction and purification of lucerne stem pectic acid.

A typical extraction of lucerne stem pectic acid is as follows (Diagram 1, p. 37). Freshly cut lucerne stem (1-2" portions), was extracted with boiling ethanol-water (4 : 1) in a Soxhlet apparatus to inactivate enzymes and remove soluble sugars, dried and then milled. The milled lucerne stem (300 g.) was extracted three times with cold water (once in a ball mill) to extract water-soluble polysaccharides (for details see below). The water extracted material was then extracted with 2% ethylenediaminetetra-acetic acid disodium salt (3 x 2.5 l.) at 70° for 3 hr. From the combined supernatant solutions crude pectin was precipitated by acetone (1 vol.). The polysaccharide was redissolved in water and precipitated as the calcium salt by the addition of 10% calcium chloride solution. The precipitate was centrifuged and converted to the ammonium pectate by heating with 0.5% ammonium oxalate (4 l.) at 90° for 30 min. The ammonium pectate was precipitated by the addition of acetone (1 vol.), washed with acetone-water (1 : 1) a few times, and it was again purified by precipitation as calcium pectate and regenerated as ammonium pectate. The ammonium pectate was finally isolated and freeze-dried (9.5 g.) [α]_D +200° (c, 0.27). [Found: uronic acid content, 73%; OMe, 2.5%].
Partial acid hydrolysis of lucerne stem pectic acid.

(Trial experiment):

Lucerne stem ammonium pectate (300 mg.) was hydrolysed in $\text{H}_2\text{SO}_4$ (20 ml.) on a boiling water bath for 8 hr. Aliquot portions (2 ml.) were removed after 3, 4, 5, 6, 7 and 8 hr. respectively and poured into acetone (8 ml.); the precipitated polysaccharide in each case was removed by centrifugation and the supernatant liquids were evaporated to remove acetone. The individual aliquot portions were diluted and neutralized with barium carbonate, the precipitates were removed at the centrifuge and washed with water (2 x 5 ml.). The supernatant liquid and water washings in the case of each individual aliquot portion were combined, the portions were deionized with Amberlite resin IR-120[H], concentrated and the syrups were examined chromatographically in solvents (A) and (D). The chromatograms showed that partial hydrolysis in each instance liberated a large amount of galacturonic acid together with neutral sugars. Visual inspection suggested that the best yield of acidic oligosaccharides could be obtained by hydrolysing the polysaccharide with $\text{H}_2\text{SO}_4$ at 100°C for 6 hr.

Large scale partial hydrolysis.

Lucerne stem ammonium pectate (17 g.) was hydrolysed in $\text{H}_2\text{SO}_4$ (560 ml.) on a boiling water bath for 6 hr. The solution was cooled and centrifuged. The
insoluble polysaccharide was washed with ethanol-water (1:1) and dried by solvent exchange to yield degraded galacturonan A (3.7 g.) \([\alpha]_D^2 +288^\circ (c, 0.46\text{ as sodium salt})\). [Found: uronic acid content, 97.5\% (potentiometric titration method)]

The supernatant solution was poured into acetone, the precipitated polysaccharide was centrifuged and dried as before to yield degraded galacturonan (B) (1.7 g.). [Found: uronic acid content, 97\%].

The supernatant solution was evaporated to remove acetone, it was neutralized with barium carbonate and centrifuged. The barium salts were washed with water (3 x 500 ml.). The supernatant liquid and washings were combined, concentrated, deionized with Amberlite resin IR-120[H] and further concentrated to yield a syrupy mixture of sugars (6.5 g.).

The sugar mixture was adsorbed on a column of diethylaminoethyl-Sephadex A-25 (40 g; 3 x 50 cm.; formate form). Elution with water afforded neutral sugars (1.8 g.). Paper chromatography of the mixture in solvents (A), (D) and (H) indicated the presence of galactose, arabinose, xylose, fucose, rhamnose, 2-\(\beta\)-methylfucose and 2-\(\beta\)-methylxylose. The column was then eluted successively with 0.05 M-formic acid, with a gradient of 0.05M \(\rightarrow\) 0.4M-formic acid, with a gradient of 0.4M \(\rightarrow\) 0.5M-formic acid and finally with 0.5M-formic acid.
Fractions (20 ml.) were collected and the contents of every tenth test tube were concentrated and examined by paper chromatography in solvents (D) or (E). Similar fractions were combined, the bulk fractions were extracted with a 5% solution of methyl-di-n-octylamine in chloroform. The aqueous solutions were evaporated and the acidic oligosaccharides were isolated as the amine salts. Further separation and purification (where necessary) was carried out on Whatman 3MM filter sheets to give individual components.

The following fractions were obtained - Table VI.
Table VI.

FRACTIONS OBTAINED FROM PARTIAL ACID HYDROLYSIS OF LUCERNE STEM PECTIC ACID

<table>
<thead>
<tr>
<th>Eluant</th>
<th>( R_{Gala} ) (Solvent D)</th>
<th>Fraction No.</th>
<th>Stain</th>
<th>Wt. (g.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formic Acid 0.05(N)</td>
<td>0.8 (major)</td>
<td>I</td>
<td>Orange</td>
<td>0.270</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td></td>
<td>Brown</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.3 (tr)</td>
<td>II</td>
<td>Brown</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td></td>
<td>Brown</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.7</td>
<td></td>
<td>Red</td>
<td>0.320</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td></td>
<td>Orange</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0 (major)</td>
<td></td>
<td>Brown</td>
<td></td>
</tr>
<tr>
<td>Formic Acid 0.05(N)</td>
<td>0.4 (N)</td>
<td>III</td>
<td>Yellow</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.30 (tr)</td>
<td></td>
<td>Brown</td>
<td>0.241</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td></td>
<td>Yellow</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0 (major)</td>
<td></td>
<td>Brown</td>
<td></td>
</tr>
<tr>
<td>Formic Acid 0.05(N)</td>
<td>1.0 (N)</td>
<td>IV</td>
<td>Brown</td>
<td>0.097</td>
</tr>
<tr>
<td></td>
<td>0.12 (tr)</td>
<td>V</td>
<td>Brown</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.55 (tr)</td>
<td></td>
<td>Orange</td>
<td>0.050</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td></td>
<td>Brown</td>
<td></td>
</tr>
<tr>
<td>Formic Acid 0.4(N)</td>
<td>0.12 (tr)</td>
<td>VI</td>
<td>Brown</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.55 (major)</td>
<td></td>
<td>Orange</td>
<td>0.060</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td></td>
<td>Brown</td>
<td></td>
</tr>
<tr>
<td>Eluant</td>
<td>$R_{GalA}$ (Solvent E)</td>
<td>Fraction No.</td>
<td>Stain</td>
<td>Wt. (g)</td>
</tr>
<tr>
<td>-------------</td>
<td>-------------------------</td>
<td>--------------</td>
<td>--------</td>
<td>---------</td>
</tr>
<tr>
<td>0.4N-Formic</td>
<td>0.5 (major)</td>
<td>VII</td>
<td>Brown</td>
<td>0.205</td>
</tr>
<tr>
<td>Acid</td>
<td>0.8</td>
<td></td>
<td>Red</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td></td>
<td>Brown</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.4 (tr)</td>
<td>VIII</td>
<td>Orange</td>
<td></td>
</tr>
<tr>
<td>0.5N-Formic</td>
<td>0.5</td>
<td></td>
<td>Brown</td>
<td>0.050</td>
</tr>
<tr>
<td>Acid</td>
<td>0.8 (tr)</td>
<td></td>
<td>Red</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td></td>
<td>Brown</td>
<td></td>
</tr>
<tr>
<td>0.5N-Formic</td>
<td>0.24 (major)</td>
<td>IX</td>
<td>Brown</td>
<td>0.110</td>
</tr>
<tr>
<td>Acid</td>
<td>0.5 (tr)</td>
<td></td>
<td>Brown</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0 (tr)</td>
<td></td>
<td>Brown</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>X</td>
<td>Brown</td>
<td>1.250</td>
</tr>
<tr>
<td></td>
<td>0.24</td>
<td></td>
<td>Brown</td>
<td></td>
</tr>
</tbody>
</table>
The following acidic oligosaccharides were obtained:

Oligosaccharide 1. (31 mg.), eluted with 0.05N-formic acid,
\[ \text{R}_{\text{Gal}} = 0.8 \quad \text{(solvent D),} \quad M_g = 0.55, \quad [\alpha]_D^0 +94^\circ \quad (c, 0.45). \]

The oligosaccharide was chromatographically and ionophoretically indistinguishable from 2-\text{O}-(\alpha-D-galactopyranosyluronic acid)-L-rhamnose and gave galacturonic acid and rhamnose on hydrolysis. The sugar was methylated by the Haworth procedure. To the material (12 mg.) in water (0.5 ml.) methyl sulphate (0.25 ml.) and sodium hydroxide (30% 0.5 ml.) were added drop-wise over a period of 1 hr. with stirring. The reaction flask was surrounded by an ice bath and an atmosphere of nitrogen maintained throughout the reaction. Five further additions of the reagents were made on five consecutive days. The solution was heated at 100° for 1 hr., twenty-four hours after the final addition, allowed to cool and the pH was adjusted to 4 with dilute sulphuric acid. Methylated spirits (8 vol.) was added, sodium sulphate was precipitated, the precipitate was centrifuged and washed with methylated spirits (4 x 10 ml.). The supernatant liquid and washings were combined, adjusted to pH 8 and concentrated. The solution was acidified and extracted with chloroform (5 x 10 ml.). The chloroform extracts were combined, dried over anhydrous sodium sulphate and concentrated. The product on recrystallisation from a chloroform-light petroleum (B.P. 100-120°) mixture yielded the methylated di-
saccharide methyl 2-O-(2,3,4-tri-O-methyl-α-D-galactopyranosyluronic acid)-3,4-di-O-methyl-L-rhamnoside dihydrate, m.p. and mixed m.p. 68-69°C, [α]_D +100°, (2, 0.2 in chloroform) and gave an x-ray powder photograph identical to that of an authentic sample.

**Oligosaccharide 2.** (25.1 mg.), eluted with 0.05N-formic acid \( R_{\text{GalA}} = 0.5 \) (solvent D), \( M_G = 0.6, [\alpha]_D -70° \) (2, 1.0). The oligosaccharide was chromatographically and ionophoretically indistinguishable from 4-O-(β-D-glucopyranosyluronic acid)-L-fucose and hydrolysis gave fucose, glucuronic acid and glucurone. The sugar was converted to the methyl ester, methyl glycosides, reduced with sodium borohydride and hydrolysed. Glucose and fucose were identified chromatographically in the hydrolysate.

The sugar was methylated by the Kuhn procedure, the methylated product was methanolysed and the methanolysis products were examined by gas-liquid partition chromatography and gave peaks with the retention times of the methyl glycosides of the following sugars.

<table>
<thead>
<tr>
<th>Sugars</th>
<th>T (column a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3-di-O-methylfucose</td>
<td>0.88, 1.17, 1.26</td>
</tr>
<tr>
<td>2,3,4-tri-O-methylglucuronic acid*</td>
<td>2.31, 3.06</td>
</tr>
</tbody>
</table>

*present as methyl ester

**Oligosaccharide 3.** (13.9 mg.), eluted with 0.05N-formic acid, \( R_{\text{GalA}} = 0.19 \) (solvent D), \( M_G = 1.02, [\alpha]_D +1.5° \) (2, 1.0). The oligosaccharide was chromatographically and ionophoretically indistinguishable from 6-O-(β-D-glucose-
pyranosyluronic acid)-\(\text{D}\)-galactose and on hydrolysis gave glucuronic acid, glucurone and galactose. The sugar methyl ester, methyl glycosides were reduced with sodium borohydride and hydrolysed. Glucose and galactose were identified chromatographically in the hydrolysate.

The sugar was methylated by the Kuhn procedure, the methylated product was methanolysed and the methanolysis products were examined by gas-liquid partition chromatography and gave peaks with the retention times of the methyl glycosides of the following sugars.

<table>
<thead>
<tr>
<th>Sugars</th>
<th>(T) (column a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3,4-tri-O-methylgalactose</td>
<td>6.76</td>
</tr>
<tr>
<td>2,3,5-tri-O-methylgalactose</td>
<td>4.01</td>
</tr>
<tr>
<td>2,3,4-tri-O-methylglucuronic acid*</td>
<td>2.32, 3.05</td>
</tr>
</tbody>
</table>

*present as methyl ester.

