

STRUCTURAL STUDIES ON
POLYSACCHARIDES OF FRUITS OF
DILLENIA INDICA LINN

BY

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DIGITIZED

Dedicated to the sacred memory
of the departed soul of
my respected father

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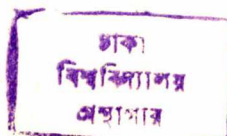
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ABSTRACT

Mucilaginous inner core and outer pulp of the fruits of Dillenia indica (Beng. Chalta) were carefully separated. A neutral polysaccharide was isolated from the aqueous extract of the inner core. The sun-dried pulp was successively extracted with petroleum ether, ethanol (95%) and finally with water. An acidic polysaccharide was isolated from the aqueous extract. The thesis deals with the structural studies of these two polysaccharides.

Examination of the neutral polysaccharide

The neutral polysaccharide was subjected to different fractionation studies and found to be homogeneous. It gave, on hydrolysis, L-arabinose (20%), D-galactose (55%) and D-mannose (18%). Methylation of the polysaccharide followed by methanolysis and hydrolysis afforded a mixture of methylated sugars from which 2,3,5-tri-O-methyl-L-arabinose, 2,3-di-O-methyl-L-arabinose, 2,3,4,6-tetra-O-methyl-D-galactose, 2,3,6-tri-O-methyl-D-galactose, 2,4,6-tri-O-methyl-D-galactose, 2,6-di-O-methyl-D-mannose, 2,4-di-O-methyl-D-galactose and 2-O-methyl-D-galactose were separated and characterised. Gas-liquid chromatographic analysis of the methanolysis products of the methylated polysaccharide provided evidence, among others, for the presence of 2,3,4-tri-O-methyl-D-galactose. The identification of these cleavage fragments provided evidence for the

presence of the following structural units in the polysaccharide

Gal_p1- ; -4Gal_p1- ; -3Gal_p1- ; -6Gal_p1- ;

$\begin{array}{c} | \\ 6 \\ -3\text{Gal}_p1- \\ | \end{array}$;
 $\begin{array}{c} | \\ 6 \\ -4\text{Gal}_p1- \\ | \end{array}$;
 $\begin{array}{c} | \\ 3 \\ -3\text{Gal}_p1- \\ | \end{array}$;

Ara_f1- ; -5Ara_f1- ; -4Man_p1- ;

Controlled acid hydrolysis of the polysaccharide removed most of the L-arabinose residues preferentially and a number of disaccharides were formed under this condition. The disaccharides were characterised as

- (1) O-D-galactopyranosyl (1→4)-D-galactopyranose
- (2) O-D-galactopyranosyl (1→3)-D-galactopyranose
- (3) O-D-galactopyranosyl (1→6)-D-galactopyranose

The characterisation of these disaccharides supported the presence of Gal_p1- ; -4Gal_p1- ; -3Gal_p1- and -6Gal_p1- residues in the macromolecule.

The controlled and graded hydrolysis of the polysaccharide afforded degraded polysaccharide consisting of only D-galactopyranose residues and thus lent support to the framework of D-galactopyranose residues and acid-labile L-arabofuranose residues in the polymer.

Repeated Smith degradation of the polysaccharide

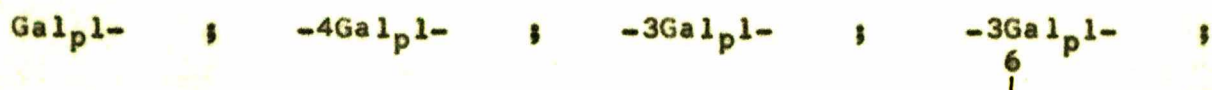
eventually degraded the polymer down to a core composed of only 1,3-linked D-galactopyranose residues.

Methylation, partial hydrolysis and Smith degradation studies provided evidence that the polysaccharide contains main chains of 1,3-linked D-galactopyranose residues to which other residues are attached as side chains and that L-arabofuranose and D-galactopyranose residues constitute end groups.

Examination of the acidic polysaccharide

The acidic polysaccharide was found to contain 34% uronic anhydride and 1% starch. The starch-free polysaccharide was also found homogeneous from various fractionation studies. It gave, on hydrolysis, L-arabinose (15%), D-galactose (45%) and D-galacturonic acid together with smaller amounts of D-mannose.

Methylation of the polysaccharide followed by methanolysis and hydrolysis gave a number of methylated sugars, from which 2,3,5-tri-O-methyl-L-arabinose, 2,5-di-O-methyl-L-arabinose, 2,3,4,6-tetra-O-methyl-D-galactose, 2,3,6-tri-O-methyl-D-galactose, 2,4,6-tri-O-methyl-D-galactose, 2,4-di-O-methyl-D-galactose, 2-O-methyl-D-galactose and 2,3,4-tri-O-methyl-D-galacturonic acid were separated and characterised. The identification of these sugars provided evidence of the following structural units in the polysaccharide





Partial and graded hydrolysis of the polysaccharide were carried out in three stages.

The first stage of the hydrolysis removed most of the L-arabinose residues and afforded the following three disaccharides;

- (1) O-L-arabofuranosyl (1→3)-L-arabofuranose
- (2) O-D-galactopyranosyl (1→3)-L-arabofuranose
- (3) O-D-galactopyranosyl (1→4)-D-galactopyranose

The formation of these disaccharides provided evidence of Ara_f1- ; -3Ara_f1- ; Gal_p1- and -4Gal_p1- residues in the polymer.

The second stage of the hydrolysis removed the rest of the L-arabinose residues and afforded four neutral and three acidic oligosaccharides.

The neutral oligosaccharides were identified as

- (1) O-D-galactopyranosyl (1→3)-L-arabofuranose
- (2) O-D-galactopyranosyl (1→4)-D-galactopyranose
- (3) O-D-galactopyranosyl (1→3)-D-galactopyranose
- (4) O-D-galactopyranosyl (1→4)-O-D-galactopyranosyl (1→3)-D-galactopyranose.

The acidic oligosaccharides were characterised as:

- (1) O-D-galactopyranosyluronic acid (1→3)-D-mannopyranose
- (2) O-D-galactopyranosyluronic acid (1→6)-D-galactopyranose
- (3) O-D-galactopyranosyluronic acid (1→3)-O-D-mannopyranosyl (1→4)-O-D-galactopyranosyl (1→3)-D-galactopyranose.

The identification of these oligosaccharides provided evidence of Gal_p1- ; -3Man_p1- ; -3Gal_p1- ; -4Gal_p1- residues.

Finally the third stage of the hydrolysis afforded three acidic oligosaccharides. They were identified as:

- (1) O-D-galactopyranosyluronic acid (1→3)-D-mannopyranose
- (2) O-D-galactopyranosyluronic acid (1→6)-D-galactopyranose
- (3) O-D-galactopyranosyluronic acid (1→6)-O-D-galactopyranosyl (1→3)-D-galactopyranose.

Repeated Smith degradation of the polysaccharide eventually degraded the polymer down to a core which was found to be composed of only 1,3-linked D-galactopyranose residues.

Thus the results of methylation, partial hydrolysis and Smith degradation studies led to the conclusion that the skeleton structure of the polysaccharide is composed of 1,3-linked D-galactopyranose residues to which other residues like L-arabofuranose, D-galactopyranose and D-galacturonic acid residues exist as side chains.

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1. POLYSACCHARIDES

Polysaccharides are components of almost all living organisms. They are most abundant in the higher orders of land plants and in seaweeds where they constitute approximately three-quarters of the dry weight.¹ They are abundant in fungi and in the exo-skeletons of insects and crustaceans. They are important constituents of the capsules of micro-organisms and of the slimes which are produced by numerous organisms. They are significant components of cartilage, of animal joint fluids, of fluid cancers, of skin and of mucosa.

Polysaccharides fill diverse roles in the physiology of plants, animals and micro-organisms. They serve living organisms in two distinct ways. One is as food reserves, as illustrated by starch, glycogen and galactomannans. The second service derives from the unique physical properties of micro-molecules.² Thus, cellulose, hemicelluloses and chitin serve obviously as structural molecules and as links that help to control cell wall permeability. They polysaccharides serve structurally in the exo-skeletons of insects and crustaceans and protectively in the armor or capsular material of certain bacteria. As surface material they partially protect tissues from desiccation and as gums they are exuded from plants to seal and protect wounds. As thickeners they serve in physical or in mechanical roles in animals and as specific substances

they are of importance in blood group specificity and in other immunological reactions. Polysaccharides are important components in normal connective tissue, in granulation tissue of healing animal wounds and probably in calcification processes.

