ABSTRACT OF THESIS

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Degree Doctor of Philosophy Date April 1958

Title of Thesis "THE POLYSACCHARIDE CONSTITUENTS OF AGAVE SISALANA"

SUMMARY

1. Extraction of sisal flesh with hot water afforded a polysaccharide which was referred to as water-soluble polysaccharide.

2. Extraction of the solid residue with ammonium oxalate aqueous solution yielded, after preliminary purification, crude ammonium pectate.

3. Attempts to separate the neutral sugar components from the ammonium polygalacturonate were unsuccessful.

4. Periodate oxidative studies on the purified ammonium pectate showed the uptake of 1 mole of oxidant per ammonium anhydro-galacturonate unit. The periodate oxidized ammonium pectate was isolated ($[\alpha]_D = - 33.8^{\circ}$, in water). Reducation of this oxypolysaccharide with potassium borohydride afforded a polysaccharide ($[\alpha]_D = + 40 \pm 2^{\circ}$) which upon hydrolysis showed chromatographically the presence of galacturonic and threonic acids as main components.

5. Purified ammonium pectate was methylated. Partial methanolysis of the highly purified and methylated methyl pectate ($[\alpha]_D = + 212.5^{\circ}$ in chloroform), reduction of the small molecular size fragments thus originated with lithium aluminium hydride, and hydrolysis, yielded a mixture of methylated neutral sugars, the fractionation of which afforded

   2:3:4:6 tetra-O-methyl-D-galactose
   2:3:4 tri-O-methyl-D-galactose
   2:3:6 tri-O-methyl-D-galactose
   2:3 di-O-methyl-D-galactose
   2:4 di-O-methyl-D-galactose
   2-O-methyl-D-galactose
   2:3:4 tri-O-methyl-L-rhamnose
   3:4 di-O-methyl-L-rhamnose

   These were characterized by the preparation of crystalline derivatives; in addition there was evidence of the presence of 2:6 di-O-methyl-O-galactose, 3-O-methyl-D-galactose, and 2:3:5 tri-O-methyl-L-arabinose.

6. The chain length of the back-bone of methylated methyl pectate was calculated from the amount of 2:3:4 tri-O-methyl-D-galactose and 2:3 di-O-methyl-D-galactose, plus mono-methyl-galactoses recovered from the fractionation. It was shown to lie between 94 and 97 units.

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7. Pectic acid was converted into the ethyleneglycol and propyleneglycol esters. The latter had a molecular weight of 37.200, corresponding to a D.P. of 168-170 units.

8. Repeated reductions with potassium borohydride, alternated with esterifications, afforded a galactan ($[\alpha]_D = + 246.7^{\circ}$) showing a molecular weight of 22.000 - 22.300, corresponding to a D.P. of 138 - 140 units. The approximate composition of this galactan was as follows:

D-galactose:	82.0 %
D-galacturonic acid:	6.0 %
L-arabinose:	6.5 %
L-rhamnose:	3.6 %
D-glucose:	2.0 %

9. Periodate oxidative studies on the galactan showed the rapid consumption of one mole of periodate per unit of anhydrohexose. Isolation of the oxypolysaccharide ($[\alpha]_D = + 45.5^{\circ}$) and hydrolysis gave threose and galactose as main components.

10. Methylation of the galactan gave a product ($[\alpha]_D = + 177^{\circ}$, water) which was hydrolyzed. The resulting mixture of methylated sugars was fractionated on a cellulose column. This showed the presence of

2:3:5 tri-O-methyl-L-arabinose
2:3:4:6 tetra-O-methyl-D-galactose
2:3:6 tri-O-methyl-D-galactose
2:4 di-O-methyl-D-galactose
2:3 di-O-methyl-D-galactose

which were characterized by the preparation of crystalline derivatives. Evidence of the presence of 2:6 di-O-methyl-D-galactose and 2-O-methyl-D-galactose was established by means of chromatographic techniques.

11. Several attempts to fractionate the water soluble polysaccharide failed to give a homogeneous material.

12. The isolation and identification of 2-O-methyl-D-xylose and 2-O-methyl-L-fucose from the water soluble polysaccharide is reported.

THE POLYSACCHARIDE CONSTITUENTS

OF

AGAVE SISALANA

by

A. CANAS - RODRIGUEZ

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INTRODUCTION

AGAVE SISALANA, a plant native to Central America, was first put into cultivation in Yucatan for the production of fibre. In 1893 it was introduced into East Africa by Germans established in Kenya, Mozambique and Angola, and it also flourishes on a large scale in Brazil, Haiti, Java and other countries.

It is a plant belonging to the Amaryllidaceae family with a large rosette of thick fleshy leaves and a stout central stem,or bole, which is hard, and usually short. The leaves, which apparently spring from the root, have a spiny margin, and they usually terminate in a sharp point.

The growth of the plant is slow and it culminates in the single blossoming after a period of from five to ten years depending on the vigour of the individual, the richness of the soil and the climate.

When the plant is about to flower a tall, pole-like stem develops in the centre of the leaf-rosette, bearing multiferous short, tubular blooms. During the growth period the plant stores the nourishment it needs to bloom, and at the time of the very rapid development of the flowering stem there is a rush of sap to the base of the spike. Finally as the fruits, which will generate new individuals, ripen, the parent plant dies.

The amount of fibre and tow extractable from the leaves is approximately only three per cent of the weight of the material as it enters the extracting machinery. Roughly ninety per cent of the weight of the fresh plant is water, and there is a proportion of from two to three per cent of sugars and acids in solution. The remaining three to four per cent consists of fleshy waste - a percentage approximately equal to the yield of marketable fibre. A variety of natural products are obtained from this waste material through appropriate manipulation consisting for the most part in extractions with the aid of different solvents and solutions.

An initial extraction made with organic solvents leads to the isolation of an important group of pigments such as chlorophyll, xanthophyll and carotenes as well as of a wax with a high melting point. This extract represents between five and six per cent of the sisal waste.

A further extraction with either hot or cold water - preferably the former - produces a solution containing organic acids (malic, citric, succinic ...), glucosides and saponines, useful for their application in the partial synthesis of cortisone, and finally water soluble polysaccharaides containing galacturonic acid, galactose, glucose, arabinose, xylose, rhamnose, 2-methyl-xylose together with other methylated sugars in small amounts. The percentage of the components of this extract constitutes 20 % of the dry waste.

After the extraction with water which brings some pectic materials also into solution, the insoluble calcium, iron and magnesium pectates can easily be removed by means of hot extractions from the fleshy residue with solutions of inorganic salts thus eliminating the metallic ions through the formation of further insoluble salts and freeing the soluble pectates. The weight of these extractable pectates represents approximately 15 % of the dried flesh.

The residue after these extractions consists of cellulose, hemicelluloses and lignin, representing about 50 % of the original quantity of sisal waste. It is a suitable raw material for the manufacture of vanillin, catechol and other important chemicals such as furfuraldehyde and cellulose esters.

NOMENCLATURE AND DEFINITIONS OF THE TERMS USED IN PECTIN CHEMISTRY

PECTIC SUBSTANCES	A group designation for those complex colloidal carbohydrates which exist in plants and are composed of a large number of anhydrogalacturonic acid units, the carboxyl group of which may be completely or partially esterified with methanol or forming salts.
PROTOPECTIN	The water-insoluble parent pectic substance which occurs in plants and which, upon restricted hydrolysis, yields pectinic acids.
PECTINIC ACIDS	The colloidal polygalacturonic acids containing a by no means negligible proportion of methyl ester groups, capable, under suitable conditions, of forming gels with sugar and acid.
PECTIN	A water-soluble pectinic acid of varying methyl ester content, with different degrees of neutralization, capable of forming gels with sugar and acid.
PECTIC ACID	The pectic substance composed of colloidal polygalacturonic acid and essentially free from methyl ester groups.

OCCURRENCE AND HISTOLOGY OF PECTIC SUBSTANCES

Pectic substances occur in all plant material, and especially in the primary cell walls and intercellular layer, being also found in small quantities in the secondary walls, as well as in the sap of fruit cells either in solution or colloidal suspension.

Pectic materials are abundant particularly in soft tissues composed mainly of primary walls, with a high water content at a stage of rapid growth (fruits and roots).(1), (2)

During the process of lignification it is usual for pectic substances contained in wood to decrease, and they are almost negligible in the mature wood.(3) In the inner bark of trees such as spruce, however, 10 % of pectic compound is to be found.(4) When fruits become over-ripe, the quantity of pectic substances tends to diminish to the point of disappearance owing to enzymic hydrolysis.

The investigation of the distribution and chemical characteristics of tissue components by separation of the various structural elements is the most suitable method leading to information on the relationship between morphology of the cell and its chemical formation. The study, involving staining techniques, though still in use, is not very reliable since the reagent most used for this purpose - Ruthenium red - is not specific. Pectic acid and Pectates are stained by the reagent but pectin is not stained appreciably.(5), (6). The formation of red colour seems to be dependent upon carboxyl groups in a free state, so that other uronic acid-containing polysaccharides, such as hemicelluloses, gums, etc., are stained by this reagent. On the other hand the reagent, even in dilute solutions, causes the coagulation of the protoplasm, cell nuclei and other cell components, which, once coagulated, are stained red by the reagent. This interferes with the detection of pectic bodies, though the colour produced by the non-pectic substances fades on heating.(7), (8). Other basic dyes can be used to detect the pectic substances (methylene blue, safranin ...).

In mature cells, the greater part of the interior, a single, large cavity, the vacuole, is filled with water containing a great variety of dissolved or dispersed substances. There is doubt as to whether the pectic substances dissolved in the cell sap are formed in this sap, being the primary source of those substances, or whether on the contrary they are originated in the cell walls and in the middle lamella and become dissolved during the ripening process. At all events, there is evidence that such a dissolution does occur when the tissue matures and disintegrates as can be observed from the pectic substances in a state of transition to be found in the tissues.

This solubility in the cell sap would appear to be governed in part by the degree of methylation, and one may support this assumption by referring to the presence of pectinic acid in a high degree of methylation in the cell sap of strawberries and raspberries.(10)

According to histological work it would seem that the primary cell walls laid down in mithosis, and the youngest and thinest cambium walls, are, in part at least, pectic in nature, while the middle lamella of later tissue has been regarded as being composed of a pectic substance. Kerr(11) and Bailey claim that the wall of a well developed cell consists of three principal layers:

1. The middle lamella or intercellular substance which is formed from the cell plate during mithosis and is shared with adjacent

cells. The main component of this layer is a pectic substance which appears to be a mixture of calcium and magnesium pectates. Molish (12) pointed out the existence of abundant calcium and magnesium in the middle lamella.

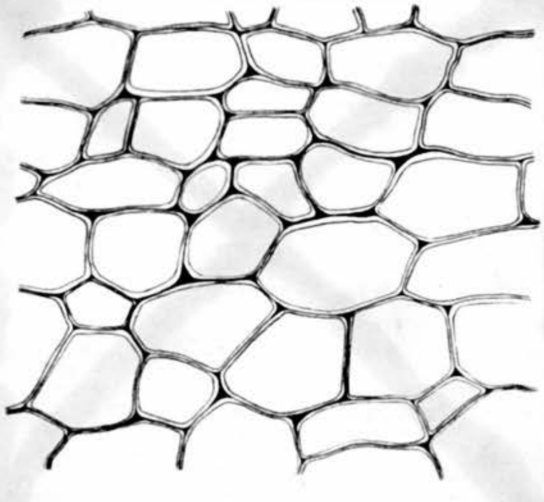
2. The primary wall, which is formed from the cambial wall, comprises cellulose, hemicelluloses, lignin and pectic materials.
3. The secondary wall, which may consist of several zones, is capable of growth by apposition and not by extension. Its components are cellulose, hemicelluloses and lignin.

The pectic substances of the middle lamella are deposited in a single or a double layer by plasma membranes, and according to Allen (13) they undergo changes in form, quantity and physical characteristics during the development of the plant. The intercellular spaces are filled by the secretion of pectic substances from the adjoining cells when they are in way of their completion, thus increasing the mass of the middle lamella.

The middle lamella may be absorbed, at least partially, by the rest of the wall. Treub (14) has suggested that during the division of the cells the protoplasmatic cell plate splits to form the plasma membrane of the two daughter cells, and the substance is secreted between these two membranes to become the middle lamella. Tupper-Carey and Priestley (15) were of the opinion that the middle lamella of a meristomatic tissue, thought primarily pectic, was not exclusively so, since considerable variation was observed both in staining reactions and in the case of extraction.

These variations in the appearance of the middle lamella are explained by Bonner (7) as being governed by corresponding variations in the calcium content. The loosening of cells and softening of the tissues of the plant during the overripening and senescence of the fruits may be retarded by an excess of calcium ions which maintain the pectic compounds in a solid and insoluble form. This fact has application in improving the texture of stored and canned fruits and vegetables which are hardened by the addition of a solution of calcium chloride. The presence of calcium ions in hard waters may be the cause of the hardening experienced by vegetables during boiling with this sort of water.

The function of the pectates in the intercellular layer is not very well known, but many workers such as Payen(16) concluded that they were a type of cement binding the cells together, and giving firmness to the tissues while the cells are in need of it, but subjected to further changes during which the rigidity was greatly reduced. Reed(17) suggested that the hydrophilic colloids of the middle lamella and the cell wall serve as a means of water translocation, this movement of water being governed by differences in water-absorbing capacity. There is little doubt that



The middle lamella in an unripe apple

pectic substances do play some role in water relations.

The suggestion that the middle lamella is composed entirely of protopectin(15) cannot be maintained as the evidence indicates that the constituent insoluble pectic material is very similar in properties and nature to calcium pectate.

There is some evidence that pectic substances are associated with protein and cellulose. Whether the protein is only an incrustation in the pectic bodies or whether there is a chemical linkage between them is not known. In some cases it is necessary to remove the protein with hypochlorite or enzymic procedures before the isolation of the pectic substances.

The use of solubilities as the only means to the characterisation of pectic materials in the middle lamella is hardly sufficient to establish their nature.

The primary cell wall, on the contrary, is richer in typical protopectin, and nearly all the available pectin from apples and citrus fruits is contained in the primary walls of parenchymous cells.

The pectic substances in the primary walls undergo changes similar to those of the middle lamella. The staining reaction with ruthenium red shows, within the cell wall of growing tissues (apple), a uniform distribution of pectic substances, while when an advanced stage of development has been reached, the pectic substances appear irregularly. During the senescence of the tissues, the cells become loose, the tissues turn soft following the separation of cells, and the cell walls become thinner and thinner. During the course of these changes the pectic substances in the cell membrane show characteristic structures under the staining processes with ruthenium red. These structures decrease however both in size and number as the senescence of the tissue goes on.

As protopectin is always found in the closest of association with cell wall constituents, and especially with cellulose, it has been suggested that it was combined with this glucan. Cross and Bevan supported this theory.(17a) Some confusing conclusions have been drawn from the treatment with Schweitzer solution of non-lignified tissues containing protopectin, whereby the cellulose is dissolved. The fate of the protopectin after such treatment is a matter for some discussion, for some workers maintained that it remained as a colourless membrane while others stated that it was dissolved simultaneously.

When one considers the very high alkalinity of this reagent and the known susceptibility of the pectin molecule to alkali, it is hardly to be expected that any very helpful information could be obtained through its use.

Protopectin is also, in von Fellenberg's opinion, a pectin-cellulose compound formed by linkages between a carboxyl group and a cellulose with the elimination of water.(18) Carre made a similar statement in observing that protopectin from different plant tissues showed considerable variations in behaviour towards hydrolysing agents.

It is possible that the retention of pectin in an insoluble form is due to mechanical difficulties of penetration, but Carre, on the contrary, has demonstrated the solubility of this type of pectin in hot diluted acids such as HCl N/100, oxalic acid 0.5 %, ammonium oxalate, and also through prolonged treatment with hot water as Ehrlich predicted.

Ripa(19) gave some experimental evidence in support of the concept of protopectin as a pectin-cellulose complex. There is no direct evidence to maintain the idea of protopectin's being built with several molecules of pectic acid linked with metallic iron through the carboxyl groups.(20)(21) The observation that pectin gives, together with small amounts of iron, a thick gelatinous precipitate, insoluble in water, is the only basis for this assumption. Henglein developed this idea, postulating that protopectin is formed by the association of polygalacturonic acid chains among themselves, and perhaps even with cellulose, exclusively through calcium linkages.(22) The transformation of protopectin into soluble pectin is due to a naturally occurring enzyme (protopectinase).(23)

EXTRACTION AND PURIFICATION OF THE PECTIC COMPLEX

It has been known for a long time that this complex consists of polygalacturonic acids, galactan and araban, and that the relationship between these three polysaccharides is very close, the methods involved in their separation being tedious and not always easily achieved.

As pectic substances occur in different forms, the methods for their extraction are varied according to the part of the plant to be extracted and the proportion of admixed materials present.

The first step to take is that of the inactivation of the enzymes which accompany pectic substances and act upon them. This may be achieved by rapid heating or by slicing the plant tissue into a large volume of boiling alcohol, the suspension being boiled again and allowed to stand for a period of twenty four hours. This treatment removes free sugars, saponins, waxes and pigments.

The material is then disintegrated into particles of small size which facilitates the further extraction of pectic compounds.(19) The soluble pectic materials contained in the cell sap of fruit can be extracted by pressing the plant tissues and collecting the liquid which contains nearly all the soluble pectinic acid. Another very efficient way of extracting the soluble pectic compounds is an extraction with cold water from the disintegrated plant. The insoluble pectates and protopectin are partially extracted by hot water, but the long periods of heating required cause degradation and loss of the jellying properties.

Dilute acids have been used for the extraction bringing about some degradation as well as de-esterification. The acids used for this purpose are hydrochloric (0.05 N at 90° for four hours), oxalic, 0.5 %,(25) which is highly effective in removing the metallic ions causing the insolubility of pectic compounds, phosphoric,(26) and sulphurous acid.

Alkalies as well as their carbonates are used for extraction(27) but they produce degradation and de-esterification as Volmert,(28) among other workers, has pointed out. More convenient is the use of salts which eliminate the calcium and magnesium ions by forming more insoluble salts through a double decomposition. Ammonium oxalate is the salt most frequently used for the extraction of insoluble forms of pectic substances. (29), (30), (31).

Difficulty in purification arises as to whether pectins are physical mixtures of polysaccharides or whether they consist mainly of highly complex polysaccharides, the constitution of which varies from source to source. The removal of starch is achieved by the use of amylolytic enzymes, while proteins are eliminated with proteolitic enzymes. Their use is of great importance in industry(32) for the clarification of pectin solutions. Salts and organic acids present in pectic materials may be removed by means of dialysis against 0.01 N HCl, but this has the disadvantage of long treatments during which the pectic substances undergo degradation.(33) Electrodialysis is more efficient(34) if this method is employed after a preliminary purification by precipitating with ethanol.

The availability of ion exchange agents makes the removal of inorganic materials a simple operation. The use of amberlite IR 120 (H) and amberlite IR 4B (OH) reduces the ash content to about 0.05 %. The solution of pectic material to be de-ashed, highly diluted (0.1 - 1 %) is treated first with Amberlite IR 120 (H) and then with Amberlite IR 4B (OH), allowing sufficient time of contact since the action of the anion-resin is rather slow.(35)

PURIFICATION OF PECTIC ACID

Ehrlich(36) discovered that some or most of the arabans and galactans which occur with the pectic substances may be removed by extraction with 70 % alcohol. The relation between the purity of the precipitates and the concentration of alcohol used was not clear in Ehrlich's publications. Schneider and Bock(37) showed that the lower the ethanol concentration employed for the purification of pectinic acids, the more admixed non-pectic material will remain unprecipitated.

In the precipitation of pectinic acids with 50 % alcohol there is a partial fractionation of pectinic acids as the lower molecular sizes may be soluble in such a concentration. In order to obtain floculation of pectinic acid by the addition of alcohol, small amounts of inorganic salts must be present as pectinic acid tends to remain colloidally dispersed. Calcium chloride is especially efficient in this way and only traces of this salt contained in the ordinary water used gives rise to rapid floculation. If 55 % alcohol is employed in the precipitation of pectinic acids, the precipitate is easily handled and filtration is reasonably efficient.

The absorption of sugars by the precipitate is very weak contrary to that of traces of acids or salts, but if 0.01 N hydrochloric acid is contained in the precipitation mixture, the absorption is diminished and the ash content decreases.

The use of acetone in the precipitation of pectinic acids from solution was recommended by Hilton.(1) A solution of pectinic acid (0.1 %) and acetone added with efficient agitation of the solution until a 50 %

concentration is reached gives excellent results and the weight of pectic acid, if the precipitation is repeated, does not decrease. Propanol-2 in place of acetone has been proposed, as well as the salting out of the solutions or the electrolytic deposition by means of a copper-tin couple.(38) (39)

The filtration of these precipitates is best carried out through muslin or any other sort of cloth, and it is important to drain and squeeze the precipitates in order to free them from the large quantity of solution they hold.

The proposed purification of pectic solutions through precipitations with the aid of metallic salts cannot be effected unless the amount of arabans and galactans present is very small and provided there is no co-existence of other acidic polysaccharides that may precipitate as well as metallic salts. Precipitation of pectin by colloidal action(40) is based on the neutralisation of electric charge of colloids. Aluminium sulphate and ammonia form a gel in the solution of pectin. The charge of the aluminium hydroxide particle neutralises the pectin charge thus causing a precipitation and rendering the filtration or centrifugation possible. The operation is carried out at a pH of 4.0 - 4.2 and the concentrations of the reagents are those empirically studied to produce a minimum viscosity. The aluminium is eliminated by converting it into the alcohol soluble chloride through the washing of the precipitate with alcohol containing hydrochloric acid.(41) Copper salts have been used for the same purpose, a very small amount of copper sulphate being necessary in order to induce precipitation. Calcium salts are used(42) when the pectic substances are extracted from the insoluble pectates.

The recovery of the pectic acid is effected by several washings with alcohol containing 0.05 % hydrochloric acid, until free from calcium ions. Ammonium oxalate in the calculated amount can be used for the removal of the calcium from these precipitates. In this case no acids are necessary, thus reducing the possibilities of degradation.

Hirst and Jones(24) prepared a pectic acid by these procedures, the equivalent weight of which - 185 - was very close to the theoretical value - 176 -. Later on, Hirst reported a preparation of pectic acid purified through copper and calcium salts, then further purifications by methods as yet unpublished(43), and the equivalent weight of this preparation was closer to 176 and showed upon hydrolysis D-galacturonic acid only.

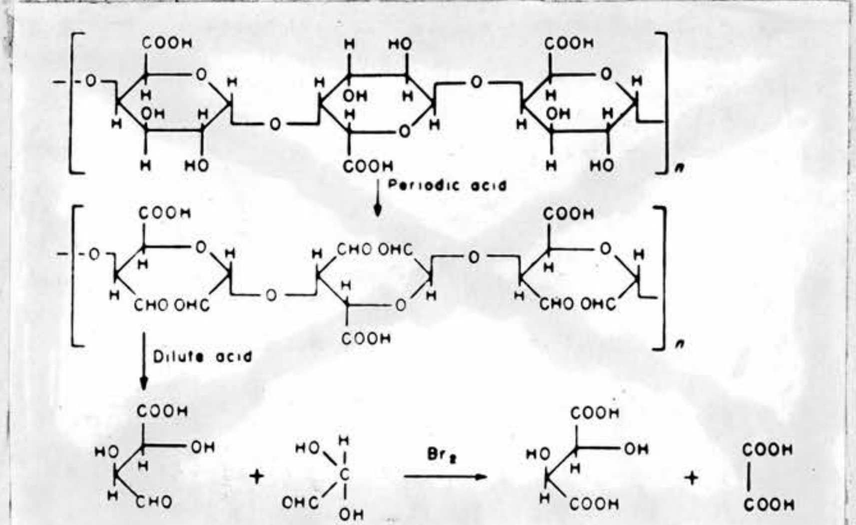
CHEMICAL STUDIES ON THE STRUCTURE OF PECTIC ACID

During the nineteenth century no definite chemical conception prevailed concerning the structure of pectic materials except that they were related to the carbohydrates and had the elementary composition typical of these compounds. B. Tollens and R.W. Tromp de Haas(44) assumed that a carbohydrate containing carboxyl groups was present in pectic substances. Smolenski,(45) in 1911, reported the presence of an uronic acid in sugar beets, though thought to be glucuronic acid. In 1917 Suarez(46) noted the occurrence in lemons of an isomer of glucuronic acid and in the same year Ehrlich(47) pointed out that pectic substances were composed principally of anhydrogalacturonic acid. Fischer and Hertz(48) described the racemic acid - galacturonic - as an aldehyde of mucic acid.