Oligosaccharide \(4\). (11.0 mg.), eluted with 0.05-0.4\(\text{N}\)-formic acid, \(R_{\text{GalA}} = 0.13\) (solvent D) = 0.55 (solvent E), \(M_G = 0.6\), \([\alpha]_D +90^\circ\) (c, 0.2).

The oligosaccharide on hydrolysis gave galacturonic acid and rhamnose; colorimetric determinations of galacturonic acid \(142\) and rhamnose \(144\) indicated their presence in the acidic oligosaccharide in the molar ratio 1.05 : 1 while the derived glycitol (sodium borohydride reduction) contained these sugars in the molar ratio 1.9 : 1. The sugar on mild acid hydrolysis gave mainly 2-O-(\(\alpha\)-\(\text{D}\)-galactopyranosyluronic acid)-\(\text{L}\)-rhamnose. Mild acid hydrolysis of the derived glycitol followed by chromato-
graphic examination (solvent G), gave this same oligosaccharide and a second major component with the mobility of 2-O-(α-D-galactopyranosyluronic acid)-L-rhamnitol.

The oligosaccharide glycitol was methylated by the Kuhn procedure, the methylated product was methanolysed and the methanolysis products were examined by gas-liquid partition chromatography and gave peaks with the retention times of 1,3,4,5-tetra-0-methylrhamnitol \([T, 1.10 \text{(column a)}]\) and the methyl glycosides of the following sugars.

<table>
<thead>
<tr>
<th>Sugars</th>
<th>(T) (column a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3,4-di-0-methylrhamnose</td>
<td>0.83</td>
</tr>
<tr>
<td>2,3-di-0-methylgalacturonic acid*</td>
<td>4.5</td>
</tr>
<tr>
<td>2,3,4-tri-0-methylgalacturonic acid*</td>
<td>6.7</td>
</tr>
</tbody>
</table>

*present as methyl ester

Oligosaccharide 5. \((87 \text{ mg.})\) eluted with \(0.4\text{N} \rightarrow 0.5\text{N}-\)formic acid, \(R_{\text{Gal}} = 0.2\) (solvent D), \(= 0.5\) (solvent E), \(M_G = 0.95\). The oligosaccharide was chromatographically and ionophoretically indistinguishable from 4-O-(α-D-galactopyranosyluronic acid)-D-galacturonic acid and gave galacturonic acid only on hydrolysis. The sugar \((20 \text{ mg.})\) was dissolved in water \((5-6 \text{ ml.})\), neutralized with calcium carbonate, centrifuged and the calcium salt was precipitated with acetone. The salt was dried with acetone and ether. Found: \([\alpha]_D +118^\circ\) \((c, 0.3 \text{ in } 0.5\text{N}-\text{hydrochloric acid})\).

The oligosaccharide \((40 \text{ mg.})\) was treated overnight with methanolic 1% hydrogen chloride \((10 \text{ ml.})\). The solution was neutralized with silver carbonate and the
silver residues were removed at the centrifuge before concentration to a syrup (45 mg.). The syrup, dried over phosphorus pentoxide in vacuo for 24 hr., was dissolved in anhydrous pyridine (10 ml.) and hexametadisilazane (1 ml.) and chlorotrimethylsilane (2 ml.) were added. The mixture was shaken vigorously for 30 seconds, allowed to stand at room temperature for one hour and was evaporated to dryness. The residue was extracted with ether (2 x 10 ml.) and the combined extracts were concentrated to a syrup (73 mg.). The syrup was redissolved in ether and refluxed with lithium aluminium hydride (150 mg.) with vigorous stirring for 2 hr. The solution was cooled and excess ethyl acetate added and the mixture was allowed to stand for several hours to ensure that all the lithium aluminium hydride had been destroyed. After evaporation of the solvent the residue was dissolved in 0.5N-sulphuric acid. The solution was neutralized with barium carbonate, deionized with Amberlite resin IR-120[H] and concentrated to a syrup (29 mg.).

The reduction product was methylated with methyl sulphate and sodium hydroxide 147 for 5 consecutive days as described for Oligosaccharide 1. The final solution was acidified and extracted with chloroform to yield methylated disaccharide (20 mg.). The methylated oligosaccharide was methanolysed and examined by gas-liquid partition chromatography and gave peaks with the retention times of the methyl glycosides of the following sugars.
<table>
<thead>
<tr>
<th>Sugars</th>
<th>T (column a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2, 3, 4, 6-tetra-Q-methylgalactose</td>
<td>1.81</td>
</tr>
<tr>
<td>2, 3, 6-tri-Q-methylgalactose</td>
<td>2.86, 3.76, 4.1</td>
</tr>
</tbody>
</table>

Oligosaccharide 6g (44 mg.), eluted with 0.5N-formic acid, $R_{\text{GalA}} = 0.1$ (solvent D) = 0.24 (solvent E), $M_G = 0.96$. The oligosaccharide was chromatographically and ionophoretically indistinguishable from $\alpha-d$-galactopyranosyluronic acid-(1 → 4)-$\alpha-d$-galactopyranosyluronic acid-(1 → 4)-$d$-galacturonic acid and gave galacturonic acid only on hydrolysis.

The oligosaccharide (10 mg.) in water (5 ml.) was neutralized with calcium carbonate, centrifuged and the calcium salt was precipitated with acetone. The salt was dried with acetone and ether and dried to give a white solid, $[\alpha]_D +135^\circ$ (c, 0.44 in 0.5N-hydrochloric acid).

The oligosaccharide (25 mg.) was treated overnight with methanolic 1% hydrogen chloride (5 ml.), the solution was neutralized with silver carbonate, the silver residues were centrifuged and the solution was concentrated to a syrup (26 mg.). The syrup, dried over phosphorus pentoxide in vacuo for 24 hr., was dissolved in anhydrous pyridine (5 ml.), hexametadisilazane (0.5 ml.) and chlorotrimethylsilane (1 ml.) were added, and the mixture was shaken vigorously for 30 seconds and allowed to stand at room temperature for one hour before evaporation to dryness.

The residue was extracted with ether (2 x 8 ml.) and the
combined extracts were concentrated to a syrup (40 mg.). The syrup was redissolved in ether and refluxed with lithium aluminium hydride (75 mg.) with vigorous stirring for 2 hr. The solution was cooled, the lithium aluminium hydride was destroyed by the addition of excess ethyl acetate, the solution was evaporated and the residue dissolved in 0.5N-sulphuric acid. The solution was neutralized with barium carbonate, deionized with Amberlite resin IR-120[H] and concentrated to a syrup (10 mg.). A small sample was hydrolyzed and shown to contain only galactose. The reduced oligosaccharide was methylated with methyl sulphate and sodium hydroxide over five consecutive days as described for Oligosaccharide 1. Finally the solution was heated on a boiling water bath for 1 hr. and cooled, the pH of the solution was adjusted to 4 and the solution was extracted with chloroform. The combined chloroform extracts were dried over anhydrous sodium sulphate and concentrated to a syrup, which was dissolved in methyl iodide (3 ml.) and refluxed with silver oxide (10 mg.) for 12 hr. The mixture was filtered, the residue was washed repeatedly with chloroform and the filtrate and washings were combined and concentrated to a syrup. The methylated, reduced oligosaccharide was methanolysed and examined by gas-liquid partition chromatography and gave peaks with the retention times of the methyl glycosides of the following sugars.
<table>
<thead>
<tr>
<th>Sugars</th>
<th>T (column a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3,4,6-tetra-O-methylgalactose</td>
<td>1.79</td>
</tr>
<tr>
<td>2,3,6-tri-O-methylgalactose</td>
<td>2.87, 3.7, 4.1</td>
</tr>
</tbody>
</table>

**Isolation and Fractionation of Water-Soluble Lucerne Polysaccharides:**

Lucerne leaf was extracted by boiling with ethanol-water (4:1) (twice), dried and then milled. Milled leaf was again extracted with boiling ethanol-water (4:1) and again dried. The milled leaf (300 g.) was extracted by stirring overnight with water (6,000 ml.). The residue was filtered on a large Buchner and re-extracted (twice) overnight with water (2 x 6,000 ml.). The combined filtered solution (17,500 ml.) was estimated to contain polysaccharide (12.1 g., as galactan by the phenol-sulphuric acid method\(^{141}\) and protein (30.0 g., by the Folin phenol reagent\(^{145}\)).

The solution of water-soluble polysaccharides was percolated through a bed of sulphoethyl-Sephadex C-50 (H-form, 60 g.) on a large Buchner to remove cations, the pH of the recovered solution (25,000 ml.) was adjusted to 4.5 by the addition of potassium acetate and the solution was concentrated at 35° to a volume of approximately 8,000 ml. This solution was now deproteinized by a phenol-H\(_2\)O distribution\(^{148}\). The solution was emulsified in two batches (4,000 ml.), with phenol (2,000 g.) for 12 hr.
The emulsions were set aside at 0°C, two layers readily formed and crystalline phenol was separated. The aqueous layers were dialysed for 4 days against several changes of distilled water. The polysaccharide solution on recovery was shown to contain polysaccharide (8 g., as galactan \(^{141}\)) and protein (5.5 g., \(^{145}\)). The solution was concentrated (4,000 ml.) and adsorbed in batches (ca. 400 ml.) on a column (30 x 13 cm., 70 g.) of diethylaminoethyl-Sephadex A-50 (formate form), the column being allowed to rest several hours after the addition of each batch. The column was eluted with several litres of water and successively with water containing 0.5, 1, 2, 4, 6, 8, 10 and 15% of formic acid. Each eluate was adjusted to pH 4.5 by the addition of potassium acetate, concentrated, dialysed against distilled water, analysed for polysaccharide and protein (as above) and freeze-dried. The details are recorded in Table II, p. 64.

Lucerne stem (1-2" portions) was extracted with boiling ethanol-water (4 : 1) in a Soxhlet apparatus, dried and then milled. The milled material was again extracted with boiling ethanol-water (4 :1) and again dried.

Milled lucerne stem (300 g.) was twice extracted by stirring overnight with water (2 x 3,000 ml.) and the residue was further extracted with water (2,500 ml.) in a ball mill. The combined extracts were adjusted to pH 4.5 with potassium acetate and concentrated. The solution
was shown to contain polysaccharide (3 g. as galactan) and protein (1.5 g.). The solution was dialysed against distilled water for 48 hr. and was then shaken three times with chloroform-pentan-1-ol (35 : 10, 1,000 ml.) the aqueous phase being separated at each stage. The combined aqueous solutions were passed through a column of sulphoneethyl-Sephadex C-50 (H form) (30 x 3 cm. 20 g.), the eluate was adjusted to pH 4.5 by the addition of potassium acetate and concentrated to a small volume, and the solution was shown to contain polysaccharide (1.82 g., as galactan) and protein (0.4 g.). This solution was adsorbed on a column of diethylaminoethyl-Sephadex A-50 (formate form) (45 x 4 cm. 25 g.). The column was eluted with distilled water and successively with water containing 0.5, 1, 2, 5, 8, 10 and 15% of formic acid. Each eluate (as in the case of those from the leaf), was adjusted to pH 4.5, concentrated, dialysed against distilled water, analysed for polysaccharide and protein and freeze-dried. The details are recorded in Table III, p. 65.

Samples of the leaf and stem fractions were analysed for hexuronic acid content by the modified carbazole procedure and for methoxyl content, specific rotations were measured and a sample of each fraction was hydrolysed and examined by paper chromatography in solvents A and D. The results are quoted in Table IV, p. 66.
Lucerne Leaf Fraction 1. Sub-fractionation and Examination of the Arabinogalactan.

A small quantity of lucerne leaf polysaccharide fraction 1 (50 mg.) was partially hydrolysed by heating with 5N-sulphuric acid (7 ml.) on a boiling water bath for 4.5 hr. The solution was neutralized with barium carbonate and centrifuged. The supernatant liquid was deionized with Amberlite resin IR-120[H] and concentrated and the hydrolysate was examined chromatographically in solvents D and E. The presence of a component with the chromatographic mobility of D-galacturonic acid was detected together with traces of acidic oligosaccharides.

Sub-fractionation of Leaf Polysaccharide Fraction 1.