Polysaccharides are classified in many ways. However, they are usually classified, based on (a) sources as in Table I, and (b) their chemical composition and structures. In such a classification, polysaccharides that, on hydrolysis, give only one kind of monosaccharide residues, are termed as homoglycans, while polysaccharides hydrolysing to two or more kinds of monosaccharide residues are termed heteroglycans. Sometimes, presence of certain constituents forms the basis of names, e.g. those containing uronic acid residues are called uronides, and those containing amino-sugar residues are called mucopolysaccharides. By far the most common practice is to take the units of sugars as the basis of names with ending of 'an'. A sugar comprised of pentoses is called a pentosan. For instance, a sugar consisting of xylose is xylan. Similarly a sugar consisting of mannose is mannan, of galactose is galactan, etc.

Polysaccharides containing only one kind of polymerized sugar units (homoglycans) are more abundant than polysaccharides which contain two or more kinds of sugar units (heteroglycans). Glucans (D-glucose polymers) are the most abundant.

Cellulose, laminaran, amylose and amylopectin are members of this class, Next in abundance is xylan. D-xylose constitute xylan, whereas D-galactose makes up the galactans of pectin and certain seaweed polymers.

Structural polysaccharides of which cellulose is the prime example, are almost always linear molecules, while polysaccharides which serve primarily as reserve foods are commonly branched or in the case of starch, a mixture of linear and branched polysaccharides with the branched type predominating. In general, branched polysaccharides are easily soluble in water and have immense thickening powers. Linear molecules, on the otherhand, are excellent structural material because they pack closely and form many intermolecular secondary valence attachments which makes the structure strong, rigid and insoluble or atleast difficultly soluble.

Table I: Sources of Polysaccharides

I. Plant Kingdom:

A. Higher plants

I. Principally cell wall constituents

- a. Cellulose
- b. Lichenan and isolichenan
- c. Pustulan
- d. Pectic substances
- e. Xylan

- f. Ivory nut mannan, wood mannan and salep mannan
- g. Uronic polysaccharide of Phormium tenax
- 2. Non-cell wall constituents
- a. Amylopectin
- b. Amylose
- c. Fructans
- d. Most gums and mucilages

B. Algae - Seaweeds.

- 1. Principally cell wall constituents
- a. Galactan of Iridophycus
- b. Carrageenans
- c. Agar
- d. Alginic acid
- e. Pectic substances
- 2. Non-cell wall constituents
- a. Floridean starch
- b. Laminaran

C. Bacteria, Fungi and Actinomyces.

- I. Cell constituents
- a. Cellulose
- b. Chitin
- c. Glycogens
- d. Glucans of Penicillium expansum, Aspergillus niger etc.

- e. Hyaluronic acid
- f. Polysaccharides of Tubercule bacilli etc.
- g. Yeast mannan
- 2. Associated with Bacteria, Fungi and Actinomyces
 - a. Crown-gall polysaccharide
 - b. Dextran of Betabacterium vermiformi
 - c. Dextran of Leuconostoc mesenteroides
 - d. Levans
 - e. Galactocarolose
 - f. Mannocarolose
 - g. Rhizobium radicum Polysaccharide
 - h. Varian
 - i. Rugulose
 - j. Capreolan
 - k. Luteic acid
 - l. Sclerotiose
 - m. Yeast glucan
 - n. Torula polysaccharide

II. Animal Kingdom

A. Non-Mammals

- 1. Chitin
- 2. Cellulose
- 3. Glycogens
- 4. Hyaluronic Acid (Fowl Sarcoma Fluid)
- 5. Ovalbumin and Ovomuroid

6. Galactan of Helix pomatia
7. Heparin, Chondroitin Sulphuric Acid and Mucoitin Sulphuric Acid
8. Carbohydrate portion of Frog-spawn Mucin

B. Mammals

1. Glycogens
2. Hyaluronic Acid
3. Heparin
4. Chondroitin Sulphuric Acid
5. Mucoitin Sulphuric Acid
6. Blood group substances
7. Galactan of Beef Lung
8. Carbohydrate residues of the Follicle-stimulating Hormone, the Luteinizing Hormone and the Gonadotropic Hormone
9. Carbohydrate residues of Submaxillary Mucin, Seromucoid and Globogyroid.

Plant Polysaccharides

The majority of plant polysaccharides are components of the cell wall. The walls of the cells in a typical plant tissue consist of three morphologically distinct layers, namely the intercellular cement or middle lamella, the primary walls and the secondary walls. The middle lamella which, when cell division is complete, serves in part, as a binder or isotropic intercellular matrix which assists in holding together the two daughter cells. In the mature tissue this

tenuous intercellular layer contains a large amount of lignin, approximately 71% in the case of micro-dissected middle lamella of Douglass fir.³ There is also present about 14% pentosan, probably xylan, uronic acid containing material and a small amount of pectin. Lignin functions as an intercellular cement but its presence in high concentrations may cause the plant tissue to become unduly brittle. The primary wall contains a high ratio of hemicelluloses. Other polysaccharides such as mannans and galactans may be present in some plants. The secondary wall is of different physiological type which adds mechanical strength to the cell. The wall is of compound nature consisting of concentric layers of cellulose and non-cellulose materials.

Since pectic substances are largely confined to the primary cell wall, the pectic content of woody tissue is low because the volume of this tenuous layer is small. In soft tissues, like those of apple and other fruits devoid of secondary walls, the primary walls and middle lamella constitute a higher proportion of the total tissue and consequently the content of pectic substances is proportionately large.

Cellulose, xylan and lignin are the predominant constituents in most woody plant tissues. Besides xylan, woody tissues possess uronic acid-containing polysaccharides (glycuronans) and other polysaccharides. Mannans, which are present in most tree woods, are especially abundant in soft

woods such as white spruce (7%). Xylan is more abundant in non-wood plants. Galactan, component of pectic triad (galacturonan, araban and galactan), is widely distributed.

Fructans are important food reserves in grasses and accumulate in the healthy mature plant. Plant gums are composed, for the most part, of dissolved or partially dissolved polysaccharides. Economic gums of commerce encompass a broad group of substances,⁴ but those of natural occurrence are limited mainly to tree exudations such as tragacanth, arabic and karaya; seaweed colloids such as agar, alginic acid and carrageenan; and seed extracts represented by locust bean, guar and quince seed gums.

Gums exhibit extreme differences in appearance and properties. All absorb water to become viscous, adhesive masses. Some dissolve completely in water, some partly and others swell and remain insoluble. All are soluble in alkaline solutions. Almost all of the exudates contain uronic acid units in more or less abundance. The polysaccharides are often of complex branched structure. Among the gums, it is common to find more than three different types of monosaccharide residues in a single polysaccharide molecule.¹

Plant Mucilages

Plant mucilages or the so-called vegetable mucilages, which appear to have much less complicated structures, are

derived from the bark, roots, leaves, fruits, seeds and in some cases, the flowers of the plants.⁵ Recently a large number of polysaccharides have been extracted and characterised from the roots,^{6,7} stem,⁸ bark,⁹ leaves,¹⁰ fruits,^{11,12} and seeds,¹³ of certain plants of Bangladesh. They are products of normal plant metabolism and may serve as food reserves¹⁴ in much the same manner as starch in many plants and glycogen in animals. The mucilages may arise from starch,^{15,16} and in certain plants at least, they seem to act, as do the polysaccharides in succulent plants (Aloe, Euphorbiaceae), as agents for holding water.¹⁷

An excellent source of mucilages composed of neutral sugar residues are the seeds of many "leguminosae" such as the locust bean or carob bean (*Ceratonia siliqua* L.), guar (*Cyamopsis tetragonolobus*), Kentucky coffee bean (*Gymnocladus dioica*) and many other. Palm seed also provide a neutral mucilaginous polysaccharide.¹

Root Mucilages

The roots of certain plants such as Iles mannan (*Amorphophallus oncophyllus*) and Konjak mannan (A Konjak) contain a polysaccharide which may be extracted with hot water or dilute alkali to give excellent mucilages that are useful in the paper or rubber latex industries.