Galacturonic acid is a component of many natural substances such as gums, mucilages and some hemicelluloses, though the most important source is to be found in pectic substances.

Apparently D-Galacturonic acid occurs in natural pectic compounds only in the pyranoside form as Morell and Link(49) concluded by measuring the velocity of the acid hydrolysis of α-methyl-D-galacto-pyranosiduronic acid and pectic acid.

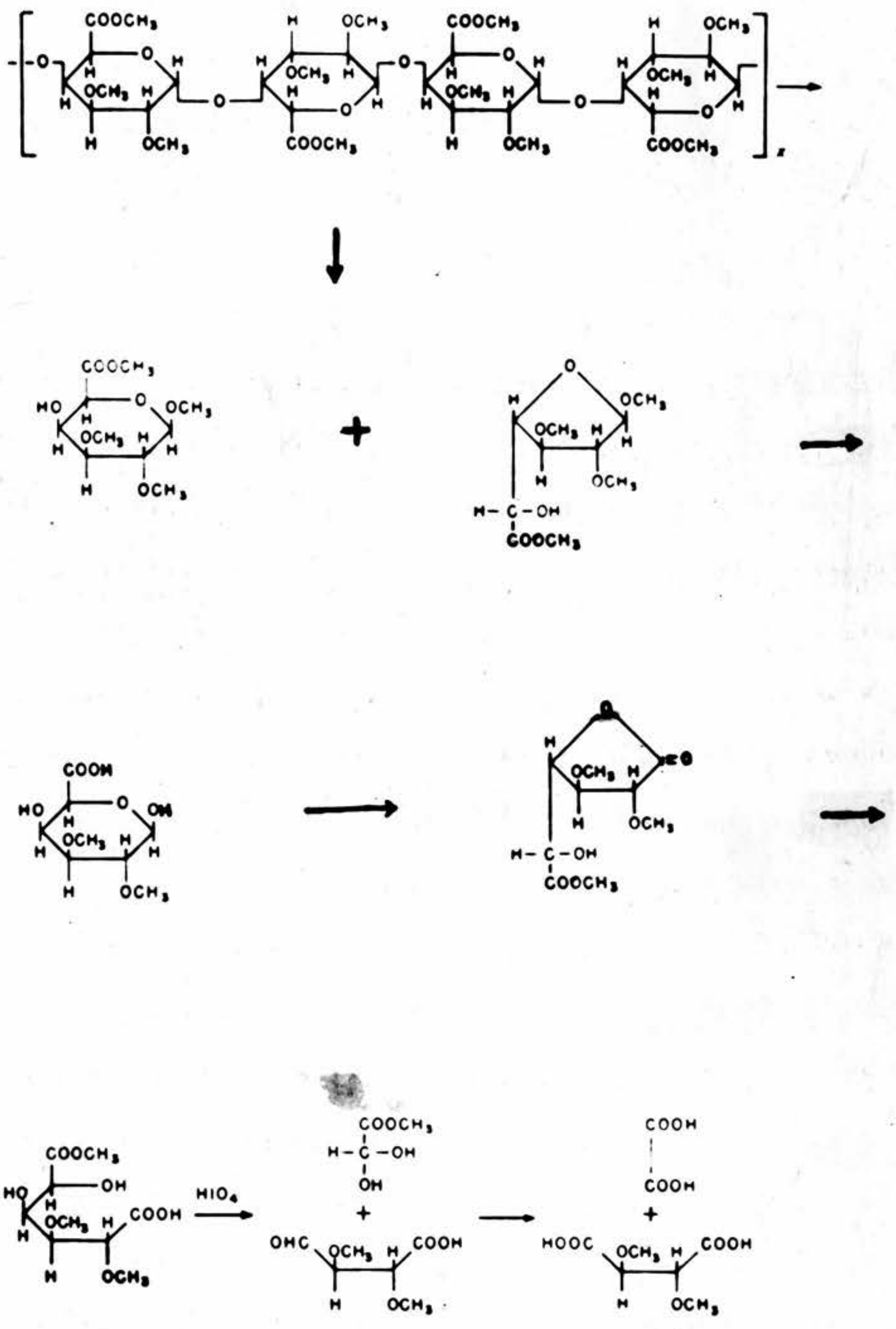
German workers were the first to approach the study of the structure of pectic acid by using the techniques of the cellulose chemistry, demonstrating that pectic acid has a long chain. Henglein and Schneider(50) prepared nitropectin by reaction of pectin with fuming nitric acid (1.52 d.) at 0° C and they found that nitropectin behaves like nitrocellulose. The tensile strength of films make of nitropectin is greater longitudinally than transversally. Accordingly the molecules seem to be elongated, thread-like. At the same time, light was thrown upon the means of linkage between the galacturonic acid units which, according to the properties of the nitroderivatives must be similar to that in cellulose. Parallel evidence came from Levene and Kreider(51) who attacked the problem from another angle by using the periodic acid as oxidant of the glycol groupings according to the techniques of Malaprade. They oxidized polygalacturonic acid with periodic acid, eliminating the excess of oxidant with ethyleneglycol and separating the inorganic ions as barium salts. The oxidized pectic acid was hydrolysed, and there followed an oxidation in order to transform the aldehydic groups into acid. The final identifiable product was D (-) tartaric acid. The cleavage of the glycol groups in the galacturonic units effected between the carbons C_2 and C_3 leaves some uncertainty as to the carbons which are engaged in the glycosidic linkage (C_4 or C_5) since there is a similar possibility of obtaining D (-) tartaric acid from the pyranose or furanose form of the galacturonic acid units.



Methylation studies involving the use of the end group estimation techniques were attempted by many investigators. These studies were carried out on a partially degraded pectic acid either through the treatment with methanolic or aqueous hydrogen chloride in order to free the pectic acid from the admixed arabans and galactans.

Hirst, Jones and Smith used dimethyl sulphate and sodium hydroxide as reagents for methylation, also the Thallium salts of the polyuronide and methyl iodide by taking advantage of the relatively acidic character of the hydroxyl groups in polysaccharides. In any of these procedures the methylation was accomplished by several Purdie's treatments.(24)(52)(53) The final product, a fully methylated methyl pectate, was subjected to hydrolysis by means of methanolic hydrogen chloride. The methanolysis mixture consisting of methylated methyl glycosides was resolved by fractional distillation. All the above mentioned workers obtained methyl 2:3 di-O-methyl-D-galacturonoside methyl ester as a repeating unit in methylated pectic acid, but all of them failed to separate from the methanolysis product any methyl 2:3:4 tri-O-methyl-D-galacturonoside methyl ester which would correspond to the end group. The finding of the furanose form of methyl 2:3 di-O-methyl-D-galacturonoside methyl ester by Smith(54) in the methanolysis products of methylated pectic acid was interpreted by Jones and Beaven(55) as due to some transformations which may occur during the process of methylation since drastic conditions are used in this operation. The most probable explanation is that during methanolysis the equilibrium between the two tautomeric forms is established, leading in this way to the misinterpretation of the results.

Hirst and Jones(43)(53) characterised the methyl 2:3 di-O-methyl-D-galacturonoside methyl ester through a series of steps:


COOCH₃
OCH₃
H
O
HIO₄
OHC
HOOC
COOH
H - C - OH
x

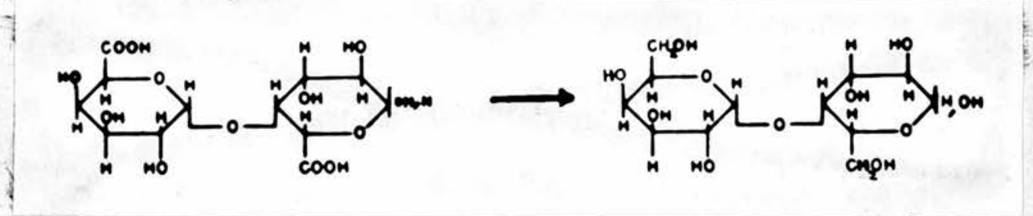
As a final product di-O-methyl-L(+) tartaric acid was obtained and characterised. Smith and Luckett(52) characterised the methyl 2:3 di-O-methyl-D-galacturonoside methyl ester by hydrolysis and oxidation to 2:3 di-O-methyl mucic acid which was compared with the authentic product obtained by synthesis. They concluded, since they were unable to find any methyl 2:3:4 tri-O-methyl-D-galacturonoside methyl ester, that the molecule either is so long that the amount of the end group is practically negligible, or possibly that the molecule forms a loop in which both ends are linked together. The reason for this failure to isolate any amount of the end group resides in the lack of accuracy and selectivity in the method of separation which they used for the resolution of the mixture.

Fractional distillation does not supply a perfect resolution, less still when the proportion of the end group is very small.

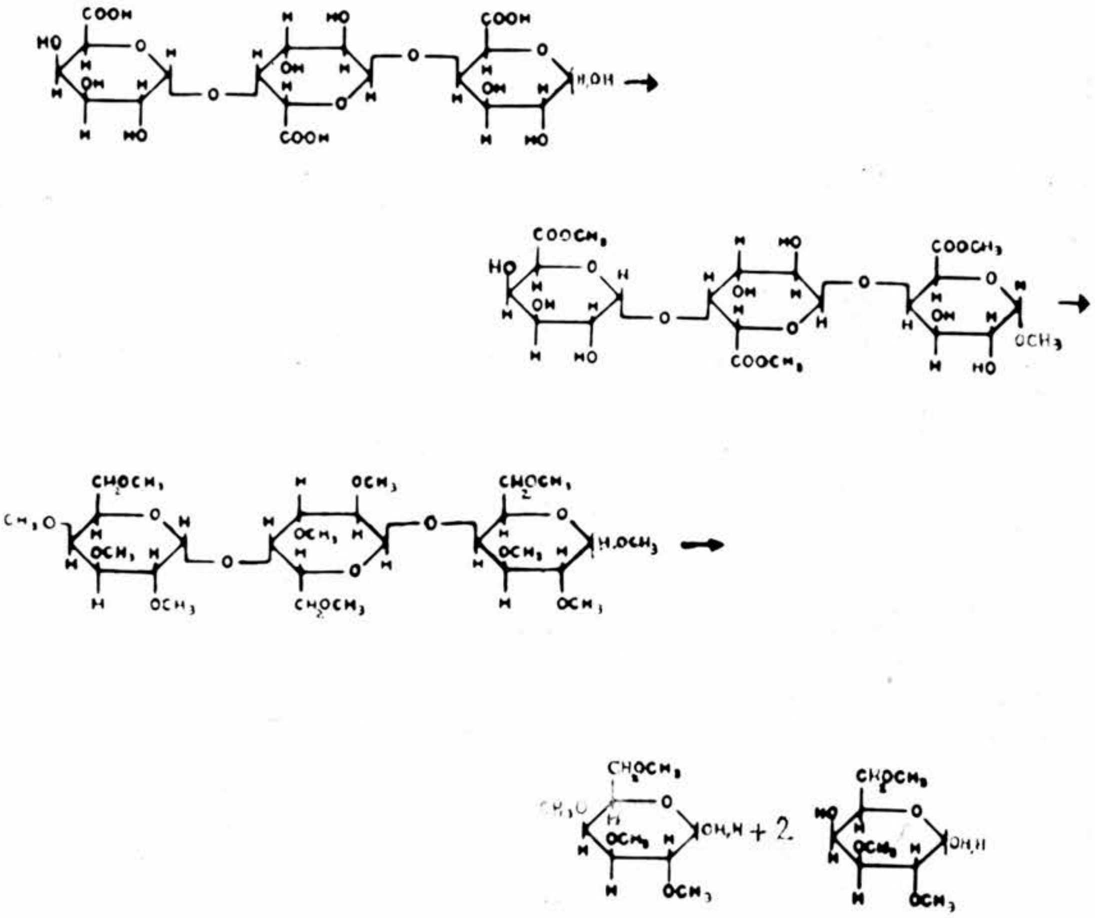
Holt (doctoral thesis) used formic acid as hydrolysing agent, and the mixture of sugars was resolved in a cellulose powder column, but the mixture of methylated galacturonic acids forms did not give successful results in the isolation of any tri-O-methyl-D-galacturonic acid. The methylation with Haworth's reagents involves a great degradation in the case of pectic acid. The amount of the end group therefore increases relatively, and the previously mentioned workers' lack of success in the isolation thereof is inexplicable.

Further evidence with regard to the means of linkage of the galacturonic acid units in pectic acid has been given by Jones and Reid(56)(57) who degraded enzymically pectic acid from apple and obtained triuronides and diuronides, which were subjected to careful investigation.

The digalacturonic acid isolated by these workers was transformed into the orthoformate by shaking with ethyl orthoformate in absolute methanol and a few drops of concentrated hydrochloric acid. The orthoformate group protects the free reducing aldehydic group and has the advantage of being free from attack by the lithium aluminium hydride. The carboxyl groups were then esterified with ethereal solution of diazomethane at 0° C, and the orthoformate methyl ester of this di-galacturonic acid was reduced with lithium aluminium hydride in tetra-hydrofuran. After the hydrolysis of the mixture, they obtained 4-O-$\underline{\alpha}$-D-galactopyranosyl-D-galactose which was identified by comparison with the authentic specimen.(58) These workers established in this way, without any doubt, the pyranosidic structure of the galacturonic acid units of pectic acid as well as the $\underline{\alpha}$ linkages. The triuronide was converted into the methyl glycoside methyl ester by refluxing with methanolic hydrogenchloride and subjected to reduction with potassium boron-hydride which transforms the ester groups into alcohol. The reduced material (methyl glycoside of a trigalactoside) showing a high rotation $[\alpha]_D = + 190°$, indicative of $\underline{\alpha}$ linkages between the galactose units, was methylated with dimethyl sulphate and sodium hydroxide, followed by Purdie's treatments, resulting in a fully methylated trisaccharide which was hydrolyzed. The breakdown products were separated in a cellulose column and characterised by obtaining crystalline derivatives. The sugars separated were 2:3:4:6 tetra-$\underline{O}$-methyl-D-galactose, 2:3:6 tri-$\underline{O}$-methyl-D-



a:)



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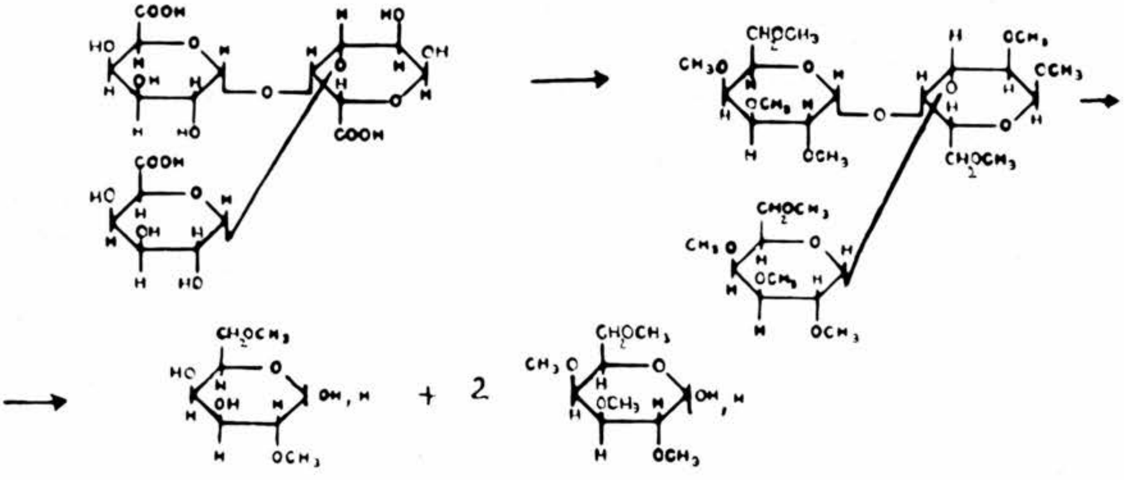


galactose and 2:6 di-*O*-methyl-D-galactose, in the relative proportions of 1 : 1.3 : 1 respectively. The isolation of these sugars suggests either:

a) that the trisaccharide is of the unbranched-chain type which on hydrolysis should furnish 1 mole of tetra-*O*-methyl-D-galactose and 2 moles of tri-*O*-methyl-D-galactose, or

b) that the trisaccharide is of the branched chain type which should give 2 moles of tetra-*O*-methyl-D-galactose and 1 mole of di-*O*-methyl-D-galactose. The deviation of the ratios of the respective sugars (from 1 to 2 and from 2 to 1) found, may be due to the formation of boric esters, or some steric factor leading to incomplete methylation.

Several attempts to reduce the labile carboxyl group have been carried out in order to avoid the difficulty encountered in the methylation and the decarboxylation which takes place during hydrolysis of the methylated material and alters the results of the methylation studies. Levene and col.[(59)] used copper chromite as a catalyst and hydrogen at 175° C and 3500 pounds per sq. inch of pressure, during six hours, and they claimed to have obtained a methylated galactan. Holt (doctoral thesis) repeated the experiments, varying the conditions, and failed to obtain any characterisable product.

PHYSICAL STUDIES ON THE STRUCTURE OF PECTIC ACID

Another approach to the study of the structure and shape of pectic acid involves the use of physical methods.

Nearly all the physical measurements of the properties of pectic compounds have been carried out on preparations containing up to one third araban and galactan, but the experimental evidence has shown that the prominent properties of pectin (gelation, film formation, high viscosity, etc.) are derived from the galacturonan chain and that the other polysaccharides act mainly as diluents.

Bonner(7) pointed out that in dealing with pectic materials several features must be borne in mind:

1. Pectins are hydrophilic colloids which possess a high negative charge varying with the number of free carboxyl groups.
2. The maximum bonding between chains occurs when calcium ions are shared between carboxyl groups from different chains and the number of carboxyl groups has reached its maximum, as in the case of calcium pectate.
3. Pectin sols differ from the typical hydrophilic sols in that they are composed of larger particles, essentially gel fragments. This furnishes an explanation in the case of the precipitation of pectins from solution by dehydrating agents.
4. Dilute solutions seem to be molecular dispersions, since they yield expected osmotic data.

Meyer and Mark(60) suggested that galacturonan contains a linear backbone - suggestion which was later confirmed by X-ray diffractive data through the work of Henglein and Schneider,(50) Astbury and Belle,(61) and more recently, of Palmer and co-workers.(62)(63)(64)

The latter group of workers interpreted the X-ray diffraction pattern obtained from oriented fibres of sodium pectate and they concluded that the chain of galacturonate units has the configuration of a three-fold screw axis, the fibre identity period containing three galacturonate units. The experimental data from birrefringence, sedimentation, diffusion and viscosity studies on various pectin derivatives show that pectinic acid molecules are rod-shaped.

OSMOTIC PRESSURE measurements provide a method of stuyding the physico-chemical behaviour of solutions of pectins. As pectic substances are usually accompanied by dissolved impurities, commonly electrolytes, even when carefully purified, their removal isof extreme importance for they produce errors in the measurements. The use of membranes which are permeable to the dispersion medium as well as to electrolytes diminishes the source of error but does not eliminate it. Edson used small collodion bags for this purpose in apple pectinic acid. Schneider and Fristchi(65) made osmotic measurements on dialysed sterile solutions of pectinic acid and obtained values corresponding to molecular weights in the range of 30.000 - 40.000, but these workers did not place much reliance on the values obtained due to the possibility of association of pectinic acid molecules in water solution. These values, obtained by the same authors from the nitro and acetyl derivatives of pectin, are, according to them, the true molecular weights, ranging from 30.000 to 100.000 depending on the source of the pectinic acid and the derivative used. The transformation of nitro-pectin into acetyl-pectin does not change the molecular weight of the samples.

Owens, Maclay and col.(66) reported osmotic pressure measurements on both pectinic acid and pectin propionates, claiming that the preparation of these derivatives does not degrade the pectic molecule. The average molecular weight calculated from the observed osmotic pressures ranged from 50.000 to 100.000. Vollmert(28) showed by means of the osmotic pressure measurements that the alkaline de-esterification of pectin was accompanied by depolymerisation even in an oxygen free atmosphere. As pectic acid itself was not affected similarly, Vollmert suggested that the degree of depolymerisation depended upon the percentage of methoxyl groups present in the polysaccharide. The values for the molecular weights of the native apple and flax pectins - 140.000 and 100.000 - fell to 50.000 and 80.000 respectively when the substances were de-esterified. This behaviour was thought to be due to the splitting of the acetal linkage of the galacturonic acid under the influence of the ester carbonyl group. The results obtained for pectic substances by osmotic methods are far from reliable as minute impurities have a great influence, and the osmotic behaviour of colloidal solutions deviates markedly from ideal solutions.

ULTRACENTRIFUGE measurements are regarded by many workers as the most dependable means of determining the size of large molecules.

The high speed ultracentrifuge developed by Svedberg(67) and his collaborators produces field intensities up to a million times that of gravity and records the measurements of sedimentation equilibrium or velocity in a centrifugal field obtained from up to 150.000 r.p.m.

The first observations on the behaviour of pectic solutions in ultracentrifuge were reported by T. Svedberg and N. Gralen(68) who used juices extracted from various fruits and lily bulbs without further purification. The results indicated molecules of asymmetrical shapes or strong hydration. The approximate molecular weights of pectinic acids from the juice of apples, pears, and plums were in the range 25.000 - 35.000, while the orange albedo gave 40.000 - 50.000.

Saverborn(69) prepared pectinic acids by a number of different methods, and subjected them to heating for two hours at 94° C in 0.05 N sulphuric acid in order to observe the effect of partial hydrolytic degradation. From the sedimentation curve of lemon pectin, Saverborn noted that it was unusually pointed indicating the presence of a great cohesion among the structural components of the macromolecule and preventing the occurrence of normal diffusion. The same is indicated by the steadily increasing values of the sedimentation constants upon decreasing the pectinic acid concentration.

The apparent weight - molecular - depended upon the pH and the method of extraction, for the difference in behaviour between the water and acid extracted samples is rather striking. The free pectins from plant juices have a considerably higher value of molecular weight (200.000 - 400.000) than those extracted even with mild reagents (60.000). Tiselius and Ingelman(70) reported some observations on the behaviour of beet pectinic acid solutions in ultracentrifuge. The preparation of this pectinic acid contained 56.8 % uronic anhydride and 8.2 % methoxyl content, and it indicated elongated molecules in the ultracentrifuge. The molecular weight calculated (90.000) is within the same range as the values given by

Saverborn for apple and lemon albedo pectins. Saverborn stated that, on account of charge effects, at concentrations of 0.3 %, pectinic acids will hardly sediment, and in some cases such as those with acid and neutral apple pectins, the substance had not come off the meniscus even after four hours of run in the ultracentrifuge at the maximum speed. The velocity of sedimentation in the presence of electrolytes is greater. At lower concentrations (0.05 %) the sedimentation becomes measurable even in water. The same author effected measurements of the sedimentation equilibrium (18.000 r.p.m.) by choosing a suitable low gravitational field in which a state of equilibrium is reached when the amount of substance sedimenting through a unit area at any point of the cell is equal to that diffusing in the opposite direction. From such measurements average molecular weights were calculated to be in the range of 40.000 - 93.000 for apple, beet and citrus pectins, which corresponds with those calculated from sedimentation velocity and diffusion measurements.

By VISCOMETRIC MEASUREMENTS, Schneider and co-workers(50)(65)(71) determined the average molecular weights of nitro-pectin and acetyl-pectin, prepared in order to avoid the anomalous behaviour due to charge effects of the unsubstituted polysaccharides. They found that both methods gave molecular weights in the range of 30.000 - 100.000, measurements indicating that there was little difference in size between the two derivatives.

Maclay and co-workers(72) who studied the viscometric behaviour of citrus and apple pectin in 0.115 N NaCl as a function of the pH and temperature found molecular weights of 23.000 - 71.000 and their measurements depicted the behaviour of the pectin molecules as rigid rods in the solution.

