ABSTRACT OF THESIS

Name of Candidate Mr. NASIR-UD-Din

Address Chemistry Department, University of Edinburgh

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Title of Thesis Structural Studies of Plant Gums, with Particular Reference to Sterculia urens Gum.

The gum exudate of Sterculia urens was deacetylated and purified by dissolution in sodium hydroxide solution (or in aqueous ammonia) and precipitation with ethanol acidified with concentrated hydrochloric acid. The purified gum had $[\alpha]_D$ +62 and galacturonic acid, glucuronic acid, galactose, rhamnose and a trace of arabinose as component sugars. The purified gum was examined for heterogeneity by the various available methods. The results obtained indicated the presence of a homogeneous polymer.

The gum acid was partially hydrolysed with N-sulphuric acid when the following acidic sugars were identified.

D-Galacturonic acid.
2-O-(α-D-Galactopyranosyluronic acid)-L-rhamnose.
4-O-(D-Galactopyranosyluronic acid)-D-galactose.
O-β-D-Glucopyranosyluronic acid (1→3)-O-α-D-galactopyranosyluronic acid (1→2)-L-rhamnose.

The neutral sugars in the hydrolysate were galactose and rhamnose together with a trace of arabinose.

The polysaccharide was fully methylated and reduced with lithium aluminium hydride to give a neutral methylated polysaccharide. The reduced methylated gum was hydrolysed with hydrochloric acid and the products were separated on a cellulose column. The following methylated sugars, obtained after separation from the column, were characterised.

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-O-methyl-D-galactose.
3-O-methyl-D-galactose.
2,3,4-Tri-O-methyl-D-glucose.
2,3,4-Tri-O-methyl-L-rhamnose.
3,4-Di-O-methyl-L-rhamnose.
3-O-methyl-L-rhamnose.

The gum acid was oxidised with sodium metaperiodate and the resulting polyaldehyde was reduced with potassium borohydride. The polyalcohol was hydrolysed with N-sulphuric acid at room temperature to give a degraded polymer and a low molecular weight material. The degraded polymer was partially hydrolysed with N-sulphuric acid at 100° to give the following acidic sugars.

D-Galacturonic acid.
2-O-(α-D-Galactopyranosyluronic acid)-L-rhamnose.
O-D-Galactopyranosyluronic acid (1→?)-O-α-D-galactopyranosyluronic acid (1→2)-L-rhamnose.

The gum acetate was reduced with diborane formed in situ with boron trifluoride and lithium borohydride. Although this procedure has resulted in the reduction of most of the hexuronic acid residues, a few ethylated sugars were obtained as artifacts.

Use other side if necessary.

From the combined results of partial acid hydrolysis, methylation and Smith degradation studies a number of partial structures for the gum molecule have been proposed.

STRUCTURAL STUDIES OF PLANT GUMS,

WITH PARTICULAR REFERENCE TO

STERCULIA URENS GUM.

BY

NASIR-UD-DIN.

Thesis submitted to the Faculty of Science
of the University of Edinburgh for the
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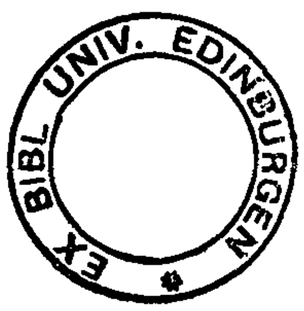

TO MY PARENTS

C O N T E N T S

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I N T R O D U C T I O N

INTRODUCTION

A wide variety of tropical and sub-tropical plants have a tendency to exude viscous and adhesive substances. Latex, terpenoids and carbohydrate exudates, which are viscous and adhesive have been classified as plant gums. This script will exclusively deal with carbohydrate exudates and they will be referred to as gums or gum exudates. Gum exudates may be obtained from various parts of the plant such as fruit, leaves, stem and roots although the principal source is the stem. The gums may be exuded spontaneously by the plant itself or as the result of a mechanical impetus. The mechanical injury may be an incentive for the production itself or it may accelerate the rate of the production. The gums may therefore be defined as "those carbohydrate materials which are spontaneously exuded from the outer portions of the trees and fruits after mechanical injury or after attack by micro-organisms" (1).

When exposed to the atmosphere and allowed to dry gums form glossy nodules or flakes which are usually of brown or yellow colour. The nodules obtained from a tree or from different trees of the same botanical species are generally similar in physical and chemical behaviour.

The gum producing trees, in particular those of commercial importance, grow predominantly in the continents of Asia and Africa indicating the climatic requirements for their growth. Gum exudates are produced in abundance by leguminous plants although a variety of other gums are produced by the plants of

the families Anacardiaceae, Biscaceae, Burseraceae, Cactaceae, Combretaceae, Liliaceae, Maringaceae, Rutaceae, Sapotaceae, Sterculiaceae etc.

The origin of the gums is still obscure. They may arise from normal plant metabolism or in abnormal conditions as a result of pathological imbalance. There is evidence to support each of these theories. For instance, after mechanical injury to the plant, the immediate release of gum tragacanth suggests that gum formation is a metabolic phenomenon. On the other hand, gums are produced in larger quantities by trees growing under dry conditions, brought about by elevated temperatues and the resulting lack of moisture. Furthermore, the inocculation of bacteria into trees with the ensuing process of gummosis (2), indicates that the gums may have been produced under pathological conditions. The phenomenon of gum formation has also been attributed to the invasion of the plant tissue by micro-organism (3) and to fungus growth (4). The fungus is believed to liberate an enzyme which induces complex polymer formation.

Although in the light of the available information it is not possible to come to any definite conclusion as to the origin of the gums, it seems probable that the formation of gums is due to more than one cause.

The most likely function of gum formation is to prevent the invasion of the plant tissue and to conserve moisture (1). The latter function is more favoured as in the majority of cases the tapping of the plants immediately results in the sealing off of

the exposed area by the gum. The fact that these plants continue to live and propagate and show no sign of being diseased after such a mechanical treatment, indicates the possibility that the gums are produced mainly to conserve moisture.

It is important to note in this context that pneumococcus polysaccharides which are chemically and structurally related to various gums are known to protect pneumococcus organisms. It is very likely that gums like these polysaccharides may perform a similar protective function and thus provide shelter to the injured tissue.

The gums are believed to be formed either at the injured site or at some other point and then rapidly mobilised to the injured spot. The source of the gums inside the plant is a formidable problem for the gum scientist. A number of carbohydrate materials have been considered as precursors of the gums but the available evidence does not permit any conclusion as to the precise source of the gums. It is, however, possible to develop some understanding of this problem if the structural relation between various carbohydrates such as cellulose hydrocellulose, hemicellulose, starch, pectin and the gum present in the plants could be discovered.

Regardless of the origin or source of the gums it is reasonable to believe that the gums are formed by an enzymic mechanism rather than by a direct chemical reaction. This assumption leads to the possibility of the polymerised gum being contaminated with the enzymes that have escaped inactivation

during the process of synthesis. As the enzymes are in contact with the synthesised gum, it is possible that under suitable conditions they may hydrolyse the synthesised product. Thus, cherry gum has been reported to undergo slow autolysis to give a mixture of reducing sugars (5). However, it is known that a majority of the gums are stable to autolysis. This resistance of the gums to autolysis has been attributed to the presence of peroxidases, which inactivate the degrading enzymes (6).

The gum polysaccharides have highly branched molecular structures and are very similar to mucilages in chemical composition. The general means of distinction is not the structure but the function and the biological origin. Plant mucilages from intercellular spaces are considered to be the product of normal metabolism. They are often described as storehouses of food. The gums and mucilages have also been differentiated on the basis of their solubility in water, the gums generally dissolving to give a clear solution while the mucilages form colloids. However, in certain cases gums such as those from Prunus species form jelly-like mucilages in water and give translucent viscous solutions. The physical properties of the gums and mucilages are largely dependent on their molecular structure and consequently there can be no accurately defined classification. It is, however, possible to group gums and mucilages polysaccharides on the basis of their structural similarities. This of course, will be of limited success since our knowledge of the chemistry of many of the mucilages is limited.

Gums are high molecular weight substances and rank amongst the most complicated chemical systems which have been studied. Molecular weights ranging from 2-300,000 for gum arabic and 9,500,000 for Karaya gum have been quoted (7). The gums may consist of one or more polysaccharides. The gum polysaccharide may comprise anything from two to four neutral sugars and an uronic acid residue. The common neutral residues are those of D-galactose, L-arabinose, L-rhamnose, D-mannose and D-xylose, whereas fucose and tagatose are rare sugar components. The common uronic acid components are D-glucuronic acid, 4-O-methyl-D-glucuronic acid and D-galacturonic acid. It is very probable that each of these sugars is involved in more than one type of linkage in the same polysaccharide.

The gums are generally marketed in groups following their similarities in physical properties. For example, gums from over one hundred species of Acacia are collectively marketed as gum arabic. It is, therefore, desirable to ensure the botanical purity of the sample in advance of the structural investigations.

Gums find their extensive commercial use largely in the textile, printing, paper and pharmaceutical industries as adhesive and emulsifiers.

It is not only the industrial application and similarity to bacterial polysaccharides that have attracted the scientific investigations of the gums, and perhaps the most inportant object is that "it leads to some of the fundamental problems in the carbohydrate chemistry, namely, the mechanism by which the

primary products of photosynthesis which appear to be based on D-glucose, are transformed into other hexoses, uronic acids, methyl pentoses and pentoses" (8).

PURIFICATION AND FRACTIONATION:

The gum polysaccharide usually exists as neutral or acid salts of the cations such as calcium, magnesium, sodium or iron and may be contaminated by various materials such as bark, dust and terpinoid resins.

Purification of the gum exudates is carried out by dissolving the powdered gum in water or dilute alkali followed by removal of the mechanical impurities. The free polysaccharide is precipitated by the addition of organic solvents such as ethanol, methanol, acetone or acetic acid. The polysaccharide may be further purified by repeated precipitation, treatment with ion exchange resins, dialysis and electrodialysis to remove inorganic ions. The purified gum may be dried by freeze drying or by trituration with acetone or ether followed by drying in a vacuum oven.

It has been observed that carbohydrate materials retain organic solvents used either for precipitation or for trituration even under drastic drying conditions (9). Also, the solvents used for purification of the polysaccharides are reported to produce artifacts (10).

One of the major problems in the study of the gum chemistry concerns the purity of the starting material and the preparation of homogeneous polymer. The usual procedure to ensure homogeneity is to subject the sample to rigorous purification by fractional precipitation or dissolution.

The heterogeneity of the gum polymers like other natural

polymers may be of three different types:

i. Polymers containing the same structural units linked in the same way but in different proportions.

ii. Polymers consisting either of different structural units or the same structural units linked in different ways.

iii. Polymers composed of the same sugar units linked in the same way but differing in molecular sizes.

The complete separation of the polymers into chemically pure fractions is particularly arduous, and in the first two types of heterogeneity the separation must be effected before proceeding to structural investigations. In the event of incomplete separation the results obtained will be ambiguous and of limited structural significance. The last type of heterogeneity is not very serious since it permits correct interpretation of the chemical investigations. Thus the primary process of structural studies involves the examination of the purified gum for the molecular purity. This can be carried out in a number of ways such as by electrophoresis, ultracentrifugation, precipitation with antipneumococcus serum, gel filtration and ion exchange chromatography. When used in conjunction with one another, the above methods may provide an analytical criteria of homogeneity. In those cases where the evidence obtained in this way suggests the presence of more than one polysaccharide, separation on a preparative scale may be carried out by various methods based on the principle of fractional precipitation.

Criteria of Homogeneity:

As it is fundamentally important to ensure purity of the polysaccharide before commencing the structural investigations, the best that can be done is to assess the nature of the heterogeneity of the polymer by various available methods. It is, however, important to exercise care as some of the techniques are so precise that they may lead to misinterpretation even if the starting material contains minute quantities of the contaminated polysaccharide. The methods described below may be applied as a test of homogeneity or in some cases to the separation of small amounts of polysaccharides.

The separation of gum polysaccharides using zone electrophoresis has only been partially successful. Gum arabic was shown to be heterogeneous by this method (11). Ionophoresis using filter paper and borate buffer has been used to distinguish certain polysaccharides (12,13). This procedure is handicapped because of the absorption of the polysaccharide by the paper and as it requires spray reagents for detecting the polysaccharides that are not interferred with by the cellulose paper. This difficulty is conveniently overcome by an inert support such as glass fibre paper. Using this technique and 2N sodium hydroxide as electrolyte and alkaline permanganate as spray reagent, Smith (13) has shown that a number of previously purified polysaccharides appear to be heterogeneous. This method can possibly be used for the separation of polysaccharides on a preparative scale if thick glass fibre sheets or a column

of powdered glass could be used.

Sedimentation analysis using the ultracentrifuge (14) provides a powerful analytical procedure to study the molecular weight distribution of a mixture of polysaccharides. The method also furnishes information regarding the molecular weight of the components.

Gel filtration, based on the principle that low molecular solutes can diffuse freely through the gel matrix, the solutes with large molecular dimensions will not diffuse or their diffusion through the grains will be restricted according to the porosity of the gel substance, and molecules with still larger dimensions are completely prevented from the diffusion through the gel grains have been extensively used to separate polysaccharides, particularly of the dextran types (15). Since gel substances with a large variety of porosity are available, this technique may be of value in determining the molecular heterogeneity of the gums.

Another diagnostic test for the homogeneity of the gums depends on the fact that specific antipneumococcus sera will give precipitation reactions with polysaccharides which are structurally similar to the corresponding pneumococcus polysaccharides. Gum arabic has been shown to give a precipitate with Type ii antipneumococcus serum, from which a polysaccharide having less rhamnose than the original gum can be isolated (16).

Recently Neukom et al. (17) have developed an elegant chromatographic technique to examine the heterogeneity of the

polymer. The method makes use of anion exchange cellulose and was developed by analogy with the precipitation of acidic and neutral polysaccharides with quaternary ammonium salts. The acidic polysaccharides are readily absorbed by anion exchange cellulose on neutral pH values, whereas neutral polysaccharides are, if at all, weakly absorbed. They fractionated wheat starch, dextrin, a mixture of sugar beet araban and pectic acid by using DiEthyAmino-Ethyl(DEAE) cellulose in different forms (borate and phosphate) together with a suitable elution medium at different pH values and electrolyte concentration. Aspinall and Young (18) have completely separated Khaya senegalensis gum into two components of different chemical structure using this technique whereas fractional precipitation gives only partial fractionation.

Furthermore, Neukom and collaborators (19) showed in the case of pectic acid that the extent of absorption on ion exchange cellulose depends on the degree of esterification, the degree of polymerisation and the proportion of side chains.

A number of other methods such as ultrafiltration, differential extraction and selective enzymolysis which have been used for purification and examination of homogeneity in the case of simple polysaccharides are less commonly used in the study of the gums.

Fractionation on Preparative Scale:

Although the methods outlined above are efficient and precise, and can be successfully applied to test the homogeneity

of the polysaccharide and in some cases may be used for small-scale separation of polysaccharides, fractional precipitation is still the main method for effecting separation on a preparative scale. Fractional precipitation may be carried out in three different ways, precipitation by the addition of non-solvent, precipitation of the polysaccharide by salting out procedures, and precipitation of the polysaccharides as salts or complexes.

Precipitation by the gradual addition of non-solvents such as ethanol, methanol, acetone or acetic acid which is essentially a process of purification has been frequently used to effect purification and fractionation simultaneously. Thus in the cases of Khaya senegalensis gum (20) and gum tragacanth (21) fractional precipitation has led to the isolation of fractions with entirely different molecular structure. Usually there is a marked tendency for co-precipitation and unless there is reasonable difference in solubility, only a rough separation is possible.

Addition of ionic salts such as ammonium sulphate to aqueous solutions of some of the polysaccharides gives a fractionation in a manner similar to the fractionation of the proteins.

The tendency of certain polysaccharides to complex with various copper compounds such as Fehling's solution (22), cupric-sulphate (23), cupric acetate (24), and cupriethylene diamine (25) has been considerably used for fractionation. Acidic polysaccharides have been separated from neutral

polysaccharides as calcium (26) and barium salts (27). More recently cetyl trimethyl ammonium bromide has been used to separate acidic polymers from neutral polymers (28). In conjunction with borate, cetavlon may be used to separate certain neutral polymers, (29).

STRUCTURAL INVESTIGATION:

The problems encountered in the elucidation of the molecular structure of a polymer involves the assessment of the nature of the component units, their relative proportions, the order in which the residues are joined together and the mode of linkage with one another.

Information regarding the component units and the relative proportions in which they occur can be obtained by the total hydrolysis of the polymer into basic units. The order of the components and the anomeric cenfiguration of the linkages in the polysaccharide can be resolved by graded hydrolysis followed by characterisation of the resulting oligosaccharides. The mode of the linkage of each residue, the proportion of non-reducing terminal groups and the nature of the ring structure can be assessed by the classical methylation technique.

Additional and more selective information can be made available by subjecting the polysaccharide to periodate oxidation, alkaline degradation, enzymic hydrolysis and immunological studies.

An idea as to the general character of the polysaccharide in advance of chemical investigation can be obtained by carrying out a number of analyses such as the determination of the neutralisation equivalent, uronic acid anhydride content, methoxyl content, the proportion of acetyl groups, and of physical constants such as optical rotation and viscosity.

Component Analysis:

The total hydrolysis of the polysaccharide to give the component sugars may be carried out by heating with dilute mineral acid. Hydrolysis with N-sulphuric acid or hydrochloric acid at 100° for six to eight hours generally liberates the monosaccharides together with acidic oligosaccharides. In the case of acidic polysaccharide, the glycosidic link of an uronic acid is relatively stable and the breakdown of this link requires vigorous hydrolysing conditions which may result in considerable decomposition of the sugars. Thus an accurate quantitative measure of the various sugars present in a polysaccharide containing a high proportion of glycosiduronic acid linkages is not possible.

The sugars liberated from hydrolysis of a polysaccharide can be separated into component sugars by chromatography on a cellulose column (30) and the monosaccharide thus obtained can be characterised by preparing crystalline derivatives.

Graded Hydrolysis:

A good deal of information regarding the molecular structure of the polysaccharide can be derived from the examination and characterisation of the products of partial hydrolysis. This procedure leads to the determination of the order of linkage of some of the sugar residues and of the anomeric configuration of the linkages in the polysaccharide from which they are derived.

Graded hydrolysis may be effected directly by heating the polysaccharide with various concentrations of mineral acid.

The direct graded hydrolysis in certain cases results in considerable decomposition of sugars, removal of funtional groups and in acid catalysed polymerisation. An elegant experimental variation of direct graded hydrolysis has been developed by Painter (31). This involves the hydrolysis of the polysaccharide in a dialysis bag with continuous removal of the products of hydrolysis. This procedure is experimentally cleaner and eliminates some of the above disadvantages. Painter, using this method obtained high yields of oligosaccharides in the hydrolysis of inulin with water soluble non-dialysable polystyrene sulphonic acid (32).

The variable stability of the glycosidic linkages towards acid may be advantageously exploited to bring about stepwise degradation of the polysaccharide. The gradual degradation liberates fragments which are simpler in structure and easily amenable to constitutional studies.

The glycosidic linkages of the sugars in the furanose form are particularly acid labile. Sugars such as L-arabinose, which is frequently found in this form, may be completely liberated by heating the polysaccharide with dilute mineral acid or in the case of acidic polysaccharides just by heating the aqueous solution. This process has often been described as autohydrolysis. The use of weak mineral acid (0·01-0·1N), apart from removing furanose sugars, may give reasonable yields of di- and higher oligosaccharides, leaving behind a less complex degraded polysaccharide. Comparative methylation studies of

the acid degraded and original polysaccharides will give information regarding the location of acid labile residues (33,34). The degraded polymer will be mainly composed of sugar residues in the pyranose form and glycosidically linked uronic acid residues. The pyranoside linkages can be broken by increasing the concentration of mineral acid to give monosaccharides and neutral oligosaccharides. The hydrolysis of glycosiduronic acid linkages to liberate acidic oligosaccharides and monosaccharides requires more vigorous hydrolysing conditions (N-2N).

The variable stability of the glycosidic linkages which permits remarkable selectivity has its disadvantages too. Thus it is very difficult to ascertain the points of attachment of acid labile units since they are the first to be cleaved during hydrolysis. It is, therefore, desirable to resort to other methods to obtain information concerning these linkages. A masterly technique to locate certain acid sensitive residues has been developed by Aspinall et al. (35). This involves the conversion of acid labile furanoside linkages to the acid resistant furanosiduronic acid linkages by oxidation with oxygen in the presence of a platinum catalyst. Thus it is possible to isolate and characterise aldobiouronic acids from the oxidised polysaccharide, maintaining and indicating the nature of the original glycosidic linkages.

Graded hydrolysis using non-aqueous reagents provides an effective tool to obtain higher yields of those oligosaccharides

which are difficult to obtain or difficult to obtain in workable quantities by aqueous graded hydrolysis. Partial acetolysis which is essentially a variation of aqueous graded hydrolysis, involves the treatment of the polysaccharide with acetic anhydride, concentrated sulphuric acid and sometimes acetic acid in the cold for a few days to give oligosaccharides, after deacetylation of oligosaccharide acetates. Mercaptolysis and methanolysis are other available techniques which have not frequently been used in the study of the gums.

The hydrolysis of polyuronides such as alginic acid, pectic acid and gum exudates with high percentages of uronic acid units results in considerable degradation. These poly-uronides are more easily hydrolysed if they are converted to the corresponding neutral polysaccharide by reduction. The conversion of hexouronic acid residues to neutral hexose residues can be carried out by first treating the polymer with ethylene oxide, followed by the reduction of the ethylene glycol ester with potassium borohydride (36). An alternative method involves the conversion of the polysaccharide into its acetate or propionate followed by reduction with diborane (37). Thus the rhamnose residues in gum arabic which are acid sensitive have been shown to be linked to the position 4 of glucuronic acid by isolation of the oligosaccharide 4-O-L-rhamnopyranosyl-D-glucose from the products of partial acetolysis of the carboxyl reduced gum arabic (38).

Enzymolysis has been frequently used for the selective

hydrolysis of certain polysaccharides, however, its use in the study of the gums is restricted perhaps due to the complex nature of the latter.

The examination of the products of partial hydrolysis involves the separation of acidic components from neutral components, the separation of monosaccharides from oligosaccharides, the separation of oligosaccharides from one another and finally their identification and characterisation. The neutral components may be separated from acidic components by absorption on weakly basic anion exchange resin. Separation and elution of acidic units can be carried out simultaneously either by eluting with solutions of increasing concentrations or by gradient elution. Neutral components may be fractionated by chromatography on cellulose (30) or charcoal-celite (39) columns. The homogeneity of the oligosaccharides, thus obtained may be assessed by paper chromatography and on ionophoresis.

By hydrolysis of the oligosaccharide, the component sugars may be identified and, by hydrolysis of the reduced oligosaccharide, the sugar in the reducing end group can be located. The mode of the linkage of the component sugars can be established by methylating the oligosaccharide followed by the hydrolysis and the identification of the resulting methylated sugars.

Additional information regarding the structure of the oligosaccharide may be obtained by periodate degradation (40). The consumption of the reagent and the estimation of the

resulting products such as formaldehyde and formic acid may indicate the type of substitution of the reducing units and the mode of the linkage. A better assessment of the periodate oxidation may be made possible by carrying out the reaction on oligosaccharide derivatives such as osazone (41), alcohol or aldonic acid (42). Information regarding the anomeric configuration of the linkage in the disaccharide may be obtained by lead tetra acetate oxidation. Using this procedure Perlin and co-workers (43) have been able to assign anomeric configuration to disaccharides by degrading them to glycitol glycosides and comparing them with authentic samples.

Care must be used, however, in assessing the structural significance from oligosaccharides obtained in minute quantities during partial acid hydrolysis since it has been demonstrated that acid reversion of monosaccharides results in the formation of oligosaccharides (44). However, a possible way of recognising polymerised oligosaccharides from acid reversion depends on the fact that they reach an equilibrium concentration and do not disappear on prolonged heating (45). Small quantities of artefacts arising from acid catalysed transglucolysation during acid hydrolysis have also been reported (46). It is, therefore, important to use caution while interpreting or ignoring results obtained from oligosaccharides isolated in small quantities.

METHYLATION

Partial hydrolysis at best will give an idea as to the order in which the sugar units are joined together and to a limited extent their linkages. An elaborate assessment of the linkages present in the polysaccharide can be elegantly carried out by blocking the free hydroxyl groups with non-reactive methyl ether groups. The methylated polymer on depolymerisation will liberate monomers carrying hydroxyl groups at those carbon atoms which were involved in the linkages between the residues. Characterization of the partly methylated sugars by preparing crystalline derivatives will provide evidence as to the nature of the sugar residues and to their linkages. The methylation studies will also suggest, whether the fragments are present as non-reducing terminal groups, at branch points or as constituents of inner chains.

The classical procedure for the methylation of polysaccharides is still that of Haworth (47), in which the polymer is treated with dimethyl sulphate and aqueous sodium hydroxide (30-40%). In the majority of the cases, however, even the repeated application of Haworth's procedure does not result in complete methylation. The widely accepted technique to effect complete methylation is that of Purdie and Irvine (48), which involves the treatment of partly methylated polysaccharide with methyl iodide and silver oxide. Kuhn and co-workers (49) have improved this method by methylating the polysaccharide in N.N dimethylformamide with methyl iodide, barium oxide and barium hydroxide, thus replacing the silver oxide which may

cause oxidative degradation.

An important modification of Haworth's method is to treat the polymer in N.N dimethylformamide or in dimethyl sulphoxide, with dimethyl sulphate, barium oxide and barium hydroxide. Kuhn and collaborators (50) using this technique have completely methylated hydrocarbons, nitrogen containing compounds, polyhydroxy compounds and natural products containing sialic acid in a single operation.

Other methods available to introduce methyl ether groupings involve the use of thallous oxide and methyl iodide, methyl iodide and sodium in liquid ammonia, and diazomethane.

The fully methylated polysaccharides are frequently insoluble in hot aqueous mineral acids and so cannot be hydrolysed directly. Partial acid catalysed depolymerisation in the cold followed by gradual heating to 100° may lead to complete hydrolysis. Alternative treatments involve preliminary methanolysis followed by hydrolysis of the methyl glycosides and formolysis followed by hydrolysis of the formyl esters. The separation of the products of hydrolysis may be effected by various chromatographic techniques available. The one which demands mention is the recently developed gas-liquid partition chromatography (51). This has been lately introduced for the separation and identification of carbohydrates and is being widely accepted as the most reliable analytical tool (52). As well as being reliable and sensitive gas liquid partition chromatography is experimentally neat and the results are remarkably reproducible. This process may efficiently be

adopted for the quantitative separation of pure fractions on a preparative scale by the recovery of the components from the effluent gas stream and thus facilitates the characterisation of the sugars by preparing crystalline derivatives.