Polysaccharides of seaweeds and other algae

The algae are of the most primitive groups in the plant kingdom and even with the passage of time they have retained their primitive characters. They have a great diversity of form ranging from tiny unicellular to giant multicellular organisms. They grow in soil, fresh and salt water, but occur in greatest profusion in the latter.

There are four classes of marine algae known commonly by their dominant colours;¹⁸ the chlorophyceae or green algae; phaeophyceae or brown algae; Rhodophyceae or red algae; and cyanophyceae or blue-green algae.

Various commercial materials have been extracted from seaweeds of which Alginate, Agar and Carrageenin are the most important. The products of these industries find many applications in the food, cosmetic, pharmaceutical, textile and paper industries.

Most of the polysaccharides isolated from seaweeds are probably those of the cell wall. Cellulose is sometimes absent from seaweeds but usually it is found in the inner cell-wall where it occurs in amounts ranging from 1 to 20%. The red seaweed "Iridophycus" contains no cellulose though there is present a galactan which constitutes 40% of the plant bulk. The brown seaweed "Laminaria" contains about 60% alginic acid derivatives as components of the outer cell wall.

Polysaccharides of Fungi

Fungi do not have a characteristic composition in respect to individual constituents or types of compounds. Chitin serves in many fungi as the main or, at least, as an important frame substance in the cell wall. Formation of this ideal structural polysaccharide, which is rich in nitrogen, may be accounted for when one considers the heterotropic life of fungi which, like animals, have so much nitrogen to draw upon that some of it is available for polysaccharide synthesis and deposition in the cell wall. Often chitin content increases progressively with age of the fungi. Mannans are absent from most fungi but a number of yeasts and yeast-like fungi have relatively large amounts of mannans in the cell wall and little or no chitin. Cellulose is the principal cell wall component in some fungi.¹⁹ Pentosans seem to be widely distributed among the lower fungi. Glycogen has also been found in some fungi.

E. Bacterial Polysaccharides

A great variety of polysaccharides are produced by bacteria. While some of these glycans serve as reserve foods, many are fabricated into the capsular sheath, which serves, in part, to protect the organism from its environment. Polysaccharides which have particularly engrossed the biochemist are those of immunological importance which occur in capsules

of the pneumococci and tubercle bacilli. Many organisms produce glycans with antigenic properties. These exact a specific response when injected into the animal body. Most polysaccharides synthesized by microorganisms are branched molecules and many are strongly hydrophilic or gum-like materials. In general, glycans produced by micro-organisms do not contain more than three different monosaccharide types in a particular polysaccharide molecule.¹

F. Animal Polysaccharides

Animals contain far fewer polysaccharide types than plants. Yet, the small number of polysaccharides, which are produced by animals play an extremely important part in the physiology of the organism.¹ Only one polysaccharide, glycogen, is formed as a food reserve but it is found generally throughout the animal kingdom. Hyaluronic acid is important as a part of the intercellular cement, particularly of mesenchyme tissue, and its destruction can open tissue to invading micro-organisms or the spreading of introduced systemic poisons. The polysaccharide plays an important role in synovial fluids and in certain cancers such as mesothelioma. Chondroitin sulphuric acid is of structural importance in animal cartilage and connective tissues. Lower animals use chitin as their principal frame substance. Heparin, a blood anticoagulant, is found in small amounts in blood and in larger amounts in various tissues.²⁰

Its presence has been demonstrated in various tissues of beef. Greatest quantities are found in muscle, liver and lung and lesser amounts in heart, thymus, spleen and blood. Heparin has demonstrated value in the treatment of frostbite and may have possible value in acute rheumatic fever and atherosclerosis.

II. STRUCTURAL INVESTIGATIONS OF POLYSACCHARIDES

Structural investigations of a polysaccharide are associated with many problems.²¹ Some of them are:-

- (1) Seasonal variation. The proportions in which the main constituents are present are affected by seasonal variation. For this reason, before an investigation is started, it is necessary to ascertain the source and time of collection of the raw material.
- (2) Purification. Purification of polysaccharides from colouring matter and protein contaminants is very difficult.
- (3) Heterogeneity. A further obstacle in the determination of structures of polysaccharides is attributed to their heterogeneity. Many polysaccharides contain a large proportion of uronic acid residues which greatly enhance the difficulties of structural studies. When acidic hydrolysis of the uronosyl linkage is attempted, its stability compared with that of normal glycosides is such that drastic conditions must be used and the uronic acid produced is simultaneously degraded. Such polymers are also very difficult to be completely methylated and must often be esterified and reduced before this can be achieved. Ofcourse, recently methods have been discovered by which complete methylation of polysaccharide containing higher proportion of uronic acid, can be achieved. The

uronosyl linkage has often the advantage that using controlled acid hydrolysis it may be possible to obtain stable fragments (e.g. aldobiouronic acid, triouronic etc. acids) and elucidation of the structure of these products by reduction and methylation yields valuable information regarding the structure of the main polysaccharide.

Many of the polysaccharides of marine algae, in contrast with those of the higher plants, contain sulphate ester groups. Their presence in polysaccharides gives rise to additional problems in structural investigation. Unequivocal proof of their location in the individual sugar units is difficult.

Methods of Purification of Polysaccharides

The methods that have developed for studying the fine structures of polysaccharides have reached such a degree of precision that the purity of the starting products becomes of great importance. The structural determinations made from an impure polysaccharide will obviously be erroneous. It is, therefore, of the utmost importance to establish the homogeneity of the starting material.²² Heterogeneity in polysaccharides is more common than hitherto believed and it is appropriate to consider methods available for purification of polysaccharides.

(1) Fractional Precipitation

(a) by "crystallization" or precipitation from water - Certain polysaccharides can be purified by dissolution in water and allowing the solution to cool when the polymer precipitates, e.g., inulin, agar, and xylans from birch wood²³ have been purified in this manner.

(b) With Alcohol or other organic liquids - This has been the main method of purifying polysaccharides, as well as their acetates, nitrates and methylated derivatives. This technique consists in dissolving the polysaccharide in water, for example, and adding ethanol gradually to effect precipitation. The arabans²⁴ and fructans²⁵ have found to respond particularly well to this type of fractionation. Fructans may also be purified by precipitation from pyridine with acetone²⁶. More recently dimethyl sulphoxide has shown promise as a solvent for preferential extraction and precipitation²⁷ of polysaccharides.

(c) with salts - Polysaccharides can be fractionated by precipitation from aqueous solution with salts. Thus, β -D-glucan (Barley Lichenin) can be separated from α -D-glucan and a pentosan present in the aqueous extract of barley flour (barley gum) by addition of ammonium sulphate. Acidic polysaccharides such as alginic acid and pectic acid can be separated almost quantitatively from neutral polysaccharides

as their insoluble calcium²⁸ and barium²⁹ salts. The technique has been recommended for the determination of alginic acid in seaweed gums.²⁸

(d) with organic molecules - The classical example is the separation of amylose from amylopectin by complexing the former with such polar organic molecules as 1-butanol or pentasol.³⁰ A wide variety of other agents besides alcohols may be used for this purpose including fatty acids, nitro-paraffins, ketones, esters, phenols and aromatic nitro-compounds.³¹

(e) with copper salts - Fehling solution has been widely employed for purifying polysaccharides, especially those containing a high proportion of mannopyranose units joined by $1 \rightarrow 4-\beta$ -D-glycosidic bonds.³² Most, if not all, galactomannan gums extracted from the seeds of the "Leguminosae" give copper complexes with Fehling solution and this offers a means of their purification and identification.

(f) with borates - In spite of the fact that almost all galactomannan form gelatinous complexes with sodium tetraborate (borax) and that the reaction is used for classification and identification purposes, it does not appear to have been used for the purification of polysaccharides.

(g) with aluminium hydroxide - Since aluminium hydroxide

has been used³³ to separate the linear and branched chain components of starch, this and other polyvalent inorganic substances may be considered in connection with the resolution of mixtures of polysaccharides.

(h) with other polysaccharides - The observation that polysaccharides having similar structures are difficult to separate, suggests that one polysaccharide can be used to purify another, providing that the new polysaccharide complex formed can be split either by chemical or enzymic means. An example of this technique is the use of cellulose to form a complex with amylose whereby it is separated from amylopectin.³⁴

(i) with proteins - This is perhaps the most selective method at present available and holds out considerable promise for the preparation of pure polysaccharides.