The relationship between methyl ester content and viscosity was reported by von Fellenberg(73) and other workers, but their statements would not seem to be of great importance as the viscosity may drop without a change in the methyl ester content as Myers and Baker(74) have shown. Many factors do indeed intervene in the viscosity measurements in the case of pectic substances, and the validity of molecular weight determination by this method is altogether questionable, even if such data conform to a given equation. Vollmert(28) and Speiser and Eddy(76) reported that de-esterification of pectin by acid or enzymes also brings about degradation as in the case of alkaline de-esterification.

Esterification of the pectins with diazomethane at low temperatures was said to cause little degradation and the esters formed are suitable for viscometric determinations in aqueous solutions.(28)

Baker and Goodwin(75) calculated the value of 280.000 for the molecular weight of a sample of apple pectinic acid. Saverborn(69) calculated from viscosities'measurements the molecular weights (average) of 67.000, 89.000 and 62.000 for commercial samples of apple, lemon and beet pectins. The determinations made by Owens, Maclay and co-workers(66) on the intrinsic viscosities of a series of pectinic acids lead these authors to conclude that the polyuronide chain is not affected in its flexibility by enzymic removal of the methoxyl groups.

More significance may be attached to molecular weight determinations based on the viscosity of non-aqueous solutions of various derivatives.(50)

ARABANS AND GALACTANS ACCOMPANYING PECTIC SUBSTANCES

These two polysaccharides form part of the pectin complex and the resolution of the mixture is somehow laborious and not always efficient.

The proportion of these two polysaccharides in the pectic materials is variable and dependent upon the source used for their extraction. There is no concrete evidence as to whether they are attached to the polyuronide chain or form a physical mixture.

Norris and Resch(77) pointed out, as a result of their work, that some araban and galactan present in pectin may occur free as ballast, while a portion of the molecules is chemically attached to the galacturonan. Speiser, Eddy and Hills(78) made a similar statement when they found that after dissolution of loose ballast in 70 % alcohol, substantial amounts of galactan and araban remain and require, for their removal, conditions of acid hydrolysis. From the activation energy involved in this hydrolysis (18.500 cal.) they assumed that the linkage of these two polysaccharides to the galacturonan chain was through ester bond (18.000 cal.). From electrophoretic mobility measurements evidence is withdrawn indicating that these polysaccharides are in some way together forming a whole. It is possible that some araban and galactan are attached to the polyuronan chain by ester linkages.

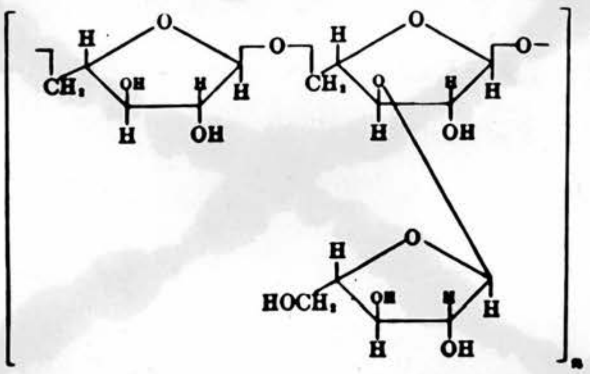
The galactan is resistant to mild hydrolysis with 0.05 N sulphuric acid, while the araban is completely degraded. Both polysaccharides are precipitated with high concentrations of alcohol (70 %) but some quantities of them may be coprecipitated with pectin at lower concentrations. By careful choice of the source of pectin it was possible to isolate araban and galactan, in each case their proportion being abnormally high.

The preparation of araban through separation of the pectin and galactan is normally difficult, but good results may be obtained when the proportions of araban in the plant tissues are high. An example has been provided in peanuts (arachis hypogea) with the aid of which Hirst and Jones(24)(79)(80)(81)(82)(83) carried out the investigation of its structure.

In this case the galactan content is very small, although its complete removal from the crude araban is tedious. The procedure followed by the above mentioned workers is, briefly :

> Extraction of the oil with organic solvents, removal of protein by extraction with 10 % solution of sodium chloride, solubilization of pectin contained with 0.2 % potassium hydroxide solution, precipitation of the pectin complex by neutralisation and addition of alcohol.
>
> The crude pectin contained 40 % araban and was extracted with 70 % alcohol for several weeks. The extracted material was recovered by evaporation of the solvent. A further extraction with pyridine containing 2 % water carried out on this isolated material dissolved only the araban and galactan. The resolution of this mixture was achieved by acetylation, separation of the acetates by differences of solubilities and de-acetylation.

The separation of araban in other fruits in which the porportion of araban is not so high is more difficult. In these cases, resolution of the complex mixture is accomplished by taking advantage of the solubilities of the methylated derivatives, as well as the rate of methylation. Araban is easily methylated in strong alkali, pectic acid being largely destroyed under these conditions, and galactan is methylated slowly. The methylated araban is soluble in ether while the corresponding derivate of galactan is not. Another interesting observation helpful in the separation of araban is that when solutions of pectin containing araban are heated with alkalies the proportion of araban increases owing to the degradation of pectic acid.(84) Various structures of araban have been proposed, based on methylation studies. The repeating unit of one of them is shown below:



Possible structure of araban (after Hirst and Jones).

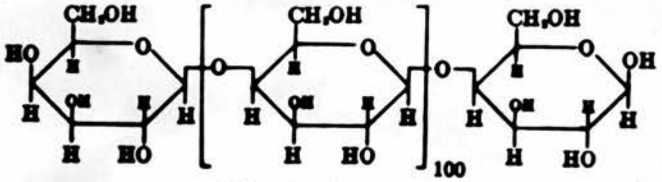
The proportions of 2-mono-*O*-methyl-L-arabinose, 2:3 di-*O*-methyl-L-arabinose and 2:3:5 tri-*O*-methyl-L-arabinose - approximately identical - leads to the conclusion that araban is built from a chain of arabinofuranose units linked through positions 1 - 5' (α), and half of these units have a side chain composed of one arabinofuranose unit linked to the back-bone (main chain) through carbons 1 - 3' (α).

The well known lability of arabans towards acids bringing about the hydrolysis of the polysaccharide even under very mild conditions indicates clearly the furanose nature of its arabinose units.

Schneider and Fritschi[(85)] suggested that the failure to obtain the nitrated araban during nitration of pectin was due to the hydrolysis experimented by the polysaccharide during the process. The high negative rotation of this polysaccharide suggests that the type of linkage involved in its structure is of the $\underset{=}{\alpha}$ sort between the L-arabinose units.

The preparation of a galactan in a pure state from the pectin complex is more difficult to achieve than that of araban. The only fruit in which a galactan could be isolated in a sufficient state of purity was the seed of white lupin (Lupinus albus).(86)(87) After extraction of the pectin complex, two precipitations from the aqueous solution with methanol, followed by precipitation of the pectic acid with the aid of calcium ions, the crude galactan was methylated by Menzies' procedure(88). The methylated galactan was separated from the araban also methylated by dissolution in ether in which the latter is completely soluble.

The methylated galactan has a specific rotation of - 17° which is indicative of B linkages. The hydrolysis of this material, requiring drastic conditions - long heating with 5 % HCl - gave 2:3:6 tri-O-methyl-D-galactose and 2:3:4:6 tetra-O-methyl-D-galactose in the proportion 100 - 120 to 1, which suggests that the polysaccharide has a linear chain of galactopyranose units linked through carbons 1 - 4'.



Galactan of pectic substances

Recently Jones and col.(89) published the finding of a galactan extracted from the Strichnos nux vomica, the rotation of which $[\alpha] = + 69 + 2$, indicates that some of the linkages present are of *α* type though some branching occurs in the polysaccharide. But this does not seem to have a close relationship with the galactan isolated from *Lupinus albus.* The proportion of methylated sugars isolated after hydrolysis of the methylated galactan is as follows: 2:3:4:6 tetra-*O*-methyl-D-galactose: 2:3:6 tri-*O*-methyl-D-galactose: 2:6 di-*O*-methyl-D-galactose: 2:4 di-*O*-methyl-D-galactose: :1 : 24 : 3 : 1.

Concerning the relationship between galactans and pectic acids, some workers(90)(91) assumed that the latter would be derived from the former by oxidation of the terminal primary alcohol group in each galactose unit. It is clear from the structures put forward for pectic acid, galactan and araban, that the conversion of one polysaccharide into another is not possible unless a previous hydrolysis and rearrangement take place.(43)

OTHER SUGARS FOUND IN THE PECTIC GROUP OF POLYSACCHARIDES

From the hydrolysis of pectic substances some other sugars have been isolated. Ehrlich and Haensel(92) isolated from ramie bast D-fucose. This assumption was doubted by the rest of the workers in the pectin field. Kertesz mentioned the low uronic content in such preparations, indicative of heterogeneity or low purity of the pectic preparations.

Xylose was reported to form part of the pectic group of polysaccharides by Bauer(93), and in 1926 Ehrlich and Schubert(94) described xylose as a component of flax pectin. Norris, later on,(95) in a more highly purified preparation of the same pectin, did not confirm the presence of this sugar. 2-mono-O-methyl-D-xylose has been, recently, separated in small quantities from pectic substances.(96)

EXPERIMENTAL

GENERAL METHODS USED THROUGHOUT THE WORK

Unless otherwise stated :

Evaporations were carried out at 40° under reduced pressure.

Samples for analysis were dried at 60° over-night in vacuo over phosphorus pentoxide.

Ash. The quantity of ash was determined by ignition to constant weight of a weighed quantity of the material in a silica or platinum crucible.

Methoxyl determinations were carried out by a micro-modification of the Zeisel method.

Small scale hydrolyses were performed by treating samples of the material (ca. 40 - 100 mg.) in sealed tubes with N sulphuric acid for four hours at 100°. The hydrolysates were neutralised in the cold with barium carbonate and the filtrates evaporated to syrups which were deionised by repeated concentration and extraction with alcohol. The hydrolysis can be carried out with hydrochloric acid of the same normality using silver carbonate for the neutralization.

Demethylations were carried out on partly methylated derivatives by treatment with hydrobromic or hydroiodic acid in a sealed tube at 100° for 5 - 10 minutes, followed by neutralisation with silver carbonate and deionisation of the filtrate by alcohol extraction. The free sugars produced were identified chromatographically.

Paper chromatography was performed on Whatman N° 1 filter paper using the following solvent systems (v/v) :

A) Butan-1-ol : benzene : pyridine : water (5 : 1 : 3 : 3) upper layer.

B) Butan-1-ol : ethanol : water (4 : 1 : 5) Upper layer.

C) Butan-2-one, half saturated with water containing 1 % ammonia.

D) Ethyl acetate : acetic acid : water (3 : 1 : 3) Upper layer.

E) Butan-1-ol : acetic acid : water (4 : 1 : 5) Upper layer.

F) Ethyl acetate : pyridine : water (10 : 4 : 3)

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

Chromatography of methylated sugars was carried out in solvent *B*, and R_g values refer to the rate of movement relative to 2,3,4,6 tetra-*O*-methyl-D-glucose in that solvent. R_f is the rate of movement relative to the solvent front.

Papers were sprayed with saturated aniline oxalate solution and the spots developed at 120°.

Paper ionophoresis was carried out in borate buffer at pH 10 at a potential of 500 volts on a water-cooled apparatus. After running for four hours the paper was air dried and the sugars located by aniline oxalate (+ 5 % acetic acid) spray at 100°.

Optical rotations were observed at 18 ± 2°.

N-phenyl-glycosylamines of methylated sugars were obtained for identification of the sugar by refluxing an alcoholic solution of freshly distilled aniline with the sugar for half an hour, protecting the reaction flask from the light by covering it with a black paper. Longer heating causes darkening of the reaction mixture. After allowing the mixture to cool, the crystalline product was recrystallised from a suitable solvent.

Lactones of methylated aldonic acids were made by the oxidation of the sugars with bromine water, followed by elimination of the excess oxidant by bubbling nitrogen through the solution. The hydrobromic acid formed was neutralised with silver carbonate. The solution was then filtered and treated with H_2S to free the aldonic acid. Again the solution was filtered and evaporated to a syrup, continuing the heating in vacuo at 100° for two to three hours to lactonize the acid. This can be accomplished also by distilling the acid in high vacuum in a cold finger.

The crystalline lactones were recrystallized from suitable solvents.

Methylated aldonoamides. The lactone was treated with dry methanolic solution of ammonia in the cold. The solvent was evaporated by blowing air into the flask and the amide recrystallized.

Estimation of uronic acid content.

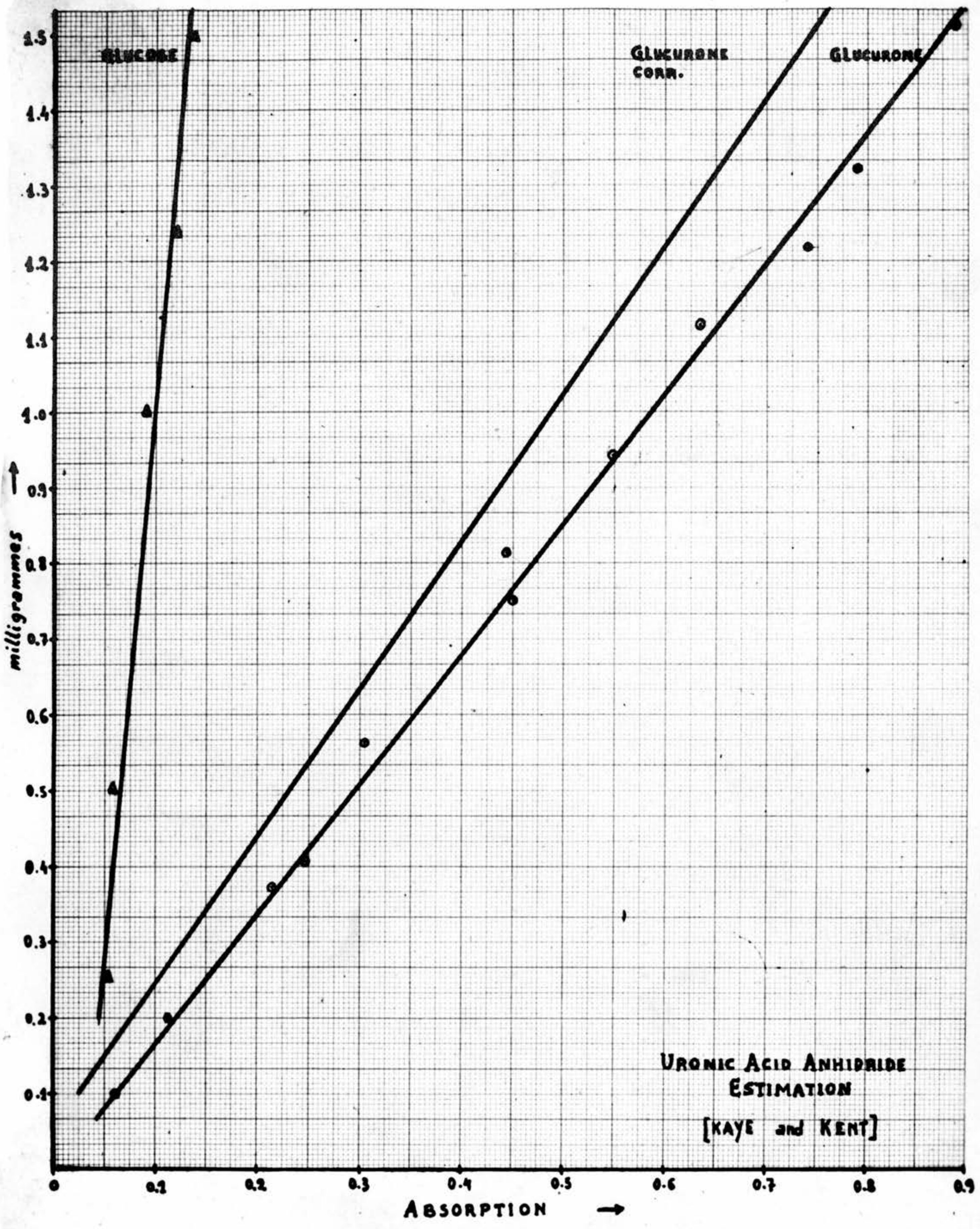
Iodometric titration. This method applicable to acids with dissociation constants of about 10^{-6} can be used with pectic acid. A sample of freeze-dried pectic acid or free acid form of a polysaccharide (0.2 - 0.5 g.) was dissolved in 20 ml. of water and this solution was mixed with 1.0 g. of potassium iodide, 5 ml. of 3 % potassium iodate and 25 ml. of 0.1 N sodium thiosulphate. The mixture was allowed to stand from fifteen to thirty minutes and it was then titrated with 0.1 N iodine solution. The results may be calculated in terms of neutralization equivalent.

Colorimetric estimation. The method used by Kaye and Kent(97) was applied in the estimation of uronic acid in pectin and polysaccharides contained in Sisal successfully.

The calibration of the curve was carried out by preparing standard solutions of recrystallized D-glucurone in such a way that 2 ml. of any solution contained an amount of D-glucurone in the range 0 - 10 micromoles. The recrystallisation of D-glucurone was effected in ethanol (96°). Aliquots of each solution (2 c.c.) were diluted with water (1 c.c.) and mixed with a freshly prepared solution of hydroxylamine (1 c.c. of 2 M hydroxylamine hydrochloride and 1 c.c. of 3.5 M sodium hydroxide). After four minutes at room temperature the solutions were treated with HCl (3.34 N, 1 c.c.) and ferric chloride (0.37 M in 0.1 N HCl, 1 c.c.). The resulting orange-brown solutions were shaken under reduced pressure (12 mm.) for 30 seconds to remove dissolved gases and then transferred to the cells of an Unicam S.P. 500 spectrophotometer. A blank solution was prepared as above but using water in place of D-glucurone solution.

After compensating for the absorption of the blank solution (freshly prepared in each case) the values of log I./I for the glucurone solution at E_{5050} and E_{4750} were read off. The following results were obtained :

mg.	moles	Glucurone		Glucose	
		E_{4750}	E_{5050}	mg.	E_{5050}
1.51	8.5	0.91	0.89	1.5	0.14
1.32	7.5	0.81	0.79	1.24	0.12
1.216	6.9	0.79	0.69	1.0	0.09
1.13	6.4	0.67	0.635	0.5	0.06
0.94	5.3	0.60	0.55	0.25	0.05
0.811	4.6	0.55	0.45	-	-
0.75	4.2	0.50	0.45	-	-
0.56	3.15	0.40	0.305	-	-
0.405	2.3	0.30	0.215	-	-
0.37	2.1	0.265	0.215	-	-
0.203	1.15	0.20	0.114	-	-
0.1014	0.56	-	0.063	-	-



Uronic Acid Anhydride Estimation
[Kaye and Kent]

In order to correct the absorption of the aldehyde group (oxime) present in the glucurone, another set of readings with solutions of D-glucose were taken, following the same procedure as above, and the readings were subtracted from those of the D-glucurone at E_{5050}.

__Procedure for polysaccharides.__ Polysaccharide (2 - 3 mg.) was heated at 100° with methanolic HCl (1-2 %, 0.5 c.c.) in a sealed tube for 30 minutes. After then time the contents of the tube were added to water (2.7 c.c.), the reagents added as before, and the reading at E_{5050} noted. Then this value was interpolated in the curve.

__Equivalent weight determination on the glycol ester of acidic polysaccharides.__ The acidic polysaccharide was converted into the glycol ester by reacting with ethylene oxide in water solution.(98)

A weighed amount of the dried and purified glycol ester was subjected to saponification with a measured amount of 0.5 N solution of sodium hydroxide and the mixture was allowed to stand in stoppered flasks for several hours to complete the saponification. The resulting sodium salt of the polysaccharide was then precipitated with alcohol, filtered off, washed with a little alcohol, and the filtrates were collected.

Titration of the excess of sodium hydroxide with acid gave the equivalent weight value of the polysaccharide glycol ester.

In an aliquot of the neutralized filtrate, the amount of ethylene-glycol present was determined by periodate uptake in the usual way.

PERIODATE - OXIDATION OF METHYLATED SUGARS

This was carried out by the Lemieux and Bauer method.(99) The syrup (1 - 5 mg.) was dissolved in 0.5 N sodium metaperiodate solution (0.12 ml.) and maintained at 0° for one hour. Ethylene glycol (2 - 5 mg.) was added, the solution was warmed to room temperature and, after five minutes, made alkaline to phenolphtalein with 0.5 N sodium hydroxide. The product was examined chromatographically in solvent D. The results obtained for some standard sugars were as follows:

Sugar	R_f	Oxidation product (Colour)
2:3 di-O-methyl-D-galactose	0.66	Grey
	0.78	Brown
	0.87	Grey
2:4 di-O-methyl-D-galactose	0.37	Pink (unchanged sugar)
2:6 di-O-methyl-D-galactose	0.15	Brilliant yellow
2-O-methyl-aldoses	0.15	Brilliant yellow
3-O-methyl-glucose	0.35	Wine red
4-O-methyl-D-mannose	0.60	Brown
6-O-methyl-D-galactose	0.65	Diffuse yellow

QUANTITATIVE ESTIMATION OF METHYLATED SUGARS

This was carried out by the hypoiodite method.(100)

QUANTITATIVE ESTIMATION OF NEUTRAL SUGARS

The quantitative estimation of neutral sugars was carried out by Somogyi's techniques.(101) It was applied to the eluates of the paper chromatography separation of sugars, as in the case of the hypoiodite estimation.(100)

EXTRACTION OF SISAL FLESH

Fractions of 1 kg. of Sisal flesh were extracted with water (6 lit.) at 90° for 3 hours. The extracts were filtered through a cloth and the plant residue was extracted twice more under similar conditions.

The extracts were combined, centrifuged in a Sharples centrifuge and concentrated under reduced pressure to 1 litre at 40°.

This solution was poured, with stirring, into equal volume of acetone, and a precipitate separated and collected by centrifuging.

The precipitate was redissolved in water (200 ml.) and reprecipitated with the same volume of acetone, washed successively with 60 % aqueous acetone, dry acetone, alcohol and ether.

The powdery solid was finally freed from traces of organic solvents in a vacuum desiccator. The yield was 20 g. of polysaccharide.
Ash content : 17.2 %.

From now onwards this polysaccharide will be referred to as water soluble polysaccharide.

After the removal of the water soluble polysaccharide contained in the sisal flesh by means of hot water, the material (2 kg.) was extracted five times with 10 litres (each time) of 0.5 % solution of ammonium oxalate by heating at 80 - 90° for two hours. The extracts were concentrated to a small volume (2 l.) under reduced pressure at 40°, and methylated spirit (2 - 3 l.) was added to the solution of ammonium pectate. The gelatinous solid separated was then filtered through a muslin cloth and squeezed as dry as possible. The solid was washed with 55 % methylated spirit and again pressed dry.

The solid was dissolved in water (2 % solution), and calcium chloride solution (5 %) was added until no more solid was precipitated. The solid was separated by filtration through a cloth and pressed dry. Then it was washed with water and freed from the washings as much as possible.

A sample was hydrolysed and the chromatographic examination showed the presence of rhamnose, xylose, arabinose, galactose and galacturonic acid. The solid was suspended in water and a slight excess of ammonium oxalate was added to the suspension. The whole was then heated at 80° for one hour. The solution was freed from the calcium oxalate precipitate by filtration and dialysed against distilled water until no positive test for oxalic acid was obtained with diphenylamine. Hydrolysis on this solution showed that xylose was removed, but the other sugars still remained. Another precipitation as the calcium salt of pectic acid, followed by conversion into ammonium salt by treatment with ammonium oxalate and dialysis, gave 180 g. of crude pectate (ammonium) when the solution was freeze-dried. Hydrolysis and chromatographic examination on a small sample showed that rhamnose, arabinose and galactose, plus galacturonic acid, were present. A sample of ammonium pectate was dissolved in water (2 % solution) and passed through a column of Amberlite resin IR-120 (H), and the solution of free pectic acid was finally freeze-dried.

$[\alpha]_D = + 238^\circ$ $c = 0.574$ % in water.

Uronic acid anhydride content : = 77.1 % (by decarboxylation).

Equivalent weight = 232.8, equivalent to 75.6 % of Uronic acid anhydride.

PURIFICATION OF AMMONIUM PECTATE (A)

Crude ammonium pectate (20 g.) was suspended in methylated spirit (450 ml.) and sodium hydroxide (10 g.), dissolved in a small amount of water, was added. The mixture was maintained at 0° for 40 hours with occasional shaking. The solid was then filtered and thoroughly washed with methylated spirit (50 %) thrice, 500 ml. each time, and shaken for one hour. Chromatographic examination of the hydrolysate of 50 mg. of material showed galactose, arabinose, rhamnose and galacturonic acid.