Lucerne leaf polysaccharide fraction 1 was dissolved in water (15 ml.). 0.2M-Sodium hydroxide was added drop-wise with stirring at 0°C until pH 12 was reached. The solution was kept at 0°C for 2 hr. and further sodium hydroxide was added as required to maintain the pH at 12. The solution was acidified with dilute hydrochloric acid and the polysaccharide was precipitated by the addition of acetone, separated by centrifugation, washed free from acid, re-dissolved in water and placed on a column of diethylaminoethyl-cellulose (25 x 2 cm.; 10 g.; phosphate form). The column was eluted successively with 0.005M, 0.05M, 0.15M and 0.5M-sodium dihydrogen phosphate buffer at pH 6. The fractions were dialysed and con-
trated, the polysaccharides were precipitated with acetone, redissolved in water and isolated by freeze-drying to yield the following fractions (Table VII). Each sample was analysed for hexuronic acid content and polysaccharide content (as galactan).

Table VII Sub-fractionation of Leaf Polysaccharide

<table>
<thead>
<tr>
<th>Eluant</th>
<th>Fraction No.</th>
<th>Polysaccharide (%) as Gal.</th>
<th>Uronic A (%)</th>
<th>[α]D</th>
<th>Wt. (mg.) isolated</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.005M NaH₂PO₄</td>
<td>1a</td>
<td>55</td>
<td>0</td>
<td>-22°</td>
<td>107</td>
</tr>
<tr>
<td>0.05M NaH₂PO₄</td>
<td>1b</td>
<td>45</td>
<td>5</td>
<td>n.d.</td>
<td>8.0</td>
</tr>
<tr>
<td>0.15M NaH₂PO₄</td>
<td>1c</td>
<td>36</td>
<td>7.5</td>
<td>n.d.</td>
<td>7.7</td>
</tr>
<tr>
<td>0.5M NaH₂PO₄</td>
<td>1d</td>
<td>40</td>
<td>36</td>
<td>n.d.</td>
<td>25.0</td>
</tr>
</tbody>
</table>

Fractions 1b-1d containing hexuronic acid were not examined further.

Examination of Leaf Polysaccharide Fraction 1a.

Polysaccharide fraction 1a was hydrolysed and examined chromatographically in solvents B and D, and the examination indicated the presence of galactose and arabinose together with a trace of rhamnose.

The polysaccharide (25 mg.) was heated on a boiling water bath in 0.5M-sulphuric acid (3 ml.) for 1 hr. The solution was neutralized with barium carbonate, centrifuged,
deionised with Amberlite resin IR-120[H] and examined chromatographically in solvents A and D. By comparison the authentic samples oligosaccharides ((i) to (iii), Table VIII) were tentatively identified.

Table VIII Neutral Oligosaccharides from Lucerne Neutral and Acidic Arabinogalactans

<table>
<thead>
<tr>
<th>Oligosaccharides</th>
<th>R&lt;sub&gt;Gal&lt;/sub&gt; (Solvent A)</th>
<th>R&lt;sub&gt;Gal&lt;/sub&gt; (Solvent D)</th>
<th>Lucerne Leaf Fractions</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-0-β-D-galactopyranosyl-D-galactose (i)</td>
<td>0.5</td>
<td>0.29</td>
<td>1a, 2, 3</td>
</tr>
<tr>
<td>4-0-β-D-galactopyranosyl-D-galactose (ii)</td>
<td>0.57</td>
<td>0.37</td>
<td>1a, 2, 3</td>
</tr>
<tr>
<td>6-0-β-D-galactopyranosyl-D-galactose (iii)</td>
<td>0.34</td>
<td>0.21</td>
<td>1a, 2, 3</td>
</tr>
<tr>
<td>3-0-β-L-arabinopyranosyl-L-arabinose (iv)</td>
<td>0.80</td>
<td>-</td>
<td>2</td>
</tr>
</tbody>
</table>

The arabinogalactan (55 mg.) was methylated with methyl iodide and sodium hydride in methyl sulfoxide and furnished methylated polysaccharide (38 mg.), [α]<sub>D</sub> -19.5° (c, 0.43 in chloroform). [Found: OMe, 41.0%].

A portion of the methylated arabinogalactan was methanolysed in a sealed tube with methanolic 4% hydrogen chloride at 100° for 16 hr. The resulting methyl glycosides were examined by gas-liquid partition chromatography on columns (a) and (b). The results are quoted in Table IX.

A further portion of the methylated polysaccharide
was methanolyzed with methanolic 4% hydrogen chloride. The solution was neutralized with silver carbonate, filtered and concentrated. The resulting methyl glycosides were dissolved in water, the pH of the solution was adjusted to 10 with sodium carbonate, and the solution was extracted overnight with chloroform in a liquid-liquid extractor. The aqueous solution was treated with Amberlite resin IR-120[H], concentrated and hydrolysis of the resulting syrup gave a single sugar which was chromatographically indistinguishable from 2,4-di-0-methyl-D-galactose in solvents H and I. The chloroform extract was concentrated, the methyl glycosides were hydrolysed and the sugars were oxidized in bromine (10-15 drops) and water (5 ml.) in the dark for 4 days. Excess bromine was removed by aeration, the solution was neutralized with silver carbonate and centrifuged, silver salts were precipitated from the supernatant with hydrogen sulphide and the solution was again centrifuged and concentrated. The resulting aldonolactones were examined by gas-liquid partition chromatography on column b (see Table IX).
Table IX. Gas Chromatography of Methylated Sugar Derivatives from Methylated Lucerne Leaf Arabinogalactan (Fraction 1a).

<table>
<thead>
<tr>
<th>Sugars</th>
<th>Column a</th>
<th>Column b</th>
<th>Aldonolactones</th>
<th>Rel. Propor. (approx.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Column a</td>
<td>Column b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Column a</td>
<td>Column b</td>
<td>Rel. Propor. (approx.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SUGARS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2,3,4-Me&lt;sub&gt;3&lt;/sub&gt; rhamnose</td>
<td>0.45</td>
<td>0.46</td>
<td>-</td>
<td>tr</td>
</tr>
<tr>
<td>2,3,4-Me&lt;sub&gt;3&lt;/sub&gt; arabinose</td>
<td>(0.89)</td>
<td>(1.04)</td>
<td>3.64</td>
<td>+++</td>
</tr>
<tr>
<td>2,3,5-Me&lt;sub&gt;3&lt;/sub&gt; arabinose</td>
<td>0.50</td>
<td>0.56</td>
<td>0.72</td>
<td>++</td>
</tr>
<tr>
<td>2,5-Me&lt;sub&gt;2&lt;/sub&gt; arabinose</td>
<td>1.48</td>
<td>1.44</td>
<td>7.98</td>
<td>++</td>
</tr>
<tr>
<td>3,5-Me&lt;sub&gt;2&lt;/sub&gt; arabinose</td>
<td>(0.89)</td>
<td>(1.04)</td>
<td>2.35</td>
<td>++</td>
</tr>
<tr>
<td>2,3,4,6-Me&lt;sub&gt;4&lt;/sub&gt; galactose</td>
<td>1.76</td>
<td>(1.77)</td>
<td>6.60</td>
<td>++</td>
</tr>
<tr>
<td>2,3,4-Me&lt;sub&gt;3&lt;/sub&gt; galactose</td>
<td>6.48</td>
<td>6.86</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>2,3,6-Me&lt;sub&gt;3&lt;/sub&gt; galactose</td>
<td>(2.69)(3.50)(4.20)</td>
<td>(2.96)(4.08)(4.47)</td>
<td>13.2</td>
<td>++</td>
</tr>
<tr>
<td>2,4,6-Me&lt;sub&gt;3&lt;/sub&gt; galactose</td>
<td>(3.50)(4.20)</td>
<td>(3.90)(4.47)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>2,4-Me&lt;sub&gt;2&lt;/sub&gt; galactose</td>
<td>13.3</td>
<td>15.2</td>
<td>15.2</td>
<td>17.6</td>
</tr>
</tbody>
</table>

Figures in parenthesis indicate T values for components which were incompletely resolved.
Lucerne Leaf Polysaccharide Fractions 2 and 3. Partial Acid Hydrolysis.

Leaf polysaccharide fraction 2 (250 mg.) and leaf fraction 3 (250 mg.) were separately hydrolysed with 0.5 N-sulphuric acid on boiling water baths for 2 hr. Aliquot portions were removed after 0.5, 1.0, 1.5 and 2.0 hr. periods from both hydrolysates, the portions were neutralized with barium carbonate, centrifuged, deionized with Amberlite resin IR-120[H] and examined chromatographically in solvents A and D. By comparison with authentic samples the oligosaccharides 3-0-1, 4-0 and 6-0-β-D-galactopyranosyl-β-D-galactose ((i) to (iii), Table VIII) were tentatively identified from both polysaccharide fractions 2 and 3.

A further portion of leaf polysaccharide fraction 2 (25 mg.) was hydrolysed with 0.1N-sulphuric acid on a boiling water bath for 1 hr. The solution was neutralized with barium carbonate, deionized as before and examined chromatographically in solvent A. A component was detected which was chromatographically indistinguishable from 3-0-β-L-arabinopyranosyl-L-arabinose ((iv), Table VIII).

Lucerne Leaf Polysaccharide Fractions 2 and 3. Methylations.

The polysaccharide fractions 2 (500 mg.) and 3 (500 mg.) were methylated with methyl iodide and sodium hydride in methyl sulphoxide and furnished methylated polysac-
charide fraction 2 (290 mg.), $[\alpha]_D^{24} -40^\circ$ (c, 0.30 in chloroform). [Found OMe, 41.9%]; and methylated polysaccharide fraction 3 (249 mg.), $[\alpha]_D^{24} +23^\circ$ (c, 0.36 in chloroform). [Found: OMe, 42.0%].

Samples of the methylated polysaccharide fractions 2 and 3 were methanolysed with methanolic 5% hydrogen chloride for 18 hr., and the resulting methyl glycosides were examined by gas-liquid partition chromatography on columns (a) and (b). The components identified are listed in Table X.

Samples of the methylated polysaccharides 2 (25 mg.) and 3 (26 mg.) were hydrolysed by refluxing with 2N-sulphuric acid for 18 hr. The solutions were neutralized with barium carbonate, deionized and concentrated to a small volume (5 ml.). Bromine (10-15 drops) was added to the solutions which were left in the dark for 3 days. The solutions were worked up as previously described and afforded aldonolactones which in both cases were examined by gas-liquid partition chromatography on column (b). The components identified from methylated polysaccharide fractions 2 and 3 are listed in Table X.
### Table X. Gas Chromatography of Methylated Sugar Derivatives from Methylated Lucerne Leaf Polysaccharide, Fractions 2 and 3.

<table>
<thead>
<tr>
<th>Derivatives</th>
<th>Methyl Glycosides</th>
<th>Aldonolactones</th>
<th>Relative Prop. (approx.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Column a</td>
<td>Column b</td>
<td>Column b</td>
</tr>
<tr>
<td>Sugars</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2,3,4-Me₃ rhamnose</td>
<td>T</td>
<td>T</td>
<td>T</td>
</tr>
<tr>
<td>2,3,4-Me₃ xylose</td>
<td>0.45</td>
<td>0.46</td>
<td>-</td>
</tr>
<tr>
<td>2,3,4-Me₃ 3-Me rhamnose</td>
<td>2.71</td>
<td>3.37</td>
<td>-</td>
</tr>
<tr>
<td>2,3,4-Me₃ arabinose</td>
<td>(0.89)</td>
<td>(1.03)</td>
<td>3.71</td>
</tr>
<tr>
<td>2,3,5-Me₃ arabinose</td>
<td>0.5</td>
<td>0.66</td>
<td>0.55</td>
</tr>
<tr>
<td>2,3,5-Me₃ arabinose</td>
<td>1.26</td>
<td>(1.48)</td>
<td>1.45</td>
</tr>
<tr>
<td>2,5-Me₂ arabinose</td>
<td>(1.50)</td>
<td>(2.69)</td>
<td>(1.79) 3.23</td>
</tr>
<tr>
<td>3,5-Me₂ arabinose</td>
<td>(0.89)</td>
<td>1.98</td>
<td>1.03</td>
</tr>
<tr>
<td>2,3,4,6-Me₄ galactose</td>
<td>1.74</td>
<td>(1.79)</td>
<td>6.77</td>
</tr>
<tr>
<td>2,3,4-Me₃ galactose</td>
<td>6.7</td>
<td>7.01</td>
<td>-</td>
</tr>
<tr>
<td>2,3,6-Me₃ galactose</td>
<td>(2.79)</td>
<td>(3.63)</td>
<td>(4.20)  (3.06)</td>
</tr>
<tr>
<td>2,4,6-Me₃ galactose</td>
<td>(3.67)(4.30)</td>
<td>3.94</td>
<td>(4.53)</td>
</tr>
<tr>
<td>2,4-Me₂ galactose</td>
<td>14.0</td>
<td>15.9</td>
<td>15.2</td>
</tr>
<tr>
<td>2,3,4-Me₃ glucuronic acid*</td>
<td>2.30 (3.19)</td>
<td>(2.40)</td>
<td>(3.06)</td>
</tr>
</tbody>
</table>

*Present as methyl ester. Figures in parenthesis indicate T values for components which were incompletely resolved.
Lucerne Leaf Polysaccharide Fraction 3. Examination of Acidic Glycoside Fractions.