Polysaccharides and especially gums, like gum arabic, containing uronic acid groups, form complexes with proteins such as gelatin.³⁵ Since only the free gum acid shows this property, the reaction is due largely to salt formation. Whereas this might be useful for separating acid polysaccharides from neutral ones, its use is somewhat restricted and the more specific reactions between carbohydrates and proteins appear to offer greater promise for polysaccharide purification.

(2) Chromatographic Separation

Only the smaller molecular weight oligosaccharides can be separated by the usual methods of paper chromatography either directly or in the form of derivatives, such as the N-glycosides. However, displacement chromatography using a charcoal column³⁶ may sometimes be used, as in the case of yeast mannan, which can be purified by this technique using 15 to 20 per cent alcohol as the eluting solvent.³⁷ Hyaluronic acid may be similarly purified.

(3) Separation by means of enzymes

Mixtures of polysaccharides can be resolved by destroying the undesirable components with specific enzymes. Thus the wheat pentosan gum has been freed from starch by amylolysis³⁸ and yeast gum has been separated from glycogen in a similar manner by autolysis.³⁹ Similarly, the glucomannan in lles mannan can be freed from the amylose like impurity by preferential decomposition of the latter with α - or β - amylase.

(4) Fractional Precipitation of Derivatives

Polysaccharides that are difficult to separate by complex formation or by fractional precipitation from aqueous solution with ethanol, because of very close similarity of

structure or association by hydrogen bonding, may sometimes be fractionated after acetylation, because by this means the hydroxyl character is obliterated and there is much less tendency for molecular association. For example, cerealium pentosan can readily be separated from contaminating glucans such as starch, since the pentosan acetates are much less soluble in water than the hexosan acetates.⁴⁰ After resolution of the polysaccharides in this way, the acetyl groups can readily be saponified and the polysaccharide recovered unchanged.

(5) Separation by Electrophoresis and Ionophoresis

The separation of polysaccharides using the Tiseleus apparatus has only been partially successful. A pentosan constituent of pearcell walls has been purified in this manner, but application of this method to other quite divergent groups of polysaccharides⁴¹ met with little success. More recently, however, using a borate buffer, some progress has been made in the separation of glycogen, starch and yeast mannan⁴² and there is good evidence to show that gum arabic is heterogeneous. Electrophoresis (ionophoresis, zone electrophoresis)⁴³ using filter paper and a borate buffer has provided some indications that certain polysaccharides can be separated and distinguished.⁴⁴

(6) Separation by Ultrafiltration

The method of ultrafiltration, using membranes or filters of controlled porous size was developed.⁴⁵ The technique has been shown to be applicable to such cellulose derivatives as the nitrate⁴⁶ and the acetate but because of the coiling of linear chains, it has been suggested that the method is not suitable for linear polymers.⁴⁷ The method has successfully been used by Jones and co-workers⁴⁸ for the fractionation of dextrans.

(7) Other Methods

Using distribution between chloroform and water, Jones and associates^{49,50} have separated an acetylated mixture of neutral polysaccharide, which dissolved in the chloroform and an acidic polysaccharide, which formed an emulsion between the two phases. Such mixtures were shown by Scott⁵¹ to be easily separated by precipitation of the acidic component with such quaternary ammonium salts as cetyl trimethyl ammonium (Cetavolon) or cetyl pyridinium halides. Polysaccharides which differ in acidity can also be separated by this method.

The structural study of polysaccharides involves chemical⁵² and enzymic methods. Some chemical methods are given below:-

(1) Hydrolysis - The usual way of determining the sugar residues

present in a polysaccharide is to hydrolyze the material and separate the sugars obtained by paper or column chromatography. The polysaccharide hydrolysis should be done under the mildest conditions possible, as the hydrolysis is always accompanied by some degradation.

(2) Methylation - The methylation technique is of outstanding importance in structural polysaccharide chemistry. The procedure involves the preparation of the exhaustively methylated polysaccharide, hydrolysis to a mixture of monomers, and the separation, identification and quantitative estimation of the components of this mixture; the original points of substitution will correspond to the unsubstituted hydroxyl groups in these monomeric methyl ethers. Although laborious and time-consuming, the method gives valuable information about the structural units that make up a polysaccharide. Often, the results obtained by this method can be summarized in terms of a "repeating unit", that is, the simplest structure of the polysaccharide consistent with these products. It may, however, be possible to construct various alternatives for this repeating unit, since the method gives no information about the mutual arrangement of various monomers. Other methods must then be used for deciding between these alternatives. It should be emphasized that a "repeating unit" is merely the simplest way of representing a set of observations and should not be given any deeper significance.

(3) Partial hydrolysis - The partial hydrolysis of polysaccharides by acids or enzymes, to give di-, tri-, and oligosaccharides has been developed as one of the principal methods for structural study of polysaccharides. Elucidation of the structures of the oligosaccharides formed provides information, both about the main structural features and about minor details of the parent polysaccharide that in many cases could not be obtained by other available methods.

The technique of partial hydrolysis has, of course, become important only since the development of the various chromatographic methods essential for the fractionation of the hydrolysates. The methods used for structural investigations on oligosaccharides are, in general, analogous to those used for polysaccharides, the only difference being that, with an oligosaccharide, it is possible to obtain a definite solution.

(4) Periodate oxidation - Oxidation with periodate ion, resulting in 1,2-glycol scission, is one of the most widely used reactions in carbohydrate chemistry.⁵³ Since its discovery by Malaprade in 1928, the periodate reaction has been applied to a large variety of problems in all fields of organic chemistry. This reaction has been used most spectacularly, however, in the study of the carbohydrates, because of their polyhydroxylic nature. The mild conditions of the reaction are especially well adapted for application to the sensitive carbohydrate structures.

Another feature contributing to the wide usage of the periodate reaction is its simplicity of application.

When vic-glycols are treated with periodic acid and its salts, cleavage of the carbon chain takes place with the formation of two aldehydic groups, one molecular proportion of periodate being consumed.⁵⁴ In the case of $\alpha\beta$ - triols, a double cleavage of the carbon chain takes place with the formation of two aldehydic groups and the liberation of one molecular proportion of formic acid, the process involving the consumption of two molecular proportions of periodate.⁵⁵ Consequently, non-reducing terminal units in a polysaccharide or (1 $\rightarrow$ 6)-linked non-terminal units having three adjacent hydroxyl groups will be cleaved by two molecular proportions of periodate to give one molecular proportion of formic acid. Non-terminal units joined by (1 $\rightarrow$ 2) or (1 $\rightarrow$ 4) bonds undergo cleavage by one molecular proportion of periodate, but no formic acid is generated. Units which do not possess adjacent hydroxyl groups such as non-terminal units joined by (1 $\rightarrow$ 3) bonds or units involved in branching at C-2 and C-4 are not affected by periodate.

Thus oxidation of a polysaccharide and quantitative determination of the periodate consumed, the formic acid generated, and the determination of the proportion of surviving sugar units will give information concerning the nature and proportion of the glycosidic linkages present in the polysaccharide.^{56,57}

Formic acid production may also be used to calculate the

degree of polymerization of a linear (1→4) - linked polymer since three molecular proportions of formic acid are liberated per linear chain, one from the non-reducing end and two from the reducing end. Likewise, in the case of branched polysaccharides, the formic acid provides a measure of the ratio of terminal to non-terminal sugar residues in the average repeating unit. In the case of highly branched polysaccharide, for example glycogen, the formic acid produced from the reducing end becomes insignificant. The presence of (1→6)-linked residues within the chain invalidates this method since they too will give rise to formic acid.

Usually the polysaccharide is oxidized by a dilute solution of sodium periodate in the cold, and the formic acid production and periodate consumption are followed at intervals. The periodate concentration is determined by titrimetric procedures⁵⁸ or by spectrophotometric method on a micro scale.⁵⁹ Formic acid is determined by direct titration with standard alkali⁶⁰, indirectly by liberation of iodine from a solution of potassium iodide and iodate^{60,61} or by a manometric procedure⁶² involving liberation of carbon dioxide from a hydrogen carbonate buffer.