Two precipitations from aqueous solution were carried out on the above mentioned material in order to eliminate araban and galactan. Sodium pectate was precipitated from 2 % solution with the same volume of methylated spirit, redissolved in water, and reprecipitated with acetone. The precipitate was filtered through a cloth and pressed dry to obtain an almost compact cake which was treated with alcohol (95 %) followed by acetone. It rendered a white powder which was dried in a vacuum desiccator over P_2O_5 for two days.

Chromatographic examination of the hydrolysis of a small sample showed the same sugars as before.

The reprecipitated sodium pectate was extracted twice with 50 % boiling ethanol for one hour. The insoluble material was dissolved in water and to this solution calcium chloride solution was added. A white gel of calcium pectate was separated and filtered through muslin. This precipitate was converted into the ammonium salt by treating an aqueous suspension with ammonium oxalate. The filtrate was dialysed to remove inorganic salts, and the solution was decolourised by means of the addition of a few drops of sodium hypochlorite solution. Concentration and freeze-drying gave ammonium pectate in the form of a bright, white and fluffy solid.

$[\alpha]_D = + 213^\circ$ c = 0.826 % in water.

Uronic acid anhydride content : = 75.2 % (by decarboxylation)

Chromatographic examination on hydrolysis mixture gave galactose, arabinose, rhamnose and galacturonic acid.

This sample of ammonium pectate was used for methylation studies.

PURIFICATION OF AMMONIUM PECTATE BY PRECIPITATION OF THE CETAVLON COMPLEX (B) (102)

To a solution of ammonium pectate (25 g.) in water (1.2 l.) the same volume of aqueous solution of cetyltrimethyl ammonium bromide (10 %) was added with stirring. A white precipitate appeared immediately, and it was centrifuged off and washed with water several times to remove adhering polysaccharides. The precipitate was then treated with acetic acid (5%) with stirring for five hours. The mixture was poured into a large volume of ethanol, and the precipitate was removed at the centrifuge and washed with ethanol-acetic acid, ethanol and finally ether. Since the polysaccharide contained some adhering cetavlon, an aqueous solution was shaken for two hours with Amberlite resin IR-120 (H). The resin was separated from the polysaccharide suspension by filtering through a muslin cloth and the filtrate was freeze-dried to give pectic acid.

Recovery : 20 g.

$[\alpha]_D = + 215^\circ$ c = 0.4 % in water (ammonium salt)

Uronic acid anhydride content: = 79.0 % (by decarboxylation)

Chromatographic examination of the hydrolysate of a small sample gave rhamnose, arabinose, galactose and galacturonic acid, indicating that little apparent fractionation had occurred.

PURIFICATION OF AMMONIUM PECTATE (C)

Crude ammonium pectate (20 g.) was dissolved in water (1 litre) and acetone (1 litre) was added with stirring. The resulting gel was filtered through a cloth, redissolved in water, and a further precipitation was carried out in the way previously indicated.

The precipitate was extracted twice with 50 % ethanol (1 litre) under reflux for one hour. The insoluble material was filtered, dissolved in water, and the solution was filtered through Celite and freeze-dried.

Recovery : = 15 g.

$[\alpha]_D = +213^\circ$ c = 1.045 % in water.

Uronic acid anhydride content : = 74.06 % equivalent to 79.7 % in the free pectic acid.

Chromatographic examination showed galactose, arabinose, rhamnose and galacturonic acid.

REACTIONS OF AMMONIUM PECTATE FROM SISAL WITH SODIUM METAPERIODATE

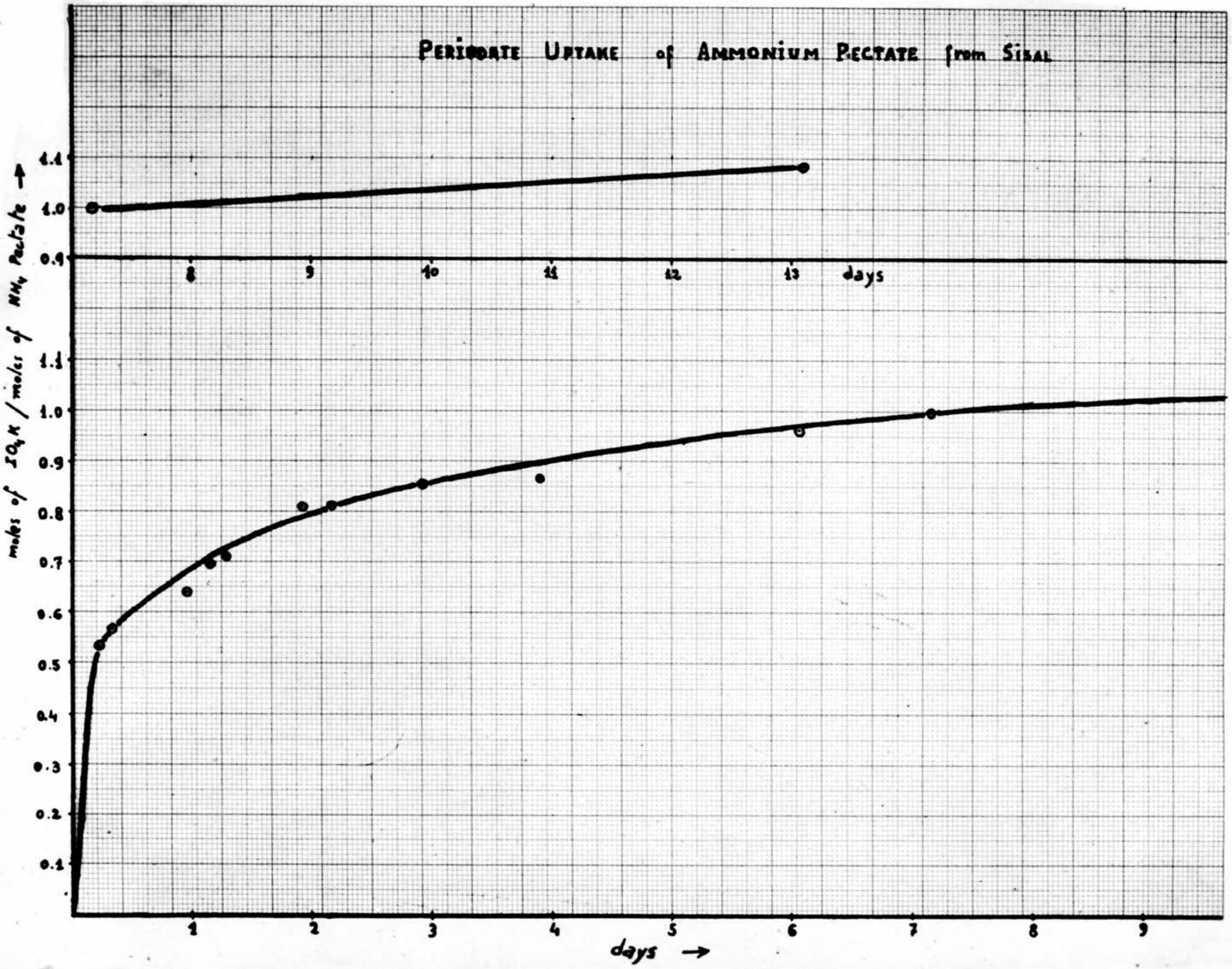
PERIODATE UPTAKE OF AMMONIUM PECTATE.

Ammonium pectate ($[\alpha] = + 213^{\circ}$) (0.6743 g.) was dissolved in distilled water (100 ml.). To this solution 0.25 M solution of sodium metaperiodate (25 ml.) was added and the mixture diluted up to 250 ml. in a graduate flask. A blank with the same amount of sodium metaperiodate was run parallel. Samples of 5 ml. were withdrawn from time to time and a known amount of standard solution of arsenite 0.09986 N was added after the previous addition of 1.5 g. of sodium bicarbonate. A solution of potassium iodide (1 ml. 20 %) was then added and the mixture allowed to stand in the dark for 15 minutes before the back titration with standard iodine solution (0.1008 N) and starch as indicator.

Operating in a similar manner with the blank, the difference between iodine titres of the ammonium pectate solution and the blank was equivalent to the amount of ammonium pectate which had been oxidised. The following table shows the results :

Time (hours)	Iodine (ml.)	R = Moles of $NaIO_4$ used / Moles NH_4 galacturonate anhyd.
5.30	0.75	0.537
8.00	0.79	0.568
22.45	0.89	0.64
27.05	0.93	0.67
30.50	0.99	0.71
46.00	1.03	0.81
70.00	1.09	0.856
93.00	1.20	0.864
145.00	1.34	0.964
171.00	1.40	1.000
314.00	1.51	1.08

PERIODATE UPTAKE of AMMONIUM PECTATE from SISAL



PREPARATION OF SODIUM METAPERIODATE OXIDIZED AMMONIUM PECTATE

Ammonium pectate ($[\alpha]_D = + 214^\circ$) (20 g.) was dissolved in water (1 l.) and sodium metaperiodate (27.5 g.) was dissolved in water (1.1 litres). From this last solution 100 ml. were withdrawn for use as a blank, and the remaining litre of periodate solution was mixed with the solution of ammonium pectate. The mixture was 0.1001 M in relation to the metaperiodate and 0.05 M to the ammonium pectate. The mixture was kept in the dark, and the course of the reaction followed titrimetrically.

5 ml. of the mixture were withdrawn every 8 hours and titrated as usual, and equivalent treatment was applied to a similar amount of the blank solution. When, after four days, the theoretical uptake had been reached, the excess of oxidant was destroyed by the addition of ethylene glycol. The solution was concentrated to 200 ml. under diminished pressure and dialysed until free from inorganic material - state tested by reaction with potassium iodide and acid, starch being employed as an indicator. The solution was further concentrated under reduced pressure to 150 ml., and the oxypolysaccharide precipitated through the addition of a large excess of ethanol. The white precipitated polysaccharide was washed with aqueous ethanol, alcohol-ether and finally with ether. Traces of organic solvents were removed in vacuo. The yield was 16.7 g. The material reduced Fehling's solution and ammoniacal silver nitrate.

$[\alpha]_D = - 33.8^\circ$ c = 4.8 % in water.

REACTION OF PERIODATE-OXIDISED AMMONIUM PECTATE WITH ISO-NICOTINYLHYDRAZIDE (103)

Periodate-oxidised ammonium pectate (1 g.) was dissolved in water (25 ml.), and iso-nicotinylhydrazide (0.75 g.) in water (10 ml.) was added. After 20 hours at room temperature, the mixture was acidified with acetic acid and a yellowish solid separated out.

The precipitate was filtered off, washed with water, alcohol and ether, and finally dried in vacuo over P_2O_5. On standing for 15 hours more the solution yielded more precipitate, which was treated as before.

Recovery : 1.2 g.

Nitrogen content: found 13.4 %, calculated for one glycol fission per anhydro-galacturonic acid unit : 13.6 %.

REACTION OF PERIODATE-OXIDISED AMMONIUM PECTATE WITH THIOSEMICARBAZIDE (103)

Periodate-oxidised ammonium pectate (1 g.) was dissolved in water (12 ml.), and thiosemicarbazide (1 g.) in water (10 ml.) was added. After standing at room temperature for 24 hours, the mixture was acidified with acetic acid and allowed to stand for a further 24 hours. The precipitate which appeared was filtered off, washed with water, alcohol and ether successively, and dried in vacuo.

Recovery : 1.1 g.

Nitrogen content: found, 16.2 %, calculated for one glycol fission per anhydro-galacturonic acid unit : 15.9 %.

REDUCTION OF PERIODATE-OXIDIZED AMMONIUM PECTATE

Periodate-oxidised ammonium pectate (14 g.) was dissolved in water (150 ml.), and potassium borohydride (8 g.) in water (50 ml.) was added with stirring. When the bubbling ceased, the mixture was neutralised with acetic acid and poured into a large volume of ethanol. The precipitated polysaccharide was washed with ethanol to remove the potassium acetate which is soluble, and the boric acid was eliminated by successive evaporations with small portions of methanol in vacuo. The dry material isolated weighed 11.0 g.

This material was dialysed in distilled water in order to fractionate it, and after three days, 4 g. of it remained inside the dialysis bag (A), and 7 g. had passed into the distilled water. The latter were recovered by concentrating the solution at low pressure (B).

Fraction (A) showed : $[\alpha]_D = + 41.9^\circ$ c = 1.074 % in water.

Fraction (B) showed : $[\alpha]_D = + 38.2^\circ$ c = 2.174 % in water.

Neither fraction reduced the Fehling's solution.

Chromatographic examination of fractions (A) and (B) using solvent A and aniline oxalate spray showed :

$R_{rhamnose}$	Colour	Intensity	Identity
0	Brown	Strong	Acidic material
0.42	Brown	Very weak	Galactose
1.13	Yellow	Strong	Threose
1.4	Brownish	Medium	?

In solvent E :

$R_{rhamnose}$	Colour	Intensity	Identity
0.	Brown	Strong	Unhydrolized material
0.35	Red-orange	Strong	Galacturonic acid
0.88	Yellowish	Weak	Erithronic acid?
1.23	Brown	Strong	Threonic acid
2.0	Yellowish	Weak	Glyceric acid ?
2.4	Yellowish	Very weak	?

Different sprays (sodium metaperiodate + bencidine, potassium iodide + potassium iodate + starch, silver nitrate) were used for detecting the non reducing components in chromatograms which were developed in solvent C containing D-galacturonic acid, D-threonic and glyceric acid as standards. In all cases the presence of these three acids in the hydrolysate was confirmed.

METHYLATION OF AMMONIUM PECTATE FROM SISAL

Methyl sulphate (500 ml.) and sodium hydroxide (230 g.) in water (350 ml) were added to a stirred solution of ammonium pectate (20 g.) in water (350 ml.), under nitrogen, the temperature being kept below 30°. Three further additions of reagents were carried out under similar conditions. The reaction mixture was then neutralized with acetic acid and dialysed against running water from the tap. The solution was concentrated to a small volume and four further additions of reagents were carried out. The mixture was neutralized, dialysed, and the solution concentrated to a reduced volume to give partially methylated sodium pectate. Methoxyl determination was performed on the freeze-dried material obtained from 10 ml. of solution.

Methoxyl content : found = 22.6 % OMe.

The solution containing the partially methylated sodium pectate was reduced to dryness under diminished pressure and the residue was extracted with methanol to remove neutral methylated polysaccharides. The solid residue was dissolved in water (1 %) and this solution was passed through a column containing Amberlite resin IR-120 (H) to remove sodium ions. The solution was neutralized with silver oxide and evaporated to dryness under reduced pressure at 30°. The silver salts were ground to a fine powder and then refluxed with methyl iodide (150 ml.) containing 5 % dry methanol. Silver oxide (50 g.) was then added in 5 g. portions over a period of four hours, and the refluxing continued for a further four hours. Another Purdie's treatment under similar conditions was carried out, after which the silver salts were separated at the centrifuge, extracted with chloroform and alcohol. The solvents were evaporated to give a crisp solid. Another Purdie's treatment was administered to the solid, the isolation of which was carried out as above.

Recovery : 10.5 - 11.0 g.

$[\alpha]_D = + 191^\circ$ c = 0.63 % in chloroform.

Methoxyl content : 39.4 % OMe.

Uronic acid determination: Di-O-methyl-methyl-D-galacturonate anhydride: 67.15 % corresponding to Uronic acid anhydride in free polysaccharide : 67.5 %.

Hydrolysis and chromatographic examination on this material, using solvent B gave the following spots :

R_G	Colour	Intensity	Probable Identity
0.00	Pink	Strong	Uronic acids
0.67	Red-brown	Weak	tri-O-Me* Galactose
0.96	Red	Weak	Tetra-O-Me Galactose

Using solvent E for developing the chromatograms :

R_G	Colour	Intensity	Probable Identity
0.00	Pink	Strong	Uronic acids
0.26	Pink	Medium	2-O-Me GaU**
0.47	Pink	Medium	2:3 di-O-Me GaU
0.59	Pink	Weak	2:3:4 tri-O-Me GaU
0.94	Red	Weak	tetra-O-Me Galactose

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* Me = Methyl

** GaU = Galacturonic acid.

FRACTIONATION OF FULLY METHYLATED METHYL PECTATE

Fully methylated methyl pectate (3 g.) was dissolved in chloroform (50 ml.) and to this solution portions of petrol ether (40° - 60°) (5 or 10 ml.) were added. There resulted a turbidness, and a flocculent precipitate separated out. This was centrifuged, separated from the supernatant, washed with petrol ether and finally dried in vacuo over paraffin wax. To the supernatant liquid a new portion of petrol ether was added and the whole process repeated. Fourteen fractions were collected in this way, the weights of which are recorded in the table below, together with rotations and uronic acid contents :

Fraction	Petrol ether added (ml.)	Weight (mg.)	$[\alpha]_D$	c g/100 ml. $CHCl_3$	Di-O-methyl, methyl galact-uronate (anhydride) %
1	15	227	+ 209°	0.2 %	-
2	5	85	-	-	-
3	5	28	-	-	-
4	5	50	-	-	-
5	5	56	-	-	-
6	10	118	-	-	-
7	10	694	+ 211°	0.5 %	81.5 %
8	10	209	-	-	-
9	10	140	-	-	-
10	10	140	+ 197°	0.25	-
11	10	130	+ 187°	0.45	-
12	10	95	-	-	-
13	10	12	-	-	-
14	10	600	+ 177°	0.5	98.8 %
Total	125 ml.	2584 mg.			

The methylated methyl pectate, which is insoluble in a mixture of petrol ether ($40^\circ - 60^\circ$) and chloroform (2; 1) (concentration : 2 %), was used in subsequent experiments.

PURIFICATION OF FULLY METHYLATED METHYL PECTATE

Methylated methyl pectate (fractions 1 to 12) was dissolved in ethanol (50 ml.). Sodium hydroxide (2 g.) in a few ml. of water was added, and after a few minutes' stirring the mixture was placed in the refigerator for eight hours. The methylated sodium pectate, in the form of a gelatinous precipitate, was separated at the centrifuge and washed thoroughly with alcohol until free from alkali, then with acetone and ether. The washings were discarded, and the solid was dissolved in water (250 ml.). The solution was then passed through a column containing Amberlite resin IR-120 (H) and neutralized with silver oxide. The silver salt of methylated pectic acid was converted into the methyl ester by refluxing with methyl iodide. The methyl ester was submitted to one Purdie's treatment and isolated in the usual way as a crisp solid weighing 1.5 g.

Methoxyl content : 40.3 %

Uronic acid anhydride content in free polysaccharide : 90.3 % by the Kaye and Kent method.

$[\alpha]_D = + 212.5^\circ$ $c = 0.4$ % in chloroform.

Chromatographic examination of this material using solvent B gave evidence of two spots :

R_G	Colour	Intensity	Probable Identity
0.00	Pink	Strong	Uronic acids
0.94	Red	Weak	Tetra-O-Me galactose

Reduction of fully methylated methyl pectate (A)

Fully methylated methyl pectate (OMe % = 39.2 %) (1 g.) was dissolved in water (20 ml.) and potassium borohydride (1.5 g.) in water (10 ml.) was added. The mixture was stirred for ten hours, then acidified with acetic acid and deionized by treatment with Amberlite resins IR-120 (H) and IR 4B (OH). The solution was concentrated to a syrup under reduced pressure. The syrup was extracted with chloroform and the extracts were evaporated to give a yellowish glassy solid.

Recovery : 0.6 g.

Equivalent weight determination : 846 corresponding to a uronic acid anhydride (di-O-methyl-) content of : 24.1 %.

Reduction of fully methylated methyl pectate (B)

Fully methylated methyl pectate (6 g.) was dissolved in water (100 ml.) and potassium borohydride (3 g.) was added in small portions with stirring. The mixture was allowed to stand for two days at room temperature. At the end of this period, the mixture was acidified with acetic acid and three additions of Haworth's reagents were carried out under nitrogen with stirring. The product of methylation was isolated by dialysis in a cellophane bag against distilled water. The solution was concentrated under reduced pressure to give a glassy solid which was partially soluble in acetone or alcohol. The aqueous solution of this solid was passed through a column containing Amberlite resin IR-120 (H) and then poured into a large volume of acetone. The white precipitate which separated was collected at the centrifuge, dissolved in a little water, and the solution freeze-dried. Fraction A

recovery : 2.0 g.

The acetonic solution or supernatant was evaporated at room temperature under reduced pressure and the residue taken up in a little water. This solution was freeze-dried. Fraction : B recovery : 2.5 g.

Fraction	Methoxyl %	Equivalent Weight	Uronic acid anhydride (free polys.)	Di-O-methyl uronic acid anhydride
A	22.1 %	450	45.7 %	45.4 %
B	24.2 %	585	35.2 %	34.9 %

Reduction of fully methylated methyl pectate (C)

Fully methylated methyl pectate (2.2 g.) was reduced with potassium borohydride (1.0 g.) in 30 % aqueous methanol at room temperature. The mixture was allowed to stand overnight, and then treated with Amberlite resin IR-120 (H) in order to remove potassium ions. The solution containing partially reduced methylated pectic acid and boric acid was neutralised with silver oxide. After centrifuging, the solution was evaporated to dryness under reduced pressure, the residue was taken up in a little methanol and refluxed with methyl iodide (30 ml.). The solvent was removed by distillation and the silver salts by dissolving the residue in methanol and centrifuging. The solution containing the methyl ester of a partially reduced and methylated pectic acid was reduced with potassium borohydride as before. The entire process was repeated once more. Recovery : 1.7 g.

Equivalent weight : 815 corresponding to 24.9 % of uronic acid anhydride in the free polysaccharide.

Reduction of fully methylated methyl pectate. (D) (104)

Fully methylated methyl pectate (2.2 g) was added to an ice-cold solution of potassium borohydride (0.25 g.) and soldium (0.002 g.) in dry methanol (30 ml.) placed in a conical flask fitted with a magnetic stirrer and calcium chloride tube. The stirring was continued until the methylated methyl pectate went into solution. The contents of the flask were then allowed to stand at room temperature for 18 hours. The alcohol was evaporated with a stream of air, while the residue was dissolved in water, and this solution was passed through a column containing Amberlite resin IR-120 (H). The solution and eluates were freeze-dried and the solid residue was dissolved in methanol and evaporated under reduced pressure. This treatment with methanol was repeated until no positive test for boric acid with turmeric paper was obtained. The aqueous solution of the residue was passed through a column containing a mexture of resins Amberlite IR-120 (H) and Duolite A4 (5 ml.), the liquids being concentrated to a thick syrup under reduced pressure.

Recovery : 1.8 g.

Uronic acid anhydride content : 55.0 % (by the Kaye and Kent method).

Reduction of fully methylated methyl pectate (E)

Fully methylated methyl pectate (2.2 g) was dissolved in tetrahydrofuran (150 ml.), and lithium aluminium hydride (0.5 - 0.7 g.) in a few ml. of the same solvent, was added. The mixture was refluxed for one to two hours. The solvent was partially recovered after allowing the reaction mixture to stand for two hours. The contents of the flask were cooled and ethyl acetate (20 ml.) was added drop-wise through the reflux condenser. When the violent evolution of gas ceased, the solvents were removed under reduced pressure and water (50 ml.) was added to replace them. In order to isolate the reduced, partially methylated pectic acid (galactan) various procedures were used unsuccessfully to obtain a characteristic product.

A) The aluminium hydroxide was extracted with alcohol.

Recovery : 0.7 g. (syrup) $[\alpha]_D = +49^\circ$ c = 1 % in water.

B) The solid mixture of polysaccharide and aluminium salts was extracted with dimethylformamide, the extracts were evaporated in vacuo (1 mm.) and the residue extracted with acetone.

Recovery : 0.9 g. (brown syrup) $[\alpha]_D = +14^\circ$ c = 1 % water.

C) The aluminium salts were extracted with n-butanol. The solvent was evaporated and the residue dissolved in water. The solution was filtered and heated to 100° in order to flocculate the partially methylated polysaccharide, which was filtered and dissolved in water. Its solution was freeze-dried.

Recovery : 0.6 g. (solid) $[\alpha]_D = +75^\circ$ c = 0.9 % in water.

D) The aqueous suspension of the solid salts was shaken with an excess of Amberlite resin IR-120 (H) for one hour, the solids removed by filtration, and the solution evaporated under reduced pressure at 30°.

