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THE MOLECULAR STRUCTURE OF PLANT GUMS,
WITH SPECIAL REFERENCE TO GUMS PRODUCED
BY TREES OF THE GENUS KHAYA

by

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A thesis presented for the degree of Doctor of Philosophy.

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TO MY PARENTS AND MAROLYN

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

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General

Many different chemical substances occurring in nature have been termed gums. The word gum has been applied to terpenoid resins, rubber, water-soluble hemicelluloses, seed extracts, seaweed polysaccharides and the polysaccharide exudates from plants. In this thesis, when the word gum is used, the reference will be to the last of the types mentioned. Gums may then be defined as: "Those polysaccharides which are obtained as exudates from plants either spontaneously or after some kind of external influence."

Little definite proof is as yet to hand, for the formation and origin of plant gums. Most theories indicate, however, that they are enzymatically synthesised and not produced by a direct chemical polymerisation process. The gums could be the product of normal plant metabolism or they could arise from pathological conditions, and there is evidence to support both these views. In support of the first suggestion, it has been observed (1) that the chemical structures of gums are to some extent related to the botanical origin of the tree from which they are exuded. Gums with chemical structures having strikingly

similar general features were found to be yielded by different trees of the same type. If the second theory were correct, however, one might expect different bacteria to produce different gums on trees of the same species; and since this is not so, the first suggestion has the most support (2). The suggestions that gums could arise from pathological conditions is supported by experiments carried out by innoculating bacteria into trees. Thus it has been observed that the parasite Stereum purpureum on plum trees causes the formation of considerable amounts of gum at the actual site where the parasite grows (3). It has also been noted that healthy Acacia trees do not produce any gums, whereas ones growing in undesirable conditions such as high elevation and drought yield gums in great quantities. This, however, could be explained in that under such conditions the bark tends to crack, resulting in gum formation.

It is not possible at the moment to give any definite answer to this problem and it is more than likely that there are several reasons for the production of gums by plants. It seems probable that the gums are synthesised by the trees themselves but their formation is stimulated by mechanical injury or attack by micro-organisms. Thus they serve the purpose of preventing loss of moisture and in addition prevent further injury by forming a protective shield at a vulnerable place.

The plants, after injury, exude a sticky, viscous substance which slowly hardens to give a hard and brittle nodule. These are composed almost entirely of carbohydrate material with traces of terpenoid resins (which give the nodules their varying colours of yellow, brown and black), dust and pieces of bark. Recently certain detailed analyses, such as uronic anhydride and acetyl contents, have been carried out on different nodules of the same gum: slight variations, greater than can be explained by analytical error, have been shown to exist (4, 5). Some of the polysaccharides in gum exudates are partly acetylated and these smell faintly of acetic acid due to decomposition. The majority of gums dissolve in water to give highly viscous solutions and others swell to give gels. The purified polysaccharide material, generally known as the 'gum acid' can be precipitated from aqueous solution by addition of organic solvents such as ethanol. Almost all gums can be dissolved in dilute alkali with concomitant deacetylation. Molecular weights of plant gums vary greatly but are generally very high, as high as 9,500,000 has been quoted for Karaya gum (6). The usual means of measurement is by sedimentation techniques, but not many molecular weight determinations have been carried out.

Plant gums may consist of one or more polysaccharides and these are heteropolysaccharides since each molecule

comprises two to four neutral sugars and one or two uronic acid residues. The neutral sugars which so far have been detected are D-galactose, D-xylose, L-arabinose, L-rhamnose, D-mannose and L-fucose. The uronic acids in gums are D-glucuronic acid, 4-O-methyl-D-glucuronic acid and D-galacturonic acid. Of course, each of these sugars can occur in different types of linkage within the one polysaccharide. A typical gum possesses a highly branched structure, resembling closely the polysaccharides obtained from the mucilages and the bacterial polysaccharides. In fact, there are no general structural differences between the gums and the mucilages and they have usually been reviewed together (2, 7-12). The only distinction lies in their mode of origin, the one resulting from exudation after injury and the other being found in the root, bark, cortex, leaves, stalks, flowers, endosperm and seed coating. Mucilages are normal products of metabolism and they are used as water reservoirs and food reserves.

The collection and distribution of plant gums is an important branch of commerce, for example the yearly sales of gum from Acacia trees (gum arabic) exceeds 20 million kg. Gums have been used since civilisation began. Egyptians used them for paint thickening and embalming purposes and today these materials serve a wide variety of purposes, including sizing, the printing and finishing of textiles, paper-making, the preparation of water colours,

pharmaceutical preparations and in the confectionery trade.

The study of these gums "leads us directly to some of the fundamental problems in 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 pentose sugars." (2).

Purification and Fractionation.

Before starting the investigation of a gum, an enquiry should be made as to its origin. Wherever possible, samples should be used which have been collected by a reliable botanical or chemical expert in preference to gums from commercial sources.

The polysaccharides in the crude gum usually exist in the form of the neutral or slightly acid salts of cations such as calcium, magnesium, sodium and potassium; in addition a few of the hydroxyl groups may be acetylated. Purification is carried out by dissolving the gum in water, followed by precipitation with an organic solvent. Generally acidified ethanol is used but methanol and acetic acid (13) have been also used. If the gum is not, or only slightly soluble in water, alkali can be used to effect dissolution, with concomitant removal of any acetyl groups which may be present. Great care should be taken in controlling the reaction conditions, however, since alkali also tends to degrade the polysaccharide. This occurs mainly at the reducing end (14) but it has also been reported (15,16) that cleavage of the chain can be achieved with alkali at the non-reducing end of a uronic acid residue, if the latter is present as the methyl ester. The purity of carbohydrate material obtained is assessed by measurement of nitrogen and ash content.

The next stage in the structural study of a polysaccharide is examination for heterogeneity. This is usually done by the simple method of fractional precipitation, which involves the addition of a precipitant to an aqueous solution of the gum and the subsequent isolation of components of different solubilities. Co-precipitation of two fractions often occurs and so several reprecipitations may be necessary to obtain pure fractions. The polysaccharides from Khaya senegalensis gum were fractionated in this way (17), by preferentially precipitating the less soluble major fraction, a polysaccharide of high uronic acid content similar to the polysaccharide from Khaya grandifolia gum (18). The minor fraction, a low uronic acid containing polysaccharide having many similar structural features to gum arabic was isolated from the supernatant liquors as the calcium salt. Olibanum gum (19) was also separated into two different polysaccharides by means of fractional precipitation.

Another method now available for separating polysaccharide mixtures makes use of the fact that some polysaccharides form insoluble salts or complexes with certain reagents. The commonest of the complexing agents are cupric acetate (20), Fehling's solution and long chain quaternary ammonium salts, such as cetyl trimethylammonium bromide (Cetavlon). The latter give precipitates with many acidic polysaccharides (21,22) and may be used

to separate them (23). In this laboratory (24) fractionation of Anogeissus schimperi gum into two polysaccharides differing considerably in their uronic acid anhydride content, has been effected by means of complex formation with Cetavlon.

By means of preferential solubility, one of the polysaccharides from gum tragacanth has been obtained in a homogeneous state (25). The arabinogalactan was separated from the acidic polysaccharide by dissolving it in 70% ethanol. It is feasible that more use might be made of this technique in the future.

The fractions obtained by fractional precipitation and complex formation can then be examined for homogeneity by means of chromatography, gel filtration or electrophoresis. The first two of these techniques have also been used on a preparative scale.

The separation of polysaccharide mixtures by chromatography has recently taken great steps forward with the introduction of ion exchange celluloses such as diethyl amino ethyl (DEAE)-cellulose (26) and ECTEOLA-cellulose (27). Anion exchange cellulose, in different forms (phosphate, borate etc.) retains acidic polysaccharides at neutral pH values, whereas neutral polysaccharides are not or only weakly absorbed. Thus by using a suitable elution medium at differing pH values and electrolytic concentrations, various polysaccharide

mixtures have been fractionated. The homogeneity of the polysaccharide fractions obtained from Khaya senegalensis gum has been assessed in this way.

Separations of polysaccharide mixtures can be effected by filtration through a bed of packed grains of a cross-linked polysaccharide, known as Sephadex (28). This technique, known as 'gel filtration' should prove to be a useful tool in assessing polymer homogeneity, in the field of plant gums. A clear-cut separation of some acid mucopolysaccharides has been obtained by ion-exchange chromatography on DEAE-Sephadex (29).

The main advantage of electrophoresis (ionophoresis) in assessing polymer homogeneity is its speed and also the fact that very little material is required. Smith and Lewis (30) have examined several polysaccharides by electrophoresis on glass fibre paper in 2N-sodium hydroxide. Several of these preparations were claimed to be heterogeneous, but it is possible that some apparent heterogeneity may have resulted from degradation under the strongly alkaline conditions used. When possible, electrolytes which are unlikely to degrade polysaccharides, should be used.

Examples of the various kinds of heterogeneity which occur in gums can now be discussed and shown to range from the kind in which the constituent polysaccharides have slight differences in proportions of certain residues to

the kind of heterogeneity in which the polysaccharides are of a completely different nature.

a) It has been reported (31) that many hemicelluloses may be considered to be members of a family of closely-related polysaccharides possessing a backbone of constant structure, to which side chains of varying constitution are attached. This type of micro-heterogeneity has been found amongst the polysaccharide components of Combretum leonense gum (32). The gum was separated into fractions which differed in uronic acid content, and probably also in the relative proportions of neutral sugar residues. Structural investigations on these polysaccharide fractions, however, failed to reveal any major differences in the nature of the sugar units or of the types of linkage by which they are joined. The presence in Combretum leonense gum of closely-related polysaccharides, which differ slightly only in the relative proportions of the various structural units, probably accounts for the inter-nodular differences (4), already mentioned. The heterogeneity in samples of gum arabic, which was demonstrated by Heidelberger (33) is probably also of this type.

b) In recent work (24) on Anogeissus schimperi gum, two polysaccharides, differing considerably in uronic acid anhydride content, have been obtained. Although the two fractions have not yet been compared in great structural detail, there are indications of common structural features

in the two polysaccharides and it would seem that this type of heterogeneity is intermediate between the kind of micro-heterogeneity occurring in Combretum leonense gum and the gross heterogeneity found in Khaya senegalensis gum and gum tragacanth.

As has been mentioned previously, two polysaccharide fractions have been isolated from deacetylated Khaya senegalensis gum (17). Structural investigations have shown that these two polysaccharides are of entirely different types and that the gum is grossly heterogeneous. Similarly, fractionation of gum tragacanth furnishes two structurally unrelated polysaccharides, a highly acidic polysaccharide, tragacanthic acid, and an arabinogalactan which contains only a very small proportion of acidic sugar units. Two completely different polysaccharides have also been isolated from Olibanum gum (19).

Structural Investigations of Gums

In investigating the structure of a polysaccharide, there are available several long established, but still invaluable, methods of attack which coupled with the latest techniques of chromatography etc., can give us an insight into the chemical layout of the polymeric molecule. The first step is to determine what the monosaccharide residues contained in the polysaccharide are, and this is achieved by a total acid hydrolysis. Information is then obtained as to the mode of linkage of each sugar unit, the proportion of non-reducing end-groups and in some cases the ring structure of the sugars. This is achieved by the methylation procedure. Partial hydrolysis is used to determine the sequence of the monosaccharide constituents, by the isolation of the oligosaccharides formed during the breakdown of the polysaccharide molecule. Oxidation of the polysaccharide with periodate yields a polysaccharide of lower molecular weight which, on investigation, will give us some information as to the structure of the original polysaccharide.

After total acid hydrolysis of the polysaccharide, the monosaccharides are separated by column chromatography (34), characterised and their relative proportions determined. The latter determination can be carried out

rapidly on a micro scale by paper chromatography, using the method of Flood, Hirst and Jones (35) in conjunction with some micro-analytical technique for estimating sugars such as the anthrone (36) or phenol-sulphuric acid (37) methods. Errors may arise due to incomplete breakdown of the polymer and degradation of the monosaccharide units liberated: so care must be taken in choosing conditions which will reduce these errors to a minimum.

METHYLATION STUDIES

The method of structural investigation, most useful generally, is still probably the classical method of methylation, followed by hydrolysis and identification of the hydrolysis products. The methoxyl group was chosen as the ether group to be introduced because of its relatively small bulk thus reducing the effects of steric hindrance to a minimum. The etherifying of hydroxyl groups in sugar residues occurs quite readily and the methoxyl groups when in position are chemically very stable.

The principle underlying the method of methylation, is that after complete methylation and subsequent hydrolysis of the methylated polysaccharide, only those hydroxyl groups involved in glycosidic linkages will remain free. Thus by identifying the various methylated sugars obtained we can get an understanding of their modes

of linkage and whether they are derived from the end group of a chain, a member of a straight chain or a branch point.

The methylation of the polysaccharide is usually carried out by Haworth's procedure using dimethyl sulphate and aqueous alkali (38). The partly methylated material obtained is methylated to completion using Purdie's reagents, silver oxide and methyl iodide (39).

Kuhn has attempted to improve on the Haworth and Purdie methylations by carrying out the reaction in non-aqueous solvents. The sugar is dissolved in dimethyl sulphoxide and dimethyl formamide and methylation effected with methyl iodide, barium oxide and barium hydroxide (40,41). Recently, using dimethyl sulphate as methylating agent (42), he has managed to methylate mono- and oligo-saccharides to completion in one step.

Fully methylated polysaccharides cannot be directly hydrolysed since they are insoluble in hot organic acids. This difficulty may be overcome by preliminary treatment with cold acid, in order to lower the chain length (43) or by boiling with a methanolic solution of hydrogen chloride (44): in both cases, hydrolysis is afterwards completed by treatment with hot dilute mineral acid. Another method involves the use of hot 90% formic acid (45) after which the polysaccharide is boiled with dilute sulphuric acid for a short time to hydrolyse the formyl esters.

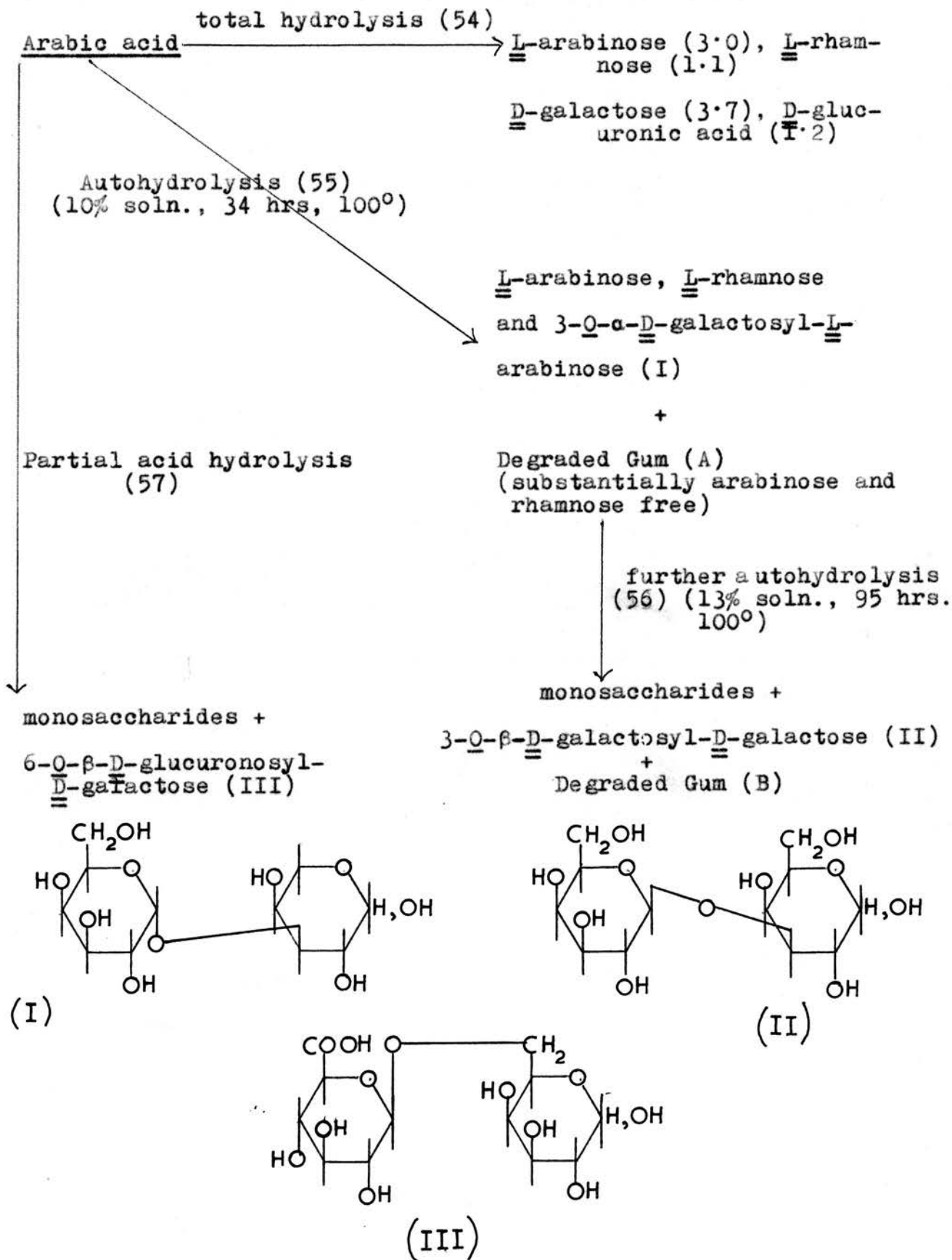
When a methylated acidic polysaccharide is being investigated, the methylated acidic sugars can be separated from the methylated neutral sugars by absorption on ion exchange resins (46). The products of hydrolysis can be separated by chromatography on cellulose columns (47) supplemented by charcoal celite columns (48).

A preliminary indication as to the nature of the individual methylated sugar can be obtained by its chromatographic mobility (49), periodate oxidation (50) and demethylation (51). Gas liquid partition chromatography for the separation of methylated and partly methylated methyl glycosides was first reported by Bishop (52). This provides a highly selective method for the analysis of individual methylated sugars and of the cleavage products from methylated oligo- and polysaccharides. The relative retention times of a large number of such methyl glycosides are now available (53). The methylated sugars are finally completely characterised by the formation of crystalline derivatives.

Linkage Analysis

The problem of determining the sequence of sugar units in a polysaccharide is an extremely complex one. Partial hydrolysis is by far the most useful for this purpose and probably the only unambiguous method available. This involves the characterisation of di- and oligosaccharides

Scheme showing graded hydrolysis of gum arabic



which have resulted from the incomplete breakdown of the polymeric molecule.

The stability of glycosidic links to acids varies considerably according to the class of the sugar, and according to the ring form. Furanosides are in general much more acid-labile than pyranosides; in fact the acidity of the hot aqueous solution of a gum may be sufficient to strip off arabinose end-groups, which usually exist in the furanose form. The process, which is known as auto-hydrolysis, is a very useful one, since the degraded polysaccharide produced has a simpler structure than the original gum, and is more easily identified. The use of weak mineral acids in aqueous solution will generally give reasonable yields of neutral di- and higher oligosaccharides. Aldobiouronic acids may be isolated after hydrolysis of the polysaccharide under more vigorous conditions. An example of the use of partial acid hydrolysis is shown in the accompanying diagram where the degradation of gum arabic is illustrated.

Another very important method of linkage analysis is acetolysis using a mixture of acetic anhydride, acetic acid and sulphuric acid as the hydrolytic agent, the rates of hydrolysis of the different linkages are altered; linkages readily broken by partial acid hydrolysis may be found to remain intact. Using this technique, an oligosaccharide of great structural importance, 2-O-α-L-fucosyl-D-xylose,

was isolated from gum tragacanth (25). This enabled the point of attachment of the L-fucose end groups to the polysaccharide to be assigned.

In comparison with acid hydrolysis, enzymic hydrolysis has the advantage of specificity. When the 1-4 linked unbranched glucan, amylose, is hydrolysed by means of β -amylase the fragmentation occurs in a systematic fashion to yield maltose as the final product. In contrast, acid hydrolysis, cleaves glycosidic linkages at random. In the case of homopolysaccharides such as starch and glycogen, enzymolysis has been used with success. But in the case of heteropolysaccharides which contain a diversity of building units and linkages, the use of enzymes has been restricted.

Recently attempts have been made to increase the stability of certain very acid-labile linkages, by first modifying the polysaccharide before attempting the partial hydrolysis. So far, this has been done in two ways. The method devised by Aspinall (58) involves oxidising these end groups with oxygen in the presence of a platinum catalyst and thus producing a stable glycosiduronic acid linkage. It is then possible to isolate and characterise the aldobiouronic acids, indicating the nature of the original glycosidic linkage. The polysaccharide to be oxidised, must contain only a few primary hydroxyl groups for this method to be of maximum value, e.g. in arabinoxylans

or hexose containing polysaccharides with a large proportion of 1,6 linkages.

The second method of modifying the polysaccharide is to reduce the uronic acid residues to the corresponding alcohols. The linkage at the reducing end of the residue, which was previously a uronic acid residue, then becomes of ordinary stability and can be much more readily cleaved. In this way 4-O-α-L-rhamnosyl-D-glucose was isolated from the products of acetolysis of reduced gum arabic (59).

The structural significance of oligosaccharides present in trace quantities in the hydrolysis products is doubtful, because acid hydrolysis is a reversible reaction and it has been shown that on heating a solution of monosaccharides with acid, oligosaccharides are formed (60,61). However these can be distinguished since they reach an equilibrium concentration and do not disappear on prolonged heating (62).

Another difficulty encountered in partial acid hydrolysis is the breakdown of the oligosaccharides as the reaction proceeds. This difficulty can be overcome by carrying out the hydrolysis in a dialyses bag and continually removing the low molecular weight material formed (63). This method was first used for autohydrolysis but by using a water-soluble acidic resin as the hydrolytic agent, its scope can be greatly increased (64). An advantage of this method is that it eliminates the

possibility of acid reversion products.

The mixture of oligosaccharides obtained from partial hydrolysis of the polysaccharide can be separated on charcoal-celite columns (65). Aldobiouronic acids can be successfully separated from weakly basic anion exchange resin columns (66).

Several oligosaccharides and their derivatives are themselves crystalline and so can be identified directly. In the majority of cases, however, to completely characterise the unknown oligosaccharide, much more work is involved. An indication as to the identity of the oligosaccharide can be obtained from its hydrolysis products, its chromatographic mobility in various solvents, ionophoretic behaviour and optical rotation. The mode (S) of linkage is established by methylation, followed by hydrolysis and characterisation of the methylated derivatives. Identification of the oligosaccharide can be carried out on a micro-scale, by complete methylation and examination of the methanolysis products by gas/liquid partition chromatography (53). Another useful tool in ascertaining the nature of an oligosaccharide is periodate oxidation (67).

Measurement of the optical rotation will give an indication as to the anomeric configuration of the disaccharide. By degrading the oligosaccharide to the glyceritol glycosides with lead tetra-acetate and comparing with authentic specimens, Perlin (68) has been able to assign the definite anomeric configuration of disaccharides.

PERIODATE OXIDATION

Periodic acid and its salts are capable of cleaving the carbon-carbon bonds in 1,2-diol and 1,2,3-triol groups.

The scission of each linkage requires one molecule of the oxidant and the reaction seems to occur via an intermediate complex in which the two hydroxyl groups are held in a cis position. Thus it has been observed that in a polysaccharide, certain sugar residues might not undergo cleavage by periodate, if the stereochemical arrangement of the polysaccharide molecule forces them to assume a rigid conformation in which the adjacent hydroxyl groups take up a fixed trans position with respect to each other (69). Generally, however, all α - β glycols are cleaved. In most instances the arrangement of diol or triol groups may be deduced from measurements of the consumption of oxidant and determination of the nature of oxidation products (formaldehyde, formic acid, carbon dioxide).

The periodate oxidation is influenced by temperature, pH, concentration of reactants and light. It is possible to utilise abnormal conditions to achieve controlled over-oxidation (70) but in general the reaction conditions are carefully regulated to avoid this excess oxidation.

In the application of periodate oxidation to polysaccharides, advantage is taken of the fact that some of the sugar units are not susceptible to attack by the

reagent since they do not possess adjacent hydroxyl groups; the unoxidisable residues of each type of monosaccharide can be characterised and their relative proportions determined (71). This method has been improved on, by selectively removing the fragments of cleaved residues, thus leaving the unoxidised residues open to further oxidation. So in the case of a complex polysaccharide, it is possible to cause stepwise degradation by a series of oxidations, followed by elimination of the fragments of the cleaved sugars. In the Barry degradation (72), the polyaldehyde is treated with phenylhydrazine and the fragments removed as the phenylosazones. Using this procedure, as shown in the accompanying diagram, gum arabic was found to have a backbone of 1,3 linked galactose units (71).

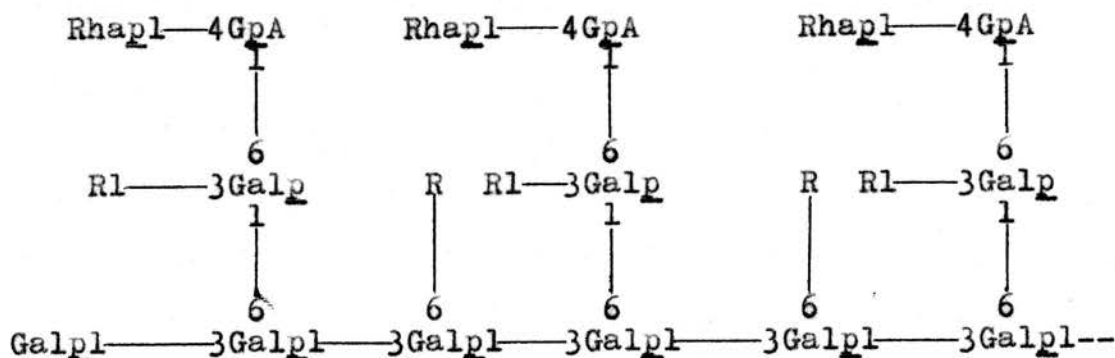
Recently, a modified periodate degradative procedure has been devised, known as the Smith degradation and based on the mild acid hydrolysis of the polyalcohol obtained after borohydride reduction (73). The oxidised polysaccharide is converted to the polyalcohol since the polyaldehydes yield interfering products on hydrolysis (74). The conditions of hydrolysis are such that true acetal systems are cleaved whereas glycosidic bonds are stable. The low molecular weight alcohols and glycosides may then be examined and the precipitated polysaccharide can be treated again with periodate or subjected to partial hydrolysis, methylation or other means of structural

investigation. By carrying out a Smith degradation on degraded gum arabic, a linear 1,3 linked galactan was obtained (75) similar to the one isolated by Dillon from the Barry degradation of arabic acid.

Difficulties are encountered when applying the Smith degradation to acidic polysaccharides since the oxidised fragments which contain uronic acid groups are less readily removed by mild acid hydrolysis than the other acetal systems. So it is preferable to reduce the uronic acid residues to the corresponding neutral sugar residues before commencing with the oxidation. There are several methods available for the reduction of these uronic acid residues but best results have been obtained by reducing the fully acetylated polysaccharide with diborane (76).

Barry Degradation of Gum Arabic (71).

R = Araf-- or Arapl-3Araf-- or Galpl-3Araf-- or Arafl-3Araf
Arabic Acid



↓ (I) IO₄'
↓ (II) phenylhydrazine
Degraded Polysaccharide A [contains 50% less arabinose and 1% less galactose, plus no rhamnose or glucuronic acid].

polysaccharide (78). Several of these bacterial polysaccharides have been subjected to structural investigations and have been found to contain some of the groupings known to be present in gum exudates. It has in fact been shown (79) that many gums give co-precipitation reactions with antipneumococcus sera. As this field of knowledge is expanded, it will become increasingly easy to predict structural features of a polysaccharide of unknown constitution, if it is known with which bacterial antisera it precipitates.

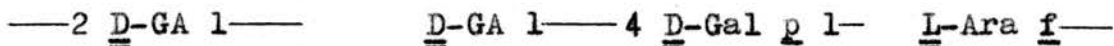
The cross reaction which occurs between certain polysaccharides and pneumococcus antisera to form a complex which precipitates has been used (33) to obtain two fractions from gum arabic, one with a smaller proportion of rhamnose residues than the other. The co-precipitation observed to take place between Cochlospermum gossypium gum and antipneumococcus serum Type II suggested the presence of, among other things, D-glucuronic acid end groups in the polysaccharide (78). This was later confirmed to be present in the gum by Aspinall and Johnston (80).

Object of the Present Investigations

I. Khaya senegalensis gum

The sample of gum used was the same as that investigated by Aspinall, Johnston and Stephen (17). This was shown to be heterogeneous by the results of fractional precipitation which gave two markedly different polysaccharides.

The major fraction (ca. 70% of the crude gum) was examined in detail and found to be a polysaccharide of high uronic acid content having many of the structural features of Khaya grandifolia gum (18). From the results of methylation studies and from a knowledge of the order of linkage of sugar residues in the aldobiouronic acid units, which had been isolated, the following structure was proposed. The back bone (I) of the polysaccharide molecule consists of 1,4 linked D-galacturonic acid and 1,2 linked L-rhamnopyranose residues, side-chains being attached to it through position 4 of the rhamnose residues. D-Galactopyranose residues (II) are present as end groups, as are 4-O-methyl-D-glucuronic acid residues, the latter being linked to position 4 of D-galactose in aldobiouronic acid units (III). The units (II) and (III) are therefore present as side-chains in the polysaccharide and it is probable that they are directly attached to position 4 of L-rhamnopyranose residues in the main chain.



There were also indications of the presence of trace amounts of the residues:-



Since these latter residues are important structural features of the major polysaccharide fraction from the gum, they could have arisen from contaminating major fraction. We intended to examine this problem, after re-fractionating the minor fraction by chromatography to obtain as homogeneous a polysaccharide as possible.

The uronic acid content of this polysaccharide was much lower than that of the major fraction and it was suggested that the structure of the minor component is that of a basic framework of galactose units, the characteristic linking points being carbon atoms 1,3 and 6 with arabinose and uronic acid residues occupying peripheral positions. The object of our research was to make a complete structural investigation of the minor fraction and see if this polysaccharide showed any resemblance to gums from another species.

2) Acacia senegal gum (gum arabic).

It is known that L-rhamnopyranose residues in gum arabic occur mainly, if not exclusively, as non-reducing end-groups (82). Since L-rhamnose and D-glucuronic acid

residues occur in almost equal proportions it has been suggested (83) that the rhamnose is linked to the non-reducing end of the glucuronic acid residue. Methylation studies indicated the presence of 2,3 Me₂ glucuronic acid (82) and it is probable therefore that the rhamnose is linked to position 4 of the glucuronic acid. In addition, alkaline degradation of the gum at 160° results in the loss of both glucuronic acid and rhamnose residues, leaving an arabinogalactan (84). Since this type of reaction appears to result in the selective cleavage of glycosiduronic acid linkages, sugar units attached to glucuronic acid would also be lost, thus supporting the above theory.

A. J. Charlson tackled this problem in this laboratory by first reducing the gum arabic with diborane (76) and then subjecting the reduced polysaccharide to acetolysis. Deacetylation of the resulting mixture of acetylated sugars gave an assortment of oligosaccharides among them a rhamnosyl-glucose which he partially characterised. It was our task to completely characterise this disaccharide and, in addition, see if any other structurally significant oligosaccharides could be found among the acetolysis products.

In order to show that this sample of gum arabic contained similar structural features to those of the sample used by Smith in his investigations (82), it was intended

to methylate the gum and to examine the methanolysis products from the methylated polysaccharide by gas/liquid partition chromatography (53).

3) Acacia mollissima gum.

Slight differences in structure have been found to exist between samples of the same gum when these samples are of different origin. Since there was available a sample of Acacia mollissima gum (black wattle gum) of West Indian origin and since a report was available (85) of a preliminary investigation of the same gum of South African origin, we intended to investigate this matter by carrying out an examination of the structure of the gum of West Indian origin, in conjunction with J. J. Carlyle and R. Stirling.

In Stephen's investigation, the gum was found to have the same constituents as gum arabic on acid hydrolysis, but in different proportions. L-Arabinose (6), L-rhamnose (1), D-galactose (5) and D-glucuronic acid (1) have been shown to be combined in the approximate molecular ratios indicated. The aldobiouronic acid, 6-O- β -D-glucuronosyl-D-galactose, which is found on partial hydrolysis of the gum, is the same as that isolated from gum arabic.

SECTION I

KHAYA SENEGALENSIS GUM

HETEROGENEITY OF KHAYA SENEGALENSIS GUM

The sample of Khaya senegalensis gum used, was the same as that investigated by Aspinall and co-workers (17). The gum was in the form of nodules of varying size and colour and, in addition, there was still an appreciable quantity of bark adhering.

Previous work on this gum (17,81) indicated the presence in the gum of two polysaccharide fractions, differing considerably in molecular structure. The gum was investigated by DEAE-cellulose chromatography to ascertain how many polysaccharides were, in fact, present. Dissolution of the crude, crushed nodules was effected using sodium hydroxide with concomitant deacetylation of the polysaccharides. Five fractions were obtained.

Fraction I was freed from inorganic material by treatment with ion exchange resins, followed by a desalting process on charcoal. No dialysis was attempted since the carbohydrate material was thought to be of low molecular weight. Investigation of the syrup obtained showed it to be mainly arabinose with a trace of galactose; no polysaccharide material was present in Fraction I.