Enzymetic Method

Enzymes are proving increasingly valuable in the elucidation of the constitution of polysaccharides. The chemical

methods, such as methylation, periodate oxidation, for the determination of the structures of polysaccharides have some apparent limitations. It is, for example, difficult to effect complete methylation of a highly branched polysaccharide⁶³ and the interpretation of the results of periodate oxidation of a polysaccharide is frequently complicated by under or over-oxidation.⁶⁴ But the 'in vitro' degradation of polysaccharides by purified hydrolytic enzymes (polysaccharases) on occasions provides the decisive answer to the structural problem.

The enzyme, using a polysaccharide as a substrate, hydrolyses the glycosidic linkages and the course of this hydrolysis can be followed by (i) the decrease in viscosity or turbidity of the polysaccharide, (ii) the change in optical rotation, (iii) the increase in reducing power, and (iv) chromatography of the polysaccharide-enzyme mixture.⁶⁵ If the substrate, ^{is insoluble} the last two methods can be used.

The complete acid hydrolysis of a polysaccharide is usually a satisfactory method for determining the component monosaccharides. In a number of instances, enzymic experiments have been of value, e.g., the presence of L-galactose in an enzymic hydrolysate of snail galactogen has confirmed the presence of both isomers of galactose in this polysaccharide. In general, enzymic hydrolysis is more specific than acidic hydrolysis; the presence of D-glucose in acid hydrolysates of inulin does not exclude the possibility

that glucose has arisen from a contaminating glucosan; however, the liberation of glucose from inulin by inulase provides evidence in favour of a glucofructosan structure for inulin.

Identification of the end-products of polysaccharase action usually enables determination of the configuration and the carbon atoms involved in the inter-residue linkage. The end products depend on the action pattern of the polysaccharase. An enzyme catalysing the stepwise hydrolysis of every glycosidic linkage produces only the constituent monosaccharides, e.g., glucose is the sole product of "amyloglucosidase" (a polysaccharase from Aspergillus niger) action on amylose.⁶⁶ If, however, a polysaccharase catalyses a stepwise hydrolysis of alternate glycosidic linkages, the sole end-product is a disaccharide containing the same glycosidic linkage as the polysaccharide. For instance, certain samples of amylose are quantitatively converted into maltose by β -amylase; the inter-residue linkage in amylose is, therefore, of the α -1:4-glucosidic type. The most common type of polysaccharase action involves random hydrolysis of the substrate thereby producing a series of oligosaccharides; e.g., pectic acid (a polymer of galacturonic acid) is degraded by a purified fungal polygalacturonase to give a mixture of mono-, di-, tri-, and tetra-galacturonic acids.) Random and stepwise hydrolysis may be distinguished by means of viscometric and reductometric measurements; in the former action, the viscosity of the substrate falls sharply with concomitant production of only a few

reducing groups, whereas in stepwise hydrolysis, reducing groups are quickly liberated, and the viscosity decreases slowly and regularly.

Branched polysaccharides contain more than one type of glycosidic linkage; the majority of these are "inter-residue" linkages which unite the monosaccharides in the "unit-chains"; the remainder are "inter-chain" linkages. Thus amylopectin contains ca. 4% of α -1:6-linkages which inter-link the constituent unit-chains, each of which comprises ca. 20 α -1:4-linked glucose residues. Random enzymic hydrolysis will, therefore, produce a mixture of oligosaccharides belonging to more than one homologous series. The majority of these will contain inter-residue linkages; the remainder will consist either of a disaccharide containing the inter-chain linkage and one or two more inter-residue linkages, depending on the specificity of the polysaccharase. Salivary α -amylase, for example, cannot hydrolyse α -1:4-linkages which are adjacent to 1:6-linkages in amylopectin, accordingly, the products of salivary amylolysis include a series of branched oligosaccharides (α -dextrins) which contain a 1:6-linkage and three or more α -1:4-linkages. In contrast, Aspergillus oryzae α -amylase can hydrolyse α -1:4-linkages adjacent to a 1:6-linkage, and the products of amylolysis, therefore, include isomaltose.

Unfortunately the action of polysaccharases is not quite so simple as this, since under certain conditions they are also

capable of synthesis. Peat and his colleagues⁶⁷ have shown that emulsin in the presence of excess glucose synthesises gentiobiose and other β -linked disaccharides. Furthermore, carbohydrases and transglycolases are often associated with polysaccharases, and the presence of a particular oligosaccharide in an enzyme hydrolysate may be due not to the action of polysaccharase, but to the presence of a contaminating transglycolase.

It two purified enzymes are used together in structural analysis they may affect the specificity of one another (synergic reaction).

Before a polysaccharase can be used in structural investigations a thorough knowledge of both its synthetic activity and synergic reactions is necessary. At the sametime care must be exercised to ensure the homogeneity and purity of all enzyme preparations before utilising them in the elucidation of polysaccharide structure.

STRUCTURES OF SOME POLYSACCHARIDES

CELLULOSE

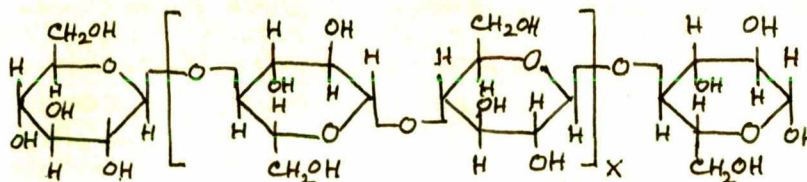
This is the most widely distributed skeletal polysaccharide, and its constitution is the same regardless of the source although its fine "structure" may vary.

The empirical formula is $C_6 H_{10} O_5$ and its molecular weight varies between 200,000 and 800,000 (that is 1,300-5,000 $C_6 H_{10} O_5$ units), depending upon the source, method of extraction, and treatment. Acid hydrolysis gives a quantitative yield of D-glucose. Hence cellulose is a homoglucon. Acetylation, nitration and methylation indicate that the $C_6 H_{10} O_5$ unit possesses three free hydroxyl groups. Hydrolysis of the methylated cellulose gives 2,3,6-^{tri-o-}methyl-D-glucose (86%) and 2,3,4,6-tetra-O-methyl-D-glucose (0.6%). The yield of the latter corresponds to a chain length of about 100-200 units. No di-O-methyl-D-glucose is isolated, and so the chain is linear.

Acetolysis (simultaneous hydrolysis and acetylation) of cellulose with acetic anhydride and sulphuric acid gives a 50% yield of octa-O-acetylcellubiose. This is not a reversion product, as glucose is not converted to octa-O-acetylcellobiose under these conditions. The yield of this product is high enough to justify the belief that cellulose is made up of cellobiose units.⁶⁸ On this evidence it is possible that cellulose is made up of a chain of cellubiose units, but these

could be linked to each other by α - or β - glycosidic links. Howorth found that when fully methylated cellulose is subjected to acetolysis at 15°C at 2 hours (mild conditions) followed by methylation of the exposed hydroxyl groups, the following compounds could be isolated: (a) Methyl 2,3,4,6-tetra-O-methyl-D-glucopyranoside, (b) Methyl hepta-O-methyl cellobioside, (c) a methylated trisaccharide and (d) a methylated tetrasaccharide, each containing the 1,4- β -D-glucosidic linkage of cellobiose.

Thus it clear that cellulose consists of D-glucopyranose units linked through C-1 and C-4 by β -links (1) and that the chain is linear and atleast 100-200 units in length. A mathematical analysis of the rate of hydrolysis of cellulose shows that the results are consistent with the cleavage of one type of link only - the β - link.



I. Cellulose

HEMICELLULOSES

The exact nature of the hemicelluloses has not been disclosed. They are a mixture of (1) unmodified glycans, some composed of pentose and others of hexose sugar units, and of (2) modified glycans (polyuronides) which contain one or more glycuronic acid units joined in the polysaccharide molecule. A wide range of molecular sizes and molecular shapes exist among them. Xylan constitutes the bulk of the hemi-cellulose mixture. In soft woods, mannan partially replaces the xylan or xylan-like polysaccharides. Arabans and galactans may be present to some extent in all hemicellulose preparations. It seems almost certain that low molecular weight cellulose molecules are also present in all hemicelluloses.

Information regarding the properties and constitution of the hemicelluloses is at best fragmentary. Lack of a clear picture may be ascribed to : (1) differences in composition of the hemicelluloses extracted from different plants (2) the fact that the hemicellulose fraction investigated by different workers have not been comparable, and (3) the lack of good methods for the unequivocal separation of the various molecular species.