Methylated polysaccharide fraction 3 (65 mg.) was methanolysed with methanolic 4% hydrogen chloride in a sealed tube at 100° overnight. The solution was neutralized with silver carbonate, centrifuged and concentrated. The resulting methyl glycosides were saponified by treatment with sodium hydroxide at pH 12 at 0° for 2 hr. Subsequently the solution was neutralized with Amberlite resin IR-120[H], the solution was adjusted to pH 10 and extracted with chloroform in a liquid-liquid extractor for 24 hr. The chloroform extract was concentrated. The aqueous extract, containing the acidic glycosides, was recovered, treated with Amberlite resin IR-120[H], concentrated and adsorbed on a small column of diethylaminoethyl-Sephadex A-25 (20 x 2 cm; 5 g.; formate form). Elution with water removed neutral oligosaccharides and elution with 0.1N- and 0.5N-formic acid gave acidic glycoside fractions (i) and (ii).

Acidic glycoside fraction (i) was concentrated to dryness, the syrup was re-methanolysed with methanolic hydrogen chloride, and a portion of the products were examined by gas-liquid partition chromatography on columns (a) and (b). The components identified are listed in Table XI.

The remaining portion of the products was reduced with lithium aluminium hydride in tetrahydrofuran and re-methanolysed with methanolic hydrogen chloride. A portion of
the reduced products was examined by gas-liquid partition chromatography on column a. The components identified are listed in Table XI.

Acidic glycoside fraction (ii) was treated with methanolic 4% hydrogen chloride and the resulting glycosides were examined by gas-liquid partition chromatography on columns a and b. The components identified are also listed in Table XI.
Table XI. Gas Chromatography of Methylated Sugar Derivatives from Methylated Lucerne Leaf Polysaccharide Fraction 3, Acidic Glycoside Sub-fractions (i) and (ii).

ACIDIC SUB-FRACTION (i)

<table>
<thead>
<tr>
<th>Derivatives</th>
<th>Methyl Glycosides</th>
<th>Relative Amounts</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Columns</strong></td>
<td><strong>Column a</strong></td>
<td><strong>Column b</strong></td>
</tr>
<tr>
<td>Sugars</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3,4-Me₂ rhamnose</td>
<td>0.86</td>
<td>0.96</td>
</tr>
<tr>
<td>3-Me rhamnose</td>
<td>2.71</td>
<td>3.34</td>
</tr>
<tr>
<td>2,3,4-Me₃ glucuronic acid*</td>
<td>2.30</td>
<td>3.10</td>
</tr>
<tr>
<td>2,3-Me₂ galacturonic acid*</td>
<td>4.29</td>
<td>4.88</td>
</tr>
</tbody>
</table>

ACIDIC SUB-FRACTION (ii)

<table>
<thead>
<tr>
<th>Derivatives</th>
<th>Methyl Glycosides</th>
<th>Relative Amounts</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Columns</strong></td>
<td><strong>Column a</strong></td>
<td><strong>Column b</strong></td>
</tr>
<tr>
<td>Sugars</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3,4-Me₂ rhamnose</td>
<td>0.86</td>
<td>0.96</td>
</tr>
<tr>
<td>3-Me rhamnose</td>
<td>2.71</td>
<td>3.34</td>
</tr>
<tr>
<td>2,3-Me₂ galacturonic acid*</td>
<td>4.29</td>
<td>4.88</td>
</tr>
</tbody>
</table>

ACIDIC SUB-FRACTION (i). DERIVATIVES AFTER REDUCTION

<table>
<thead>
<tr>
<th>Derivatives</th>
<th>Methyl Glycosides</th>
<th>Relative Amounts</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Columns</strong></td>
<td><strong>Column a</strong></td>
<td><strong>Column b</strong></td>
</tr>
<tr>
<td>Sugars</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3,4-Me₂ rhamnose</td>
<td>0.85</td>
<td>-</td>
</tr>
<tr>
<td>3-Me rhamnose</td>
<td>2.71</td>
<td>-</td>
</tr>
<tr>
<td>2,3,4-Me₃ glucose</td>
<td>2.28</td>
<td>3.22</td>
</tr>
<tr>
<td>2,3-Me₂ galactose</td>
<td>8.19</td>
<td>10.62</td>
</tr>
<tr>
<td></td>
<td>11.61</td>
<td>13.20</td>
</tr>
</tbody>
</table>

*Present as methyl ester.
Lucerne Leaf Polysaccharide Fraction 5. Partial Acid Hydrolysis.

Lucerne leaf polysaccharide fraction 5 (1.5 g.) was heated in $\text{H}_2\text{SO}_4$-sulphuric acid on a boiling water bath for 5 hr. The cooled solution was poured into acetone, degraded galacturonan precipitated and was centrifuged and dried by solvent exchange (0.47 g.). The supernatant solution was concentrated to remove acetone, neutralized with barium carbonate, centrifuged, deionised with Amberlite resin IR-120[H] and further concentrated to yield a syrupy mixture of sugars (0.55 g.). The sugar mixture was adsorbed on a column of diethylaminoethyl-Sephadex A-25 (25 x 2 cm.; 8 g.; formate form) and the column was eluted with water to remove neutral sugars. The column was then eluted successively with 0.05$\text{N}$-formic acid to give acidic fractions (i)-(iv), with 0.4$\text{N}$-formic acid to give acidic fractions (v) and (vi) and finally with 0.5$\text{N}$-formic acid to give acidic fraction (vii). The fractions were concentrated and examined chromatographically and by comparison with authentic samples oligosaccharides I-VI (Table XII) were tentatively identified. The detailed results were tabulated in Table XII.

Fraction (i). This fraction contained a single component which was methylated by the Kuhn procedure, the methylated product was methanolysed and the resulting glycosides were examined by gas-liquid partition chromatography and
gave peaks with the retention times of the methyl glycosides of following sugars.

<table>
<thead>
<tr>
<th>Sugars</th>
<th>T (Column a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3,4-di-O-methyl rhamnose</td>
<td>0.87</td>
</tr>
<tr>
<td>2,3,4-tri-O-methyl galacturonic acid*</td>
<td>6.30 6.60</td>
</tr>
</tbody>
</table>

*Present as methyl ester.

Fraction (iii) was further hydrolysed and examined chromatographically in solvents B and D. By comparison with authentic samples, glucuronic acid, glucurone, fucose and galactose were identified in addition to the galacturonic acid already present.
<table>
<thead>
<tr>
<th>Eluant</th>
<th>Fraction No.</th>
<th>( R_{Gala} )</th>
<th>Stain (Aniline Oxalate)</th>
<th>Components identified by comparison with authentic samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05N-Formic Acid</td>
<td>(i)</td>
<td>0.8 (Solvent D)</td>
<td>Orange</td>
<td>I 2-O-(( \alpha )-D-galactopyranosyluronic acid)-L-rhamnose</td>
</tr>
<tr>
<td></td>
<td>(ii)</td>
<td>0.19</td>
<td>Yellow</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.50</td>
<td>Brown</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.80 (tr)</td>
<td>Orange</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(iii)</td>
<td>0.19</td>
<td>Yellow</td>
<td>II 6-O-(( \beta )-D-glucopyranosyluronic acid)-D-galactose</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.50</td>
<td>Yellow</td>
<td>III 4-O-(( \beta )-D-glucopyranosyluronic acid)-L-fucose</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.00</td>
<td>Brown</td>
<td>D-galacturonic acid</td>
</tr>
<tr>
<td></td>
<td>(iv)</td>
<td>1.00</td>
<td>Brown</td>
<td>D-galacturonic acid</td>
</tr>
<tr>
<td>0.4N-Formic Acid</td>
<td>(v)</td>
<td>0.56 (Solvent E)</td>
<td>Orange</td>
<td>IV Q-( \Omega )-L-rhamnoppyranosyl-(1 ( \rightarrow ) 4)-O-( \Omega )-galactopyranosyluronic acid -(1 ( \rightarrow ) 2)-L-rhamnose</td>
</tr>
<tr>
<td></td>
<td>(vi)</td>
<td>0.5</td>
<td>Brown</td>
<td>V 4-O-(( \alpha )-D-galactopyranosyluronic acid)-D-galacturonic acid</td>
</tr>
<tr>
<td>0.5N-Formic Acid</td>
<td>(vii)</td>
<td>0.23 (tr)</td>
<td>Brown</td>
<td>VI Q-( \alpha )-D-galactopyranosyluronic acid-(1 ( \rightarrow ) 2)-O-( \alpha )-D-galactopyranosyluronic acid-(1 ( \rightarrow ) 4)-D-galacturonic acid</td>
</tr>
</tbody>
</table>
Lucerne Stem Polysaccharide Fraction 5. Partial Acid Hydrolysis.

Lucerne stem polysaccharide fraction 5 (0.8 g.) was partially hydrolysed with N-sulphuric acid using the exact procedure as described for leaf fraction 5, and afforded degraded galacturonan (0.2 g.) and a syrupy mixture of sugars (0.31 g.) which was fractionated on a column of diethylaminoethyl-Sephadex A-25 (2 x 20 cm.; 5 g.; formate form). Neutral sugars were eluted with water and the column was then eluted successively with 0.05N-formic acid to give acidic fractions (i) and (ii), with 0.4N-formic acid to give acidic fraction (iii) and with 0.5N-formic acid to give acidic fraction (iv). The fractions were concentrated and examined chromatographically.

Acidic fraction (i) contained one component (R_{Ga1A} = 0.8, solvent D), which gave galacturonic acid and rhamnose only on hydrolysis and which was chromatographically indistinguishable from 2-0-(α-D-galactopyranosyluroniec acid)-L-rhamnose.

Acidic fraction (ii), in addition to galacturonic acid, contained two components (R_{Ga1A} = 0.19 and 0.50, solvent D), which were chromatographically indistinguishable from 6-0-(β-D-glucopyranosyluroniec acid)-D-galactose and 4-0-(β-D-glucopyranosyluroniec acid)-L-fucose respectively.

Acidic fraction (iii) contained one component (R_{Ga1A} = 0.2, solvent D, R_{Ga1A} = 0.5, solvent E), which gave galacturonic acid only on hydrolysis and which was chromatographically
indistinguishable from \(4-O-(\alpha-D\)-galactopyranosyluronic acid\)-\(D\)-galacturonic acid.

Acidic fraction (iv) contained one component \(R_{GalA} = 0.23\), solvent E) which was chromatographically indistinguishable from the trisaccharide, \(O-\alpha-D\)-galactopyranosyluronic acid-(1 \(\rightarrow \) 4)-\(O-\alpha-D\)-galactopyranosyluronic acid-(1 \(\rightarrow \) 4)-\(D\)-galacturonic acid.

Sodium Acetate Fractionation of the Pectic Acid.

Lucerne leaf polysaccharide fractions (5-9) and stem polysaccharide fractions (5-8) were combined (3.25 g.) and dissolved in water (400 ml.). Insoluble material was removed by centrifugation, dried by solvent exchange (0.23 g.) and shown to be predominately protein. This portion was rejected.

De-esterification of the Pectic Acid.\(^5\)

The polysaccharide solution was adjusted to pH 12 with dilute sodium hydroxide and kept at 0°C for 2 hr. Sodium hydroxide was added where necessary throughout this period to maintain the pH at 12.