Periodate Oxidation:

Important structural information may be obtained by subjecting the polysaccharide to periodate oxidation. The periodate ion is capable of cleaving the -C-C- bond in 1,2 diol and 1,2,3 triol systems. The estimation of periodate consummed, the formaldehyde and formic acid release, and the structural studies on the periodate resistant molecule will give an idea as to the linkages, the extent of branching and the backbone in the polysaccharide.

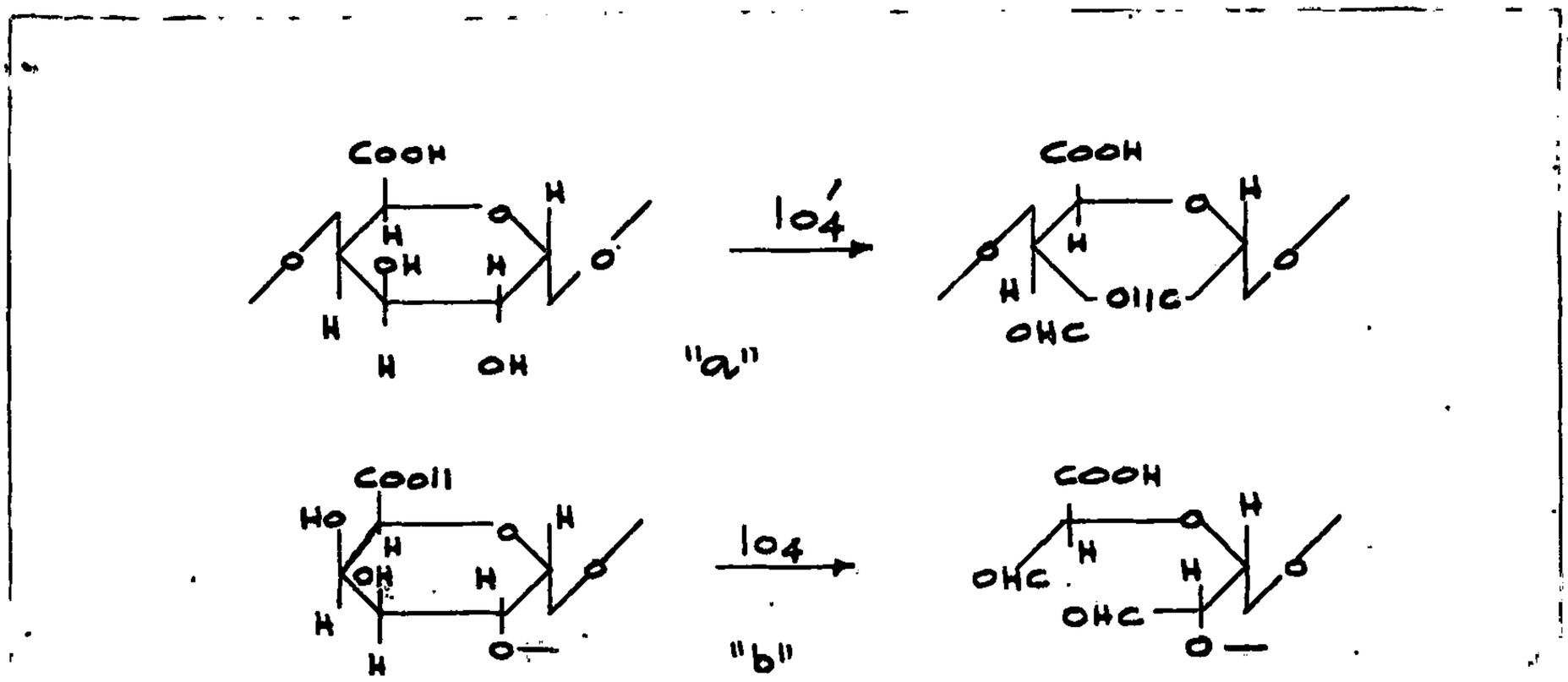
The specificity of the periodate oxidation reaction and the cleaner experimental procedure has resulted in the reaction being widely accepted as a standard analytical method for the structural investigations of the polysaccharide.

The cleavage of the -C-C- bond by the periodate ion readily takes place at room temperature and is stoichimetrically complete in a relatively short period. The periodate oxidation reaction is influenced by temperature, pH, buffers, concentration of periodate, solvents, light and the duration of oxidation (40). In many cases higher temperature, alkaline pH of the solution and higher concentration of the periodate ion have been reported to give products different from those obtained

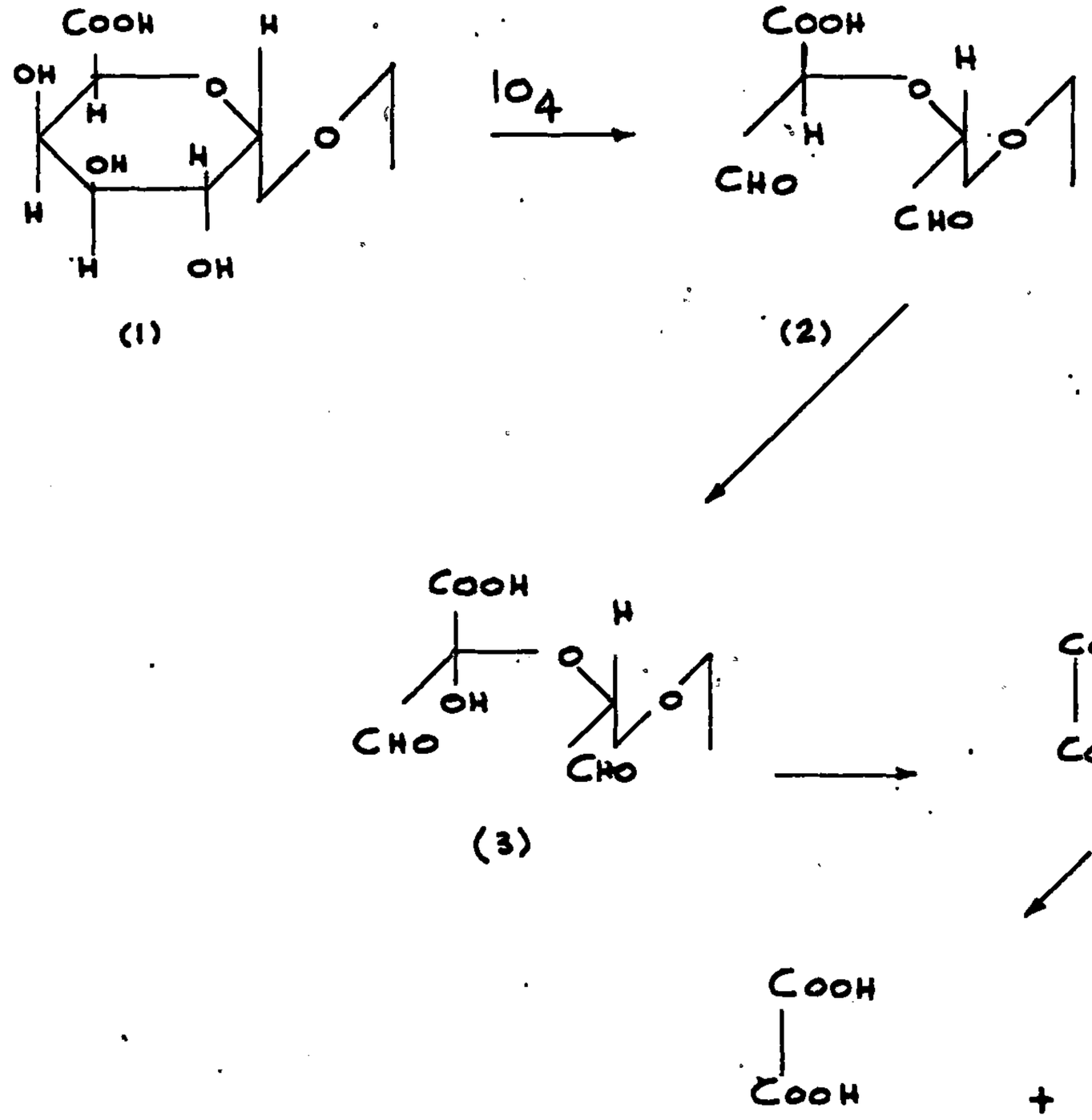
under normal reaction conditions, and thus give ambiguous results. This process which results from the over consumption of the periodate has often been described as over oxidation and involves the further oxidation of products of the glycol-cleaving reaction.

The over consumption of the periodate in the case of neutral polysaccharide can be controlled by the adjustment of pH, temperature, light and the concentration of periodate (4).

In the case of acidic polysaccharides the normal course of reaction is rather exceptional since the over-oxidation is a characteristic feature of the uronic acid residues. They behave abnormally and undergo complete oxidation to formic and oxalic acid. The uronic acid residues present as non-reducing terminal groups, joined by 1 , 4 (figure "a") and 1 , 2 (figure "b") linkages undergo cleavage to give a dialdehyde which is succeptible to complete oxidation (53).



An illustration of over-oxidation of non-reducing terminal uronic acid residues is given below:



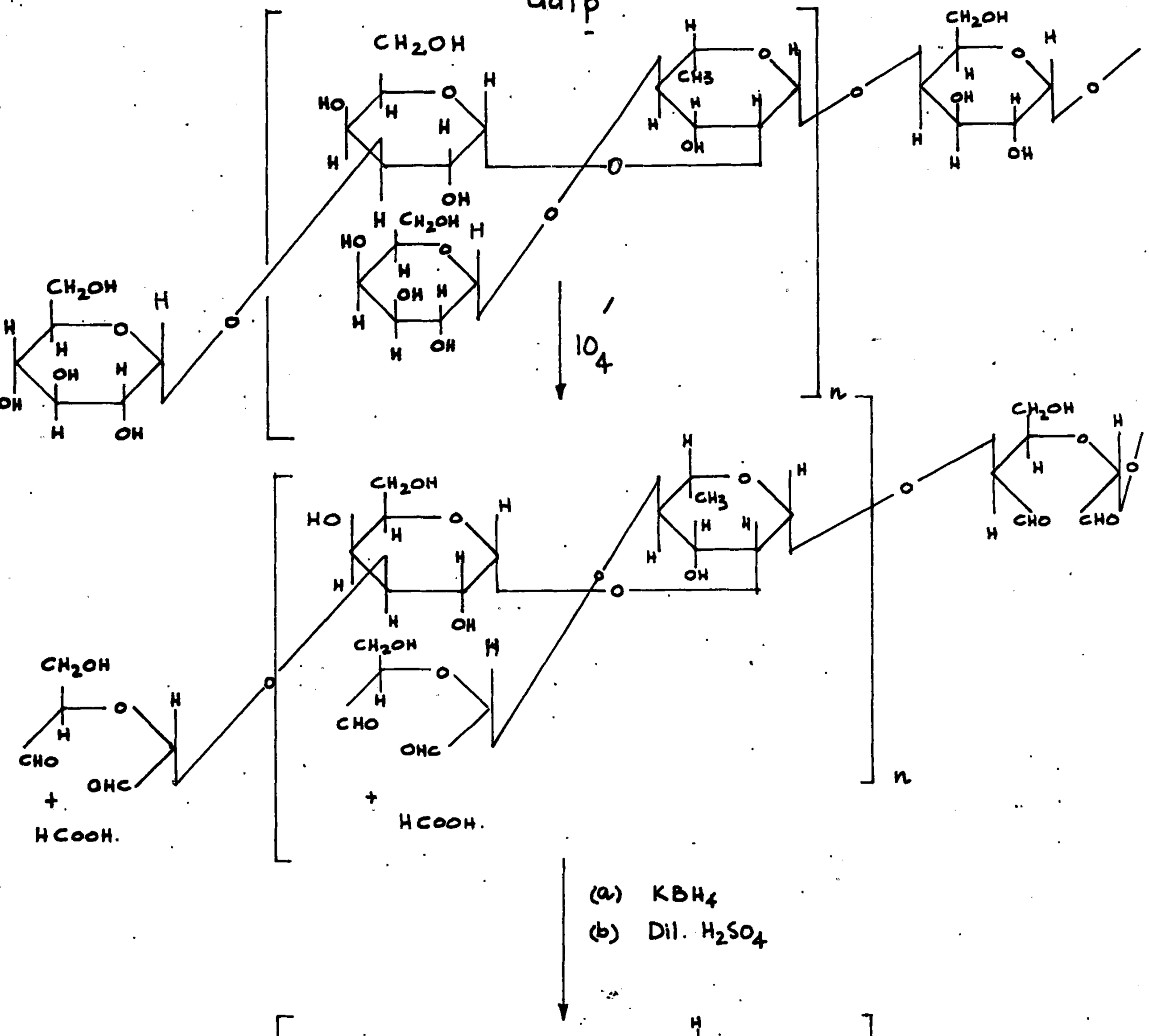
The extent of over-oxidation of uronic acid residues may be reduced, and the stage "2" in the above illustration and the dialdehyde stage in cases of 1, 4 and 1, 2 linked uronic acid units may be obtained by the use of buffer, control of temperature and pH (53).

The periodate oxidation of acidic polysaccharides is apparently a complex reaction and the results obtained from this reaction are open to many different interpretations. Thus in case of these complex polymers the periodate oxidation

Gpl — [→ 3 Gal p l → 2 Rha p l]→ 4 Gal p l —

4 ↑

Gal p



Other Methods for Structural Studies.

Alkaline degradation is an important chemical method for the structural investigation of the polysaccharides and although this reaction has not been frequently applied to the study of the gums, valuable information may be deduced from it. The degradation is believed to start at the reducing end and proceed stepwise. The saccharinic acids produced are characteristic of the linkages in the polysaccharide and thus supply information as to the structure (57).

The idea of cross reactions between bacterial antisera and polysaccharide is becoming increasingly important in the study of the gums. Heidelberger (58) has shown that many gums can be co-precipitated with antipneumococcus sera and attributed this phenomenon of mutual precipitation to the presence of characteristic multiple groupings of sugar residues. For example, the reactivity, in the case of pneumococcus Type I is possibly due to galacturonic acid, in Type II to glucuronic acid end groups, glucose branch points and 1,3-linked rhamnose residues and in Type III to multiple units of cellobiose and cellobiouronic acids.

The knowledge of the reactive groups which are responsible for precipitation is limited since only a few cases have been studied elaborately. It is possible that with the comprehensive studies of antipneumococcus capsular polysaccharide it may become easier to predict or confirm the presence of certain structural features in other polysaccharides.

Object of Present Investigation.

The plant gums comprise one of the most complicated groups of plant polysaccharides and although, with detailed chemical investigations it is possible to evaluate important structural features, the assignment of a complete chemical formula is not possible at the moment. The chemical methods discussed in this thesis can be used to locate the sugar residues and to provide information regarding the adjoining units, but the finer details of the structure of this complex group of polysaccharides are rather difficult to assess by the available procedures. However, chemical investigations of a number of gums show that gums on the basis of structural similarities may be grouped or arranged in the following three groups.

(a) Gums based on inner chains of D-galactose residues.

(b) Gums based on inner chains of D-xylose residues.

(c) Gums based on inner chains of D-galacturonic acid and L-rhamnose residues.

Studies on the gums of the type "c" such as Khaya senegalensis gum, Cochlospermum gossypium gum and Tragacanthic acid in this laboratory suggested that investigations into the structure of the industrially important Sterculia urens gum, commonly known as Karaya gum would be desirable. Sterculia urens gum an exudate of a genus Sterculia is structurally similar to the gums based on the inner chains of D-galacturonic acid and L-rhamnose residues. Preliminary investigations on this gum (59,60,61) showed the presence of galacturonic acid,

galactose and rhamnose residues. The other gums of the Sterculia family, namely, Sterculia setigera gum and Sterculia caudata gum have been investigated in detail. Sterculia setigera gum was found to contain galacturonic acid, galactose and rhamnose residues (61), whereas Sterculia caudata gum has been reported to contain glucuronic acid, galactose and rhamnose residues (62). The variations in the constituent sugar units in the gums of Sterculia family indicate the possibility of reasonable differences in the chemical structure of member gums.

D I S C U S S I O N

DISCUSSION.

The tree Sterculia urens, which grows in India, produces a gum, commonly known as Karaya gum, as a result of artificial stimulation. The gum nodules are hard and are brown to pale yellow in colour. Like other gums of the family Sterculiaceae, this gum absorbs large quantities of water and swells in the cold to give a translucent gel. The gum dissolves when its aqueous dispersion is heated. The sample of the gum under investigation was obtained from Clarke and Smith (India) Ltd., London.

Preliminary investigations showed that the crude gum occurs as partly acetylated derivative (acetyl content ca.7-8·5%). The presence of acetyl groups was shown by preparing a crystalline derivative of the distilled acetic acid.

Purification of the gum.

The powdered gum was dissolved in sodium hydroxide solution and in aqueous ammonia. The latter base seems to effect deacetylation more conveniently and thus results in the easy dissolution of the gum. The free gum acid was precipitated by the addition of ethanol acidified with concentrated hydrochloric acid. The precipitated gum was dissolved in water, the solution was reduced in volume and freeze dried to give purified gum. The purified gum was found to have specific rotation +62, uronic acid anhydride 39·5%, neutralisation equivalent 398-400, acetyl content and methoxyl content - nil.

Hydrolysis of the gum and subsequent chromatography of the hydrolysate showed the presence of galacturonic acid, galactose, rhamnose and a trace of arabinose.

A sample of the gum was hydrolysed with N-sulphuric acid for six hours and the products were methanolysed. Reduction of the derived methyl esters methylglycosides with potassium borohydride followed by hydrolysis and chromatography of the hydrolysate gave galactose, glucose, rhamnose and a trace of arabinose. The presence of glucose in the reduction products indicates that glucose has arisen from glucuronic acid residues. The previous investigations on the gum had shown that gum consists mainly of D-galacturonic acid, D-galactose and L-rhamnose residues (59,60).

Examination of the gum for heterogeneity.

The purified polysaccharide was examined for heterogeneity using various available techniques such as ionophoresis on glass fibre paper, DEAE-cellulose column chromatography and fractional precipitation.

Addition of calcium chloride to the gum solution gave complete precipitation. The polysaccharide was regenerated by treating the insoluble calcium salt with ammonium oxalate. The calcium precipitated polysaccharide had sugar components similar to those of purified gum. The changes in specific rotation and uronic acid anhydride of the calcium precipitated polysaccharide were not beyond experimental variation. Although

the possibility of co-precipitation of acidic polymers cannot be overlooked, it is expected that a neutral polymer would remain in the solution under these conditions.

Copper acetate has been used to fractionate a mixture of acidic polysaccharides (29). Using this procedure a precipitate of the polysaccharide was obtained. The polysaccharide was regenerated from the precipitated copper complex by treatment with hydrochloric acid. The free polysaccharide had the specific rotation +58.5°, uronic acid anhydride 39.5% and this suggests that there was no significant change in the polysaccharide obtained by this method and the purified polysaccharide.

Smith (13) using glass fibre ionophoresis has shown that many purified polysaccharides previously thought to be homogeneous are in fact heterogeneous. When the polysaccharide under investigation was examined for heterogeneity by this analytical method only one component was observed on the developed ionophoretograms. This shows that Sterculia urens gum may consist of a homogeneous polymer.

The purified polysaccharide was placed on a DEAE-cellulose column in the phosphate form. Elution of the column with increasing concentration of phosphate buffer gave very small amounts of polymer estimated by the anthrone colorimetric method. The eluted polysaccharide was in too small a quantity to be subjected to further investigations. The gradient elution of the column with sodium hydroxide and subsequent

examination of the eluant fractions by the carbazole colorimetric method showed that most of the polysaccharide was eluted by the alkali over the range of 0·07-0·11N sodium hydroxide. A plot of the polysaccharide concentration against volume of the eluant showed the presence of a single polymer. The polysaccharide containing fractions were combined, reduced in volume, dialysed and the resulting inorganic ions free solution was freeze dried. The polysaccharide obtained showed very little change in uronic acid anhydride and no change in component sugars. The remaining fractions eluted by alkali were combined, dialysed and freeze dried. The quantity of the polysaccharide obtained was very small and was possibly contaminated with inorganic material. The values obtained for specific rotation and uronic acid anhydride content are therefore, of little significance.

Another small scale experiment was carried out on a DEAE-cellulose column using potassium chloride as an eluant (in place of sodium hydroxide). A plot of polysaccharide concentration against the volume of the eluant showed the presence of two fractions.

The two fractions, however, had the same percentage of uronic acid anhydride and the same component sugars.

Cetavlon has been used in many cases to resolve mixtures of polysaccharides (28). Cetavlon precipitates acidic polysaccharides and treatment of the precipitated complexes with acetic acid liberates the free polymers. Sterculia urens

gum readily gave a precipitate with cetavlon which was regenerated by treatment with acetic acid to give a polysaccharide A_1. The polysaccharide A1 had virtually the same specific rotation, uronic acid anhydride and component sugars as the purified gum.

The polysaccharide A1 was subjected to a further cetavlon treatment to give a twice cetavlon precipitated polysaccharide A_2. The polymer A_2 had no significant variation in specific rotation, uronic acid anhydride and component units. The polymer A_2 was once more treated with cetavlon and the regenerated polymer A_3 had optical rotation, uronic acid anhydride content and component units similar to that of polymer A_2.

The polysaccharide A3 was placed on a DEAE-cellulose column and the column was eluted with increasing concentration of phosphate buffer followed by a gradient elution with sodium hydroxide. A plot of the polysaccharide concentration against the volume of eluate again suggested that a very little amount of the polymer was eluted with phosphate buffer and most of the polysaccharide was eluted by the alkali. The alkali eluted polysaccharide (fractions of the peak) had specific rotation, uronic acid anhydride and component units similar to that of polysaccharide A_3 and virtually the same as those of the purified gum.

The evidence provided by the different fractionation methods shows that Sterculia urens gum either consists of a single polymer or if it is heterogeneous, the polymers must be structurally similar and differ only in the proportions of

the neutral sugar units rather than in the nature of constituent sugar units.

Graded Hydrolysis.

Graded hydrolysis of the gum acid was carried out with N-sulphuric acid at 100° for five and a half hours. Examination of the products of hydrolysis showed the presence of acidic sugars, galactose, rhamnose and a trace of arabinose.

The acetone:water soluble sugar mixture which contained acidic and neutral sugars was separated on an anion exchange resin column into acidic and neutral sugars. The neutral sugar fraction was examined chromatographically and was found to contain galactose and rhamnose together with traces of arabinose and galacturonic acid.

The acidic sugars which were eluted from the column by water containing increasing amounts of formic acid, were further fractionated by chromatography on thick paper. A total of six acidic sugar fractions were obtained, of which two were present in relatively small quantities.

The fraction 1 which had the chromatographic mobility of galacturonic acid was characterised as D-galacturonic acid by preparing a crystalline derivative, D-galacturonic acid, 2,5-dichlorophenylhydrazone.

The second component isolated was the main aldobiouronic acid, 2-O-(α-D-galactopyranosyluronic acid)-L-rhamnose. The sugar was methylated by treatment with Haworth reagents. The methylated sugar crystallised and had specific rotation,

melting point and X-ray powder diagram characteristic of methyl-2-O-(α-D-galactopyranosyluronic acid)-L-rhamnoside pentamethyl ether dihydrate.

The third acidic component was isolated in very small quantity and only preliminary investigations were possible. The sugar was chromatographically pure. Hydrolysis of the sugar and subsequent chromatography of the hydrolysate gave galactose and galacturonic acid. Reduction of the derived methyl ester methylglycosides with potassium borohydride followed by hydrolysis gave only galactose.

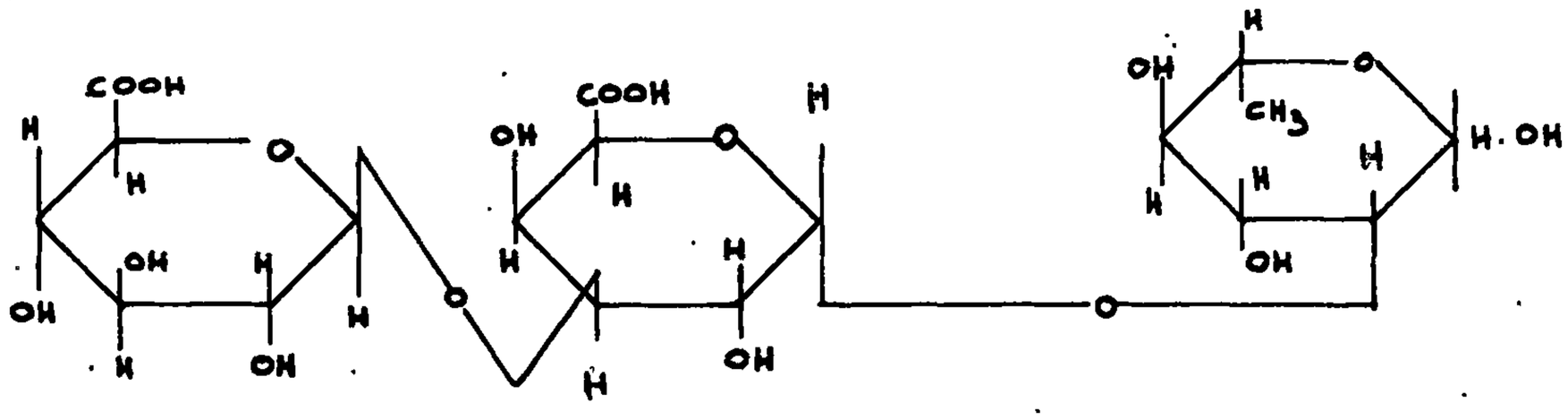
The fourth acidic component was chromatographically and ionophoretically pure and appeared to be a trisaccharide. The sugar afforded galacturonic acid, glucuronic acid and rhamnose on hydrolysis with N-sulphuric acid. Reduction of the derived methyl ester methylglycosides of the sugar with potassium borohydride followed by hydrolysis gave galactose, glucose and rhamnose. Reduction of the acid to the corresponding glycitol with potassium borohydride followed by hydrolysis showed the presence of galacturonic acid, glucuronic acid, rhamnitol and the absence of rhamnose.