Recovery : 0.87 g. $[\alpha]_D = +42^\circ$ c = 1.8 % in water.

HYDROLYSIS OF FULLY METHYLATED METHYL PECTATE WITH FORMIC ACID AND REDUCTION

Fully methylated methyl pectate (1 g.) was dissolved in formic acid (10 ml., 98 %) and refluxed on a boiling water bath for ten hours. The solvent was evaporated under reduced pressure, water was added to the syrup and then evaporated. This treatment was repeated twice in order to remove traces of formic acid. The residue left after the final evaporation was dissolved in a few ml. of a methanol-acetone mixture (1 : 10) and this solution was cooled at 0° and neutralized with an ethereal solution of diazomethane, then allowed to stand in the refrigerator for twelve hours. The solvents were removed under reduced pressure and the resulting syrup was refluxed with 50 ml. of 1 % methanolic hydrogen chloride for seven hours. It was then neutralized with an ethereal solution of diazomethane, the solution being concentrated in vacuo. The syrup was dissolved in anhydrous ether (50 ml.) and lithium aluminium hydride (1.2 g.) was added. The refluxing of the mixture was carried out over a period of four hours. After cooling the contents of the flask, ethyl acetate (10 ml.) was added to destroy the excess of lithium aluminium hydride. Water was then added, the mixture was acidified with 2N sulphuric acid, and aluminium ions were precipitated with barium hydroxide solution. Barium and lithium ions were removed as carbonates by passing CO_2 through the solution and by centrifuging. Evaporation of the solution yielded a syrup which was extracted with boiling chloroform. Elimination of the solvent in vacuo left a syrup which was refluxed with methanolic hydrogen chloride and reduced with lithium aluminium hydride a second time, as above. Recovery : 0.41 g.

Chromatographic examination of the syrup in solvent *B* showed the following spots :

R_G	Colour	Intensity	Probable identity
0.12	Brown	Weak	mono-O-methyl galactose
0.47	Brown	Very strong	2:3 di-O-methyl galactose
0.67	Brown	Weak	tri-O-methyl galactose ?
0.76	Red brown	Weak	tri-O-methyl galactose
0.9	Red	Weak	tetra-O-methyl galactose

Partial methanolysis of fully methylated methyl pectate and subsequent reduction.

Fully methylated methyl pectate (2 g.) ($[\alpha]_D$ = + 210.6, OMe % = 42.0) was dissolved in dry methanol (40 ml.), and methanolic hydrogen chloride (10 ml. 25 %) was added, resulting in a solution which contained 5 % of HCl. The mixture was placed in a Carius tube and sealed. The tube was maintained at 115° for eighteen hours. After cooling the contents of the tube, they were neutralized with dry silver carbonate. The silver salts were removed by filtration, and the solution was taken to dryness under reduced pressure. The syrupy residue was dissolved in dry acetone, and the solvent evaporated in vacuo. The addition of dry acetone was repeated as well as the filtration in order to eliminate the colloidal silver salt which is coagulated in that way. Finally the solution was evaporated and dried to constant weight.

Recovery : 1.59 g.

Reduction: The syrup was dissolved in dry tetrahydrofuran (50 ml.) and a solution of Lithium aluminium hydride (0.7 g.) in tetrahydrofuran (15 ml.) was added drop-wise through the reflux condenser.

After standing half an hour at room temperature, the mixture was heated in a water bath for a further half hour, and then cooled. The excess of reducing agent was destroyed with ethyl acetate after recovering most of the tetrahydrofuran. Water was then added to the reaction mixture and the solvents eliminated under reduced pressure. The sugars were extracted with dry acetone from the solid residue.

Recovery : 1.45 g.

Hydrolysis: The syrupy material left after the evaporation of the solvent was refluxed in 1 N hydrochloric acid (15 ml.) for four hours in a boiling water bath, after which the solution was neutralized with silver carbonate, and deionized with ion-exchange resins.

Recovery : 1.19 g.

Chromatographic examination of the syrup using solvent C as mobile phase gave the following spots :

R_G	Colour	Intensity	Probable Identity
0.04	Brown	Medium	mono-O-methyl galactose
0.203	Brown red	Very strong	2:3 di-O-methyl galactose
0.377	Red	Medium	tri-O-methyl galactose ?
0.471	Red	Weak	tri-O-methyl galactose
0.751	Yellow	Weak	di-O-methyl rhamnose
0.984	Red	Weak	tetra-O-methyl galactose

METHANOLYSIS OF FULLY METHYLATED METHYL PECTATE AND SUBSEQUENT REDUCTION

(Large scale experiment on a product made from commercial sodium pectate)

Fully methylated methyl pectate (6 g.), divided into two batches of three grammes each, was subjected to methanolysis for seven hours in a Carius tube with methanolic hydrochloric acid (50 ml., 5 %). The temperature in the oven in which the tubes were placed was maintained at 118°. At the end of the heating period, the contents of the tube were cooled, combined and neutralised with an ethereal solution of diazomethane. The resulting solution was evaporated down to a syrup under reduced pressure.
Recovery : 4.54 g.

Saponification of the syrup was carried out in aqueous solution of sodium hydroxide (50 ml., 4 %), heating the reaction mixture in a water bath at 60° for two hours. The fully methylated volatile neutral glycosides were removed from the alkaline solution by continuous extraction for 24 hours with petroleum ether (40° - 60°). The sugars contained in the petrol ether solution were recovered by evaporation at room temperature in vacuo.

Recovery : 0.0711 g. <u>Fraction A.</u>

The aqueous alkaline solution was passed through columns containing Amberlite resins IR-120 (H) and IR-4B (OH). The eluent was evaporated at 30° in vacuo.

Recovery : 3.5134 g. <u>Fraction B.</u>

The acidic material absorbed in the resin Amberlite IR-4B (OH) was eluted with N sodium hydroxide and the free acid regenerated immediately with Amberlite resin IR-120 (H). The aqueous solution was then concentrated to a syrup, which was esterified with an acetone-ether (1 : 10) solution of diazomethane.

Recovery : 0.7725 g. *Fraction C.*

Fraction A : This fraction was hydrolysed with N HCl for four hours over a boiling water bath. The solution was neutralized with silver carbonate, filtered and evaporated to a syrup.

Recovery : 55 mg.

Chromatographic examination in solvent *C* showed :

R_G	R_f	*Colour*	*Intensity*	*Probable identity*
0.62	0.55	Pink	Weak	tri-O-methyl galactose
0.71	0.63	Red	Weak	tri-O-methyl galactose ?
0.84	0.73	Brown orange	Medium	?
0.92	0.81	Red	Strong	tetra-O-methyl galactose
0.99	0.88	Pink red	Strong	?
1.03	0.91	Grey	Medium	tri-O-methyl arabinose

Fraction A was not examined further.

Fraction B : Chromatographic examination of the hydrolysate of this fraction, using solvent *C*, gave the following results :

R_G	R_f	*Colour*	*Intensity*	*Probable identity*
0	0	Pink	Strong	Uronic acids
0.19	0.16	Brown	Weak	di-O-methyl galactose
0.53	0.45	Red brown	Weak	tri-O-methyl galactose ?
0.78	0.65	Brown orange	Medium	?

Fraction C : Examination of this fraction showed that no neutral sugars were present in its hydrolysate.

Reduction of Fractions B and C : Fractions B and C were combined, and the mixture was treated with methanolic hydrochloric acid (50 ml. 1 %) for five hours under reflux. The solution was neutralized with dry silver carbonate, filtered and evaporated to a syrup which was dissolved in tetrahydrofuran (50 ml.). To this solution, placed in a flask fitted with a reflux condenser, a solution of lithium aluminium hydride (1.0 g.) in tetrahydrofuran (10 ml.) was added drop-wise.

The reaction mixture was heated over a boiling water bath for one hour after having been left to stand for half an hour at room temperature. After destroying the excess of lithium aluminium hydride with ethyl acetate, the solvents were removed, and water was added to the mixture. The water was then evaporated in vacuo, and the reaction product recovered by extraction with acetone and dry methanol. The solution was taken to dryness and dissolved in dry methanol in order to eliminate the aluminium hydroxide. Finally the syrup was dissolved in water and treated with Amberlite IR-120 (H), and the solution evaporated in vacuo at 30°.

Recovery : 4.0794 g.

Hydrolysis : The syrup thus obtained was hydrolysed with N sulphuric acid for six hours over a boiling water bath. The brown solution was then neutralized with barium carbonate and filtered, and barium ions were removed by treatment with Amberlite resins.

Recovery : 3.7847 g.

FRACTIONATION OF METHYLATED SUGARS

The mixture of methylated sugars isolated after hydrolysis (3.7847 g.) was fractionated on a cellulose column (70 x 3.5 cm.), methylethyl ketone two thirds saturated with water containing 1 % ammonia being used as an eluent. Four fractions were collected:

Fraction	Tubes	Weight (mg.)	R_G	Colour	Probable Identity
I a.	1- 10	64.1	1.01	Yellow	Tri-O-methyl rhamnose
b.			0.88	Yellow	Di-O-methyl rhamnose
c.			0.78	Orange	A
II a.	11- 28	762.8	0.91	Red	Tetra-O-methyl galactose
b.			0.88	Yellow	Di-O-methyl rhamnose
c.			0.78	Orange	A
d.			0.72	Brown	Tri-O-methyl galactose
e.			0.64	Red	Tri-O-methyl galactose
f.			0.50	Brown	Di-O-methyl galactose
III	29- 79	2144.1	0.5	Brown	2:3 di-O-methyl galactose
IV a.	80-157 + washings	223.5	0.5	Brown	2:3 di-O-methyl galactose
b.			0.34	Brown	di-O-methyl galactose ?
c.			0.25	Brown	Mono methyl galactose
d.			0.2	Brown	Mono methyl galactose

Total recovered: 3.1945 g. = 84.5 % recovery.

<u>FRACTION I</u> was refractionated on thick paper using solvent B.

Fraction Ia. R_G = 1.01 (16.1 mg.) was identified as 2:3:4 tri-O-methyl-L-rhamnose by oxidation to the corresponding aldonic acid, and the formation of the phenylhydrazide : M.P. : 175 - 177°. Authentic specimen has an M.P.: 177°.(105)

Fraction Ib: R_g = 0.88 (18.7 mg.), was identified by the formation of the lactone of 3:4 di-O-methyl-L-rhamnonic acid: M.P. : 76 - 79°. C.f.: 76 - 78°.[106]

Fraction Ic: R_g = 0.78 (29.0 mg.). Demethylation of this sugar (A) gave galactose.

FRACTION II (762.8 mg.) This fraction was hydrolyzed with N HCl for six hours on a boiling water bath. After neutralisation with silver carbonate and evaporation of the solution, 619.8 mg. were recovered and fractionated in a cellulose column (70 x 2.5 cm.) using as an eluent n-butanol, two thirds saturated with water.

Sub-fraction	Tubes	Weight (mg.)	R_g	Colour	Probable Identity
a.	1- 78	65.6	0.88	Yellow	di-O-methyl rhamnose
b.	79- 94	45.2	0.88 0.73	Yellow Orange	di-O-methyl rhamnose A
c.	95-110	12.9	0.73 0.70	Orange Red Brown	A tri-O-methyl galactose
d.	111-120	8.7	0.70	Red	tri-O-methyl galactose
e.	121-149	112.0	0.70 0.63	Red brown Brown	tri-O-methyl galactose tri-O-methyl galactose
f.	150-189	277.7	0.40	Brown	2:3 di-O-methyl galactose
g.	190-290	11.6	0.40 0.38	Brown Brown	2:3 di-O-methyl galactose di-O-methyl galactose ?
h.	231-329	60.5	0.40 0.26	Brown Brown	di-O-methyl galactose ? mono-O-methyl galactose

Total recovered : 594.2 mg. equivalent to a 96 % recovery.

Fraction IIa: R_G = 0.88 (65.6 mg.)

$[\alpha]_D$ = + 18.85° c = 1.75 % in water.

Oxidation with bromine water of this fraction yielded the lactone of 3:4 di-O-methyl-L-rhamnonic acid, which was recrystallised from acetone-petrol ether.

M.P. : 76 - 78°. quoted in the literature : 76 - 78°.(106)

Fraction IIb: R_G = 0.88 and 0.73 (45.2 mg.), was refractionated in thick paper using solvent B, and the relative proportions of the components as deduced from the weight recovered on elution of the chromatograms were as follows:

3:4 di-O-methyl-L-rhamnose	R_G = 0.88	5 mg.
A	R_G = 0.73	40 mg.

Fraction IIc: R_G = 0.78 (12.9 mg.)

The main component of this fraction was the sugar (A) giving an orange colour with aniline oxalate spray (bright yellow under U.V. light). It was used for demethylation experiments which showed the presence of galactose only. In subsequent experiments it showed, upon hydrolysis 2:3 di-O-methyl-D-galactose.

Fraction IId: R_G = 0.7 (8.7 mg.) crystallized on standing, and was recrystallized from alcohol M.P.: 75 - 78°. An X-ray powder photograph, using the crystalline sugar, showed identity with 2:3:4 tri-O-methyl-D-galactose.

Fraction IIe: R_G = 0.7 and 0.63 (112 mg.).

Demethylation gave galactose.

Chromatography, using solvent C, indicated three components, the weights of which were determined by the hypoidite method.(100)

R_G	Colour	Identity	Weight
0.63	Pink red	2:3:4 tri-O-methyl-D-galactose	19.3 mg.
0.55	Brown	2:3:6 tri-O-methyl-D-galactose	86.6 mg.
0.45	Brown	tri-O-methyl galactose ?	6.1 mg.

Treatment of the syrup with aniline gave a mixture of N-phenyl-glycosylamines from which a small amount of 2:3:4 tri-O-methyl-N-phenyl-D-galactosylamine could be isolated. M.P.: 163 - 165°.

2:3:6 tri-O-methyl-D-galactose was identified chromatographically by comparison with the authentic specimen.

Fraction IIf: R_G = 0.48 (277.7 mg.), was characterised as 2:3 di-O-methyl-D-galactose by its conversion into the corresponding N-phenyl-galactosylamine. M.P.: 153 - 155°.

Fraction IIg: R_G = 0.48 and 0.38 (11.6 mg.)
Chromatograms developed in solvent B, using this fraction and authentic specimens of 3:4 di-O-methyl-D-galactose and 2:3 di-O-methyl-D-galactose, showed the identity in the travelling rates. Demethylation gave only galactose.

Fraction IIh: R_G = 0.40 and 0.26 (60.5 mg.)
Two main components were travelling at the same rate as 2- and 3-O-methyl-D-galactoses (0.26) together with traces of another sugar (0.40). Chromatograms were run in n-butanol-ethanol-water (10-3-5) and sprayed with

p-anisidine hydrochloride. Three spots appeared in the chromatograms:

$R_{galactose} = 1.27$	Yellow green	corresponding to 3-O-methyl-D-galactose.
$R_{galactose} = 1.73$	Purple	corresponding to 2-O-methyl-D-galactose.
$R_{galactose} = 2.24$	Purple	unidentified.

The first two components are present in nearly the same proportions as inferred from the chromatograms. The third is present in small quantities.

FRACTION III $R_g = 0.48 - 0.5$ (2.1441 g.)

Chromatographic examination of this fraction showed it to be nearly pure 2:3 di-O-methyl-D-galactose.

$[\alpha]_D = + 81.2^\circ$ c = 0.493 % in water.

Methoxyl content: 29.4 %.

Further evidence as to the identity of this fraction was ascertained by the conversion of the sugar into the corresponding N-phenyl-galactosylamine.

Melting point: 153 - 155°. The sugar was transformed into the corresponding aldonic acid with bromine water, which yielded, following lactonisation and treatment with methanolic ammonia, 2:3 di-O-methyl-D-galactonoamide M.P.: 139 - 140°, undepressed on admixture with an authentic specimen.

FRACTION IV $R_g = 0.5, 0.34, 0.25, 0.2$ (223.5 mg.)

On standing this fraction partially crystallised. The crystals were separated on a tile, and recrystallised from ethanol and proved to be 2-O-methyl-D-galactose M.P.: 155 - 156°. Mixed M.P.: 154 - 156°. Periodate oxidation of the original syrup and chromatography of the oxidation products(99) gave evidence of the presence of 2-O-methyl-D-galactose by the yellow spot R_f = 0.22 (solvent D) due to methoxy malon-dialdehyde, and of 3-O-methyl-D-galactose by the wine red spot R_f = 0.4 of 2-methyl-D-xylose.

Summary of the fractionation of methylated sugars from reduction and hydrolysis of methylated methyl pectate:

Sugar	Column I	Column II (x 1,288)	Total	Milli-moles	Moles %
2:3:4 tri-O-methyl-Rhamnose	16.1	...	16.1	0.077	0.5
3:4 di-O-methyl-Rhamnose	18.7	91.0	109.7	0.572	3.8
A (Yellow orange spot)	29.0	67.2	96.2	0.463	3.0
2:3:4 tri-O-methyl-Galactose	...	30.2	30.2	0.136	0.9
2:3:6 tri-O-methyl-Galactose	...	90.2	90.2	0.406	2.7
(0.64) incompletely identified	...	36.1	36.1	0.162	1.1
2:3: di-O-methyl-Galactose	2166.4	300.0	2466.4	11.870	78.3
3:4 di-O-methyl-Galactose ?	22.3	15.2	37.5	0.179	1.2
mono-methyl-Galactose	178.4	70.0	248.4	1.282	8.5
Total	2430.9	699.9	3130.8	15.147	

METHANOLYSIS OF METHYLATED METHYL PECTATE AND SUBSEQUENT REDUCTION.

(from ammonium pectate extracted from sisal flesh with ammonium oxalate: BATCH A)

Methylated methyl pectate (5 g.) was partially methanolyzed with 5 % methanolic HCl (100 ml.) in a Carius tube at 100° for a period of four hours. The contents of the tube were neutralized with dry silver carbonate, and filtered, after which the solvent was evaporated under reduced pressure to a syrup which was dissolved in tetrahydrofuran (150 ml.). The solution was refluxed gently in a water bath while a solution of lithium aluminium hydride (2 g.) in tetrahydrofuran (50 ml.) was added drop-wise through the reflux condenser. After refluxing for one hour, the mixture was allowed to stand for two hours at room temperature. The excess of reducing agent was eliminated with ethyl acetate, water was added to the mixture, and the solvents evaporated in vacuo. The residue was extracted several times with acetone and alcohol and the extracts were evaporated to dryness. Water was then added and the solution was treated with Amberlite resins IR-120 (H) and IR 4B (OH). The solution was evaporated near to dryness, and the residue hydrolyzed with N HCl for five hours on a boiling water bath. After neutralization of the mixture with silver carbonate and treatment with H_2S, the solution was evaporated to a thick syrup which contained methylated sugars.

Recovery : 3.6611 g.

FRACTIONATION OF THE METHYLATED SUGARS The syrup containing the methylated sugars was fractionated in a cellulose column (70 x 3.9 cm.) by using methylethylketone two-thirds saturated with water as an eluent. After collecting 245 tubes and washing the column through with water the recovery was 3.2379 g., equivalent to 88.3 %.

Only one fraction (tubes 90 - 170), which proved to be pure 2:3 di-O-methyl-D-galactose (1.9981 g.), was characterized.

$[\alpha]_D = + 80^\circ$ c = 0.63 % in water.

Methoxyl content: 29.9 %.

Treatment of this sugar with aniline furnished the 2:3 di-O-methyl-N-phenyl-D-galactosylamine M.P.: 154 - 155°. Mixed M.P.: unchanged.

The remaining fractions, which were mixtures, were combined (1.2398 g.) and refractionated in a cellulose column (70 x 2.5 cm.) with n-butanol and petrol ether (100-120°) (30: 70) as mobile phase.

The following table gives an account of the fractionation:

Fraction	Tubes	Weight (mg.)	R_g	Colour	Probable identity
1	1- 25	22.6	1.01	Yellow	Tri-O-methyl-rhamnose
2	26- 60	134.1	0.95 0.90	Grey Red	Tri-O-methyl-arabinose Tetra-O-methyl-galactose
3	61- 80	18.9	0.92	Red	Tetra-O-methyl-galactose
4	81-120	16.0	0.92 0.88	Red Yellow	Tetra-O-methyl-galactose Di-O-methyl-rhamnose
5	121-145	20.7	0.90 0.88 0.50	Red Yellow Brown	Tetra-O-methyl-galactose Di-O-methyl-rhamnose 2:3 di-O-methyl-galactose
6	146-220	49.7	0.90 0.88 0.78	Red Yellow Orange yellow	Tetra-O-methyl-galactose Di-O-methyl-rhamnose A
7	221-294	42.0	0.78	Orange yellow	A
8	295-419	95.1	0.78 0.72	Orange yellow Brown	A Tri-O-methyl-galactose
9	420-444	17.7	0.70	Red brown	Tri-O-methyl-galactose
10	445-500	46.9	0.72 0.69	Brown Red	Tri-O-methyl-galactose Tri-O-methyl-galactose
11	501-535	53.9	0.76 0.64	Red brown Brown	Tri-O-methyl-galactoses Tri-O-methyl-galactose ?
12	536-580	64.8	0.64 0.50	Brown Brown	Tri-O-methyl-galactose ? 2:3 di-O-methyl-galactose
13	581-625	38.4	0.56	Brown Redish	2:3 di-O-methyl-galactose 2:4 di-O-methyl-galactose
14	626-670	31.4	0.50 0.42	Brown Grey brown	2:3 di-O-methyl-galactose Unknown
15	671-725	26.0	0.88 0.70 0.43 0.38	Yellow (traces) Brown Red brown Redish (traces)	Di-O-methyl-rhamnose Tri-O-methyl-galactose Unknown Di-O-methyl-galactose ?
16	726-776	12.5	0.41	Grey brown	Unknown
17	777-868 + washings	319.6	0.25	Brown	2-O-methyl-galactose 3-O-methyl-galactose

Total . . . . 1010.2 - equivalent to an 81.5 % recovery.

(Loss in fraction No. 7 due to an electrical cut which paralysed the turn-table)

Fraction 1: $R_g = 1.01$ (22.6 mg.)

$[\alpha]_D = + 24.8^\circ$ c = 0.2 % in water.

Authentic 2:3:4 tri-O-methyl-L-rhamnose : $[\alpha]_D = + 26^\circ$ (water).(107)

Demethylation gave rhamnose.

One attempt to make the 2:3:4 tri-O-methyl-L-rhamnonolactone in order to obtain the crystalline derivative proved unsuccessful.

Fraction 2: $R_g = 0.95$ and 0.90 (134.1 mg.)

$[\alpha]_D = + 48.7^\circ$ c = 1.6 % in water.

Demethylation performed on this fraction indicated the presence of arabinose and galactose.

The optical rotation corresponds to a mixture of:

2:3:5 tri-O-methyl-L-arabinose ($[\alpha]_D = - 39.5^\circ$) (108)	50.4 mg.
2:3:4:6 tetra-O-methyl-D-galactose ($[\alpha]_D = + 109.5^\circ$) (109)	83.7 mg.

Fraction 3: $R_g = 0.92$ (18.9 mg.)

$[\alpha]_D = + 108^\circ$ c = 0.18 % in water.

Methoxyl content: 49.8 %.

The sugar was characterized by conversion into the 2:3:4:6 tetra-O-methyl-N-phenyl-D-galactosylamine M.P.: 187 - 190°. Mixed M.P.: 188 - 191°.

Fraction 4: $R_g = 0.92$ and 0.88 (16.0 mg.)

$[\alpha]_D = + 54.7^\circ$ c = 0.8 % in water.

Demethylation gave evidence of galactose and rhamnose derivatives.

The optical rotation corresponds to a mixture of :

2:3:4:6 tetra-O-methyl-D-galactose ($[\alpha]_D = + 109.5^\circ$) (109)	6.0 mg.
3:4 di-O-methyl-L-rhamnose ($[\alpha]_D = + 18.5^\circ$) (110)	10.0 mg.

Fraction 5: R_g = 0.9, 0.88 and 0.50 (20.7 mg.)

This fraction was refractionated on thick paper (Benzene: ethanol: water:: 169:47:15) and the proportions of the different components were obtained by the hypoiodite method(100):

Sugar	Weight in fraction
2:3:4:6 tetra-O-methyl-D-galactose	5.4 mg.
3:4 di-O-methyl-L-rhamnose	11.4 mg.
2:3 di-O-methyl-D-galactose	4.1 mg.