Afterwards the solution was acidified (0.5\(M\)-hydrochloric acid) and an acid insoluble component precipitated. The precipitate was washed with ethanol-water (60 : 40, containing 5\% hydrochloric acid), with ethanol-water (60 : 40), with ethanol and it was finally redissolved in water and freeze-dried (2.4 g.). \(\alpha\) \(D\) \(+227^\circ\) (c, 0.423 as sodium salt). \(\alpha\) \(D\) \(+227^\circ\) (c, 0.423 as sodium salt).

[Found: uronic acid content (potentiometric titration), 81\%].\(^1\)
Acid soluble polysaccharide was precipitated by ethanol (4 vol.), washed with ethanol-water (80:20 containing 5% hydrochloric acid), with ethanol-water (80:20, 9 vol. containing 5% hydrochloric acid), dissolved in water and was freeze-dried (0.14 g.). \([\alpha]_D^\text{+} +86^\circ (E' 0.4).\) (Found: uronic acid content (carbazole procedure 143), 38%).

Fractionation of the Pectic Acid 14

The acid insoluble pectic acid (2 g.) was dissolved in water (225 ml.) containing 5% sodium hydroxide (5 ml.) to give a solution having pH 6.5-6.7. 2M-NaOH solution (14 ml.) was added with vigorous stirring to give a molarity of 0.12M sodium acetate. The mixture was left at 0° for 18 hr. The precipitate was then centrifuged, washed with 0.12M sodium acetate, with ethanol-water (60:40) and finally redissolved in water and freeze-dried. Further quantities of 2M sodium acetate were added to the supernatant solution to give net molarities of 0.14M, 0.16M, 0.18M, and 0.21M-sodium acetate. In each case the precipitate (if any) was centrifuged, purified and washed as described above and finally freeze-dried.

In all, five fractions were obtained, all of which contained polysaccharides. A further polysaccharide fraction was isolated by precipitation with ethanol, washed, dissolved in water and freeze-dried.
were examined by moving boundary electrophoresis in
0.05M-sodium tetraborate buffer (pH 9.25), specific
rotations of their sodium salts were measured and
uronic acid contents were determined. The following
results were obtained:

I  Pectic acid fraction I (0.45 g.), precipitated
at 0.12M-sodium acetate; \([\alpha]_D +267^\circ (c, 0.35)\);
uronic acid, 94% by potentiometric titration.\(^{14}\)

II Pectic acid fraction II (0.05 g.), precipitated
at 0.14M-sodium acetate; \([\alpha]_D +254^\circ (c, 0.29)\);
uronic acid, 90% by the carbazole procedure.\(^{143}\)

III Pectic acid fraction III (0.25 g.), precipitated
at 0.16M-sodium acetate; \([\alpha]_D +237^\circ (c, 0.5)\);
uronic acid, 87% by potentiometric titration.

IV Pectic acid fraction IV (0.016 g.), precipitated
at 0.18M-sodium acetate; \([\alpha]_D +230^\circ (c, 0.2)\);
uronic acid, 83% by the carbazole method.
No precipitate was obtained at 0.21M-sodium acetate.

V Pectic acid fraction V (0.55 g.), precipitated
with ethanol; \([\alpha]_D +170^\circ (c, 0.4)\); uronic acid,
61% by potentiometric titration.

Hydrolysis of fractions I to V gave galacturonic
acid as the chief component, but all contained neutral
sugars, including galactose, arabinose, rhamnose to-
gether with traces of xylose and fucose.
SECTION II

THE EXTRACELLULAR POLYSACCHARIDES FROM Sycamore CAMBIAL CELLS

DISCUSSION
SECTION II

THE EXTRACELLULAR POLYSACCHARIDES FROM SYCAMORE CAMBIAL
CELLS. FRACTIONATION OF THE POLYSACCHARIDE MIXTURE.

The extracellular polysaccharide mixture, cultured
in media lacking yeast extract, was supplied as a
pale white powder by Dr. P. Albersheim, University of
Colorado, Boulder. The insolubility in water of one-
third of the polysaccharide mixture suggests that some
of the mixture had undergone a change in physical state
during the drying process. Both soluble and insoluble
fractions were shown on hydrolysis to contain similar
mixtures of galacturonic acid, galactose, glucose, arabi-
nose, xylose, fucose, rhamnose together with a trace of
mannose.

The fractionation scheme carried out on the poly-
saccharide mixture is summarised in Diagram 2. The in-
soluble fraction on extraction with 2% ethylenediamine-
tetra-acetic acid (disodium salt) yielded a small quan-
tity of pectic acid. The water-soluble polysaccharide
fraction contained both acidic and neutral polysaccharides
and the acidic polysaccharides were precipitated as their
copper salts to give a quantity of pectinic acid.
Fractionation of the neutral polysaccharide mixture was
achieved by chromatography on a cellulose column.
Elution of the column with water afforded a pure arabino-
galactan, $[\alpha]_D -57^\circ$ while elution with 7M-urea and 0.5M-sodium hydroxide afforded two xyloglucan fractions which were judged to be similar. Both fractions contained glucose, xylose and fucose and had, $[\alpha]_D +27^\circ$. 
External Polysaccharide Mixture, (I)

Cold Water

Soluble material (II)

7% copper acetate

Copper ppt. Acidic material
(Pectinic Acid) (V)

(III) Insoluble material

2% E.D.T.A.

E.D.T.A. Extract
(Pectic Acid) (IV)

Crude

Neutral (VI)

Polysaccharides

Cellulose
Chromatography

Elution with water
Arabinogalactan (VII)

Elution with 7M-urea
Xyloglucan (VIIIa)

Elution with 0.5N-NaOH
Xyloglucan (VIIIb)
In the previous investigation of the extracellular polysaccharides from sycamore cambial cells by Aspinall and Craig, the polysaccharide mixture was fractionated by a procedure very similar to that used in the present study. These authors isolated a pure pectinic acid and a pure arabinogalactan together with a xyloglucan or xylan and glucan mixture. However, the extracellular polysaccharide mixture was also shown to contain a mannan, which was not a product of the sycamore cambial cells but which was a constituent of the yeast which was at that time used as a nutrient for the sycamore cells. Nevertheless, the authors concluded that the pectinic acid and arabinogalactan were genuine products of the sycamore cambial cells, and this conclusion has been confirmed by the present investigation of the external polysaccharides from cells grown in a medium devoid of yeast extract.

In the investigation of the arabinogalactan and xyloglucan described below, polysaccharide samples from the extracellular mixture supplied by Dr. Albersheim were used for the greater part, but extracellular polysaccharide mixtures from sycamore cells supplied by Dr. Loewus, State University of New York, Buffalo, were examined and fractionated in accordance with the procedure outlined in Diagram 2. Two distinct batches of extracellular polysaccharide mixtures were supplied by
Dr. Loewus, both grown in liquid Murashige and Schoog medium, containing myo-inositol and deficient in myo-inositol respectively. From both of the samples, an arabinogalactan and xyloglucan were isolated pure as described above (Diagram 2), but the samples contained only minute quantities of pectinic acid. It is to be noted, as mentioned earlier, that during their investigation of the extracellular polysaccharides in the media of sycamore suspension-callus tissue, Northcote and co-workers noted an absence of strongly acidic components and reported that pectic polysaccharides in the medium are associated with old cultures, where cell necrosis is likely.

**The Arabinogalactan.**

The arabinogalactan contained galactose : arabinose in the ratio 2.2 : 1. Partial hydrolysis of the arabinogalactan with 0.5N-sulphuric acid gave two oligosaccharide components which were chromatographically indistinguishable from 3-O-β-D-galactopyranosyl-D-galactose and 6-O-β-D-galactopyranosyl-D-galactose.

The highly branched nature of the polysaccharide was established by methylation and by the detection by gas chromatography of (a) the methyl glycosides formed on methanolysis and of (b) the aldonolactones formed by hydrolysis of the methylated polysaccharide followed by oxidation with aqueous bromine. The following methylated
sugars as structural units in the methylated polysaccharide were identified:

<table>
<thead>
<tr>
<th>Sugars</th>
<th>Relative Amounts</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3,4-tri-O-methylrhamnose or</td>
<td>tr</td>
</tr>
<tr>
<td>2,3,4-tri-O-methylxylose</td>
<td>++</td>
</tr>
<tr>
<td>2,3,5-tri-O-methylarabinose</td>
<td>+++</td>
</tr>
<tr>
<td>2,3-di-O-methylarabinose</td>
<td>++</td>
</tr>
<tr>
<td>2,3,4,6-tetra-O-methylgalactose</td>
<td>+</td>
</tr>
<tr>
<td>2,4,6-tri-O-methylgalactose</td>
<td>++</td>
</tr>
<tr>
<td>2,3,4-tri-O-methylgalactose</td>
<td>++</td>
</tr>
<tr>
<td>2,4-di-O-methylgalactose</td>
<td>+++</td>
</tr>
</tbody>
</table>

The arabinogalactan has a highly branched structure. In view of the high proportion of 2,3,5-tri-O-methylarabinose obtained from the methylated polysaccharide, most of the arabinose residues must be present as end groups, which together with the absence of branching through arabinose residues, confirms that the arabinose must be a constituent of a heteropolysaccharide and not of a discrete arabinan. On the basis of the above evidence the galactose is present as structural units XXIV, XXIX, XXXI and XXXII.

\[
\text{Galp} 1 \rightarrow 3 \text{Galp} 1 \rightarrow \text{Galp} 1 \rightarrow
\]

\[
\text{XXIV} \quad \text{XXIX} \quad \text{XXXI}
\]

\[
\uparrow 3 \text{Galp} 1 \rightarrow 6 \text{Galp} 1
\]

\[
\text{XXXII}
\]
Thus it is apparent that there is present in the extracellular polysaccharide mixture from sycamore cambial cells an arabinogalactan in which the galactose residues are linked by $1,3$ and $1,6$ linkages. This polysaccharide is similar to the water-soluble, highly branched arabinogalactans from coniferous woods and is of the same structural type as the arabinogalactans from Western Larch $^{70-73}$ or European Larch $^{79-80}$, which have been discussed earlier, and differs from the pectic type of arabinogalactan (i.e. the arabinogalactan from soybeans $^{24,25}$) which contain galactose residues linked through the $1$ and $4$ positions only. The results here coincide with and confirm those of Aspinall and Craig $^{27}$ in respect of sycamore extracellular arabinogalactan, which is the first arabinogalactan of this type to have been isolated from a deciduous wood.

The Xyloglucan. The polysaccharide was shown to contain glucose : xylose : fucose in the ratio of $4 : 4 : 1$. Information on the structure of the polysaccharide was provided by methylation and by the detection by gas chromatography of (a) the methyl glycosides formed on methanolysis and of (b) the aldonolactones formed by hydrolysis of the methylated polysaccharide followed by oxidation with aqueous bromine. The following methylated sugars as structural units in the methylated polysaccharide were identified:
<table>
<thead>
<tr>
<th>Sugars</th>
<th>Relative Amounts</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3,4-tri-0-methylxylose</td>
<td>++++</td>
</tr>
<tr>
<td>3,4-di-0-methylxylose</td>
<td>+</td>
</tr>
<tr>
<td>2,3-di-0-methylxylose</td>
<td>++</td>
</tr>
<tr>
<td>2,3,4-tri-0-methylfucose</td>
<td>+</td>
</tr>
<tr>
<td>2,3,4,6-tetra-0-methylglucose</td>
<td>tr</td>
</tr>
<tr>
<td>2,3,6-tri-0-methylglucose</td>
<td>++</td>
</tr>
<tr>
<td>2,3-di-0-methylglucose</td>
<td>+++</td>
</tr>
</tbody>
</table>

For comparative purposes a list of the methylated sugars identified as structural units in mustard seed amyloid is here included:

<table>
<thead>
<tr>
<th>Sugars</th>
<th>Relative Amounts</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3,4-tri-0-methylxylose</td>
<td>++++</td>
</tr>
<tr>
<td>2,3-di-0-methylxylose</td>
<td>++</td>
</tr>
<tr>
<td>2,4-di-0-methylxylose</td>
<td>++</td>
</tr>
<tr>
<td>2-0-methylxylose</td>
<td>+</td>
</tr>
<tr>
<td>2,3,4-tri-0-methylfucose</td>
<td>+</td>
</tr>
<tr>
<td>2,3,4,6-tetra-0-methylglucose</td>
<td>++</td>
</tr>
<tr>
<td>2,3,6-tri-0-methylglucose</td>
<td>+++</td>
</tr>
<tr>
<td>2,3-di-0-methylglucose</td>
<td>++</td>
</tr>
<tr>
<td>2,3,4,6-tetra-0-methylgalactose</td>
<td>tr</td>
</tr>
</tbody>
</table>

**Enzymic Degradation.** The xyloglucan was subjected to enzymic degradation using a purified *Streptomyces* preparation devoid of β-glucosidase, to give a degraded polysaccharide and a mixture of oligosaccharides which were further fractionated by paper partition chromato-
Graphy. Three mobile components were isolated together with an appreciable quantity of immobile oligosaccharides. The first component was the chromatographically pure oligosaccharide O₁, which was obtained crystalline and was identical to 4-O-β-D-xylopyranosyl-β-D-xylose in its m.p. and mixed m.p., in its specific rotation, in its chromatographic mobility and in its x-ray powder photograph.