The polysaccharides from Fractions II and III were investigated and found to be identical. The two fractions were combined to give polysaccharide B, $[\alpha]_D +8^\circ$ and uronic anhydride content 21%. The constituent sugars of this

polysaccharide were shown to be galactose, arabinose, 4-O-methyl-glucuronic acid and a trace of glucuronic acid. Polysaccharide B was estimated to constitute 23% of the crude gum.

Polysaccharide A was obtained on dialysing and freeze-drying Fraction IV. This polysaccharide, which represented 77% of the crude gum, had $[\alpha]_D +136^{\circ}$ and uronic anhydride content 55%. Hydrolysis gave rhamnose, galactose, galacturonic acid, 4-O-methyl-glucuronic acid and a trace of arabinose.

Fraction V, which was obtained when the column was washed with stronger sodium hydroxide, must have been cellulose bleeding from the column as it had no uronic anhydride content and on chromatographic examination of the hydrolysis products only glucose was obtained.

DEAE-Cellulose chromatography of the crude gum shows, therefore, that Khaya senegalensis gum, is composed of two polysaccharides:- polysaccharide A, with the high uronic acid content, present as the main component, and polysaccharide B with the much lower uronic acid content.

Isolation of polysaccharide B on a preparative scale.

A fractional precipitation experiment was carried out on the crude gum, using the same conditions as reported by Aspinall (17). The two polysaccharide fractions obtained were examined for heterogeneity by DEAE-cellulose

chromatography.

The major fraction was found to consist mainly of polysaccharide A, but with an appreciable amount of contaminating polysaccharide B still present. The minor fraction was grossly heterogeneous consisting of almost equal amounts of the two polysaccharides.

Several fractional precipitations of the crude gum were carried out, using varying concentrations of ethanol and water, until a system was devised for obtaining a pure sample (checked by DEAE-cellulose chromatography) of polysaccharide B. This method, however, gave a low yield (ca. 4%) and so extensive extraction of crude gum had to be carried out. After every extraction, the polysaccharide material obtained was analysed for rhamnose, since the presence of this sugar indicated the presence of contaminating polysaccharide A.

Isolation of homogeneous polysaccharide A.

a) Methylation. A sample of polysaccharide A, obtained from DEAE-cellulose chromatography of the crude gum, was fully methylated. The mixture of methyl glycosides obtained by methanolysis was examined by vapour phase chromatography in two different systems. A sample of the methylated gum was reduced and this polysaccharide examined in the same way. Evidence was obtained for the presence of all the methylated sugars previously reported

to be present in polysaccharide A from Khaya senegalensis gum. However, since arabinose was not a sugar residue which had been previously reported to be a constituent of gums, having the same structural features as polysaccharide A, such as those of the Sterculia genera, it was suspected that this sugar may have arisen from contaminating polysaccharide B.

b) Large-scale DEAE-cellulose chromatography. A sample of polysaccharide A obtained by the fractional precipitation technique was chromatographed on a DEAE-cellulose column. Large quantities of the effluent known to desorb polysaccharide B, was used until the eluate was sugar free. Elution with more concentrated phosphate buffer gave fraction ii which corresponded to pure polysaccharide A.

c) Methylation of fraction ii. This fraction was fully methylated and the derived methyl glycosides examined by gas phase chromatography. The results obtained were exactly the same as those obtained for the previous sample of methylated polysaccharide A. Most importantly, there was still an appreciable amount of 2,3,5-tri-O-methyl-arabinose present.

d) 2 x DEAE-Cellulose chromatography. Fraction ii from DEAE cellulose chromatography of polysaccharide A material, was refractionated on DEAE-cellulose chromatography. It was shown to be heterogeneous, containing about 4% of contaminating polysaccharide B. So again the fraction

corresponding to pure polysaccharide A was isolated and methylated as before. Examination of the methanolysis products by vapour phase chromatography in both solvent systems showed the absence of any methylated arabinose derivatives. So it would appear that we have at last obtained a sample of homogeneous polysaccharide A. It is interesting to note that in the methylated polysaccharide, there was a large amount of 2,4-di-O-methyl-galactose present which had previously been thought to have arisen from contaminating polysaccharide B and which was not reported previously to be present as a constituent of methylated Khaya grandifolia gum (18).

PARTIAL HYDROLYSIS STUDIES ON POLYSACCHARIDE B

By carrying out several trial experiments, it was ascertained that the best conditions for formation of acidic oligosaccharides from polysaccharide B, was hydrolysis of the polysaccharide with N sulphuric acid for 4 hours at 100°. A small scale partial hydrolysis under these conditions, after removal of the neutral sugars, yielded two acidic oligosaccharides, aldobiouronic acid A (R_{GAL} 0.50 in solvent C) and aldobiouronic acid B (R_{GAL} 0.77), together with acidic monosaccharide material.

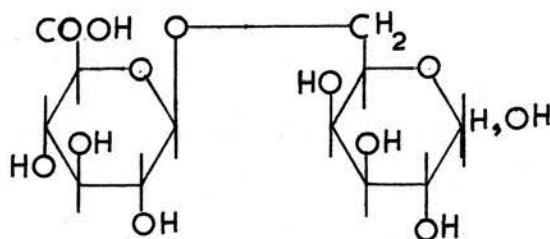
A sample of polysaccharide B was hydrolysed with N sulphuric acid for 4 hours. After neutralisation, the sugar solution was allowed to soak into an anion exchange resin column, generated to the formate form, the neutral sugars were removed with water and the acidic sugars eluted with increasing concentrations of aqueous formic acid. The fractions obtained were not homogeneous and had to be subfractionated on filter sheets. Aldobiouronic acid A and aldobiouronic acid B were isolated, together with some glucuronic acid, 4-O-methyl-glucuronic acid and glucuronolactone.

Identification and characterisation of aldobiouronic acid A.

This acidic oligosaccharide had $[\alpha]_D -3^\circ$ and R_{GAL} 0.19 in solvent B and 0.50 in solvent C. It was chromatographically pure and indistinguishable from 6-O- β -D-glucuronosyl-

D-galactose and gave only galactose and glucuronic acid on hydrolysis. Reduction of the derived methyl ester, methyl glycoside with potassium borohydride followed by hydrolysis gave galactose and glucose. The latter sugar must have arisen from reduction of glucuronic acid. Methylation afforded a crystalline derivative which was identical (mixed m.p., specific rotation, OMe content and X-ray powder diagram) with an authentic sample of the methyl ester of methyl 6-(hexa-O-methyl- β -D-glucopyranosyl uronic acid)- β -D-galactoside.

The above evidence proves that aldobiouronic acid A, derived from polysaccharide B, has the following structure:-



Identification and characterisation of aldobiouronic acid B.

This acidic oligosaccharide had $[\alpha]_D +48^\circ$ and was homogeneous in several solvent systems, R_{GAL} 0.61 in solvent B and 0.77 in solvent C. Hydrolysis and examination of the products by paper chromatography indicated the presence of galactose and an acidic monosaccharide, with the same chromatographic mobility as 4-O-methyl-D-glucuronic acid. Reduction of the derived methyl ester methyl glycoside with borohydride followed by hydrolysis gave D-galactose

and 4-O-methyl-D-glucose. These two sugars were separated on a filter sheet and characterised, the latter giving a crystalline phenylosazone which was identical in mixed m.p., optical rotation and X-ray powder photograph with authentic 4-O-methyl-D-glucose phenylosazone.

Although aldobiouronic acid B was chromatographically homogeneous, the fact that it had a rotation of $+48^\circ$, compared with $+8^\circ$ already reported (89) for the 6-linked aldobiouronic acid, suggested that it might be a mixture of the 6- and either the 2,3, or 4-linked anomers. The presence of 2,3,4- and 2,3,6-tri-O-methyl-D-galactoses in the reduced methylated acidic sugars (see next section) indicated that there might be a 1-4 linked aldobiouronic acid present in the polysaccharide. The presence of the 4-linked anomer in aldobiouronic acid B would be consistent with an increase in positive rotation, since its reported rotation was $+86^\circ$ (89).

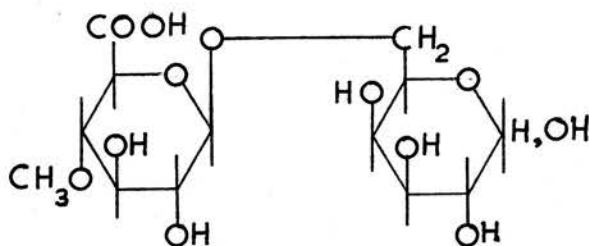
To clear up this question it was decided to subject a small portion of the syrup to periodate oxidation and observe if any formaldehyde was released. This of course can only result from a 2,3 or 4-linked aldobiouronic acid and not from a 6-linked, as the latter has no free primary hydroxylgroups (90).

Since the formaldehyde released was to be measured colorimetrically by the chromotropic acid method (91) a standard curve of optical density against amount of

X

formaldehyde, was prepared by the action of periodate on erythritol. A 0.5% periodate solution was used and the solution buffered at pH 8, while with the aldobiouronic acid, a 1% periodate solution was used at the same pH. A definite release of formaldehyde was observed in amount indicating that a 2,3 or 4-linked anomer was present as ca. 23% of the total fraction.

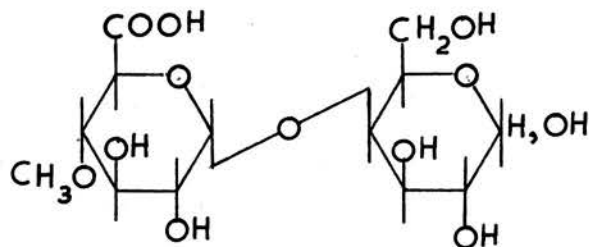
The main component in aldobiouronic acid B was characterised as 6-O-β-(4-O-methyl-D-glucopyranosyl uronic acid)-D-galactose by conversion into the fully methylated derivative, which was identical (mixed m.p., optical rotation, OMe content and X-ray powder photograph) to an authentic sample. Therefore this component has the following structure:-



To ascertain the mode of linkage of the minor component in aldobiouronic acid B, the remaining syrup was fully methylated. A portion of the methylated material was reduced and the methanolysis products of both the reduced and unmodified methylated disaccharides examined by vapour phase chromatography. Evidence was obtained in two systems for the presence of 2,3,6-tri-O-methyl-galactose. This

proves that the minor component in aldobiouronic acid B is 1-4 linked. An attempt was made to isolate this tri-O-methyl-galactose on a charcoal column but this proved unsuccessful as only a small amount of material remained.

Thus the third aldobiouronic acid present in polysaccharide B must have the following structure:-



N.B. An α -glycosidic linkage is postulated here due to the high positive rotation of aldobiouronic acid B. All aldobiouronic acids of this structure so far isolated have had an α -linkage.

METHYLATION STUDIES ON POLYSACCHARIDE B

A sample of polysaccharide B was methylated with dimethyl sulphate and sodium hydroxide. The partially methylated polysaccharide obtained was converted to the silver salt with silver carbonate and the methylation was completed by several treatments with methyl iodide and silver oxide. The fully methylated product had $[\alpha]_D = -26.5^\circ$ and -OMe, 41.1%.

From a preliminary vapour phase chromatographic examination of the methanolysis products of the methylated polysaccharide, the methylated polysaccharide after reduction with lithium aluminium hydride and the reduced methylated polysaccharide after remethylation, evidence was obtained for the presence in the methylated polysaccharide of residues of tetra-, tri- and di-O-methyl-galactoses, tri-O-methyl-arabinose and tri-O-methyl-glucuronic acid.

The fully methylated polysaccharide was depolymerised with methanolic hydrogen chloride (3%) and after neutralisation, the methyl ester of the acidic component was saponified with aqueous barium hydroxide. Continuous extraction of this solution with boiling light petroleum gave fraction X. The residual aqueous solution was treated with Amberlite resin 1R120, and passed through an anion exchange resin Duolite A4. The acidic component was selectively removed and the neutral methylated sugars eluted

with water to give fraction Y. The acidic component Z was isolated from the column by displacement with sodium hydroxide.

Examination of the fractions by gas phase chromatography showed that the separation between acid and neutral methylated sugars had been adequate but that there was not a sufficient difference between fractions X and Y to warrant working them up separately so they were combined to give fraction W.

After hydrolysis of the methyl glycosides, Fraction W, consisting almost entirely of neutral sugars, was further fractionated on cellulose, complemented by charcoal column and filter sheet separations, giving the following methylated sugars.

	<u>Approximate Weight</u>
2,3,5-Tri- <u>O</u> -methyl- <u>L</u> -arabinose	120 mg.
2,3,4,6-tetra- <u>O</u> -methyl- <u>D</u> -galactose	100 mg.
2,3,4-tri- <u>O</u> -methyl- <u>D</u> -galactose	130 mg.
2,4,6-tri- <u>O</u> -methyl- <u>D</u> -galactose	65 mg.
2,3,6-tri- <u>O</u> -methyl- <u>D</u> -galactose	55 mg.
2,4-di- <u>O</u> -methyl- <u>D</u> -galactose	310 mg.
2,6-di- <u>O</u> -methyl- <u>D</u> -galactose	95 mg.
2-mono- <u>O</u> -methyl- <u>D</u> -galactose	75 mg.

All of these methylated sugars were characterised by means of crystalline derivatives. In addition to the above major components, traces of the following were detected chromatographically:- 2,5- and 2,3-di-O-methyl-

arabinoses and 4-mono-O-methyl-galactose.

A small sample of fraction Z was treated with dilute sulphuric acid to hydrolyse the methyl glycosides and was deionised to convert the barium salts to free acid. An investigation was made as to the possibility of isolating the methylated aldobiouronic acids present in this fraction, in an attempt to ascertain how the uronic acid residues were linked to the main chain. The chromatographic mobilities of the methylated acidic sugars were too similar for a separation to be possible, however.

Fraction Z was then reduced with lithium aluminium hydride, followed by total hydrolysis. Fractionation on charcoal, aided where necessary by filter paper separations, gave the following methylated sugars.

	<u>Approximate Weight.</u>
2,4-Di- <u>O</u> -methyl- <u>D</u> -galactose	12 mg.
2,6-di- <u>O</u> -methyl- <u>D</u> -galactose	10 mg.
2,3,4-tri- <u>O</u> -methyl- <u>D</u> -galactose	25 mg.
2,3,6-tri- <u>O</u> -methyl- <u>D</u> -galactose	35 mg.
3,4-di- <u>O</u> -methyl- <u>D</u> -glucose	20 mg.
2,3,4-tri- <u>O</u> -methyl- <u>D</u> -glucose	90 mg.

All of these methylated sugars, save one, were characterised by means of crystalline derivatives. 3,4-Di-O-methyl-D-glucose was identified by optical rotation, by gas chromatography of the methyl glycosides, by paper chromatography

of the sugar and of the products of demethylation and of periodate oxidation, and by the positive staining reaction of the sugar with triphenyl tetrazolium hydroxide which indicated the presence of an unsubstituted hydroxyl group at C(2).

The methylation results on polysaccharide B are tabulated below. The proportions of the methylated sugars given are only very approximate since many fractions were mixtures and several re-fractionations had to be performed to separate the components.

Hydrolysis products of methylated polysaccharide B from *Khaya senegalensis*.

	Approx. molecular proportions.
2,3,4,6-Tetra-O-methyl-D-galactose	4.3
2,3,4-tri-O-methyl-D-galactose	7.2
2,4,6-tri-O-methyl-D-galactose	3.0
2,3,6-tri-O-methyl-D-galactose	4.1
2,4-di-O-methyl-D-galactose	15.8
2,6-di-O-methyl-D-galactose	5.3
2-mono-O-methyl-D-galactose	4.0
2,3,4-tri-O-methyl-D-glucose *	4.1
3,4-di-O-methyl-D-glucose *	1.0
2,3,5-tri-O-methyl-L-arabinose	6.4
2,3- or 2,5-di-O-methyl-L-arabinose	trace

* Arise from the corresponding glucuronic acid derivatives,

AUTOHYDROLYSIS OF POLYSACCHARIDE B

Polysaccharide B was hydrolysed with sulphuric acid, the monosaccharides fractionated on thick paper and the sugar proportions estimated, by the p-amino benzoic acid method (106) as follows:- L-arabinose (25%), D-galactose (55%) and uronic acids (20%).

A small scale autohydrolysis was carried out on polysaccharide B, and the reaction followed by paper chromatography and change in optical rotation of the reaction mixture. The rotation, which increases due to the release of L-arabinose residues, rose from $+8^{\circ}$ to $+32^{\circ}$ after 32 hours and was then stationary. The only hydrolysis product for the first 30 hours was arabinose and thereafter a trace of galactose appeared. Examination of the degraded polysaccharide by hydrolysis and subsequent paper chromatography, showed that all the L-arabinose residues were not hydrolysed until the reaction had been in progress for 48 hours.

A 2% aqueous solution of polysaccharide B was refluxed for 42 hours. After cooling, the solution was poured into ethanol, thus precipitating out the degraded polysaccharide. After several reprecipitations this polysaccharide was taken up in water and freeze-dried. The combined filtrates were evaporated to dryness giving the low molecular weight sugars which had been hydrolysed from the original polysaccharide.

The low molecular weight sugars, on paper

chromatographic examination were found to consist of galactose and arabinose. A sample of the mixture was fractionated on thick paper and the proportion of arabinose: galactose found to be 3.3 : 1. These results show that if we assume that all the arabinose residues have been removed from polysaccharide B, the degraded polysaccharide will contain 7 out of every 8 galactose units present in the unmodified polysaccharide. Since this is the ratio of galactose units present as end groups, as indicated by methylation studies, it seems likely that only end-group galactose units have been hydrolysed, leaving the galactan framework intact.

The degraded polysaccharide $[\alpha]_D^{20}$ and uronic acid content - 28%, gave galactose, uronic acids and a trace of arabinose on hydrolysis. Partial acid hydrolysis gave 1,3- and 1,6-galactobiose in addition to monosaccharides. The polysaccharide was methylated to give methylated degraded polysaccharide, $[\alpha]_D^{-18}$ and OMe, 43.0%. Reduction with lithium aluminium hydride in tetrahydrofuran gave reduced methylated degraded polysaccharide, $[\alpha]_D^{-16}$ and OMe, 41.2%. The methanolysis products of the reduced and unmodified methylated polysaccharides were examined by gas-chromatography in both solvent systems. The results obtained, in addition to the results obtained by paper chromatographic examination of the hydrolysed methylated degraded polysaccharide, showed that methylated degraded polysaccharide B

contained the methylated sugars shown in table A. A comparison is drawn between the relative proportions of these sugars in the methylated polysaccharide and the methylated degraded polysaccharide

TABLE A.

Sugar	Relative Proportions	
	Methylated polysaccharide B	Methylated degraded polysaccharide B
2,3,5-Tri- <u>O</u> -methyl arabinose	+++	trace
2,3,4,6-tetra- <u>O</u> -methyl galactose	+++	++
2,3,4-tri- <u>O</u> -methyl galactose	+++	+++
2,4,6-tri- <u>O</u> -methyl galactose	+	++++
2,3,6-tri- <u>O</u> -methyl galactose	++	++
2,4-di- <u>O</u> -methyl galactose	+++++	++++
2,6-di- <u>O</u> -methyl galactose	+++	+++
2- <u>O</u> -monomethyl galactose	+++	++
2,3,4-tri- <u>O</u> -methyl glucuronic acid	++	+++

Smith Degradation studies on polysaccharide B.

The occurrence of 2,4,6-tri-O-methyl-D-galactose residues in the methylated polysaccharide, indicated the presence of 1,3-linked D-galactose units in the polysaccharide. The polysaccharide was therefore subjected to a Smith degradation in order to obtain information on the location in the polysaccharide of these periodate resistant fragments. Difficulties are encountered when applying the Smith degradation to acidic polysaccharides, since the oxidised fragments which contain uronic acid groups are less readily removed by mild acid hydrolysis than the other acetal systems (109). The polysaccharide was, therefore, first converted into the acetate and reduced with diborane (76).

A trial experiment was carried out by degrading reduced polysaccharide B with sodium metaperiodate until no more reagent was consumed. It was observed that 0.82 moles of the oxidant was consumed per sugar unit.

A large quantity of reduced polysaccharide B was oxidised with sodium metaperiodate until no more reagent was consumed. 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 fragments resulting from

cleaved residues. This procedure is referred to as the Smith degradation.

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 galactose, arabinose and 4-O-methyl glucose together with the low molecular weight alcohols.

The degraded polysaccharide B obtained, by precipitation with ethanol, on hydrolysis gave galactose and a trace of arabinose. Partial hydrolysis of this degraded polymer gave 1,3-galactobiose, 1,3-galactotriose, and higher oligosaccharides, with only a trace of 1,6-galactobiose. A small sample of the degraded polysaccharide was subjected to periodate attack; it was observed that 0.26 moles of the oxidant were consumed per sugar unit. This indicated that the polymer was approximating to a 1,3 linked galactan, the galactose and arabinose end-groups plus a few remaining 1,6-linked galactose residues being responsible for the small amount of periodate that was consumed. The postulation was confirmed by methylation of the degraded polysaccharide and subsequent gas chromatographic examination of the methanolysis products. The methylated polysaccharide was shown to contain a large amount of

2,4,6-tri-O-methyl galactose with small amounts of 2,3,4,6-tetra-, 2,3,4-tri- and 2,4-di-O-methyl galactose and traces of 2,3,5-tri-O-methyl arabinose.

STRUCTURAL FEATURES OF POLYSACCHARIDE B

(ex. Khaya senegalensis gum)

The structural features of the gum based on methylation, partial acid hydrolysis and Smith degradation studies are discussed below, by considering in turn the galactan framework, the acidic fragments and the acid-labile pentose units.

The galactan framework.

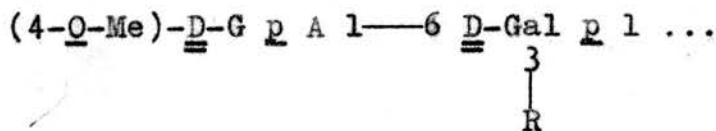
It is clear from methylation studies that polysaccharide B is highly branched and that D-galactopyranose residues are substituted at positions 3 and/or 6. Galactose residues are mutually joined by 1→3 and 1→6 linkages since disaccharides having the chromatographic mobility of 3- and 6-O-β-D-galactopyranosyl-D-galactoses were detected as products of partial acid hydrolysis. Although 4-O-substituted D-galactose residues are present in the polysaccharide, the 1,4-linked galactobiose was not detected as a partial hydrolysis product, and it is probable that the majority of such galactose residues are substituted by 4-O-methyl-D-glucuronic acid residues.

When carboxyl-reduced polysaccharide B was degraded by Smith's procedure a degraded galactan was isolated, which contained only a very small proportion of arabinose residues and in which galactose residues were mainly 1,3-linked. It follows that the majority of D-galactose residues which

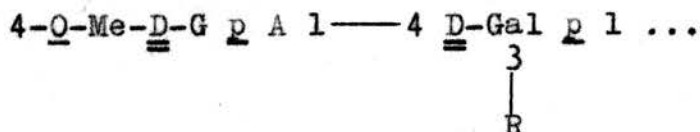


Again, it is difficult to predict on the present evidence, how these aldobiouronic acid units are incorporated in the general molecular structure of the polysaccharide. It is likely, however, that the two 1,6-linked aldobiouronic acids terminate the 1,6-linked galactose side-chains, as is the case in gum arabic. No differentiation in structural position in the polysaccharide can be made between these two acidic units (II) and (III). It is probable, since the majority of the glucuronic acid units are end-groups, that the 1,4-linked aldobiouronic acid units are also present as side-chains, but it is impossible to say how these fragments are attached to the 1,3-linked galactose backbone.

The fact that appreciable quantities of 2,4- and 2,6-di-O-methyl-D-galactoses were found in the acidic methylated sugar fraction, indicates that some of the galactose residues in the aldobiouronic acids must be substituted. Fragments V and VI must be of structural significance in the polysaccharide.



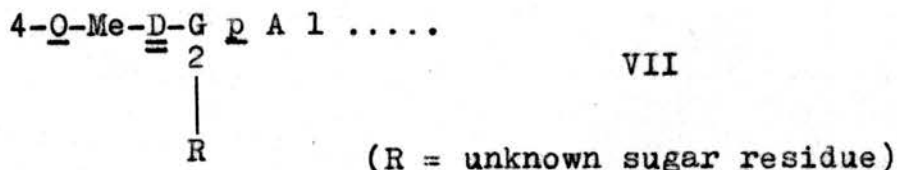
V



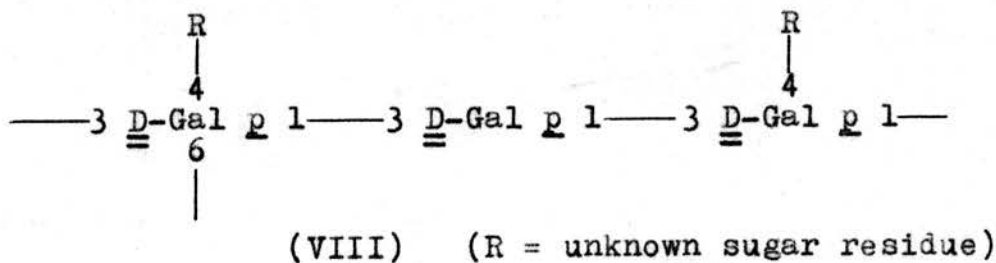
VI

(R = unknown sugar residue).

Although there is no substitution of the glucuronic acid residues at position 4 in this polysaccharide, as is the case in gum arabic (59), 1 in 5 of the uronic acid residues carry a substituent at position 2; this is indicated by the presence of 3,4-di-O-methyl-D-glucuronic acid in the methylated polysaccharide. It is impossible to say which, if not all, of the aldobiouronic acids are so substituted. Fragment VII must, therefore, be present in the polysaccharide.



The presence in the methylated polysaccharide of appreciable amounts of 2,6-di-O- and 2-O-mono-methyl-D-galactose indicates that there must be branch points at position 4 in the 1,3-linked D-galactose units composing the main chain as shown in (VIII). There is little evidence available for the nature of grouping R.

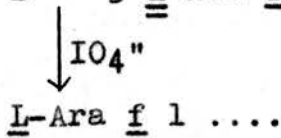
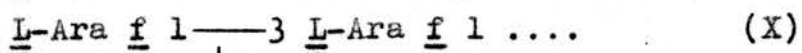
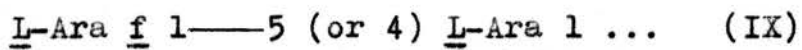


The acid-labile pentose units.

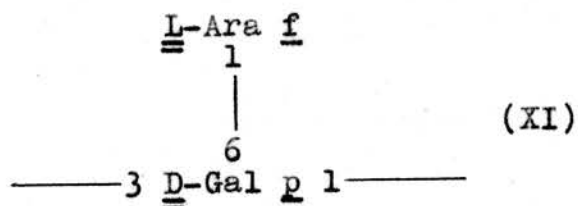
L-Arabinose residues have been found to constitute 25%

of the total polysaccharide. The majority of these residues are in the form of L-arabinofuranose end-groups, L-Ara f l ... This is indicated by methylation studies and by the fact that almost all the L-arabinose units are removed by mild acid hydrolysis. The mild acid hydrolysis also removes a small proportion of D-galactose residues; the inference that these galactose units are in the furanose form is refuted by methylation studies. Bouveng has, in fact, shown recently (114) that D-galactopyranose end groups are hydrolysed with mild acid at a comparable speed to the rate of hydrolysis of L-arabinofuranose end-groups, thus explaining the release of D-galactose residues during autohydrolysis of polysaccharide B.

There is also evidence for the presence of a few non-terminal arabinose units, probably 1,3- and 1,5-linked, as shown in (IX) and (X). The evidence for this lies in the presence of traces of 2,3- and 2,5-di-O-methyl-L-arabinoses in the methylated polysaccharide. The presence of fragment (X) in the polysaccharide is confirmed by the occurrence of L-arabinofuranose end-groups in the Smith degraded polysaccharide, which must have arisen from cleavage of the arabinobiose as shown. The substituent attached to the non-terminal arabinose units is probably L-arabinofuranose since if it had been D-galactose, the relatively stable galactosyl-arabinose should have been detected as a partial hydrolysis product.



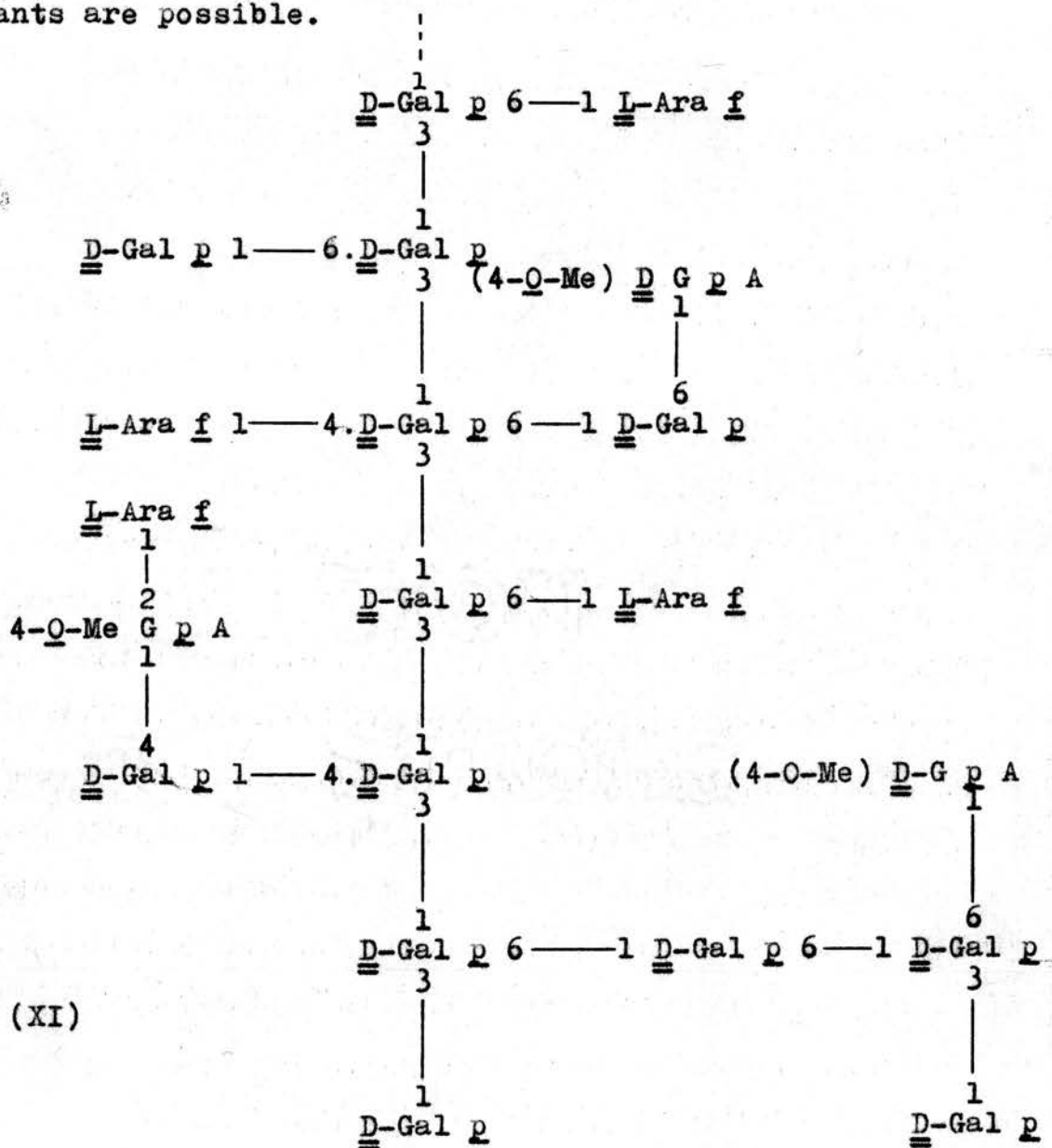
The points of attachment of the acid-labile residues to the galactose residues in the polysaccharide, follow from a comparison of the proportion of D-galactose methyl ethers formed on hydrolysis of the methylated polysaccharide and of the methylated degraded polysaccharide. The most striking difference is the large increase in the proportion of 2,4,6-tri-O-methyl-D-galactose from the methylated degraded polysaccharide and the corresponding decrease in the proportion of 2,4-di-O-methyl-D-galactose. It follows that the majority of the acid-labile groups are attached to position 6 of 1→3 linked galactose residues in the main chain of the molecule and so fragment (XI) must be a major structural feature in the polysaccharide.



It is also feasible that a few of the acid-labile groups may be attached to position 3 in the 1,6-linked galactose side-chains.

By incorporating the above fragments (I) to (XI), a structure for the polysaccharide can be put forward as

shown in (XII), which is consistent with the experimental results. It should be stressed, however, that other variants are possible.



Comparison of the structural features of polysaccharide B (ex. Khaya senegalensis 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 following gums belong to groups (b); polysaccharide A (ex. Khaya senegalensis gum), Khaya grandifolia gum (18), Sterculia setigera gum (115, 116), Sterculia urens gum (117) and Cochlospermum gossypium gum (118, 80). The aldobiouronic acid 2-O-(α -D-galactopyranosyluronic acid)-L-rhamnose has been isolated from all the gums of this group, and all of these gums have a high uronic acid content (ca. 50%). Polysaccharide B does not belong to this group and has few structural features in common with these gums, except that aldobiouronic acid (IV) has also been isolated from Khaya grandifolia gum and Cochlospermum gossypium gums.