Fraction 6: R_g = 0.9, 0.88 and 0.78 (49.7 mg.)

The mixture was resolved on thick paper using benzene:ethanol:water (169:47:15)

The proportions of the sugars were estimated by the hypoiodite method(100):

Sugar	Weight in fraction
2:3:4:6 tetra-O-methyl-D-galactose	16.1 mg.
3:4 di-O-methyl-L-rhamnose	29.0 mg.
(0.78) Sugar A	4.4 mg.

The N-phenyl-glycosylamine of the tetra-O-methyl-D-galactose was prepared, the melting point of which was 189 - 191°. Authentic specimen: 190 - 191°.(116)(117)

The di-O-methyl-L-rhamnose was identified as the 3:4 di-O-methyl-L-rhamnose by ionophoretic examination on filter paper.

Fraction 7: R_g = 0.78 (42.0 mg.)

$[\alpha]_D = +32 \pm 4^\circ$ c = 0.38 % in water.

Upon demethylation this fraction showed galactose. The colour of the sugar with aniline oxalate spray is orange brown, and bright yellow under ultra-violet light. Upon hydrolysis with N HCl for four hours, this fraction gave 2:3 di-O-methyl-D-galactose and some monomethylgalactose. (Chromatography in solvent B).

Fraction 8: R_S = 0.78 and 0.72 (95.1 mg.)

$[\alpha]_D = + 36.3 \pm 1°$ c = 1.9 % in water.

Upon demethylation this fraction gave galactose. The proportions of the two components were calculated from the rotation:

R_S = 0.78 (Sugar A) ($[\alpha]_D = + 32^{0}$) ... 86.5 mg.

R_S = 0.72 (2:3:6 tri-O-methyl-D-galactose) ($[\alpha]_D = + 80^{0}$)(118) ... 8.6 mg.

Attempts to characterize the 2:3:6 tri-O-methyl-D-galactose were unsuccessful.

Fraction 9: R_S = 0.70 (17.7 mg.)

$[\alpha]_D = + 111.2^{0}$ c = 0.354 % in water.

Methoxyl content: 40.5 %.

The 2:3:4 tri-O-methyl-N-phenyl-D-galactosylamine was prepared for identification purposes. M.P.: 163 - 165°. Mixed M.P.: unchanged.

Fraction 10: R_S = 0.72 and 0.69 (46.9 mg.)

$[\alpha]_D = + 85.5^{0}$ c = 0.61 % in water.

Methoxyl content: 39.7 %.

Preparation of the 2:3:6 tri-O-methyl-D-galactonolactone gave a crystalline solid M.P.: 98 - 99° after two sublimations. Cf. (97 - 98°)(112) (99°)(111).

The optical rotation corresponds to a mixture of:

R_G = 0.72 (2:3:6 tri-O-methyl-D-galactose) ($[\alpha]_D$ = + 80°)(118) ...41.7 mg.

R_G = 0.69 (2:3:4 tri-O-methyl-D-galactose) ($[\alpha]_D$ = + 114.5°)(113) ... 5.2 mg.

Fraction 11: R_G = 0.76 and 0.64 (53.9 mg.)

$[\alpha]_D$ = + 86 ± 2° c = 0.9 % in water.

Demethylation on this fraction gave galactose.

The proportion of each of the two components was ascertained by hypoiodite estimation(100) (solvent B).

R_G = 0.76 (2:3:6 tri-O-methyl-D-galactose)	24.5 mg.
R_G = 0.64 (tri-O-methyl galactose ?) (incompletely identified)	29.4 mg.

Fraction 12: R_G = 0.72 (traces), 0.64 and 0.50 (64.8 mg.)

Two main components were present, together with traces of 2:3:6 tri-O-methyl-D-galactose. Demethylation showed that this fraction contained galactose derivatives. Resolution of the mixture on paper, in solvent C, and hypoiodite estimation(100) of the sugars gave :

R_G = 0.64 (incompletely identified)	45.8 mg.
R_G = 0.50 {2:3 di-O-methyl-D-galactose plus 2:6 di-O-methyl-D-galactose ?	19.0 mg.

Chromatography of the periodate oxidation products(99) was consistent with the presence of 2:3 and 2:6 di-O-methyl-D-galactoses.

Fraction 13: R_G = 0.56 (38.4 mg.)

$[\alpha]_D$ = + 83.3° c = 0.67 % in water.

This fraction showed, upon chromatographic examination in solvent C :

R_G = 0.2 (2:4 di-O-methyl-D-galactose)

R_G = 0.3 (2:3 di-O-methyl-D-galactose, possibly with some 2:6 di-O-methyl

R_G = 0.45 (traces of disaccharide 0.78)

Periodate oxidation of the mixture and chromatographic examination of the oxidation products(99) using solvent *B*, gave the characteristic canary yellow spot indicating the presence of 2:6 di-O-methyl-D-galactose, plus unchanged 2:4 and 2:3 di-O-methyl galactoses, plus the pattern of spots corresponding to the latter. It was possible to prepare and separate from this mixture the 2:4 di-O-methyl-N-phenyl-D-galactosylamine by taking advantage of its relative insolubility in comparison with the others. M.P.: 208 - 209.5°. *Cf*: (209 - 210°) (115)

Fraction 14: R_G = 0.72 (traces), 0.50 and 0.42 (31.4 mg.)

$[\alpha]_D$ = + 81.2° c = 0.43 % in water.

Traces of 2:3:6 tri-O-methyl-D-galactose were discovered with the principal component of this fraction (R_G 0.50). This was identified by preparing the 2:3 di-O-methyl-N-phenyl-D-galactosylamine M.P.: 152 - 154°. *Cf*: (153 - 154°).(114)

Fraction 15: R_G = 0.88, 0.70, 0.43 and 0.38 (26.0 mg.)

The main components in this fraction were the sugars with R_G = 0.88 (di-O-methyl rhamnose) and 0.43 (2:4 di-O-methyl-galactose ?). Periodate oxidation and chromatography of the oxidation products(99) gave the typical canary yellow spot indicative of a 2-O-methyl sugar.

Examination of this fraction in solvent *C* gave the following spots:

R_G	Colour	Intensity	Identity	Visual proportion
1.01	Yellow	Strong	Di-O-methyl-rhamnose	2
0.48	Red brown	Medium	Tri-O-methyl-galactose	1
0.21	Red	Strong	2:4 di-O-methyl-galactose	2
0.16	Brownish	Medium	Unknown	1

<u>Fraction 16</u>: R_G = 0.41 (12.5 mg.)

The principal component was the sugar with R_G = 0.41. A small trace of a component which gave methoxy-malon dialdehyde upon periodate oxidation was present.

<u>Fraction 17</u>: R_G = 0.25 (319.6 mg.)

Examination of this fraction in butanol-ethanol-water (10:3:5) and p-anisidine HCl spray, gave three spots:

$R_{galactose}$	Colour	Intensity	Identity	Proportion
1.75	Purple	Strong	2-O-methyl-galactose	1.5
1.48	Yellow	Strong	3-O-methyl-galactose	1
1.00	Purple	Traces	Galactose	...

Examination in methylethylketone:acetic acid:water (9:1:1), saturated with boric acid, gave the same spots as above, the $R_{galactoses}$ of which are 2-O-methyl-galactose (3.17), and 3-O-methyl-galactose (2.37).

Periodate oxidation of the mixture and chromatography of the oxidation products in solvent B gave two spots:

a yellow (canary) spot corresponding to methoxy-malon dialdehyde and indicative of 2-O-methyl sugar;

a dark brown one, corresponding to the degradation of a 3-O-methyl sugar.

Separation on thick paper, using the second solvent, gave 2-O-methyl-D-galactose crystalline mixed with some syrup. The crystals were separated on a tile. M.P.: 150 - 154°.

Estimation of the proportions was carried out by the hypoiodite method(99) on one strip of the chromatogram.

2-O-methyl-D-galactose	192.2 mg.
3-O-methyl-D-galactose	113.4 mg.
Galactose	14.0 mg.

SUMMARY OF THE FRACTIONATION OF METHYLATED SUGARS FROM THE HYDROLYSIS OF REDUCED METHYLATED METHYL PECTATE.

Sugar	Column I	Column II	Total (x 1.227)	Millimoles	Moles % (x 6.58)
2:3:4 tri-O-methyl-L-rhamnose	-	22.6	27.7	0.134	0.88
2:3:5 tri-O-methyl-L-arabinose	-	50.4	61.8	0.322	2.12
2:3:4:6 tetra-O-methyl-D-galactose	-	130.1	159.6	0.676	4.45
3:4 di-O-methyl-L-rhamnose	-	59.0	72.4	0.376	2.48
Sugar A	-	132.9	162.8	0.784	5.16
2:3:6 tri-O-methyl-D-galactose	-	79.1	97.0	0.436	2.87
2:3:4 tri-O-methyl-D-galactose	-	22.9	28.1	0.126	0.83
(0.64) incompletely identified sugar	-	75.2	80.0	0.360	2.37
2:3 di-O-methyl-D-galactose	1998.1	35.5	2041.7	9.768	63.62
2:6 di-O-methyl-D-galactose 2:3 di-O-methyl-D-galactose 2:4 di-O-methyl-D-galactose	-	66.0	81.0	0.389	2.56
2-O-methyl-D-galactose	-	192.2	212.0	1.092	7.18
3-O-methyl-D-galactose	-	113.4	139.2	0.717	4.72
Total			3.1633 g.	15.180	

ESTERIFICATION OF PECTIC ACID WITH ETHYLENE OXIDE(98)

Ethylene oxide (100 ml.) was added to a suspension of pectic acid (33 g.; Uronic acid anhydride content: 79.0 %, $[\alpha]_D$ = + 215°, BATCH D), in water (330 ml.), and the mixture was shaken for nine days at room temperature. At the end of this period, the esterification was complete with the formation of a viscous solution and the change of the pH from 2 to 7. The glycol ester was recovered by precipitation with acetone, and the gelatinous precipitate was washed with acetone-alcohol. Finally it was dissolved in water and the solution was freeze-dried.

Recovery : 39 g.

$[\alpha]_D$ = + 194 ± 5°   c = 0.586 % in water.

Uronic acid determination:

<u>Uronic acid anhydride content</u>: 65.1 % - equivalent to 78 % in free polysaccharide.
(decarboxylation)

<u>Equivalent weight</u>: 263 equivalent to 66.8 % uronic acid anhydride in the glycol ester, and 78.7 % in the free polysaccharide.

<u>Glycol estimation</u>: 67.5 % uronic acid anhydride equivalent to 80.9 % in the free polysaccharide.

ESTERIFICATION OF PECTIC ACID WITH PROPYLENE OXIDE

Purified pectic acid from BATCH C ($[\alpha]_D$ = 213°; uronic acid anhydride content: 74.06 % as ammonium pectate) to the amount of 5 g. was suspended in water (50 ml.), and propylene oxide (15 ml.) was added. The mixture was shaken in a stoppered bottle for eight days until the solution was neutral.

The isolation of the ester was carried out as in the case of the ethyleneglycol ester.

Recovery : 7 g.

$[\alpha]_D = + 168 \pm 2^\circ$ $c = 0.893$ % in water.

Uronic acid anhydride content: 61.7 %, corresponding to 78 % in the
(decarboxylation)
free polysaccharide.

Glycol estimation: 63.5 % uronic acid anhydride, corresponding to 79.7 % in the free polysaccharide.

Molecular weight determination on this ester by ultracentrifuge methods gave a value of 37,200. Degree of polymerization : 168 - 170 units.

$S = 1.5 \times 10^{-13}$; $D = 2.5 \times 10^{-7}$; $\bar{v} = 0.6$.

REDUCTION OF ETHYLENEGLYCOL PECTATE WITH POTASSIUM BOROHYDRIDE

Ethyleneglycol pectate (11 g.) was dissolved in water (250 ml.) containing glycerol (4.5 g.). The solution was cooled to 0° and potassium borohydride (3.5 g.) in water (20 ml.) was added with stirring.

The mixture was allowed to stand overnight. Inorganic salts were removed by passing the solution through columns containing Amberlite resins IRA-400 (OH) and IR-120 (H), and the solution, freed from inorganic ions, was then freeze-dried. The polysaccharide was washed with alcohol, redissolved in water and freeze-dried again.

Recovery : 7.3 g.

The polysaccharide was esterified by the same procedure as above using the corresponding amount of ethylene oxide.

The glycol ester of this partially reduced pectic acid showed:

$[\alpha]_D = + 247^\circ$ $c = 0.327$% in water.

Uronic acid anhydride content: 18.4 % (Kaye and Kent method) equivalent
to 19.3 % in the free polysaccharide.

After repeating the esterification and the reduction with potassium borohydride, three times, the uronic acid anhydride content was:

Uronic acid anhydride content: 12.4 % (Kaye and Kent method) in the
free polysaccharide.

Two further esterifications and reductions were carried out, and the product was isolated.

$[\alpha]_D = + 246.7^\circ$ c = 1.297 % in water.

Uronic acid anhydride content: 5.0 % (decarboxylation)
6.06 % (Kaye and Kent method)
5.74 % (Kaye and Kent method)

The recovery of polysaccharide was 5 g. after all these series of reductions and esterifications.

Molecular weight determination on this galactan was carried out by ultracentrifuge methods, giving the following results: 22.000 - 22.300.

Degree of polymerization : 138 - 140 units.

$S = 1.42 \times 10^{-13}$; $D = 4.0 \times 10^{-7}$; $\bar{v} = 0.6$.

DETERMINATION OF THE PERCENTAGE OF SUGARS PRESENT IN THE REDUCED PECTIC ACID (GALACTAN), BY SOMOGYI'S PROCEDURE(101)

Galactan ($[\alpha]_D$ = + 247°, uronic acid anhydride : 6 %) to the amount of 19.41 mg., and ribose (3.87 mg.) were hydrolyzed with N sulphuric acid for four hours on a boiling water bath. The hydrolysate was neutralized with barium carbonate, filtered and evaporated in vacuo to a syrup, which was spotted on chromatographic paper. The chromatograms were developed in solvent A for forty hours. The sugars were estimated by Somogyi's procedure following the technique described by Hirst and co-workers.(100)

Sugar	$Na_2S_2O_3$ N/100 ml.	Factor $Na_2S_2O_3$ ml/mg anhydrosugar	Anhydro-sugar (mg.)	Anhydro-sugar %	Average
D-ribose	5.02	0.1355	0.77	...	
	4.88		0.75	...	
D-galactose	21.60	0.1386	2.98	78.5	77.8
	21.44		2.97	77.1	
L-arabinose	2.46	0.1188	0.29	7.5	6.6
	1.84		0.22	5.8	
L-rhamnose	1.10	0.1507	0.17	4.26	4.2
	1.05		0.16	4.2	
D-glucose	0. 68	0.1143	0.08	2.0	2.2
	0.85		0.10	2.5	

D-galacturonic acid anhydride 6.0

D-galactose attached to D-galacturonic acid
forming aldobiouronic acids 5.5

Total 102.3

Another set of titrations was carried out, without using ribose as a reference, calculating the percentages whilst bearing in mind the fact that the aldobiouronic acids originated during hydrolysis constitute about 11.5 % of the polysaccharide (as calculated from the uronic acid content of the polysaccharide.).

Sugar	$Na_2S_2O_3$ N/100 ml.	Factor $Na_2S_2O_3$ ml/mg anhydrosugar	Anhydro-sugar (mg.)	Anhydro-sugar %
D-galactose	33.6	0.1386	4.650	76.7
L-arabinose	3.0	0.1295	0.388	6.4
L-rhamnose	1.5	0.1507	0.181	3.0
D-glucose	0.9	0.1143	0.103	1.8
D-galacturonic acid anhydride				6.0
D-galactose attached to D-galacturonic acid as aldobiouronic acid				5.5
Total				99.4 %

The results of the first set of titrations are a little high as some destruction of the reference sugar took place during hydrolysis.

PERIODATE UPTAKE OF THE GALACTAN

Galactan (0.3595 g.) (22.18 millimoles of anhydrohexose) were dissolved in water (10 ml.) and 0.25 M solution of sodium metaperiodate (40 ml.) was added. A blank was run parallel with a similar amount of metaperiodate solution. Both solutions were kept in the dark and 1 ml. samples were withdrawn at suitable intervals. Sodium bicarbonate (1 g.), standard solution of arsenite (5 ml.) and 20 % solution of potassium iodide (1 ml.) were added in the indicated order to the samples. After allowing the mixtures to stand for fifteen minutes in the dark, they were titrated with standard solution of iodine (0.09868 M) using starch as an indicator.

Time (hrs.)	Iodine (ml.)	$NaIO_4$ uptake μ- moles	$R = \dfrac{NaIO_4\ \mu\text{- moles}}{\text{Anhydrohexose}\ \mu\text{- moles}}$
2	0.86	42.37	0.953
5	0.88	43.36	0.975
9	0.89	43.85	0.990
25	0.93	45.83	1.032
56	0.95	46.77	1.055
74	0.96	47.31	1.066
145	1.02	50.27	1.133

ISOLATION OF THE OXIDIZED GALACTAN

The oxygalactan was recovered from the solution containing inorganic salts by destroying the excess of periodate with ethylene glycol and carrying out dialysis against distilled water. After concentrating the remaining solution, the oxypolysaccharide was precipitated with acetone, redissolved in a little water, precipitated again with acetone, separated at the centrifuge, washed with acetone-ether and ether, finally to be dried in vacuo.

The white powder recovered weighed 0.1 g.

$[\alpha]_D = +45.5^\circ$ $c = 0.967$ % in water.

Hydrolysis was performed on a little of this material with N Sulphuric acid for four hours in a boiling water bath. Chromatographic examination of the hydrolysate in solvent A showed: threose and galactose as the main components, small amounts of erythrose and traces of xylose, arabinose and rhamnose.

REACTION OF PERIODATE-OXIDIZED GALACTAN WITH ISONICOTINYLHYDRAZIDE

The oxy-galactan (90 mg.) in a little water was treated with isonicotinylhydrazide (150 mg.), and the mixture was allowed to stand for 48 hours. The complex was precipitated with a little potassium iodide and the supernatant after centrifuging off the precipitated complex was taken to dryness and extracted with n-butanol. The remaining powdery material was washed with alcohol-ether, and ether, and finally dried in vacuo.

Recovery : 100 mg.

Nitrogen content: Found: 11.9 % Required for 1 glycol fission.

10.5 %

Per hexose unit: 14.15 %

The nitrogen content suggests that 80 ± 6 % of the sugar residues were attacked by periodate.

METHYLATION OF PARTIALLY REDUCED PECTIC ACID

Partially reduced pectic acid (8 g.) ($[\alpha]_D$ = + 247°; Uronic acid anhydride = 18.5 %) was methylated with dimethyl sulphate (160 ml. x 5) and sodium hydroxide (70 g. x 5 - 30 % solution), under nitrogen. After adding the last portion of reagents the mixture was stirred for five hours. It was then partially neutralized (pH 8) and heated to 80 - 90°. Since no polysaccharide separated, the solution was cooled and dialysed to remove inorganic salts. The solution remaining in the cellophane bag was concentrated to 250 ml., and treated with Amberlite resin IR 120 (H) to give a solution of the free acid of a partially methylated polysaccharide. This was neutralised with silver carbonate, then evaporated under reduced pressure at 30°. The dark silver salts were ground and treated with methyl iodide together with a little dry methanol under reflux for half an hour. More methyl iodide was added (150 ml.) and silver oxide (2 g.) was added at intervals of fifteen minutes for a period of six hours until it totaled 50 g. The solvent was separated from the silver salts at the centrifuge, and evaporated under reduced pressure to give a crisp yellowish solid. The silver salts were extracted with hot chloroform and the extracts evaporated. The crisp solid was combined with the one previously isolated. Recovery: 5.2 g. OMe = 41.3 %.

REDUCTION OF THE METHYLATED ACIDIC POLYSACCHARIDE WITH $LiAlH_4$

The methylated polysaccharide (5.2 g.) was dissolved in tetrahydrofuran (150 ml.) and a solution of lithium aluminium hydride (3 g.) in tetrahydrofuran (100 ml.) was added drop-wise. The mixture was refluxed for one hour, the excess of lithium aluminium hydride being destroyed by the addition of ethyl acetate. Water was then added, and the supernatant liquid was concentrated.

The insoluble residue was extracted with alcohol and the extracts were taken to dryness. The residue of the evaporation was combined with the one obtained from the supernatant liquid. This combination, containing the polysaccharide and some inorganic salts, was treated in aqueous solution with Amberlite resins IR 120 (H) and the solution was neutralized with silver carbonate. After taking it to dryness, the solid residue was twice treated with methyl iodide (150 ml.) and silver oxide (50 g.)
Isolation of the methylated polysaccharide gave 4.1 g.
Methoxyl content: 42.5 %.

A further treatment with the methylating reagents was administered to this material, but the methoxyl content was not increased.
Recovery 3.8 g.

$[\alpha]_D = + 177 \pm 1^\circ$ c = 3.02 % in water.

$[\alpha]_D = + 158 \pm 2^\circ$ c = 3.17 % in chloroform.

HYDROLYSIS OF THE FULLY METHYLATED GALACTAN

Fully methylated galactan (3.5 g.) was hydrolysed with a mixture of methanol (75 ml.) and hydrochloric acid 2N (75 ml.) in a boiling water bath under reflux for five hours. The methanol was then evaporated under reduced pressure at 30° and replaced by water.

The refluxing was continued for three further hours, at the end of which the solution was cooled and neutralised with silver carbonate, filtered, evaporated in vacuo to a syrup and taken up with dry methanol.

After filtration eliminating all the coagulated silver salts, the solution was concentrated to a syrup and dried in vacuo to constant weight. Recovery: 3.1250 g.

FRACTIONATION OF THE METHYLATED SUGARS FROM METHYLATED GALACTAN HYDROLYSIS

A cellulose column (75 x 3.9 cm) was used for the fractionation of the mixture of methylated sugars.

The eluant was petrol ether (100-120°) / n-butanol (70 / 30), saturated with water. The syrup obtained in the previous hydrolysis (3.1250 g.) was placed in the column with the aid of a few ml. of n-butanol. The following table shows the weight of the different fractions and their composition:

Fraction	Tubes	Weight	R_G	Colour	Probable Identity
1	1- 15	20.0	1.00 - 0.98	Pink red	Unknown (A)
2	16- 25	434.5	1.02	Pink red	Unknown (A)
			0.98	Grey	Tri-O-methyl-arabinose
3	26- 30	184.8	0.99	Pink red	Unknown (A)
			0.93	Grey	Tri-O-methyl-Arabinose
			0.86	Red	Tetra-O-methyl-galactose
4	31- 45	119.6	0.99	Pink red	Unknown (A)
			0.96	Grey-red	Tri-O-methyl-arabinose
			0.87	Red	Tetra-O-methyl-galactose
5	46- 65	498.6	0.93	Red	Tetra-O-methyl-galactose
			0.81	Brown	Tri-O-methyl-galactose
6	66- 90	278.5	0.91	Red	Tetra-O-methyl-galactose
			0.79	Brown	Tri-O-methyl-galactose
7	91-165	706.7	0.85	(traces) Red	Tetra-O-methyl-galactose
			0.73	Brown	Tri-O-methyl-galactose
8	166-200	52.2	0.86	(traces) Red	Tetra-O-methyl-galactose
			0.74	Brown	Tri-O-methyl-galactose
			0.67	(traces) Brown	Tri-O-methyl-galactose ?
			0.61	(traces) Brown	Tri-O-methyl-galactose ?
9	201-330	211.3	1.02	(traces) Pink red	Unknown
			0.89	(traces) Grey red	Tri-O-methyl-arabinose
			0.85	Red	Tetra-O-methyl-galactose
			0.72	Brown	Tri-O-methyl-galactose
			0.53	Brown	Di-O-methyl-galactose
10	331-440	26.1	0.74	(traces) Brown	Tri-O-methyl-galactose
			0.51	Brown	Di-O-methyl-galactose
11	Washings	184.4	0.72	(traces) Brown	Tri-O-methyl-galactose
			0.41	Red	Di-O-methyl-galactose
			0.32	Brown	Di-O-methyl-galactose
			0.24	Brown	Mono-methyl-galactose

Total recovered 2716.7 mg. - equivalent to an 87.2 % recovery.