The sugar was methylated by treatment with Haworth and Purdie reagents. The fully methylated acid was reduced with lithium aluminium hydride. Hydrolysis of the reduced oligosaccharide with N-sulphuric acid and subsequent examination of the hydrolysate by chromatography and on ionophoresis showed the presence of 2,3,4-tri-O-methylglucose, 3,4-di-O-methyl-

rhamnose and 2,4-di-O-methylgalactose.

Gas-liquid partition chromatography of the derived methylglycoside of the sugar indicated the presence of methylglycosides of 2,3,4-tri-O-methylglucose, 3,4-di-O-methylrhamnose and 2,4-di-O-methylgalactose. The remaining methylated reduced oligosaccharide was hydrolysed and the component sugars were separated chromatographically and ionophoretically on filter sheets. The 2,4-di-O-methyl-D-galactose and 3,4-di-O-methyl-L-rhamnose were characterised by their crystallinity. The 2,3,4-tri-O-methyl-D-glucose was characterised by preparing a crystalline derivative, 2,3,4-tri-O-methyl-N-phenyl-D-glucosylamine.

The identification of the glucuronic acid as a non-reducing end group (as shown by the methylation studies) and the detection of the rhamnose as a reducing group indicates that the galacturonic acid is located in between the above two residues. It, therefore, follows that the trisaccharide is linear and may be assigned the following formula:



The glucuronic acid in the trisaccharide has been assigned the β-configuration after comparison of the molecular rotation of the trisaccharide (229°) with that of its fragment 2-O-(α-D-galactopyranosyluronic acid)-L-rhamnose (306°).

The fifth fraction when examined chromatographically, streaked in all the solvents but appeared homogeneous to visual inspection. However, the stain of the sugar developed with aniline oxalate spray gave a heterogeneous fluorescence in ultra violet light. This observation was further supported when the sugar was examined ionophoretically.

Hydrolysis of the sugar with N-sulphuric acid and subsequent chromatography of the hydrolysate gave galactose, rhamnose, galacturonic acid and glucuronic acid. Reduction of the derived methyl esters methylglycosides with potassium borohydride followed by hydrolysis gave galactose, glucose and rhamnose. Reduction of sugar to the corresponding glycitols with potassium borohydride, followed by hydrolysis and subsequent chromatography of the hydrolysate showed the presence of galactitol, rhamnitol, galacturonic acid, glucuronic acid and the absence of rhamnose and galactose. The presence of two reducing groups in the mixture indicates that the sugar may be a mixture of at least two acidic oligosaccharides.

A portion of the acid mixture was methylated by treatment with Haworth and Purdie reagents. Hydrolysis of the methylated acid and subsequent chromatography of the hydrolysate showed the presence of 2,3,6-tri-O-methylgalactose, 3,4-di-O-methyl-

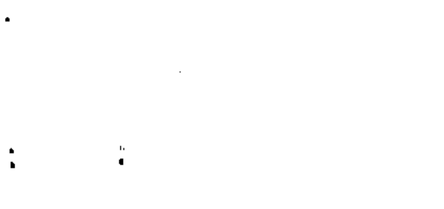
rhamnose and a mixture of methylated acids. A portion of the methylated acids were methanolysed and the products were examined by gas-liquid partition chromatography. Methylglycosides of 2,3,4-tri-O-methylgalacturonic acid, 2,3,4-tri-O-methylglucuronic acid, 2,3,6-tri-O-methylgalactose and 3,4-di-O-methylrhamnose were detected.

A portion of the hydrolysed methylated acid mixture was converted into the methyl esters methylglycosides and the products were reduced with potassium borohydride. Hydrolysis of the reduced glycosides and subsequent chromatography of the hydrolysate showed the presence of 2,3,6-tri-O-methylgalactose, 2,3,4-tri-O-methylgalactose, 2,4-di-O-methylgalactose and 2,3,4-tri-O-methylglucose and 3,4-di-O-methylrhamnose. Vapour phase chromatography of the derived methylglycosides of the methylated reduced mixture of the acids showed the presence of the methylglycosides of 2,3,6-tri-O-methylgalactose, 2,3,4-tri-O-methylgalactose, 2,4-di-O-methylgalactose, 2,3,4-tri-O-methylglucose and 3,4-di-O-methylrhamnose.

The remaining mixture of hydrolysed sugars (methylated acids) was separated into neutral and acidic sugars by chromatography on filter sheets. The neutral sugar 2,3;6-tri-O-methyl-D-galactose was characterised by conversion into a crystalline aldonolactone derivative while 3,4-di-O-methyl-L-rhamnose was characterised by its crystallinity.

The experimental evidence so far obtained indicates the presence of two acidic fragments in the mixture; a tri-

saccharide structurally similar to component 4(i) and a di-saccharide with components similar to those of fraction 3 and whose structure may be formulated as follows (ii)



(1)

D-Gal A 1→4 D-Gal (ii)

A small portion of the acid mixture was subjected to degradation by alkali in the cold and in the absence of oxygen with the hope that the acid (i) in the mixture would be resistant to alkali. Chromatographic examination of the products showed the presence of a sugar with chromatographic mobility of component 4 and a trace of galacturonic acid. This corresponds to the fact that non-reducing sugars and sugars substituted at C2 (as in the case of acid (i)) will not be attacked by cold dilute alkali whereas a fragment like acid (ii) will be attacked giving isosaccharinates and galacturonic acid which in turn will give saccharinic acids.

The remaining mixture of acids was degraded with alkali and the product was methylated by treatment with Haworth and Purdie reagents. The methylated acid was reduced with lithium aluminium hydride. A portion of the reduced methylated acid mixture was methanolysed and the products were examined by gas-liquid partition chromatography. The methyl glycosides of the following sugars were detected.

2,3,4-Tri-O-methylglucose.

2,4-Di-O-methylgalactose.

3,4-Di-O-methylrhamnose.

In addition to the above sugars gas-liquid partition chromatography also indicated, traces of 2,3,6-tri-O-methyl-galactose and 2,3,4-tri-O-methylgalactose. The presence of these sugars in traces indicates that the alkaline degradation of acid (ii) was incomplete.

It may, therefore, be concluded from the above experimental evidence that the mixture consists of the following two acidic sugars:

a. D-GAl $\xrightarrow{\beta}$ 3 D Gal A 1 $\xrightarrow{\alpha}$ 2 L-Rha

b. D-Gal A 1 → 4 D-Gal.

It may be noted in this context that Cochlospermum gossypium which is structurally very similar to the gums of Sterculiaceae family furnished an acidic fragment on partial acid hydrolysis which was similar to fraction five and its separation into component acids was not possible (79).

The fraction 6 was isolated only in small quantity and hydrolysis of this sugar indicated the presence of galacturonic acid, galactose and rhamnose as component sugars.

Methylation results.

The purified gum acid was methylated with methyl sulphate and sodium hydroxide solution. The partly methylated gum acid was converted into the silver salt and the methylation was

completed by two treatments with methyl iodide and silver oxide. The fully methylated polysaccharide had $[\alpha]_D$+66° and OMe, 41%.

Gas-liquid partition chromatography of the methanolysed polymer and paper chromatography of the hydrolysed methylated polysaccharide showed the presence of the following methylated sugars together with unidentified methylated acids.

2,3,4,6-Tetra-O-methylgalactose.

2,3,6-Tri-O-methylgalactose.

2,3,4-Tri-O-methylrhamnose.

3,4-Di-O-methylrhamnose.

Rhamnose.

2,3,4-Tri-O-methylglucuronic acid.

The methylated gum acid was reduced with lithium aluminium hydride to give a neutral polymer. Small portions of the carboxyl reduced methylated gum were methanolysed and hydrolysed. Vapour phase and paper chromatography of the products of methanolysis and hydrolysis showed the presence of the following methylated sugars.

2,3,4,6-Tetra-O-methylgalactose.

2,3,6-Tri-O-methylgalactose.

2,3-Di-O-methylgalactose.

3-O-Methylgalactose.

2-O-Methylgalactose.

2,3,4-Tri-O-methylrhamnose.

3,4-Di-O-methylrhamnose.

3-O-methylrhamnose.

Rhamnose.

2,3,4-Tri-O-methylglucose.

Since galactose is a component sugar of the gum acid it is, therefore, necessary to distinguish between methylated derivatives arising from this galactose and the galactose formed by the reduction of galacturonic acid. Comparing the sugars obtained after methylating the gum acid and the sugars obtained after reducing the methylated gum acid it may be concluded that the following sugars arose from the acidic components of the polysaccharide.

2,3,4-Tri-O-methylglucose.

2-O-Methylgalactose.

3-O-methylgalactose.

2,3-Di-O-methylgalactose.

The fully methylated (carboxyl reduced) gum was hydrolysed with hydrochloric acid and the hydrolysate was neutralised by treatment with silver carbonate. The syrup containing the sugars was placed on a cellulose column and the column was eluted with petroleum ether: butan-1-ol (70:30), petroleum ether: butan-1-ol (50:50), butan-1-ol half saturated with water and finally with water.

The following sugars were eluted from the column and were characterised by preparing crystalline derivatives.

2,3,4,6-Tetra-O-methyl-D-galactose.

2,3,6-Tri-O-methyl-D-galactose.

2,6-Di-O-methyl-D-galactose.

2,3-Di-O-methyl-D-galactose.

2-O-Methyl-D-galactose.

3-O-Methyl-D-galactose.

2,3,4-Tri-O-methyl-L-rhamnose.

3,4-Di-O-methyl-L-rhamnose.

3-O-Methyl-L-rhamnose.

L-Rhamnose.

2,3,4-Tri-O-methyl-D-glucose.

In addition to the above sugars gas liquid partition chromatography indicated the presence of 2,3-di-O-methylrhamnose.

Smith degradation studies on the gum acid.

The gum acid was subjected to Smith degradation in order to obtain information on the periodate resistant part of the gum molecule.

A trial experiment was carried out by degrading the gum acid with sodium metaperiodate until no more reagent was consumed. It was observed that 0·87 mole of the oxidant was consumed per sugar unit. The polyaldehyde was reduced with potassium borohydride to the polyalcohol. The acetal linkages in the polyalcohol were cleaved with mineral acid at room temperature and the degraded polymer was precipitated with ethanol leaving in solution the cleaved residues. This procedure is referred to as Smith degradation.

The degraded gum acid obtained, by precipitation with ethanol, on hydrolysis gave galacturonic acid, galactose and

rhamnose. A sample of the polysaccharide was hydrolysed with N-sulphuric acid for five hours and the products were methanolysed. Reduction of the derived methyl esters methylglycosides with potassium borohydride followed by hydrolysis gave galactose and rhamnose.

Another sample of the degraded gum was hydrolysed for five hours and subsequent chromatography of the hydrolysate showed the presence of galacturonic acid, galactose, rhamnose and 2-O-galacturonosylrhamnose.

The soluble portion obtained by evaporation of the supernatant mother liquor contained non-reducing sugars together with the low molecular weight material such as glycerol and threitol. Hydrolysis of this fraction with N-sulphuric acid and subsequent chromatography of the hydrolysate showed the presence of galacturonic acid and rhamnose together with the low molecular weight material.

A large quantity of the gum acid was subjected to Smith degradation and the degraded gum acid was precipitated with ethanol.

The Smith degraded gum was partially hydrolysed and the hydrolysate was found to contain three acidic sugars together with galactose and rhamnose. The syrup containing acidic and neutral sugars was placed on a column of Amberlite resin CG 45 in the formate form. The neutral sugars were eluted with formic acid. The acidic sugars were further separated on filter sheets.

Component 1 had the chromatographic mobility of galacturonic acid. Reduction of the derived methyl ester methylglycosides of the sugar with potassium borohydride followed by hydrolysis gave only galactose.

Component 2 had the chromatographic mobility and the fluorescence, when stained with aniline oxalate, which are characteristic of 2-O-galacturonosylrhamnose. Hydrolysis of the sugar followed by chromatography gave galacturonic acid and rhamnose. Reduction of methyl ester methylglycosides of the sugar with potassium borohydride followed by hydrolysis gave galactose and rhamnose. The sugar was characterised as 2-O-(α-D-galactopyranosyluronic acid)-L-rhamnose by preparing a crystalline methylated derivative.

Component 3 was isolated in very small quantity and gave galacturonic acid and rhamnose on hydrolysis. Reduction of the derived methyl ester methylglycosides followed by hydrolysis gave galactose and rhamnose. Reduction of the acid to the corresponding glycitol with potassium borohydride followed by hydrolysis and subsequent chromatography of the hydrolysate showed the presence of galacturonic acid and rhamnitol. Partial hydrolysis of the sugar with 0·1N sulphuric acid gave galacturonic acid, rhamnose and 2-O-galacturonosylrhamnose.

The sugar was methylated by treatment with methyl sulphate and sodium hydroxide solution, and methyl iodide and silver oxide. The methylated acid was reduced with lithium aluminium hydride and a small portion of the reduced product was methanolysed. Gas-liquid partition chromatography of

methanolysis products showed the presence of methylglycosides of 2,3,4-tri-O-methylgalactose, 3,4-di-O-methylrhamnose and an unidentified sugar.

The evidence provided by the above experiments is not enough to formulate the structure of the sugar. However, it indicates a possibility of the following structure for the trisaccharide.

Gal A 1⟶? Gal A 1⟶ 2 Rha.

Structural features of Sterculia urens gum.

The structural features of the gum based on methylation, partial acid hydrolysis and Smith degradation studies are discussed below.

The neutral methylated sugars isolated from the methylated polysaccharide show the presence of the following sugar residues in the gum.

D-Gal 1- D-Gal 1- -3 D-Gal 1-
4 4

L-Rha 1- -2L Rha 1- -2L Rha 1- -2L Rha 1-
4 3,4

Although 2,6-di-O-methyl-D-galactose was isolated only in a small quantity from the methylated gum, detection of galactose in the Smith degraded polymer indicates the presence of periodate resistant galactose in the gum.

The acidic portion of the gum as shown by the methylation studies consists of 1,4-linked D-galacturonic acid, having

branch points at C_2 and C_3, and glucuronic acid residues present only as non-reducing terminal groups as shown below.

4 D-Gal Al- -4 D Gal Al- -4 D-Gal Al-
2 3

D GAl-

The main aldobiouronic acid obtained from the gum is 2-O-(α-D-galactopyranosyluronic acid)-L-rhamnose. This acid was isolated from the products of partial acid hydrolysis of the gum and also from the Smith degraded gum. It is obvious that this fragment is arising from the periodate resistant backbone of the gum.

-4 D Gal Al $\xrightarrow{\alpha}$ 2 L-Rha 1-
(2) 4

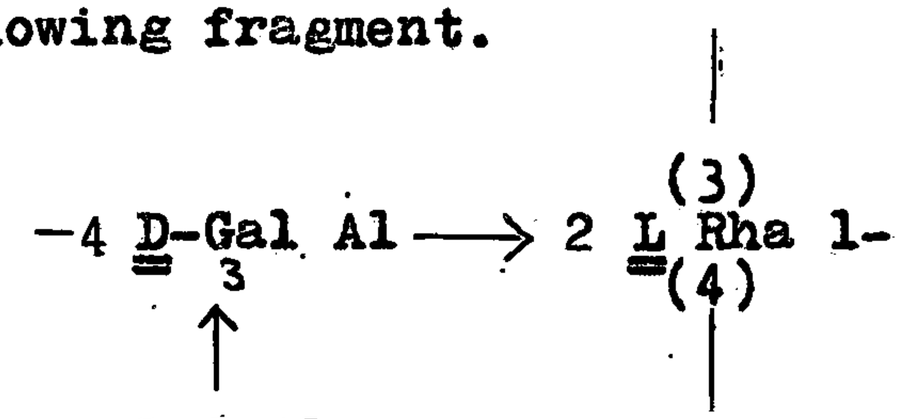
The partial acid hydrolysis of the gum also gave 4-O-(D-galactopyranosyluronic acid)-D-galactose. It is difficult to say how this fragment is incorporated in the gum.

... D-Gal Al→4 D Gal 1-

The partial acid hydrolysis of the gum afforded a trisaccharide with the following structure.

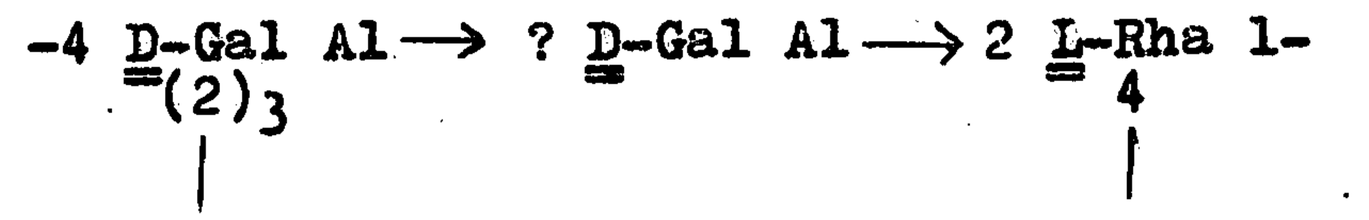
D-G Al $\xrightarrow{\beta}$ 3D-Gal Al $\xrightarrow{\alpha}$ 2L Rha

Methylation studies on the gum have shown the presence of 1,3,4- but no 1,3-linked galacturonic acid. It, therefore, follows that the galacturonic acid unit of the trisaccharide in the gum is present as a branch point. The gum was found to contain 2-, 2,4- and 2,3,4-substituted rhamnose residues. It is, therefore, not possible to assess how the rhamnose unit of the trisaccharide is incorporated in the gum. The point of attachment of glucuronic acid is in agreement with the above studies where only D-glucuronic acid terminal groups were isolated. The gum, therefore, contains the trisaccharide as a following fragment.



Hydrolysis of the Smith degraded polymer liberated a trisaccharide. Although this trisaccharide has not been fully characterised it contains centiguous galacturonic acid residues linked to a rhamnose unit.

Since this sugar arises from the periodate resistant portion of the gum molecule, it is probable that all the three units are present in the gum as branching points and arise from the excessively branched inner core of the gum as indicated in the following partial structure.



On the basis of the above results it can be concluded that the gum polysaccharide contains the following sequences which may or may not overlap in the molecule of the gum.

In addition the gum molecule is found to contain sugar residues, V and VI, the point of their attachment is yet to be accounted for.

i. -4 D-Gal Al $\xrightarrow{\alpha}$ 2,(3) L-Rha 1-
3
↑
D-GAl

ii. -4-D-Gal Al $\xrightarrow{\alpha}$ 2,(3) L-Rha 1-
(2)$_3$ (4)

iii. ..... D-Gal Al $\longrightarrow$ 4 D-Gal 1-

iv. -4 D-Gal Al —— ? D Gal Al $\longrightarrow$ 2,4 L-Rha 1-
(2)$_3$

v. D-Gal 1- L-Rha 1-

vi. -4 D-Gal 1- -4 D-Gal 1-
3

In formulating a structure for the gum it is difficult to incorporate the above fragments, but it is very likely that the gum polysaccharide consists of a main chain of D-galacturonic acid and L-rhamnose. All the D-glucuronic acid and some of the L-rhamnose and D-galactose residues are present

as end groups attached as side chains to D-galacturonic acid and L-rhamnose residues in the interior chains, which must provide the branching points in the polysaccharide.

Comparison of the Structural features of Sterculia urens gum with those of other gums.

Although a large number of gums have received preliminary investigations, but relatively a few have been investigated in detail. The structural evidence so far obtained indicates that the gums in general may be arranged in the following two groups.

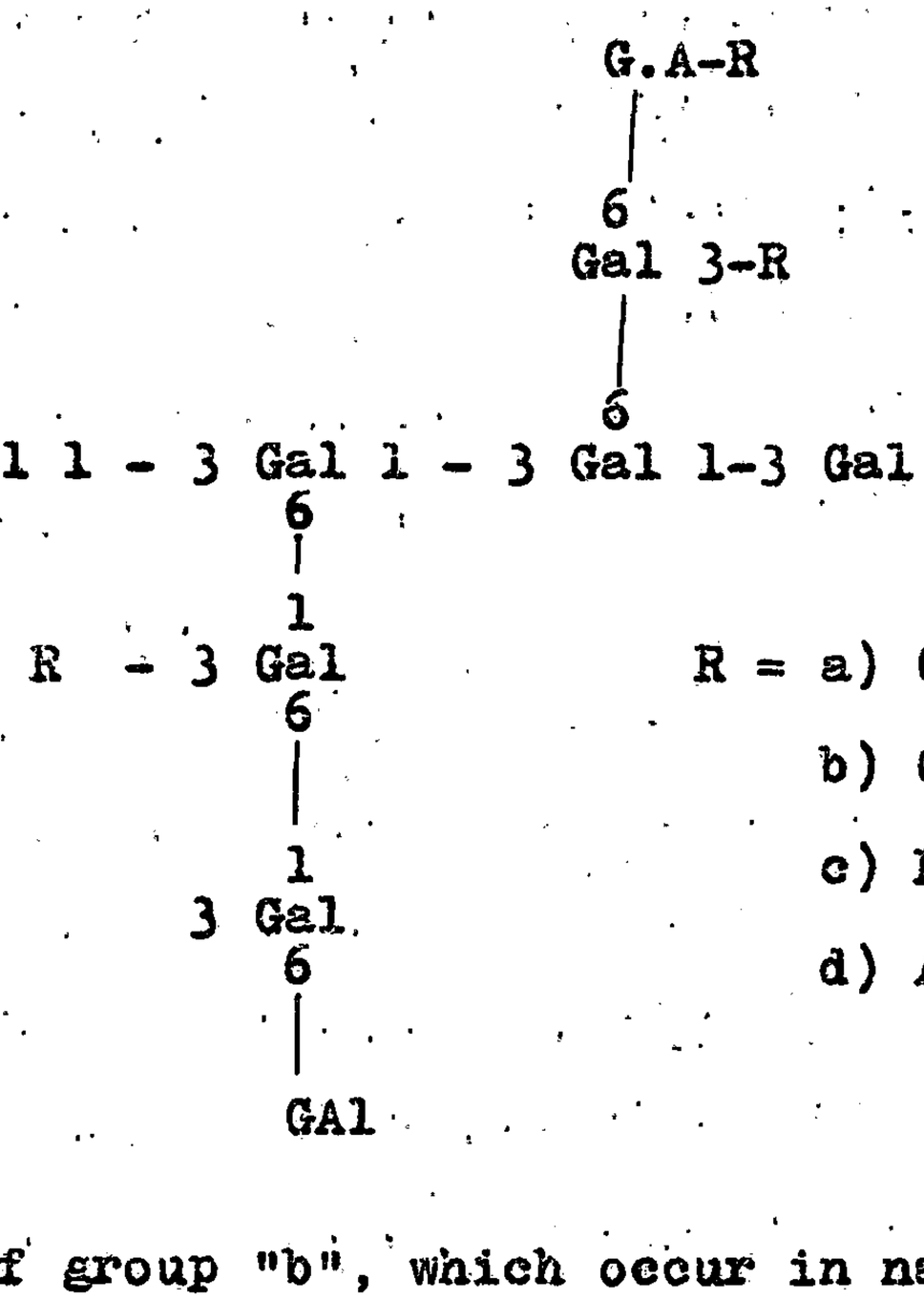
a) Gums based on inner chains of D-galactose residues.

b) Gums based on inner chains of D-galacturonic acid and L-rhamnose residues.

The structural investigations of a number of Acacia (81-91), Prunus (92-101) and Cumbretaceae (109-113) together with Citrus lemon (103,104), Prosopisjuliflora (105-107), Asafoetida (102) and Cholla (94,99) gums, which belong to the group "a", have indicated that they have inner chains of 1,3- and 1,6-linked D-galactose residues to which a complex periphery is attached. The periphery which is generally acid labile, is composed mainly of pentose units. The uronic acid content of these gums is generally low (10-20%) and the common acidic component with the exception of Combretum leonense and Cholla gums is D-glucuronic acid, which may occur sometimes wholly or partly as its 4-O-methyl ether. These gums generally have three types of linkages with distinctly different rates of hydrolysis. This property provides an efficient method to effect stepwise degradation of the polymer and advantage has been taken of this in the study of a number of gums. The

gums of type "a" are similar in many respects to gum arabic from Acacia senegal, which has been investigated in detail. They all contain almost the same component units although in different proportions. Since gum arabic is well investigated and typifies most of the general characteristics of the gums of group "a", its structural features are compared with those of Sterculia urens gum.

Gum arabic had been stepwise degraded to liberate L-arabinose, L-rhamnose, 3-O-α-D-galactopyranosyl-L-arabinose and a degraded polymer. The degraded polymer was further hydrolysed to give 3-O-β-D-galactopyranosyl-D-galactose and an aldobiouronic acid (6-O-(β-D-glucopyranosyluronic acid)-D-galactose. Such a stepwise degradation to liberate neutral oligosaccharides, a degraded polymer and then acidic oligosaccharides is generally not possible in the case of gums of group "b" and was not achieved for Sterculia urens gum. The structural investigations of gum arabic indicated that most of the galactose residues are either 1,6- or 1,3-linked. In Sterculia urens gum there is diversity of linkages and each of the sugar residues except those of D-glucuronic acid residues are involved in linkages through various positions. The two gums differ fundamentally in their basal structure too, one having the inner chains of D-galacturonic acid and L-rhamnose residues whereas the other has D-galactose residues as the only component of the inner chains. A partial structure of gum arabic is shown below.



R = a) Gal 1-

b) Gal 1-3 Ara f 1-3 Ara f 1-

c) Rha 1-

d) Ara f 1-

The gums of group "b", which occur in nature as partly acetylated polysaccharides are characterised by having high uronic acid anhydride content, usually of the order 40-50%. These gums differ from the gums of type "a" in having 1,4-linked galactose residues rather than 1,3- or 1,6-linked residues. Furthermore, these gums have lower proportions of acid labile groups, so that autohydrolysis to give a degraded polymer is generally not possible.

Sterculia urens gum has a high percentage of uronic acid residues, the galactose residues are 1,4-linked and the inner chains are composed only of D-galacturonic acid and L-rhamnose residues. These structural features of Sterculia urens gum resemble those of the gums of type "b". For the purpose of structural comparison of Sterculia urens gum with the gums of

type "b", such as Khaya, Sterculia and Cochlospermum gossypium gums, their known structural features are summarised in Table 1.

Although more elaborate information is required before detailed structural comparisons of these gums can be made, a number of similarities and variations are outlined below.

D-Galactose residues in all the six gums are present as 1,4-linked and end groups. In the case of Sterculia urens and Cochlospermum gossypium gums some of the galactose residues have also been found as branching points. Each of these gums contains L-rhamnose residues in the inner chains and as branching points. Sterculia urens and Cochlospermum gossypium gums differ from the rest of these gums in having some of the L-rhamnose units as non-reducing terminal groups.