Sugars most frequently observed among the hydrolytic products of hemicelluloses are the hexoses D-glucose, D-galactose, D-mannose and D-fructose; the pentoses D-xylose and

L-arabinose; and the glycuronic acids D-glucuronic acid, D-galacturonic acid and D-mannuronic acid. Less often there occur the methyl pentoses or 6-deoxy hexoses, particularly L-rhamnose.

XYLANS

From the structural points of view most of the land plant xylans are composed of chains of β -1,4-D-xylopyranose residues to which some or all of three sugar residues L-arabinose, D-glucuronic acid and 4-O-methyl-D-glucuronic acid may be attached as side chains.⁶⁹ Algal xylans contain β -1,3-D-xylopyranose units in addition to the usual 1,4-linked residues.⁷⁰

Xylan from espartograss (*Stipatenacissima*) appears to be a structure containing approximately 75 D-xylopyranose units joined by β -1,4-linkages with a single branch formed by a 1,3-union at some points.

A pure water-soluble xylan from *Rhodymenia palmata*, a species of red algae, thought to be a food reserve, has been studied extensively⁷¹⁻⁷³ and found to have a linear structure,⁷⁴ the chain containing β -1,4-linked and β -1,3-linked D-xylose units. Enzymic studies suggest that these two types of linkages are arranged in random manner in the macromolecule. Extraction with other solvents⁷⁵ led to the isolation of essentially similar xylans containing different proportions of the two types of linkages,



X = β -xylopyranosyl.

On the other hand, xylans from Porphyra umbilicalis and Bangia fuscopurpurea are said to be built up entirely of 1,3-linked xylose units.⁷⁶

The dilute alkali extract of L. pinnatifida and the chlorite extract of P. umbilicalis are similar to one another and to that from R. palmata. Each contains 1,3- and 1,4-links in different proportions and only a small amount of branching as judged by the yield of 2-O-methylxylose. The two fractions of xylan from R. floridulum, however, indicated a considerable degree of branching.

The xylans constitute the main cell-wall materials of a number of green algae, although small proportions of polysaccharides comprising other sugar units may also be present.⁷⁷ The pure xylan, obtained by alkaline extraction of C. filiformis,⁷⁷ on methylation and hydrolysis gave largely 2,4-di-O-methyl xylose indicating 1,3-linkage. Periodate oxidation studies confirmed the results of methylation. These results proved that the xylan of C. filiformis consists essentially a linear chain of 1,3-linked xylose units. Enzymic studies⁷⁸ on H. cuneata xylan confirmed the presence of 1,3-linkage and the absence of 1,4-linkage.

MANNANS

The most convenient source of D-mannose is vegetable ivory, a name given to the endosperm of the seed of the lagoon palm. As indicated from film, methylations and other investigative works, the alkali soluble polymer is a linear chain of β -D-mannose residues involving 1,4-linkage.

The mannan prepared from the tubers of various orchids is similar in structure to the mannan of ivory nuts. The polysaccharide commonly known as salep mannan, was first⁷⁹ prepared in 1924. Acid hydrolysis of the methyl mannan yields 84% of 2,3,6-tri-O-methyl-D-mannose and 1.7% of 2,3,4,6-tetra-O-methyl-D-mannose, indicating a 1,4-union of the D-mannose residues. The relative amount of the tetramethyl derivative suggests a molecular weight of about 12,000.

A polysaccharide isolated⁸⁰ from the red algae Porphyra umbilicalis is essentially linear polymers of 1,4-linked D-mannose units. This is the first reported occurrence of a mannan in seaweeds, which is of the branched chain type with about one branch for each 12 D-mannose residues. A mannuronan is, of course, abundant as alginic acid.

The mannans comprise the main component in some Siphonaceous algae such as Acetabularia calyculus and Halicoryne wrightii.⁸¹ A mannan from Codium fragile⁸² has been studied in

detail. Enzymic degradation gave mannubiose (4-O- α -D-mannopyranosyl-D-mannose), triose, tetra-ose and a small quantity of oligosaccharides, Acetolysis further substantiated these results. Methylation and periodate oxidation studies together with the negative rotation established that the C. fragile mannan comprises β -1,4-linked mannose units.

The mannan isolated from yeast is of interest because its heavily branched structure is in direct contrast to the linear configuration of the mannan isolated from ivory nuts. Yeast mannan and glycogen are the polysaccharide constituent of the yeast cell.

PECTIC SUBSTANCES

A fascinating triad of polysaccharides called the pectic group is found in the cell walls and intercellular layers of all plant tissues. The pectic triad consists of galacturonan, arabinan and galactan of which the galacturonan, a linear polymer of α -1,4-linked D-galacturonic acid units either fully or partially esterified. The alcohol participating in the ester is predominantly methanol.

Pectic substances occur without exception in all higher plants. They have been detected in beech wood,⁸³ box wood⁸⁴, black locust,⁸⁵ lemon, mesquite, white pine⁸⁶ and cotton wood.⁸⁷

According to latest concepts, pectin is looked upon as a

mixture of methyl esterified galacturonan, galactan and araban in which some of the galacturonan molecules are chemically linked to some of the galactan and araban molecules.

Total acid hydrolysis of pectin leads to D-galacturonic acid, D-galactose and L-arabinose and in some cases minor proportions of L-rhamnose,⁸⁸⁻⁹⁰ D-xylose, L-fucose,⁹¹ D-glucose,⁹⁰ 2-O-methyl-L-fucose⁹¹ and 2-O-methyl-D-xylose.⁹¹ In some pectins galactose occurs as a β -1,4-linked galactan⁹² and arabinose as the highly branched arabinan.⁹³⁻⁹⁵ In recent work Northcote⁹⁶ has shown that pectic polysaccharides from apple can be fractionated into a neutral arabinan-galactan complex and two acidic polysaccharides, one with a high uronic acid content (98%) and the other containing 31% uronic acid and 51% arabinose.

ARABANS

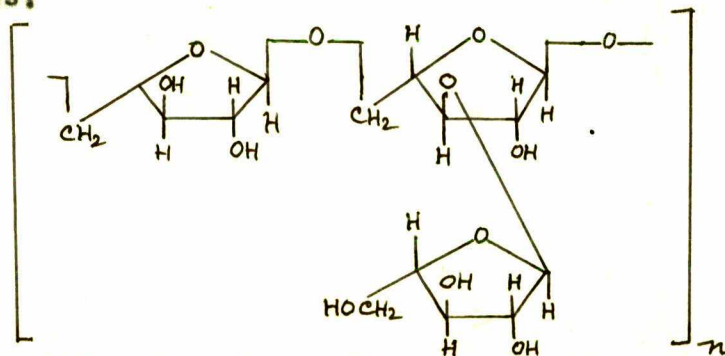
Araban is a pure polymer of L-arabinose. The molecule is of low molecular weight and very likely has a structure consisting of a chain of α -1,5-linked L-arabofuranose units, half of which bear an additional α -1,3-linked L-arabofuranose residues as a one unit side chain. Araban, a member of the pectic triad, is found in nature wherever pectic substances occur and is consequently widely distributed throughout the plant world.

A relatively pure araban was apparently separated from sugar beets as early⁹⁷ as 1894, although it was not recognized

as such at the time. For the most part, araban has been isolated directly from pectin or plant tissue high in pectin content. Ehrlich and co-workers⁹⁸ have examined araban prepared in this manner but the most extensive investigation of the isolation and characterization of araban has been made by Hirst and Jones.⁹⁹⁻¹⁰⁴

Serious difficulties are encountered in attempts to prepare pure araban, and many preparations reported in the literature still retain impurities in which galactan predominates. A pure araban can be prepared in small quantities from peanuts pectin, which is low in galactan content, by a rather tedious process.