Fraction 1: $R_g = 0.98 - 1.00$ (20.0 mg.)

This fraction, upon hydrolysis, yielded 2:3:6 tri-O-methyl-D-galactose, which was identified chromatographically.

Fraction 2: $R_g = 1.02$ and 0.98 (434.5 mg.)

Demethylation gave arabinose and galactose.

This fraction was hydrolyzed with N HCl for five hours, and chromatographic examination with solvent B indicated the presence of three spots:

$R_g = 0.95$ (Grey black) 2:3:5 tri-O-methyl-L-arabinose
$R_g = 0.9$ (Red) 2:3:4:6 tetra-O-methyl-D-galactose
$R_g = 0.72$ (Brown) 2:3:6 tri-O-methyl-D-galactose.

The weight of each component was ascertained by separation on paper (benzene: ethanol: water : (169:47:15)) and hypoiodite estimation on a strip of the chromatogram.(100)

Tri-O-methyl-L-arabinose : 118.4 mg.
Tetra-O-methyl-D-galactose: 97.9 mg.
Tri-O-methyl-D-galactose : 218.2 mg.

The sugars were eluted from the thick paper, and the following crystalline derivatives were prepared:

2:3:5 tri-O-methyl-Larabonamide M.P.: 137 - 139°. Mixed M.P.: unchanged.
2:3:4:6 tetra-O-methyl-N-phenyl-D-galactosylamine M.P.: 194 - 196°.
2:3:6 tri-O-methyl-D-galactonolactone M.P.: 97 - 98°. Mixed M.P.: unchanged.

Fraction 3: $R_g = 0.99 - 0.93$ and 0.86 (184.8 mg.)

$[\alpha]_D = + 52 \pm 2^\circ$ c = 3.42 % in water.

The syrup was hydrolysed with N HCl for four hours, and chromatographic examination of the resulting syrup showed:

R_G = 0.93 (Grey black) Tri-O-methyl-L-arabinose

R_G = 0.89 (Red) Tetra-O-methyl-D-galactose

R_G = 0.73 (Brown) Tri-O-methyl-D-galactose.

The weight of each component was obtained from the hypoiodite method(100) using the same solvent as for the previous fraction:

2:3:5 tri-O-methyl-L-arabinose : 52.8 mg.

2:3:4:6 tetra-O-methyl-D-galactose : 23.4 mg.

2:3:6 tri-O-methyl-D-galactose : 108.6 mg.

The presence of tri-O-methyl-L-arabinose was confirmed by the preparation of the 2:3:5 tri-O-methyl-D-arabonamide M.P.: 137 - 140°. Mixed M.P.: 137 - 139°.

The 2:3:6 tri-O-methyl-D-galactonolactone was obtained for identification purposes. M.P.: 97 - 99°. Mixed M.P.: unchanged.

Fraction 4: R_G = 0.99, 0.96 and 0.87. (119.6 mg.)

$[\alpha]_D = +47 \pm 1^\circ$ c = 0.392 % in water.

Demethylation of the mixture gave arabinose and galactose.

Hydrolysis was carried out on this fraction with N HCl for four hours. Chromatographic examination and hypoiodite estimation of the components(100) gave:

R_G = 0.95	(Black grey)	2:3:5 tri-O-methyl-L-arabinose	29.7 mg.
R_G = 0.89	(Red)	2:3:4:6 tetra-O-methyl-D-galactose	19.9 mg.
R_G = 0.73	(Brown)	2:3:6 tri-O-methyl-D-galactose	70.0 mg.

Fraction 5: R_G = 0.93 and 0.81 (498.6 mg.)

$[\alpha]_D = +82.2^\circ$ c = 3.6 % in water.

The main component of this fraction was the sugar with $R_g = 0.81$.

Demethylation of the mixture gave galactose.

The weight of each component was calculated from the rotations.

2:3:4:6 tetra-O-methyl-D-galactose ($[\alpha]_D = + 109.5^\circ$) (109) 38.3 mg.
2:3:6 tri-O-methyl-D-galactose ($[\alpha]_D = + 80^\circ$) (118) 460.3 mg.

The principal component was identified as the 2:3:6 tri-O-methyl-D-galactonolactone M.P.: 98 - 99°, undepressed upon admixture with authentic specimen.

Fraction 6: $R_g = 0.91$ and 0.79 (278.5 mg.)

$[\alpha]_d = + 82.0^\circ$ c = 4.7 % in water.

The weights of both components were calculated from the rotation :

2:3:4:6 tetra-O-methyl-D-galactose ($[\alpha]_D = + 109.5^\circ$) (109) 17.8 mg.
2:3:6 tri-O-methyl-D-galactose ($[\alpha]_D = + 80^\circ$) (118) 260.7 mg.

The 2:3:6 tri-O-methyl-D-galactonolactone was prepared M.P.: 98 - 99°, undepressed upon admixture with authentic specimen.

Fraction 7: $R_g = 0.85$ and 0.73 (706.7 mg.)

$[\alpha]_D = + 79.4^\circ$ c = 4.82 % in water.

The component with $R_g = 0.85$ was present in traces, as was deduced from the chromatographic examination of the mixture.

Demethylation on this fraction gave only galactose.

The 2:3:6 tri-O-methyl-D-galactonolactone was prepared M.P.: 97 - 99°.
Mixed M.P.: unchanged.

Fraction 8: $R_g = 0.86$, 0.74, 0.67 and 0.61 (52.2 mg.)

The main component in this fraction was the sugar with $R_g = 0.74$.

Demethylation gave galactose, and periodate oxidation and saponification of the formyl esters, followed by chromatographic examination using solvent B gave the same components unchanged.

Fraction 2: R_g = 1.02 - 0.94, 0.89, 0.85, 0.72 and 0.53 (211.3 mg)
The main component in this fraction was the sugar with R_g = 0.53.
Demethylation gave galactose. Periodate oxidation and chromatographic examination of the oxidation products gave the yellow spots corresponding to methoxy-malon dialdehyde and indicative of 2-6 di-O-methyl sugar, plus the spots corresponding to the unchanged components (R_g = 0.51, 0.79, 0.91 and 1.01).

Resolution of the mixture was carried out on thick paper, using solvent C. Hypoiodite estimation on one strip of the chromatogram gave the components in the following proportions. The actual weights in the fraction were calculated from them:

R_g	Probable identity	Weight
0.51	Di-O-methyl-galactoses	129.6 mg.
0.64	Tri-O-methyl-galactose ?	10.8 mg.
0.72	Tri-O-methyl-galactose	21.6 mg.
0.85	Tetra-O-methyl-galactose	
0.94	Tri-O-methyl-arabinose	48.6 mg.
1.01	Yellow spot	

After extracting the different components from the thick paper, only the component R_g = 0.51 was investigated. It resulted to be a mixture of di-O-methyl-galactoses. Chromatographic examination in solvent C for thirty-six hours showed the presence of 2:3 di-O-methyl-D-galactose and

2:4 di-O-methyl-D-galactose. The fact that this fraction gives the typical canary yellow spot on periodate oxidation and chromatographic examination of the oxidation products suggested the existence of 2:6 di-O-methyl-D-galactose, but it was not possible to isolate any, or to prepare any derivative of this sugar. The 2:4 di-O-methyl-N-phenyl-D-galactosylamine was obtained (M.P: 207 - 209°) by reacting the mixture with aniline. The two components could be separated partially by differences in the solubility in ether-petrol ether mixtures.

Fraction 10: $R_g = 0.74$ and 0.51 (26.1 mg.)
$[\alpha]_D = + 82.1 \pm 2^\circ$ c = 0.52 % in water.

The main component of this fraction was the sugar $R_g = 0.51$.

Demethylation showed galactose, and periodate oxidation and chromatographic examination of the oxidation product gave the typical canary yellow spot corresponding to methyoxy malon dialdehyde, indicative of 2:6 di-O-methyl-galactose within the mixture.

Fraction 11: $R_g = 0.72$ (traces), 0.41, 0.32 and 0.24 (184.4 mg.)
Demethylation on this fraction showed galactose. Periodate oxidation and chromatographic examination of the oxidation products gave the yellow spot characteristic of 2-O-methyl-malonodialdehyde, indicative of 2-O-methyl or 2-6 di-O-methyl sugar. Chromatographic examination of the syrup with standards of 2-O-methyl-D-galactose and 2:4 di-O-methyl-D-galactose showed the identity of their travelling rates.

No estimation of the sugars by hypoiodite was carried out owing to the poor separation obtained in the chromatograms.

Summary of the fractionation:

Sugar	Weight	Millimoles	Moles %
2:3:5 tri-O-methyl arabinose	200.9 mg.	1.087	8.93
2:3:4:6 tetra-O-methyl-galactose	197.3 mg.	0.835	6.86
2:3:6 tri-O-methyl-galactose	1918.3 mg.	8.650	71.10
0.64 (tri-O-methyl-galactose?)	10.8 mg.	0.086	0.71
Di-O-methyl-galactoses	314.0 mg.	1.507	12.38
	2641.3 mg.	12.165	

PURIFICATION OF THE WATER-SOLUBLE POLYSACCHARIDE

A 1 % solution of the water soluble polysaccharide (10 g.) was passed through Amberlite ion exchange resins (IR - 120 (H) and IR 4B (OH)) to remove inorganic salts. The solution was neutralized with ammonia and concentrated under reduced pressure at 40°, after which the polysaccharide was precipitated with acetone. Dialysis of the aqueous concentrated solution of the polysaccharide in a cellophane bag against distilled water gave a solution which, after freeze-drying, yielded 6 g. of purified polysaccharide.

Ash content : 0.7 % (as sulphate)

$[\alpha]_D = + 55^\circ$ c = 0.108 % in water.

Hydrolysis of a few milligrammes showed in the chromatograms the existence of galacturonic acid, galactose, glucose, arabinose, xylose, rhamnose and a sugar travelling faster than rhamnose (probably a methyl sugar.)

PARTIAL HYDROLYSIS OF WATER-SOLUBLE POLYSACCHARIDE

Purified water-soluble polysaccharide (200 mg.) was hydrolysed with 0.05 N HCl in a boiling water bath under reflux.

Samples of the solution were drawn off every half an hour, and they were neutralized with barium carbonate and filtered. The filtrates were concentrated to a syrup. Chromatographic examination of the syrups showed:

Time (hours)	0.5	1	1.5	2	2.5
Sugar	Ga; Ar.	Ga; Ar.	Ga; Ar.	Ga; Ar; Rh.	Ga; Ar; Rh.

A further hydrolysis was performed using 0.01 N oxalic acid in place of hydrochloric acid :

Time (hours)	0.5	1	1.5	2	2.5	3
Sugar	Ar:(traces)	Ar.	Ar.	Ar.	Ar.	Ga; Ar.

ATTEMPTED FRACTIONATIONS OF THE WATER SOLUBLE POLYSACCHARIDE

A) Fractional precipitation with AMMONIUM SULPHATE: Water soluble polysaccharide (8 g.) was dissolved in water (800 ml.), and the solution was freed from insoluble material by centrifuging. To this solution ammonium sulphate was added in portions of 80 grammes with stirring to help dissolution. It was then centrifuged to separate any precipitated polysaccharide, and the same operation was repeated until the concentration of ammonium sulphate reached 70 %. At concentrations of 50, 60 and 70 % ammonium sulphate, a slight precipitation occurred. The total amount of precipitated polysaccharide was approximately 0.5 g. Chromatographic examination of the hydrolysates of these fractions showed that the same sugars were present in approximately the same proportions - galactose, glucose, arabinose, xylose, rhamnose, plus a pink spot further than rhamnose.

The remaining solution was dialysed against distilled water until free from inorganic salts, then freeze-dried.

Recovery : 5.5 g.

An electrophoresis measurement was carried out on this material by using 0.05 g. of polysaccharide dissolved in borate buffer pH 10, (10 ml.) in the Antweiler microelectrophoresis apparatus (1.5 mA, 35 V, 10 minutes). It revealed a single symmetrical peak.

B) Fractionation of the water-soluble polysaccharide with ACETONE :

Crude polysaccharide (20 g.) was dissolved in water (400 ml.), and acetone (400 ml.) was added with manual stirring. The precipitate which appeared was centrifuged off and washed with aqueous acetone (55 %) several times, and finally dissolved in water and freeze-dried.

Fraction 1.

To the supernatant of the previous centrifugation, acetone (200 ml.) was added, and the precipitate was separated, washed with acetone (65 %) and its aqueous solution was freeze-dried. *Fraction 2.*

To the supernatant after the removal of the second fraction more acetone was added until a concentration of 75 - 80 % was reached. The precipitate was treated in the same way as the previous ones.

Fraction 3.

The weights of each fraction are tabulated below, as well as their optical rotations. The solutions used for this purpose were decolourized with a few drops of sodium hypochlorite solution.

Fraction 1 (50 % acetone)	:	14.7 g.	$[\alpha]_D = + 74.2^\circ$	c = 0.108 %
Fraction 2 (60 % acetone)	:	2.5 g.	$[\alpha]_D = - 18.6^\circ$	c = 0.214 %
Fraction 3 (75 % acetone)	:	1.3 g.	$[\alpha]_D = + 47.2^\circ$	c = 0.170 %

Determination of the uronic acid anhydride content on *fraction 1* was carried out by the Kaye and Kent method.

Uronic acid anhydride content : 31.9 %.

C) Fractionation of the water-soluble polysaccharide with CETAVLON :

Fraction 1, isolated in the previous experiment, was used for this fractionation. Polysaccharide (5 g.) was dissolved in water (250 ml.), and an aqueous solution of Cetavlon (250 ml., 5 %) was added with stirring.

The precipitate which appeared was separated at the centrifuge, washed with water and dissolved in 2 N acetic acid (20 ml.). This solution was poured into a large excess of alcohol and the precipitate was successively washed with alcohol-acetic acid, alcohol, ether, and then dried in vacuo. (Fraction A_1).

A solution of borate buffer pH = 10 (250 ml.) was added to the supernatant of the centrifugation, and the precipitate was separated - again by centrifuging - dissolved in 2 N acetic acid (100 ml.). This solution was poured into a large volume of alcohol. The precipitate was washed in the same manner as previously. (Fraction A_2).

The supernatant was neutralized with acetic acid and concentrated under reduced pressure to 150 ml., and this solution was poured into a large volume of alcohol. The precipitate was treated as before. Fraction A_3).

The table below shows the characteristics of these precipitates:

Fraction	Rotation	Weight	Uronic acid	Sugars on hydrolysis
A_1	&[*]	0.4 g.	35.3 %	Ga; Glu; Ar; Xy; Rh; 2MXy
A_2	+ 47°	3.2 g.	24.5 %	Ga; Glu; Ar; Xy; Rh; 2MXy
A_3	&	1.0 g.	22.1 %	Ga; Glu; Ar; Xy; Rh; 2MXy

Another batch of water-soluble polysaccharide was fractionated in the same way. Ten grammes of polysaccharide were used for this purpose, and the following table shows the results of the fractionation :

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[*] & = no rotation could be read due to cloudiness.

Fraction	Rotation	Weight	Uronic acid	Sugars
B_1	& *	4.2 g.	33.1 %	Ga; Glu; Ar; Xy; Rh; 2MXy
B_2	&	1.6 g.	26.5 %	Ga; Glu; Ar; Xy; Rh; 2MXy
B_3	+ 10°	3.0 g.	28.2 %	Ga; Glu; Ar; Xy; Rh; 2MXy

(* & = no rotation could be read due to cloudiness)

Fractions A_1 and B_1 were combined, as were (A_2, A_3, B_2, B_3). Chromatographic examination on the hydrolysates of these fractions showed that the same sugars were present in approximately the same proportion.

HYDROLYSIS OF THE WATER-SOLUBLE POLYSACCHARIDE

Water-soluble polysaccharide (1.8374 g.) ($[\alpha]_D$ = + 74.2°) was dissolved in N. H_2SO_4 (100 ml.), and the solution was heated in a boiling water bath, under reflux, for four hours. Neutralisation was carried out with barium carbonate. After filtrating and washing the inorganic salts with water, the solution was concentrated under reduced pressure to dryness. The weight of the hydrolysis product was 1.7016 g. (0.1253 g. was put aside for use in chromatograms). The remainder was dried in a vacuum desiccator until constant weight 1.5763 g.

The material (1.5763 g.) was dissolved in 10 ml. of distilled water, and the solution was absorbed in a few grammes of washed cellulose powder. The mixture was then carefully freeze-dried. The fluffy powder was suspended in a little butanol, and the suspension quantitatively transferred to the top of a cellulose powder column. A piece of cotton wool was placed on top.

The column was developed with n-butanol, half saturated with water.

Six different fractions were collected in this way, and one further fraction was obtained by elution of the column with water.

Fraction	Tubes	Sugar Identity
1	1 - 41	2-O-methyl xylose 2-O-methyl fucose tri-O-methyl hexose ?
2	42 - 57	L-Rhamnose
3	58 - 72	D-Xylose
4	73 - 105	L-Arabinose
5	106 - 145	D-Glucose + D-Galactose
6	146 - 270	D-Galactose
7	-	Barium Galacturonate

IDENTIFICATION OF THE FRACTIONS.

Fraction 1 Chromatographic examination of the syrup in solvent B showed the presence of three components:

R_G	Colour with Aniline Oxalate	Intensity	
0.39 - 0.45	Purple	Strong	(a)
0.55 - 0.59	Yellow	Weak	(b)
0.70 - 0.76	Red	Very weak	(c)

The main component was separated in a filter sheet by using solvent B, and chromatographically it travelled at the same rate as 2-O-methyl-D-xylose. Paper ionophoresis examination and comparison with authentic 2-O-methyl-D-xylose and 3-O-methyl-D-xylose showed its identicalness with 2-O-methyl-D-xylose. The other components were examined later with a larger amount of hydrolysate.

Fraction 2 (105.2 mg.) On standing, the syrup crystallised in arborescent forms.

$[\alpha]_D = + 8.6^\circ$ c = 1.0 % in water after 24 hours.

The sugar travelled at the same rate as L-Rhamnose in solvent A. Identity with L-Rhamnose was ascertained by the preparation of the benzoylhydrazone. About 50 mg. of this sugar were dissolved in water (2 ml.) and this solution was treated with an alcoholic solution of benzoylhydrazine (10 ml. 5 %), and the mixture was kept at room temperature for two days, and then cooled in the refrigerator at 3° for a further day. The crystalline product was filtered, washed with ice-cold alcohol, and dried at 100° for 30 minutes.

Melting point and mixed melting point : 180 - 181° .

Fraction 3 (46.7 mg.)

$[\alpha]_D = + 19.2^\circ$ c= 0.4 % in water.

The component of this fraction travelled at the same rate as D-xylose in solvent A. The benzylidene-dimethylacetal derivative was prepared for the purposes of identification. One ml. of reagent, prepared by mixing freshly redistilled benzaldehyde (40 ml.) and methanolic HCl (20 ml.) (2.5 % by volume) and methanol (120 ml.), was allowed to react with the dried syrup (0.04 g.) in a stoppered flask for seven days at room temperature with occasional shaking. The crystalline product separated was filtered off, washed successively with water (15 ml.) and methanol (4 ml.) and dried. Recrystallization was carried out from chloroform-petrol ether (40 - 60°).

Melting point and mixed melting point : 210 - 211°.

Fraction 4 (202.3 mg.)

$[\alpha]_D = + 106^\circ$ c = 2.0 % in water.

Chromatographic examination showed that this sugar travelled at the same rate as the authentic L-arabinose. The preparation of the benzoylhydrazone, in the manner previously indicated, confirmed the presence of L-arabinose in this fraction.
Melting point: 185 - 190°; mixed melting point: 185 - 189°.

Fraction 5 (299.9 mg.)

This fraction contained some galactose together with glucose. The ratio of both sugars was determined by polarimetry. The sugars were separated on filter paper by using solvent A.

D-glucose was characterized by the formation of the p-nitrophenylhydrazone in aqueous solution containing a drop of acetic acid. The derivative crystallized out as a mass of yellow crystals which melted at 184-185°. Mixed melting point: 183-185°.

The specific rotation of D-glucose was obtained after separating the D-galactose: $[\alpha]_D = + 57.4^\circ$ c = 1.6 % in water.
Weight of D-glucose present in this fraction: 235.9 mg.
Weight of D-galactose present in this fraction: 64.0 mg.

Fraction 6 (130.6 mg.)

$[\alpha]_D = + 81^\circ$ c = 1.3 % in water.

The methyl-1-phenylhydrazone was prepared by dissolving the sugar in water (1 ml.) and adding a little 1-methyl-1-phenylhydrazine sulphate and sodium acetate. The mixture was kept for 12 hours at 35° and then cooled to 0° for a further 12 hours. The crystals were collected and recrystallised from water.

Melting point: 187 - 189° undepressed on admixture with authentic specimen.

Fraction 7 (0.6654 g.)

This fraction was present as the barium salt. Barium ions were removed from the aqueous solution of this fraction by using Amberlite IR-120 (H).

$[\alpha]_D = + 84.5^\circ$ $c = 1.7$ % in water, after deionizing.

The 2,5 dichlorophenylhydrazone was prepared by mixing, in equal amounts, the acid and the 2,5 dichlorophenylhydrazine in methanol with approximately 0.3 % of $NaHSO_3$. The whole was heated over the water bath until the methanol had boiled away. A little ether was immediately added to remove the excess of hydrazine. The product was recrystallized from dioxan.

Melting point and mixed melting point : 179 - 181°.

Further evidence of the presence of D-galacturonic acid in this fraction was ascertained by the conversion into mucic acid by oxidation with bromine water.

Melting point and mixed melting point: 205 - 206°.

Chromatographic examination of the syrup in solvent D showed some traces of an aldobiuronic acid together with D-galacturonic acid.

SUMMARY OF THE FRACTIONATION OF THE SUGARS FROM THE WATER SOLUBLE POLYSACCHARIDE.

Sugar	Weight (mg.)	Millimoles	Moles (%)
2-O-methyl-D-xylose	27.1	0.165	2.23
L-rhamnose	105.2	0.643	8.67
D-xylose	46.7	0.313	4.22
L-arabinose	202.3	1.35	18.23
D-glucose	235.9	1.31	17.70
D-Galactose	194.6	1.081	14.60
Barium D-galacturonate	665.4	2.543	34.30

TOTAL RECOVERED : 1.4772 g. - equivalent to a 93.7 % recovery.

IDENTIFICATION OF THE METHYLATED SUGARS PRESENT IN THE WATER-SOLUBLE POLYSACCHARIDE

Crude water soluble polysaccharide (10 g.) was hydrolysed with N sulphuric acid (500 ml.) for six hours in a boiling water bath. After neutralization with barium carbonate, the solution was deionised and concentrated to a syrup under reduced pressure. The syrup was placed in a charcoal column made in a Büchner in the way described by F. Andrews, L. Hough and D. B. Powell. (96)

The elution was carried out with water until xylose and rhamnose began to come out of the column, whereupon the eluent was changed by aqueous alcohol of different concentrations in order to collect the methylated sugars. Fractions of 500 ml. were collected and evaporated under reduced pressure. They were examined chromatographically.

A fraction (14) was collected containing rhamnose, a methylxylose and some other methylated sugar travelling faster than monomethyl xylose and showing a greenish yellow colour with the aniline oxalate spray. Its components were separated on filter paper using solvent B as mobile phase. After eluting the strips of paper, 45 mg. of mono-O-methyl-D-xylose (A) and 18 mg. of another methylated sugar (b) were obtained, and they were recrystallized from alcohol.

Identification was carried out as follows:

Sugar (A) travelled at the same rate as 2-O-methyl xylose and was identified as a crystalline sugar. Melting point: 132 - 134°; mixed melting point with authentic specimen: 131 - 133°.

$[\alpha]_D = +34.3 \pm 1^\circ$ c = 0.25 % in water.

Comparison of an X-ray powder photograph with the authentic specimen showed identity with 2-O-methyl-D-xylose.

Oxidation of a few mg. of the sugar with sodium metaperiodate and oxidation stopped before the hydrolysis of formyl esters showed (99) chromatographically the existence of methoxy-malon-dialdehyde by the lemon spot (R_G : 0.22; solvent B) after spraying with aniline oxalate. A similar result was obtained with the authentic 2-O-methyl-D-xylose under the same conditions. An attempt to prepare the crystalline anilide proved unsuccessful.

Sugar (B) was identified as the 2-O-methyl-L-fucose as follows:

$[\alpha]_D = - 85.4^{\circ}$ c = 0.36 % in water.