All the gums given in the Table 1 except Sterculia setigera have residues of D-glucuronic acid or of its 4-O-methyl ether present exclusively as end groups. The D-galacturonic acid residues are only found in the inner chains. Differences between these gums are obvious in the nature of the units involved in the branching points, both L-rhamnose and D-galacturonic acid are involved in Sterculia urens and Cochlospermum gossypium gums, but only L-rhamnose units in the Khaya gums.

The acidic fragments which have been derived from these gums are shown in Table 2. Direct evidence for the presence of an aldobiouronic acid, 2-O-(α-D-galactopyranosyluronic acid)-L-rhamnose is available for the first four gums (in Table 2) and its presence in Sterculia setigera has also been indicated.

Investigations on Sterculia caudata (Brachychiton diversifolium) gum so far carried out show that the gum contains D-glucuronic acid residues as the only acidic component and these units are present only as non-reducing terminal groups. Since the D-glucuronic acid residues are present entirely as end groups, it is very likely that hexuronic acid residues may also be present in the inner chains. Although evidence for the presence of the D-galacturonic acid is not available, the indications are that the hexuronic acid residues are present since reduction of the gum results in an increase of the galactose content. Furthermore, this gum differs from the rest of the gums of group "b" in that it liberates on hydrolysis 2-O-(α-D-glucopyranosyluronic acid)-L-rhamnose in contrast to 2-O-(α-D-galactopyranosyluronic acid)-L-rhamnose, which is a common constituent of the rest of the gums.

Sterculia urens and Cochlospermum gossypium gums like the Khaya gums contain D-glucuronic acid end groups. In the case of Khaya gums, the isolation of an aldobiouronic acid, 4-O-(4-O-methyl-D-glucopyranosyluronic acid)-D-galactose has shown that D-glucuronic acid end groups are joined to D-galactose residues. In the present investigations of Sterculia urens gum, however, it has been shown that D-glucuronic acid terminal groups are linked directly to D-galacturonic acid residues present in the inner chains, by the isolation of a trisaccharide (No.5 in Table 2).

Although, in the case of Cochlospermum gossypium gum the

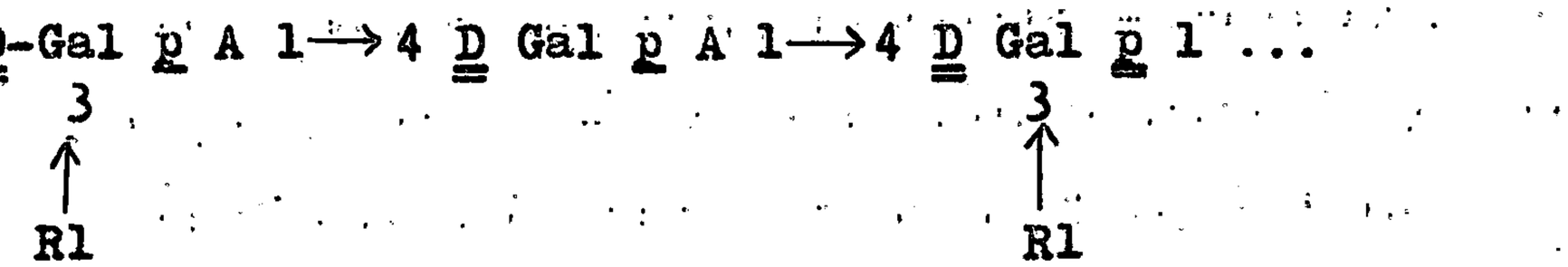
point of attachment of D-glucuronic acid residues has not been directly demonstrated, the results obtained are in consistence with the possibility that the D-glucuronic acid residues are joined to D-galacturonic acid residues (79).

Sterculia urens gum afforded 4-O-(D-galactopyranosyluronic acid)-D-galactose. The presence of this acid in the products of hydrolysis of Sterculia setigera and Cochlospermum gossypium gum is also indicated.

Gum tragacanth was one of the first D-galacturonic acid gums to receive comprehensive investigation. The gum was fractionated into three fractions (21). More recently the water soluble portion has been separated into two polysaccharides (119). The acidic polysaccharide (tragacanthic acid) was investigated in detail and has been shown to contain α-D-galacturonic acid with side chains attached to position of galacturonic acid residues. Although this acid (tragacanthic acid) differs considerably from Sterculia urens gum, in the component units and the components of the inner chains, but the presence of 1,4-linked galacturonic acid residues in the inner chain is a common feature of the gums of group "b".

The gums of group "b" in general differ from tragacanthic acid, as they (except Sterculia caudata) consist of inner chains of D-galacturonic acid and L-rhamnose residues to which are attached D-glucuronic acid, D-galactose and in some cases L-rhamnose residues. On the other hand, tragacanthic acid consists of the inner chains of 1,4-linked D-galacturonic acid

residues to which are attached side chains containing L-fucose and D-xylose residues (as shown in the partial structure).



R = D-xyl p , D-Gal p 1 2 D-xyl p

or L-Fuc p 1 2 D-xyl p

The evidence so far obtained suggests strong structural similarities between Sterculia urens and Cochlospermum gossypium gums. However more elaborate structural assessment of this group ("b") of gums, which is indeed arduous, is required before detailed structure can be postulated.

Table 1.

	Khaya grandifolia	Khaya senegalensis	Sterculia setigera	Sterculia caudata	Sterculia urens	Cochlospermum gossypium
Gal 1..	+	+	+	+	+	+
..4 Gal 1..	+	+	+	+	+	+
..4 Gal 1..3					trace	trace
Rha 1-					+	+
..2 Rha 1..			?+	+	+	+
..2 Rha 1..4	+	+	?+	+	+	+
(Me) GAl..	(Me)+	(Me)+	-	+	+	+
4 GalAl..	+	+			trace	trace
4 GalAl..2					+	+
4 GalAl..3			+		+	+
References	111, 117	20	61, 118	62		23, 79

Table 2.

	Khaya grandifolia	Khaya senegalensis	Cochlospermum gossypium	Sterculia urens	Sterculia setigera	Sterculia caudata
D-GalpAl $\xrightarrow{\alpha}$ 2L Rhap	+	+	+	+	?+	
D-GpAl $\rightarrow$ 2L Rhap						+
4-MeD-GpAl $\rightarrow$ 4D-Galp	+	+				
D-GalpAl $\rightarrow$ 4D-Galp			?+	+	??+	
D-GpAl $\rightarrow$ 3D-GalpAl $\rightarrow$ 2L Rhap			??	+		
D-GalAl $\rightarrow$ D-GalA			??		??	
References	11, 117	20	79		61, 118	62

E X P E R I M E N T A L

General Methods.

Small scale hydrolyses on 1-10 mg. of substance were carried out at 100° in sealed glass tubes with dilute mineral acid (given normality). Where sulphuric acid was used, the solution was treated with a saturated solution of barium hydroxide until the solution was almost neutral and neutralisation was completed by the addition of solid barium carbonate. The excess barium carbonate and barium sulphate was filtered off, the filtrate was treated with Amberlite resin IR-120 (H) and evaporated to dryness. Hydrochloric acid hydrolysates were neutralised with silver carbonate and the solution was filtered. The silver ions from the solution were removed with hydrogen sulphide, filtered and evaporated to dryness. The sugars were extracted with acetone and the extract was taken to dryness.

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

Paper chromatography. Qualitative work was carried out on Whatman No.1, 541 and 3MM paper. Descending development with the following solvent systems (v/v) was used.

(A) Ethylacetate : Acetic acid : Formic acid : Water (18:3:1:4).

(B) Ethylacetate : Pyridine : Water (10:4:3).

(C) Ethylacetate : Acetic acid : Formic acid : Water (18:8:3:9).

(D) Ethylacetate : Acetic acid : Water (10:5:6).

(E) Ethylacetate : Pyridine : Acetic acid : Formic acid (5:5:1:3).

(F) Ethylacetate : Acetic acid : Formic acid : Water (18:8:3:4).

(G) Butan-1-ol : Ethanol : Water (4:1:5, upper layer).

(H) Butan-1-ol : Acetic acid : Water (4:1:5, upper layer).

(I) Benzene : Ethanol : Water (169:47:15, upper layer).

(J) Butan-2-one half saturated with water.

(K) Butan-2-one : Acetic acid : Water (9:1:1 saturated with boric acid).

The chromatograms after being developed for the required length of time, were air dried and the sugars were located using the following spray reagents.

1. Aniline oxalate.

Reducing sugars were located by spraying the dried chromatograms with saturated aqueous solution of aniline oxalate and heating at 130-140° for 3-5 minutes.

2. Silver nitrate (66).

Polyhydroxy compounds were located by dipping the chromatograms in the silver nitrate solution (1 ml. of saturated aqueous silver nitrate solution added to 20 ml. of acetone). The chromatograms were allowed to stand for about 5-10 minutes followed by dipping in ethanolic sodium hydroxide. After development of the sugar stains the chromatograms were treated with aqueous sodium thiosulphate and then washed with water. The developed sugars were of grey to black colour.

3. Periodate-Benzidine (67).

Reducing and non-reducing sugars were detected by spraying the chromatograms with an aqueous solution of sodium periodate (0·2%), drying in air for five minutes and spraying with ethanolic solution of benzidine (0·25 g. in 80 ml. of ethanol and 20 ml. of acetic acid). Sugars which react with periodate

show up as white spots on a blue background, the blue colour fades.

4. Hydroxylamine-Ferric chloride (68).

Esters and lactones were located by spraying the dried chromatograms with alkaline hydroxyl amine reagent prepared by mixing equal volumes of methanolic N hydroxyl amine hydrochloride and methanolic N potassium hydroxide and filtered to remove insoluble solids. The chromatograms were allowed to stand for ten minutes and then sprayed with 1% hydrochloric acid solution containing 1·6% ferric chloride. Lactones and esters show up as mauve spots on a pale yellow background.

5. Bromothymol blue indicator (69).

Acidic sugars were detected by spraying the dried chromatograms with bromothymol blue solution (0·1% in 80% ethanol) adjusted to pH 10.

Chromatograms were allowed to stand at room temperature for four days, the dried chromatograms were warmed in an oven at 40° for five minutes and then sprayed with bromothymol blue indicator. The acidic sugars appeared as yellow spots on a blue background.

The sugars were removed from chromatograms by cutting out areas containing acidic sugars and eluting the sugars and indicator from the paper with water. The indicator was removed from the solution by the addition of small quantity of charcoal. The solution was filtered, deionised with Amberlite resin IR-120 (H) and the resulting solution was evaporated to dryness.

Unless otherwise stated, the $R_{Gal\ A}$ values for acidic sugars refer to the rate of movement relative to galacturonic acid on Whatman No.1 paper in solvent system A.

The R_G values of methylated sugars refer to the rate of movement relative to 2,3,4,6-tetra-O-methyl-D-glucose on Whatman No.1 paper in solvent system G.

The R_{Gal} values for neutral sugars refer to the rate of movement relative to galactose on Whatman No.1 paper in solvent system B.

Quantitative paper chromatography.

Thick paper - Whatman 3MM and 31MM extra thick papers were used. The sugars were located either by cutting thin side strips and spraying the strips with spray reagent 1 or by spraying the dried chromatograms with spray reagent 5 (in case of acidic sugars).

Column chromatography.

Charcoal-celite columns, were used to separate methylated sugars, and monosaccharides from oligosaccharides and from one another. Charcoal was washed four times with boiling distilled water. Celite was washed with hydrochloric acid : water (1:1), allowed to stand for six hours, filtered and washed with water till free from chloride ions. The mixture (1:1) was packed as a slurry into columns and washed with water.

The mixture of sugars was dissolved in small quantity of water and was allowed to adsorb on the column. Monosaccharides were eluted with water, the oligosaccharides were then stepwise

eluted with aqueous ethanol.

Fractionation of methylated sugars was carried out by gradient elution with water : butan-2-one. Fractions were collected automatically and examined for sugars paper chromatographically.

Anion-exchange resin columns were used to separate neutral sugars from acidic sugar, and to fractionate acidic sugars.

Amberlite resin CG-45 was suspended in water and packed into column. The column was washed with N sodium hydroxide solution (6 bed volumes) and then washed with water till free from alkali. The resin was regenerated in the formate form with 10% formic acid (6 bed volumes) and washed free of acid.

The sugar solution was run on to the resin column in small quantity of water and allowed to stand overnight. Neutral sugars were eluted with water and acidic sugars were eluted with a gradient of 0-2N formic acid (2 litre).

A cellulose column was used to separate methylated sugars. The column was packed dry and washed with water followed by the solvents to be used. The packing of the column was checked by the even flow of the universal indicator.

The solvents were purified as below:

Light petroleum was shaken overnight with concentrated sulphuric acid (10% v/v of light petroleum), washed free of acid and distilled.

Butan-1-ol was refluxed for 1·5-2 hours with potassium hydroxide (1% w/v) and distilled.

The sugar mixture was placed on the column in the given solvent and the column was eluted with the given solvents. A small volume of the eluate was removed from every fifth tube, evaporated to dryness and the resulting syrup was examined paper chromatographically. The similar fractions were combined and evaporated, residue was dissolved in water and the solution was warmed and filtered through a bed of charcoal. The filtrate was concentrated to a syrup, the syrup was dried over phosphorous pentoxide and weighed.

DEAE-cellulose columns were used for fractionation of polysaccharide (17). DEAE-cellulose was washed three times, alternatively with 0·1N hydrochloric acid and 0·1N sodium hydroxide solution. Finally the cellulose was washed free of alkali. A perforated disc was placed at the bottom of the column, then a layer of glass wool and a cm. layer of acid washed celite. The cellulose in water was poured as a slurry and air pressure was applied at the top. The DEAE-cellulose was generated in phosphate form by elution with 0·5M sodium dihydrogen phosphate buffer (pH 6; 1 litre). The cellulose was then equilibrated with 0·05M sodium dihydrogen phosphate buffer (pH 6; 1 litre). The rate of flow of the column was adjusted to 40 ml. an hour.

Gas-liquid partition chromatography (52) was carried out on a "Pye Argon chromatograph." Separations were made on the following columns (120 x 0·5 cm.).

a) 15% by weight of butan 1,4-diol succinate polyester on

acid washed celite (80-100 mesh) at 175°.

b) 10% by weight of polyphenyl ether (m-bis(m-phenoxy-phenoxyl)benzene) on acid washed celite at 200°.

Methylglycosides of sugars were prepared by heating the sugar on a boiling water bath with 3% hydrogen chloride for 4-6 hours. Longer reaction periods (ca. 18-24 hrs.) were used for the methanolysis of the methylated polysaccharide.

The retention times (T) of the methyl ethers methyl-glycosides are relative to that of methyl-2,3,4,6-tetra-O-methyl-β-D-glucopyranoside. The T values given in the brackets indicate the presence of methylglycosides of methylated sugars with similar retention time.

Ionophoresis (70) on filter sheets was carried out in borate buffer (pH 10) at 500 volts for 5-6 hours. The ionophoretograms were allowed to dry and the sugars were located by spraying with saturated solution of aniline oxalate acidified with glacial acetic acid.

Ionophoresis on glass fibre paper (13) was used to examine the homogeneity of the polysaccharide. 2N-Potassium hydroxide solution was used as an electrolyte and a potential of 220 volts was applied.

The dried ionophoretograms were sprayed with alkaline potassium permanganate.

Optical rotations were observed in aqueous solutions (unless otherwise stated) at ca. 20±2° using sodium D-line as light source.

Methoxyl contents were estimated by means of the semimicro Zeisel method (71). A 10% solution of sodium antimony tartarate was used as scrubber. Zeisel's infra red modification (72) was used to estimate and distinguish between methoxyl and ethoxyl groups.

Demethylations (73) were carried out by dissolving the methylated sugars in dry dichloroethane and treating with borontrichloride at -70° for 30 minutes. The solution was allowed to evaporate at room temperature under anhydrous conditions, methanol was added and the solution was evaporated. The syrup was examined chromatographically.

Methylglycosides and methylester methylglycosides of the sugars were prepared by heating the sugar in a sealed tube at 100° with dry methanolic hydrogen chloride. The solution was neutralised with silver carbonate, filtered, silver carbonate washed with methanol and the combined filtrate and washings were taken to dryness.

Borohydride reductions were carried out by addition of potassium borohydride to an aqueous solution of the sugar and allowing the solution to stand overnight. Excess borohydride was destroyed and cations were removed by the addition of Amberlite resin IR-120 (H). The solution was filtered, the filtrate was evaporated to dryness and borate was removed by repeated addition and evaporation of methanol.

Small scale periodate oxidation of methylated sugars (74) was carried out by dissolving the sugar (1-2 mg.) in 0·5M

sodium metaperiodate solution (0·2 ml.) and allowing the solution to stand at 0°C for an hour. The excess of periodate was destroyed by the addition of ethylene glycol (1 drop) and the solution was made alkaline to phenolphthalein with 0·5N sodium hydroxide solution. The solution was evaporated to dryness, extracted with acetone, reduced in volume and examined chromatographically in solvent system G.

In some cases the periodate oxidation was conveniently carried out by converting the methylated sugars into the corresponding sugar alcohols with potassium borohydride.

Anthrone method for estimation of sugars (75). The reagent was prepared by dissolving anthrone (20 mg.) in 100 ml. of Analar sulphuric acid (70% v/v). The anthrone solution was aged for two hours before use and discarded after twenty-four hours. The reagent was kept at 0° when not in use.

Various volumes of the sugar solution (D-galactose, 0·2-1 ml.) were pipetted into large boiling tubes and diluted to 1 ml. with distilled water. Each tube was placed in an ice bath and agitated while the anthrone reagent (10 ml.) was slowly added. The tubes were stoppered and after their contents were thoroughly mixed, transferred to a vigorously boiling water bath and the heating was allowed for seven minutes. The tubes were then placed in a cold water bath and stored in the dark before the colour produced was measured using an "Unicam" spectrophotometer (at 620). A blank consisting of water (1 ml.) and the anthrone reagent was included

in each batch of analysis to eliminate colour effects due to anthrone. The standard curve was obtained by plotting concentration of galactose/ml. against the optical intensity.

The polysaccharide content of the fractions from DEAE-cellulose column eluted by phosphate buffer was then estimated by treating 1 ml. of the fraction as above and measuring the optical intensity.

Roe method (63). This method was used for the quantitative measure of ketoses in the polysaccharide.

The acid reagent was prepared by adding glycerol (130 g.) to Analar hydrochloric acid (100 ml.) containing Analar copper sulphate pentahydrate (45 mg.) and water (50 ml.).

The resorcinol reagent was prepared by dissolving resorcinol (4·5 g.) in water (100 ml.).

Various volumes (0·1 to 2 ml.) of the sucrose solution were pipetted into tubes and diluted to 2 ml. Acid reagent (5 ml.) and resorcinol reagent (1 ml.) were added to each tube. The tubes were shaken and heated in a boiling water bath for 12 minutes. The tubes were cooled in a running water bath. The absorption of the colour developed was measured using 1 cm. cells and violet 601 filter in a Hilger absorptiometer. A standard curve over the range 0·1 to 0·5 mg. sucrose was obtained.

The ketose content of the polysaccharide was estimated by treating 2 ml. of the polysaccharide (various concentrations) solution as above.

Dische method (64) for estimation of ketohexoses. The reagent was prepared by dissolving cysteine-hydrochloride (2·5 g.) in water (10 ml.).

Various volumes of the tagatose solution (containing about 250 /1 ml.) were pipetted into tubes and diluted to 0·5 ml. Cysteine hydrochloride solution (0·1 ml.) and sulphuric acid (75% v/v, 6 ml.) were added to the sugar solution and the mixture was shaken. The tubes were allowed to stand at room temperature when a yellow colour appeared. The intensity of the colour produced was measured using an "Unicam" spectrophotometer at 412 . The standard curve was obtained by plotting the tagatose concentration/0·5ml. against the optical intensity. The ketose content of the polysaccharide was estimated as above.

Phenol sulphuric acid method for estimation of sugars (76).

The reagent was prepared by dissolving Analar phenol (5 g.) in 100 ml. of copper free distilled water.

Varying volumes (0·1-1 ml.) of the polysaccharide solution was pipetted in tubes and diluted to 1 ml. Phenol solution (1 ml.) and Analar sulphuric acid (5 ml.) were added to the polysaccharide solution. The tubes were allowed to stand for 10-20 minutes, shaken and heated on a water bath at 25-30° for 10-20 minutes. The intensity of the colour developed was measured using an "Unicam" spectrophotometer at 485 . The standard curve was obtained by plotting the polysaccharide concentration against the optical intensity. The polysaccharide

content eluted from the DEAE-cellulose column was estimated by treating 1 ml. of the fraction as above and measuring the optical intensity.

Carbazole method for estimation of uronic acid (77).

The reagent was prepared by dissolving recrystallised carbazole (1·5 g.) in purified alcohol (100 ml.). Various concentrations of galacturonic acid (5-100 /2 ml.) were added to ice cooled tubes containing 12 ml. of concentrated sulphuric acid. The tubes were further cooled to 5°, and then heated on a boiling water bath for ten minutes and cooled. Carbazole reagent (1 ml.) was added, the tubes were shaken and allowed to stand at room temperature for twenty-five minutes. The optical intensity of the colour developed was measured on a spectrophotometer at 520 . The uronic acid anhydride content of the polysaccharide eluted from the DEAE-cellulose column was estimated as above, and calculated using a standard curve based on galacturonic acid.

Uronic acid anhydride determinations were carried out by (a) decarboxylation and titremetric determination of carbon dioxide evolved (78) and (b) by the carbazole method.

Purification of methyl iodide for use in methylation was carried out by refluxing over silver oxide and distilling in a dry system.

Preparation of dry silver oxide. The silver oxide was prepared by the addition of sodium hydroxide solution (80 g. in 500 ml.) to a solution of silver nitrate (340 g. in 500 ml.). The precipitated silver oxide was repeatedly washed with cold

and hot water. The precipitate was ground with acetone and finally with ether, dried in a vacuum oven and stored in a coloured bottle.

Aniline derivatives of the methylated sugars were prepared by refluxing the sugar in ethanolic redistilled aniline (equimolecular) for half hour in the dark. Evaporation of the solvent gave the aniline derivative which crystallised and was recrystallised from the given solvent.

Lactones of aldonic acids of methylated sugars were prepared by oxidation with bromine water. The sugar was dissolved in water, bromine was added, and the mixture was kept in the dark at room temperature for two days. Excess bromine was removed by aeration, and the solution was neutralised with silver carbonate, filtered and the filtrate was evaporated to dryness. The sugar was extracted with acetone, filtered and evaporated to dryness. The lactone crystallised and was recrystallised from the given solvent.

Aldonamide: The lactone prepared as above was dried in a vacuum desiccator dissolved in dry methanolic ammonia and allowed to stand at 0^{o} for two days. The excess methanolic ammonia was evaporated to give the crystalline amide. The aldonamide derivative was recrystallised from the given solvent.

Tetrahydrofuran for lithium aluminium hydride reductions was purified by allowing to stand over sodium wire and distilled from lithium aluminium hydride.

Preliminary Investigations.

The pale brown nodules of the gum were crushed to small pieces and powdered mechanically. The powdered gum was analysed and found to have: acetyl content, 7.0-8.5%, alkoxyl content, 1.4% (by Ziesel's titremetric method), ethoxyl content 0.3% (by Ziesel's infra red method) and uronic acid anhydride 37.2%.

A sample (0.12 g.) of the crude gum was saponified with 4N sodium hydroxide solution (20 ml.) for 0.5 hr. at 150-160°, the solution was cooled, acidified with sulphuric acid (1:2) and distilled. The distillate was neutralised with alcoholic potassium hydroxide and the neutral solution was evaporated to dryness. The residue was dissolved in water (5 ml.), alcohol (10 ml.) and p-Nitrobenzylbromide (0.08 g.) was added. The mixture was refluxed for 1.5 hr. and the clear green solution was allowed to cool when a crystalline precipitate appeared. The acid derivative was recrystallised thrice from ethanol, m.p. 76-77° and mixed m.p. 77° (with authentic p-Nitrobenzyl acetic acid).

Another sample of the gum was hydrolysed with 2N-sulphuric acid at 100° for twenty hours. Chromatography of the hydrolysate in solvents A and B showed the presence of galacturonic acid, galactose, rhamnose together with traces of arabinose and sugars with $R_{rhamnose}$ 1.1 and 1.25 (in solvent system B).

Purification of the gum.

The finely powdered gum (25 g.) was dispersed in N-sodium hydroxide solution (1250 ml.) and stirred for ten hours when most of the gum had dissolved. The solution was centrifuged to remove mechanical impurities. The supernatant liquid was treated with ethanol (3750 ml.) acidified with concentrated hydrochloric acid (375 ml.). The precipitated polysaccharide was allowed to settle at room temperature. The supernatant liquid was decanted off and the precipitate was removed at the centrifuge. The precipitated gum acid was repeatedly washed with ethanol (5 x 50) and finally with ether and dried in a vacuum oven to give a white amorphous substance. The dried gum acid was again dissolved in alkaline solution (0·5N sodium hydroxide solution, 1 litre) and gradually precipitated with acidified ethanol (3 litres). The process of dissolution of gum acid in alkaline solution and precipitation with acidified ethanol was repeated five times with decreasing concentrations of alkali required to dissolve the gum. Finally, the gum was dissolved in water and the solution was deionised with Amberlite resins IR 120 (H) and IR 4B (OH), reduced in volume and freeze dried to give the purified gum acid (18 g.). The gum acid was found to have $[\alpha]_D$ +62° (c, 1.8 in 1% sodium hydroxide solution), uronic acid anhydride 39.5%, neutralisation equivalent 398-400 and alkoxyl content 0·0% (by Ziesel's infra red method).

A sample of the purified gum was hydrolysed with 2N

sulphuric acid at 100° for eighteen hours and the resulting solution was neutralised with barium carbonate, deionised with Amberlite resin IR 120 (H) and evaporated to dryness. Chromatography of the hydrolysate in solvent systems A and B showed the presence of galacturonic acid, galactose, rhamnose and a trace of arabinose.

Another sample of the polysaccharide was hydrolysed with N sulphuric acid for six hours at 100°and the product was methanolysed. The resulting methyl esters methyl glycosides were treated with potassium borohydride and the product was hydrolysed with N sulphuric acid at 100° for four hours. Chromatography of the hydrolysate in solvent systems A and B showed the presence of galactose, glucose, rhamnose and arabinose.