Acid hydrolysis of araban leads only to L-arabinose¹⁰⁵ which may be obtained crystalline in yields¹⁰⁰ of 96%. Methanolysis of the methylated product gives equal molar quantities of methyl 2,3,5-tri-O-methyl-L-arabofuranoside, methyl 2,3-di-O-methyl-L-arabofuranoside and methyl 2-O-methyl-L-arabofuranoside. From these observations alongwith specific rotation, the general type of structure in the araban was suggested¹⁰³ as follows:



Possible structure of araban (after Hirst & Jones)

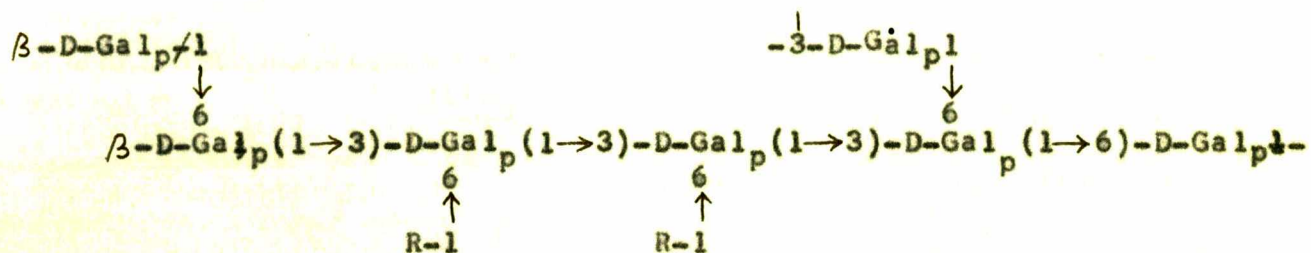
GALACTANS

A linear galactan constitutes one component of the pectic triad of substances and hence is widely distributed in the plant world. Although it is present in relatively large quantities in numerous plant tissues it has so far been obtained in reasonable purity only from the seeds of Lupinus albus.¹⁰⁶⁻¹⁰⁸ Structural work indicates that it is a polymer of β -1,4-linked D-galactopyranose units.

Aqueous extraction of larch wood removes a polysaccharide material designated in the literature either as E-galactan or as arabinogalactan. The principal source of the polysaccharide material has been the wood of western larch¹⁰⁹ (Larix occidentalis) in which it occurs to the extent¹¹⁰ of 8-18%. The polysaccharide material can also be obtained in smaller yield from Eastern, European and Siberian larches¹¹¹ and pulps from larches of Hokkaido.¹¹² Likewise, it may occur to a small extent in southern yellow pine¹¹³ and in Abelmoschus manihot, Medic.¹¹⁴ Other water-soluble polysaccharides which contain galactose units have been isolated from bean seeds,¹¹⁵ timothy pollen¹¹⁶ and young beets.

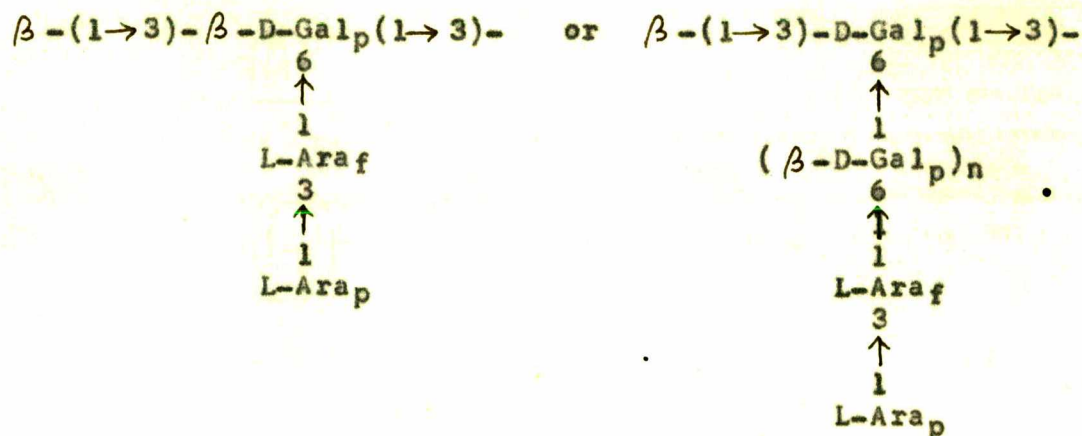
Hirst and co-workers¹¹⁷ isolated an arabinogalactan from European larch, but concluded that the product was not homogeneous. In the series of brilliant investigations, White¹¹⁸ suggested that the arabinogalactan from larch wood contains (1 $\rightarrow$ 3)-linked, (1 $\rightarrow$ 6)-linked galactose and the arabinose units attached to C-6

of a (1→3)-linked D-glactose.



where R = D-Gal_p or L-Araf.

Hirst and co-workers¹¹⁷ extracted an arabinogalactan from the wood of European larch (Larix decidua). The polysaccharide is a highly branched polymer, containing a frame work of (1→3)-linked β-D-galactopyranose residues each of which carries through C-6 side chains consisting, on the average, of two (1→6)-linked β-D-galactopyranose residues. Additional evidence for the attachment of the D-galactopyranose and 3-O-β-L-arabopyranosyl-L-arabinofuranose side chains was obtained by catalytic oxidation of the arabinogalactan.¹¹⁹



The arabinogalactan present in the wood of tamarak (Larix laricina) has been extensively studied by Adams and co-workers.^{120,121} This polysaccharide contains more L-arabinose residues than most arabinogalactans from larch wood.¹²⁰ Methylation and periodate oxidation data indicate the presence of both (1→3)- and (1→6)-linked D-galactose residues in the backbone and the presence of (1→3)-linked terminal side-chains of D-galactopyranose units.

Jones and Reid¹²² have investigated an arabinogalactan from alpine larch (Larix lyalli). On the basis of methylation, periodate oxidation, Smith degradation and partial hydrolysis the authors concluded that the polysaccharide molecule consists of a backbone of (1→3)-linked D-galactose residues, substituted at C-6 by terminal groups or by a chain of (1→6)-linked D-galactose residues. All the D-glucuronic acid (not less than 6.8%) groups are present as terminal, non-reducing residues and are, at least partly, linked to C-6 of D-galactose residues.

An arabinogalactan present in the Scots pine wood has been investigated by Aspinall and Wood.¹²³ It has been shown to be a highly branched polysaccharide containing main chains of 1,3-linked β -D-galactopyranose residues to which are attached side chains carrying more D-galactopyranose residues present as end groups or linked through positions 1 and 6 and L-arabofuranose residues present mainly as end groups.

STARCHES

Carbohydrates are stored as food reserves by all plants, but by far the most abundant and widely distributed carbohydrate reserves are starches. Starches are not single substances but, except in very rare instances, are mixtures of two structurally different glucans; one a linear, thread-like molecule termed amylose and the other a branched or bush-shaped structure termed amylopectin.

Evidence regarding the manner in which D-glucose units are joined in the starch components has been obtained largely from hydrolysis and methylation experiments.

Studies on the kinetics of starch hydrolysis in acid solution led several workers¹²⁴ to assume that practically all the glycosidic links in the chains open with the same reaction constant and are of the α -glucosidic type; that is, they behave like maltose linkages. Optical rotatory considerations and comparisons of the rotations of methylated starches to the rotations of methylated D-glucose, maltose, maltotriose and maltotetraose also suggest the presence of α -1,4-glucosidic linkages. Further support for the prevalence of α -1,4-links in the starch components is given by the actual isolation, during starch cleavage, of maltose in high yield.

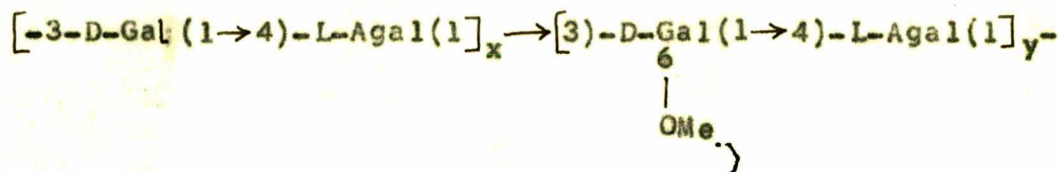
Methylation, periodate oxidation and partial hydrolysis lead to the conclusion that the amylose contains 1,4-linked

α -glucopyranose units and has a degree of polymerization of at least 250 units, and possibly as high as 980 units while amylopectin has 1,4-linked α -glucopyranose units with the chain branching taking place through an α -1,6-linkage and has one end group for each 25-27 units.

AGAR

Agar, a polysaccharide of seaweed is most important and is widely known today because of its use as a gel-forming agent in media for culturing micro-organisms.