Melting point 154 - 159°; mixed melting point: 155 - 159°.

R_G in solvent B: 0.49 - 0.53 (green yellow colour with aniline oxalate spray).

An X-ray powder photograph was identical with the authentic specimen.

Further evidence of the presence of a 2-methyl aldose was shown by periodate oxidation as mentioned above. A lemon yellow spot was obtained indicating the formation of methoxy malon-dialdehyde. (99)

METHYLATION OF THE WATER SOLUBLE POLYSACCHARIDE

Fractions A_1 and B_1 from the fractionation of the water soluble polysaccharide with Cetavlon were combined to form fraction "I". Fractions A_2, A_3, B_2 and B_3 were similarly combined to constitute fraction "II". These two fractions, "I" and "II", were methylated separately following the procedure described for "I".

Fraction "I" (4.6 g.) was dissolved in water (50 ml.). A solution of thallium hydroxide (36 g.) in water (200 ml.) was then added, and the resulting suspension was freeze-dried at room temperature. The powdery material was refluxed with methyl iodide until the yellow suspension was no longer alkaline. The solution was separated from the thallium iodide at the centrifuge and the solvent evaporated under reduced pressure. The thallium salts were extracted several times with ethanol, and then with water. Upon evaporation these extracts left a crisp yellow solid which was combined with the solid residue remaining after evaporation of the methyl iodide solution. The material was treated twice more under the same conditions with thallium hydroxide solution and methyl iodide, finally to be isolated by extraction with chloroform and acetone, and by evaporation of the solvents. Two Purdie's treatments were given to this partially methylated polysaccharide in the usual way, 1.5 g. being recovered at the end of the operations.

$[\alpha]_D = + 34 \pm 1^\circ$ c = 0.946 % in chloroform.

Methoxyl content: 43.1 %.

Uronic acid anhydride content: 19.1 % (by decarboxylation)

20.6 % (by the Kaye and Kent method)

Fraction "II" was methylated in the same way as fraction "I". After one Purdie's treatment, the methylated polysaccharide was fractionated by precipitation with petrol ether from its chloroformic solution (50 ml.) The following table shows the different fractions together with their characteristics :

Fraction	Weight (g)	$[\alpha]_D^{\circ}$	c g % in $CHCl_3$	Uronic acid anhydride % (decarboxylation)	Methoxyl %	Petrol ether added (ml.)
1	1.2	$+28 \pm 1^{\circ}$	0.7	15.7	40.2	75
2	2.2	$+2 \pm 1^{\circ}$	0.82	12.9	41.8	150
3	1.2	$-8 \pm 1^{\circ}$	1.15	13.3	43.1	200
4	0.6	-1.4°	1.5	15.6	43.9	225
5	0.3	$+18^{\circ}$	1.02	16.2	44.0	250
6	1.2	syrup	-	-	-	300

This fractionation, as well as the previous ones, shows that the polysaccharide is not homogeneous, the separation of the different components being unsuccessful owing to the close similarity of their physical and chemical properties. These studies were discontinued.

DISCUSSION

EXTRACTION OF THE PLANT

The plant was extracted successively with water and with aqueous solution of ammonium oxalate. The former extracts a polysaccharide which is referred to as water-soluble polysaccharide, and the latter brings the pectic substances into solution. The extracts from the pectic materials were concentrated, centrifugated in a Sharples super-centrifuge, then dialysed against distilled water to give a solution of crude ammonium pectate which was shown, upon hydrolysis, to contain galactose, arabinose, rhamnose, xylose, together with galacturonic acid.

FRACTIONATION

The concentrated solution containing ammonium pectate plus the possible galactans and arabans which usually accompany pectic compounds was treated with an equal volume of acetone or alcohol in order to eliminate the former. Ammonium pectate was precipitated in the form of a slightly coloured gel which was pressed dry and redissolved in water to form a solution of approximately 2 %. This was treated several times in the same way as above, and it was not expected that galactose and arabinose would appear on the chromatograms upon hydrolysis since it has been found in other cases that galactan and araban components of pectic substances are soluble in 45 - 50 % acetone or alcohol.

As no effective separation of polysaccharides was achieved with this procedure, fractionation was attempted through the precipitation of the calcium salts. Even after repeated precipitation, the isolated acidic polysaccharide still gave neutral sugars upon hydrolysis. It has been suggested that neutral polysaccharides are attached to pectic acid through ester linkages.(78) The sisal pectic material was therefore treated with sodium hydroxide in order to bring about the saponification of any such esters. Yet after this treatment the acidic polysaccharide still gave arabinose and galactose upon hydrolysis.

Another method of fractionation which has proved useful in other cases(102) was employed to resolve the mixture of polysaccharides, with the aid of the Cetavlon complex. Cetavlon (cetyltrimethylammonium bromide) precipitates the acidic polysaccharides from the dilute solutions of their alkaline salts, and treatment of the precipitate with acetic acid (5 N) regenerates the polysaccharide. Determination of the optical rotation and the uronic acid anhydride of the polysaccharide, as well as chromatographic examination of the hydrolysates, revealed scarcely any change in the recovered material's properties and composition throughout all these purifications. Subsequently sisal ammonium pectate obtained from the crude material, following two preliminary precipitations with acetone from its aqueous solution, were twice extracted with boiling 45 - 50 % aqueous ethanol. The extracted material was shown to possess the same properties as the highly purified ammonium pectate after repeated precipitations. $[\alpha]_D = +213^\circ$, uronic acid anhydride content: 74.06 %, equivalent to 79.7 % in the free pectic acid.

The alcoholic extracts were evaporated under reduced pressure to a crisp solid. Chromatographic examination of the hydrolysate indicated the presence of the same sugars as in purified ammonium pectate (galactose, arabinose, rhamnose and galacturonic acid). The optical rotation ($[\alpha]_D = 159 \pm 4^\circ$), and the uronic acid anhydride content (56.6 % in the free polysaccharide) were considerably lower than the corresponding values in the ammonium pectate.

From none of these experiments was it possible to obtain a pure polygalacturonic acid. It is not yet clear whether these neutral sugars are constituents of sisal pectic acid or physically associated polysaccharides.

PERIODATE OXIDATIONS ON AMMONIUM PECTATE

Periodate oxidation studies on ammonium pectate showed that after 314 hours the polysaccharide consumed slightly more than one mole of sodium metaperiodate per unit of ammonium anhydrogalacturonate. The periodate-oxidized ammonium pectate was isolated ($[\alpha]_D = -33.8^o$) and reduced with potassium borohydride. The reduced material had $[\alpha]_D = +40 \pm 2^o$, and upon hydrolysis with N H_2SO_4 it gave galacturonic acid, threonic acid and traces of glyceric acid together with small quantities of unidentified substances. Threonic acid would result from 1:4' linked galacturonic acid units, glyceric acid from the non-reducing end groups, while galacturonic acid would arise either from the incomplete oxidation or from galacturonic acid units present as branching points in the molecule. These results are consistent with what might be expected from a 1:4' linked polygalacturonic acid of the type known to be present in pectic substances.

The reaction between the periodate-oxidized ammonium pectate and isonicotinylhydrazide or thiosemicarbazide furnished complexes, the nitrogen content of which (13.4 % for the isonicotinylhydrazide complex, and 16.2 % for the thiosemicarbazide) would indicate that nearly all the galacturonic acid units had been attacked by periodate.

METHYLATION OF AMMONIUM PECTATE

Ammonium pectate was methylated under the conditions normally used with Haworth's reagents. The isolation of the partially methylated material after one set of treatments was carried out be means of dialysis thus removing all the inorganic salts as well as low molecular-weight carbohydrates resulting from the degradation of the pectic molecule. Evaporation under diminished pressure gave a partially methylated sodium pectate, which was repeatedly extracted with alcohol and acetone in order to remove any neutral methylated polysaccharide. Upon evaporation these extracts left a residue which was methylated by two treatments with Purdie's reagents to give a product having $[\alpha]_D = - 10.2^\circ$ (chloroform) with a uronic acid anhydride content of 21.1 %. Chromatographic examination of the hydrolysate (solvent B) indicated the presence of di-, tri-, tetra-O-methyl galactoses, tri-O-methyl arabinose and di-O-methyl rhamnose.

The partially methylated sodium pectate was then transformed into methyl ester with the aid of silver salts, and the ester was treated with silver oxide and methyl iodide in order to bring about the completion of the methylation.

This process resulted in a product having $[\alpha]_D = + 191^\circ$ (chloroform) with a methoxyl content of 39.4 %, which was subjected to fractionation by precipitation from its chloroformic solution with petrol ether (40-60°). The fractions soluble in chloroform-petrol ether (1 : 2) were discarded, for although they contained nearly 100 % of uronic acid anhydride (di-O-methyl, methyl ester), their solubility in such a composition of solvent suggested that they were of small molecular size. The fractions 1 - 12 (see page 56) were used for further experiments after a preliminary purification involving alkaline saponification, extraction of the sodium salt with organic solvents to remove any neutral methylated polysaccharide, and reconversion into the methyl ester. These series of processes gave a methylated methyl pectate, $[\alpha]_D = +212.5^\circ$ (chloroform) with a methoxyl content of 40.3 %. Chromatographic examination of the hydrolysate products showed the persistant presence of neutral methylated sugars.

REDUCTION STUDIES ON METHYLATED METHYL PECTATE

Since the hydrolysis of pectic acid and its methylated derivative can only take place with difficulty and is accompanied by considerable decomposition (decarboxylation, formation of furan derivatives, etc.), attention was directed towards methods for the reduction of the carboxyl group to primary alcohol. The neutral polysaccharide obtained in this manner was expected to undergo hydrolysis without any difficulty. Many attempts to reduce methylated methyl pectate were made by adding a solution of lithium aluminium hydride in tetrahydrofuran to a solution of the methylated polysaccharide in the same solvent. It proved impossible to obtain any characterizable product from these reductions.

The isolation of a partly reduced polysaccharide brought more satisfactory results after reduction with potassium borohydride of the methylated methyl pectate. Different conditions were used for the reduction but in each case a 50 - 70 % reduction was achieved. Attempts to remethylate the partially reduced polysaccharide failed to increase the methoxyl content, possibly due to the formation of boric esters. Similar difficulties have been encountered by J. K. N. Jones and co-workers (56) in the methylation of trisaccharides derived from the enzymic degradation of pectic acid and reduced with potassium borohydride.

Controled hydrolysis of methylated methyl pectate in formic acid (90%) and reduction with lithium aluminium hydride of the hydrolysis products after conversion into the methyl glycoside methyl esters yielded a mixture of methylated sugars amounting to 43 %. The principal component of this mixture was identified chromatographically as 2:3 di-O-methyl-D-galactose. It would arise from the backbone of 1:4' linked galacturonic acid units in the pectic acid molecule. Monomethylgalactoses, tri- and tetra-O-methyl galactose were also present in smaller proportions.

This experiment showed the possibility of reducing the methylated galacturonic acid residues to methylated galactoses following hydrolysis or after conversion of the methylated methyl pectate into low molecular fragments. Accordingly methylated methyl pectate was subjected to partial methanolysis with methanolic HCl in a sealed tube at 100 - 118° for a few hours. After the neutralisation of the contents of the tube and evaporation of the solvent, the syrup was dissolved in dry tetrahydrofuran and reduced with lithium aluminium hydride in the normal way. Extraction of the reduced methylated material and hydrolysis gave a mixture of methylated sugars which was fractionated in a cellulose column. This process was practised on two samples of methylated methyl pectate. One of them was obtained from commercial sodium pectate (sisal) and the other was ammonium pectate extracted from sisal waste with ammonium oxalate. ($[\alpha]_D$ = + 210.6°, methoxyl content : 42.0 %, and $[\alpha]_D$ = + 212.5°, methoxyl content : 40.3 %, respectively).

The following sugars were characterised from the first sample of methylated methyl pectate :

Sugars found and characterised by crystalline derivatives:

2:3:4 tri-O-methyl-L-rhamnose ... as 2:3:4 tri-O-methyl-L-rhamnonophenyl hydrazide.

3:4 di-O-methyl-L-rhamnose ... as 3:4 di-O-methyl-L-rhamnonolactone.

2:3:4 tri-O-methyl-D-galactose ... as 2:3:4 tri-O-methyl-N-phenyl-D-galactosylamine and crystalline sugar.

2:3 di-O-methyl-D-galactose ... as 2:3 di-O-methyl-N-phenyl-D-galactosylamine
and 2:3 di-O-methyl-D-galactonamide.

2-O-methyl-D-galactose ... as crystalline sugar.

2:3:4:6 tetra-O-methyl-D-galactose and 3-O-methyl-D-galactose were identified chromatographically.

The main component of the hydrolysis products was 2:3 di-O-methyl-D-galactose which would be formed from the 2:3 di-O-methyl-D-galacturonic acid methyl ester units of methylated methyl pectate. Similarly non-reducing galacturonic acid end groups would give rise to 2:3:4 tri-O-methyl-D-galactose. If all the methylated galactoses arise from galacturonic acid residues in pectic acid, the result would indicate that there is one non-reducing group per 97 units. (Some, at least, of the monomethylgalactoses arise from incomplete methylation or demethylation during hydrolysis).

The following sugars were characterised as crystalline derivatives from the fractionation of the hydrolysis products of the second sample of methylated methyl pectate obtained from the extraction of sisal waste with ammonium oxalate :

2:3:4:6 tetra-O-methyl-D-galactose ... as the corresponding N-phenyl-galactosylamine.

2:3:4 tri-O-methyl-D-galactose ... as the corresponding N-phenyl-galactosylamine.

2:3:6 tri-O-methyl-D-galactose ... as the lactone of the corresponding aldonic acid.

2:3 di-O-methyl-D-galactose ... as the N-phenyl-galactosylamine.

2:4 di-O-methyl-D-galactose ... as the N-phenyl-galactosylamine.

2-O-methyl-D-galactose ... as crystalline sugar.

In addition chromatographic and electrophoretic evidence indicated the presence of 2:3:4 tri-O-methyl-L-rhamnose; 3:4 di-O-methyl-L-rhamnose; 2:3:5 tri-O-methyl-L-arabinose; 2:6 di-O-methyl-D-galactose and 3-O-methyl-D-galactose.

Again, in this mixture of methylated sugars, the main component is the 2:3 di-O-methyl-D-galactose, and if one considers the monomethylgalactoses to have arisen from galacturonic acid residues in pectic acid, there is one non-reducing group (2:3:4 tri-O-methyl-D-galactose) per 94 units. Since the results yielded only a small proportion of non-reducing groups in the methylated polysaccharide, it may be assumed that the monomethylgalactoses are the result of incomplete methylation or demethylation during hydrolysis rather than branching points.

The following methylated sugars :

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

must arise from neutral sugar residues in the methylated polysaccharide, but it is not yet possible to indicate their structural significance.

ESTERIFICATION OF PECTIC ACID IN AQUEOUS SOLUTION

Pectic acid can be esterified in aqueous solution by using ethylene oxide or propylene oxide as esterifying reagents.(98) On stirring the suspension of pectic acid and ethylene oxide for eight or nine days the mixture became viscous and the pH dropped from 2 to neutral as the esterification became complete at the end of this period. The esters were recovered by freeze-drying the solution, washing the residue with alcohol, redissolving the material in water and freeze-drying the solution once more. The uronic acid anhydride content in the ethylene and propylene glycol esters corresponds to approximately 78 % in the free acidic polysaccharide : $[\alpha]_D = + 194 \pm 5^\circ$ and $[\alpha]_D = + 168 \pm 2^\circ$ respectively.

MOLECULAR WEIGHT DETERMINATION

This was carried out on the propylene glycol pectate by ultra-centrifuge methods. The measurements indicated that the molecular weight was 37.200, which corresponds to a degree of polymerization of 168 - 170 units. ($S = 1.5 \times 10^{-13}$; $D = 2.5 \times 10^{-7}$; $\bar{v} = 0.6$)

REDUCTION OF ETHYLENE GLYCOL PECTATE TO GALACTAN

The esters of pectic acid furnish a suitable and easily obtained material for the reduction of pectic acid to galactan. Reduction with potassium borohydride(119)(120) is not normally complete since the reagent gives rise to alkaline solutions which cause the saponification of the esters and the formation of the potassium salts of the acid which cannot be reduced by the reagent. In order to minimise ester hydrolysis glycerol was added to the reaction mixture. This maintains the pH of the solution at about 8 by forming glyceroboric acids, stronger than boric itself. The reduction was carried out in aqueous solution at 0°. The problem of the isolation of the reduced polysaccharide was resolved by the use of Amberlite resins IR-120 (H) and IRA-400 (OH). The former removed all the potassium ions from the solution, the latter the boric acid. This strong alkaline resin was selected because of its rapid action and efficiency even with such weak acids as boric. Although reducing sugars are degraded by strong basic resins, it is unlikely that significant degradation occurs during this treatment as the potential reducing group of the polysaccharide has been reduced to galactitol during the reduction of the ester groups. The same could be said of the acidic resin. It is unlikely that a short treatment with cation-exchange resins in the cold would cause the hydrolysis of the polysaccharide.

After the first esterification it was possible to isolate the polyester by precipitation from the aqueous solution with acetone. After reduction, however, the polysaccharide needed higher concentrations of acetone for its precipitation, especially when glycerol was present. In such cases the polysaccharide was best isolated by freeze-drying the solution after the removal of inorganic salts. The residue remaining after this operation was rendered impure by the presence of glycerol, which was removed by washing the polysaccharide with ethanol, dissolving it in a little water and freeze-drying the solution again. The uronic acid anhydride content after the first reduction and re-esterification was 18.4 %, which corresponds to 19.3 % in the free polysaccharide. This means that the reduction was 75.7 % of the theoretical value. By repeating the reductions with potassium borohydride after re-esterification three times more, the uronic acid anhydride content was brought down to 12.4 % (reduction 32.6 %). The whole process was repeated twice more when the uronic acid anhydride content was 5.0 to 6.0 % ($[\alpha]_D = + 246.7^\circ$ in water). This polysaccharide differed completely from its parent pectic acid in viscosity, solubility, precipitability with organic solvents, etc. Ultra centrifuge measurements on this polysaccharide (galactan) showed a molecular weight of 22.000 - 22.300, corresponding to a degree of polymerization of 138 - 140 units. ($S = 1.42 \times 10^{-13}$; $D = 4.0 \times 10^{-7}$; $\bar{v} = 0.6$).

The percentage composition of this polysaccharide was obtained by making use of the Somogyi's technique. The results are tabulated below :

	Mean value
D-galactose	82.0 %
L-arabinose	6.5 %
L-rhamnose	3.6 %
D-glucose	2.0 %
D-galacturonic acid	6.0 %

PERIODATE OXIDATIVE STUDIES ON THE GALACTAN

A consumption of one mole of sodium metaperiodate per anhydrohexose unit of the galactan was reached after twenty hours. The periodate-oxidised galactan ($[\alpha]_D = + 45.5^\circ$ in water) was isolated and upon hydrolysis, it showed chromatographically the presence of threose and galactose as its main components. The oxy-galactan isonicotinylhydrazide complex was obtained, the nitrogen content of which (10.5 - 11.9 %), since the required nitrogen content for one glycol fission per anhydrohexose unit is 14.15 %, suggests that only 80 ± 6 % of the sugar residues were attacked by periodate. It is probable, however, that this value is low.

METHYLATION OF GALACTAN

In order to obtain a fully methylated galactan a partially reduced pectic acid (uronic acid anhydride : 18.4 %) was used for methylation, carried out in the usual way according to Haworth's procedure. The partially methylated polysaccharide was isolated by means of dialysis, converted into the methyl ester through silver salts. This methylated methyl ester was then remethylated with Purdie's reagents. The fully methylated methyl ester polysaccharide was reduced in tetra-hydrofuran with lithium aluminium hydride. The isolation of the partially methylated galactan presented no difficulties as in the case of the reduction of the methylated methyl pectate. It was readily soluble in organic solvents. The residue remaining after evaporation of the extracts was methylated by two treatments with Purdie's reagents. A further treatment with the same reagents did not increase the methoxyl content of the polysaccharide (42.5 %) ($[\alpha]_D = +177 \pm 1^\circ$ in water).

HYDROLYSIS AND FRACTIONATION OF THE METHYLATED SUGARS

The methylated polysaccharide was hydrolysed with N HCl, and the mixture of methylated sugars originated was fractionated in a cellulose column using n-butanol-petrol ether (100 - 120°) (30 - 70) as eluent. The following sugars were characterised:

2:3:5 tri-O-methyl-L-arabinose ... as 2:3:5 tri-O-methyl-L-arabonamide.

2:3:4:6 tetra-O-methyl-D-galactose... as N-phenyl-glycosylamine.

2:3:6 tri-O-methyl-D-galactose ... as 2:3:6 tri-O-methyl-D-galactonalactone.

2:4 di-O-methyl-D-galactose ... as N-phenyl-glycosylamine.

2:3 di-O-methyl-D-galactose ... as N-phenyl-glycosylamine.

2:6 di-O-methyl-D-galactose ... chromatographically.

2-O-methyl-D-galactose ... chromatographically.

It is clear that the principal component of this mixture (2:3:6 tri-O-methyl-D-galactose) arises from 1-4' linked methylated galactan formed, as described, from a polysaccharide containing 1-4' galacturonic acid units. It has been observed that arabinose occurs only as the 2:3:5 tri-methyl ether, and no evidence has been obtained as to the presence of di- or mono-methyl-L-arabinose. The arabinose residues, therefore, cannot arise from an araban of the type found in other pectic substances, but must be linked to the polysaccharide based on galactose or galacturonic acid units.

PURIFICATION AND FRACTIONATION OF THE WATER-SOLUBLE POLYSACCHARIDE

The crude water-soluble polysaccharide isolated from the water extractions of the sisal waste was freed from inorganic salts and other impurities by means of several precipitations with acetone from the aqueous solution. Treatment with Amberlite resins IR-120 (H) and IR4B (OH), neutralisation with ammonia and dialysis of the concentrated solution gave a product with an ash content of 0.7 % (as sulphate). ($[\alpha]_D = + 55^\circ$).

Upon hydrolysis this material gave : galactose, glucose, arabinose, xylose, rhamnose, plus two unidentified sugars.

Mild hydrolysis with 0.05 N HCl or 0.01 N oxalic acid showed that the arabinose is easily removed under hydrolytic conditions which cause very little degradation of the other sugars present.

Fractionation of this water-soluble polysaccharide was carried out with different procedures.

1°) Fractionation with ammonium sulphate was unsuccessful, as none, or very little polysaccharide was precipitated during the whole process.

2°) Fractionation by precipitation with acetone gave three fractions which, although differing in the optical rotations, presented, upon hydrolysis and chromatographic examination, the same sugars as mentioned above. The rotations of these fractions are quoted for comparison:

Fraction 1) $[\alpha]_D$ = + 74.2° (uronic acid anhydride content : 31.9 %)
Fraction 2) $[\alpha]_D$ = - 18.6°
Fraction 3) $[\alpha]_D$ = + 47.2°

A further fractionation was attempted with Cetavlon, but although the different fractions showed different uronic acid anhydride contents, they gave the same sugars upon hydrolysis.

Water-soluble polysaccharide ($[\alpha]_D$ = + 74.2°) was hydrolyzed and the following sugars were separated and identified :

2-O-methyl-D-xylose ... by ionophoresis
L-rhamnose ... as benzoylhydrazone
L-arabinose ... as benzoylhydrazone
D-xylose ... as benzyliden dimethylacetal
D-glucose ... as p-nitrophenylhydrazone
D-galactose ... as 1-methyl-1-phenyl-hydrazone
D-galacturonic acid ... as mucic acid and 2.5 dichlorophenylhydrazone.

The identification of the methylated sugars was achieved first by hydrolysing 10 g. of crude water-soluble polysaccharide, then by fractionating the mixture of sugars on a charcoal column. Elution of the column with water removed most of the unmethylated sugars, and the column was then eluted with mixtures of water - alcohol. A fraction containing the methylated sugars was collected and refractionated by means of chromatography on thick paper, using solvent B. Identification of the components involving X-ray powder photographs, optical rotations, melting points of the crystalline sugars and periodate oxidation and chromatography of the oxidation products gave evidence of 2-O-methyl-D-xylose and 2-O-methyl-L-fucose.

These sugars have been found also in the polysaccharides contained in plum leaves.(96)(121)

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