The supernatant liquid and the washings from all the precipitations were combined and neutralised with calcium carbonate. The filtrate was reduced in volume and ethanol (5 volumes) was added. The addition of ethanol did not give any precipitate.

In subsequent purifications, deacetylation and dissolution of the gum was carried out in 1% (w/v) aqueous ammonia.

Tests for ketoses.

The gum acid was examined for the presence of ketosugars by Roe (63) and Dische (64) colorimetric methods. The absorption obtained by the above methods suggested the absence of ketosugars.

Examination for Heterogeneity of the Gum.

Electrophoretic examination of the gum.

The purified polysaccharide in potassium hydroxide solution was examined by electrophoresis on glass fibre sheets in potassium hydroxide solution (2N). The developed electrophaetograms showed the presence of a single component at a distance of 1 cm. (after six hours) from the starting line.

Precipitation as calcium salt.

The crude gum (5 g.) was dissolved in sodium hydroxide solution (250 ml., 1% w/v) and calcium chloride solution (5% w/v) was gradually added until the precipitation was complete. The solution was allowed to stand for 1 hr. at room temperature and then centrifuged. The precipitate was repeatedly washed with water till free from adhering chloride ions. The supernatant liquid obtained after removing the precipitated polysaccharide did not give any further precipitate with ethanol.

The polysaccharide was regenerated by heating the calcium salt with ammonium oxalate (0·3%) for 0·5 hr. at 80-90°. The solution was centrifuged and to the supernatant acetone was gradually added. Only one precipitate was obtained. The precipitated polysaccharide was dissolved in sodium hydroxide solution (0·5%, 150 ml.), the solution was deionised with Amberlite resins IR-120 (H) and IR 4B (OH), reduced in volume and freeze dried to give free gum acid (3·2 g.). The gum acid had $[\alpha]_D$ +58° (c, 1·6 in 1% sodium hydroxide solution) and uronic acid anhydride 38%.

A sample (10 mg.) of the freeze dried polysaccharide was hydrolysed with 2N sulphuric acid at 100° for sixteen hours. Chromatography of the hydrolysate in solvent systems A and B showed the presence of galacturonic acid, galactose, rhamnose and a trace of arabinose.

Precipitation as copper complex.

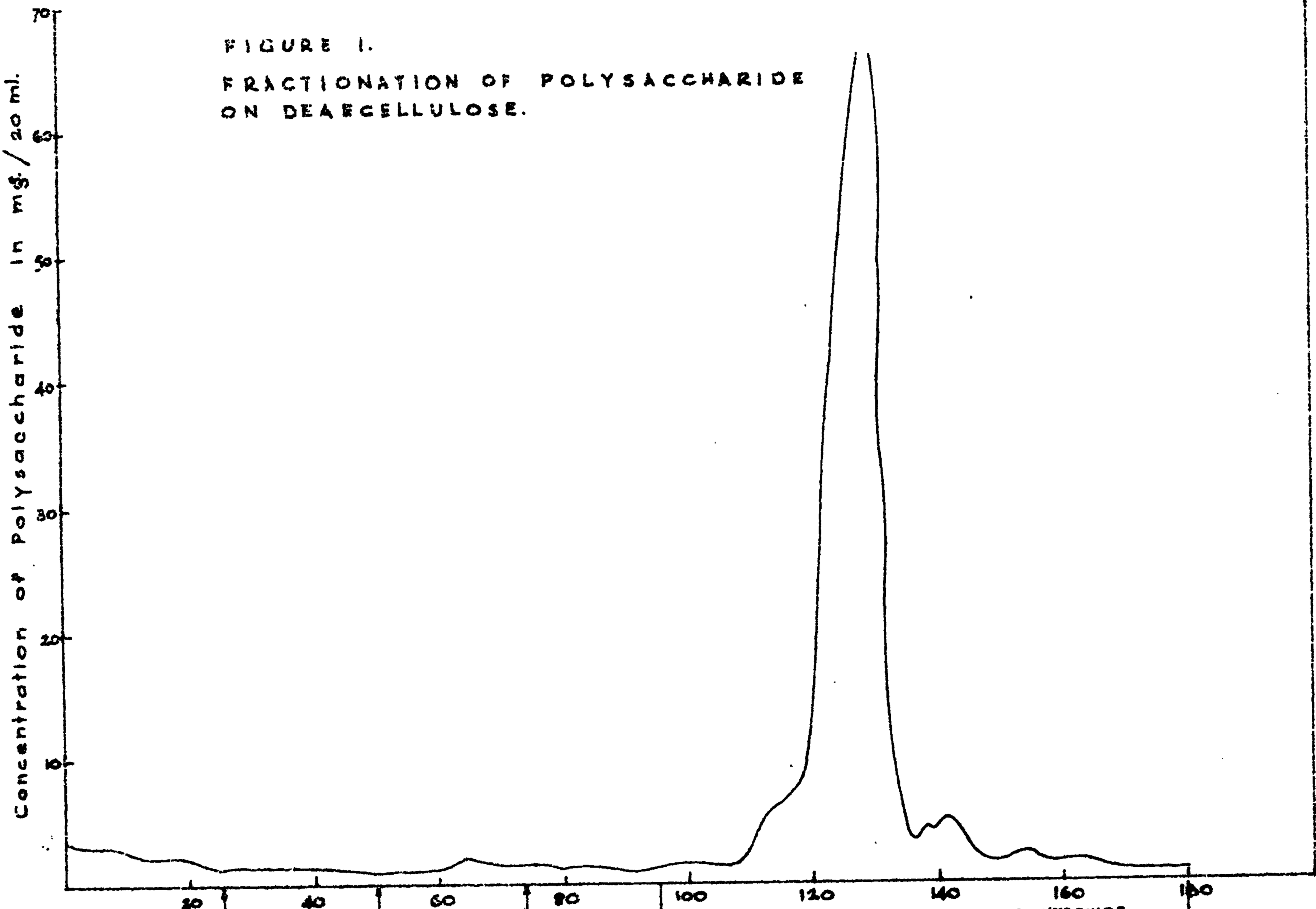
The purified polysaccharide (4 g.) was dissolved in aqueous ammonia (400 ml., 1% w/v). The solution was deionised with Amberlite resin IR-120 (H) and cupric acetate solution (7%) was added until the precipitation was complete. The precipitate was allowed to stand at room temperature for 0·5 hr. and then centrifuged. Further addition of cupric acetate solution followed by ethanol to the supernatant liquid did not give any precipitate.

The polysaccharide precipitated as copper complex was regenerated twice by dissolution in N hydrochloric acid and precipitation with ethanol. The precipitated polysaccharide was repeatedly washed with ethanol till free from chloride ions. The polysaccharide was dissolved in water and the solution was deionised with Amberlite resins IR-120 (H) and IR 4B (OH), reduced in volume and freeze dried. The freeze dried polysaccharide (2·8 g.) had $[\alpha]_D$ +58·5° (c, 1·8) and uronic acid anhydride, 39·5%.

A sample (10 mg.) of the polysaccharide was hydrolysed with 2N sulphuric acid at 100° for eighteen hours. Chromatography of the hydrolysate in solvent systems A and B showed

FIGURE I.

FRACTIONATION OF POLYSACCHARIDE ON DEAECELLULOSE.



the presence of galacturonic acid, galactose, rhamnose and a trace of arabinose.

DEAE-Cellulose column chromatography.

Experiment 1.

The purified polysaccharide (250 mg.) was dissolved in sodium hydroxide solution (15 ml. 1%), the solution was deionised with Amberlite resin IR-120 (H) and reduced in volume. The solution was run on to the diethyl aminoethyl cellulose column (40 x 1·5 cm.) and left to stand overnight so that the polysaccharide was completely adsorbed on the ion exchange cellulose. The column was eluted with:

a, 0·025 M (500 ml.)
b, 0·05 M (500 ml.)
c, 0·1 M (500 ml.)
d, 0·25 M (500 ml.)

sodium dihydrogen phosphate buffer at pH 6. Elution was continued with a linear gradient of sodium hydroxide solution (0·0-0·3M, 2000 ml.). Fractions (20 ml.) were collected every half hour. 1 ml. sample was removed from every fraction and the polysaccharide present in the fractions was estimated by the anthrone colorimetric method using a calibration curve based on D-galactose as a reference sugar.

The uronic acid content of the polysaccharide eluted with sodium hydroxide was determined by the carbazole colorimetric method using a calibration curve based on D-galacturonic acid. A plot of the polysaccharide concentration per 20 ml. fraction against the volume of eluate (figure 1) indicated that the phosphate buffer eluted a very small amount of the

polysaccharide. Most of the polysaccharide was eluted by sodium hydroxide solution over the range of 0·07-0·11M. The fractions containing the polysaccharide (eluted by sodium hydroxide solution over the range of 0·07-0·11M) were combined, dialysed and reduced in volume. The solution was treated with Amberlite resins IR-120 (H) and IR 4B (OH) and freeze dried. The polysaccharide (0·195 g.) had uronic acid anhydride 42% (by decarboxylation).

Hydrolysis of the polysaccharide with 2N sulphuric acid at 100° for sixteen hours and chromatography of the hydrolysate in solvent systems A and B showed the presence of galactose, rhamnose, galacturonic acid and a trace of arabinose.

The remaining fractions eluted by sodium hydroxide solution were combined and dialysed. The dialysate was treated with Amberlite resins IR-120 (H) and IR 4B (OH), reduced in volume and freeze dried. The freeze dried polysaccharide (0.020 g.) had $[\alpha]_D$ +32° (c, 0.42 in 1% sodium hydroxide solution) and uronic acid anhydride content 28%.

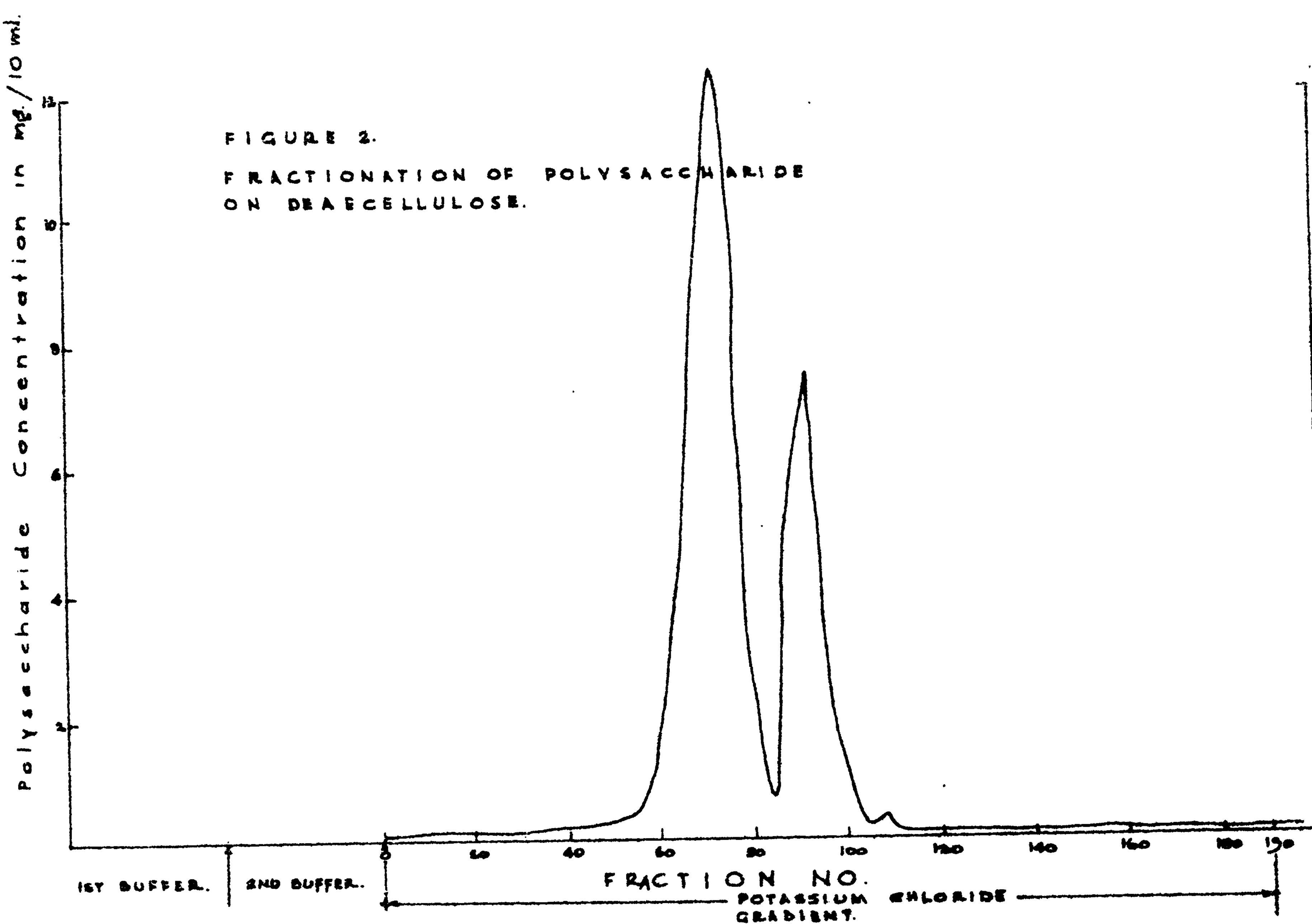
A sample of this polysaccharide was hydrolysed with 2N sulphuric acid at 100° for sixteen hours. Chromatography of the hydrolysate showed the presence of galactose, rhamnose, galacturonic acid and a trace of arabinose.

Experiment 2.

The purified gum acid (0·05 g.) was run on to the DEAE-cellulose column and allowed to stand overnight. The column was eluted with:

FIGURE 2.

FRACTIONATION OF POLYSACCHARIDE ON DEAECELLULOSE.



a, 0·1M (150 ml.) b, 0·25M (150 ml.)

sodium dihydrogen phosphate buffer at pH 6. Examination of the eluates "a" and "b" by the phenol-sulphuric acid colorimetric method showed the absence of polysaccharide.

Elution was continued with a gradient of potassium chloride (0·0-0·5M, 800 ml.). Fractions (5 ml.) were collected every 10 minutes. Aliquot samples (1 ml.) were removed from every second tube and the polysaccharide present in the fractions was determined by the phenol-sulphuric acid colorimetric method using a calibration curve based on the polysaccharide.

A plot of the polysaccharide concentration per 10 ml. fraction against the volume of eluate (figure 2) showed two peaks. The fractions corresponding to each peak were combined and dialysed. The dialysates were reduced in volume, deionised with Amberlite resins IR-120 (H) and IR 4B (OH) and precipitated with ethanol. The precipitated polysaccharides were centrifuged and dried in a vacuum oven.

Fraction I. The polysaccharide (21 mg.) had $[\alpha]_D$ +60° (c, 0.40 in 1% sodium hydroxide solution) and uronic acid anhydride content 41%. Hydrolysis of the polysaccharide and chromatography of the hydrolysate in solvent systems A and B showed the presence of galacturonic acid, galactose, rhamnose and a trace of arabinose.

Fraction II. The polysaccharide (12 mg.) had uronic acid anhydride content 40% and galactose, rhamnose, galacturonic acid and a trace of arabinose as component sugars.

Precipitation with cetyl trimethyl ammonium bromide.

Experiment 1.

The polysaccharide (6 g.) was dissolved in aqueous ammonia (1% w/v) and the solution was deionised with Amberlite resin IR-120 (H). Cetavlon solution (5% w/v) was gradually added to the polysaccharide until the precipitation was complete. The precipitated complex was removed at the centrifuge and washed with water until free from adhering cetavlon. The polysaccharide was recovered by stirring the complex with 5N acetic acid (100 ml.) for four hours. The free polysaccharide was precipitated by the addition of ethanol (5 volumes). The poly saccharide was dissolved in sodium hydroxide solution (200 ml. 1%) and the solution was treated with Amberlite resins IR-120 (H) and IR-4B (OH). The solution was reduced in volume and freeze dried to give the purified polysaccharide A_1 (4·8 g.). The polysaccharide A_1 had $[\alpha]_D$ +59° (c, 0.84 in 1% sodium hydroxide solution) and uronic acid anhydride 39%.

A sample of the polysaccharide A_1 was hydrolysed with 2N sulphuric acid at 100° and the hydrolysate was examined by chromatography in solvents A and B. The presence of galacturonic acid, galactose, rhamnose and a trace of arabinose was indicated.

Experiment 2.

The polysaccharide A_1 (4 g.) was dissolved in aqueous ammonia (400 ml.) and the solution was deionised with Amberlite resin IR-120 (H). The polysaccharide was precipitated by the

gradual addition of aqueous cetavlon (5% w/v) and the precipitated complex was processed as described in Experiment 1. The freeze dried polysaccharide A_2 (3·6 g.) had $[\alpha]_D$ +58° (c, 4·31 in 1% sodium hydroxide solution) and uronic acid anhydride 38%.

Hydrolysis of the polysaccharide with 2N sulphuric acid followed by chromatography of the hydrolysate in solvents A and B showed the presence of galacturonic acid, galactose, rhamnose and a trace of arabinose.

Experiment 3.

The polysaccharide A_2 (3·4 g.) was dissolved in aqueous ammonia (300 ml. 1% w/v) and the solution was deionised with Amberlite resin IR-120 (H). The polysaccharide was precipitated with aqueous cetavlon (5% w/v) and the precipitated complex was worked up as described in Experiment 1. The freeze dried polysaccharide A_3 (3·2 g.) had $[\alpha]_D$ +58° (c, 0·38 in 1% sodium hydroxide solution) and uronic acid anhydride 38%.

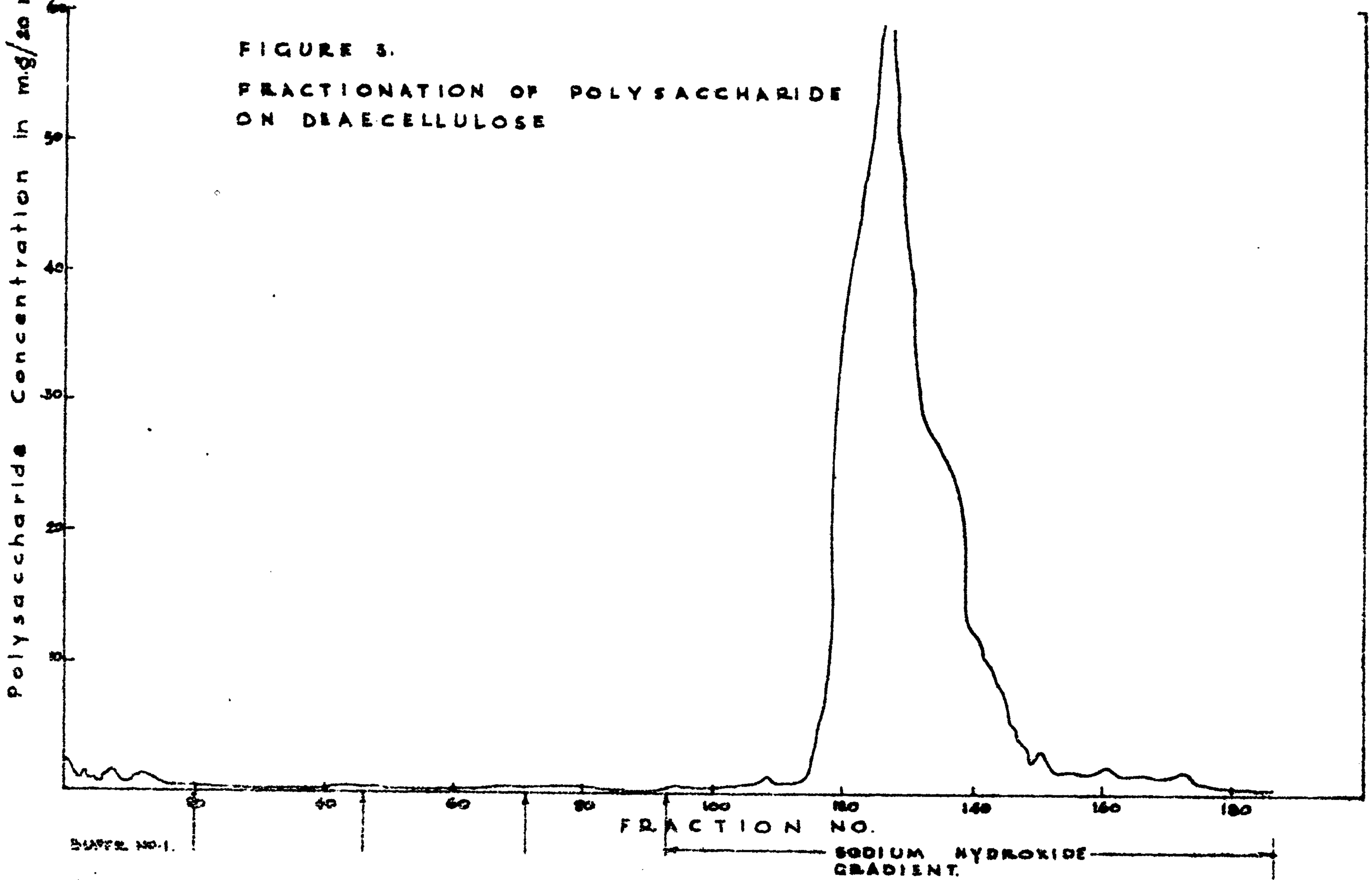
Hydrolysis of the polysaccharide A_3 with 2N sulphuric acid at 100° for sixteen hours and chromatography of the hydrolysate in solvent systems A and B showed the presence of galacturonic acid, galactose, rhamnose and a trace of arabinose.

DEAE-Cellulose column chromatography of the Polysaccharide A_3.

The purified polysaccharide A_3 (0·275 g.) obtained after a third cetavlon precipitation was dissolved in sodium hydroxide solution (30 ml., 0·5%), the solution was deionised with

FIGURE 3.

FRACTIONATION OF POLYSACCHARIDE ON DEAE-CELLULOSE



Amberlite resins IR-120 (H) and IR-4B (OH), reduced in volume (15 ml.) and run onto the DEAE-cellulose column (60 x 1·5 cm.). The column was eluted and the fractions were examined in a manner similar to that described under diethylaminoethyl cellulose column chromatography in Experiment 1.

The curve obtained by plotting the polysaccharide concentration per 20 ml. fraction is shown in figure 3. This showed that again a very little amount of the polysaccharide was eluted by the phosphate buffer (over the range of 0·025M) and the major portion of the polysaccharide was eluted by the alkali over the range of 0·08-0·13M. Fractions containing the polysaccharide (as indicated by the peak of the graph) were mixed, dialysed, reduced in volume, treated with Amberlite resins IR-120 (H) and IR-4B (OH) and freeze dried. The polysaccharide (0·205 g.) had $[\alpha]_D$ +58° (c, 0·687 in 1% sodium hydroxide solution) and uronic acid anhydride 38·5%.

Hydrolysis of the polysaccharide and subsequent chromatography of the hydrolysate in solvent systems A and B showed the presence of galacturonic acid, galactose, rhamnose and a trace of arabinose.

The remaining fractions eluted by sodium hydroxide solution were combined and dialysed. The dialysate was treated with Amberlite resin IR-120 (H) and IR-4B (OH), reduced in volume and freeze dried. The polysaccharide (0·021 g.) had $[\alpha]_D$ +59° (c, 0·42 g. in 1% sodium hydroxide solution) and uronic acid anhydride 38%.

Hydrolysis of the polysaccharide and subsequent chromatography of the hydrolysate in solvent systems A and B indicated the presence of galactose, rhamnose, galacturonic acid and a trace of arabinose.

Partial Hydrolysis.

Trial Experiment.

Gum acid (0·3 g.) was dissolved in water (10 ml.) and 2N sulphuric acid (10 ml.) was added, and the solution was heated on a boiling water bath for eight hours. Aliquot portions (1 ml.) were removed every 0·5 hr., diluted to 5 ml. and their optical rotations were recorded. Each sample was neutralised with a saturated solution of barium hydroxide to pH 6, solid barium carbonate was added to complete the neutralisation, and the resulting solution was filtered, deionised with Amberlite resin IR 120 (H) and evaporated to dryness. The syrup was spotted as one spot on Whatman 3MM paper and examined in solvent system A. Visual inspection of the chromatograms suggested that the best yield of the acidic oligosaccharides could be obtained by hydrolysing the polysaccharide with N sulphuric acid at 100° for five and a half to six hours when the optical rotation was constant ($[\alpha]_D$+79°).

Large Scale Hydrolysis.

Gum acid (10 g.) was dissolved in N-sulphuric acid (250 ml.) and the solution was heated at 100° for six hours. The solution was cooled and acetone (5 volumes) was added. The addition of acetone did not give any precipitate. The resulting solution was reduced in volume, neutralised with a saturated solution of barium hydroxide followed by barium carbonate. The solution was filtered, deionised with Amberlite resin IR-120 (H) and

evaporated to dryness. Chromatography of the syrup in solvent systems A, B, C and D using spray reagent 1 indicated the presence of four sugars with $R_{Gal\ A}$ 0·98, 0·78, 0·39, 0·27 and(0·0).

Separation of acidic sugars.

The syrup (6.1 g.), which contained neutral and acidic sugars, was placed on an Amberlite resin CG 45 column (70 x 2·5 cm.) in the formate form. The column was washed with water until free from neutral sugars. The eluate was evaporated to dryness and the syrup (3·1 g.) was examined chromatographically in solvent systemsA and B. The presence of galactose, rhamnose and traces of arabinose and galacturonic acid was observed.

The acidic sugars which remained on the column were eluted with a gradient of 0-2N formic acid (2 litre). The eluate was collected in 20 ml. fractions and every fifth fraction was examined by paper chromatography in solvent system A. The similar fractions were combined and in all five fractions were obtained:

Fraction	Weight	$R_{Gal\ A}$
A	1·29 g.	0·98, 0·78, 0·28-0·39, 0·0
B	0·329 g.	0·98, 0·78, 0·27-0·39, 0·26
C	0·29 g.	0·28-0·39, 0·26, 0·0
D	0·098 g.	0·39, 0·26
E	0·029 g.	0·0.

Fraction A.

Chromatography of this fraction in solvent systemsA, C and D

indicated the presence of at least four sugars. This fraction was separated into four fractions on Whatman 3MM extra thick paper in solvent system A using spray reagent 5.

Sub-fraction A_1.

This fraction (0·31 g.), $R_{Gal\ A}$ 0·99 was chromatographically identical to galacturonic acid and had a trace of a sugar $R_{Gal\ A}$ 0·78.