The gums belonging to group (a), in general have a backbone of galactose residues which carry side-chains containing aldobiouronic acid units and have acid-labile (mainly pentose) units on the periphery of the molecule.

The gums of this group may be further divided into three sub groups on the basis of the nature of the linkages in the galactan backbone.

(i) Gums which have highly branched core of D-galactose residues which are involved in 1,3- and 1,6-linkages.

(ii) Gums having main chains composed of 1,3-linked D-galactopyranose residues, with 1,6-linked D-galactose incorporated in the side-chains.

(iii) Gums which have main chains composed of 1,6-linked D-galactopyranose residues.

The Prunus gums (Damson (119), Cherry (120) and golden apple (121, 122)) and other gums such as Lemon (62, 111-113), Cholla (123) and Mesquite (124-127) belong to sub group (i). These gums have been classified together since it is not yet clear what the nature of their galactose backbone is. If a Smith degradation were carried out on each gum it could be shown whether the gums belonged to sub group (ii) or whether indeed they were a separate group. Apart from the question of the galactose backbone, the gums, notably Cholla gum and golden apple gum, differ from polysaccharide B in that they have acid-labile xylose residues, in addition to arabinose residues, on the periphery of the molecule. Lemon gum and grapefruit gum (111-113) are similar to polysaccharide B in that aldobiouronic acid (IV) has been isolated from them.

Anogeissus latifolia gum (gum ghatti) (128-130),

Anogeissus schimperi gum (131), Combretum leonense gum (32) and Virgilia oroboides gum (108 and 132) all belong to sub group (iii). These gums differ considerably from polysaccharide B in their galactan backbone and in that most of them contain D-mannose residues, and do not contain 4-O-methyl glucuronic acid.

Polysaccharide B is most akin to the gums in sub group (ii) which includes the Acacia gums and Asofoetida gum (105). The Acacia gums which have been extensively investigated, such as gum arabic, Acacia mollissima gum (85) and Acacia pycnantha gum (103) can be compared, along with Asofoetida gum, to polysaccharide B. All of the gums contain the same galactan framework as shown in (I). The differences amongst them occur in the nature of the acidic fragments and the acid-labile units, as can be seen in table I.

The main difference between polysaccharide B and the rest lies in the fact that the former contains a 1,4-linked aldobiouronic acid (IV) and that a higher proportion of the 1,3-linked D-galactose residues in the main chain are 4-O-substituted. In addition the Acacia gums contain L-rhamnopyranose end-groups, which is not a structural feature of polysaccharide B. Another structural feature which is unique to polysaccharide B is substitution of a few of the uronic acid residues at position 2.

TABLE I.

Structural units	<u>Asofoetida</u> gum	<u>Acacia</u> <u>senegal</u> (gum arabic)	Polysac- charide B (ex. <u>Khaya</u> <u>senega-</u> <u>lensis</u>)	<u>Acacia</u> <u>molli-</u> <u>ssima</u>	<u>Acacia</u> <u>pycnan-</u> <u>tha</u>
<u>L</u> -Ara <u>f</u> 1	+	+	+	+	+
<u>L</u> -Ara <u>f</u> 1 3 <u>L</u> -Ara			trace		+
<u>D</u> -Gal <u>p</u> 1 3 <u>L</u> -Ara		+			
<u>L</u> -Ara <u>p</u> 1 3 <u>L</u> -Ara		+		+	
<u>L</u> -Rha <u>p</u> 1	trace	+		+	trace
4- <u>O</u> -Me- <u>D</u> - <u>Gp</u> A1 6 <u>D</u> -Gal	+		+	+	
<u>D</u> -G <u>p</u> A 1 6 <u>D</u> -Gal	+	+	+	+	+
4- <u>O</u> -Me- <u>D</u> - <u>Gp</u> A1 4 <u>D</u> -Gal			+		

EXPERIMENTAL

GENERAL METHODS

PAPER CHROMATOGRAPHY - Descending chromatography, used for qualitative work, was carried out on Whatman No. I, unless otherwise stated. The following solvent systems (v/v) were employed:

- A) ethyl acetate - pyridine - water (10:4:3)
- B) ethyl acetate - acetic acid - formic acid - water (18:3:1:4)
- C) ethyl acetate - acetic acid - formic acid - water (18:8:3:9)
- D) butan-1-ol - ethanol - water (4:1:5, upper layer)
- E) butan-1-ol - acetic acid - water (4:1:5, upper layer)
- F) benzene - ethanol - water (169:47:15, upper layer)
- G) benzene - acetic acid - water (169:47:15, upper layer)
- H) methyl ethyl ketone saturated with water (+ trace of ammonia)
- I) butan-1-ol - acetic acid - water (9:1:1, saturated with boric acid).

Explanation of symbols used in connection with paper chromatography.

$$R_F = \frac{\text{Rate of movement of substance}}{\text{Rate of movement of solvent front}}$$

$$R_{GAL} = \frac{\text{Rate of movement of substance}}{\text{Rate of movement of D-galactose}}$$

$$R_G = \frac{\text{Rate of movement of substance}}{\text{Rate of movement of 2,3,4,6-tetra-O-methyl-D-glucose (in solvent D)}}$$

The chromatograms were run for a predetermined length of time, air-dried and the sugars located by spraying with one of the following reagents.

I Aniline Oxalate - Unless otherwise stated, non-methylated reducing sugars were detected by spraying with a saturated aqueous solution of aniline oxalate and heating at 140-160° for about 5 minutes.

II p-Anisidine HCl - A 1% solution of p-anisidine HCl in butan-1-ol was used to detect methylated reducing sugars. Examination of the chromatogram in u.v. light revealed the presence of trace components, by their fluorescence.

III Silver Nitrate (92) - This reagent was used to detect the presence of sugar alcohols as well as reducing sugars.

IV Periodate Benzidine (93) - Glycitol were also detected, by this method, as white spots on a blue background.

Preparative separation of sugars on thick paper.

Whatman 3MM and 31 (extra thick) were used. The position of the sugars was determined by cutting off narrow side-strips to which a chromatographic spray was applied. The appropriate parts of the filter sheet were then cut out and eluted. Usually, cold water was used for this purpose, until the eluate gave a blank reading with the phenol-sulphuric acid reagent (37).

Cellulose columns.

The cellulose was washed thoroughly with water and dried for several days in a vacuum oven. The column was packed dry and washed with water, followed by the solvent to be used. Butan-1-ol for use on columns was purified by refluxing for 1-2 hours with potassium hydroxide (10 g./litre), and distilling; light petroleum was shaken overnight with concentrated sulphuric acid (10% v/v), washed free of acid with distilled water and distilled.

Fractionation was achieved by collecting the eluate in tubes on an automatic turntable. At suitable intervals, the contents of a tube were evaporated to dryness and examined by paper chromatography. Fractions were bulked accordingly, and evaporated to dryness, and the syrups obtained were cleaned by dissolving in water and treating with charcoal. The final filtrate was taken to dryness, dried over phosphorus pentoxide and weighed.

Charcoal-Celite Columns.

Preparation of charcoal-celite columns was carried out as follows.

Activated charcoal was washed thrice with boiling distilled water. Celite (grade 545) was washed with concentrated hydrochloric acid:water (1:1), allowed to stand overnight, filtered, washed till free of chloride with tap water and finally washed with distilled water. Equal

weights of charcoal and Celite were mixed well in a slurry which was poured into a glass column. Before applying the sugar mixture, the column was washed thoroughly with water. The columns can be used for separating (a) mixture of oligosaccharides (65), and (b) mixture of methylated sugars (48).

a) The mixture of sugars was allowed to soak into the column and the monosaccharides were eluted with water.

The oligosaccharides were then separated by stepwise elution with aqueous ethanol of increasing concentration (0 - ca.15%).

b) The mixture of methylated sugars was fractionated by eluting the column with a gradient of aqueous methyl ethyl ketone (0 - 6.5%). The less fully methylated sugars are eluted first followed by the completely methylated residues. In some cases the elution pattern was followed by evaporating tubes to dryness at suitable intervals, forming the methyl glycosides and examining the products by gas/liquid partition chromatography.

In all instances, when the fractions were bulked together and evaporated to dryness, quantities of Celite were obtained along with the carbohydrate material. In the case of the methylated sugars, the sugars were dissolved in A.R. acetone and the solution filtered, leaving the Celite behind; with oligosaccharides, the residues had to be taken up in water and evaporated to dryness several

times, leaving the Celite on the walls of the flask.

DEAE-Cellulose Columns.

Diethyl amino ethyl cellulose (26) was washed alternately with 0.1N HCl and 0.1N NaOH (500 ml. for each washing). The cellulose was stirred with acid (or base) for 5-10 mins., centrifuged, and the turbid solution decanted off. The cellulose was then washed twice with distilled water. A perforated disc was placed at the bottom of the column, then a layer of glass wool, followed by a 1 cm. layer of silver sand and finally 1 cm. layer of acid washed Celite. The cellulose was poured in as a water slurry and slight air pressure applied at the top. The solvent level was always kept above the cellulose to avoid air bubbles. A layer of glass wool was placed at the top as well. The initial generation and subsequent re-generation of the cellulose in the phosphate form was done by eluting the column with 2 litres of 0.5M sodium dihydrogenphosphate (adjusted to pH 6 by adding sodium hydroxide), followed by equilibration with 1 litre of 0.005M sodium dihydrogen phosphate solution of the same pH. The polysaccharide mixture was taken up in a little water and allowed to soak into the column overnight. The elution pattern was followed by analysing a small fraction of each tube by the phenol-sulphuric acid method (37).

Optical Rotation.

The optical rotation of a substance was measured at $18^{\circ} \pm 2^{\circ}$ using water as the solvent, unless otherwise stated.

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

Gas-Liquid Partition Chromatography (52,53).

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

Methyl glycosides were formed by refluxing the sugar with methanolic hydrogen chloride (3%) for ca. 6 hr. Longer reaction times (ca. 18-24 hr.) were employed during the methanolyse of methylated polysaccharides in order to achieve adequate depolymerisation.

Small-scale hydrolyses.

Hydrolyses were carried out by heating the sample with the given normality of acid on a boiling water-bath. Where sulphuric acid was used, barium hydroxide solution was added

until the solution was almost neutralised; this was completed by addition of solid barium carbonate. The solution was filtered and the filtrate evaporated to dryness. If uronic acids were present the filtrate was first stirred with cation exchange resin for ca. $\frac{1}{2}$ hour to convert the barium salt of the acid into the free acid.

Hydrochloric acid hydrolysates were neutralised with excess silver carbonate and filtered. The residue was washed thoroughly with water, filtered and the filtrates combined. The solution was evaporated to dryness. The products of methanolysis were also neutralised with silver carbonate.

Methoxyl determinations.

The determination of methoxyl content was carried out by means of the semi-micro Zeisel method (94).

Demethylations (51)

The sugar derivative (1-10 mg.) was dissolved or suspended in dry, purified methylene chloride (1-2 ml.) and cooled in acetone/dry ice. Boron trichloride (1-2 g.) cooled in the same way, was added. The mixture was kept at -80° for 30 mins., allowed to warm to room temperature and kept for 16 hours under anhydrous conditions. Any solvent or BCl_3 remaining was removed at low pressure. Boric acid was removed by adding methanol (3 x 3 ml.) and evaporating.

Small-scale periodate oxidations.

This was carried out by the method of Lemieux and Bauer (50). Methylated sugar (1-2 mg.) was dissolved in 0.5M sodium metaperiodate solution (0.2 ml.) and kept at 0°C for 1 hour. The excess of periodate was destroyed by the addition of ethylene glycol (1 drop) and the solution, after 5 mins., was made alkaline to phenolphthalein. The solution was taken to dryness, extracted with acetone, taken to a syrup and examined chromatographically in solvent D. The following results were obtained for some standard sugars.

<u>Sugar</u>	<u>R_G</u>	<u>Oxidation Products</u>
		<u>Colour with p-anisidine HCl</u>
2- <u>O</u> -Me-aldohexoses	0.20	brilliant yellow
3- <u>O</u> -Me-glucose	0.35	plum
4- <u>O</u> -Me-glucose	0.53	citrine
6- <u>O</u> -Me-glucose	0.71	canary yellow
2,4-di- <u>O</u> -Me- <u>D</u> -galactose (unaffected)	0.50	pink/brown
2,6-di- <u>O</u> -Me- <u>D</u> -galactose	0.20	brilliant yellow
2,3-di- <u>O</u> -Me- <u>D</u> -galactose	0.66, 0.78 0.86	grey, brown, grey
3,4-di- <u>O</u> -Me- <u>D</u> -galactose	0.93	pink

Aniline derivatives

The methylated sugar was refluxed in ethanolic

redistilled aniline (equimolar quantities) for 30 mins., in the dark. Evaporation of the solvent gave the aniline derivative, which was generally recrystallised from ethyl acetate.

Aldonolactones.

The methylated sugar (10-100 mg.) was dissolved in water (2-3 ml.), bromine (5-20 drops) was added, and the mixture was kept in the dark at room temperature for three days. Excess bromine was removed by aeration and the solution evaporated to dryness. The residue was extracted with ether and the lactone recrystallised by slow evaporation of an ethanolic solution.

Aldonamides.

The lactone, prepared as above, was dried in a vacuum desiccator, dissolved in dry methanolic ammonia (prepared by bubbling ammonia gas into dry, ice-cooled methanol, until saturation) and left in the ice box for 2 days. Evaporation of the solvent gave the crystalline amide which was recrystallised from the given solvent.

Di-No₂-benzoates.

The methylated sugar (15 mg.) was dissolved in dry pyridine (2 ml.) and treated with p-nitro benzoyl chloride (recrystallised from pyridine) (50 mg.) for 30 mins. at 65-70°C. After leaving overnight at room temperature,

a saturated solution of sodium bicarbonate was added dropwise to the reaction mixture until no further effervescence occurred. Water (5 ml.) was added, and the product extracted with chloroform (3 x 15 ml.). The chloroform extract was dried (anhydrous sodium sulphate) and evaporated to small volume. Addition of light petroleum and leaving in the ice-box for a time induced crystallisation of the derivative, which was filtered off and recrystallised from methanol.

Phenylosazones.

Sugar (10 mg.) was heated for 30 mins. on a boiling water bath with 0.01 ml. each of phenylhydrazine (redistilled) and glacial acetic acid, water (0.25 ml.), and a drop of saturated sodium bisulphite solution. On cooling and adding water (ca. 1.5 ml.), the phenylosazone was precipitated and was recrystallised from boiling water.

Acetyl determinations.

The acetyl content of an acetylated polysaccharide was determined by saponifying with sodium hydroxide, acidifying and distilling off the acetic acid, which was then titrated with standard alkali (94).

Reductions.

a) Methylated polysaccharide. An aliquot of tetrahydrofuran was purified by allowing to stand over sodium wire

for 48 hours, distilling over fresh sodium and finally distilling from lithium aluminium hydride. Methylated gum (100 mg.) was dissolved in tetrahydrofuran (3 ml.) and lithium aluminium hydride (100 mg.) in tetrahydrofuran (3 ml.) was added. After 0.5 hrs. at room temperature, the mixture was refluxed for 3 hours. Excess of hydride was destroyed with ethyl acetate and water. The resulting mixture was shaken with dilute sulphuric acid and the reduced methylated polysaccharide extracted with chloroform.

b) Aldobiouronic acid. The acidic material was converted to the methyl ester methyl glycoside by heating in a sealed tube with methanolic hydrogen chloride (2.5%) for 3 hours. After dissolving in the solvent, lithium aluminium hydride was added, and the mixture refluxed, with exclusion of moisture, for 1 hour; more hydride was then added and refluxing continued for a further hour. The mixture was worked up as above.

c) Sugar to sugar alcohols. Equal weights of sugar and sodium borohydride were dissolved in water and the solution allowed to stand overnight at room temperature. Excess hydride was then destroyed and potassium ions removed by shaking the solution with Amberlite resin 1R-120(H⁺) till the solution was just acidic. The filtrate was taken to dryness and the borate ions were removed by repeated evaporation with methanol.

Preparation and Purification of Purdie's reagents.

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

The silver oxide was prepared by addition of sodium hydroxide (40 g. in 500 ml.) to a solution of silver nitrate (170 g. in 500 ml.) and washing the product with cold water (4 l.) and hot water (1 l.). After filtering with suction and draining the water completely, the silver oxide was ground with acetone (1 l.), filtered and finally washed with ether to dry. The silver oxide was further dried in vacuum desiccator and stored in a dark bottle.

Estimation of sugars.

a) Anthrone method (36). A standard curve for this reagent was made from the polysaccharide A. Disadvantages of the method were that the reagent had to be prepared fresh every day and that the colours produced were only stable for a limited period.

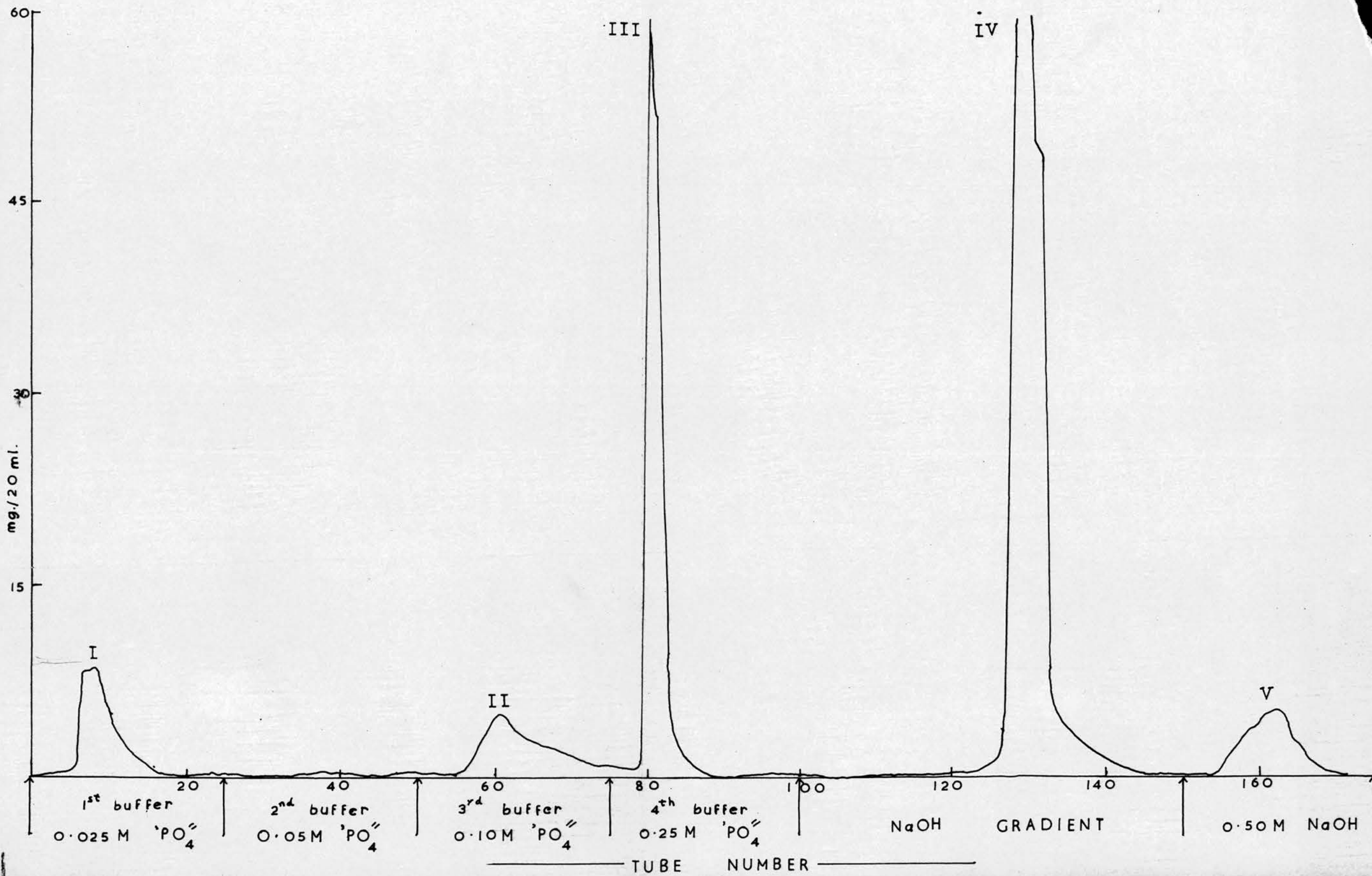
b) Phenol-sulphuric acid method (37). This was the method used for sugar estimations, unless otherwise stated.

Uronic acid anhydride determinations.

a) Carbazole method (95). Uronic acid anhydride contents estimated by this reagent, were not altogether reliable but useful as an indication on a small scale.

b) Decarboxylation method (96). This method is much more accurate but more material is required.

FRACTIONATION OF KHAYA SENEGALENSIS GUM ON DEAE-CELLULOSE



Purification and Fractionation of the Gum.

DEAE-cellulose chromatography of Khaya senegalensis gum.

Crude gum (1.2 g.) was left to soak overnight in water (35 ml.). Sodium hydroxide (1.4 g.) was added, i.e. a N solution was now obtained, and the solution stirred for about 8 hours. After filtering through muslin, the solution was stirred with Amberlite resin 1R-120, until the pH was 6 (ca. 4 hours). The sugar concentration of the solution was estimated (26 mg. per ml.) and an aliquot (30 ml.) of this polysaccharide solution pipetted onto the DEAE-cellulose column, which had been prepared in the usual way. The gum (780 mg.) was allowed to soak into the column overnight. The column was eluted successively with

- a) 0.025 M (500 ml.)
 - b) 0.05 M (500 ml.)
 - c) 0.10 M (500 ml.)
 - d) 0.25 M (500 ml.)
 - e) aqueous sodium hydroxide gradient (0-0.3M) (2 litres)
 - f) sodium hydroxide (0.5M).
- } of sodium dihydrogen phosphate
} buffer, pH 6

Fractions (10 ml.) were collected every half hour and the amount of polysaccharide present in the fractions was determined by the phenol-sulphuric acid method. The curve obtained by plotting the polysaccharide content of each fraction against the fraction number is shown in Figure I. Five fractions were obtained.

Fraction I, (tubes 6-15). This fraction was bulked as indicated, and after evaporating to small volume was stirred with mixed resins and freeze dried (550 mg.). No dialysis was attempted since the carbohydrate material was suspected to be of low molecular weight. On analysis of this material for sugar content, it was found that the majority of the product was ionic material and the remainder carbohydrate material (40 mg.).

The freeze-dried product was dissolved in water (20 ml.) and placed on a column (9 x 2 cm.) of Ultrasorb charcoal (86) which had been prepared by washing with water (500 ml.) to remove alkaline water-soluble matter. Elution was effected with water until the eluent was phosphate free (silver nitrate test). Desorption of the carbohydrate material was carried out using 25% aqueous ethanol (200 ml.). The sugar solution obtained was evaporated to dryness and examined chromatographically. The main constituent was found to be arabinose, with traces of galactose and some unidentified oligosaccharides.

Fraction II (tubes 56-75) and Fraction III (Tubes 80-85) were bulked as indicated. The resulting sugar solutions were dialysed against tap water for 2 days, stirred with mixed resins, evaporated to small volume and freeze-dried to give two polysaccharide products (40 and 80 mg. respectively). These two polysaccharides had the same specific rotation, $[\alpha]_D = +11$ (c, 1.0) and the same uronic anhydride

content (21.5%). On chromatographic examination of the products of acid hydrolysis (2N acid, 6 hrs.) they were shown to be made up of the same monosaccharide residues - galactose, arabinose and 4-O-methyl-glucuronic acid. No differences could be detected between these two polysaccharide fractions and so they were combined.

Fraction IV (tubes 125-155) gave a polysaccharide (400 mg.) having $[\alpha]_D +136^\circ$ (c , 0.54) and uronic acid anhydride content = 55%. Hydrolysis of this polysaccharide with 2N acid for 6 hours gave rhamnose, galactose, galacturonic acid, 4-O-methyl-glucuronic acid and a trace of arabinose.

Fraction V was obtained on bulk elution of the column with 0.5 N sodium hydroxide. This was shown to be cellulose bleeding from the column since it had no uronic acid content (carbazole method) and since on hydrolysis and subsequent chromatographic examination, only glucose was obtained.

Khaya senegalensis gum, therefore must consist of only two polysaccharides, polysaccharide A (Fraction IV) and polysaccharide B (Fractions II and III).

Fractional Precipitation of the Gum (17).

Finely ground gum (20 g.) was stirred with N-sodium hydroxide (500 ml.) for 6 hrs., and the resulting solution, after being kept overnight, was poured into ethanol containing concentrated hydrochloric acid (ethanol concentration of resulting solution was 69%). The gel, which separated,

was removed at the centrifuge and the supernatant liquid (as also from subsequent reprecipitations) was immediately neutralised by the addition of calcium carbonate. The gel was dispersed in water and reprecipitated 5 times from aqueous solution by pouring this solution into ethanol (net ethanol concentration was 72% for the first and 62% for subsequent reprecipitations). This procedure furnished fraction I (7.5 g.) $[\alpha]_D + 136^\circ$ (c , 0.095) uronic acid anhydride 55%.

From the combined supernatant liquors neutralised with calcium carbonate there was isolated (as insoluble calcium salt) fraction 2. (4.3 g.), $[\alpha]_D + 7.5$ (c , 1.06), uronic acid anhydride content 27-33%.

Fraction 1 was examined for homogeneity by chromatography on a DEAE-cellulose column in the same way as for the crude gum. A small peak was obtained corresponding to polysaccharide B but the majority of the fraction corresponded to polysaccharide A. The supposedly pure polysaccharide A fraction was recovered and found to have $[\alpha]_D 140^\circ$ (c , 1.01) and uronic anhydride 55%.

DEAE-cellulose chromatographic examination of fraction 2 showed it to be heterogeneous consisting of approximately equal amounts of polysaccharides A and B. Polysaccharide B was isolated and had $[\alpha]_D + 11^\circ$ (c , 0.52) and uronic acid content 20.9%. Hydrolysis of a small sample gave galactose,

arabinose and 4-O-methyl-glucuronic acid but no trace of rhamnose could be detected.

Method for obtaining homogeneous polysaccharide B.

Since the samples of polysaccharide B, isolated by DEAE-cellulose chromatography have been shown to have no rhamnose on hydrolysis, the absence of this sugar residue was now used as a ready criterion of purity; corroborated by the value obtained for the uronic acid anhydride content.

Fractional precipitation experiments were carried out using varying concentrations of ethanol and water until a method could be devised for obtaining pure polysaccharide B on a preparative scale. This was achieved as follows.

The gum (100 g.) was allowed to swell in water (1350 ml.) overnight. Sodium hydroxide (53 g.) was added slowly and stirring continued for 7 hours, after which time most of the gum had gone into solution. After filtering through muslin, a white gel was obtained on adding the filtrate with stirring to ethanol (4240 ml.) containing HCl (200 ml.). This was allowed to settle and coagulate overnight at 0°C and separated from the supernatant liquid by decantation and centrifugation. From the supernatant liquor, neutralised with calcium carbonate there was isolated (as insoluble calcium salt) polysaccharide B (4.0 g.). The gum acid was obtained on stirring with cation exchange resin. No rhamnose was detected on hydrolysis of this polysaccharide

and DEAE-cellulose chromatography gave only one peak.

Since the yield of polysaccharide B was so small (ca. 4%) extensive extraction of crude gum had to be carried out. In every instance the sample of polysaccharide B obtained was checked for homogeneity by examination of the polysaccharide for the presence of rhamnose and if found to be heterogeneous was discarded.

Attempt to obtain pure polysaccharide A.

A sample (800 mg.) of polysaccharide A, isolated by DEAE-cellulose chromatography, was subjected to several Haworth methylations, with the usual precautions being taken. Aliquots of dimethyl sulphate (12 ml.) and 30% sodium hydroxide (24 ml.) were added on five successive days. On working up, a syrup (490 mg.) of partly methylated polysaccharide was obtained. Conversion of the methylated polysaccharide to its silver salt, was followed by two Purdie methylations, using methyl iodide (10 ml.) and silver oxide (5 g.) in methanol (5 ml.). The methylated polysaccharide obtained (350 mg.) had OMe, 40.3%.

TABLE 2.

Methyl glycosides of	<u>T</u> in system a		<u>T</u> in system b	
	Methylated Gum	Reduced Methylated Gum	Methylated Gum	Reduced Methylated Gum
2,3,5-Tri-O-methyl-L-arabinose	0.55 & (0.72)	0.55 & (0.72)	(0.46) & (0.60)	(0.46) & (0.60)
2,3,4-Tri-O-methyl-L-rhamnose	0.45	0.45	(0.46)	(0.46)
3,4-Di-O-methyl-L-rhamnose	(0.72) & 1.01	(0.72) & 1.02	(0.60)	(0.61)
3-O-Methyl-L-rhamnose	3.60	(3.72)	1.01	1.02
2,3,4,6-Tetra-O-methyl-D-galactose	1.80	1.80	(1.57) & 1.52	(1.59)
2,3,6-Tri-O-methyl-D-galactose	(3.21) & (4.66)	3.21 & 4.70	(1.57) & (2.49)	(1.59) & 2.50
2,3,4-Tri-O-methyl-D-glucuronic acid	(3.21) & 2.49		2.18 & 1.72	
2,3-Di-O-methyl-D-glucuronic acid			3.18 & (2.49)	
2,3-Di-O-methyl-D-galacturonic acid	5.30		6.2 & 7.35	
2,3,4-Tri-O-methyl-D-galactose	7.5	7.5	2.88	2.88
2,4,6-Tri-O-methyl-D-galactose	(4.70)			2.08
2,4-Di-O-methyl-D-galactose			4.40	4.40
2,3-Di-O-methyl-D-galactose				2.50 & 4.20
2,3,4-Tri-O-methyl-D-glucose		(3.72) & 2.62		1.82 & 1.34
2,3-Di-O-methyl-D-glucose				3.27

When a T value corresponds to more than one methyl glycoside it is given within parenthesis.

A portion (200 mg.) of methylated polysaccharide A was reduced with lithium aluminium hydride in tetrahydrofuran.

The methanolysis products of the methylated polysaccharide and the reduced methylated polysaccharides were examined by vapour phase chromatography in both systems. It is not possible to interpret unambiguously the complicated patterns obtained but since we already have an idea of the possible methylated sugars present, from previous studies (17) on methylated polysaccharide A, we can assign the major peaks with fair accuracy. Table 2 gives the \underline{T} values of the methyl glycosides of the methylated sugars present.

DEAE-Cellulose chromatography of Polysaccharide A.

A sample (1.30 g.) of polysaccharide A, obtained by fractional precipitation, was chromatographed on a DEAE-cellulose column. The column was eluted successively with the following:

- a) 0.25 \underline{M} sodium dihydrogen phosphate buffer pH 6 (700 ml.)
- b) 0.50 \underline{M} sodium dihydrogen phosphate buffer pH 6 (1000 ml.)
- c) 0.50 \underline{M} potassium chloride (500 ml.).

The eluate was changed after making sure, by testing a few ml. of the eluate with phenol-sulphuric acid, that no more polysaccharide was coming off with the preceding eluant.

The eluates were collected separately in bulk and after carrying out the usual procedure of dialysis, deionisation and freeze-drying, three polysaccharide fractions were

obtained as shown in table 3.

TABLE 3

Buffer	Fraction	Yield (mg.)	$[\alpha]_D$
0.25 <u>M</u> phosphate	i	225	+130° (<u>c</u> , 1.1)
0.50 <u>M</u> phosphate	ii	812	+139° (<u>c</u> , 1.01)
0.50 <u>M</u> KCl	iii	41	+140° (<u>c</u> , 1.05)

Methylation of Fraction ii.

Fraction ii (250 mg.) was methylated by the Kuhn procedure (42) using barium oxide (1.7 g.), barium hydroxide (1.7 g.) and dimethyl sulphate (3.5 ml.) in dimethyl sulphoxide (10 ml.) and dimethyl formamide (10 ml.). Examination of the cleavage products of the methylated product (175 mg.) by paper and gas phase chromatography indicated that the reaction had not gone to completion and that further methylation was necessary. Methylation of partly methylated polysaccharide (165 mg.) was completed with methyl iodide (1 ml.) and silver oxide (1.0 g.) in dimethyl formamide (3 ml.) (87).

The methylated polysaccharide (144 mg.) had $-OMe = 40.9\%$. This indication of complete methylation was confirmed on examination of the methanolysis products of a small sample by gas/liquid chromatography. These gas phase chromatographic

results were almost identical to those obtained from the previous methylation of polysaccharide A. Most importantly, there was still an appreciable amount of 2,3,5-tri-O-methyl-L-arabinose present. Retention times of the methyl glycosides of this methylated derivative were:-

Column a:- 0.54 and 0.70

Column b:- 0.47 and 0.61

DEAE-Cellulose chromatography of Fraction ii (i.e. 2 X DEAE-cellulose fractionation of polysaccharide A).