The second component was the chromatographically pure oligosaccharide O₂, which was indistinguishable from cellobiose, 4-O-β-D-glucopyranosyl-β-D-glucopyranose, in its specific rotation and its chromatographic mobility. Hydrolysis and methylation studies confirmed this view. A portion of the oligosaccharide was characterised as cellobiose β-octa-acetate which was identical to an authentic sample in its m.p., mixed m.p. and specific rotation.

The third component was not chromatographically homogeneous and insufficient material was available for proper characterisation. Hydrolysis indicated the presence of fucose, xylose and glucose while hydrolysis of the derived glycitols gave glucitol (but not xylitol) in addition to the three sugars already present, indicating that glucose was the sole reducing sugar present.

Characterisation of the Constituent Sugars. The degraded polysaccharide from the enzymic degradation was
shown to contain glucose : xylose : fucose in the proportions 3 : 3.5 : 1. The degraded polysaccharide was hydrolysed and the sugars were purified by paper partition chromatography and were identified as follows:

- **D-glucose** was characterised as D-glucose p-nitrophenyl-hydrazone;
- **D-xylose** was characterised as di-O-benzylidene-D-xylose dimethyl acetal;
- **L-fucose** was characterised as L-fucose toluene-p-sulphonyl hydrazone.

Sycamore extracellular xyloglucan represents another example of a hemicellulose preparation containing both xylose and glucose residues. The enzyme used for the degradation of the xyloglucan was a cellulase from Streptomyces sp. QM B814, the specificity of which has been studied by Perlin and co-workers,¹⁵² (the enzyme hydrolysese glycosyl linkages in a "(1 → 4)-\(\beta-(1 → 4)\) arrangement), and the isolation from the enzymic degradation of cellobiose, 4-\(\alpha-\beta\)-D-glucopyranosyl-D-glucopyranose, indicates that areas occur in the molecule composed of a sequence of at least four 1,4 linked D-glucopyranose residues (XXXVIIa).

XXXVIIa → \(4 \text{Gp}_1 \rightarrow 4 \text{Gp}_1 \rightarrow 4 \text{Gp}_1 \rightarrow 4 \text{Gp}_1 \rightarrow \)

XXXVIIb → \(4 \text{Gp}_1 \rightarrow 4 \text{Gp}_1 \rightarrow 4 \text{Gp}_1 \rightarrow 4 \text{Gp}_1 \rightarrow \)

\[ \begin{array}{c}
\text{Xyl}_2 \downarrow \\
6 \\
\text{Xyl}_2 \downarrow \\
6 \\
\text{Xyl}_2 \downarrow \\
6 \\
\text{R} \\
\end{array} \]
From the methylation study (apart from the presence of a trace of glucose present as non-reducing end groups), the results indicate the presence of glucose as residues linked through positions 1 and 4 and as residues linked through positions 1, 4 and 6 and thus there is possibly present in the polysaccharide a core of 1,4 linked glucose residues, many of which residues are substituted at C₆ (XXXVIIb).

Because of the high proportion of 2,3,4-tri-β-methyl-xylose detected in the methylation study, much of the xylose must be present as non-reducing end groups, which together with the absence of branching through xylose residues, confirms that many of the xylose residues must be constituents of a heteropolysaccharide. Although definite evidence is lacking, it is suggested that the end-group xylose residues are attached at C₆ of the tri-substituted glucose residues in the glucan chain as indicated in partial structure XXXVIIb. These are the only available points of attachment for accommodating the end-group xylose residues and this type of substitution would correspond to the arrangement in Tamarindus-amyloid 100 where xylose residues are attached by 1,6 linkages to glucose residues in the main chain of the polysaccharide.
Also from the enzymolysis study of sycamore xyloglucan $4\text{-}O\text{-}\beta\text{-D-}$xylopyranosyl-$\beta\text{-D}$-xylose was isolated. It was demonstrated that the enzyme preparation used hydrolyses $1,4$ linked linear xylans, and assuming the same specificity as in the case of $\beta$-glucans, the isolation of xylobiose indicates the presence in the polysaccharide of areas composed of a sequence of $1,4$ linked $\beta$-xylose residues (XXXVIIIa). This structural feature (XXXVIIIa) could arise from a separate $1,4$ linked xylan admixed with a xyloglucan, but this is considered unlikely from the mode of preparation (linear xylans are eluted from cellulose with extreme difficulty) and such xylans are very insoluble whereas the polysaccharide under investigation was water soluble. Thus it seems more likely that the xylan portion (XXXVIIIa) is linked to the glucan portion (XXXVIIb) in sycamore xyloglucan but evidence for the mode of linkage is lacking and the structural problem remains unsolved on the basis of the present evidence.

A partial structure for sycamore xyloglucan must also accommodate a proportion of $1,2$ linked xylose residues and also a proportion of fucose present as non-reducing end-groups and from the methylation study the proportions of these two sugars present were approximately the same. In the absence of evidence for the mode of attachment of these two structural units, it is suggested with reservation by analogy with other polysaccharides
(i.e. tragacanthic acid\textsuperscript{128}) that a fucosyl (1 → 2) xylose arrangement (XXXVIIIb) may occur in the polysaccharide, which structural unit is possibly attached by a 1,6 linkage to glucose residues in the main glucan chain. Some evidence for the attachment of xylose and fucose to glucose residues was provided by the isolation of an oligosaccharide component containing all three sugars. The component was not homogeneous and proper characterisation was not possible. However, it was established that glucose was the sole reducing sugar present and, therefore, the xylose and fucose were attached (directly or indirectly) to glucose residues. Thus it is concluded that the existing evidence can best be interpreted in terms of partial structures XXXVII and XXXVIII (which are not unique) but further structural investigation of sycamore xyloglucan is obviously required before a more complete structure for the polysaccharide can be defined.

The xyloglucan from mustard seed is the only other fucose containing polysaccharide of this type which has been reported\textsuperscript{102}. The mustard seed xyloglucan was examined by a methylation study and the methylated components identified have been listed above. There are obvious structural differences between sycamore xyloglucan and mustard seed xyloglucan, which latter polysaccharide contains 1,3 linked xylose residues, certain of the xylose residues are branch points and the poly-
saccharide also contains traces of galactose. Moreover, mustard seed xyloglucan is classed as an amyloid since it gives the colour reaction characteristic of amyloids, whereas sycamore xyloglucan did not give the distinctive amyloid reaction and it is, therefore, in this respect a type of xyloglucan distinct from the amyloid class.

Sycamore extracellular xyloglucan is similar in some respects to the type of xyloglucan reported recently by Hay and co-workers from the roots of sugar maple or to the xyloglucan isolated by Timell from the bark of Engelmann Spruce. This latter polysaccharide contained sequences of 1,4 linked β-D-glucopyranose residues most of which units carried branches through C\textsubscript{6}. Xylose occurred as a sequence of units linked through positions 1 and 4, but here also it was not established whether the xylan was linked to the glucan. Engelmann spruce xyloglucan contained a proportion of galactose. Sycamore xyloglucan represents a new addition to the class of cell-wall xyloglucans and quite some variation within this class of polysaccharide is already apparent.
EXPERIMENTAL
Fractionation of the Extracellular Polysaccharide Mixture from Sycamore Cambial Cells.

The details of the fractionation of the extracellular polysaccharides from sycamore cambial cells supplied by Dr. Albersheim are as follows. This was the typical fractionation procedure used throughout the investigation. The polysaccharide mixture (I) (4.8 g.) (for details of hydrolysis pattern see Table XIII below) was stirred in cold water (2,000 ml.) for 24 hr. The insoluble polysaccharides were removed at the centrifuge and dried by solvent exchange to yield insoluble polysaccharide mixture (III) (1.6 g.). A small portion of sample III (200 mg.) was extracted with 2% ethylenediamine-tetra-acetic acid disodium salt (pH 4.5) at 90°C for 4 hr. The insoluble residue was again removed by centrifugation and acidic polysaccharide was precipitated from the supernatant solution with acetone (1 vol.). The precipitate was removed by centrifugation, redissolved in water, neutralized with ammonia solution, reprecipitated with acetone and finally dissolved in a little water and freeze-dried. A small quantity (50 mg.) of acidic polysaccharide was obtained (IV), (for details of hydrolysis patterns of samples (III) and (IV), Table XIII).

The supernatant solution from the original aqueous extraction was concentrated, poured into acetone (4 vols.), the polysaccharide was removed by centrifugation, re-
dissolved in water and freeze-dried to give polysaccharide mixture (II) (2.8 g.), a portion of which was hydrolysed and examined chromatographically (Table XIII).

The mixture of soluble polysaccharides (2.7 g.) was redissolved in water (1.5 l.) and 7% copper acetate solution (250 ml.) was added. An acidic polysaccharide was precipitated as its copper salt, the precipitate was decomposed by dissolution in a minimum of dilute hydrochloric acid, and the polysaccharide was precipitated with ethanol (4 vol.) washed free of hydrogen chloride with ethanol-water (4:1), redissolved in water and freeze-dried to give pectinic acid fraction (V) (0.8 g.), a portion of which was hydrolysed and examined chromatographically (Table XIII).

The supernatant solution from the copper acetate precipitation, was poured into ethanol (4 vol.) and the neutral polysaccharide mixture was centrifuged, washed with ethanol containing 1% hydrogen chloride and with ethanol, and was finally redissolved in water and freeze-dried to give neutral polysaccharide mixture (VI) (1.4 g.), the hydrolysis pattern of which is given in Table XIII.

Finally the neutral polysaccharide mixture (1.4 g.) was dissolved in the minimum amount of water (50 ml.) and applied to a cellulose column (45 g.). Elution of the column with water afforded polysaccharide fraction VII, which was precipitated with ethanol, redissolved in water and freeze-dried (0.5 g.), and elution of the column with
7M-urea and 0.5M-sodium hydroxide afforded fractions VIIIa and VIIIb respectively. The sodium hydroxide eluate was neutralized with 5M-hydrochloric acid and was then dialysed against running water for two days, as was the urea eluate. The two fractions were precipitated with ethanol (4 vol.), redissolved in water and freeze-dried to yield fractions VIIIa (0.3 g.) and VIIIb (0.35 g.). All three fractions were hydrolysed (Table XIII), fraction VII is seen to constitute an arabinogalactan and fractions VIIIa and VIIIb to constitute a xyloglucan or xylan and glucan mixture.

The samples of sycamore extracellular polysaccharides supplied by Dr. Loewus were also fractionated by the above procedure with similar results, except that in these cases very minute quantities of soluble pectinic acid were obtained.
Table XIII. Constituent Sugars of Fraction I-VIII obtained from Sycamore Extracellular Polysaccharide Mixture.

<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>I 25.5</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>tr</td>
</tr>
<tr>
<td>II n.d.</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>III n.d.</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>tr</td>
<td>tr</td>
</tr>
<tr>
<td>IV n.d.</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>tr</td>
<td>-</td>
</tr>
<tr>
<td>V  n.d.</td>
<td>+++</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>tr</td>
<td>-</td>
</tr>
<tr>
<td>VI -</td>
<td>tr</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>VII -</td>
<td>-</td>
<td>+++</td>
<td>tr</td>
<td>+++</td>
<td>tr</td>
<td>tr</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>VIII -</td>
<td>-</td>
<td>tr</td>
<td>+++</td>
<td>tr</td>
<td>+++</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

The Arabinogalactan. $\left[\alpha\right]_D^{57^\circ} -57^\circ (c, 0.4)$.