Sub-fraction A_2.

This fraction (0·162 g.) $R_{Gal\ A}$ 0·78 was chromatographically pure and gave an orange fluorescence with spray reagent 1 in ultra-violet light.

Sub-fraction A_3.

This fraction (0·128 g.) $R_{Gal\ A}$ 0·28-0·39 was chromatographically pure and the stain with spray reagent 1 was homogeneous to visual inspection.

Sub-fraction A_4.

This fraction (0·021 g.) $R_{Gal\ A}$ 0·0 was examined chromatographically in solvent system D in which it had $R_{Gal\ A}$ 0·2.

Fraction B.

Chromatography of this fraction in solvent systems A, C and D indicated the presence of three sugars. This fraction like fraction A was separated into four fractions on Whatman 31MM extra thick paper in solvent A using spray reagent 5.

Sub-fraction B_1.

Chromatography of this fraction (0·079) $R_{Gal\ A}$ 0·98, 0·78

in solvent systems A, C and D showed the presence of mainly a sugar ($R_{Gal\ A}$ 0.98) and traces of another sugar ($R_{Gal\ A}$ 0.78).

Sub-fraction B_2.

This fraction (0.075 g.) $R_{Gal\ A}$ 0.78 was chromatographically pure and gave an orange fluorescence with spray reagent 1 in the ultra-violet light.

Sub-fraction B_3.

This fraction (0.105 g.) $R_{Gal\ A}$ 0.27-0.39 was chromatographically pure and the stain developed with spray reagent 1 was homogeneous to visual inspection.

Sub-fraction B_4.

This fraction (0.060 g.) $R_{Gal\ A}$ 0.26 was chromatographically pure and gave an orange-brown stain with spray reagent 1.

Fraction C.

This fraction like fractions A and B was separated in solvent system A using spray reagent 5 into three fractions.

Sub-fraction C_1.

This fraction (0.021 g.) was chromatographically pure and had $R_{Gal\ A}$ 0.27-0.39.

Sub-fraction C_2.

This fraction (0.118 g.) $R_{Gal\ A}$ 0.26 was chromatographically pure and gave an orange-brown stain with spray reagent 1.

Sub-fraction C_3.

This fraction (0.019 g.) like sub-fraction A_4 did not move in solvent A and had $R_{Gal\ A}$ 0.2 in solvent system D.

Fraction D.

This fraction like fractions A, B and C was separated into two fractions.

Sub-fraction D_1.

This fraction (0·018 g.) $R_{Gal\ A}$ 0·39 was chromatographically pure and gave a brown stain with spray reagent 1.

Sub-fraction D_2.

This fraction (0·028 g.) $R_{Gal\ A}$ 0·26, was chromatographically pure and gave an orange-brown stain with spray reagent 1.

The above fractions with similar chromatographic mobilities were combined to give the following six fractions:

	Weight	$R_{Gal\ A}$
1.	0·38 g.	0·99
2.	0·22 g.	0·78
3.	0·018 g.	0·39
4.	0·196 g.	0·26
5.	0·230 g.	0·27-0·39
6.	0·045 g.	0·0

Fraction 1. D-Galacturonic acid.

Chromatography of this fraction (0·375 g.) in solvent systems A and B indicated the presence of galacturonic acid and traces of galactose and a sugar $R_{Gal\ A}$ 0·78. The sugars were run onto the Duolite A_4 resin column in the hydroxyl form. The column was washed with water till free from galactose. The acid was eluted with 0·05 M sodium hydroxide solution

(500 ml.). The eluate was deionised with Amberlite resin IR-120 (H) and evaporated to dryness. Chromatography of the syrup in solvent systems A and B showed the presence of only galacturonic acid.

The syrup (0·315 g.) had $R_{Gal\ A}$ 1·0 and $[\alpha]_D$ +54° (c, 2·4). A sample of the sugar was converted into the methyl ester methyl glycosides, reduced with potassium borohydride and the glycosides of the reduced sugar was hydrolysed with N-sulphuric acid for two hours. Chromatography of the hydrolysate in solvent system B showed only galactose.

The sugar (20 mg.) was dissolved in water (1 ml.) and 2,5 dichlorophenylhydrazone (20 mg.) in methanol (1 ml.) was added, the syrup on evaporation crystallised. The D-galacturonic acid 2,5 dichlorophenylhydrazone had m.p. 178-180° and mixed m.p. 180-181°.

Fraction 2. 2-O-(α-D-galactopyranosyluronic acid)-L-rhamnose

This fraction had a trace of galacturonic and was purified by chromatography in solvent system A on Whatman 3MM paper.

The purified sugar (0·196 g.) had $R_{Gal\ A}$ 0·78 and $[\alpha]_D$ +90·5° (c, 3·5).

A sample of the sugar was hydrolysed with N sulphuric acid for four hours. Chromatography of the hydrolysate in solvent systems A and B showed the presence of galacturonic acid and rhamnose. The sugar (5 mg.) was heated with 2·5% methanolic hydrogen chloride and the product was reduced with potassium

borohydride. The reduced sugar was hydrolysed with N sulphuric acid for four hours. Chromatography of the hydrolysate in solvent system B showed the presence of rhamnose and galactose.

A sample of the sugar (5 mg.) was dissolved in potassium carbonate (1 ml., 1% w/v), potassium borohydride (5 mg.) added and the reduced sugar was hydrolysed with N sulphuric acid for four hours. Chromatography in solvent system K using spray reagent 3 showed the presence of rhamnitol, galacturonic acid and the absence of rhamnose.

Methylation of fraction 2.

The acid (0·11 g.) was dissolved in water (2 ml.), dimethyl sulphate (1 ml.) and sodium hydroxide solution (1 ml.) were added dropwise over a period of one hour with vigorous stirring. The reaction flask was kept in ice and an atmosphere of nitrogen was maintained during the methylation. A further batch of dimethyl sulphate (4 ml.) and sodium hydroxide solution (9 ml.) was added over a period of four hours. Three more additions of the reagents were made during the next three days. After the final methylation the solution was heated at 80° for thirty minutes, allowed to cool, and made acidic to pH 4 with dilute sulphuric acid. Sodium sulphate was precipitated by the addition of methylated spirit (six volumes). The precipitate was filtered and repeatedly washed with methylated spirit (4 x 10). The filtrate and the washings were made alkaline to pH 8 and reduced in volume. The solution was acidified with dilute sulphuric acid and extracted with cold

chloroform (4 x 15). The chloroform extract was dried over anhydrous sodium sulphate and filtered. The filtrate was evaporated to dryness. The sugar (0.072 g.) had $[\alpha]_D$ +92.5° (c, 1·39 in chloroform). The sugar crystallised and was re-crystallised from light petroleum (b.p. 100-120°). The recrystallised 2-O-(α-D-galactopyranosyluronic acid)-L-rhamnoside penta-methyl ether dihydrate had m.p. 67-68° and mixed m.p. 65-66° (with authentic sample).

An X-ray powder photograph of the methylated acid was recorded which was identical to that of authentic sample.

Fraction 3. 4-O-(D-Galactopyranosyluronic acid)-D-galactose.

This fraction (0·018) $R_{Ga\ A}$ 0·39 was chromatographically pure and gave a brown stain with spray reagent 1.

A sample of the sugar was hydrolysed with N-sulphuric acid for four hours. Chromatography of the hydrolysate in solvent systems A and B showed only galacturonic acid and galactose.

Another sample of the sugar (3 mg.) was heated at 100° with methanolic hydrogen chloride for four hours and the product was reduced with potassium borohydride. The reduced sugar was hydrolysed with N-sulphuric acid for four hours. Chromatography of the hydrolysate in solvent system B showed only galactose.

Fraction 4. O-D-Glucopyranosyluronic acid-(1→3)-O-α-D-galactopyranosyluronic acid-(1→2)-O-L-rhamnose.

This fraction (0·19 g.) had $R_{Gal\ A}$ 0·27, $[\alpha]_D$ +44°(c, 3·6)

and was chromatographically and ionophoretically pure.

A sample of the sugar was hydrolysed with N sulphuric acid for four hours and the hydrolysate was examined in solvent systems A, B and E. The presence of galacturonic acid, glucuronic acid and rhamnose was indicated.

Another sample of the sugar (5 mg.) was converted into the methyl ester methylglycosides and reduced with potassium borohydride. Hydrolysis of the reduced sugar and subsequent chromatography of the hydrolysate in solvent system B indicated the presence of galactose, glucose and rhamnose.

A small quantity of the sugar (5 mg.) was dissolved in water and potassium borohydride (5 mg.) was added. The reduced sugar was hydrolysed with N-sulphuric acid for four hours. Chromatography of the hydrolysate in solvent system K using spray reagent 3 showed the presence of rhamnitol, galacturonic acid (glucuronic acid had a chromatographic mobility similar to galacturonic acid) and the absence of rhamnose.

Methylation of (the trisaccharide) fraction 4.

The sugar (0·13 g.) was dissolved in water (2 ml.), dimethyl sulphate (1 ml.) and sodium hydroxide solution (1 ml., 30%) were added dropwise over a period of one hour with vigorous stirring in nitrogen atmosphere. A further batch of dimethyl sulphate (5 ml.) and sodium hydroxide solution (11 ml.) was added over a period of five hours. Three more additions of the reagents were made and the methylated sugar was processed as described under fraction 2.

Hydrolysis of a sample (2 mg.) of the methylated trisaccharide with N sulphuric acid for six hours followed by chromatography of the hydrolysate in solvent system G showed that the methylation was incomplete. The partially methylated sugar was neutralised with solid silver carbonate and filtered. The filtrate was reduced in volume and freeze dried. The silver salt was dissolved in methyl iodide (4 ml.) and the solution was refluxed for half an hour. Silver oxide (0·25 g.) was added to the mixture in four batches over a period of four hours. The reaction mixture was allowed to reflux overnight. The solution was cooled, filtered and the silver oxide was extracted with boiling chloroform in a Soxhlet extractor. The combined filtrate and the extract was dried over anhydrous sodium sulphate and evaporated to dryness to give the methylated trisaccharide (0·095 g.).

Reduction of the methylated (trisaccharide) fraction 4.

The methylated sugar (95 mg.) was dissolved in dry tetrahydrofuran (5 ml.) and lithium aluminium hydride (0·1 g.) in dry tetrahydrofuran (5 ml.) was added portionwise. The mixture was allowed to stand at room temperature for 0·5 hr. and then refluxed for 1.5 hr. A further addition of lithium aluminium hydride (50 mg.) in dry tetrahydrofuran (3 ml.) was made and the heating was continued for a further period of 0·5 hr. The solution was cooled and excess lithium aluminium hydride was destroyed with ethyl acetate. The solution was made acidic to pH 4 and centrifuged. The supernatant liquid was evaporated

to dryness and extracted with chloroform. The residue was neutralised with sodium bicarbonate solution and evaporated to dryness. The residue was dispersed in water and extracted with boiling chloroform in a liquid-liquid extractor. The chloroform extracts were combined, dried over anhydrous sodium sulphate and evaporated to dryness to give the methylated reduced trisaccharide (0·081 g.).

A sample of the reduced sugar was hydrolysed with N-sulphuric acid for four hours and the hydrolysate was examined by chromatography in solvent G. The presence of 3,4-di-O-methylrhamnose, 2,3,4-tri-O-methylglucose and 2,4-di-O-methyl-galactose was indicated.

A sample of the methylated trisaccharide was methanolysed and the products were examined by gas-liquid partition chromatography. The methylglycosides of the following sugars were detected.

	T in system b.	
3,4-Di-O-methyl-L-rhamnose	0·61	
2,3,4-Tri-O-methyl-D-glucose	1·83	1·35
2,4-Di-O-methyl-D-galactose	4·6	
3-O-methyl-L-rhamnose (trace)	1·01	

Hydrolysis of the methylated trisaccharide.

The methylated trisaccharide (75 mg.) was dissolved in N hydrochloric acid and the solution was heated on a boiling water bath for six hours. The solution was allowed to cool, neutralised with silver carbonate and filtered. Hydrogen

sulphide gas was passed through the solution and the solution was filtered. The filtrate was evaporated to dryness and extracted with acetone. The acetone extract was evaporated to dryness and the syrup (0·068) obtained was separated into two fractions on Whatman 3MM paper in solvent system G.

Fraction i). 2,4-Di-O-methyl-D-galactose.

The sugar (0·018 g.) R_G 0·5, $[\alpha]_D$ +89° (c, 0·4) was chromatographically pure and indistinguishable from 2,4-di-O-methyl-D-galactose. Demethylation of the sugar gave only galactose. The sugar was unattacked by the periodate. The sugar crystallised and was recrystallised from ethyl acetate and had m.p. 99-100°, mixed m.p. 98-99° (with authentic 2,4-di-O-methyl-D-galactose monohydrate).

Fraction ii). 2,3,4-Tri-O-methyl-D-glucose and 3,4-di-O-methyl-L-rhamnose.

A sample of the sugar was demethylated and chromatography of the products in solvent system G showed the presence of glucose and rhamnose.

The sugar mixture was separated into 3,4-di-O-methyl-L-rhamnose and 2,3,4-tri-O-methyl-D-glucose by ionophoresis in borate buffer (pH 10).

Sub-fraction iia). 3,4-Di-O-methyl-L-rhamnose.

The sugar (0·016 g.) R_G 0·91, $[\alpha]_D$ +22·5°(c, 0·12) was chromatographically and ionophoretically pure and was

indistinguishable from 3,4-di-O-methyl-L-rhamnose. Demethylation of the sugar gave rhamnose. The sugar crystallised and was recrystallised from light petroleum and had m.p. 95-96° and mixed m.p. 95° (with authentic 3,4-di-O-methyl-L-rhamnose),

Sub-fraction iii b). 2,3,4-Tri-O-methyl-D-glucose.

This fraction (0·017) R_G 0·91, $[\alpha]_D$ +69° (c, 0·17) was chromatographically and ionophoretically pure and identical to 2,3,4-tri-O-methyl-D-glucose. Demethylation of the sugar gave glucose.

The sugar was characterised by conversion into an aniline derivative, which crystallised and was recrystallised from ether-light petroleum, m.p. 134-135°, mixed m.p. 135-136° (with authentic 2,3,4-tri-O-methyl-N-phenyl-D-glucosylamine).

Fraction 5. O-D-Glucospyranosyluronic acid-(1→3)-O-α-D-galactopyranosyluronic acid-(1→2)-O-L-rhamnose and 4-O-(D-galactopyranosyluronic acid)-D-galactose.

Chromatography of this fraction (0·26 g.) $R_{Gal\ A}$ 0·28-0·39, $[\alpha]_D$ +49° (c, 3·9) in solvent system A, B, C, D, E, F and K on Whatman 1, 4, 541, 31 and 3MM paper indicated the presence of two sugars with similar mobilities. The sugar streaked in all solvents and $R_{Gal\ A}$ of the two visually distinct spots could be taken as 0·28 0·39 in solvent A. Ionophoresis of the sugar using borate buffer indicated the presence of two sugars with similar mobilities.

A sample of the sugar mixture was hydrolysed with N sulphuric

acid and the hydrolysate was examined by chromatography in solvent systems A, B and E. The presence of galacturonic acid, glucuronic acid, galactose and rhamnose was indicated.

A small sample of this fraction was methanolysed and the products were reduced with potassium borohydride. The reduced sugar was hydrolysed with N sulphuric acid for four hours. Chromatography of the hydrolysate in solvent system B showed the presence of galactose, glucose and rhamnose.

Another sample of the sugar (5 mg.) was dissolved in water and potassium borohydride (5 mg.) was added. The reduced sugar was hydrolysed and chromatography of the hydrolysate in solvent system K using spray reagent 3 showed the presence of galactitol, rhamnitol, galacturonic acid and the absence of rhamnose and galactose.

Methylation of (the sugar mixture) fraction 5.

The sugar (0·128 g.) was dissolved in water (2 ml.) and methylated by three additions of Haworth reagents and a treatment with Purdie's reagents. The methylated sugar was processed as described under fraction 2, and fraction 4.

A sample (5 mg.) of the methylated sugar mixture (0·102 g.) was hydrolysed with N-sulphuric acid for four hours. Chromatography of the hydrolysate in solvent system G indicated the presence of 2,3,6-tri-O-methylgalactose, 3,4-di-O-methylrhamnose and two acidic sugars (R_G 0·12, 0·09).

Another sample of the methylated sugar was methanolysed and the products were examined by gas liquid partition

chromatography.

The methylglycosides of the following sugars were detected:

Methylglycosides of:	T in system a.	T in system b.	
2,3,4-Tri-O-methyl-D-galacturonic acid	7·25	3·91	4·25
2,3,4-Tri-O-methyl-D-glucuronic acid	(3·23) 2·50	1·75	2·22
2,3,6-Tri-O-methyl-D-galactose	(3.23) 4.69	1.61	2.53
3,4-Di-O-methyl-L-rhamnose	1·00	0·61	

The rest of the methylated sugar (0·091 g.) was hydrolysed with N sulphuric acid for four hours, neutralised with saturated solution of barium hydroxide, solid barium carbonate and filtered. The filtrate was deionised with Amberlite resin IR-120 (H) and evaporated to dryness.

A small sample of the hydrolysate was converted into the methyl esters methylglycosides and reduced with potassium borohydride. A portion of the reduced sugars was hydrolysed with N sulphuric acid for two hours and the product was examined by chromatography in solvent system G. The presence of 2,3,6-tri-O-methylgalactose, 2,3,4-tri-O-methylgalactose, 2,3,4-tri-O-methylglucose, 3,4-di-O-methylrhamnose and 2,4-di-O-methyl-galactose was indicated. The remaining reduced sugar was again heated with methanolic hydrogen chloride (2·5%) and the product was examined by gas-liquid partition chromatography. The following methylglycosides were detected.

	T in system a.	T in system b.
2,3,6-Tri-O-methyl-D-galactoside		2·53 2·27, 1·62
2,3,4-Tri-O-methyl-D-galactoside		2·9
2,3,4-Tri-O-methyl-D-glucoside		1·86 1·36
3,4-Di-O-methyl-L-rhamnoside		0·60
2,4-Di-O-methyl-D-galactoside		3·9 4·59

The remaining hydrolysate was separated into three fractions on Whatman 3MM paper using solvent system G.

Sub-fraction 1. 3,4-Di-O-methyl-L-rhamnose.

This sugar (0·014) R_G 0·91, $[\alpha]_D$ +22° (c, 0·105) was chromatographically pure and indistinguishable from 3,4-di-O-methyl-L-rhamnose. Demethylation of the sugar gave rhamnose. The sugar crystallised and was recrystallised from light petroleum (b.p. 80-100°), m.p. 95-96° and mixed m.p. 94-95°.

Sub-fraction 2. 2,3,6-Tri-O-methyl-D-galactose.

This fraction (0·012 g.) R_G 0·76; $[\alpha]_D$ +79° (c, 0·09) was chromatographically pure and identical to 2,3,6-tri-O-methyl-D-galactose. Demethylation of the sugar gave galactose. The sugar was characterised by preparing 2,3,6-tri-O-methyl-D-galactonolactone which crystallised and was recrystallised from ether, m.p. 96-97°, mixed m.p. 95-96° (with authentic sample).

Sub-fraction 3.

This fraction (46 mg.) was a mixture of acidic sugars and had a trace of 2,3,6-tri-O-methyl-D-galactose.

Alkaline degradation of the fraction 5 (acid mixture).

Trial experiment.

The acid mixture (20 mg.) was dissolved in saturated lime water (5 ml.) in an atmosphere of nitrogen and allowed to stand for ten days. The solution was deionised with Amberlite resin IR-120 (H) and evaporated to dryness. The alkaline degraded sugar was examined by chromatography in solvent systems A and B using spray reagents 1 and 4. The presence of a sugar $R_{Gal\ A}$ 0·27 and a trace of galacturonic acid was indicated using spray reagent 1.

A sample of the alkaline degraded sugar was hydrolysed with N-sulphuric acid for four hours. Chromatography of the hydrolysate in solvent systems A and B indicated the presence of galacturonic acid and rhamnose.

Another sample of the alkaline degraded sugar was converted into methyl ester methylglycosides, reduced with potassium borohydride and the product was hydrolysed with N-sulphuric acid for four hours. Chromatography of the hydrolysate in solvent B showed the presence of galactose, glucose and rhamnose.

A small quantity of the alkaline degraded sugar was reduced with potassium borohydride and the product was hydrolysed with N-sulphuric acid for four hours. Chromatography of the

hydrolysate in solvent system K using spray reagent 3 showed the presence of rhamnitol, galacturonic acid, glucuronic acid and the absence of rhamnose.

Methylation of alkaline degraded (acid mixture) fraction 5.

Acid mixture (70 mg.) was dissolved in saturated lime water (15 ml.) in an atmosphere of nitrogen and was allowed to stand for ten days. The resulting solution was deionised with Amberlite resin IR-120 (H) and evaporated to dryness. The sugar (46 mg.) was methylated by a procedure similar to that used for fraction 4.

A sample of the methylated alkaline degraded sugar was hydrolysed with N-sulphuric acid and the products were examined by chromatography in solvent system G. The presence of 3,4-di-O-methylrhamnose and two acidic sugars (R_G 0·12, 0·08) was indicated.

Another sample of the methylated alkaline degraded sugar was methanolysed and the products were examined by gas-liquid partition chromatography. The methylglycosides of the following sugars were detected.

	T in system a.		T in system b.	
2,3,4-Tri-O-methyl-D-glucuronic acid	3·25	2·51	2·23	1·75
2,3,4-Tri-O-methyl-D-galacturonic acid (trace)	7·4		3·91	4·25
2,3,6-Tri-O-methyl-D-galactose(trace)	4·34		2·52	1·63
3,4-Di-O-methyl-L-rhamnose	1·01		0·60	
3-O-Methyl-L-rhamnose (trace)	3·64		1·03	

Reduction of the methylated alkaline degraded (acid mixture) fraction 5.

The same procedure was followed as for the reduction of fraction 4 except in that 20 mg. of lithium aluminium hydride in 2 ml. of tetrahydrofuran was used.

A small portion of the reduced sugar (15 mg.) was hydrolysed with N-sulphuric acid for four hours and the products were examined in solvent systems G and I. The presence of 2,3,4-tri-O-methylglucose, 3,4-di-O-methylrhamnose and 2,4-di-O-methylgalactose was indicated. The methylglycosides of the following sugars were detected.

	T in system b.	
2,3,4-Tri-O-methyl-D-glucose	3·23	1·36
2,4-Di-O-methyl-D-galactose	4·58	3·85
3,4-Di-O-methyl-L-rhamnose	6·6	
2,3,4-Tri-O-methyl-D-galactose (trace)	2·57	2·90
2,3,6-Tri-O-methyl-D-galactose	2·52	1·63

Fraction 6.

This fraction (0·045) ($R_{Gal\ A}$ 0·0) had a chromatographic mobility of 0·2 relative to galacturonic acid in solvent system D.

A sample of the sugar was hydrolysed with N-sulphuric acid for six hours and the products were examined in solvent systems A and B. The presence of galacturonic acid, galactose and rhamnose was indicated. Another sample of the sugar was converted to methyl ester methylglycosides, reduced with potassium

borohydride and the product was hydrolysed with N-sulphuric acid for four hours. Chromatography of the hydrolysate in solvent system B showed the presence of galactose, glucose and rhamnose.

Another sample of the sugar was reduced with potassium borohydride and the product was hydrolysed with N-sulphuric acid for four hours. Chromatography of the hydrolysate in solvent system K using spray reagent 3 indicated the presence of galactitol, rhamnitol, galacturonic acid and the absence of galactose and rhamnose.

METHYLATION OF THE GUM.

The purified gum acid (10 g.) was dissolved in water (350 ml.) and dimethyl sulphate (20 ml.) and sodium hydroxide solution (20 ml., 30%) were added dropwise over a period of two hours. The reaction flask was kept in ice water and an atmosphere of nitrogen was maintained. A further addition of dimethyl sulphate (80 ml.) and sodium hydroxide solution (180 ml.) was made. Six further batches were added in six days. Vigorous stirring was maintained during the methylation. No precipitation was observed during the methylation. Periodically a drop or two of secondary octyl alcohol was added to the reaction mixture to cease the frothing. After the final methylation the reaction mixture was heated to 80° over a water bath for an hour. The reaction mixture was extracted with chloroform (5 x 100). The chloroform extracts were combined, dried over anhydrous sodium sulphate and reduced in volume. The methylated gum acid was precipitated with light petroleum (b.p. 60-80°) and dried in a vacuum oven. The methylated gum acid (6·4 g.) had $[\alpha]_D$ +66° (c, 1·98 in chloroform) (Found: OMe, 28·1%).

The partially methylated gum acid (6·3 g.) was neutralised with silver carbonate and filtered. The filtrate was reduced in volume and freeze dried. The silver salt of the methylated gum acid was refluxed with freshly distilled methyl iodide (100 ml.). Silver oxide (8 g.) was added to the refluxing mixture in four portions. The mixture was allowed to reflux for twelve hours,

cooled and filtered. The residual silver oxide was extracted with hot chloroform in a Soxhlet extractor. The combined filtrate and the extract was dried over anhydrous sodium sulphate and reduced in volume. The methylated gum acid was precipitated with light petroleum (b.p. 60-80°) and dried in a vacuum oven (Yield = 3·6 g., OMe, 38·6%).

The methylated gum acid was subjected to another treatment with Purdie reagents and was recovered as described above. The methylated gum acid (3·4 g.) had $[\alpha]_D$ +66·8° (c, 1·8 in chloroform) (Found: OMe, 41·0%).

Once more the methylated gum was methylated using methyl iodide and silver oxide and processed as described above. The methylated polysaccharide was fractionally precipitated from chloroform solution with light petroleum (b.p. 60-80°). Two fractions were obtained.

Weight	Methoxyl Content
0.19 g.	39·8%
2.9 g.	41·3%

$[\alpha]_D$ =+66·6° (c, 1·2 in chloroform).

A small sample of the fully methylated polysaccharide was methanolysed and the methyl glycosides were examined by gas-liquid partition chromatography. The methyl glycosides of the sugars given in the table below were detected.

Methyl glycosides of:	T in system a.	T in system b.
2,3,4-Tri-O-methyl-L-rhamnose	0·45	0·47

Methyl glycosides of:	T in system a.		T in system b.	
3,4-Di-O-methyl-L-rhamnose	1·01	0·69	0·61	
3-O-Methyl-L-rhamnose	3·60		1·03	
2,3,4,6-Tetra-O-methyl-D-galactose	1·80		1·52	(1·61)
2,3,6-Tri-O-methyl-D-galactose	4·68	4·29	2·55	2·25
	3·91	3·22	2·07	(1·61)
2,3,4-Tri-O-methyl-D-glucuronic acid	3·22	2·49	1·78	2·25
Unidentified sugar			1·26	

Trial reduction of the methylated gum acid.