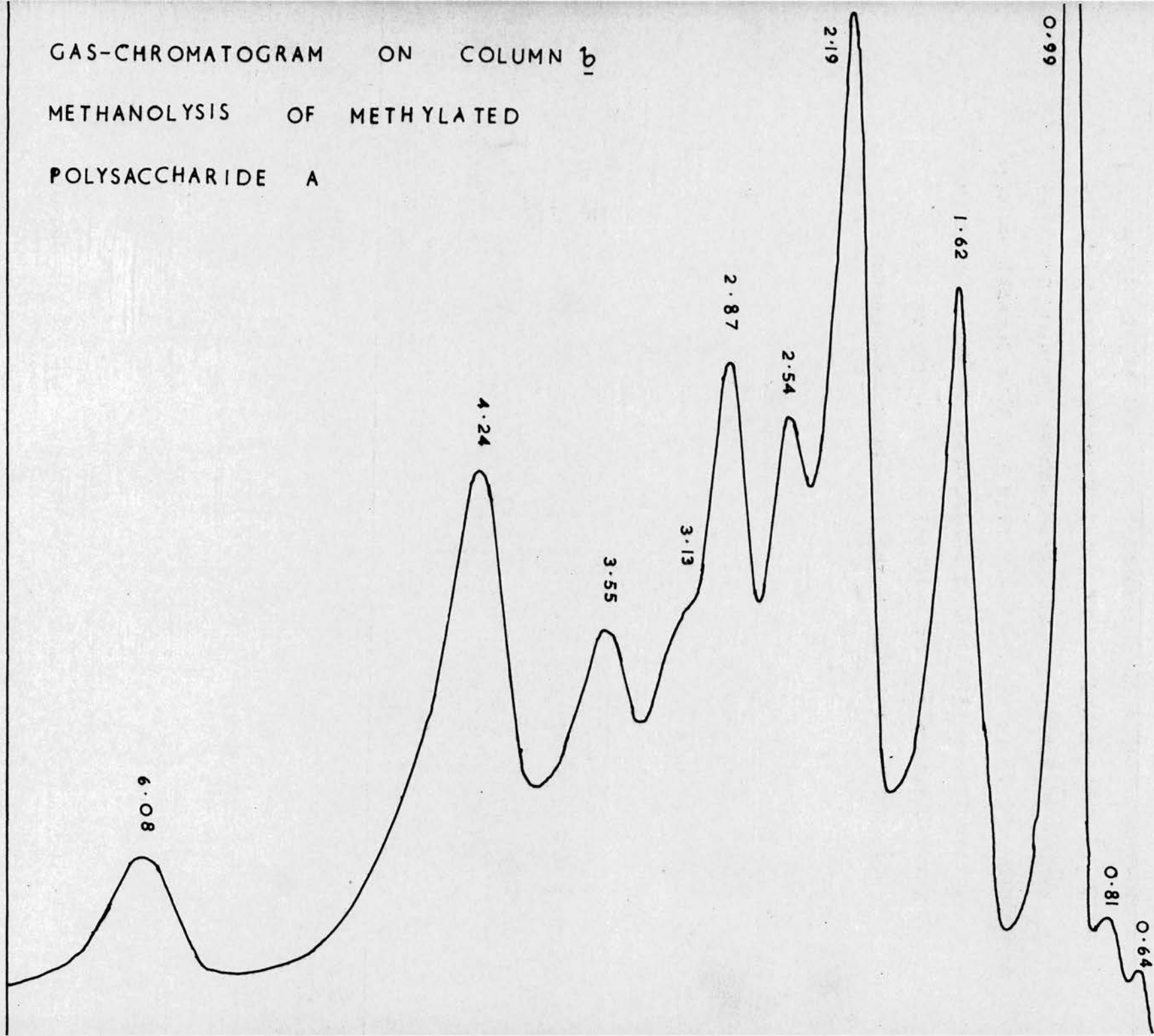
Fraction ii (150 mg.) from the DEAE-cellulose chromatographic fractionation of polysaccharide A was further fractionated on DEAE-cellulose. Two peaks were obtained. A small one, corresponding to contaminating polysaccharide B, represented ca. 4% of the whole. The fraction containing polysaccharide A was worked up in the usual way and again chromatographed on DEAE-cellulose. This time only one peak was obtained, indicating that this fraction was pure, homogeneous polysaccharide A. This polysaccharide was methylated in exactly the same way as fraction ii (i.e. by two Kuhn methylations) to give methylated polysaccharide A (102 mg.), OMe 41.1%.

A small sample of the methylated polysaccharide was depolymerised with methanolic hydrogen chloride and the derived methyl glycosides examined by vapour phase chromatography in both systems. No evidence for the presence of

GAS-CHROMATOGRAM ON COLUMN b

METHANOLYSIS OF METHYLATED

POLYSACCHARIDE A



2,3,5-tri-O-methyl-arabinose could be seen, confirming that the sample of polysaccharide A was homogeneous. It is interesting to note that there was an appreciable amount of 2,4-di-O-methyl-D-galactose present (T = 4.24 and 3.55 in column b) since this had been thought to have arisen from contaminating polysaccharide B. The vapour phase chromatogram on column b for homogeneous polysaccharide A is shown in the accompanying diagram.

PARTIAL HYDROLYSIS STUDIES ON POLYSACCHARIDE B

Samples (100 mg.) of polysaccharide B were hydrolysed at 100°C with a) 0.5 N sulphuric acid, b) N sulphuric acid and c) 2N sulphuric acid. Aliquots (5 ml.) were removed from the hydrolysates (initially 20 ml.) at regular intervals, neutralised in the usual way, cations removed with Amberlite resin LR120 and examined by paper chromatography in solvents A and B. The optimum conditions for the formation of acidic disaccharides appeared to be hydrolysis with N sulphuric acid for 4 hours.

A small scale partial hydrolysis was carried out on a sample (750 mg.) of polysaccharide B with N sulphuric acid for 4 hours. After neutralisation and removal of the cations with cation exchange resin, the hydrolysate was separated into a neutral and an acidic component by chromatography on anion exchange resin.

Examination of the acidic sugars by paper chromatography indicated the presence of two oligosaccharides, aldobiouronic acid A. R_{GAL} 0.19 in solvent B and 0.50 in solvent C and aldobiouronic acid B R_{GAL} 0.63 in solvent B and 0.77 in solvent C. In addition there was a large amount of acidic monosaccharides present.

Isolation and Characterisation of the Acidic Oligosaccharides

Polysaccharide B (4.0 g.) was hydrolysed with N-sulphuric

acid (100 ml.) for 4 hours at 100°C. The hydrolysate was cooled, neutralised with barium hydroxide and barium carbonate and the barium ions removed by stirring with cation exchange resin. The sugar solution obtained was evaporated to small volume (5 ml.) and placed on an Amberlite CG45 resin column (350 ml. resin) which had been regenerated with 4% sodium hydroxide (6 bed vols.), converted to the formate form with 15% formic acid (6 bed vols.) and finally washed with CO₂ free water, until the eluate was acid free. After allowing the sugars to soak well in over two nights, the neutral sugars were eluted with CO₂ free water until the eluate gave a blank reading with phenol-sulphuric acid reagent. The solution containing the neutral sugar residues was evaporated to a syrup (2.33 g.).

The acidic sugars were desorbed from the column with increasing concentration of formic acid. The following elution pattern was used:-

	Eluent	Total vol.	Size of Fraction	No. of tubes
1	0-5% formic acid gradient	1000 ml.	10 ml.	1-100
2	5% formic acid	500 ml.	10 ml.	101-150
3	5-15% formic acid gradient	1000 ml.	20 ml.	151-200
4	15% formic acid	500 ml.	20 ml.	201-225
5	25% formic acid	500 ml.		Bulk recovery

Samples were removed from every fifth tube and examined by paper chromatography in solvent C (developed for 16 hours). All the sugars were eluted into tubes 40-130, i.e. all were eluted with 5% formic acid. The different acidic sugars were not completely resolved however and so the tubes could only be divided into three fractions, none of these fractions being homogeneous. The different fractions obtained are shown in table 4. The recovery from the column was 92%

During the small scale partial hydrolysis, formyl esters (detected by alkaline hydroxylamine, ferric chloride (88)) were formed when the formic acid, sugar containing solutions were being evaporated to dryness. To avoid this here, before evaporating, as much of the formic acid as possible was removed by means of repeated extractions with ether.

Fractions I, II and III, from the resin column fractionation of the acidic oligosaccharides, were sub-fractionated on filter sheets (Whatman No.31) by developing in solvent C for $3\frac{3}{4}$ hours (for Fractions II and III). Difficulty was encountered in locating the position of the different sugars; side strips could not be used, since the chromatographic mobilities of the different fractions were too similar. Several indicator sprays were experimented with, chiefly bromo-thymol blue and bromocresol green, but inconclusive results were obtained.

TABLE 4

Fractions obtained from column	Wt. (in mg.)	Sub-fractions from filter-sheets	Wt. (in mg.)	Constituent Sugars
I (tubes 41-75)	110 (some material used for testing sprays)	a	10	unknown ($R_{GAL} 0.11$) + glucuronosyl-galactose
		b	20	glucuronosyl-galactose
		c	33	4-O-methyl-glucuronosyl-galactose
II (tubes 76-86)	223	a	17	unknown ($R_{GAL} 0.11$) + glucuronosyl-galactose
		b	34	glucuronosyl-galactose
		c	151	4-O-methyl-glucuronosyl-galactose
III (tubes 87-130)	832	a	75	glucuronosyl-galactose
		b	349	4-O-methyl-glucuronosyl-galactose
		c	36	contain mixtures of glucuronic acid, 4-O-methyl-glucuronic acid and glucuronolactone
		d	64	
		e	38	

The sugars were finally located by placing the filter sheets in the oven at 160°C for 3-4 mins. and immediately examining under u.v. light. The bands corresponding to the different sugars, fluoresced and were seen to be very irregular, but could be easily marked and cut out. The

fluorescence is believed to be due to the charring of the sugars. Fractions I, II and III were thus sub-fractionated as shown in table 4.

Fractions Ib, IIb and IIIa were combined to give the aldobiouronic acid A, detected during the small-scale partial hydrolysis. Fractions Ic, IIc and IIIb were similarly combined to give aldobiouronic acid B.

Identification and Characterisation of Aldobiouronic Acid A.

Aldobiouronic acid A ($R_{GAL} = 0.50$ in solvent C) had $[\alpha]_D -3^\circ$ ($c, 2.0$). Hydrolysis with 2N sulphuric acid for 4 hours and subsequent paper chromatographic examination of the products gave galactose and glucuronic acid ($R_{GAL} 1.60$), in equal proportions (visual examination only). The aldobiouronic acid (5 mg.) was converted to the methyl ester, methyl glycoside by heating with 2% methanolic hydrogen chloride for 2.5 hours. The glycoside was dissolved in water (1 ml.) and sodium borohydride (25 mg.) in water (1 ml.) added. After leaving at room temperature overnight the excess hydride was destroyed and the resulting neutral disaccharide hydrolysed with N sulphuric acid for 4 hours. On examination of the products by paper chromatography in solvent A, the presence of galactose and glucose was indicated. The latter must have arisen from reduction of glucuronic acid. Although no information was yet available as to the position of linkage between the glucuronic acid and

galactose residues, the aldobiouronic acid was chromatographically identical to 6-O- β -D-glucopyranosyl uronic acid-D-galactose in solvents B and C.

Methylation of aldobiouronic acid A.

A sample (100 mg.) of aldobiouronic acid A was dissolved in water (5 ml.) and 1 ml. each of dimethyl sulphate and 30% sodium hydroxide added during a period of 1-2 hours. The reaction mixture was kept in an ice-bath and the solution stirred vigorously (magnetic stirrer) in an atmosphere of nitrogen. Aliquots of dimethyl sulphate (10 ml.) and 30% sodium hydroxide (20 ml.) added over a period of 5 hours; similar additions were made on four successive days. The mixture was heated on a boiling water bath for 30 mins. to destroy unreacted dimethyl sulphate, acidified with dilute sulphuric acid till just acidic and the sodium sulphate precipitated by addition of methylated spirits. After filtering, the residue was washed thoroughly with methylated spirits and the filtrate and washings combined. After making the solution slightly alkaline, it was concentrated to a small volume (50 ml.), made acidic and extracted with chloroform (4 x 100 ml.) in the cold. The chloroform extracts were concentrated, filtered and the methylated disaccharide precipitated by addition of light petroleum (b.p. 60-80°). The partly methylated product (80 mg.) was methylated to completion with methyl iodide (4 ml.)

and silver oxide (2 g.) in dry methanol (3 ml.). The mixture was filtered and the residue continuously extracted with boiling chloroform overnight. The extracts and filtrate were combined and evaporated to a syrup. Another Purdie methylation was carried out in an analagous manner, but the methoxyl content was not increased.

The methylated derivative (50 mg.) had $-OMe = 52.5\%$ and was recrystallised from acetone-light petroleum (b.p. $40-60^{\circ}$). A minimum of acetone was used, and after leaving at $0^{\circ}C$ for several weeks, rosette like crystals were obtained which had m.p. = 90° unchanged on mixing with authentic methyl ester of methyl hexa-O-methyl-6-O- β -D-glucopyranosyl uronic acid-D-galactoside. These crystals had $[\alpha]_D = -40^{\circ}$ (c , 0.42 in $CHCl_3$) and gave an identical powder photograph to the one obtained from the authentic specimen.

Identification and Characterisation of Aldobiouronic Acid B

Aldobiouronic acid B ($R_{GAL} 0.63$ in solvent B) had $[\alpha]_D +48^{\circ}$ (c , 1.0). Hydrolysis of this disaccharide with 2N sulphuric acid for 5 hours and subsequent paper chromatographic examination of the products in solvent B gave galactose and 4-O-methyl glucuronic acid ($R_{RHA} 0.98$) in equal proportions (visual examination only).

The acidic disaccharide (150 mg.) was converted into the methyl ester, methyl glycoside by heating with 3%

methanolic hydrogen chloride for 4 hours. The resulting syrup was dissolved in water (3 ml.) and sodium borohydride (150 mg.) was added. After leaving the mixture at room temperature overnight, the excess hydride was destroyed and cations were removed with Amberlite resin 1R120. Boric acid was removed by evaporating the solution several times to dryness with methanol. The resulting methyl glycoside of the neutral disaccharide was hydrolysed with 2N sulphuric acid for 4 hours. After neutralisation and evaporation, a syrup (100 mg.) was obtained, which on paper chromatographic examination in solvent A was found to be composed of galactose and 4-O-methyl-glucose (R_{RHA} 0.87). The latter must have arisen from reduction of 4-O-methyl-glucuronic acid.

The syrup was fractionated on a filter sheet in solvent A. 4-O-Methyl-D-glucose (32 mg.) had $[\alpha]_D +60^\circ$ (c , 0.53). It was converted to the phenylosazone in the usual way by treatment with freshly distilled phenylhydrazine (0.2 ml.) in glacial acetic acid (0.2 ml.). On recrystallisation from aqueous alcohol orange crystals were obtained m.p. 156°C , not depressed on mixing with authentic 4-O-methyl-D-glucose phenylosazone. This derivative had $[\alpha]_D = -20^\circ$ (c , 0.31 in pyridine) and its X-ray powder photograph was identical to that obtained from the authentic sample.

D-Galactose (43 mg.) had $[\alpha]_D = +76$ (equil.) (c , 0.40) and was characterised by recrystallisation from absolute ethanol, m.p. and mixed m.p. 156° .

Periodate oxidation of aldobiouronic acid B.

A standard curve for formaldehyde estimation was prepared using a standard erythritol solution. 1 Mole of erythritol, when oxidised with periodate yields quantitatively 2 moles of formaldehyde. The reaction was buffered at pH 8 and a 0.5% sodium periodate solution was employed. The amount of formaldehyde released was estimated by treating with chromotropic acid reagent (91) and measuring the optical density of the resulting solution at $570 \text{ m}\mu$.

A sample (4.00 mg.) of aldobiouronic acid B was dissolved in water (3 ml.). To this solution, 0.5 M phosphate buffer (1 ml.) and 1% sodium periodate solution (1 ml.) were added. The mixture was left in the dark for 24 hours to ensure that the reaction had gone to completion, after which time an aliquot (1 ml.) was removed and the amount of formaldehyde present estimated.

The formaldehyde released ($15.7 \mu \text{ g.}$) corresponded to an amount ($186 \mu \text{ g.}$) of a 2, 3 or 4 linked disaccharide; so the latter must be present as 23% of the mixture.

Methylation of aldobiouronic acid B.

1) Aldobiouronic acid B (151 mg.) was converted to its fully methylated derivative by the same methylation procedure as was used for the aldobiouronic acid A. The methylated derivative was recrystallised from acetone-light petroleum (b.p. 40-60°) giving rosette-like crystals, m.p. 89°, not depressed on mixing with an authentic sample of the methyl ester of methyl hexa-O-methyl-6-β-D-glucopyranosyluronic acid-D-galactoside. These crystals had OMe = 51.9%, $[\alpha]_D = -35^\circ$ (c, 1.23 in CHCl₃) and gave an identical powder photograph to that obtained from the authentic sample.

2) The remainder (105 mg.) of aldobiouronic acid B was methylated with additions of dimethyl sulphate (5 ml.) and 30% sodium hydroxide (10 ml.) on five successive days. After working up in the usual way, the methylated disaccharide mixture was obtained. A sample was methanolised with 5% methanolic hydrogen chloride overnight and the derived methyl glycosides examined by vapour phase chromatography in both systems.

The methylated aldobiouronic acid mixture (75 mg.) was converted into the methyl ester, methyl glycosides by heating with methanolic hydrogen chloride (2.5%) for 2 hours in a sealed tube at 100°. After neutralisation, the derived methyl esters were reduced with lithium aluminium hydride in tetrahydrofuran. The methanolysis products of this reduced material were examined by vapour

phase chromatography. The results for the reduced methylated aldobiouronic acid mixture and for the unmodified derivatives are shown in table 5.

TABLE 5.

Methyl Glycosides of	<u>T</u> for methyl glycosides from methylated aldobiouronic acids	<u>T</u> for methyl glycosides from reduced methylated aldobiouronic acids
2,3,4-Tri-O-methyl-galactose	2.85 & 2.58	2.86 & 2.58
2,3,6-tri-O-methyl-galactose	1.63	1.66 & 2.24
2,3,4-tri-O-methyl-glucose		1.80 & 1.33
2,3,4-tri-O-methyl-glucuronic acid	2.18 & 1.74	

T is retention times for column b.

Methyl Glycosides of	<u>T</u> for methyl glycosides from methylated aldobiouronic acids	<u>T</u> for methyl glycosides from reduced methylated aldobiouronic acids
2,3,4-Tri-O-methyl-galactose	7.4	7.4
2,3,6-tri-O-methyl-galactose	4.68	3.19 & 4.65
2,3,4-tri-O-methyl-glucose		3.70 & 2.59
2,3,4-tri-O-methyl-glucuronic acid	3.22 & 2.49	

T is retention times for column a.

An attempt was made to isolate the 2,3,6-tri-O-methyl-galactose from the remainder (60 mg.) of the reduced methylated aldobiouronic acid B. Gradient elution with aqueous methyl ethyl ketone (0-5%) on charcoal Celite column was used and the elution pattern followed by gas phase chromatography. A pure sample of the required methylated sugar could not be obtained.

METHYLATION STUDIES ON POLYSACCHARIDE B

Methylation of Polysaccharide B.

A sample (10 g.) of polysaccharide B was dissolved in N sodium hydroxide (250 ml.) and methylated in an atmosphere of nitrogen at 0-5°C. Dimethyl sulphate (120 ml.) and sodium hydroxide solution (62 g. in 140 ml. H₂O) were added dropwise with stirring over a period of about 8 hours and continued stirring overnight. Four additions of dimethyl sulphate (120 ml.) and 30% sodium hydroxide solution (240 ml.) were made on four successive days. Secondary octyl alcohol was added occasionally to control frothing. The reaction mixture was heated on a boiling water-bath for 30 mins. On cooling, a quantity of sodium sulphate precipitated out; this was filtered and the filtrate dialysed in running water for 3 days. The partly methylated polysaccharide (4.5 g.), which was isolated by concentrating the aqueous solution to small volume and freeze-drying, had OMe, 32.3%.

The methylated polysaccharide (4.5 g.) was dissolved in water (150 ml.), stirred with Amberlite resin 1R120 to remove sodium ions, treated with silver carbonate, filtered, concentrated and freeze-dried. Yield of silver salt - 5.0 g.

The silver salt (5 g.) was dissolved in the minimum volume (20 ml.) of dry methanol. Methyl iodide (65 ml.) was added and the mixture refluxed for 10 hours with the

addition of silver oxide (8 g.) at intervals. The residue was filtered and extracted repeatedly with boiling chloroform. The combined filtrate and extract were concentrated to a small volume and the methylated gum was precipitated by addition of excess light petroleum (b.p. 60-80°). The methylated polysaccharide (3.8 g.) had OMe, 37.1%.

Another Purdie methylation was carried out in a similar manner and the isolated methylated product had an increased methoxyl content. Another Purdie methylation could not increase this any further.

Methylated polysaccharide B (3.5 g.) had $[\alpha]_D = -26.5^\circ$ (c , 0.49 in CHCl_3) and OMe, 41.1%.

A sample (100 mg.) of the methylated polysaccharide was dissolved in tetrahydrofuran (3 ml.) and reduced with lithium aluminium hydride (100 mg.) in the usual way. The resulting reduced methylated polysaccharide was remethylated in dimethyl formamide (3 ml.) with methyl iodide (1.0 ml.) and silver oxide (1.0 g.)

The methanolysis products of the methylated polysaccharide and the reduced methylated polysaccharide after remethylation were examined by gas phase chromatography in both solvent systems. The results obtained are shown in table 5. When a \underline{T} value corresponds to more than one methyl glycoside it is given within parenthesis.

TABLE 5.

Methyl glycosides of	<u>T</u> in system <u>a</u>		<u>T</u> in system <u>b</u>
	Methylated polysaccharide	Methylated Red. ^a Methylated Polys.	
2,3-Di-O-methyl-L-arabinose	1.58		0.64
2,4-di-O-methyl-L-arabinose		2.30	1.10
2,5-di-O-methyl-L-arabinose			0.70
2,3,4-tri-O-methyl-L-arabinose	1.04	1.11	0.83
2,3,5-tri-O-methyl-L-arabinose	0.55 & 0.74	0.56 & 0.72	0.46 & 0.59
2,3,4,6-tetra-O-methyl-D-galactose	1.80	1.80	1.57 & 1.61
2,3,4-tri-O-methyl-D-galactose	7.5	7.42	2.62 & 2.92
2,3,6-tri-O-methyl-D-galactose	(3.24) & (4.77)	3.26 & (4.71)	(1.61)
2,4,6-tri-O-methyl-D-galactose	4.16 & (4.77)	4.23 & (4.71)	2.09 & 2.39
2,4-di-O-methyl-D-galactose			(3.70) & 4.40
2,6-di-O-methyl-D-galactose			(3.70)
2,3,4-tri-O-methyl-D-glucuronic acid	2.49 & (3.24)		1.78 & 2.23
2,3,4,6-tetra-O-methyl-D-glucose		1.0 & 1.44	
2,3,4-tri-O-methyl-D-glucose *		3.71 & 2.59	

0.46

0.59

0.64

0.70

0.82

1.01

1.51

1.61

1.79

2.09

2.23

2.39

2.62

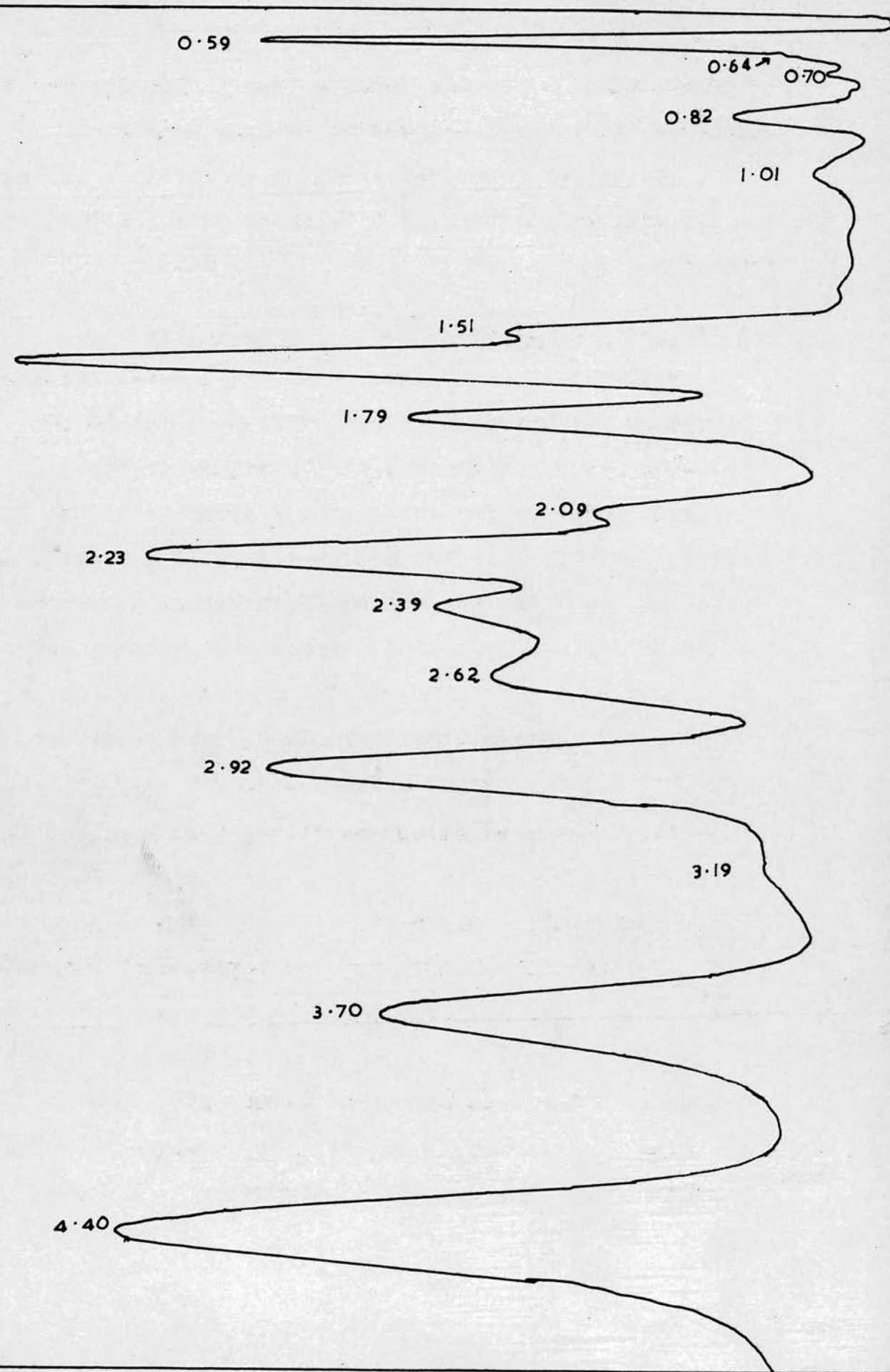
2.92

3.19

3.70

4.40

GAS-CHROMATOGRAM ON COLUMN b
METHANOLYSIS OF METHYLATED
POLYSACCHARIDE B



* This methyl glycoside results from incomplete remethylation of the reduced methylated polysaccharide.

The vapour phase chromatogram on column b for fully methylated polysaccharide B is shown in the accompanying diagram.

Hydrolysis of the methylated polysaccharide.

Methylated polysaccharide B (2.5 g.) was refluxed in 3% methanolic hydrogen chloride (65 ml.) for 16 hours. The solution was neutralised with silver carbonate, filtered and evaporated to dryness giving a syrup of methyl glycosides. To saponify the methyl esters of the acidic components, the syrup was dissolved in barium hydroxide solution (32 ml. saturated at room temperature) and heated for 2 hours at 60°. The cooled aqueous solution was continuously extracted with boiling light petroleum (b.p. 40-60°) (150 ml.) to give fraction X (55 mg.).

The aqueous solution was filtered from traces of solid, passed through a column of Amberlite resin 1R120 and the resin washed until sugar free.

A column of purified and regenerated Duolite A4 resin was prepared and the effluent from the cation exchange resin passed through. The acidic component was selectively removed and the neutral sugars eluted with water. A large quantity of water (ca. 2 l.) was used to wash the column and the eluate and washings were evaporated to a syrup,

fraction Y (1.378 g.).

The acidic component was isolated from the column by displacement with N sodium hydroxide (250 ml.) and the eluate passed through a column of Amberlite resin 1R120 to remove the cations. Evaporation of the eluate and washings gave fraction Z (0.777 g.).

A small sample of fraction Z was refluxed overnight with 2.5% methanolic hydrogen chloride and the products, along with fractions X and Y, examined by gas phase chromatography. The results obtained indicated that the separation between acid and neutral methylated sugars had been adequate but since fractions X and Y contained similar mixtures of neutral methylated glycosides, they were combined to give fraction W (1.41 g.).

The mixture of methyl glycosides in fraction W was heated in N hydrochloric acid (30 ml.) at 100° for 5 hours. The solution was neutralised with silver carbonate, filtered and the residue extracted with hot methanol. The filtrate and washings were combined to give on evaporation the neutral methylated sugars (1.17 g.).

Fractionation of neutral methylated sugars.

The neutral methylated sugars (1.17 g.) were separated by chromatography on cellulose column (3 x 55 cm.). The column was eluted successively with the following solvents:-

Light petroleum (b.p. 100-120): butan-1-ol (3:7, saturated with water).

Butan-1-ol, half saturated with water.

Eleven fractions were collected in all and the recovery was 92.1% (1.078 g.).

TABLE 6

Frac- tion	Tubes	Wt. (in mg.)	<u>T</u> values		R _G	Contents
			<u>Column</u> <u>a</u>	<u>Column</u> <u>b</u>		
1	21-34	231	0.45		1.02	Unknown (trace)
			0.55 & 0.71	0.47 & 0.59	0.98	2,3,5-tri-O-methyl- arabinose
			1.79	1.60	0.89	2,3,4,6-tetra-O-methyl- galactose
			7.5	-	0.73	2,3,4-tri-O-methyl- galactose (trace)
2	35-43	206	7.4	2.89 & 2.60	0.71	2,3,4-tri-O-methyl- galactose
			4.14 & (4.68)	2.36 & 2.07	0.72	2,4,6-tri-O-methyl- galactose
			3.19 & (4.68)	1.62	0.73	2,3,6-tri-O-methyl- galactose
			1.88	0.70	0.83	2,5-di-O-methyl- arabinose (trace)
3	44-67	54	7.4	2.90 & 2.65	0.71	2,3,4-tri-O-methyl- galactose
			4.70 & 4.16	2.36 & 2.08	0.72	2,4,6-tri-O-methyl- galactose (trace)

TABLE 6 (cont.)

Fraction	Tubes	Wt. (in mg.)	T values		R _G	Contents
			Column a	Column b		
4	68-91	127		4.55 & 3.85	0.53	2,4-di-O-methyl- galactose
5	92-105	67		4.56 & 3.85	0.53	2,4-di-O-methyl- galactose
				2.60	0.55	2,6-di-O-methyl- galactose (trace)
6	106-280	112		4.51 & 3.80	0.53	2,4-di-O-methyl- galactose
				2.55	0.58	2,6-di-O-methyl- galactose
7	281-304	135		2.56	0.58	2,6-di-O-methyl- galactose
				4.52 & 3.83	0.53	2,4-di-O-methyl- galactose
					0.30	2-O-methyl-galactose
8	305-310	35			0.30	2-O-methyl-galactose
9	311-324	29			0.30	2-O-methyl-galactose
					0.27	4-O-methyl-galactose
10	325-350	28			0.30 streak	2-O-methyl-galactose acidic methylated sugars
11	351-420	54			streak	acidic methylated sugars

Examination of the Fractions.

Fraction 1 - mainly 2,3,5-tri-O-methyl-arabinose and 2,3,4,6-tetra-O-methyl-galactose.

This fraction (231 mg.) was shown by paper chromatography in solvent D to be a mixture of methylated sugars as shown in table 6. The presence of these derivatives was confirmed by examination of the methanolysis products of a small sample by gas phase chromatography in both solvent systems. Fraction 2 had $[\alpha]_D +34^\circ$ (c, 1.0 in CHCl_3) indicating that there was almost equal quantities of the tri-O-methyl-arabinose and tetra-O-methyl-galactose present.

The syrup was fractionated on a cellulose column (1.8 x 55 cm.) using light petroleum (b.p. 100-120°):butan-1-ol (8:2, saturated with water) as eluent and finally washing the column with butan-1-ol (half saturated with water). The results are shown in table 7.

Subfraction a.

This fraction had the same R_G value in solvents D and F as 2,3,4-tri-O-methyl-L-rhamnose and gave the characteristic green stain when sprayed with p-anisidine HCl. No peaks were obtained, however, on gas chromatograms of the derived methyl glycoside, suggesting that most of the weight was made up of extraneous mechanical material. On decolorising the syrup with charcoal, this was shown to be the case since there was only a trace (3 mg.) of the sugar left. No sugars

TABLE 7

Fraction	Tubes	Wt. (in mg.)	Colour with p-anisidine HCl	R _G	Contents
a	6-16	10	Green	1.02	Unknown
b	17-28	35	Green	1.02	Unknown
			Black	0.98	2,3,5-tri-O-methyl-arabinose
c	29-44	80	Black	0.98	2,3,5-tri-O-methyl-arabinose
d	66-114	100	Red-brown	0.87	2,3,4,6-tetra-O-methyl galactose
e	176-244	22	Brown-pink	0.76	2,3,4-tri-O-methyl-galactose
			Brown	0.82	mixture of di- and tri-O-methyl-arabinoses
			Red-brown	0.89	2,3,4,6-tetra-O-methyl-galactose
f	300 ml. bulk	13	Red-brown	0.89	2,3,4,6-tetra-O-methyl-galactose

could be detected on chromatographic examination of the products from demethylation.