The sugar proportions were determined using Wilson's procedure and the ratio of galactose to arabinose was estimated as 2.2 : 1.

Partial Hydrolysis. A sample of the polysaccharide (25 mg.) was heated on a boiling water bath with 0.5N-sulphuric acid (5 ml.) for 1 hr. The solution was neutralized with barium carbonate, centrifuged, deionised with Amberlite resin IR-120[H] and examined chromatographically in solvents A and D. By comparison with authentic samples the oligosaccharides $3-O-\beta-D$-galactopyranosyl-$D$-galactose and $6-O-\beta-D$-galactopyranosyl-$D$-galactose were identified.
Methylation. The arabinogalactan (400 mg.) was methylated with methyl iodide and sodium hydride in methyl sulfoxide and furnished methylated polysaccharide (249 mg.) \( [\alpha]_D -75^\circ \) (c, 1.5 in chloroform). [Found: OMe 42%].

A portion of the methylated polysaccharide (25 mg.) was methanolysed in a sealed tube with methanolic 5% hydrogen chloride for 16 hr. The resulting methyl glycosides were examined by gas-liquid partition chromatography on columns (a), (b) and (d). The components recognized are listed in Table XIV.

A further sample of the methylated polysaccharide (26 mg.) was hydrolysed by refluxing with 2N-sulphuric acid for 18 hr. The solution was neutralized with barium carbonate, centrifuged, deionised and concentrated to a small volume (5 ml.). Bromine (10-15 drops) was added to the solution which was left in the dark for 3 days. The solution was worked up as previously described and afforded aldonolactones which were examined by gas-liquid partition chromatography on column b. The components recognized are listed in Table XIV.
Table XIV. Gas Chromatography of Methylated Sugar Derivatives from Methylated Sycamore Extracellular Arabinogalactan.

<table>
<thead>
<tr>
<th>Derivatives</th>
<th>Methyl Glycosides</th>
<th>Aldonolactones</th>
<th>Relative Amounts Approx.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Column a</td>
<td>Column b</td>
<td>Column c</td>
</tr>
<tr>
<td>2,3,4-Me&lt;sub&gt;3&lt;/sub&gt; Rhamnose or 2,3,4-Me&lt;sub&gt;3&lt;/sub&gt; Xylose</td>
<td>-</td>
<td>0.46 (0.58)</td>
<td>0.49 (0.54)</td>
</tr>
<tr>
<td>2,3,5-Me&lt;sub&gt;3&lt;/sub&gt; Arabinose</td>
<td>0.51 0.63</td>
<td>0.44 0.71</td>
<td>0.54 0.68</td>
</tr>
<tr>
<td>2,3-Me&lt;sub&gt;2&lt;/sub&gt; Arabinose</td>
<td>1.21 1.49</td>
<td>1.52 1.88</td>
<td>1.59 1.78</td>
</tr>
<tr>
<td>2,3,4,6-Me&lt;sub&gt;4&lt;/sub&gt; Galactose</td>
<td>1.70</td>
<td>1.79</td>
<td>(1.58)</td>
</tr>
<tr>
<td>2,4,6-Me&lt;sub&gt;3&lt;/sub&gt; Galactose</td>
<td>3.42 3.98</td>
<td>3.88 4.52</td>
<td>3.57 3.92</td>
</tr>
<tr>
<td>2,3,4-Me&lt;sub&gt;3&lt;/sub&gt; Galactose</td>
<td>6.19</td>
<td>7.11</td>
<td>6.50</td>
</tr>
<tr>
<td>2,4-Me&lt;sub&gt;2&lt;/sub&gt; Galactose</td>
<td>12.84 15.09</td>
<td>16.50 18.73</td>
<td>-</td>
</tr>
</tbody>
</table>

Figures in parenthesis represent T values for components which were incompletely resolved.
The Xyloglucan. \([\alpha]_D +27^\circ (c, 0.6)\).

The polysaccharide was shown on hydrolysis to contain glucose, xylose and fucose (Table XIII). The sugar proportions were determined using Wilson's procedure and the ratio of glucose to xylose to fucose was estimated as 4:4:1.

Methylation. The polysaccharide fraction (VIIIb) (220 mg.) was methylated with methyl iodide and sodium hydride in methyl sulfoxide and furnished methylated polysaccharide (140 mg.). \([\alpha]_D +20^\circ (c, 1.1 \text{ in chloroform})\). [Found: OMe 40.9%].

A portion of the methylated polysaccharide was methanolysed in a sealed tube with methanolic 5% hydrogen chloride for 16 hr. The resulting methyl glycosides were examined by gas-liquid partition chromatography on columns (a), (b) and (d). The results are given in Table XV. Another sample of the methylated polysaccharide (30 mg.) was hydrolysed by refluxing with 2N-sulphuric acid for 18 hr. The solution was neutralized with barium carbonate, deionized and concentrated to a small volume (5 ml.). Bromine (10-15 drops) was added to the solution, which was left in the dark for 3 days. The solution was worked up as previously described and afforded aldonolactones which were examined by gas-liquid partition chromatography on column (d). The components recognized are also listed in Table XV.
Table XV. Gas Chromatography of Methylated Sugar Derivatives from Methylated Sycamore Xyloglucan.

<table>
<thead>
<tr>
<th>Derivatives</th>
<th>Methyl Glycosides</th>
<th>Aldonolactones</th>
<th>Relative Amounts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Column a</td>
<td>Column b</td>
<td>Column d</td>
</tr>
<tr>
<td>2,3,4-Me$_3$ Xylose</td>
<td>0.45 0.54</td>
<td>0.46 0.58</td>
<td>0.56 0.63</td>
</tr>
<tr>
<td>3,4-Me$_2$ Xylose</td>
<td>1.0 1.17</td>
<td>1.29 1.53</td>
<td>1.22 (1.49)</td>
</tr>
<tr>
<td>2,3-Me$_2$ Xylose</td>
<td>1.13 1.30</td>
<td>(1.41) 1.70</td>
<td>(1.45) 1.63</td>
</tr>
<tr>
<td>2,3,4-Me$_3$ Fucose</td>
<td>0.65 0.74</td>
<td>0.73 1.49</td>
<td></td>
</tr>
<tr>
<td>2,3,4,6-Me$_4$ Glucose</td>
<td>(1.0) (1.42)</td>
<td>1.0 (1.42)</td>
<td>1.00 (1.37)</td>
</tr>
<tr>
<td>2,3,6-Me$_3$ Glucose</td>
<td>2.70 3.60</td>
<td>3.2 4.42</td>
<td>2.90 3.63</td>
</tr>
<tr>
<td>2,3-Me$_2$ Glucose</td>
<td>7.00 9.51</td>
<td>- 11.72</td>
<td>14.00 -</td>
</tr>
</tbody>
</table>

Figures in parenthesis represent T values for components which were incompletely resolved.
The Amyloid Reaction.

A portion of the xyloglucan (Fraction VIIIa) (10 mg.) was dissolved in water and tested with the potassium tri-iodide and sodium sulphate reagent in accordance with the reaction procedure of Kooiman. The reaction was, however, negative and the characteristic extinction of amyloids with a maximum at 650 mμ was not observed.

Enzymic Degradation of the Xyloglucan (Fraction VIIIa).

Enzymic degradation was carried out using a purified Streptomyces preparation, devoid of β-glucosidase, which had been kindly supplied by Dr. E. T. Reese. In a trial experiment the xyloglucan (10 mg.) in water (5 ml.) was incubated with the Streptomyces preparation (5 mg.) at 50°C. Aliquot portions (0.25 ml.) were removed occasionally and the reducing power estimated by the Nelson reagent by comparison with a standard curve for D-glucose. The reaction appeared to have reached completion after 48 hr. by which time, as estimated by the increase in reducing power, approximately 1 unit in 12 of the polysaccharide had been hydrolysed. Moreover, aliquot portions (0.2 ml.) of the hydrolysate were removed at intervals and examined chromatographically in solvent C, and by visual estimation it was determined that the best yield of oligosaccharides could be obtained by incubating the polysaccharide with the Streptomyces preparation at 50°C for 48 hr.
Enzymic Degradation - Large Scale Experiment.

The xyloglucan (500 mg.) was incubated with the Streptomyces preparation (50 mg.) in aqueous solution (50 ml.) at 50° for 48 hr., at which time the reaction was judged to have reached completion. The resulting solution was heated on a boiling water bath for 15 min., the small quantity of insoluble material was centrifuged and discarded, the aqueous solution was shaken with chloroform-pentan-l-ol (35 : 10, 40 ml.) in accordance with the Sevag procedure and the aqueous layer was separated. To the solution ethanol (4 vol.) was added and polysaccharide precipitated. The degraded polysaccharide was removed by centrifugation, redissolved in water and freeze-dried (180 mg.).

The supernatant solution was evaporated to dryness and examined chromatographically in solvents A, B and C. The presence of two distinct oligosaccharides was apparent together with a possible third and an appreciable quantity of immobile material. The oligosaccharides were separated on Whatman 3MM filter sheets in solvent C to yield the following, oligosaccharide $O_1$ (20 mg.), oligosaccharide $O_2$ (25 mg.), oligosaccharide $O_3$ (5 mg.) and immobile oligosaccharides (148 mg.).

Oligosaccharide $O_1$ was chromatographically indistinguishable from xylotriose, $4-O-\beta-\text{D}-\text{xylopyranosyl-}\text{D}-\text{xylose}$ and yielded xylose only on hydrolysis. The oligosaccharide
was crystallised from methanol, m.p. and mixed m.p. 181-182°, \([\alpha]_D -24^\circ\) (equil.)(\(c\), 0.4). The x-ray powder photograph of the crystalline oligosaccharide was identical with that of 4-O-\(\beta\)-D-xylopyranosyl-D-xylose.

**Oligosaccharide** \(O_2\) was chromatographically indistinguishable from cellobiose, 4-O-\(\beta\)-D-glucopyranosyl-D-glucopyranose and gave glucose only on hydrolysis. [Found: \([\alpha]_D +33.5^\circ\), (equil.)(\(c\), 0.5)]. A small quantity of the oligosaccharide (2-3 mg.) was methylated by the Kuhn procedure, the methylated product was methanolysed and the methanolysis products were examined by gas-liquid partition chromatography using column a. Peaks were detected with the retention times of methyl glycosides of 2,3,4,6-tetra-O-methyl glucose \((T = 1.0, 1.42)\) and 2,3,6-tri-O-methyl-D-glucose \((T = 2.70, 3.60)\). A portion of the oligosaccharide was converted into cellobiose \(\beta\)-octa-acetate,\(^{155}\) m.p. and mixed m.p. 200-201°, \([\alpha]_D -13^\circ\) (\(c\), 0.5 in chloroform).

**Oligosaccharide** \(O_3\) appeared homogeneous in solvent C \((R_G = 0.21)\), and on hydrolysis gave fucose, xylose and glucose with xylose and glucose predominating.

Hydrolysis of the derived glycitol gave glucitol in addition to fucose, xylose and a reduced proportion of glucose and no xylitol was detectable in the hydrolysate. Re-examination in solvent B (3°) indicated that \(O_3\) was not in fact homogeneous but contained at least two components. Material was not available for further characterisation.
Characterisation of the Constituent Sugars.

The degraded polysaccharide (175 mg.) from the enzymic degradation of the xyloglucan was hydrolysed with N-sulphuric acid for 6 hr. The solution was neutralized with barium carbonate and centrifuged, the supernatant liquid was deionized with Amberlite resin IR-120[H] and concentrated. A portion of the hydrolysate was examined using Wilson's procedure and the proportion of glucose : xylose : fucose was estimated 3 : 3.5 : 1.