Methylated acid (0·10 g.) was dissolved in dry tetrahydrofuran (4 ml.) and lithium aluminium hydride (0·10 g.) in dry tetrahydrofuran (4 ml.) was added portion wise. The solution was kept at room temperature for half an hour and then refluxed for two hours. A further addition of lithium aluminium hydride (0·05 g.) in tetrahydrofuran (2 ml.) was made and the heating was continued for a further half hour. The solution was cooled, excess lithium aluminium hydride was destroyed by the simultaneous addition of ethyl acetate and water. The solution was acidified (pH 4) and the precipitate was centrifuged. The residue was neutralised with sodium bicarbonate solution and filtered. The filtrate and the supernatant liquid were combined, reduced in volume and repeatedly extracted with warm chloroform (5 x 15). The chloroform extract was dried over anhydrous sodium sulphate and reduced in volume.

The carboxyl reduced methylated gum was precipitated with light petroleum (b.p. 80-100°), centrifuged and dried in a vacuum oven at 30-35°.

A sample of the methylated carboxyl reduced polysaccharide was hydrolysed with N-sulphuric acid and the products were examined by chromatography in solvent systems G and 1. The presence of 2,3,4-tri-O-methylrhamnose, 3,4-di-O-methylrhamnose, 3-O-methylrhamnose, rhamnose, 2,3,4,6-tetra-O-methylgalactose, 2,3,6-tri-O-methylgalactose, 2,3-di-O-methylgalactose, mono-O-methylgalactose, trace of galactose and 2,3,4-tri-O-methyl-glucose was indicated.

Another sample of the methylated carboxyl reduced gum was methanolysed and the resulting methyl glycosides were examined by gas-liquid partition chromatography. The methyl glycosides of the following sugars were detected.

Methyl glycosides	T in system a.	T in system b.
2,3,4-Tri-O-methyl-L-rhamnose	0·45	0·47
3,4-Di-O-methyl-L-rhamnose	1·00-1·01	0·61
3-O-methyl-L-rhamnose	(3·68)	1·02
2,3,4,6-Tetra-O-methyl-D-galactose	1·80	(1·61)
2,3,6-Tri-O-methyl-D-galactose	3·25 4·31 4.70	(1·61) 2·54 2·25 2·09
2,6-Di-O-methyl-D-galactose		(2.54)(3·15)(3·71)
2,3-Di-O-methyl-D-galactose		(2·54)(3·15)(3·71) 4·25
2,3,4-Tri-O-methyl-D-glucose	2·59 (3·68)	1·36 1·83

Large scale reduction of the methylated gum acid.

Methylated gum acid (3.3 g.) was dissolved in dry tetrahydrofuran (100 ml.) and lithium aluminium hydride (4 g.) in dry tetrahydrofuran (100 ml.) was batchwise added to the methylated acid solution. The mixture was allowed to stand at room temperature for 0·5 hr. and then refluxed for two hours. The solution was allowed to cool and a further quantity of lithium aluminium hydride (1·5 g.) in tetrahydrofuran (25 ml.) was added. The solution was allowed to reflux, cool and the methylated acid was isolated as described under trial experiment (yield, 3·1 g., Found: OMe, 35·8%, $[\alpha]_D$=+41·0° (c, 0·9 in chloroform).

A sample of Sterculia urens gum (different batch) obtained from the same source which supplied the material (Sterculia urens gum) under investigation was methylated and reduced as described above and was found to have: $[\alpha]_D$=+42° (c, 1·4 in chloroform), OMe, 36·4%.

Remethylation of the carboxyl reduced methylated gum.

The carboxyl reduced methylated gum (0·105 g.) was dissolved in dimethyl formamide (3·3 ml.), barium oxide (0·66 g.), barium hydroxide (0·026 g.) and methyl iodide (0·35 ml.) were added. The mixture was stirred and heated at 40° for two hours. The mixture was allowed to stir overnight and finally filtered. The filtrate was evaporated to dryness and the residue was extracted with chloroform (5 x 15). The chloroform extract

was dried over anhydrous sodium sulphate and evaporated to dryness. A portion of the syrup (90·0 mg.) was hydrolysed with N sulphuric acid and the hydrolysate was examined chromatographically in solvent systems I and G. The presence of 2,3,4,6-tetra-O-methylglucose, 2,3,4-tri-O-methylglucose, 2,3,4-tri-O-methylrhamnose, 3,4-di-O-methylrhamnose, 3-O-methylrhamnose, rhamnose, 2,3,4,6-tetra-O-methylgalactose, 2,3,6-tri-O-methylgalactose, di-O-methylgalactose, mono-O-methylgalactose and a trace of galactose was indicated.

A sample of the methylated carboxyl reduced remethylated gum was methanolysed and the resulting products were examined by gas-liquid partition chromatography. The methylglycosides of the following sugars were detected.

Methyl glycosides	T in system a.	T in system b.
2,3,4-Tri-O-methyl-L-rhamnose	0·44	0·46
3,4-Di-O-methyl-L-rhamnose	(1·00)	0·62
3-O-Methyl-L-rhamnose	(3·65)	1·02
2,3,4,6-Tetra-O-methyl-D-galactose	1·78	(1·62)
2,3,6-Tri-O-methyl-D-galactose	3·22 4·31 4·68	(1·62)
2,3,4,6-Tetra-O-methyl-D-glucose	(1·00) 1·44	(1·35) 1·02
2,3,4-Tri-O-methyl-D-glucose	2·58 (3·65)	1·83 (1·35)

The remethylation of the carboxyl reduced methylated gum was considered to be incomplete as the presence of 2,3,4-tri-O-methyl-D-glucose was detected.

Trial hydrolysis of the methylated reduced gum.

Methylated reduced gum (0·05 g.) was dissolved in 2N hydrochloric acid (5·0 ml.) and allowed to stand for 48 hr. at room temperature. The solution was heated overnight at 40° and the temperature was gradually raised to 60° over a period of six hours. The heating at 60° was continued for a further period of six hours. The temperature was now gradually raised to 80° over a period of four hours, and heating at 80° was continued for eight hours. Finally the temperature was raised to 100° and the heating was continued for eight hours when the optical rotation was constant. The solution was diluted to 10 ml., neutralised with silver carbonate, silver ions were precipitated by hydrogen sulphide gas and the filtrate was evaporated to dryness. Chromatography of the hydrolysate in the solvents G and I indicated the presence of 2,3,4-tri-O-methylrhamnose, 3,4-di-O-methylrhamnose, 3-O-methylrhamnose, rhamnose, 2,3,4,6-tetra-O-methylgalactose, 2,3,6-tri-O-methylgalactose, di-O-methylgalactose, mono-O-methylgalactose and 2,3,4-tri-O-methylglucose.

Large scale hydrolysis.

Methylated carboxyl reduced gum (2·7 g.) was dissolved in 2N hydrochloric acid (250 ml.), heated and processed as described under trial hydrolysis.

Separation of the methylated sugars.

The syrup (2·5 g.) in butan-1-ol half saturated with water

(4 ml.) was placed on a cellulose column and the column was eluted successively with the following solvents:

Light petroleum (b.p. 100-120°):butan-1-ol (70:30, saturated with water).

Light petroleum (b.p. 100-120°):butan-1-ol (200 ml.).

Light petroleum (b.p. 100-120°):butan-1-ol (50:50, saturated with water).

Butan-1-ol (200 ml.)

Butan-1-ol half saturated with water and finally with water. The eluate was collected on an automatic turntable in fractions of suitable volume. Every fifth fraction was examined by chromatography in the solvent systems G and I and the similar fractions were combined to give a total of twenty-five fractions.

Fraction	Tube No.	R_G	Stain with spray reagent I	Weight in mg.	$[\alpha]_D$	Probable identity
1	1-15	1·02 1·1	Khaki/ Brown	6		2,3,4-tri-O-methyl rhamnose & unknown
2	16-26	1·04	Khaki	125	+21·8°	2,3,4-tri-O-methyl rhamnose
3	27-33	1·0 1·04	Khaki/ Pink	19		2,3,4-tri-O-methyl rhamnose, 2,3,4-tri-O-methylglucose, 2,3,4,6-tetra-O-methylgalactose and 3,4-di-O-methyl-rhamnose.
4	34-52	0·94	Brown/ Pink	938	+72°	2,3,4-tri-O-methyl-glucose, 2,3,4,6-tetra-O-methyl-galactose and 3,4-di-O-methylrhamnose
5	53-77	0·9 0·89	Pink/ Yellow	43	+46-50°	2,3,4-tri-O-methyl glucose & unknown

Fraction	Tube No.	R_G	Stain with spray reagent 1	Weight in mg.	$[\alpha]_D$	Probable identity
6	78-90	0·89 0·92 0·78	Pink/ yellow	15		2,3,4-tri-O-methyl glucose, 2,3,6-tri-O-methylgalactose and unknown.
7	91-117	0·87 0·76	Brown/ yellow	18		2,3,6-tri-O-methyl galactose & unknown
8	118-120	0·75	Brown	15		2,3,6-tri-O-methyl galactose & unknown
9	121-140	0·76	Brown	167	+70°	2,3,6-tri-O-methyl galactose & unknown
10	141-195	0·76	Brown	20		2,3,6-tri-O-methyl galactose & unknown
11	196-239	0·76 0·62	Brown/ khaki	21	+41°	2,3,6-tri-O-methyl galactose and 3-O-methylrhamnose.
12	240-305	0·61	Khaki	182	+27·2°	3-O-methylrhamnose.
13	306-327	0·62 0·54	Brown/ khaki	21		3-O-methylrhamnose and 2,6-di-O-methyl galactose.
14	328-343	0·54	Brown	28		2,6-di-O-methyl galactose
15	344-360	0·54 0·50	Brown	16		2,6-di-O-methyl galactose and 2,3-di-O-methylgalactose.
16	361-391	0·50	Brown	38	+71°	2,3-di-O-methyl galactose.
17	392-400	0.50 0.44	Brown/ pink	19	58	2,3-di-O-methyl galactose & unknown
18	401-438	0.50 0.44	Brown Khaki	26	+58°	2,3-di-O-methyl galactose & rhamnose
19	439-481	0.42	Khaki	28		rhamnose
20	482-547	0.42 0.33	Khaki/ brown	28	+38°	2-O-methylgalactose and rhamnose

Fraction	Tube No.	R_G	Stain with spray reagent 1	Weight in mg.	$[\alpha]_D$	Probable identity
21	548-615	0.32	Brown	129	+55-82°	2-O-methylgalactose
22	616-634	0.31 0.30	Brown pink	38		2-O-methylgalactose and 3-O-methyl-galactose.
23	635-655	0·30	Pink	198		3-O-methylgalactose.
24	656-670	0·28	Pink/ brown	19		3-O-methylgalactose and galactose.
25	671-770	0·24	Pink/ brown	18		3-O-methylgalactose, galactose and a trace of galacturonic acid.

Fraction 1. 2,3,4-Tri-O-methyl-L-rhamnose and an unknown.

Chromatography of this fraction (0·006) in solvent systems G, I and K indicated the presence of two sugars (R_G, 1·1 and 1·04). One of the sugars (R_G, 1·04) had the chromatographic mobility similar to 2,3,4-tri-O-methyl-L-rhamnose.

Fraction 2. 2,3,4-Tri-O-methyl-L-rhamnose.

This fraction (0·125 g.) had R_G, 1·04 and $[\alpha]_D$+21° (c, 1·38). Chromatography in solvent systems G, I and K showed this fraction to be pure and indistinguishable from 2,3,4-tri-O-methyl-L-rhamnose. A sample of the sugar was converted into the methyl glycoside and examined by gas chromatography in system a. and b. Only methyl-2,3,4-tri-O-methyl-L-rhamnoside was detected. Demethylation of the sugar gave rhamnose. The anilide derivative of the sugar was prepared which crystallised and was recrystallised from light-petroleum, m.p. 112-113° and mixed m.p. 111-112°

(with authentic sample of 2,3,4-tri-O-methyl-N-phenyl-L-rhamnosyl amine).

Fraction 3. 2,3,4-Tri-O-methyl-L-rhamnose, 2,3,4-tri-O-methyl-D-glucose, 2,3,4,6-tetra-O-methyl-D-galactose, 3,4-di-O-methyl-L-rhamnose.

Chromatography of this fraction (0·01 g.) in solvent systems G, I, J and K indicated the presence of at least four sugars which had the chromatographic mobilities similar to 2,3,4-tri-O-methyl-L-rhamnose, 2,3,4-tri-O-methyl-D-glucose, 2,3,4,6-tetra-O-methylgalactose and 3,4-di-O-methylrhamnose.

Fraction 4. 2,3,4,6-Tetra-O-methyl-D-galactose, 2,3,4-tri-O-methyl-D-glucose and 3,4-di-O-methyl-L-rhamnose.

This fraction (0·938 g.) had R_G, 0·93 and $[\alpha]_D$+72° (c, 1·48). Paper chromatography of this fraction in solvent systems G, I, J and K showed this fraction to be a mixture of at least two sugars. Ionophoresis using borate buffer indicated the presence of three sugars.

A sample of the sugars was converted into the methylglycosides and the products were examined by gas liquid partition chromatography. The methyl glycosides of the following sugars were detected.

Methylglycosides of:	T in system a.	T in system b.
3,4-di-O-methyl-L-rhamnose	1·00	0·62
2,3,4-tri-O-methyl-D-glucose	3.75 2.58	1.36 1.82
2,3,4,6-tetra-O-methyl-D-galactose	1.80	1·60

Demethylation of the sugars gave galactose, glucose and rhamnose.

The syrup (0·27 g.) was separated into two fractions on Whatman 3MM paper in solvent system I.

Fraction 4A. 2,3,4,6-Tetra-O-methyl-D-galactose.

This fraction (0·112 g.) had R_G 0·88 and $[\alpha]_D$ +91° (c, 1·3). Paper chromatography of this fraction in four different solvents showed a single component. Methylglycosides of the sugar were examined by vapour phase chromatography and only methyl-2,3,4,6-tetra-O-methyl-D-galactoside was detected. The anilide derivative of the sugar was prepared which crystallised and was recrystallised from ethyl acetate, m.p. 197-198° and mixed m.p. 197° (with authentic 2,3,4,6-tetra-O-methyl-N-phenyl-D-galactosyl amine).

Fraction 4B. 2,3,4-Tri-O-methyl-D-glucose and 3,4-di-O-methyl-L-rhamnose.

Paper chromatography of this fraction (0·126 g.) in solvent systems G, I, J and K showed either one spot or a streak with overlapping stains. Ionophoresis using borate buffer indicated the presence of two sugars with ionophoretic mobilities similar to 2,3,4-tri-O-methyl-D-glucose and 3,4-di-O-methyl-L-rhamnose. Demethylation of the sugar gave glucose and rhamnose. A sample of the sugars was converted into the methylglycosides and the glycosides were examined by vapour phase chromatography. The methyl glycosides of 2,3,4-tri-O-methyl-D-glucose and 3,4-di-O-

methyl-L-rhamnose were detected.

The syrup (0·120 g.) was separated into two fractions by ionophoresis using borate buffer. The sugars were located by cutting the side strips and developing these with spray reagent I acidified with glacial acetic acid. The sugars after being eluted from the paper with water were treated with Amberlite resin IR120 (H) and evaporated to dryness. The sugars were repeatedly dissolved in methanol (5 x 5) and evaporated to dryness.

Sub-fraction I. 3,4-Di-O-methyl-L-rhamnose.

This fraction (0.058 g.) was chromatographically and ionophoretically pure and had R_G, 0·91 and $[\alpha]_D$+22·9° (c, 2·9 g.). Demethylation of the sugar gave rhamnose. A sample of the sugar was converted into the methylglycoside and the product was examined by vapour phase chromatography. The methylglycoside of only 3,4-di-O-methyl-L-rhamnose was detected. The syrup, on allowing to stand crystallised and was recrystallised from light petroleum (b.p. 40-60°), m.p. 96-97° and mixed melting point 95-96°. An X-ray powder photograph was recorded which was identical to that of 3,4-di-O-methyl-L-rhamnose.

Sub-fraction II. 2,3,4-Tri-O-methyl-D-glucose.

This fraction (0·042 g.) had R_G, 0·9 and $[\alpha]_D$+44 - 68° (c, 0.84). The sugar was chromatographically and ionophoretically pure and was indistinguishable from 2,3,4-tri-O-methyl-D-glucose. Demethylation of the sugar gave glucose. A sample of the sugar was converted into the methylglycosides and the

product was examined by gas-liquid partition chromatography. Methyl 2,3,4-tri-O-methyl-D-glucosides were detected. The sugar was characterised by conversion into 2,3,4-tri-O-methyl-N-phenyl-D-glucosylamine, m.p. 129-130° and mixed m.p. 127-128°.

Fraction 5. 2,3,4-Tri-O-methyl-D-glucose and unknown.

This fraction (0·043 g.) had $[\alpha]_D$+46 - 56° (c, 0·81) and R_G, 0·93 and 0·88. Paper chromatography in solvents G, I, J and K showed the presence of two sugars with pink and yellow stains using spray reagent I. A sample of the sugar was converted into the methyl glycosides and the glycosides were examined by vapour phase chromatography. Methylglycosides with the following retention times were detected.

Methylglycoside	T in system a.		T in system b.	
i.	2·59	3·69	1·84	1·38
ii.	1·78-1·79	1·48	0·74 - 0·75	

The methylglycoside (I) has the retention times characteristic to 2,3,4-tri-O-methyl-D-glucose. The methylglycoside (II) with retention times 1.78-1.79 and 0.74-0.75 in systems a. and b. respectively could possibly be 2,3-di-O-methyl-L-rhamnoside.

This fraction was separated in two fractions using solvent system A on Whatman 3MM paper.

Sub-fraction I. 2,3,4-Tri-O-methyl-D-glucose.

This fraction (0·012 g.) had R_G, 0·91 and $[\alpha]_D$+69° (c, 0·60). Paper chromatography of this fraction in solvent systems A, B,

G, I, J and K showed it to be pure and indistinguishable from 2,3,4-tri-O-methyl-D-glucose. Demethylation of the sugar gave glucose.

Sub-fraction II. Unknown.

This sugar (0.014 g.) had R_G0.87 and gave a yellow/golden stain with spray reagent I. Demethylation of the sugar gave glucose. Periodate oxidation of the sugar gave a pattern similar to that of a 2,3-di-O-substituted sugar.

Fraction 6. 2,3,4-Tri-O-methyl-D-glucose, unknown and 2,3,6-tri-O-methyl-D-galactose.

This fraction (0.018 g.) had R_G 0.92, 0.88, 0.80 and gave pink/yellow stain with spray reagent I. Demethylation of the sugars gave glucose, galactose and two unidentified sugars, ($R_{rhamnose}$ 0.98, 1.29 probably a mono or a di-methyl derivative of glucose or galactose produced as a result of incomplete demethylation).

Fraction 7. 2,3,6-Tri-O-methyl-D-galactose and unknown.

This fraction (0.018 g.) had R_G 0.86 and 0.76. Chromatography of this fraction in solvent systems A, G, I, J and K showed it to be a mixture of 2,3,6-tri-O-methyl-D-galactose and an unidentified sugar. Demethylation of the sugars gave galactose, glucose and a sugar with $R_{rhamnose}$ 0.98.

Fraction 8. 2,3,6-Tri-O-methyl-D-galactose and unknown.

This fraction (0.015 g.) was chromatographically pure and

indistinguishable from 2,3,6-tri-O-methyl-D-galactose. The methylglycosides of the sugar was prepared and examined by gas liquid partition chromatography. The methylglycosides with the following retention times were detected.

Methylglycoside	T in system a.		T in system b.	
I	3·21	4·3	1·61	2·49
		4·69	2·21	2·06
II			3·41	

The methylglycoside I had the retention times characteristic to 2,3,6-tri-O-methyl-D-galactoside. The methylglycoside II with retention time 3·41 in system b. could possibly be of a dimethyl hexose.

Demethylation of the sugar gave only galactose.

Fraction 9. 2,3,6-Tri-O-methyl-D-galactose.

This fraction had R_G, 0·76 and $[\alpha]_D$+72° (c, 0·94). Chromatography of this fraction in solvent systems G, I and J showed it to be pure and indistinguishable from 2,3,6-tri-O-methyl-D-galactose. A portion of the sugar was converted into the methylglycosides and the product was examined by gas-liquid partition chromatography. The methylglycosides with the following retention times were detected.

Methylglycoside	T in system a.		T in system b.	
I	3·21	4·30	1·61	2·49
			2·21	2·06
II			3·41	

The methylglycoside I had the retention times characteristic

to 2,3,6-tri-O-methyl-D-galactose whereas methylglycoside II could possibly be of di-O-methyl hexose.

Demethylation of this fraction gave only galactose. Ionophoresis of the sugar using borate buffer showed the presence of only one component. A sample of the sugar was hydrolysed with N-hydrochloric acid for four hours and the products were examined in solvents G, I, J and K. The presence of a sugar identical to 2,3,6-tri-O-methyl-D-galactose was indicated.

A sample of the sugar was reduced with potassium borohydride to 2,3,6-tri-O-methylgalactitol and treated with sodium meta-periodate solution at 0° for 1·5 hr. The product on chromatography gave a pattern similar to the one obtained after periodate oxidation of 2,3,6-tri-O-methyl-D-glucitol.

The sugar was identified as 2,3,6-tri-O-methyl-D-galactose by oxidation to 2,3,6-tri-O-methyl-D-galactonolactone which crystallised and was recrystallised from ether, m.p. 97-98° and mixed m.p. 98°. (with authentic 2,3,6-tri-O-methyl-D-galactonolactone). The 2,3,6-tri-O-methyl-D-galactonolactone was examined in solvent systems A, G, H and K using spray reagent 4. The developed chromatograms showed the presence of a single component.

Fraction 10. 2,3,6-Tri-O-methyl-D-galactose and unknown.

Chromatography of this fraction (0·02 g.) in solvent systems B, G, I, J, and K showed the presence of a sugar,

indistinguishable from 2,3,6-tri-O-methyl-D-galactose. Demethylation of the sugar gave galactose only. Vapour phase chromatography of the methylglycosides of this fraction showed the presence of two methylglycosides with the following retention times.

Methylglycosides	T in system a.	T in system b.
I	3.21 4.3	1.61 2.49 2.21 2.06
II		3.4

This fraction, like fraction 8 and 9 showed the presence of mainly methyl-2,3,6-tri-O-methyl-D-galactoside and an unidentified methylglycoside which could possibly be a di-O-methylglycoside.

Column chromatography of Fractions 8, 9 and 10.

Fractions 8, 9 and 10 were combined and run on to the charcoal-celite column (1·5 x 25 cm.). The column was eluted with a gradient of 0-5% butan-2-one. Fractions (5 ml.) were collected and every third fraction was examined by paper chromatography. Similar fractions were combined, reduced in volume and a sample of each fraction was converted into the methylglycosides. The methylglycosides were examined by gas liquid partition chromatography.

Two fractions were obtained.

Fraction a. This fraction (0·118) was paper chromatographically and vapour phase chromatographically pure and was indistinguishable from 2,3,6-tri-O-methyl-D-galactose.

Fraction b. This fraction (0·128) was a mixture of two sugars and had R_G, 0·76 and 0·38. The stain from the sugar with R_G 0·38 on chromatograms disappeared when heated at 120-130° for three minutes. Gas-liquid partition chromatography of the methylglycosides of this fraction showed the presence of only methyl 2,3,6-tri-O-methyl-D-galactosides.

Fraction 11. 2,3,6-Tri-O-methyl-D-galactose and 3-O-methyl-L-rhamnose.

This fraction (0·021 g.) had $[\alpha]_D$+41° (c, 0·58) and R_G, 0·76 and 0·62. Chromatography of this fraction in solvents G, I and J showed it to be a mixture of 2,3,6-tri-O-methyl-D-galactose and 3-O-methyl-L-rhamnose. Demethylation of the sugars gave galactose and rhamnose.

Fraction 12. 3-O-Methyl-L-rhamnose.

This sugar (0·182 g.) had $[\alpha]_D$+27·2° (c, 0·71), R_G 0·61 and was chromatographically pure. A sample of the sugar was converted into the methylglycosides and the product was examined by gas-liquid partition chromatography. Methyl-3-O-methyl-L-rhamnoside only was detected. The sugar was allowed to stand in a desiccator when it crystallised and had m.p. 114-115° and mixed m.p. 115-116°. An X-ray powder photograph of the sugar was recorded which was identical to that of authentic 3-O-methyl-L-rhamnose.

Fraction 13. 3-O-Methyl-L-rhamnose and 2,6-di-O-methyl-D-galactose.

Chromatography of this fraction (0·021 g.) in solvent systems G, I and B showed it to be a mixture of 3-O-methyl-L-rhamnose and 2,6-di-O-methyl-D-galactose. Demethylation of the sugars gave galactose and rhamnose.

Fraction 14. 2,6-Di-O-methyl-D-galactose.

This fraction (0·028 g.) had R_G, 0·54 and $[\alpha]_D$ +58 - 81·8° (c, 0·56). Chromatography in solvent systems G, I, J and K showed this fraction to be pure and indistinguishable from 2,6-di-O-methyl-D-galactose. Demethylation of the sugar gave only galactose. A sample of the sugar was subjected to periodate oxidation and the products were examined by chromatography in solvent G. Development of the chromatogram showed the presence of a yellow spot, R_G 0·24. The sugar was allowed to stand in the desiccator when it crystallised and was recrystallised from chloroform-petroleum ether as monohydrate, m.p. 86-87° and mixed m.p. 85-86°.

Fraction 15. 2,6-Di-O-methyl-D-galactose and 2,3-di-O-methyl-D-galactose.

Chromatography of this fraction (0·016 g.) in solvent systems A, B, G, I and J showed the presence of two sugars identical to 2,6-di-O-methyl-D-galactose and 2,3-di-O-methyl-D-galactose (R_G, 0·54 and 0·51). Demethylation of the sugars

gave only galactose. Periodate oxidation of the sugars followed by examination in solvent system G showed the presence of a yellow spot (R_G 0·24), a grey spot (R_G 0·82), a brown spot (R_G 0·93) and a grey spot (R_G 1·06).

Fraction 16. 2,3-Di-O-methyl-D-galactose.