Subfraction b.

Paper chromatography of this fraction, $[\alpha]_D -40^\circ$ (equil.) (c, 0.35), showed it to be mainly 2,3,5-tri-O-methyl-arabinose

with a trace of the same unknown sugar, found in subfraction a. Demethylation gave only arabinose. The main component in the fraction was characterised by conversion into 2,3,5-tri-O-methyl-L-arabonamide, which was recrystallised from ethyl acetate, and had m.p. and mixed m.p. 135° (with an authentic sample melting at 136°).

Subfraction c.

This fraction (80 mg.), R_G 0.98, $[\alpha]_D -41^\circ$ (equil.) (c , 0.75) gave a characteristic black stain with aniline oxalate, which appears red in v.v. light. Paper chromatography in solvents D and F showed it to be homogeneous and identical to 2,3,5-tri-O-methyl-L-arabinose. Demethylation yielded arabinose only. The sugar was characterised by conversion into 2,3,5-tri-O-methyl-L-arabonamide, m.p. and mixed m.p. 134-6°, with authentic sample melting at 136°.

Subfraction d.

This fraction (100 mg.), R_G 0.89, $[\alpha]_D +100^\circ$ (c , 0.50) was chromatographically identical to 2,3,4,6-tetra-O-methyl-D-galactose in solvents D and F. The derived 2,3,4,6-tetra-O-methyl-N-phenyl-D-galactosylamine, after recrystallisation from ethanol, had m.p. and mixed m.p. 194-5° (with authentic sample, m.p. 195°).

Subfraction e.

Paper chromatography of this fraction (22 mg.) in solvents D, F and H gave three spots, corresponding to the methylated

sugars given in table 7. Demethylation gave galactose and arabinose in equal proportions (visual examination only).

Gas phase chromatography of the derived methyl glycosides, gave the results listed below.

Methyl glycosides of	<u>T</u> in column <u>a</u>	<u>T</u> in column <u>b</u>
2,3,4-Tri- <u>O</u> -methyl-galactose	7.5	2.91 & 2.65
2,3,4,6-tetra- <u>O</u> -methyl-galactose	1.82	1.58
2,3,4-tri- <u>O</u> -methyl-arabinose	1.04	0.82
2,3-di- <u>O</u> -methyl-arabinose	1.51	
2,4-di- <u>O</u> -methyl-arabinose	2.24	1.04
2,5-di- <u>O</u> -methyl-arabinose		0.76

Subfraction f.

This fraction (13 mg.) resulted from bulk elution of the column with butan-1-ol (half saturated with water). It had the same chromatographic mobility as 2,3,4,6-tetra-O-methyl-D-galactose in solvents D and H. Demethylation gave only galactose.

Fraction 2 - mixture of tri-O-methyl-galactoses.

Paper chromatography in solvents D, F and H showed this fraction (206 mg.) to consist of tri-O-methyl-galactoses. The results of gas phase chromatography showed the mixture to have roughly equal proportions of 2,3,4-, 2,3,6- and

2,4,6-tri-O-methyl-D-galactoses with a trace of di-O-methyl-L-arabinose. The retention times are given in table 6.

Gradient elution with aqueous methyl ethyl ketone (1.5-6%) on a charcoal-Celite column (45 x 2 cm.) gave the following fractionation.

Fraction	Tubes	Wt. (in mg.)	Sugars present
a	45-58	51	2,3,4-tri- <u>O</u> -methyl- <u>D</u> -galactose + di- <u>O</u> -methyl- <u>L</u> -arabinose
b	59-65	26	2,3,4- and 2,4,6-tri- <u>O</u> -methyl- <u>D</u> -galactose
c	66-74	29	2,4,6-tri- <u>O</u> -methyl- <u>D</u> -galactose
d	75-89	40	2,4,6- and 2,3,6-tri- <u>O</u> -methyl- <u>D</u> -galactose
e	90-120	30	2,3,6-tri- <u>O</u> -methyl- <u>D</u> -galactose
f	121-150	7	2,3,6-tri- and 2,3,4,6-tetra- <u>O</u> -methyl- <u>D</u> -galactose
g	151-190	5	2,3,4,6-tetra- <u>O</u> -methyl- <u>D</u> -galactose

Subfraction a.

This fraction (51 mg.) had $[\alpha]_D +134^{\circ} \rightarrow +105^{\circ}$ (equil.) (c, 0.51). Gas phase chromatography indicated that the syrup was substantially pure 2,3,4-tri-O-methyl-galactose with a trace of 2,3-di-O-methyl-arabinose. Demethylation gave galactose and a trace of arabinose. The main component was characterised as 2,3,4-tri-O-methyl-D-galactose by conversion into the aniline derivative, which, after

recrystallisation from ethyl acetate, had m.p. and mixed m.p. 167-8°.

Subfraction c.

The syrup (29 mg.), $[\alpha]_D +91^\circ$ (c , 0.29), crystallised and was recrystallised from acetone:ether:light petroleum (1:1:1) to give 2,4,6-tri-O-methyl-D-galactose, m.p. and mixed m.p. 98-100°. The aniline derivative, after recrystallisation from ethyl acetate, had m.p. 163° and mixed m.p. 167° on mixing with an authentic sample m.p. 168°.

Subfraction e.

Subfraction e (30 mg.) had $[\alpha]_D +20^\circ \rightarrow +81^\circ$ (equil.) (c , 0.30) and was characterised as 2,3,6-tri-O-methyl-D-galactose by conversion into 2,3,6-tri-O-methyl-D-galactono-lactone, which after recrystallisation from ether, had m.p. 97-9°, unchanged on mixing with an authentic sample m.p. 98°.

Fraction 3 - mainly 2,3,4-tri-O-methyl-D-galactose.

The fraction (54 mg.) had $[\alpha]_D +109^\circ$ (c , 0.46) and on demethylation gave galactose only. Gas phase chromatography (as shown in table 6) indicated that the main component was 2,3,4-tri-O-methyl-galactose with a trace of 2,4,6-tri-O-methyl-galactose. The main component was characterised as the aniline derivative, which was recrystallised from ethyl acetate to give m.p. 158° not depressed on mixing an authentic specimen (m.p. 168°).

Fractions 4 and 5 - mainly 2,4-di-O-methyl-D-galactose.

These two fractions were combined (190 mg.), since gas phase and paper chromatography showed them to be the same, consisting almost entirely of 2,4-di-O-methyl-D-galactose with traces of 2,3,4-tri-O-methyl-D-galactose and 2,6-di-O-methyl-D-galactose. Demethylation gave galactose and Lemieux Bauer (50) periodate oxidation gave one spot (R_G 0.53) in solvent D, corresponding to unchanged 2,4-di-O-methyl-galactose.

The sugar had $[\alpha]_D +104^\circ \rightarrow +91^\circ$ (equil.) (c , 1.0) and after recrystallisation from aqueous acetone, it had m.p. and mixed m.p. 94-95°. The sugar was finally characterised by conversion into 2,4-di-O-methyl-N-phenyl-D-galactosylamine m.p. and mixed m.p. 210° (with an authentic sample 212°).

Fraction 6. 2,4- and 2,6-di-O-methyl-D-galactoses.

Chromatography of this fraction (112 mg.) in solvents D and H R_G 0.53 and 0.58, showed it to be a mixture of the above two sugars. This was confirmed by the results obtained from gas chromatographic examination (shown in table 6). Demethylation gave galactose only. Periodate oxidation of a small sample, and examination of the products in solvent D, gave two spots R_G 0.50 and 0.22, corresponding to unchanged 2,4-di-O-methyl-galactose and methoxymalonaldialdehyde (from 2,6-di-O-methyl-galactose).

Separation of the two components was achieved by

chromatography on filter sheets in solvent H, using the technique of stepwise development (3 x 6 hours), and giving subfractions a and b.

Subfraction a.

This fraction (37 mg.) was shown by gas phase and paper chromatography and by its resistance to periodate oxidation to be identical to 2,4-di-O-methyl-D-galactose. It crystallised on standing to give 2,4-di-O-methyl-D-galactose monohydrate m.p. and mixed m.p. 93-94°, $[\alpha]_D +103^\circ \rightarrow +87^\circ$ (equil.) (c , 0.30), and the identity of the sugar was confirmed by conversion into the aniline derivative, which, after recrystallisation from ethyl acetate, had m.p. 212° (unchanged on mixing with an authentic sample m.p. 212°).

Subfraction b.

Chromatography of this fraction (41 mg.), R_G 0.58, $[\alpha]_D +40^\circ \rightarrow +86^\circ$ (equil.) (c , 0.41) in three solvent systems, D, F and H indicated that it was homogeneous and identical to 2,6-di-O-methyl-D-galactose. Chromatography of the periodate oxidised product gave a brilliant yellow spot (R_G 0.22), which would be consistent for a 2,6 substituted hexose. Finally, on demethylation, only galactose was obtained.

The syrup crystallised and was recrystallised by dissolving in a few drops of chloroform and adding light

petroleum (b.p. 40-60°), m.p. and mixed m.p. 113-115° (with 2,6-di-O-methyl-D-galactose). The sugar was finally characterised by conversion into the anilide, which was recrystallised from ethyl acetate. The derived 2,6-di-O-methyl-N-phenyl-D-galactosylamine had m.p. and mixed m.p. 119°.

Fraction 7. 2,4- and 2,6-di-O-methyl-galactoses and 2-O-methyl-galactose.

Chromatography of this fraction (135 mg.) in solvents D and H, showed it to be a mixture of the above sugars. The mono-O-methyl derivative was separated from the di-O-methyl-galactoses by fractionation on filter sheets in solvent D. The di-O-methyl-galactoses were separated from each other by chromatography on filter sheets in solvent H as in fraction b. Three subfractions were obtained.

Subfraction a.

This fraction (25 mg.) had R_G 0.30 and $[\alpha]_D +82^\circ$ (equil.) (c , 0.25). It was chromatographically indistinguishable from 2-O-methyl-D-galactose in solvents A, D and H. Demethylation gave galactose and periodate oxidation gave a bright yellow spot (R_G 0.20), which is correct for a 2-substituted hexose.

Subfraction b.

Gas phase and paper chromatography showed this fraction

(60 mg.) to be pure 2,4-di-O-methyl-galactose. The sugar was resistant to attack by periodate and on demethylation gave galactose. On standing, the syrup crystallised and was recrystallised from aqueous acetone to give 2,4-di-O-methyl-D-galactose monohydrate, m.p. and mixed m.p. 96° , $[\alpha]_D +104^{\circ} \rightarrow +87^{\circ}$ (equil.) (c , 0.50).

The sugar was further characterised by formation of the aniline derivative which after recrystallisation from ethyl acetate, had m.p. 213° (unchanged on mixing with authentic 2,4-di-O-methyl-N-phenyl-D-galactosylamine, m.p. 212°).

Subfraction c.

This fraction (37 mg.) was proved to be a homogeneous sample of 2,6-di-O-methyl-galactose by paper chromatography in solvents A, D and H. Periodate oxidation products gave a spot (R_G 0.22) in solvent D. The syrup crystallised after several weeks and recrystallisation gave needles m.p. $114-116^{\circ}$, $[\alpha]_D +40^{\circ} \rightarrow +80^{\circ}$ (equil.) (c , 0.25).

Attempts to make the aniline derivative, however, met with no success.

Fraction 8. 2-O-Methyl-D-galactose.

Chromatography of this fraction (35 mg.) showed it to be homogeneous and with an identical mobility (R_G 0.30) to 2-O-methyl-D-galactose in solvents A and D. Demethylation

gave only galactose and chromatographic examination of the periodate oxidation products gave a bright yellow spot (R_G 0.22) corresponding to methoxymalonaldehyde.

The sugar was recrystallised from aqueous acetone and had m.p. and mixed m.p. 150° $[\alpha]_D +53^\circ \rightarrow +82^\circ$ (equil.) (c , 0.20).

Fraction 9. 2-O- And 4-O-mono-methyl-D-galactoses.

This fraction (29 mg.) gave two spots, R_G 0.30 and 0.27 on paper chromatographic examination in solvent D. Galactose was the only sugar obtained on demethylation. Chromatography of the periodate oxidised product gave a bright yellow spot (R_G 0.20) and a citrine spot (R_G 0.58). The latter corresponded to 2-O-methyl-erythrose and must have arisen from a 4-substituted hexose.

Examination of the acidic methylated sugars.

The acidic methylated sugars (0.777 g.), fraction Z, were hydrolysed with N hydrochloric acid for 5 hours, to remove the methyl glycosides, neutralised with silver carbonate, filtered and evaporated to small volume. The solution was deionised with Amberlite resin 1R120, decolorised with charcoal and taken to a syrup (0.536 g.).

A small portion (30 mg.) of this syrup was chromatographed on a filter sheet in solvent A, with 2,4-di- and 2,3,4-tri-O-methyl-D-galactoses as standards. The acidic

sugars were recovered by cutting out the strip around the starting line, eluting the strip with water and evaporating to a syrup. This acidic material was examined by paper chromatography in solvents B, D, E and G, with 6-O- β -D-glucuronosyl-D-galactose hexamethyl ether as standard. Best separation was achieved in solvent G, when three spots were obtained. A spot corresponding to the standard (R_F , 0.45) was obtained but it could not be resolved from the spot (R_F , 0.51) corresponding to 2,3,4-tri-O-methyl-D-glucuronic acid. A chromatographic separation of these two sugars would have been impractical. The third spot (R_F 0.35) may have arisen from 4-O-D-glucuronosyl-D-galactose hexa-methyl ether or from a methylated aldobiouronic acid, in which one of the hydroxyls of the galactose moiety was unsubstituted or it may correspond to 3,4-di-O-methyl-D-glucuronic acid.

A small portion (50 mg.) of the acidic methylated sugars was retained for further investigation of this matter.

The remaining acidic methylated sugar (425 mg.) was refluxed with 2% methanolic hydrochloric acid for 4 hrs., neutralised with silver carbonate and concentrated to a syrup. The product was dissolved in anhydrous tetrahydrofuran (30 ml.) and lithium aluminium hydride (400 mg.) in tetrahydrofuran (10 ml.) was added dropwise. The mixture was refluxed for 2 hrs. and further additions (50 mg.) of hydride were made. Excess hydride was destroyed by

successive addition of ethyl acetate and water. Dilute sulphuric acid was added until the solution was pH 3 and the solution extracted with chloroform (5 x 100 ml.) in the cold. The extracts were combined, dried with anhydrous sodium sulphate, and on evaporation to dryness a syrup (265 mg.) of the reduced acidic methylated sugars was obtained. The reduced material was hydrolysed with N-hydrochloric acid (40 ml.) at 100° for 6 hr., neutralised with silver carbonate and concentrated to a syrup (237 mg.).

This mixture of methylated sugars was separated into seven fractions by chromatography on a charcoal-Celite column (35 x 2.5 cm.) using a gradient elution of aqueous methyl ethyl ketone (0-5%, 2 litres). Fractions (8 ml.) were collected and the elution pattern followed by paper chromatography in solvent D. Positions of breakdown, in many cases, however, required the evidence of gas phase chromatography.

Fraction	Tubes	Wt. (in mg.)	R _G	<u>T</u> in column <u>b</u>	Contents
1	96-108	26	0.28		2- <u>O</u> -methyl- <u>D</u> -galactose
			0.47	4.29 & 3.61	2,4-di- <u>O</u> -methyl- <u>D</u> -galactose
			0.50	2.44	2,6-di- <u>O</u> -methyl- <u>D</u> -galactose

Fraction	Tubes	Wt. (in mg.)	R _G	<u>T</u> in column <u>b</u>	Contents
2	109-122	267	0.47	4.30 & 3.61	2,4-di-O-methyl-D-galactose
			0.50		2,6-di-O-methyl-D-galactose
			0.65	2.82 & 2.56	2,3,4-tri-O-methyl-D-galactose
3	123-136	13	0.65	2.89 & 2.61	2,3,4-tri-O-methyl-D-galactose
			0.54	2.42	3,4-di-O-methyl-D-glucose
4	137-143	26	0.54	2.36	3,4-di-O-methyl-D-glucose
			0.67	1.63 & 2.54	2,3,6-tri-O-methyl-D-galactose
5	144-175	25	0.67	1.63 & 2.54	2,3,6-tri-O-methyl-D-galactose
6	176-180	3	0.67	1.63 & 2.55	2,3,6-tri-O-methyl-D-galactose
			0.74	1.24	2,3,4-tri-O-methyl-D-glucose
7	181-254	89	0.74	1.25 & 1.83	2,3,4-tri-O-methyl-D-glucose

Total weight recovered 208 mg. (88%).

Fraction 1. 2-O-Methyl-D-galactose and 2,4- and 2,6-di-O-methyl-D-galactoses.

Paper chromatography in solvent D indicated the presence of three components (R_G 0.28, 0.47 and 0.50) in this fraction

(26 mg.). Demethylation of a small sample gave only galactose. Separation of the fraction into mono-methyl hexose and dimethyl hexoses was effected on a filter sheet in solvent D.

Subfraction 1a.

Subfraction 1a (5 mg.) contained the mono-methyl galactose derivative. The hexose was shown to be 2-O-methyl-galactose since on periodate oxidation, a bright yellow spot (R_G 0.20) was obtained.

Subfraction 1b.

This fraction (17 mg.) contained the di-O-methyl-D-galactoses and was combined with subfraction 2a.

Fraction 2. 2,4- And 2,6-di-O-methyl-D-galactoses and
2,3,4-tri-O-methyl-D-galactose.

Paper chromatographic examination of this fraction (26 mg.) gave three spots (R_G 0.47, 0.50 and 0.65). Only galactose resulted from demethylation. The syrup was separated into di-methyl-galactoses (7 mg.), (subfraction a), and 2,3,4-tri-O-methyl-galactose (subfraction b), by chromatography on a filter sheet in solvent D.

Subfractions 1b and 2a.

These two fractions were combined (24 mg.), since they both contained a mixture of di-methyl-galactoses. The mixture was fractionated on a filter sheet in solvent H.

2,4-Di-O-methyl-D-galactose. The first fraction (12 mg.) had the same chromatographic mobility as the above sugar in solvents D and H. The syrup crystallised out on standing and was recrystallised from ethyl acetate m.p. and mixed m.p. 93° (with authentic sample of 2,4-di-O-methyl-D-galactose monohydrate) $[\alpha]_D^{+100} \rightarrow +79^{\circ}$ (equil.) (c , 0.10).

2,6-Di-O-methyl-D-galactose. The second fraction (10 mg.) was shown by paper chromatography in solvents D and H, to be homogeneous and identical to the above sugar. Chromatographic examination of the periodate oxidised product in solvent D gave a brilliant yellow spot (R_G 0.20), confirming that the sugar was a 2,6 substituted hexose. Attempts to get the syrup crystalline failed.

Subfraction 2b.

This fraction (17 mg.) had $[\alpha]_D^{+109}$ (c , 0.17) and R_G 0.65. It was shown by paper and gas-liquid phase chromatography to be identical to 2,3,4-tri-O-methyl-D-galactose. The aniline derivative was prepared in the usual way and recrystallised from ethyl acetate m.p. 167° , not depressed on mixing with an authentic specimen (m.p. 168°).

Fraction 3. 3,4-Di-O-methyl-D-glucose and 2,3,4-tri-O-methyl-D-galactose.

This fraction (13 mg.) had $[\alpha]_D^{+102}$ (c , 0.13) and

on paper chromatography in solvent D, two spots (R_G 0.65 and 0.54) were obtained, which were indistinguishable from the above two sugars. The presence of these two sugars was confirmed on examination by gas phase chromatography. Demethylation gave galactose and glucose in equal quantities (visual examination only) in solvent A.

Chromatographic examination of the periodate oxidised product gave two spots (R_G 0.70 and 0.61) corresponding to unchanged 2,3,4-tri-O-methyl-galactose and 2,3-di-O-methyl arabinose, the latter arising from oxidation of 3,4-di-O-methyl glucose.

A chromatogram was run in solvent D and sprayed with 1:1 aqueous solution of triphenyl tetrazolium (2%) and N sodium hydroxide. A red spot was obtained which had exactly the same R_G value (0.54) as the methylated glucose derivative. This suggests that the sugar was 3,4-di-O-methyl-D-glucose since triphenyltetrazolium reagent (88) detects sugars with 2-position free.

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

Chromatography of this fraction (26 mg.), R_G 0.54 and 0.67, in solvents D and F showed it to be a mixture of the above two sugars. On demethylation and examination of the products in solvent A, two spots corresponding to glucose and galactose were obtained. A red spot (R_G 0.54)

was obtained with the triphenyltetrazolium spray.

A separation was achieved on thick paper in solvent H (stepwise development, 3 x 3 hrs.).

Subfraction a.

This fraction (10 mg.), $[\alpha]_D +91^\circ$ (c , 0.10), had the same mobility in solvent D (R_G 0.54) and the same T value on column b (2.39) as an authentic sample of 3,4-di-O-methyl-D-glucose. Demethylation gave glucose and a red spot was obtained with the triphenyl tetrazolium spray. Attempts to obtain the syrup crystalline have so far failed.

Subfraction b.

This fraction was chromatographically pure and identical to 2,3,6-tri-O-methyl-D-galactose. The syrup (12 mg.) was added to fraction 5.

Fraction 5. 2,3,6-Tri-O-methyl-D-galactose.

Fraction 5 (25 mg.) was combined with Subfraction 4b after preliminary chromatographic examination. Paper and gas liquid chromatography showed the syrup to be identical to 2,3,6-tri-O-methyl-D-galactose. It was characterised by conversion into 2,3,6-tri-O-methyl-D-galactonolactone which after recrystallisation from ethyl acetate, had m.p. $97-98^\circ$, unchanged on admixture with an authentic sample m.p. 98° .

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

This fraction (3 mg.) only contained enough material for an analysis by gas chromatography. Peaks were obtained with T values corresponding to the above two sugars.

Fraction 7. 2,3,4-Tri-O-methyl-D-glucose.

This fraction (89 mg.), $[\alpha]_D^{+75^\circ}$ (c, 0.89), was chromatographically indistinguishable (R_G 0.74) from an authentic sample of 2,3,4-tri-O-methyl-D-glucose and was characterised by conversion to the aniline derivative m.p. and mixed m.p. 131° .

AUTOHYDROLYSIS OF POLYSACCHARIDE B

Sugar proportions in polysaccharide B.

A sample (50 mg.) of polysaccharide B was hydrolysed with 2N sulphuric acid at 100° for 8 hours. The solution was neutralised with barium hydroxide and barium carbonate, treated with Amberlite resin 1R120 to remove barium ions and concentrated to a syrup (45 mg.). The syrup was fractionated on thick paper in solvent A. The sugars were eluted with water and estimated by the following modification of the p-amino benzoic acid method (106). Sugar solution (1 ml.), containing (20-130 μ g) was added to a 1.5% solution of p-amino benzoic acid in glacial acetic acid (3 ml.) and 1.5% sulphursalicylic acid (3 ml.). The mixture was heated at 100° for 1 hour, cooled and the optical density measured against a reagent blank at 375 $m\mu$. The amount of sugar in each fraction was obtained by reference to standard curves of optical density against sugar concentration, for galactose and arabinose. The fractions were galactose (23.1 mg.) and arabinose (11.3 mg.).

Small-scale autohydrolysis.

Polysaccharide B (500 mg.) was dissolved in water (25 ml.) and the solution was refluxed for 48 hours. Aliquots were removed at intervals and their optical rotations were measured, with the following results.

$[\alpha]_D$	+8°	+12°	+16°	+24°	+28°	+30°	+32°
Time (hours)	-	0.75	1.5	6	12	24	48

After measuring the optical rotation of each aliquot, the solution was poured into ethanol (4 volumes), the precipitated polysaccharide was removed at the centrifuge and the filtrate was concentrated to a syrup. Paper chromatographic examination of these syrups in solvent A showed that for the first 30 hours, the only hydrolysis product was arabinose and thereafter a trace of galactose was indicated. After 48 hours, the remainder of the reaction mixture was poured into ethanol (200 ml.) and the precipitate (162 mg.) was removed at the centrifuge. After washing several times with ethanol, the degraded polysaccharide was treated with 0.2N sulphuric acid for 1 hour and the products were examined by paper chromatography in solvents A and B. Spots were obtained corresponding to 1,3- and 1,6-galactobioses in addition to higher oligosaccharides. The combined filtrates and washings were concentrated and, on paper chromatographic examination of the resulting syrup (135 mg.), shown to contain arabinose with traces of galactose and 4-O-methyl glucuronic acid.

Large-scale autohydrolysis.

Polysaccharide B (2.3 g.) was dissolved in water (115 ml.) and the solution refluxed for 42 hours. After cooling, the solution was poured into ethanol (600 ml.) and the

degraded polysaccharide was precipitated out. This polymer was redissolved in water and precipitated with ethanol. After several such re-precipitations, degraded polysaccharide B (1.12 g.), $[\alpha]_D 0^\circ$ (c , 0.50) was obtained.

The combined filtrates were concentrated to small volume, treated with charcoal and evaporated to a syrup (660 mg.). A portion (40 mg.) of this syrup was fractionated on thick paper in solvent A and the fractions containing galactose and arabinose were obtained. Estimation with p-amino benzoic acid reagent showed the following quantities:- galactose (8.1 mg.) and arabinose (26.9 mg.).

Degraded polysaccharide B, uronic anhydride content 28%, gave on hydrolysis, galactose, uronic acids and a trace of arabinose. Treatment with 0.2N sulphuric acid for 1.5 hours, followed by paper chromatographic examination of the products gave 1,3- and 1,6-galactobioses in addition to galactose, uronic acids and a trace of arabinose. The degraded polysaccharide (1.0 g.) was methylated successively with dimethyl sulphate and sodium hydroxide, and methyl iodide and silver oxide to give methylated degraded polysaccharide B, $[\alpha]_D -13^\circ$ (c , 0.50 in CHCl_3) (Found: OMe, 43.0%). A sample (250 mg.) of the methylated material in tetrahydrofuran (8 ml.) was reduced with lithium aluminium hydride (250 mg.) in tetrahydrofuran (8 ml.), in the usual way to give reduced methylated degraded polysaccharide B (195 mg.) $[\alpha]_D -16^\circ$ (c , 0.77

in CHCl₃) (Found: OMe 4.2%). The two methylated polysaccharides were methanolysed and the resulting mixtures of methyl glycosides analysed by gas-chromatography. The results obtained are shown in the following table.

Methyl glycoside of	Column <u>a</u>		Column <u>b</u>	
	Methylated	Red. ^d methylated	Methylated	Red. ^d methylated
2,3,5-Tri-O-methyl arabinose	0.56 & 0.73	0.56 & 0.73	0.47 & 0.62	0.47 & 0.60
2,3,4,6-tetra-O-methyl galactose	1.80	1.81	(1.62) & 1.53	(1.62)
2,3,4-tri-O-methyl galactose	7.3	7.4	2.91 & 2.57	2.89 & 2.60
2,3,6-tri-O-methyl galactose	(3.21) & (4.66)	3.20 & (4.68)	(1.62) & (2.37)	(1.62) & (2.37)
2,4,6-tri-O-methyl galactose	4.13 & (4.66)	4.15 & (4.68)	2.09 & (2.37)	2.06 & (2.37)
2,4-di-O-methyl galactose			3.63 & 4.35	3.60 & 4.28
2,3,4-tri-O-methyl glucuronic acid*	2.49 & (3.21)		1.75 & 2.23	
2,3,4-tri-O-methyl glucose		2.62 & 3.71		1.35 & 1.81

When a T value corresponds to more than one methyl glycoside it is given within parenthesis.

* present as methyl ester.

A sample (10 mg.) of the methylated polysaccharide was

treated with 2N hydrochloric acid at 100° for 8 hours. After neutralisation, the hydrolysate was examined by paper chromatography in solvent D. Evidence was obtained for, in addition to the methylated sugars listed above, the presence of 2,6-di-O-methyl galactose (R_G 0.58) and 2-O-monomethyl galactose (R_G 0.30).

SMITH DEGRADATION OF POLYSACCHARIDE B.

Reduction of polysaccharide B.

The polysaccharide was acetylated in formamide solution with acetic anhydride and pyridine by Carson and Maclay's method (104) to give acetylated polysaccharide B, $[\alpha]_D -10^{\circ}$ (c , 1.02 in CHCl_3) (Found: OAc 36.7%). Polysaccharide B acetate (5.0 g.) was dissolved in 1,2-dimethoxyethane (100 ml.), (which had been dried twice over sodium wire and distilled from lithium aluminium hydride). Sodium borohydride (1 g.) was dissolved in the solution, and diborane was generated in situ by the addition of portions (ca. 5 ml.) of a solution of boron trifluoride-ether complex (10 g.) in 1,2-dimethoxyethane (40 ml.) during 1.5 hr. After each addition the stoppered flask was shaken gently and then vigorously to break up the gel which separated. The mixture was set aside overnight and was then poured into ice-water (500 ml.). The mixture was made just alkaline and was concentrated under reduced pressure to a thick paste. The paste was dissolved in 0.1N sodium hydroxide, and the solution was adjusted to pH 9 and heated for 1 hr. at 55° . The resulting solution was dialysed against tap water for 48 hr. and against distilled water for 24 hr., filtered, concentrated, and poured into a stirred mixture of ethanol (250 ml.) and ether (50 ml.). The gummy precipitate was triturated with ethanol and dried to give a residue (3.5 g.).

The crude polysaccharide was dissolved in water (75 ml.), and the solution was deionised by passage through columns of Amberlite resins 1R-120 (H) and 1R-45 (OH), concentrated to 30 ml., and freeze-dried to give reduced polysaccharide B (3.0 g.). No traces of acidic material could be detected on paper chromatographic examination of the hydrolysis products of the reduced polysaccharide and the neutral sugars were galactose, arabinose, 4-O-methyl glucose and glucose.

Doubts as to the reduced polysaccharide being fully deacetylated were raised, when the amount of periodate (0.26 moles) consumed per sugar unit in the polysaccharide, was found to be much lower than the anticipated value. The presence of acetyl groups in the polysaccharide was confirmed by infra-red analysis. The polysaccharide was completely de-acetylated by stirring in a 20% (w/v) ammonia solution for 24 hours. An infra-red spectrum of the polysaccharide obtained showed the absence of acetyl groups.

The reduced polysaccharide (0.50 g.) was dissolved in water (20 ml.), freshly prepared sodium metaperiodate solution (44 ml. of 0.2N) was added and the solution was made up to 100 ml. In other flask 44 ml. of the same periodate solution was diluted with distilled water to 100 ml. In a third flask 44 ml. of the periodate solution was treated with ethylene glycol (8 ml.) and the mixture diluted to 100 ml. to give a sodium iodate solution of the same molarity

as the above two solutions. 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 m (107). From the results obtained, the consumption of periodate per sugar unit after various intervals of time were calculated and are tabulated below.

Time (hrs.)	0.5	1.5	5	18	25	35
Moles of periodate consumed per sugar unit	0.63	0.79	0.80	0.82	0.82	0.82

After 35 hours the reaction was stopped by the addition of ethylene glycol (6 ml.) and the solution was dialysed against tap water for four days. The dialysate was concentrated to a small volume (30 ml.), potassium borohydride (150 mg.) was added and the solution was stirred for six hours. A further addition of potassium borohydride (150 mg.) was made and the solution was allowed to stand overnight. The excess borohydride was destroyed by the addition of Amberlite resin 1R-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 ml.) and the solution was evaporated to dryness to give the polyalcohol (284 mg.).

The polyalcohol was dissolved in N sulphuric acid

(30 ml.) and shaken. The optical rotation of the resulting solution was measured at intervals and the following results were obtained:-

Time (in hours)	0	0.5	1.5	3	4
Rotation (in 1 dm.tube)	+0.20°	+0.22°	+0.26°	+0.28°	+0.28°

The reaction was stopped here by neutralising with barium hydroxide and barium carbonate. After deionising with Amberlite resin 1R-120 (H), the solution was evaporated to small volume and poured into ethanol (4 vols.). A degraded polysaccharide (66 mg.) was obtained, which on hydrolysis gave galactose, a trace of arabinose and no sugar alcohols.

The supernatant liquid, remaining after precipitation of the degraded polysaccharide, was concentrated to give a syrup (170 mg.). Chromatography of this material in solvent systems A and B using spray reagents II and III showed the presence of glycerol and other sugar alcohols, but no reducing sugars.

A sample of the low molecular weight material was hydrolysed with N sulphuric acid for five hours. Paper chromatography of the hydrolysate indicated the presence of galactose, arabinose and a trace of 4-O-methyl glucose in addition to glycerol, erythritol and other sugar alcohols (R_{GAL} 1.15, 1.68 and 2.63).

Large-scale Smith degradation.

Reduced polysaccharide B (3.2 g.) was dissolved in water (200 ml.), sodium metaperiodate (13.2 g.) was added and the solution was diluted to 500 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-two hours by the addition of ethylene glycol (20 ml.) and the solution was dialysed against tap-water for four days.