The sugars were purified on Whatman 3MM filter sheets in solvent C and the following components were obtained:

\[ \text{D-glucose (35 mg.), } [\alpha]_D^{20} +52^\circ \text{ (equil., } c, 1.0), \text{ was characterised as } \text{D-glucose p-nitrophenylhydrazone, m.p. and mixed m.p. 186-191^\circ.} \]

\[ \text{D-xylose (42 mg.), was characterised as di-O-benzylidene-} \text{D-xylose dimethyl acetal, m.p. and mixed m.p. 207-209, } [\alpha]_D^{20} -7.5 \text{ (c, 1.5 in chloroform).} \]

\[ \text{L-fucose (18 mg.), } [\alpha]_D^{20} -75^\circ \text{ (c, 0.25) was characterised as } \text{L-fucose toluene-p-sulphonyl hydrazone, m.p. and mixed m.p. 169-170^\circ.} \]

Activity of the Streptomyces Preparation.

A sample of soybean hull xylan A (10 mg.) was incubated with the Streptomyces enzyme (5 mg.) in aqueous medium (5 ml.) for 24 hr. at 50^\circ. The hydrolysate was examined chromatographically in solvents A and C, and the presence of various oligosaccharides including an appreciable quantity of xylobiose was detected.
SECTION III

ARaucaria Bidwillii Gum

Discussion
PARTIAL HYDROLYSIS OF ARAUCARIA BIDWILLII GUM AND
CHARACTERISATION OF OLIGOSACCHARIDES.

Araucaria bidwillii gum had previously been extracted as
follows. The crude gum nodules were crushed and dis-
persed in water and the resulting suspension was extracted
with light petroleum to remove terpenoid resins. The
aqueous solution was filtered and freeze-dried to give
the carbohydrate gum, which was purified by precipitation
from aqueous solution with ethanol and again freeze-dried.

A. bidwillii gum was subjected to partial acid hydrolysis
with a view to isolating oligosaccharides containing 1,6
linked galactose residues, i.e. the disaccharide 6-O-\(\alpha\)-
galactopyranosyl-\(\alpha\)-galactose and the polymer homologous
trisaccharide, for the purpose of establishing the nature
of the configuration of the glycosidic bonds.

A. bidwillii gum was partially hydrolysed with 0.1N-
sulphuric acid to yield a mixture of monosaccharides and
neutral and acidic oligosaccharides. The acidic sugars
were removed by passing the solution through a column of
diethylaminoethyl-Sephadex A-25 (formate form) and the
neutral sugars were fractionated by chromatography on a
charcoal-celite column. Elution of the column with water
afforded monosaccharides and elution with water containing
increasing proportions of alcohol afforded the oligosac-
charides which were subfractionated where necessary by
preparative paper chromatography. Oligosaccharides 1 and 2
were obtained pure and were characterised as described below.
Oligosaccharide 1 was chromatographically indistinguishable from 6-\(\alpha\)-\(\beta\)-\(D\)-galactopyranosyl-\(D\)-galactose. Hydrolysis of the oligosaccharide gave galactose only whereas hydrolysis of the derived glycitol gave galactose and galactitol. Gas-liquid chromatography of the methanolysis products from the methylated oligosaccharide gave peaks with the retention times of the methyl glycosides of 2,3,4,6-tetra-, 2,3,4- and 2,3,5-tri-\(\alpha\)-methyl galactose. The oligosaccharide was characterised by conversion into its phenyl o-sazone.

The nature of the configuration of the glycosidic bond was determined by nuclear magnetic resonance spectroscopy of the glycitol when the signal for the anomeric proton had a \(\tau\) value (5.39) and coupling constant (7.3 c.p.s.) which indicated the presence of a \(\beta\) linkage.

Periodate oxidation of the derived glycitols of oligosaccharide 1 and of gentiobiose resulted in changes of specific rotation to the same identical final value in each case which was indicative of a \(\beta\) linkage. On the basis of this evidence oligosaccharide 1 was assigned the following structure:

6-\(\alpha\)-\(\beta\)-\(D\)-galactopyranosyl-\(D\)-galactose
Oligosaccharide 2 was obtained crystalline and appeared identical to \(\beta-D\)-galactopyranosyl-(1 \(\to\) 6)-\(\beta-D\)-galactopyranosyl-(1 \(\to\) 6)-\(D\)-galactose in its m.p. and mixed m.p., in its specific rotation, in its chromatographic mobility and in its x-ray powder photograph. Hydrolysis, partial hydrolysis and methylation studies confirmed this view and oligosaccharide 2 was assigned the following structure:

\[
\beta-D\text{-galactopyranosyl-(1 \(\to\) 6)-}\beta-D\text{-galactopyranosyl-(1 \(\to\) 6)-D-galactose}
\]

The results obtained confirm that in \(A.\) bidwillii gum the intergalactose 1,6 linkages are in the \(\beta\) configuration. No evidence was obtained to suggest the presence in the oligosaccharides isolated, or therefore in the original polysaccharide, of \(\alpha\)-1,6 intergalactose linkages.

From the acetolysis study carried out on carboxyl-reduced \(A.\) bidwillii gum in the previous investigation a series of oligosaccharides were formed, among which all those containing 1,6 linkages had high positive rotations indicating the presence of \(\alpha-D\)-galacto- and \(\alpha-D\)-gluco-pyranosyl linkages.
The 6-\(\beta\)-galactopyranosylgalactose isolated was assigned the structure 6-\(\beta\)-\(\alpha\)-\(D\)-galactopyranosyl-\(D\)-galactose on the basis of its high specific rotation \([\alpha]_D^0 +126^\circ\).

In the case of the oligosaccharide 6-\(\beta\)-\(\alpha\)-\(D\)-glucopyranosyl-\(D\)-galactose, the presence of the \(\alpha\) linkage was confirmed by n.m.r. spectroscopy and periodate oxidation studies.

The results obtained in the present study together with those of the previous investigation confirm that during the acetolysis of the carboxyl-reduced gum, acid-catalysed anomerisation at the glycosidic bond occurred affecting a high proportion of the 1,6 linkages.

The procedure of acetolysis is complementary to partial acid hydrolysis and is extremely useful for isolating oligosaccharides containing certain residues which are very labile towards partial acid hydrolysis. Acetolysis of reduced gum arabic\(^{89}\) and of carboxyl-reduced A. bidwillii gum\(^{98}\) gave oligosaccharides in which the rhamnopyranosyl bond was retained and thus allowed the site of attachment of the rhamnopyranose residues in these polysaccharides to be identified.

However, in the light of the present evidence, it is apparent that the results of acetolysis studies can be complicated by the occurrence of anomerisation at the glycosidic bond and this must be taken into account in the interpretation of the results of such studies.
EXPERIMENTAL
Araucaria bidwillii Gum: Partial Hydrolysis and Characterisation of Oligosaccharides.

Preliminary experiments. Araucaria bidwillii gum (100 mg.) was heated under reflux with 0.1N-sulphuric acid (20 ml.) on a hot plate for 6 hr. Aliquot portions (3 ml.) were removed from the hydrolysate after 1, 2, 3, 4, 5 and 6 hr. and poured into acetone. In each case the precipitated polysaccharide was removed by centrifugation, the supernatant liquids were evaporated to remove acetone, the solutions were neutralized with barium carbonate, the precipitates were centrifuged and the solutions were de-ionised with Amberlite resin IR-120[H]. The aliquot portions were passed through small columns of diethylaminoethyl-Sephadex A-25 (formate form) to remove acid components and were finally concentrated and examined chromatographically in solvents A and D. Visual examination suggested that good yields of neutral oligosaccharides could be obtained by refluxing the A. bidwillii gum with 0.1N-sulphuric acid for 4 hr.
Large Scale Partial Hydrolysis:

*Arucaria bidwillii* gum (17.5 g.) was heated under reflux with 0.1N-sulphuric acid (650 ml.) on a hot plate for 4 hr. Degraded polysaccharide was precipitated by acetone, the precipitate was centrifuged, the supernatant liquid was evaporated to remove acetone, the solution was neutralized with barium carbonate, centrifuged and de-ionised with Amberlite resin IR-120[H]. The deionised solution was concentrated and placed on a column of diethylaminoethyl-Sephadex A-25 (formate form; 3 x 40; 20 g.). Neutral mono- and oligosaccharides were eluted with water, the eluate was concentrated and placed on a column of charcoal-celite (1:1; 5 x 37 cm.). The column was eluted with water (4 l.), with water containing 0-5% ethanol (4 l.), with 5% ethanol (2 l.) and finally with water containing 5-10% ethanol (4 l.).

Elution with water gave monosaccharide fractions. These fractions were examined chromatographically in solvents A and D, and by comparison with authentic samples the presence of the following sugars was indicated, D-galactose, L-arabinose, D-xylose and L-rhamnose.

Elution with water containing 0-5% ethanol gave a major component (oligosaccharide 1) which was chromatographically indistinguishable from 6-O-D-galactopyranosyl-D-galactose and a second component which was chromatographically indistinguishable from 3-O-D-galactopyranosyl-
D-galactose. Elution with 5% ethanol gave a further quantity of the latter component while elution with water containing 5-10% ethanol gave a component (oligosaccharide 2) which was chromatographically indistinguishable from O-D-galactopyranosyl-(1 → 6)-O-D-galactopyranosyl-(1 → 6)-D-galactose, together with a component with the chromatographic mobility of O-D-galactopyranosyl-(1 → 6)-O-D-galactopyranosyl-(1 → 3)-D-galactose.

Oligosaccharides 1 and 2 were purified (where necessary) on Whatmann 3MM filter sheets.

Oligosaccharide 1 (102 mg.), $R_{Gal} = 0.36$ and 0.22 in solvents A and D, $[\alpha]_D^0 +30.2^\circ$ (c, 1.1). Hydrolysis of a sample gave galactose only, whereas hydrolysis of the derived glycitol gave galactose and galactitol on paper chromatography in solvent F. A sample of the oligosaccharide (9 mg.) was methylated by the Kuhn procedure, the methylated sugar was methanolysed and the products were examined by gas-liquid partition chromatography on column b. The presence of the methyl glycosides of the following sugars was indicated: 2,3,4,6-tetra-O-methyl galactose ($T = 1.77$), 2,3,4-tri-O-methyl galactose ($T = 7.15$) and 2,3,5-tri-O-methyl galactose ($T = 4.33$).

Samples of the glycitol from oligosaccharide 1 (10 mg.) and of gentiobiose (16 mg.) were each dissolved in water (2 ml.) containing sodium periodate (75 mg.). Measurements of the rotation were made at intervals and the observed
rotations were constant after 4 hr. 160

![Diagram of chemical structure](image)

**Gentiobiose**

Assuming conversion of gentiobiose into (i) the specific rotation of the oxidized product was \([\alpha]_D +101^\circ\). The specific rotation of oxidized disaccharide 1, assuming the same conversion was \([\alpha]_D +103^\circ\).

A sample of the glycitol from oligosaccharide 1 (55 mg.) in deuterium oxide was examined by nuclear magnetic resonance spectroscopy. The signal for the anomeric proton showed as a doublet \((\tau = 5.39; J = 7.3\) c.p.s.). 159

Oligosaccharide 1 (17 mg.) was dissolved in water (0.5 ml.) and treated with phenylhydrazine and acetic acid and heated on a steam bath for 2 hr. The osazone separated, it was washed with water, with ethanol and ether and recrystallised from ethanol. [Found: m.p. and mixed m.p. 180-182\(^\circ\). 161].

Oligosaccharide 2 (19 mg.); \(R_{Gal} = 0.24\) and 0.10 in solvents C and A, \([\alpha]_D +22^\circ\) (equil. \(C, 0.5\)). The oligosaccharide was crystallised from ethanol-water, [Found: m.p. and mixed m.p. 137-142\(^\circ\)] and gave an x-ray powder photograph identical to that of an authentic sample of \(\alpha-\beta-D-\)
1.57.

Hydrolysis of the oligosaccharide gave galactose only, while partial hydrolysis gave galactose and a component indistinguishable from 6-β-D-galactopyranosyl-D-galactose. Comparison of the colorimetric reactions of the sugar and the derived glycitol with the phenol-sulphuric acid reagent indicated that it was a trisaccharide. 162

A sample (4 mg.) of the oligosaccharide was methylated by the Kuhn procedure and the methylated sugar was methanolyzed and the methanolysis products were examined by gas-liquid partition chromatography on column b. The presence of the methyl glycosides of the following sugars was indicated: 2,3,4,6-tetra-β-methyl galactose (T = 1.77), 2,3,4-tri-β-methyl galactose (T = 7.10) and 2,3,5-tri-β-methyl galactose (T = 4.35).
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BIBLIOGRAPHY