This fraction (0·034 g.) had R_G, 0·50 and $[\alpha]_D$ +71·6°(c, 0·68). Chromatography of the sugar in solvent systems A, B, G, I and J showed it to be pure and indistinguishable from 2,3-di-O-methyl-D-galactose. A sample of the sugar was converted into the methylglycosides and the product was examined by vapour phase chromatography. Only methyl-2,3-di-O-methyl-D-galactoside was detected. Demethylation of the sugar gave galactose. Chromatography of the periodate oxidised sugar showed three spots with grey (R_G 0·82), brown (R_G 0·93) and grey (1·06) stains. The sugar was reduced with potassium borohydride to 2,3-di-O-methyl-D-galactitol and oxidised with sodium-meta periodate. The products on chromatographic examination gave a pattern identical to that obtained from the authentic sample. The aldonamide derivative of the sugar was prepared which crystallised and was recrystallised from dry ethanol-acetone, m.p. 135-136° and mixed m.p. 134° (with authentic sample of 2,3-di-O-methyl-D-galactonamide.

Fraction 17. 2,3-Di-O-methyl-D-galactose and unknown.

Chromatography of this fraction (0·019 g.) in solvent

systems A, B, G, I and K showed the presence of two sugars, R_G 0·5 and 0·44. The sugar with R_G 0.44 disappeared when the chromatogram was heated for three minutes at 130-140°. Demethylation of the sugars gave only galactose.

Fraction 18. 2,3-Di-O-methyl-D-galactose and L-rhamnose.

This fraction (0·026), R_G 0·50, 0·44, $[\alpha]_D$ +58° (c, 0·28) was chromatographically indistinguishable from 2,3-di-O-methyl-D-galactose and rhamnose. Demethylation of the sugars gave galactose and rhamnose.

Fraction 19. L-Rhamnose.

This fraction (0·028 g.) R_G 0·43, $[\alpha]_D$ +12° 7·8° (c, 0·58), was chromatographically indistinguishable from rhamnose in solvent systems A, B and G. The sugar on allowing to stand in a desiccator crystallised and was recrystallised from aqueous acetone, m.p. 91-92° and mixed m.p. 91-92° (with authentic L-rhamnose monohydrate).

Fraction 20. L-Rhamnose and 2-O-methyl-D-galactose.

This fraction (0·028 g.) R_G 0·42, 0·33, $[\alpha]_D$ +38° (c, 0·14) was examined chromatographically in solvent systems A, B, G and I. The presence of rhamnose and 2-O-methyl-D-galactose was indicated. Demethylation of the sugar gave rhamnose and galactose.

Fraction 21. 2-O-Methyl-D-galactose.

This fraction (0·129 g.) R_G 0·32, $[\alpha]_D$ +55 → 82° (c, 0·76) was chromatographically pure and identical to 2-O-methyl-D-galactose. It gave a brown stain with spray reagent I which was fluorescent in ultraviolet light. Demethylation of the sugar gave only galactose. A sample of the sugar was subjected to periodate oxidation and the products were examined by paper chromatography in solvent G. The yellow stain (R_G 0·24) developed when the chromatogram was sprayed with spray reagent I. The sugar crystallised and was recrystallised from aqueous acetone. The sugar had m.p. 156-157° and mixed m.p. 157° (with authentic 2-O-methyl-D-galactose).

Fraction 22. 2-O-Methyl-D-galactose and 3-O-methyl-D-galactose.

Chromatography of this fraction (0·038) showed it to be a mixture of 2-O-methyl-D-galactose and 3-O-methyl-D-galactose.

Fraction 23. 3-O-Methyl-D-galactose.

This fraction (0·198 g.) R_G 0·29, $[\alpha]_D$ +104° (c, 0·94) was chromatographically pure and indistinguishable from 3-O-methyl-D-galactose. Demethylation of the sugar gave galactose. Chromatography of the periodate oxidised product of the sugar gave a pink spot (R_G 0·40) corresponding to mono-O-methyl-pentose. The syrup was seeded and allowed to stand when it crystallised, m.p.

Fraction 24. 3-O-Methyl-D-galactose and galactose.

Chromatography of this fraction (0·019) in solvent systems A, B and G indicated the presence of 3-O-methyl-D-galactose and galactose.

Fraction 25. 3-O-methyl-D-galactose, galactose and galacturonic acid.

Chromatography of this fraction (0·018 g.) in solvent systems A, B and G indicated the presence of 3-O-methyl-D-galactose, galactose and a trace of galacturonic acid.

Water Eluate. Chromatography of water eluate from the column showed the absence of carbohydrate material.

Smith degradation of the gum acid.

The gum acid (1 g.) was dispersed in freshly prepared 0·1M sodium metaperiodate solution (88 ml.) and the solution was made up to 100 ml. The gum acid dissolved when the solution was shaken in the dark for two hours. In another flask 88 ml. of 0·1M sodium metaperiodate solution was diluted with distilled water to 100 ml. In a third flask 88 ml. of the same sodium periodate solution was treated with ethylene glycol (8 ml.) and the volume was made up to 100 ml. The three solutions were kept in the dark at room temperature and were periodically shaken.

After certain intervals of time aliquot samples (1 ml.) were removed from each solution, diluted to 1000 ml. and the optical densities of the resulting solutions were measured in the "Unicam" spectrophotometer at 222·5 mμ(65). From the results obtained, the consumption of periodate per sugar unit after various intervals of time were calculated and are tabulated below.

Time hrs.	4	10	14	20	24	30
Moles of periodate consumed per sugar unit.	0·7	0·8	0·82	0·87	0·87	0·87

After thirty hours the reaction was stopped by the addition of ethylene glycol (3 ml.) and the solution was dialysed against tap water for four days. The dialysate was concentrated to a small volume (20 ml.), potassium borohydride (250 mg.) was added and the solution was allowed to stand overnight. A

further addition of potassium borohydride (100 mg.) was made and the solution was allowed to stand for six hours at room temperature. The excess borohydride was destroyed by the addition of Amberlite resin IR-120 (H) and the solution was stirred for 15 minutes, filtered and concentrated to a syrup. The syrup was repeatedly dissolved in methanol (3 x 15) and the solution was evaporated to dryness to give the degraded gum acid (0·54 g.).

A sample of the periodate oxidised and reduced gum acid was hydrolysed with 2N-sulphuric acid at 100° for 16 hours. Chromatography of the hydrolysate in solvent systems A and B using spray reagents 1 and 2 showed the presence of galacturonic acid, galactose, rhamnose, glycerol and threitol or erythritol.

Another sample of the degraded gum acid was hydrolysed with N sulphuric acid for five hours. Chromatography of the hydrolysate in solvent systems A and B using spray reagents 1 and 2 showed the presence of galacturonic acid, galactose, rhamnose, galacturonosylrhamnose, glycerol and threitol or erythritol.

The rest of the degraded gum acid (0·48 g.) was dissolved in N sulphuric acid (20 ml.) and the solution was allowed to stand at room temperature for two hours. The acidic solution was repeatedly treated with dimethyl octyl amine in chloroform (10% v/v). The aqueous layer was reduced in volume and treated with Amberlite resin IR-120 (H). The solution was poured into ethanol (1:4). The precipitate was removed at the

centrifuge and was repeatedly washed with ethanol (5 x 10).

The supernatant liquid and the washings were combined and concentrated to give a syrup (0·21 g.) containing low molecular weight material.

Chromatography of this material in solvent systems A and B using spray reagents 1 and 2 showed the presence of glycerol, a tetritol (presumably threitol) and a slow moving sugar ($R_{Gal\ A}$ 0·14), but no reducing sugars.

A sample of the low molecular weight material was hydrolysed with N sulphuric acid for ten hours. Chromatography of the hydrolysate in solvent systems A and B using spray reagents 1 and 2 indicated the presence of galacturonic acid, rhamnose and a trace of galactose in addition to the above sugar alcohols.

A sample of the precipitated polysaccharide (degraded and hydrolysed) was hydrolysed with 2N sulphuric acid for eight hours and chromatography of the hydrolysate in solvent systems A and B using spray reagents 1 and 2 showed the presence of galacturonic acid, rhamnose and galactose.

Another sample of the degraded gum was hydrolysed for five hours with N sulphuric acid and the products were examined by chromatography in solvent systems A and B using spray reagents 1 and 2. The presence of galacturonic acid, galactose, rhamnose, galacturonosylrhamnose and the absence of sugar alcohols was indicated.

Degraded gum acid (15 mg.) was hydrolysed with N sulphuric acid for five hours and the products were converted to methyl

ester methyl glycosides. The methyl ester methylglycosides in aqueous solution were treated with potassium borohydride (15 mg.). The product was hydrolysed with N sulphuric acid for four hours and the hydrolysate was examined by chromatography in solvent systems A and B using spray reagents 1 and 2. The presence of galactose and rhamnose was observed.

Large scale Smith degradation.

The gum acid (9·3 g.) was dissolved in water (500 ml.), sodium metaperiodate (15·96 g.) was added and the solution was diluted to 750 ml. The solution was kept in the dark at room temperature. The periodate uptake was followed spectrophotometrically and no change in periodate concentration was observed after twenty hours. The reaction was stopped after thirty hours by the addition of ethylene glycol (25 ml.) and the solution was dialysed against tap water for four days.

The dialysate was reduced in volume, potassium borohydride (2·5 g.) was added to the dialysate and the mixture allowed to stand for a day. Another addition of potassium borohydride (1 g.) was made and the solution was allowed to stand overnight at room temperature. The solution was treated with Amberlite resin IR-120 (H) and the resulting solution was concentrated to a syrup. The syrup was repeatedly dissolved in methanol (5 x 30) and evaporated to a small volume. The solution was treated with Amberlite resins IR-120 (H) and IR 4B (OH) and the polyalcohol was precipitated by the addition of ethanol (5 volumes).

The polyalcohol (4·8 g.) had $[\alpha]_D$ +63° (c, 0·5 in water) and uronic acid anhydride 16·8% (by the decarboxylation method) and 18·4% (by the carbazole colorimetric method).

The polyalcohol (4·7 g.) was dissolved in N sulphuric acid (100 ml.) and allowed to stand at room temperature for two hours. The hydrolysate was repeatedly extracted with Dimethyl Octylamine (10% in chloroform v/v). Finally the aqueous layer was treated with Amberlite resin IR-120 (H) and the resulting solution was reduced in volume. Ethanol (5 volumes) was added to the solution and the resulting precipitate was removed at the centrifuge. The precipitate was repeatedly washed with ethanol (5 x 20), finally with ether and then dried in a vacuum oven at 35-40° to give the Smith degraded polymer (0·81 g.).

The supernatant liquid and the washings were combined, reduced in volume and the resulting syrup (2·8 g.) was examined chromatographically. The presence of components similar to that obtained in trial experiment was observed.

Partial hydrolysis of Smith degraded polymer.

Trial experiment.

A sample (40 mg.) of the Smith degraded polymer was dissolved in N sulphuric acid (10 ml.) and the solution was heated at 100° for six hours. Aliquot samples (1 ml.) were removed every hour, neutralised with saturated solution of barium hydroxide and solid barium carbonate. The solutions were filtered and the filtrates were deionised with Amberlite resin IR-120 (H) and evaporated to dryness. The syrups were examined

chromatographically in solvent system A using spray reagent 1. Visual inspection of the developed chromatograms suggested that the best yield of the acidic oligosaccharides could be obtained by hydrolysing the polymer at 100° for four to five hours.

Large scale hydrolysis.

The Smith degraded gum acid (0·65 g.) was dissolved in N sulphuric acid (60 ml.) and the solution was heated at 100° for four and a half hours. The solution was cooled and neutralised to pH 6 with saturated barium hydroxide solution and neutralisation was completed by the addition of solid barium carbonate, The solution was filtered, the filtrate was deionised with Amberlite resin IR-120 (H) and evaporated to dryness. The syrup (0·38 g.) was examined chromatographically in solvent system A using spray reagent 1. The presence of four sugars with $R_{Gal\ A}$ 1, 0·78, 0·18 and 3·8 (rhamnose) was indicated.

Separation of the sugars.

The sugars were placed on an Amberlite resin CG 45 column (30 x 1·5 cm.) in the formate form. The column was washed with water until free from neutral sugars. The eluate was evaporated to dryness and the syrup (105 mg.) was examined chromatographically in solvent systems A and B. The presence of rhamnose together with traces of galactose and galacturonic acid was indicated.

The acidic sugars which remained on the column were eluted

with 2N formic acid (500 ml.). The eluate was reduced in volume (10-15 ml.) and examined chromatographically in solvent system A. The presence of three sugars with $R_{Gal\ A}$ 1·0, 0·78 and 0·18 was indicated. The syrup (340 mg.) was separated into three fractions on Whatman 3MM paper using solvent system A.

Fraction 1. D-Galacturonic acid.

This fraction (0·058 g.) $R_{Gal\ A}$ 1·0 was chromatographically pure and indistinguishable from galacturonic acid. The sugar was converted into the methyl ester methylglycoside and the product was reduced with potassium borohydride. Hydrolysis of the reduced glycoside and subsequent chromatography of the hydrolysate in solvent B showed the presence of galactose.

Fraction 2. 2-O-(α-D-galactopyranosyluronic acid)-L-rhamnose.

This fraction (0·14 g.) had $R_{Gal\ A}$ 0·78, $[\alpha]_D$ +90° (c, 2·80) and was chromatographically pure.

A sample of the sugar was hydrolysed with N sulphuric acid for four hours and the hydrolysate was examined chromatographically in solvent systems A and B. The presence of galacturonic acid and rhamnose was indicated.

Another sample of the sugar (5 mg.) was methanolysed for four hours and the product was reduced with potassium borohydride. Hydrolysis of the reduced sugar and subsequent chromatography of the hydrolysate in solvent system B showed the presence of galactose and rhamnose.

A sample of the sugar (5 mg.) was dissolved in water (2 ml.)

and potassium borohydride (5 mg.) was added. The reduced sugar was hydrolysed with N sulphuric for four hours at 100° and the hydrolysate was examined chromatographically in solvent system K using spray reagent 3. The presence of rhamnitol, galacturonic acid and the absence of rhamnose was indicated.

Methylation of fraction 2.

The syrup (80 mg.) was dissolved in water (2 ml.) and sodium hydroxide (10 ml., 30%) and dimethyl sulphate were added dropwise over a period of four hours. The reaction flask was kept in ice and the atmosphere of nitrogen was maintained during the methylation. Three more additions of the reagents were made during the next three days. The methylated acid was isolated from the reaction mixture as described in the methylation of fraction 2 (2-O-(α-D-galactopyranosyluronic acid)-L-rhamnose) of partial hydrolysis of the gum acid.

The methylated acid (0·036 g.) had $[\alpha]_D$ +92° (c, 0·72 in chloroform). The sugar crystallised and was recrystallised from light petroleum (b.p. 80-100°). The recrystallised sugar had m.p. 67-68° and mixed m.p. 66-67° (with authentic sample of 2-O-(α-D-galactopyranosyluronic acid)-L-rhamnoside pentamethyl ether dihydrate.

An X-ray powder photograph of the methylated acid was recorded which was identical to that of authentic sample.

Fraction 3.

This fraction (0·059 g.) had $R_{Gal\ A}$ 0·18 and was

chromatographically pure. A sample of the sugar was hydrolysed with N sulphuric acid for six hours and examined chromatographically in solvent systems A and B. The presence of galacturonic acid and rhamnose was indicated.

Another sample of the sugar (5 mg.) was converted into the methyl ester methylglycosides and the product was reduced with potassium borohydride. The reduced sugar was hydrolysed with N sulphuric acid at 100° for four hours. Chromatography of the hydrolysate in solvent system B showed the presence of galactose and rhamnose.

The sugar (5 mg.) was dissolved in water (1 ml.) and potassium borohydride (5 mg.) added. The reduced sugar was hydrolysed with N sulphuric acid for four hours and the hydrolysate was examined chromatographically in solvent systems A and K using spray reagents 1 and 3. The presence of galacturonic acid, rhamnitol and the absence of rhamnose was indicated.

Another sample (5 mg.) of the sugar was partially hydrolysed with 0·1N sulphuric acid for four hours. Chromatography of the hydrolysate in solvent systems A and B showed the presence of galacturonic acid, galacturonosylrhamnose and rhamnose.

Methylation of fraction 3.

The acid (0·035 g.) was methylated by three additions of Haworth reagents and an addition of Purdie reagents. The methylated acid was reduced with lithium aluminium hydride in tetrahydrofuran to give neutral methylated sugar (0·018 g.).

A sample of the reduced acid was hydrolysed with N sulphuric acid for six hours and the hydrolysate was examined chromatographically in solvent system G. The presence of 3,4-di-O-methylrhamnose, 2,3,4-tri-O-methylgalactose and a sugar with R_G 0·45 was indicated.

Another sample of the methylated reduced acid was methanolysed and the products were examined by gas-liquid partition chromatography. The methylglycosides of the following sugars were indicated.

Methylglycosides of:	T in system b.
3,4-Di-O-methyl-L-rhamnose	0·60
2,3,4-Tri-O-methyl-D-galactose	2·91
Unidentified sugar.	4·16

Reduction of the Gum Acid.

Preparation of the gum acetate.

The gum acid (0.5 g.) was dried in a vacuum oven at 35-40° and was dispersed in formamide (20 ml.). The dispersion was stirred overnight at 25-30°, when a clear solution was obtained. Pyridine (20 ml.) was added to the gum solution in three batches, each over a period of half an hour. Vigorous stirring was maintained throughout the addition of pyridine. The reaction mixture was cooled and acetic anhydride (15·0 ml.) was added dropwise over a period of three hours. The mixture was allowed to stir overnight. A further batch of acetic anhydride (4 ml.) was added and the mixture stirred for 0·5 hr. The mixture was poured in ice and water (150 ml.) containing concentrated hydrochloric acid (2 ml.). The mixture was allowed to stir for an hour and the precipitated gum acetate was allowed to stand at room temperature for two hours. The gum acetate was washed with 0·2% hydrochloric acid followed by four water washings. The precipitate was suspended in water, allowed to stand overnight and dried in a vacuum oven at 35-40° to give the gum acetate (0·71 g.).

Trial reduction of the gum acetate.

The gum acetate (0·65 g.) was dissolved in diglyme (10 ml.) and sodium borohydride (0·25 g.) in diglyme (5 ml.) was added with stirring. Boron trifluoride etherate (0·55 g.) in diglyme (5 ml.) was added portionwise to the mixture. After each

addition the flask was stoppered and shaken by hand, gently at first and then vigorously to break the gel formed. After the final addition of the borontrifluoride the flask was stoppered and allowed to stand overnight. The mixture was poured into ice and water (150 ml.), neutralised with dilute sodium hydroxide solution and chloroform (35 ml.) was added. The resulting emulsion was evaporated to dryness, the residue was dissolved in 0·25N sodium hydroxide solution and the resulting solution was adjusted to pH 10. The alkaline solution was warmed at 60-70° for 0·5 hr., cooled and dialysed against running water for three days.

The dialysate was filtered, reduced in volume, deionised with Amberlite resins IR-4B (OH) and IR-120 (H). The syrup was poured into stirred mixture of ethanol and ether (75:25). The precipitated gum was removed at the centrifuge, repeatedly washed with ether and dried in a vacuum oven at 35-40°.

The carboxyl reduced gum had uronic acid anhydride 12%, and galactose, glucose, rhamnose and two unidentified sugars as component units.

Large scale acetylation and reduction.

Experiment 1.

The gum acid (10 g.) was dissolved in formamide (250 ml.) and redistilled analar pyridine (150 ml.) was added to the gum solution with stirring. Acetic anhydride (200 ml.) was added dropwise to the mixture over a period of six hours and the

stirring was continued overnight. The gum acetate was precipitated and processed as described in trial experiment.

The gum acetate (14 g.) was dispersed in diglyme (250 ml.) and the dispersion was shaken for a day when a clear solution was obtained. Lithium borohydride (4 g.) in diglyme (50 ml.) was added to the gum acetate solution and the solution was allowed to stand overnight at room temperature. Boron trifluoride etherate (about18 g.; 40 ml.) was added to the mixture in small batches. The mixture was periodically shaken to break the gel formed. After the final addition the flask was allowed to stand overnight at room temperature.

The reduced gum was obtained in a manner similar to that described in trial experiment.

The carboxyl reduced gum (7·3 g.) had uronic acid anhydride content 12·8%.

A sample of the reduced gum was hydrolysed with N-sulphuric at 100° for sixteen hours and the hydrolysate was examined chromatographically in solvent systems A and B. The presence of galacturonic acid, galactose, glucose, rhamnose and two unidentified sugars ($R_{rhamnose}$ 0·98, 1·1) was indicated.

As sufficient quantity of uronic acid was found in the reduced gum, it was considered necessary to reduce further the partially reduced gum.

The partially reduced gum was again acetylated and reduced using the procedure described in experiment 1.

The product (1·8 g.) obtained had uronic acid anhydride,

7·6% and galactose, glucose, rhamnose, trace of galacturonic and two unidentified sugars as component units.

Experiment 2.

The gum acetate (14 g.) was prepared by the procedure described under experiment 1.

The gum acetate (14 g.) was dissolved in 1,2-dimethoxyethane (250 ml.) which had been dried over sodium and distilled over lithium aluminium hydride. Lithium borohydride (10 g.) dispersed in 1,2-dimethoxyethane (50 ml.) and boron trifluoride (about 42 g.) in 1,2-dimethoxyethane (50 ml.) were simultaneously added to the gum acetate solution in small portions. After each addition the flask was stoppered, shaken by hand, at first gently and then vigorously. The resulting mixture after three additions solidified. Hence, more 1,2-dimethoxyethane (250 ml.) was added. After the final addition the flask was stoppered and allowed to stand overnight. The reduced gum was processed and dried as described in experiment 1. The reduced gum (7·8 g.) had uronic acid anhydride content of 11·4%.

The partially reduced gum was again acetylated and reduced as described above. The twice reduced gum had uronic acid anhydride content 6·4% and galactose, glucose, rhamnose and two unidentified sugars as component units.

Experiment 3.

The gum acid (15 g.) was acetylated as in experiment 1 to give 21 grams of the gum acetate. The gum acetate (21 g.)

was dissolved in 1,2-dimethoxyethane (400 ml.). Lithium borohydride (20 g.) in 1,2-dimethoxyethane (50 ml.) was added to gum acetate solution over a period of five hours. Boron trifluoride etherate (85 g.) in 1,2-dimethoxyethane (50 ml.) was added in small proportions to the lithium borohydride-gum solution. After the final addition the mixture was allowed to stand for six days. The reduced gum was obtained as described in experiment 1.

The reduced gum (4·8) had uronic acid anhydride content 7% and OEt., 2·8%.

Hydrolysis of the partially reduced gum with N-sulphuric acid and subsequent chromatography of the hydrolysate showed the presence of glucose, galactose, rhamnose, trace of galacturonic acid and two unidentified sugars.

Identification of unknown sugars.

Partially reduced gum (0·430 g.) was hydrolysed with N sulphuric acid for sixteen hours. The hydrolysate was neutralised with barium hydroxide and barium carbonate, filtered and the solution was deionised with Amberlite resin IR-120. The ions free solution was reduced in volume and the syrup was examined chromatographically in solvent systems A and B. The presence of galactose, glucose, rhamnose, trace of galacturonic acid and two unknown sugars was indicated. The unknown sugars which were found to have chromatographic mobility similar to rhamnose were separated from glucose and galactose on Whatman 3MM

paper in solvent system B.

The unknown sugars and rhamnose were further separated into three fractions by ionophoresis in borate buffer at pH 10.

Fraction 1. L-Rhamnose.

The sugar was chromatographically and ionophoretically pure and had the chromatographic mobility of rhamnose.

Fraction 2.

The sugar ($R_{rhamnose}$ 1·05) gave a pink stain with spray reagent 1. Demethylation of the sugar gave glucose. The sugar was examined by infra-red Zeisel method for the qualitative estimation of alkoxyl groups. The presence of ethoxyl group and absence of methoxyl group was indicated.

Fraction 3.

The sugar ($R_{rhamnose}$ 0·95) was chromatographically and ionophoretically pure and gave a pink stain with spray reagent 1. Demethylation of the sugar gave galactose. Qualitative examination of the sugar by infra-red Zeisel method showed the presence of ethoxyl group and the absence of methoxyl group.

The gum acid was reduced in order to facilitate Smith degradation studies as the periodate oxidised uronic acid residues, after reduction with potassium borohydride, are not easily cleaved on mild acid hydrolysis (80).

Since considerable quantities of the ethyl ethers of the sugars were found after reduction with diborane, Smith degradation studies were not pursued, owing to the possibility of the periodate resistance of these artefacts.

It is, however, not known how the ethyl ether is formed during the reduction, but probably it arises from the reduction of the acetyl group to the ethyl group (114,115). In order to overcome this difficulty, the diborane gas was produced in a separate reaction vessel and then transferred to the reaction mixture. No artifacts were observed but only a small proportion of uronic acid residues was reduced (116).

Examination of a second batch of Sterculia urens gum.

Another sample of the gum (Sterculia urens) was obtained from United Chemical and Allies Products, Calcutta, India. The gum was purified by dissolution in aqueous ammonia and precipitated with ethanol acidified with concentrated hydrochloric acid. The purified gum was found to have $[\alpha]_D$ +62° (c, 0·34 in 1% sodium hydroxide solution), uronic acid anhydride content 37% and galacturonic acid, glucuronic acid, galactose and a trace of arabinose as component sugars.

The gum was examined for heterogeneity by chromatography on DEAE-cellulose, ionophoresis on glass fibre paper and fractional precipitation. The procedures followed for these experiments are described under section I. The results obtained from the above examinations indicated that the polymer is homogeneous.

The gum was methylated by treatment with Haworth and Purdie reagents (details of methylation are given in the methylation of the gum in section I).

The fully methylated gum was methanolysed and the products were examined by gas-liquid partition chromatography. The presence of methyl estersmethyl glycosides of the following sugars was indicated.

Methyl glycosides of:	T in system "b"
2,3,4-Tri-O-methylrhamnose	1·47
3,4-Di-O-methylrhamnose	0·60-0·61
3-O-Methylrhamnose	1·03
2,3,4,6-Tetra-O-methylgalactose	(1·61), 1·52
2,3,6-Tri-O-methylgalactose	(1·26), 1·61, (2·25), 2·55
2,3,4-Tri-O-methylglucuronic acid	1·78 (2·25)
Unidentified	1·26

The second batch of the gum in general resembles the first batch, however, there are indications (obtained from methylation studies) that the two samples may have minor differences.

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