The dialysate was reduced in volume, potassium borohydride (600 mg.) added and the solution was stirred for six hours. Another addition of potassium borohydride (600 mg.) was made and the solution was allowed to stand overnight at room temperature. The solution was treated with Amberlite resin 1R-120 (H) and the resulting solution was concentrated to a syrup. The syrup was repeatedly dissolved in methanol (5 x 30 ml.) and evaporated to dryness. The resulting polyalcohol (2.9 g.) was hydrolysed with N-sulphuric acid (66 ml.) for 5 hours at room temperature. After neutralisation, 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 ml.) and then dried in a vacuum oven to give the Smith degraded polysaccharide (678 mg.).

The supernatant liquid and the washings were combined,

reduced in volume and the resulting syrup (1.73 g.) was examined chromatographically. The presence of components similar to that obtained in the trial experiment was observed.

Examination of the Smith degraded polysaccharide.

The degraded polysaccharide, $[\alpha]_D^{+25}(\underline{c}, 1.00)$ gave galactose and a trace of arabinose on total hydrolysis; paper chromatographic examination of a partial acid hydrolysate indicated the presence of 1,3-galactobiose ($R_{GAL} 0.51$), 1,3-galactotriose ($R_{GAL} 0.25$) and a trace of 1,6-galactobiose ($R_{GAL} 0.36$). A sample (50 mg.) of the degraded polysaccharide was treated with 0.1M sodium metaperiodate solution (100 ml.). The uptake of the oxidant was followed spectrophotometrically and found to be 0.26 moles per sugar unit.

The degraded polysaccharide (150 mg.) was methylated with barium oxide (1.35 g.), barium hydroxide (1.35 g.) and dimethyl sulphate (3 ml.) in dimethyl sulphoxide (8 ml.) and dimethyl formamide (8 ml.) according to the method of Kuhn and Trischmann (42). Methylation of the partly methylated polysaccharide (105 mg.) was completed with methyl iodide and silver oxide in dimethylformamide. Methylated Smith degraded polysaccharide (85 mg.) had $[\alpha]_D^{-10}(\underline{c}, 1.0$ in $CHCl_3$) (Found: OMe, 44.1%). Gas-chromatographic examination of the methanolysis products of this methylated

polysaccharide gave the following results.

Methyl glycoside of	<u>T</u> in column <u>a</u>	<u>T</u> in column <u>b</u>
2,4,6-Tri-O-methyl galactose	4.15 & 4.71	2.07 & 2.38
2,3,4,6-tetra-O-methyl galactose	1.81	1.64
2,3,4-tri-O-methyl galactose	7.4	2.90
2,4-di-O-methyl galactose		3.70 & 4.35

SECTION II

ACACIA SENEGAL GUM (Gum Arabic)

Acacia senegal (gum arabic).

The Location of L-Rhamnopyranose Residues.

The differing stability of glycosidic linkages in polysaccharides, which allows great degrees of selectivity in hydrolysis, has its disadvantage too. Thus it is very difficult to determine the nature of the very acid-labile linkage, since this linkage will be among the first to be cleaved on acid hydrolysis and will not be present in any of the oligosaccharides isolated. For this reason, the location of L-rhamnopyranose residues in gum arabic has not yet been definitely established.

It is known that L-rhamnose residues in gum arabic occur as non-reducing end-groups and that glucuronic acid residues are mainly substituted at position 4. Therefore, since these sugar residues occur in almost equal proportions in gum arabic, it has been suggested by several authorities (7, 83) that L-rhamnose is glycosidically linked to D-glucuronic acid residues in the polysaccharide molecule. Further evidence was obtained for this when Hamilton and Thompson (84) showed that alkaline hydrolysis of gum arabic at 160° leads to the loss of both glucuronic acid and rhamnose residues with the formation of an arabino-galactan. Since this type of reaction appears to result in the selective cleavage of glycosiduronic acid linkages, sugar units attached to glucuronic acid residues would

also be lost; so this observation would be consistent with the theory that the L-rhamnose is attached to the D-glucuronic acid. Recently (97) it has been shown that Fenton's reagents degrade gum arabic giving 10% of low molecular weight material, consisting of rhamnose, arabinose, galactose and a disaccharide. Since it is considered that Fenton's reagents cleave the glycosiduronic acid linkages, an association of rhamnose and other sugar units with glucuronic acid residues is suggested. We have confirmed these postulations by isolating oligosaccharides containing a rhamnosyl-glucose linkage, from reduced gum arabic.

Methylation Studies on Gum Arabic.

In this study Acacia senegal gum, obtained through the courtesy of Mr. Videll-Hall of the Forest Department of the Sudan Government, has been used. In order to show that this sample contained similar structural features to those of the sample investigated by Smith and co-workers (98), we methylated the gum and examined the methanolysis products by gas-liquid partition chromatography.

Arabic acid was methylated with methyl sulphate and sodium hydroxide. The partially methylated polysaccharide was methylated to completion by several treatments with methyl iodide and silver oxide. The fully methylated gum arabic had $[\alpha]_D -47.5$ and OMe, 41.3%. The methanolysis

products of a sample were examined by gas phase chromatography in both solvent systems. The results obtained showed the presence of components having the same retention times as those of the methyl glycosides of all the major cleavage products which had been characterised by Smith (99); these were:-

2,3,4-tri-O-methyl-L-rhamnose

2,3,5-tri- and 2,5-di-O-methyl-L-arabinose

2,4-di- and 2,3,4,6-tetra-O-methyl-D-galactose

2,3,4-tri- and 2,3-di-O-methyl-D-glucuronic acid

In addition there were indications of traces of the following sugars, not reported present by Smith:-

2,3,4-tri-O-methyl-arabinose

2,4,6-tri-O-methyl-galactose.

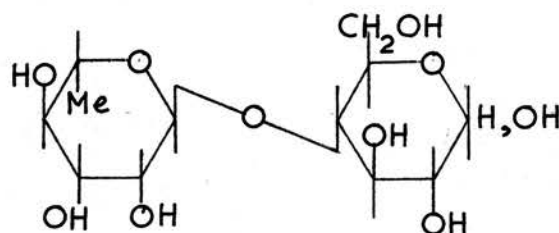
A sample of the methylated polysaccharide was reduced with lithium aluminium hydride and remethylated. On examination of the methanolysis products of reduced methylated and methylated reduced methylated gum arabic, evidence was obtained for the presence of 2,3-di- and 2,3,6-tri-O-methyl-glucose in these polysaccharides respectively. This was further proof that the D-glucuronic acid residues in the gum were 4-O-substituted. The presence, however, of traces of 2,3,4-tri- and 2,3,4,6-tetra-O-methyl-D-glucose, was an indication that not all the D-glucuronic acid residues were substituted.

Characterisation of rhamnosyl-glucose.

A. J. Charlson reduced acetylated gum arabic with diborane (76) and carried out a partial hydrolysis on the reduced gum with acetic anhydride-sulphuric acid. Column chromatography on cellulose and charcoal-Celite led to the isolation of a rhamnosyl-glucose. This was partially characterised by periodate oxidation and other means, and indications of the presence of a 1-4 glycosidic linkage obtained. From the hydrolysed methylated disaccharide, Charlson characterised 2,3,4-tri-O-methyl-L-rhamnose but failed to characterise the derived methylated glucose residue. It was at this stage that we took over from Charlson.

The rhamnosyl-glucose disaccharide from another fraction was methylated with methyl sulphate and sodium hydroxide. Methylation was completed using Purdie's reagents methyl iodide and silver oxide. The methylated disaccharide was hydrolysed and separated into two methylated derivatives on a filter sheet, 2,3,4-tri-O-methyl-L-rhamnose and 2,3,6-tri-O-methyl-D-glucose were characterised by the formation of crystalline derivatives. The configuration of the glycosidic linkage is indicated by the similarity of the optical rotation ($[\alpha]_D -6^\circ$) to that ($[\alpha]_D -0.1$) of the isomeric disaccharide, 6-O- α -L-rhamnopyranosyl-D-glucose, for whose configuration independent evidence has been obtained by Gorin and Perlin (99).

Thus the rhamnosyl-glucose disaccharide has the following structure (I).



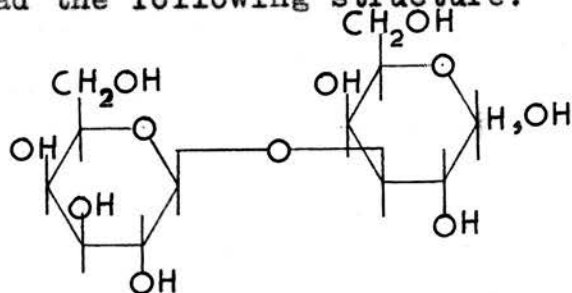
(I)

4-O-α-L-Rhamnopyranosyl-D-glucopyranose

Isolation of other oligosaccharides from acetolysis of reduced gum arabic.

Paper chromatographic investigation of another fraction from the acetolysis products of the gum indicated the presence of three components. These were separated on a filter sheet giving Fractions 1, 2 and 3.

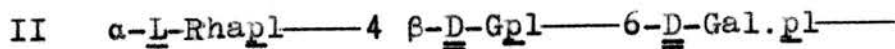
Fraction 1 - Hydrolysis and paper chromatographic examination of the products gave only galactose. The sugar had the same chromatographic mobility in several solvents as 3-O-β-D-galactosyl-D-galactose, and was characterised as such by m.p. and mixed m.p. of the recrystallised disaccharide. It gave an identical X-ray powder photograph to that obtained from an authentic sample. Thus the galactose disaccharide had the following structure.



3-O-β-D-galactosyl-D-galactose

Fraction 2 - Paper chromatography of the hydrolysis products of this fraction, indicated the presence of galactose, glucose and rhamnose in equal proportions. Thus the sugar is probably a trisaccharide. Reduction of the trisaccharide, followed by hydrolysis and paper chromatographic examination, gave rhamnose, glucose and galactitol, indicating that the galactose residue was situated at the reducing end. Chromatography of the products of mild acid hydrolysis gave three spots, corresponding to rhamnose, an unknown spot and the starting material; this indicates that the rhamnose residue is situated at either the reducing or non-reducing end of the trisaccharide. Since it has already been shown that galactose is at the reducing end, the inference is that the rhamnose occupies the non-reducing position.

Methylation of the trisaccharide followed by hydrolysis gave three methylated sugars, which were separated by column chromatography. These were characterised by the formation of crystalline derivatives, as 2,3,6-tri-O-methyl-D-glucose, 2,3,4-tri-O-methyl-L-rhamnose, and 2,3,4-tri-O-methyl-D-galactose. Thus the isolated trisaccharide has the following structure (II).



Fraction 3. The third fraction was chromatographically homogeneous and appeared to be a neutral trisaccharide.

Total hydrolysis gave galactose and mild acid hydrolysis gave 1,3-galactobiose and galactose (N.B. no other galactose disaccharides obtained). This suggests that the sugar is O-D-galactopyranosyl(1-3)-O-D-galactopyranosyl(1-3)-D-galactose could not be confirmed by the procuring of a crystalline derivative, although the R_{GAL} value and optical rotation are similar to those reported for the above trisaccharide (100).

Attempted isolation of an acidic trisaccharide.

Using the same conditions of acetolysis as for reduced gum arabic, an attempt was made to isolate the acidic trisaccharide, O-L-rhamnopyranose(1-4)-O-D-glucuronosyl(1-6)-D-galactose from the unmodified gum. Although a small amount, of what we supposed to be the above oligosaccharide, was obtained, it could not be completely characterised. Mild acid hydrolysis of this sugar gave rhamnose and a spot with the same chromatographic mobility in several solvents as 6-O-β-D-glucuronosyl-D-galactose, while total acid hydrolysis gave rhamnose, galactose and glucuronic acid in roughly equal proportions.

The characterisation of the rhamnose containing oligosaccharide (I) as a partial hydrolysis product of reduced gum arabic provides clear evidence that some of the L-rhamnopyranose residues in the gum are glycosidically linked to C-4 of D-glucuronic acid residues. Moreover since a

isolated from these gums and so their exact location has been in doubt. We have now proved that in gum arabic they are linked to position 4 of the D-glucuronic acid residues. It is possible that this is also the case in Acacia cyanophylla gum (83) and Acacia mollissima gum (85), although in our preliminary examination of the methylated sugars obtained on hydrolysis of methylated Acacia mollissima gum, only a small amount of the methylated glucuronic acid derivatives were found to be unsubstituted at position 4 suggesting that some of the rhamnose residues may have a different mode of linkage.

In other gums from this genus such as Acacia karoo (101) and Acacia sundra (102), the L-rhamnose residues are probably also attached to position 4 of glucuronic acid, but the amount of D-glucuronic acid residues is much higher than the amount of L-rhamnose residues, and therefore some of the glucuronic acid residues must be unsubstituted, have other sugar residues attached or are present as their 4-O-methyl ethers.

It is significant that in Acacia pycnantha gum, on which detailed methylation studies have been carried out (103), no 2,3-di-O-methyl-D-glucopyranosyl uronic acid was found in the hydrolysis products of the methylated polysaccharide. The L-rhamnose residues present in this gum, must therefore have a different mode of attachment to the backbone of the molecule than they have in gum arabic.

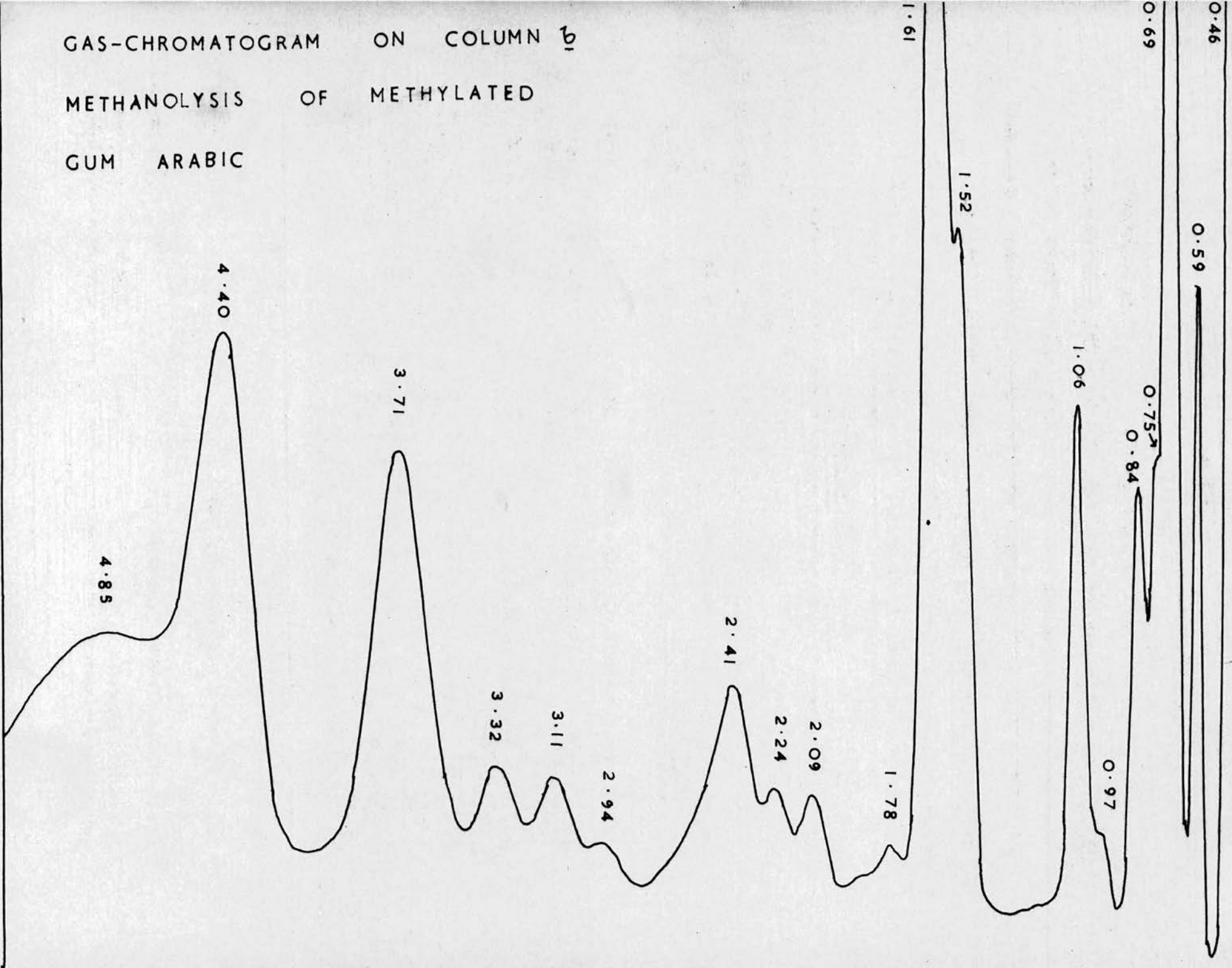
E X P E R I M E N T A L

Methylated gum arabic and derivatives.

Gum arabic (20 g.) was methylated successively with dimethyl sulphate and sodium hydroxide, and with methyl iodide and silver oxide, to give methylated gum arabic (11.2 g.), $[\alpha]_D -47.5^\circ$ (c , 1.01 in CHCl_3) (Found: OMe, 41.3%). Lithium aluminium hydride (150 mg.) in tetrahydrofuran (5 ml.) was added to methylated gum arabic (150 mg.) in tetrahydrofuran (5 ml.), and after 0.5 hr. at room temperature the mixture was refluxed for 3 hr. The excess of hydride was destroyed with ethyl acetate and water, and the resulting mixture was shaken with dilute sulphuric acid and extracted with chloroform. The chloroform extract afforded reduced methylated gum arabic (105 mg.), $[\alpha]_D -43.5^\circ$ (c , 1.06 in CHCl_3) (Found: OMe, 39.6%). Hydrolysis of a sample of the reduced methylated gum followed by chromatography of the products in solvents D and E showed that the reduction of hexuronic acid residues was complete. Reduced methylated gum (90 mg.) was methylated in NN-dimethyl formamide with methyl iodide and silver oxide, to give methylated reduced gum arabic (78 mg.), $[\alpha]_D -47.5^\circ$ (c , 1.02 in CHCl_3) (Found: OMe, 41.3%).

Samples of the methylated gum, reduced methylated gum, and methylated reduced gum were heated with methanolic 2.5% hydrogen chloride in sealed tubes at 100° for 16 hr., the

GAS-CHROMATOGRAM ON COLUMN b
METHANOLYSIS OF METHYLATED
GUM ARABIC



cooled solutions were neutralised with silver carbonate, filtered, and concentrated, and the resulting syrups were examined by gas-chromatography, with results shown in the following tables. The gas chromatogram on column b for the methyl glycosides from methylated gum arabic is shown opposite.

Relative retention times (T) of methyl glycosides
on column a

Sugar	Methylated gum arabic	Reduced methylated gum arabic	Methylated reduced gum arabic
2,3,4-Tri- <u>O</u> -methyl rhamnose	0.44	0.44	0.45
2,3,5-tri- <u>O</u> -methyl arabinose	0.55 & 0.72	0.54 & 0.71	0.55 & 0.71
2,3,4-tri- <u>O</u> -methyl arabinose	1.04	1.04	(1.02)
2,5-di- <u>O</u> -methyl arabinose	(1.84) & 3.47	(1.86) & 3.41	(1.87) & (3.50)
2,3,4,6-tetra- <u>O</u> -methyl galactose	(1.84)	(1.86)	(1.87)
2,4,6-tri- <u>O</u> -methyl galactose	4.19 & 4.80	4.15 & 4.70	4.17 & (4.82)
2,3,4-tri- <u>O</u> -methyl glucuronic acid *	2.52 & 3.28		
2,3-di- <u>O</u> -methyl glucuronic acid *	8.4 & 9.3		
2,3,4-tri- <u>O</u> -methyl glucose		2.54 & 3.70	
2,3,4,6-tetra- <u>O</u> -methyl glucose			(1.02) & (1.49)
2,3,6-tri- <u>O</u> -methyl glucose			3.50 & 4.82
Unknown sugar	1.51	1.49	(1.49)

Relative retention times (T) of methyl glycosides on column b.

Sugar	Methylated gum arabic	Reduced methylated gum arabic	Methylated reduced gum arabic
2,3,4-Tri- <u>O</u> -methyl rhamnose	(0.46)	(0.46)	(0.46)
2,3,5-tri- <u>O</u> -methyl arabinose	(0.46) & 0.59	(0.46) & 0.59	(0.46) & 0.59
2,3,4-tri- <u>O</u> -methyl arabinose	0.84	0.83	0.82
2,5-di- <u>O</u> -methyl arabinose	0.69 & 1.06	0.70 & 1.05	0.69 & (1.06)
2,3,4,6-tetra- <u>O</u> -methyl galactose	1.52 & 1.61	1.52 & 1.61	1.52 & 1.61
2,4,6-tri- <u>O</u> -methyl galactose	2.09 & (2.41)	2.10 & (2.46)	(2.21) & 2.39
2,4-di- <u>O</u> -methyl galactose	3.71 & 4.40	3.68 & 4.40	3.69 & 4.40
2,3,4-tri- <u>O</u> -methyl glucuronic acid *	1.78 & 2.24		
2,3-di- <u>O</u> -methyl glucuronic acid *	(2.41) & 3.11		
2,3,4-tri- <u>O</u> -methyl glucose		1.36 & 1.86	
2,3-di- <u>O</u> -methyl glucose		(2.46) & 3.28	
2,3,4,6-tetra- <u>O</u> -methyl glucose			(1.02) & 1.34
2,3,6-tri- <u>O</u> -methyl glucose			1.72 & 2.21

* Methyl glycosides present as methyl esters.

Figures in parentheses indicate T values of components which were incompletely resolved.

Characterisation of the rhamnosyl-glucose.

Fraction 2a from the acetolysis products of reduced gum arabic (59) had $[\alpha]_D -5^\circ$ (c , 1.02) and was found to be chromatographically homogeneous ($R_{GAL} = 1.0$). Hydrolysis with 0.5N sulphuric acid gave glucose and rhamnose

The sugar (fraction 2a; 102 mg.) was methylated successively with dimethyl sulphate and sodium hydroxide, and with methyl iodide and silver oxide, to give methylated disaccharide (55 mg.), $[\alpha]_D -21^\circ$ (c , 0.55 in $CHCl_3$). Essentially complete etherification was indicated by a) paper chromatography of the hydrolysate, which showed two main components, and b) gas-chromatography of the methanolysis products on columns a and b, which showed the presence of major components having the retention times of methyl glycosides of 2,3,4-tri-O-methyl rhamnose (T 0.46 and 0.46 on columns a and b) and 2,3,6-tri-O-methyl glucose (T 3.50 and 4.78, and 1.31 and 1.61 on columns a and b). The methylated disaccharide (50 mg.) was hydrolysed in N sulphuric acid at 100° for 4 hr., and after neutralisation with barium carbonate furnished a mixture of sugars (45 mg.) which was chromatographed on a filter sheet in solvent F, to give two fractions (R_F 0.84 and 0.50).

Fraction 1 (20 mg.) was chromatographically homogeneous and had $[\alpha]_D +18^\circ$ (c , 0.20); the sugar was characterised as 2,3,4-tri-O-methyl-L-rhamnose by conversion into the aniline

derivative, which after recrystallisation from ethyl acetate, had m.p. and mixed m.p. 111° , with authentic sample, m.p. 111° .

Fraction 2 (15 mg.) was chromatographically homogeneous and had $[\alpha]_D +68^{\circ}$ (equil.) (c , 0.15 in CHCl_3); the sugar was characterised as 2,3,6-tri-O-methyl-D-glucose by conversion into the 1,4-di-p-nitrobenzoate, which after recrystallisation from methanol, had m.p. and mixed m.p. 191° on mixing with an authentic sample.

Investigation of other oligosaccharides found in the acetolysis products of reduced gum arabic.

Paper chromatographic examination in several solvent systems of Fraction 4c (from the acetolysis products of reduced gum arabic (59)), showed it to have three components. The mixture (500 mg., containing Celite) was chromatographed on thick paper in solvent B, giving fractions A, B and C.

Examination of fraction A. Fraction A (91 mg.) had $R_{\text{GAL}} 0.51$ in solvent A and had $[\alpha]_D +58^{\circ}$ (c , 0.48). It afforded only galactose on hydrolysis and was chromatographically indistinguishable from 3-O- β -D-galactopyranosyl-D-galactose in solvents A, B and C. The sugar crystallised and was recrystallised from aqueous acetone, to give 3-O- β -D-galactopyranosyl-D-galactose monohydrate, which had m.p. and mixed m.p. 158° , and gave an X-ray powder diagram identical to that of an authentic sample.

Examination of fraction B. Fraction B (170 mg.) was chromatographically homogeneous in several solvents (R_{GAL} 0.35 in solvent A) and had $[\alpha]_D +10^\circ$ (c , 0.43). Hydrolysis gave galactose, glucose and rhamnose in equal proportions (visual examination only). Reduction of the oligosaccharide (10 mg.) with sodium borohydride (10 mg.) in water (1 ml.) gave the corresponding glycitol. The latter on hydrolysis with 0.5N sulphuric acid at 100° for 5 hr., gave rhamnose, glucose and galactitol. Treatment of the oligosaccharide with 0.1N sulphuric acid for 1 hr. and examination of the products by paper chromatography in solvent A indicated the presence of rhamnose, an unknown spot (R_{GAL} 0.45) and the original trisaccharide.

Fraction B (165 mg.) was methylated successively with dimethyl sulphate and sodium hydroxide, and methyl iodide and silver oxide to give methylated trisaccharide (140 mg.). Incomplete methylation was indicated by a) paper chromatography of the hydrolysate, which showed, in addition to the three expected methylated sugars, a spot corresponding to dimethyl hexoses, and b) gas-chromatography of the methanolysis products, which showed the presence of components having the retention times of methyl glycosides of 2,3,4-tri-O-methyl rhamnose (T 0.46 and 0.46 on columns a and b), 2,3,6-tri-O-methyl glucose (T 3.54 and 4.78, and 1.70 and 2.20 on columns a and b), 2,3,4-tri-O-methyl galactose (T 7.4, and 2.66 and 2.90 on columns a and b)

and di-O-methyl hexoses (T 3.81 on column b). Unfortunately the oligosaccharide was inadvertently hydrolysed with N sulphuric acid for 5 hours before methylation could be completed. The hydrolysis products (130 mg.) were allowed to soak into a cellulose column (60 x 2 cm.) and elution performed with light petroleum (b.p. 100-120°): butan-1-ol (70:30, saturated with water), followed by light petroleum (b.p. 100-120°): butan-1-ol (50:50, saturated with water). Five fractions were collected in all.

Frac- tion	Tubes	Wt. in (mg)	<u>T</u> values for methyl glycosides		R _g	Contents
			column <u>a</u>	column <u>b</u>		
1	11-35	40	0.46	0.46	1.01	2,3,4-tri- <u>O</u> -methyl rhamnose
2	74-199	16	3.52 & 4.76	1.72 & 2.19	0.85	2,3,6-tri- <u>O</u> -methyl glucose
3	200-225	12	3.54 & 4.76	1.72 & 2.22	0.85	2,3,6-tri- <u>O</u> -methyl glucose
			7.4	2.71 & 2.95	0.72	2,3,4-tri- <u>O</u> -methyl galactose (trace)
4	226-280	21	3.53 & 4.76	1.74 & 2.24	0.85	2,3,6-tri- <u>O</u> -methyl glucose (Trace)
			7.5	2.73 & 2.95	0.72	2,3,4-tri- <u>O</u> -methyl galactose
5	281-380	34		4.04 & 3.00	0.56	di- <u>O</u> -methyl hexoses

Fraction 1. This fraction (40 mg.), $[\alpha]_D +23^\circ$ (c, 0.40) was chromatographically identical to 2,3,4-tri-O-methyl-L-rhamnose.

It was characterised as such by conversion into the aniline derivative which, after recrystallisation from ethyl acetate, had m.p. 114° , unchanged on mixing with an authentic sample, m.p. 111° .

Fraction 2. Paper chromatography showed this fraction (16 mg.) to be mainly 2,3,6-tri-O-methyl-D-glucose; it had $[\alpha]_D +72^{\circ}$ (c, 0.16). The main component in the fraction was characterised by formation of the derived 1,4-di-p-nitrobenzoate, which after recrystallisation from methanol had m.p. and mixed m.p. 193° (on mixing with an authentic sample).

Fraction 3. This fraction (12 mg.), $[\alpha]_D +85^{\circ}$ (c, 0.12) was shown by paper chromatography to be a mixture of 2,3,6-tri-O-methyl-D-glucose and 2,3,4-tri-O-methyl-D-galactose. Demethylation gave glucose and a trace of galactose.

Fraction 4. Paper chromatography of this fraction (21 mg.), $[\alpha]_D +118^{\circ}$ (c, 0.21), showed to be almost pure 2,3,4-tri-O-methyl-D-galactose. It was characterised as such by conversion into the aniline derivative, which after recrystallisation from ethyl acetate, had m.p. 154° , unchanged on mixing with an authentic sample.

Fraction 5. Paper chromatography by this fraction (34 mg.) gave only one spot (R_G 0.56) but gas-chromatography of the methyl glycosides indicated the presence of several di-O-methyl hexoses which could not be identified. Demethylation gave glucose and galactose in equal proportions.

Examination of Fraction C. This fraction (89 mg.)

$[\alpha]_D +56^\circ$ (c , 0.89), was chromatographically homogeneous in solvents A and B ($R_{GAL} = 0.17$ in solvent A). Hydrolysis gave only galactose and treatment with 0.1N sulphuric acid for 1 hour gave galactose, 1,3-galactobiose and the starting material. Attempts to obtain the sugar crystalline, failed.

Attempted isolation of an acidic trisaccharide.

One of the acetolysis products from reduced gum arabic obtained by A. J. Charlson (69) appeared to be an acidic trisaccharide, resulting from incomplete reduction of hexuronic acid units. This was used as a chromatographic standard in an attempt to obtain this acidic trisaccharide by acetolysis of the unmodified gum. Since this trisaccharide readily breaks down to give 6-O- β -D-glucuronosyl-D-galactose, it was necessary to be able to distinguish between these two sugars by paper chromatography. This was achieved by chromatography on Whatman No.4 paper in solvent B for 5 days, when the acidic trisaccharide had $R_X 0.71$ (where $X = 6-O-\beta-\underline{D}$ -glucuronosyl-D-galactose).

Arabic acid (30 g.) was acetylated in formamide solution with acetic anhydride and pyridine (104), to give acetylated arabic acid $[\alpha]_D -21^\circ$ (c , 1.0 in $CHCl_3$). Acetolysis was carried out on the acetate (40 g.) with acetic anhydride (720 ml.) and concentrated sulphuric acid

(22 ml.) for 1 hour at 0°C and for a further 3 hours at room temperature. The mixture was worked up in the same way as for acetolysis of reduced gum arabic (59) and after deacetylation with barium methoxide, a mixture (21 g.) of sugars was obtained. The acidic sugars were selectively removed on cation exchange resin, and after elution of the neutral sugars with water, elution was carried out with increasing concentration of formic acid. Thick paper chromatography of one of the fractions from the column, gave finally a pure sample (75 mg.) of the acidic trisaccharide, $[\alpha]_D +18^\circ$ (c , 0.40).

Hydrolysis with 0.1N sulphuric acid for 1 hour afforded rhamnose and 6-O-β-D-glucuronosyl-D-galactose. Total hydrolysis with N sulphuric acid for 12 hours at 100°, gave rhamnose, galactose, glucuronic acid and a trace of the aldobiouronic acid. The acidic trisaccharide (60 mg.) was methylated successively with dimethyl sulphate and sodium hydroxide and methyl iodide and silver oxide to give methylated sugar (30 mg.). Paper chromatographic examination of the hydrolysis products indicated the presence of 2,3,4-tri-O-methyl-D-galactose and 2,3,4-tri-O-methyl-D-glucuronic acid, but provided no evidence of rhamnose derivatives. Gas-chromatographic examination of the methanolysis products confirmed the presence of the methyl glycosides of the above two sugars, together with a trace of the methyl glycoside of 2,3,4-tri-O-methyl-L-rhamnose.

SECTION III

ACACIA MOLLISSIMA GUM

ACACIA MOLLISSIMA GUM

DISCUSSION

Slight differences in structure have been found to exist between samples of the same gum, when these samples are of different origin (7). Since there was available a sample of Acacia mollissima gum of West Indian origin and since a report was available (85) of a preliminary investigation of the same gum of South African origin, we intended to investigate this matter by carrying out an examination of the structure of the gum of West Indian origin. This gum was obtained for us from the one tree, through the courtesy of Dr. Jean Tyler. Another sample of South African Acacia mollissima obtained from Professor A. Stephen, was available as the fully methylated derivative. The three samples of the gum had the following physical constants:-

Sample of <u>Acacia mollissima</u> gum	$[\alpha]_D$	Equivalent (by titration)	Equivalent (by uronic anhydride estimation)
A. Stephen's first sample of South African origin (85)	-49°	1880	-
A. Stephen's new sample of South African origin	-55°	1920	1730
Sample of West Indian origin	-52°	-	1550

The sample of West Indian Acacia mollissima gum, was methylated to give methylated polysaccharide, $[\alpha]_D -66^\circ$ and OMe, 42.4%. A sample of this was reduced with lithium aluminium hydride to give reduced methylated polysaccharide, $[\alpha]_D -66^\circ$ and OMe, 40.5%. The methanolysis products from the reduced and unmodified methylated polysaccharides were compared with the results from gas-chromatographic analysis of the methanolysis products from methylated South African Acacia mollissima, $[\alpha]_D -66^\circ$ and OMe, 42.6% and from the derived reduced methylated polysaccharide, $[\alpha]_D -66^\circ$ and OMe, 40.8%. The proportions of the different methyl glycosides were estimated by visual examination and are tabulated below, Table A.

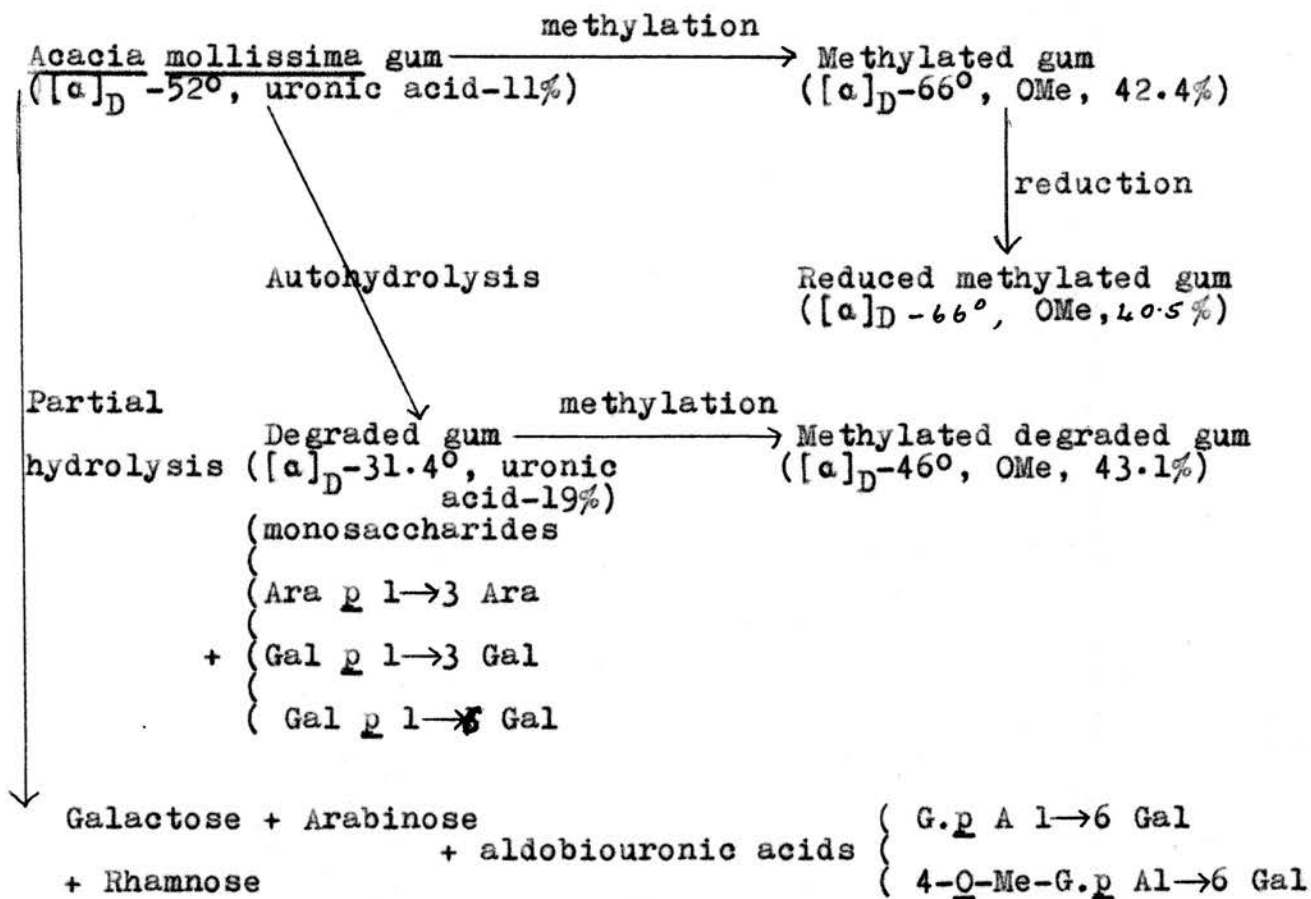
TABLE A

Sugar	Proportions of sugars	
	South African sample	West Indian sample
2,3,4-Tri-O-methyl rhamnose	+	+
2,3,5-tri-O-methylarabinose	++++	+++++
2,3,4-tri-O-methyl arabinose	+	+
2,3-di-O-methyl arabinose	+++	+++
2,5-di-O-methyl arabinose	++++	++++
2,3,4,6-tetra-O-methyl galactose	++	trace
2,4,6-tri-O-methyl galactose	++++	+++
2,3,4-tri-O-methyl galactose	+++	+
2,4-di-O-methyl galactose	+++++	+++++
2,3,4-tri-O-methyl glucuronic acid	+++	+++
2,3-di-O-methyl glucuronic acid	trace	trace

As can be seen from the table, all the methylated sugars found in the one polysaccharide were also present in the other, but significant differences were observed in the relative proportions of the methylated sugars.

The gum acid was investigated for heterogeneity by chromatography on DEAE-cellulose. Two polysaccharide fractions, equal in amount, were obtained, but no appreciable difference in uronic acid content could be detected between them. It is still uncertain whether this fractionation resulted from a difference in molecular size or from the two fractions having slightly different structural features.

A number of experiments, as shown in the flow sheet below, were carried out to obtain an insight into the structure of the gum molecule.



Hydrolysis of Acacia mollissima gum gave D-galactose, L-arabinose and L-rhamnose, all of which, after separation on thick paper, were characterised by means of crystalline derivatives, in addition to acidic material. The same sugars were obtained by Stephen from his first sample of South African Acacia mollissima gum in the proportions indicated, D-galactose (5), L-arabinose (6), L-rhamnose (1) and D-glucuronic acid (1), (85).

The gum acid was autohydrolysed by heating an aqueous solution (2%) at 90° for 64 hr. The degraded polysaccharide, $[\alpha]_D -31.4^\circ$ and uronic acid content 18.7% (corresponding to

an equivalent weight of 938), was obtained from the aqueous solution by precipitation with ethanol; evaporation of the filtrate to dryness gave the low molecular weight hydrolysis products.

Methylation of the degraded polysaccharide gave methylated degraded Acacia mollissima gum, $[\alpha]_D -46^\circ$ (c, 0.30) (Found: OMe, 43.1%). The derived methyl glycosides from this methylated polysaccharide were examined by gas-chromatography. This showed the presence of the methyl glycosides of 2,3,4,6-tetra-, 2,3,4-tri-, 2,4,6-tri- and 2,4-di-O-methyl galactose and 2,3,4-tri-O-methyl glucuronic acid.

The oligosaccharides obtained from autohydrolysis of the gum were allowed to soak into a charcoal-celite column and the monosaccharides were eluted with water. Elution with increasing strengths of ethanol gave fractions containing three oligosaccharides, which were purified by further fractionation on thick paper.

Oligosaccharide A had $[\alpha]_D +183^\circ$ and was chromatographically identical to 3-O- β -L-arabinopyranosyl-L-arabinose in solvents A and B. Hydrolysis gave only arabinose; methylation of the disaccharide and subsequent examination of the methyl glycosides, formed on methanolysis of the methylated derivative, by gas-chromatography showed the presence of the methyl glycosides of 2,3,4-tri-, 2,4-di and 2,5-di-O-methyl arabinose, indicative of a 1,3 linkage. The sugar was characterised as 3-O- β -L-arabinopyranosyl-L-

arabinose by conversion into the crystalline phenylosazone.

Oligosaccharide B had similar chromatographic mobility and optical rotation, $[\alpha]_D +50^\circ$, to an authentic sample of 3-O- β -D-galactopyranosyl-D-galactose. It was characterised as such by its crystallinity, the crystals, after recrystallisation from aqueous acetone, having m.p. 225° . (Although this m.p. is higher than the normal value reported, it is comparable with one reported by Stephen (108) for the same sugar). An X-ray powder photograph of oligosaccharide B was identical to that obtained from an authentic sample of 3-O- β -D-galactopyranosyl-D-galactose, m.p. 168° .

Oligosaccharide C, $[\alpha]_D +45^\circ$, was chromatographically identical to 6-O- β -D-galactopyranosyl-D-galactose in several solvents, and was characterised as this sugar by conversion into the phenylosazone.

Partial hydrolysis of the gum acid with N sulphuric acid at 100° for 7 hours gave a mixture of mono- and oligosaccharides. Column chromatography with anion exchange resin was used to separate the acidic from the neutral sugars. Paper chromatographic fractionation of the acidic sugars gave two aldobiouronic acids.

Aldobiouronic acid I, $[\alpha]_D -3^\circ$, was chromatographically identical to 6-O- β -D-glucopyranosyluronic acid-D-galactose in several solvents. Reduction of the derived methyl ester, methyl glycosides with sodium borohydride, followed by

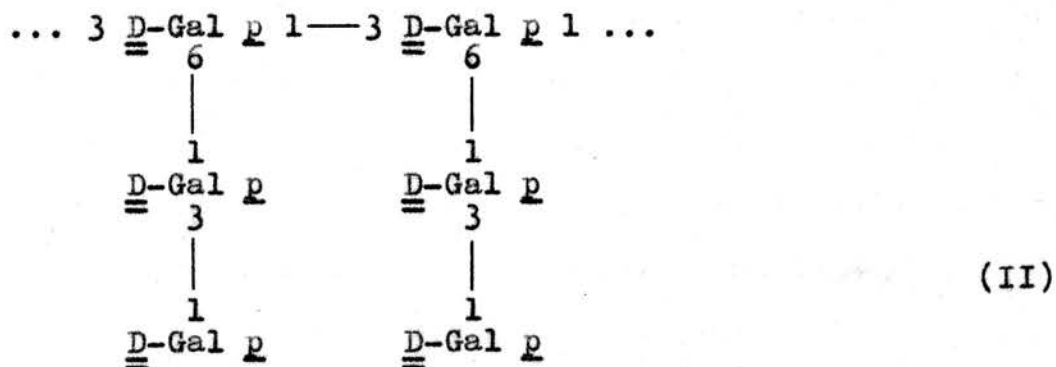
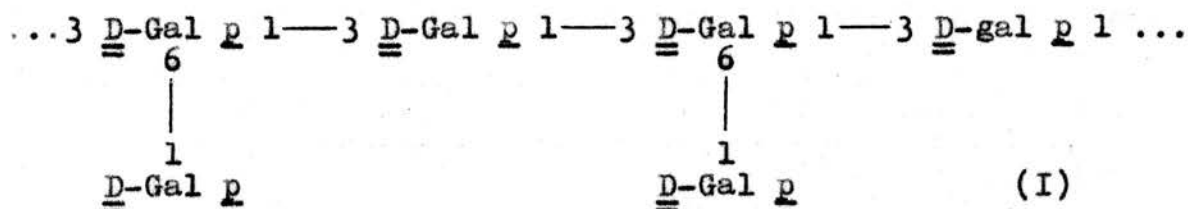
hydrolysis gave galactose and glucose; the latter must have arisen from reduction of glucuronic acid. The aldobiouronic acid was methylated but the expected methyl ester of methyl 6-O- β -D-glucopyranosyl- β -D-galactoside, hexa-methyl ether, has so far failed to crystallise.

Aldobiouronic acid II, $[\alpha]_D +6^\circ$, was chromatographically identical to 6-O-(4-O-methyl- β -D-glucopyranosyl uronic acid)-D-galactose in several solvents. Reduction of the derived methyl ester, methyl glycosides with sodium borohydride, followed by hydrolysis gave galactose and 4-O-methyl glucose; the latter must have arisen from reduction of 4-O-methyl glucuronic acid. No formaldehyde was released on periodate oxidation of the aldobiouronic acid, which is consistent with a 1,6 linkage. The methanolysis products of the methylated aldobiouronic acid were examined by gas-chromatography. Evidence was obtained for the presence of the methyl glycosides of 2,3,4-tri-O-methyl glucuronic acid and 2,3,4- and 2,3,5-tri-O-methyl galactose, thus confirming that the linkage is 1 \rightarrow 6 in this disaccharide.

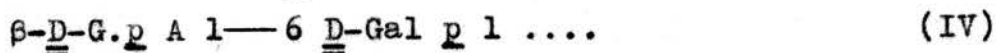
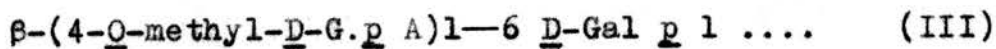
The main structural features of *Acacia mollissima* gum.

It follows, from the methylation studies on the degraded gum, and from the isolation of 1,3 and 1,6 galactobioses on partial hydrolysis of the original gum, that the degraded gum contains a highly branched stable framework of 1:3- and 1:6-linked D-galactopyranose residues for which partial

structures (I), (II) and other variants may be advanced.



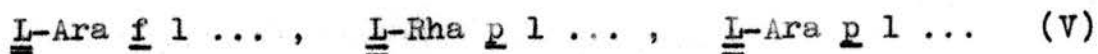
It is impossible, with the results available, to distinguish between these structures. This could be done by Smith degradation studies, as was the case in gum arabic (75), where the results obtained could only be consistent with structure (I).



The isolation of two aldobiouronic acids from Acacia mollissima gum, indicate that fragments (III) and (IV) must be important features of the gum molecule, but there is no evidence yet for the positions of linkage of these units to the galactan framework.

From methylation studies on the undegraded polysaccharide,

and from the isolation of 3-O-β-L-arabopyranosyl-L-arabinose, it is evident that the following moieties are attached to the galactan backbone.



or



The points of attachment of the acid-labile residues to galactose residues in the gum, follow from a comparison of the proportion of D-galactose methyl ethers formed on hydrolysis of the methylated gum and of the methylated degraded gum. The most striking difference is the large increase in the proportion of 2,4,6-tri-O-methyl-D-galactose from the methylated degraded gum and the corresponding decrease in the proportion of 2:4-di-O-methyl-D-galactose. It follows that a large proportion of the acid-labile groups are attached to position 6 of 1→3 linked D-galactopyranose residues in the main chains of the molecular structure. A second but significant difference is the increase in the proportion of 2,3,4,6-tetra-O-methyl-D-galactose which corresponds approximately with the decrease in the proportion of 2,3,4-tri-O-methyl-D-galactose. Further acid-labile groups are therefore linked to position 6 of D-galactopyranose residues present as side chains in the gum. It is not possible to ascribe particular points of attachment

	<u>Acacia mollissima</u>	<u>Acacia senegal</u>	<u>Acacia pycnantha</u>
<u>Proportions</u> } <u>D-Gal</u> of } <u>L-Ara</u> <u>monosaccha-</u> } <u>L-Rha</u> <u>rides</u> } <u>D-GA</u> 4- <u>O-Me-D-GA</u>	5	3	65
	6	3	27
	1	1	2
	1	1	5
<u>Fragments</u>	<u>L-Arap1-β-3L-Ara1-</u>	<u>L-Arap1-β-3L-Ara1-</u>	
<u>from</u>	<u>L-Rhap1-</u>	<u>L-Rhap1-</u>	<u>L-Rhap1-</u>
<u>periphery</u>	<u>L-Ara1-</u>	<u>L-Ara1-</u>	<u>L-Ara1-</u>
	<u>L-Arap1-</u>	<u>D-Galp1-α-3L-Ara1-</u>	<u>L-Ara1-β-3L-Ara1-</u>
<u>Fragments from</u>	<u>D-Galp1-β-3D-Gal</u>	<u>D-Galp1-β-3D-Gal</u>	<u>D-Galp1-β-3D-Gal</u>
<u>galactan framework</u>	<u>D-Galp1-β-6D-Gal</u>	<u>D-Galp1-β-6D-Gal</u>	<u>D-Galp1-β-6D-Gal</u>
<u>Acidic</u>	<u>D-GpAl-β-6D-Gal</u>	<u>D-GpAl-β-6D-Gal</u>	<u>D-GpAl-β-6D-Gal</u>
<u>fragments</u>	<u>4-O-MeD-GpAl-β-6D-Gal</u>		

All three gums contain highly branched backbones of galactose residues in which the main chain is linked 1→3 and the side-chains are attached by 1→6 linkages, but whereas all the galactose residues in the main chain of gum arabic carry galactose-containing side-chains, such side-chains are attached only to every second galactose residue in the main chains of Acacia pycnantha and Acacia mollissima gum. Although the three gums contain the same aldobiouronic acids in the side-chains, in Acacia mollissima gum some of the glucuronic acid residues are present as their 4-O-methyl ethers, as is the case in Asafoetida gum (105). Similarities between the three Acacia gums are also shown by the L-arabofuranose and L-rhamnopyranose end-groups which are removed by mild acid hydrolysis. The gums differ, however, in the nature of the more complex side-chains also removed under these conditions, as can be seen from the table. In gum arabic, the L-rhamnopyranose residues have been shown to be attached to position 4 of the glucuronic acid residues (59). This cannot be the case in the other two gums due to the absence of any appreciable amount of 2,3-di-O-methyl-D-glucuronic acid residues in the methylated polysaccharides. Thus in Acacia pycnantha gum and Acacia mollissima gum, the L-rhamnose residues must have a different mode of attachment to the backbone of the molecule.

In view of the heterogeneity present in Acacia

mollissima gum as indicated by DEAE-cellulose chromatography, care must be exercised in the interpretation of the results of structural investigation of such a complex polysaccharide. There is indeed evidence for a measure of heterogeneity in some samples of gum arabic (30, 33) and of Acacia pycnantha gum. Nevertheless it is already clear from the comparisons made, that the major components of the three gums have important structural features in common.

ACACIA MOLLISSIMA GUM

EXPERIMENTAL

Preparation of the gum acid.

Crude gum (18 g.) was crushed and allowed to swell in water (2 litres) overnight. Dissolution was achieved by heating the solution to 70°, while keeping the pH 7-9 by the addition of dilute ammonia. After cooling and centrifugation, the aqueous solution was poured into acidified methylated spirits (4 volumes) and the precipitate was removed at the centrifuge. Several reprecipitations of this residue were required to obtain the pure gum acid (12 g.).

Acacia mollissima gum acid had $[\alpha]_D -52^\circ$ (c, 1.0 in NaOH) and uronic acid anhydride content 11.1%, corresponding to an equivalent weight of 1550.

Methylation Studies on Acacia mollissima gum.

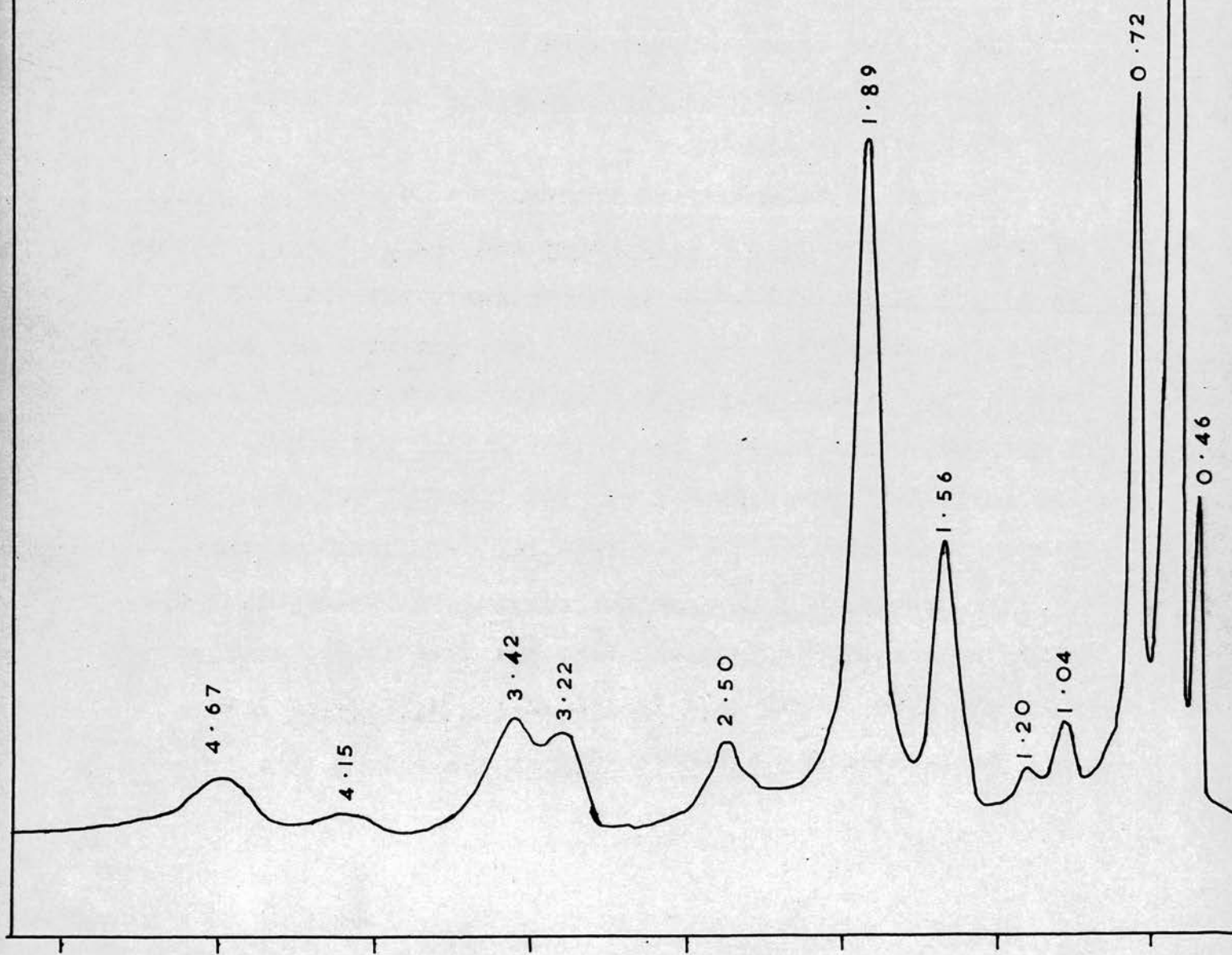
West Indian Acacia mollissima gum acid was methylated successively with methyl sulphate and sodium hydroxide, and with methyl iodide and silver oxide, to give methylated Acacia mollissima gum, $[\alpha]_D -66^\circ$ (c, 0.97 in CHCl₃) (Found: OMe, 42.4%). Lithium aluminium hydride (250 mg.) in tetrahydrofuran (8 ml.) was added to the methylated gum acid (250 mg.) in tetrahydrofuran (8 ml.), and after 0.5 hr. at

GAS-CHROMATOGRAM ON COLUMN a

METHANOLYSIS OF METHYLATED

ACACIA MOLLISSIMA GUM

0.56



room temperature the mixture was refluxed for 3 hours. The excess of hydride was destroyed with ethyl acetate and water, and the resulting mixture was shaken with dilute sulphuric acid and extracted with chloroform. The chloroform extract afforded reduced methylated Acacia mollissima gum (200 mg.), $[\alpha]_D -66^\circ$ (c, 0.80 in CHCl_3) (Found: OMe, 40.5%) Samples of the methylated gum and reduced methylated gum were treated with methanolic hydrogen chloride (2.5%) and the resulting syrups, after neutralisation, examined by gas-chromatography as shown in table I.

The vapour phase chromatogram on column a for methylated West Indian Acacia mollissima gum is shown in the accompanying diagram.

Through the courtesy of Professor A. Stephen, a sample of South African Acacia mollissima gum, $[\alpha]_D -55^\circ$ (c, 1.4 at pH 8) and equivalent 1920 (by titration), was obtained as the fully methylated derivative. The gum acid had been methylated successively with dimethyl sulphate and sodium hydroxide, methyl iodide and silver oxide, and barium oxide, barium hydroxide hydrate and dimethyl sulphate in dimethylformamide (42). We gave the methylated material another treatment with Purdie's reagents to ensure that the final methylation conditions were the same in the samples of South African and West Indian Acacia mollissima gum.

Methylated South African Acacia mollissima gum,

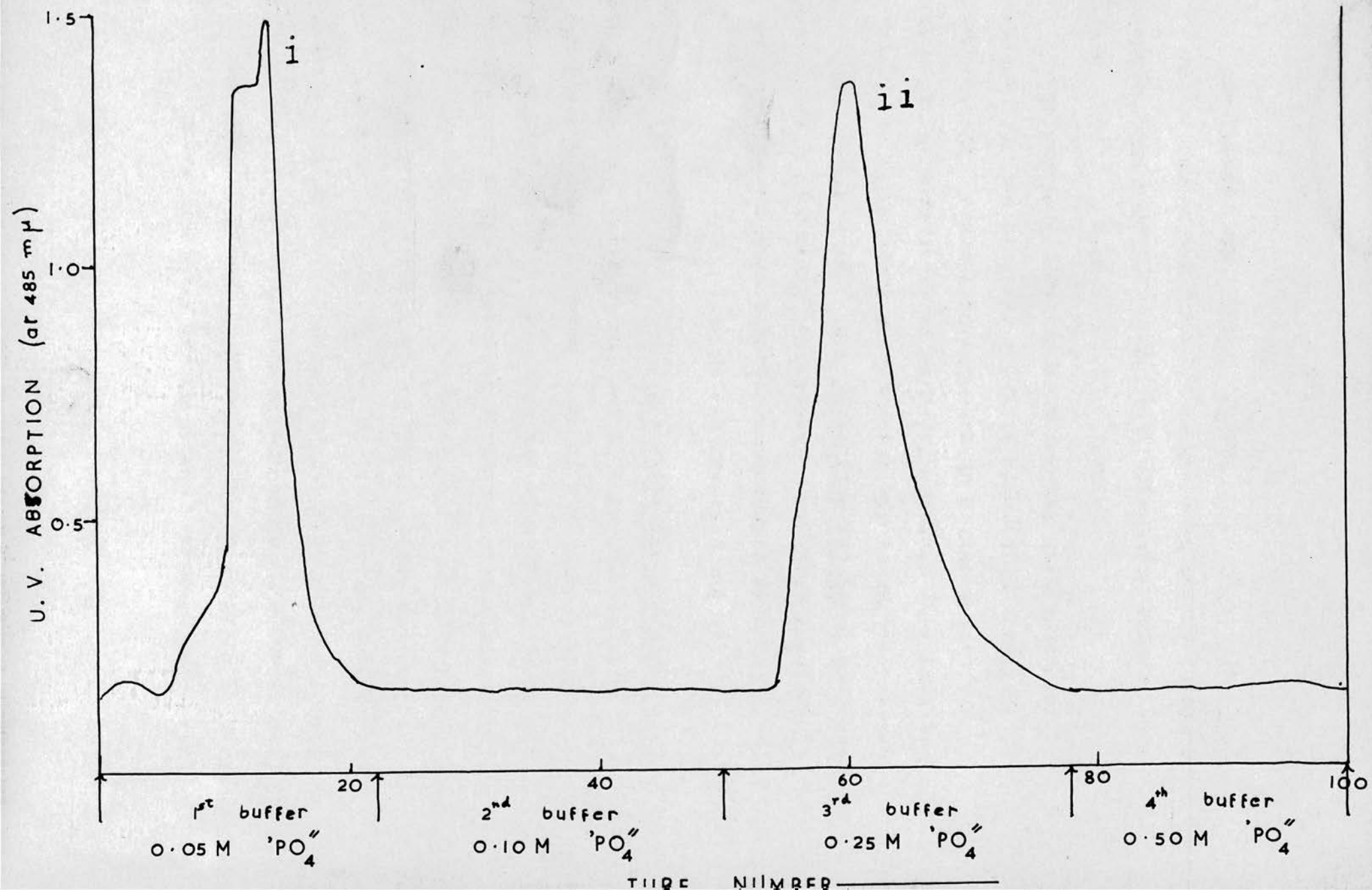
TABLE I

Methyl glycosides of	<u>T</u> in system <u>a</u>		<u>T</u> in system <u>b</u>	
	Methylated gum	Reduced methylated gum	Methylated gum	Reduced methylated gum
2,3,4-Tri-O-methyl rhamnose	0.46	0.45	(0.46)	(0.45)
2,3,4-tri-O-methyl arabinose	1.04	1.05	0.80	0.81
2,3,5-tri-O-methyl arabinose	0.56 & 0.72	0.55 & 0.73	(0.46) & 0.58	(0.45) & (0.66)
2,3-di-O-methyl arabinose	1.56 & (1.89)	1.57 & (1.89)	(0.66) & 0.96	(0.66) & 0.96
2,5-di-O-methyl arabinose	(1.89) & 3.42	(1.89) & 3.44	(0.66) & 1.02	(0.66) & 1.02
2,3,4,6-tetra-O-methyl galactose	(1.89)	(1.89)	1.62	1.61
2,4,6-tri-O-methyl galactose	4.15 & 4.67	4.15 & 4.70	2.36	2.38
2,3,4-tri-O-methyl galactose	7.4	7.4	2.98	2.90
2,4-di-O-methyl galactose			3.60 & 4.26	3.60 & 4.27
2,3,4-tri-O-methyl glucuronic acid*	2.50 & 3.22		1.76 & 2.22	
2,3-di-O-methyl glucuronic acid*	8.3 & 9.2			
2,3,4-tri-O-methyl glucose		2.59 & 3.71		1.32 & 1.79

* Methyl glycosides present as methyl esters.

Figures in parenthesis indicate T values of components which were incompletely resolved.

FRACTIONATION OF ACACIA MOLLISSIMA GUM ON DEAE-CELLULOSE



$[\alpha]_D -66^\circ$ (c , 0.99 in CHCl_3) (Found: OMe, 42.6%) was reduced, in the same way as the West Indian sample, to give reduced methylated South African Acacia mollissima, $[\alpha]_D -66^\circ$ (c , 1.0 in CHCl_3) (Found: OMe, 40.8%). Samples of the methylated gum and reduced methylated gum were treated with methanolic hydrogen chloride and the resulting syrups were examined by gas-chromatography. The results obtained were closely analagous to those obtained from gas-chromatography of the methanolysis products of methylated West Indian Acacia mollissima gum. Exactly the same constituent methylated sugars were present in each, although their relative proportions differed slightly as shown in table A (in the discussion section).

DEAE-Cellulose chromatography.

A sample (50 mg.) of the gum acid was placed on a DEAE-cellulose column (3 x 30 cm.), which had been generated to the phosphate form in the usual way. The column was eluted successively with phosphate buffers of increasing strengths, 0.05M, 0.1M, 0.25M and 0.50M, all at pH 6 (250 ml. of each). The graph of tube number against sugar concentration was calculated and is given in the accompanying diagram. Two fractions were obtained, Fraction (i) (42.2% of the whole) had uronic acid content = 13.6%, and Fraction (ii) (57.8%) had uronic acid content = 14.7%. The experiment was repeated on another sample of the gum acid, using a

different DEAE-cellulose column and exactly the same results were obtained.

Isolation and characterisation of the neutral monosaccharide constituents.

Gum acid (5 g.) was hydrolysed with N sulphuric acid for 5 hours at 100°. After neutralisation and deionisation, the solution was allowed to soak into a CG45 resin column, generated to the formate form. Elution with water gave a mixture of neutral monosaccharides, part (500 mg.) of which was fractionated on thick paper in solvent B.

Fraction (i) (78 mg.) was characterised as L-rhamnopyranose by conversion into the benzoylhydrazone, m.p. and mixed m.p. 178-9°.

Fraction (ii) (244 mg.) was characterised as L-arabinose by conversion into the benzoylhydrazone, m.p. and mixed m.p. 186°.

Fraction (iii) (106 mg.) was characterised as D-galactose by conversion into β -1,2,3,4,6-penta-acetyl-D-galactose, m.p. and mixed m.p. 142°.

Autohydrolysis of the gum acid.

A small scale test hydrolysis was carried out by heating a 2% aqueous solution (50 ml.) of the gum acid at 90°. Complete solvation was not achieved until the reaction had been in progress for 3.5 hr. Samples (5 ml.) were removed at intervals and their optical rotations were

measured with the following results:-

Time (hours)	3.5	10	25	50	65
$[\alpha]_D$	+4°	+12°	+15°	+18°	+18.5°

After measuring the rotation of each aliquot, the solution was poured into ethanol (4 volumes), thus precipitating out the degraded polysaccharide whilst keeping all mono- and oligosaccharides in solution. After removing the residue, the filtrate was concentrated to a syrup and examined by paper chromatography. The low molecular weight material, which had been removed from the polysaccharide, was composed of D-galactose, L-arabinose, L-rhamnose and unidentified oligosaccharides. The degraded polysaccharide which remained after 65 hours gave, on hydrolysis, galactose and acidic material, but no trace of arabinose or rhamnose.

Large-scale autohydrolysis. Gum acid (8 g.) was dissolved in water (400 ml.) and the mixture was heated at 90° for 64 hours. The reaction mixture was then poured into ethanol (1.5 litres) and filtered, the filtrate being concentrated to a syrup (6.5 g.) of low molecular weight sugars.

The residue was washed five times with ethanol and dried overnight in a vacuum oven to give degraded Acacia mollissima gum (1.8 g.), $[\alpha]_D -33^\circ$ (c , 0.85) and uronic acid anhydride content 19%, corresponding to an equivalent of 938.

The degraded gum (1.06 g.) was methylated successively with methyl sulphate and sodium hydroxide, and with methyl iodide and silver oxide to give methylated degraded Acacia mollissima gum (84 mg. - low yield due to accident), $[\alpha]_D -46^\circ$ (c , 0.84 in CHCl_3) (Found: OMe, 43.1%). A sample of the methylated polysaccharide was methanolised and the mixture of methyl glycosides obtained, were examined by gas-chromatography on both solvent systems as shown in table II.

TABLE II

Methyl glycoside of	<u>T</u> in column <u>a</u>	<u>T</u> in column <u>b</u>
2,3,4,6-Tetra- <u>O</u> -methyl galactose	1.75	1.68 & 1.55
2,3,4-tri- <u>O</u> -methyl galactose	7.30	2.70 & 2.97
2,4,6-tri- <u>O</u> -methyl galactose	4.08 & 4.62	2.14 & 2.45
2,4-di- <u>O</u> -methyl galactose		3.72 & 4.43
2,3,4-tri- <u>O</u> -methyl glucuronic acid*	2.44 & 3.17	1.82 & 2.28

* Methyl glycoside present as methyl ester.

The syrup (6.5 g.) containing the low molecular weight sugars removed from the polysaccharide, was dissolved in water (10 ml.) and placed on a charcoal-celite column. The monosaccharides were eluted with water and were found, by paper chromatographic examination, to consist of galactose,

arabinose and rhamnose. The oligosaccharides were desorbed by graded elution with aqueous ethanol 2½-10% (3 litres in all). The fractions obtained contained three oligosaccharides, which were purified by fractionation on thick paper in solvent B.

Oligosaccharide A. This fraction (24 mg.), R_{GAL} 0.91, $[\alpha]_D +183^\circ$ (c , 0.24) was chromatographically identical to 3-O- β -L-arabopyranosyl-L-arabinose in solvents A and B. Hydrolysis gave only arabinose. The oligosaccharide (2 mg.) was methylated with dimethyl sulphate and sodium hydroxide; the methylated sugar obtained was treated with 2.5% methanolic hydrogen chloride and the mixture of methyl glycosides obtained were analysed by gas-chromatography. The results are shown in table III.

TABLE III

Methyl glycoside of	<u>T</u> in column <u>a</u>	<u>T</u> in column <u>b</u>
2,3,4-Tri- <u>O</u> -methyl arabinose	1.04	0.82
2,4-di- <u>O</u> -methyl arabinose	2.29 & 2.18	1.14 & 1.07
2,5-di- <u>O</u> -methyl arabinose	1.86 & 3.41	0.69

The derived 3-O- β -L-arabopyranosyl-L-arabinose phenylosazone after recrystallisation from ethanol:water (1:10), had m.p. and mixed m.p. 221° and 223° respectively (on mixing with an authentic sample). An X-ray powder photograph of the

phenylosazone was identical to that obtained from an authentic specimen.

Oligosaccharide B. This fraction (20 mg.), R_{GAL} 0.51, $[\alpha]_D +50^\circ$ (c , 0.20) was chromatographically identical to 3-O- β -D-galactopyranosyl-D-galactose in solvents A and B. Hydrolysis gave only galactose. The sugar crystallised on standing and was recrystallised from acetone-water. The crystals had m.p. 225° . A wide range of recorded values for the m.p. of 3-O- β -D-galactopyranosyl-D-galactose is available. The only authentic sample we had gave a m.p. 168° and this gave an identical X-ray powder diagram to that obtained from oligosaccharide B.

Oligosaccharide C. This fraction (16 mg.) R_{GAL} 0.36, $[\alpha]_D +45^\circ$ (c , 0.16) was chromatographically identical to 6-O- β -D-galactopyranosyl-D-galactose in solvents A, B and C. Hydrolysis gave only galactose. The sugar was characterised as the above disaccharide by conversion into the phenylosazone which was identical in m.p. 179° , mixed m.p. 180° and X-ray powder diagram to an authentic sample, m.p. 181° .

Partial Hydrolysis of the gum.

Gum acid (6.3 g.) was treated with N-sulphuric acid (310 ml.) at 100° for 7 hours. The hydrolysate was neutralised with barium hydroxide and barium carbonate, treated with Amberlite resin 1R120(H) to remove barium ions,

evaporated to small volume and placed on a column of Amberlite CG45 resin (formate form, 400 ml.). The neutral sugars were eluted with water, and the eluate concentrated to give a syrup (4.47 g.) consisting of neutral monosaccharides.

The acidic sugars were desorbed from the column with 8% formic acid and the eluate was concentrated to a syrup (1.18 g.). Paper chromatographic examination in solvent B, showed this syrup to consist of a mixture of monosaccharides, acidic and neutral, plus two aldobiouronic acids, R_{GAL} 0.25 and 0.60. These two disaccharides were obtained pure by fractionation on thick paper in solvent B.

Aldobiouronic acid I. This fraction (380 mg.), R_{GAL} 0.25, $[\alpha]_D -3^\circ$ (c, 1.0) was chromatographically identical to 6-O- β -D-glucuronosyl-D-galactose in solvents B and C. Reduction of the derived methyl ester, methyl glycosides with sodium borohydride, followed by hydrolysis and paper chromatographic examination of the products, gave glucose and galactose. Hydrolysis of the unreduced disaccharide gave galactose and glucuronic acid. The aldobiouronic acid was methylated in an exactly analogous manner to the methylation of the aldobiouronic acids obtained from Khaya senegalensis gum. The expected crystalline methylated derivative has so far failed to appear.

Aldobiouronic acid II. This fraction (200 mg.), R_{GAL} 0.60, $[\alpha]_D +6^\circ$ (c , 1.02) was chromatographically identical to 6-O-(4-O-methyl- β -D-glucopyranosyl uronic acid)-D-galactose in solvents B and C. Reduction of the derived methyl ester, methyl glycosides (10 mg.) with sodium borohydride, followed by hydrolysis and paper chromatographic examination of the products gave galactose and a spot (R_{Rha} 0.91) with the same mobility as 4-O-methyl glucose. Part (5 mg.) of the hydrolysis products from the reduced disaccharide was subjected to Lemieux-Bauer periodate oxidation. Paper chromatographic examination of the products gave a spot, R_F 0.54 in solvent D which is consistent for a 4-O-substituted hexose.

Another sample of aldobiouronic acid II was treated with sodium metaperiodate but no formaldehyde was released, as detected by the chromotropic acid method (91). This is consistent with a 1,6-linked disaccharide. The remainder (108 mg.) of the fraction was methylated with barium oxide, barium hydroxide and dimethyl sulphate in dimethyl formamide and dimethyl sulphoxide. The methylated disaccharide obtained (52 mg.) was methanolysed and the products were examined by gas-phase chromatography in both solvent systems with the results shown in table IV.

TABLE IV

Methyl glycoside of	<u>T</u> in column <u>a</u>	<u>T</u> in column <u>b</u>
2,3,4-Tri- <u>O</u> -methyl glucuronic acid*	2.47 & 3.20	1.77 & 2.22
2,3,4-tri- <u>O</u> -methyl galactose	7.5	2.62 & 2.94
2,3,5-tri- <u>O</u> -methyl galactose	4.45	1.94

* Present as the methyl ester.

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317. *The Location of L-Rhamnopyranose Residues in Gum Arabic.*

By G. O. ASPINALL, A. J. CHARLSON, E. L. HIRST, and R. YOUNG.

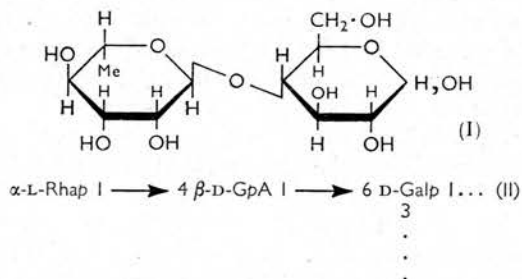
D-Glucuronic acid residues in gum arabic are reduced to D-glucose residues by treatment of the acetylated gum with diborane. Acetolysis of the reduced polysaccharide, followed by deacetylation, furnishes a mixture of oligosaccharides amongst which the disaccharide, 4-O- α -L-rhamnopyranosyl-D-glucose, has been characterised. These experiments establish the location of some of the L-rhamnopyranose residues in the gum.

L-RHAMNOPYRANOSE residues in *Acacia senegal* gum (gum arabic) are known to occur mainly, if not exclusively, as non-reducing end groups.¹ Since L-rhamnose and D-glucuronic acid residues are present in the approximate ratio of 1 : 1 in gum arabic, and also in *Acacia mollissima*² and *Acacia cyanophylla*³ gums, Charlson, Nunn, and Stephen³ suggested that this might indicate that L-rhamnose is glycosidically linked to D-glucuronic acid residues in these three gums. A similar suggestion has been made by Smith and Montgomery.⁴ Hamilton and Thompson⁵ have shown that alkaline hydrolysis of gum arabic at 160° leads to the loss of both glucuronic acid and rhamnose residues with the formation of an arabinogalactan. Since this type of reaction appears to result in the selective cleavage of glycosiduronic acid linkages, sugar units attached to glucuronic acid residues would also be lost. Although other interpretations are possible, this observation seems to support the contention that L-rhamnose is attached to D-glucuronic acid residues in gum arabic. Very recently, O'Colla and his collaborators⁶ have shown that degradation of gum arabic with Fenton's reagent yields 10% of dialysable material which includes rhamnose, arabinose, galactose, and a disaccharide. The mechanism of this degradation is not yet understood, but since glycosiduronic acid linkages are apparently cleaved an association of rhamnose and other sugar units with glucuronic acid residues is indicated. We now report definite evidence for such a rhamnose-glucuronic acid linkage.

In this study *Acacia senegal* gum, obtained through the courtesy of Mr. Videll-Hall of the Forest Department of the Sudan Government, has been used. In order to show that this sample of gum arabic contained similar structural features to those of the sample used by Smith in his investigations,¹ the gum was methylated and the methanolysis products from the methylated gum were examined by gas-liquid partition chromatography.^{7,8} This highly selective analytical technique showed the presence therein of components having the same retention times as those of the methyl glycosides of all the major cleavage products which had been characterised by Smith,¹ namely, 2,3,4-tri-O-methyl-L-rhamnose, 2,3,5-tri- and 2,5-di-O-methyl-L-arabinose, 2,3,4,6-tetra- and 2,4-di-O-methyl-D-galactose, and 2,3,4-tri- and 2,3-di-O-methyl-D-glucuronic acid; smaller relative amounts of methyl glycosides of 2,3,4-tri-O-methylarabinose and 2,4,6-tri-O-methylgalactose were also indicated. Further indications that the D-glucuronic acid residues in the gum were 4-O-substituted, and, to a smaller extent, present as end groups, were obtained by gas-chromatographic examination of the methanolysis products of the methylated gum after reduction of the glucuronic acid to glucose residues with lithium aluminium hydride, and also after subsequent remethylation. In these methanolysis products the components having the retention times of methyl glycosides of 2,3-di- and 2,3,4-tri-O-methyl-D-glucuronic acid were replaced by components having the retention times of methyl glycosides of 2,3-di- and 2,3,4-tri-O-methyl-D-glucose and of 2,3,6-tri- and 2,3,4,6-tetra-O-methyl-D-glucose, respectively.

The acetylated gum was then reduced by using the diborane procedure of Smith and Stephen;⁹ examination of the reduced polysaccharide, which was isolated in high yield after deacetylation of the reaction product, showed that *ca.* 82% of the carboxyl groups

had been reduced. Partial hydrolysis of the reduced gum was effected by acetolysis with acetic anhydride-sulphuric acid¹⁰ for 24 hours at room temperature. Paper-chromatography of the sugars formed on deacetylation of the acetolysis products showed the presence of monosaccharides and at least eight oligosaccharides. Column chromatography on cellulose and charcoal-Celite led to the isolation of a syrupy disaccharide which was characterised as its crystalline phenylosazone. The structure of the disaccharide as 4-*O*- α -L-rhamnopyranosyl-D-glucopyranose (I) follows from the following considerations. Glucose and rhamnose were detected on paper chromatograms after hydrolysis of the disaccharide, but only rhamnose was detected on hydrolysis of the derived phenylosazone. The nature of the reducing portion of the disaccharide was established by the isolation of D-glucitol on hydrolysis of the glycol formed on reduction of the disaccharide with sodium borohydride. Oxidation of the disaccharide with an excess of lead tetra-acetate in acetic acid containing 2% of water for 2 hours at room temperature¹¹ followed by hydrolysis of the product gave rhamnose and erythrose. These experiments show that the disaccharide is a rhamnoglucoside and that 1 \rightarrow 2 and 1 \rightarrow 3 linkages are absent. Oxidation of the disaccharide with periodate gave 0.55 mol. of formaldehyde, which could only have arisen from the glucose portion, thus indicating the absence of 1 \rightarrow 5 and 1 \rightarrow 6 linkages. Positive evidence in favour of a 1 \rightarrow 4 linkage was obtained by examining the cleavage products from the methylated disaccharide. The methanolysis products from the methylated disaccharide were examined by gas-liquid chromatography,^{7,8} and the presence was indicated of methyl glycosides of 2,3,4-tri-*O*-methylrhamnose and 2,3,6-tri-*O*-methylglucose. Hydrolysis of the methylated disaccharide gave 2,3,4-tri-*O*-methyl-L-rhamnose and 2,3,6-tri-*O*-methyl-D-glucose, both sugars being characterised by the formation of crystalline derivatives. The configuration of the glycosidic linkage is indicated by the similarity of the optical rotation ($[\alpha]_D -6^\circ$ in water) to that ($[\alpha]_D -0.1^\circ$) of the isomeric disaccharide, 6-*O*- α -L-rhamnopyranosyl-D-glucose, for whose configuration independent evidence has been obtained by Gorin and Perlin.¹²



The characterisation of 4-*O*- α -L-rhamnopyranosyl-D-glucopyranose (I) as a partial hydrolysis product of reduced gum arabic provides clear evidence that some of the L-rhamnopyranose residues in the gum are glycosidically linked to C-4 of D-glucuronic acid residues. Since earlier work¹ on gum arabic showed that L-rhamnopyranose residues were present as terminal groups, that the majority of D-glucuronic acid residues carried substituents at position 4, and that the majority of D-galactopyranose residues were 3,6-di-*O*-substituted, and since the aldobiouronic acid, 6-*O*-(β -D-glucopyranosyluronic acid)-D-galactose, is formed on graded hydrolysis of the gum, it may be concluded that the structural unit (II) is an important fragment of the gum molecule.

In addition to isolating the rhamnose-containing disaccharide (I), we have obtained evidence that higher oligosaccharides containing rhamnose residues are produced in the acetolysis. One of these appeared to be an acidic trisaccharide, resulting from incomplete reduction of hexuronic acid units, which gave, on graded hydrolysis, sugars with the chromatographic mobilities of L-rhamnose and 6-*O*-(β -D-glucopyranosyluronic acid)-D-galactose. It was shown subsequently (unpublished results) that this substance

is more readily formed from the unmodified gum. It is noteworthy that several oligosaccharides containing terminal 6-deoxyhexose residues have been isolated by using acetolysis for the cleavage of glycosidic linkages. Kuhn and his collaborators¹³ have isolated 2-*O*- α -L-rhamnopyranosyl-D-galactose and 2-*O*- α -L-rhamnopyranosyl-(3-*O*- β -D-galactopyranosyl-D-glucose) on acetolysis of the steroidal glycoside solanin. In this laboratory 2-*O*- α -L-fucopyranosyl-D-xylose has been recently isolated from gum tragacanth by a similar procedure.¹⁴ It is clear, therefore, that acetolysis provides a valuable alternative method for the linkage analysis of polysaccharides containing 6-deoxyhexose residues which are relatively labile towards hydrolysis with aqueous mineral acid.

EXPERIMENTAL

Paper chromatography was carried out on Whatman Nos. 1 and 3MM papers with the following solvent system (v/v): (A) butan-1-ol-ethanol-water (4:1:5, upper layer); (B) butan-1-ol-acetic acid-water (4:1:5, upper layer); (C) benzene-ethanol-water (169:47:15, upper layer); (D) butan-2-one, half saturated with water; (E) ethyl acetate-pyridine-water (10:4:3); ethyl acetate-acetic acid-formic acid-water (18:3:1:4).

Gas-liquid partition chromatography of the methyl glycosides of methylated sugars was carried out in a Pye argon chromatograph according to the procedure of Bishop and Cooper⁷ (see also accompanying paper⁸). Separations were carried out on the following columns (120 \times 0.5 cm.) at gas flow rates of 80–100 ml./min.: (a) 15% by weight of butan-1,4-diol succinate polyester⁷ on acid-washed Celite at 175°; (b) 10% by weight of polyphenyl ether [*m*-di-(*m*-phenoxyphenoxy)benzene] on acid-washed Celite at 200°; (c) 20% by weight of Apiezon M on acid-washed Celite at 150°. Retention times (*T*) are quoted relative to methyl 2,3,4,6-tetra-*O*-methyl- β -D-glucopyranoside as an internal standard.

Optical rotations were observed at *ca.* 18°.

Methylated Gum Arabic and Derivatives.—Gum arabic (20 g.) was methylated successively with methyl sulphate and sodium hydroxide, and with methyl iodide and silver oxide, to give methylated gum arabic (11.2 g.), $[\alpha]_D -48^\circ$ (*c* 1.0 in CHCl₃) (Found: OMe, 41.5%). Lithium aluminium hydride (150 mg.) in tetrahydrofuran (5 ml.) was added to methylated gum arabic (150 mg.) in tetrahydrofuran (5 ml.), and after 0.5 hr. at room temperature the mixture was refluxed for 3 hr. The excess of hydride was destroyed with ethyl acetate and water, and the resulting mixture was shaken with dilute sulphuric acid and extracted with chloroform. The chloroform extract afforded reduced methylated gum arabic (105 mg.), $[\alpha]_D -43.5^\circ$ (*c* 1.06 in CHCl₃) (Found: OMe, 39.6%). Hydrolysis of a sample of the reduced methylated gum followed by chromatography of the products in solvents A and B showed that reduction of hexuronic acid residues was complete. Reduced methylated gum (90 mg.) was methylated in *NN*-dimethylformamide with methyl iodide and silver oxide, to give methylated reduced gum arabic (78 mg.), $[\alpha]_D -47.5^\circ$ (*c* 1.02 in CHCl₃) (Found: OMe, 41.3%).

Samples of the methylated gum, reduced methylated gum, and methylated reduced gum were heated with methanolic 2.5% hydrogen chloride in sealed tubes at 100° for 16 hr., the cooled solutions were neutralised with silver carbonate, filtered, and concentrated, and the resulting syrups were examined by gas-chromatography, with results shown in the Tables.

Carboxyl-reduced Arabic Acid.—Arabic acid (equivalent weight, 1380) was prepared from *Acacia senegal* gum by precipitation from aqueous solution which had been acidified with hydrochloric acid by the addition of ethanol (4–5 vol.), and the gum acid was acetylated in formamide solution with acetic anhydride and pyridine by Carson and Maclay's method¹⁵ to give acetylated arabic acid, $[\alpha]_D -21^\circ$ (*c* 0.94 in CHCl₃). Acetylated arabic acid (30 g.) was dissolved in 1,2-dimethoxyethane (400 ml.), lithium borohydride (6.5 g.) was dissolved in the solution, and diborane was generated *in situ* by the addition of portions (*ca.* 5 ml.) of a solution of boron trifluoride-ether complex (10 g.) in 1,2-dimethoxyethane (40 ml.) during 1.5 hr. After each addition the stoppered flask was shaken gently and then vigorously to break up the gel which separated, and further solvent (200 ml.) was added during the additions when the mixture became too thick. The mixture was set aside overnight and was then poured into ice-water (1.5 l.). The mixture was made just alkaline and was concentrated under reduced pressure to a thick paste. The paste was dissolved in 0.1*N*-sodium hydroxide, and the solution was adjusted to pH 9 and heated for 2 hr. at 55°. The resulting solution was dialysed against tap water for 48 hr. and against distilled water for 24 hr., filtered, concentrated, and poured into

TABLE 1.
Relative retention times (*T*) of methyl glycosides on column (a).

Sugar	Methylated gum arabic	Reduced methylated gum arabic	Methylated reduced gum arabic
2,3,4-Tri- <i>O</i> -methylrhamnose	0.44	0.44	0.45
2,3,5-Tri- <i>O</i> -methylarabinose	0.55, 0.72	0.54, 0.71	0.55, 0.71
2,3,4-Tri- <i>O</i> -methylarabinose	1.04	1.04	(1.02)
2,5-Di- <i>O</i> -methylarabinose	(1.84), 3.47	(1.86), 3.41	(1.87) (3.50)
2,3,4,6-Tetra- <i>O</i> -methylgalactose	(1.84)	(1.86)	(1.87)
2,4,6-Tri- <i>O</i> -methylgalactose	4.19, 4.80	4.15, 4.70	4.17, (4.82)
2,3,4-Tri- <i>O</i> -methylglucuronic acid *	2.52, 3.28		
2,3-Di- <i>O</i> -methylglucuronic acid *	8.4, 9.3		
2,3,4-Tri- <i>O</i> -methylglucose		2.54, 3.70	
2,3,4,6-Tetra- <i>O</i> -methylglucose			(1.02) (1.49)
2,3,6-Tri- <i>O</i> -methylglucose			3.50, 4.82
Unknown sugar	1.51	1.49	(1.49)

* Methyl glycosides present as methyl esters.

Figures in parentheses indicate *T* values of components which were incompletely resolved.

TABLE 2.
Relative retention times (*T*) of methyl glycosides on column (b).

Sugar	Methylated gum arabic	Reduced methylated gum arabic	Methylated reduced gum arabic
2,3,4-Tri- <i>O</i> -methylrhamnose	(0.46)	(0.46)	(0.46)
2,3,5-Tri- <i>O</i> -methylarabinose	(0.46), 0.59	(0.46), 0.59	(0.46), 0.59
2,3,4-Tri- <i>O</i> -methylarabinose	0.84	0.83	0.82
2,5-Di- <i>O</i> -methylarabinose	0.69, 1.06	0.70, 1.05	0.69, (1.06)
2,3,4,6-Tetra- <i>O</i> -methylgalactose	1.52, 1.61	1.52, 1.61	1.52, 1.61
2,4,6-Tri- <i>O</i> -methylgalactose	2.09, (2.41)	2.10, (2.46)	(2.21), 2.39
2,4-Di- <i>O</i> -methylgalactose	3.71, 4.40	3.68, 4.40	3.69, 4.40
2,3,4-Tri- <i>O</i> -methylglucuronic acid *	1.78, 2.24		
2,3-Di- <i>O</i> -methylglucuronic acid *	(2.41), 3.11		
2,3,4-Tri- <i>O</i> -methylglucose		1.36, 1.86	
2,3-Di- <i>O</i> -methylglucose		(2.46), 3.28	
2,3,4,6-Tetra- <i>O</i> -methylglucose			(1.02), 1.34
2,3,6-Tri- <i>O</i> -methylglucose			1.72, 2.21

* Methyl glycosides present as methyl esters.

Figures in parentheses indicate *T* values of components which were incompletely resolved.

a mixture of ethanol (750 ml.) and ether (250 ml.). The gummy precipitate was triturated with ethanol and dried to give a residue (20 g.). The crude polysaccharide was dissolved in water (200 ml.), and the solution was deionised by passage through columns of Amberlite resins IR-120(H) and IR-45(OH), concentrated to 100 ml., and freeze-dried to give the reduced polysaccharide (15 g.), which had $[\alpha]_D -16^\circ$ (*c* 0.94 in H₂O) and equivalent weight 7800. Hydrolysis of the reduced polysaccharide followed by paper chromatography of the products showed rhamnose, arabinose, galactose, and glucose with only traces of aldobiouronic acid (6-*O*-β-D-glucuronosyl-D-galactose).

Acetolysis of Carboxyl-reduced Arabic Acid.—The reduced polysaccharide (14 g.) was added with stirring to acetic anhydride (250 ml.) and concentrated sulphuric acid (7.5 ml.) at 3°, stirring was continued for 0.5 hr. at 3° and at room temperature for 6 hr., and the mixture was kept overnight. The resulting dark solution was poured into ice-water (500 ml.), and after being stirred for 15 min. the mixture was extracted with chloroform (4 × 225 ml.). The chloroform extract was washed with water and sodium hydrogen carbonate solution, dried, and concentrated to a syrup (23.9 g.). The syrup was treated with methanolic 5% sodium methoxide (50 ml.) for 18 hr. at 3°, and the resulting mixture was neutralised with acetic acid and concentrated to a syrup, which was dissolved in water, deionised by passage through cation- and anion-exchangers and concentrated to a syrup (10 g.). A further quantity (9 g.) of the mixture of sugars was similarly formed from the reduced polysaccharide (10 g.).

The combined syrups (19 g.) were fractionated on cellulose (35 × 6.8 cm.) by elution with benzene-ethanol (5:1 in steps to 1:5) containing small amounts of water (0.5 to 5%), and later with ethanol-water (4:1) and with water. The earlier fractions contained mono-saccharides (rhamnose, arabinose, galactose, and glucose) and substances of higher chromato-

graphic mobility (probably partially acetylated sugars). Later, four fractions containing oligosaccharides were collected.

Fractionation of Oligosaccharides.—*Fraction 1.* Chromatography of the syrup (0.7 g.; eluted with 1:1 benzene-ethanol containing 1% and 2% of water) indicated galactose, oligosaccharide 1 ($R_{\text{galactose}}$ 0.69 and 0.75 in solvents A and F), and traces of arabinose and galactosylarabinose. Galactose (70 mg.) separated from the syrup and the remaining syrup was fractionated on charcoal-Celite (1:1; 29×3.8 cm.). Elution with water afforded galactose (150 mg.), elution with water containing 2% of ethanol (3×500 ml.) gave fractions 1a to 1c, and elution with water containing 4% of ethanol gave fraction 1d. Although no sugars could be detected in fractions 1a and 1d, these fractions were concentrated and combined with fraction 2 for re-fractionation. Fraction 1b was concentrated, dissolved in methanol, filtered, and taken to dryness to give oligosaccharide 1 (110 mg.), $[\alpha]_{\text{D}} -6^{\circ}$ (c 0.97 in H_2O), which was substantially pure except for a trace of galactosylarabinose. Concentration of fraction 1c gave further oligosaccharide 1 (150 mg.) probably contaminated with Celite.

Fraction 2. Chromatography of the syrup [1.62 g.; eluted with benzene-ethanol (1:2, later 1:5, containing 3% and 5% of water, respectively)] indicated galactose, arabinose, and oligosaccharides 1 and 2. Elution of the syrup from charcoal-Celite (1:1; 33×3.8 cm.) with water (1 l.) afforded monosaccharides, but further elution with water (6.5 l.), and with water (2 l.) containing 3% of ethanol, gave an unresolved mixture (1.15 g.) of oligosaccharides 1 and 2. The oligosaccharides were fractionated on cellulose (38×2.3 cm.), elution with butan-1-ol, half saturated with water, furnishing oligosaccharide 1 (fraction 2a; 0.31 g.) containing only a trace of galactosylarabinose, and elution with methanol-water (9:1) furnished oligosaccharide 2 (fraction 2b; 0.57 g.), which was chromatographically indistinguishable from 3-O- α -D-galactopyranosyl-L-arabinose, $R_{\text{galactose}}$ 0.50 and 0.72 in solvents A and E.

Fraction 3. Chromatography of the syrup [(1.16 g.; eluted with benzene-ethanol-water (1:10:1) and ethanol-water (4:1)] indicated oligosaccharides 2 and 3 ($R_{\text{galactose}}$ 0.2, 0.50, and 0.29 in solvents A, E, and F) and traces of galactose and arabinose. Oligosaccharide 3 was probably a galactobiose since a sub-fraction from chromatography on charcoal-Celite, which was rich in this component, gave mainly galactose on hydrolysis.

Fraction 4. Chromatography of the syrup (6.63 g.; eluted with water) indicated a complex mixture of higher oligosaccharides. The syrup was chromatographed on charcoal-Celite (35×6.8 cm.), elution with water removing traces of monosaccharides and elution with water containing 2, 5, 10, and 20% of ethanol furnishing fractions 4a (0.2 g.), 4b (0.7 g.), 4c (0.62 g.), and 4d (0.91 g.). Fraction 4a, $R_{\text{galactose}}$ 0.17 and 0.0 in solvents F and E, was probably an acidic trisaccharide which gave rhamnose, an aldobiouronic acid (probably 6-O- β -D-glucuronosyl-D-galactose), and a trace of galactose on hydrolysis. Fractions 4c and 4d contained higher rhamnose-containing oligosaccharides which gave rhamnose, glucose, and galactose amongst the hydrolysis products.

Examination of oligosaccharide 1. Oligosaccharide 1 had $R_{\text{galactose}}$ 0.69 and 0.75 in solvents A and F, but cochromatographed with glucose in solvent E and afforded rhamnose and glucose on hydrolysis. The sugar (fraction 2a; 0.2 g.) was reduced with sodium borohydride (0.1 g.) in water (10 ml.) for 18 hr. The solution was acidified with acetic acid, shaken with Amberlite resin IR-120(H), filtered, concentrated, and taken to dryness with methanol to remove boric acid. The product was chromatographically homogeneous, but treatment of a portion with acetic anhydride and pyridine failed to yield a crystalline acetyl derivative. The remainder of the glycol was hydrolysed with 0.5N-sulphuric acid at 100° for 5 hr., and chromatography of the hydrolysate indicated rhamnose and glucitol. The hydrolysate was heated with aqueous barium hydroxide at 100° for 3 hr. to destroy reducing sugar, and the solution was acidified with dilute sulphuric acid, filtered, neutralised with Amberlite resin IR-45 (OH), and taken to dryness. The residue was treated with acetic anhydride and pyridine, and the product after recrystallisation from ethanol furnished D-glucitol hexa-acetate, $[\alpha]_{\text{D}} +9^{\circ}$ (c 2.4 in Me_2CO), which was identified by m. p. and mixed m. p. 100° and by its infrared spectrum.

The sugar (fraction 1b; 95 mg.) was heated with phenylhydrazine hydrochloride (0.2 g.) and sodium acetate (0.3 g.) in water (2 ml.) at 100° for 2.5 hr. and furnished a phenylosazone which had m. p. $165-167^{\circ}$ after recrystallisation from ethanol-water (Found: C, 55.3; H, 6.4; N, 10.8; and after drying at 100° : C, 57.1; H, 6.9. $\text{C}_{24}\text{H}_{32}\text{N}_4\text{O}_8 \cdot \text{H}_2\text{O}$ requires C, 55.2; H, 6.6; N, 10.7. $\text{C}_{24}\text{H}_{32}\text{N}_4\text{O}_8$ requires C, 57.2; H, 6.4%). Hydrolysis of the phenylosazone gave rhamnose as the sole reducing sugar.

Lead tetra-acetate (15 mg.) in acetic acid (1 ml.) was added to the sugar (fraction 1b; 5 mg.) in acetic acid (3 ml.) and water (0.08 ml.), and the solution was kept at room temperature for 2.5 hr. The excess of lead tetra-acetate was destroyed and lead was precipitated by the drop-wise addition of 10% oxalic acid in acetic acid. The filtered solution was concentrated, the residue was hydrolysed and chromatography of the hydrolysate showed rhamnose and erythrose. Periodate oxidation of the sugar (fraction 1b; 10 mg.) in sodium hydrogen carbonate buffer¹⁶ afforded formaldehyde, identified as the dimedone derivative, m. p. 190°.

The sugar (fraction 1c; 130 mg.) was methylated successively with methyl sulphate and sodium hydroxide, methyl iodide and silver oxide, and methyl iodide and silver oxide in *NN*-dimethylformamide, to give methylated disaccharide (128 mg.), $[\alpha]_D -23^\circ$ (*c* 1.33 in CHCl_3). Essentially complete etherification was indicated by (a) paper chromatography of the hydrolysate, which showed two main components, and (b) gas-chromatography of the methanolsate on columns *a* and *c*, which showed the presence of major components having the retention times of methyl glycosides of 2,3,4-tri-*O*-methyl-L-rhamnose (*T* 0.46 and 0.46 on columns *a* and *c*) and 2,3,6-tri-*O*-methyl-D-glucose (*T* 3.50 and 4.78, and 1.31 and 1.61 on columns *a* and *c*) and only traces of other components. The methylated disaccharide (95 mg.) was hydrolysed in 0.5*N*-sulphuric acid at 90° for 4 hr., and after neutralisation with barium carbonate furnished a mixture of sugars (85 mg.) which was chromatographed on cellulose with benzene-ethanol (20:1; later, 10:1) containing a trace of water, to give three fractions. Chromatography in solvent A indicated that fraction 1 (44 mg.) contained 2,3,4-tri-*O*-methylrhamnose, fraction 2 (14 mg.) contained a mixture of sugars, and fraction 3 (12 mg.) contained 2,3,6-tri-*O*-methylglucose. Fraction 1 had $[\alpha]_D +18^\circ$ (*c* 1.1 in H_2O) and the sugar was characterised as 2,3,4-tri-*O*-methyl-L-rhamnose by conversion into the aniline derivative, which was identified by m. p. and mixed m. p. 112–114° and by its infrared spectrum. The relative yield of tri-*O*-methylglucose (fraction 3) was low and it is possible that the methylated disaccharide was incompletely hydrolysed. Attempts to characterise the sugar by the formation of a crystalline derivative failed, but in a separate experiment methylated disaccharide (from fraction 2a) was hydrolysed and the corresponding methylated sugar was characterised as 2,3,6-tri-*O*-methyl-D-glucose by conversion into the 1,4-di-*p*-nitrobenzoate, m. p. and mixed m. p. 191°.

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