The agar-type of polysaccharide is in fact a mixture of two closely related polysaccharides; agarose and agaropectin. Agarose consists of a linear chain of alternating 1,3-linked D-galactopyranose and 1,4-linked 3,6-anhydro-L-galactopyranose units. Evidence for this structural pattern are derived from partial hydrolysis,¹²⁵ methylation^{126,127} and enzymic studies.¹²⁵ Agarose is essentially devoid of half ester sulphate group and stands unique in this respect with other galactans of the class. A general formula for agarose is depicted below:



Gal = galactopyranose

Agal = 3,6-anhydrogalactopyranose

Me = Methyl

The structure of agarpectin, on the other hand, is relatively less established than agarose. It is possibly a mixture of polysaccharides mainly built up of D-galactopyranose, 3,6-anhydro-L-galactopyranose, D-glucuronic acid units and half ester sulphate groups. In addition, pyruvic acid in acetal linkage to D-galactose residues is found in agarpectin from G. amansii and G. subcostatum¹²⁸ Ahnfeltia plicata also contains L-arabinose units.¹²⁹ The contribution of the glucuronic acid residues and L-arabinose units to the structure of the macromolecule is not known. The sites of all the ester sulphate groups are not established either. It seems most likely that with the advent of newer and effective fractionating techniques agarpectin will be found to be a mixture of polymers rather than a single entity.

LAMINARAN

Laminaran, a water-soluble polymer of D-glucose, is found in some brown algae, where it serves as a reserve carbohydrate. Complete hydrolysis of laminaran generally gives solely D-glucose^{130,131} but in some cases¹³² small proportions of D-mannose have also been reported, which is believed to be structurally insignificant.

Laminaran particularly from the genus Laminaria^{130,131} has frequently been investigated. Early methylation studies¹³³ on laminaran together with its negative rotation, $[\alpha]_D = -14^\circ$,

indicated that it is essentially a linear polymer of β -1,3-linked-D-glucose units. This has been confirmed by partial hydrolysis¹³⁴ and enzymatic hydrolysis¹³⁵ which gave a homologous series of β -1,3-linked oligosaccharides from which the laminaribiose was isolated and characterized.¹³⁶

In addition to β -1,3-linked glucose units, the presence of β -1,6-glycosidic linkages in laminaran was first suggested by Peat and his co-workers.¹³⁴ They reported¹³⁴ that laminaran is essentially a linear molecule in which the main chain of β -1,3-linked glucose residues are occasionally interrupted by β -1,6-inter residue linkages. Absence of 3,6-di-O- β -D-glucosyl-D-glucose in the partial hydrolysis products was the main basis for this conclusion. Further support of 1,6-inter residue linkages in laminaran came from methylation studies.

FLORIDEAN STARCH

Floridean starch, a typical glucan was first reported by colin¹³⁷ in 1933 and is considered to be the food reserve material of many Red algae, e.g. Dilsea edulis and Furcellaria species. D-glucose is the sole monosaccharide of this type of polysaccharide and its arrangement in the macro-molecule appears to be similar to that of amylopectin^{138,139} of land plants. The main core consists essentially of α -1,4-linked D-glucopyranose residues, in some cases occasionally interspersed by α -1,3-linkages¹⁴⁰ and an average chain length¹⁴¹ of 11-15. The 1,3-linkage

and the some what shorter chain length may serve to distinguish floridean starch from amylopectin of higher plants.

Floridean starch in Dilsea edulis is accompanied by a sulphated galactan which on hydrolysis yields, D-galactose, D-glucuronic acid, D-xylose, sulphuric acid (ca 11%) and traces of 3,6-anhydro-D-galactose. Structural studies have ascertained that the main chain of the molecule consists of 1,3-linked D-galactose residues to which are attached side chain composed of D-glucuronic acid units (terminal units), 1,3-linked D-xylose residues and 1,4-linked D-galactose residues.¹⁴²

ALGINIC ACID

Alginic acid, a polyuronide, constitutes one of the principal carbohydrates of brown seaweed. It was first discovered in about 1880 by Stanford¹⁴³ and has since then become a product of commercial interest.

Early works such as methylation, periodate and bromine oxidations¹⁴⁴ as well as X-ray diffraction studies¹⁴⁵ together with the negative rotation indicated that the alginic acid is a linear polymer made up entirely of D-mannuronic acid units united by β -1,4-linkages.¹⁴⁶

However, the major advance in alginic acid chemistry came from Fischer and Dorfel.¹⁴⁷ They, with improved chromato-

graphic techniques, were able to show that guluronic acid in addition to mannuronic acid was present in alginic acid from various species of brown algae. They were able to separate two crystalline lactones;¹⁴⁸ D-mannurono- and L-guluronolactones, from the acid hydrolysate of alginic acid and concluded from infrared measurements that both the lactones were pyranose 3,6-lactones. However, further proof that guluronic acid units are indeed present in the alginic acid and have not resulted during alkaline extraction was obtained by the acidic hydrolysis of the weed itself where both acids were present in the hydrolysate.

Partial hydrolysis of a reduced sample of alginic acid has led to the isolation of a number of oligosaccharides. From these 4-O- β -mannosylgulose and β -1,4-mannubiose were isolated and characterised.¹⁴⁹ These results indicated that at least some of the polymeric molecules contain both mannuronic and guluronic acid residues and also that mannuronic acid residues are adjacent.

THE WATER-SOLUBLE
POLYSACCHARIDES OF FRUITS OF
DILLENIA INDICA LINN

INTRODUCTION

Dillenia indica is a large evergreen tree of Bangladesh, Central and South India and Burma. It is also distributed through the Eastern peninsula from Sylhet to Singapore, rare on the plains of Northern or Western India, but occurs along the base of the hills from Kamaon and Garhwal eastward and becomes plentiful from South Kanara southwards.¹⁵⁰ The tree is generally 30-80 feet high with spreading branches and a rounded crown of bright green foliage. The bark of the tree is smooth, red and moderately thick. Leaves which are lanceolate, serrate and leathery are generally situated at the ends of the branches. Blades of the leaves are 8-12 inch in diameter. Flowers are solitary, white, fragrant and 6 inch in diameter.¹⁵¹ Fruits are large, 3-5 inch in diameter, globose and hard consisting of five closely fitting sepals enclosing numerous seeds embedded in a glutinous pulp. It is an aggregate fruit which develops from an apocarpous pistil. The imbricately arranged 5 sepals encircles the aggregated fruit. The innermost sepal completely encircles the fruitlets. The stigma about 20 in number coherent in the centre are white and stiff. Innumerable number of reddish brown linear anthers are persistent and encircles the inner compactly arranged free fruitlets. Each fruitlet has a thick fleshy wall, the chamber is filled with white gummy substance and each fruitlet has a few hard seeds. Seeds are small compressed and reniform with hairy margins. The fruit attains maturity in the month of Decem-

Different aspects of Chalta fruit



A. As seen from pedicel side



B. As seen from distal side



C. Succulent sepals stretched apart



D. Showing fruitlets and styles



E. Fruitlets cut to show seeds and mucilage



F. Mass of seeds and mucilage

ber. Different parts of a mature fruit are shown in Fig.-1.

Ripe fruits have an agreeably acid taste and are eaten by the locals, either raw or cooked - chiefly cooked in curries. They are also made into pleasant jams and jellies. The acid juice, sweetened with sugar and water, forms a cooling drink.¹⁵² The fruit is said to possess tonic and laxative properties and is used in abdominal pains.

A survey of the literature reveals that very little work has so far been done on chalta. Bhattacharjee et.al¹⁵³ reported the isolation and characterization of betulic acid and betulin, the triterpenoid compounds, from the bark of the tree.

Earlier investigation¹⁵⁴ on the carbohydrate constituent of the fruit revealed the presence of an arabinogalactan in the water soluble extract of the fruit (2.6% of the fresh fruit). The polysaccharide (uronic anhydride, 39%), on hydrolysis, gave D-galactose, D-glucose, L-arabinose and D-galacturonic acid. An aqueous solution of the polysaccharide gave a blue colour with iodine, indicating the presence of starch. The starch (ca.1%) was separated from the extract by precipitation as the starch-iodine complex and the residual polysaccharide was recovered in 61% yield. Fractional precipitation of an aqueous solution of the starch-free polysaccharide with ethanol gave four different fractions I - IV at alcohol concentrations of 48%, 60%, 80% and 95% respectively. The yields of different fractions were 40.9%, 34.4%, 12.3% and 4.1% respectively.

On hydrolysis fraction I gave mainly D-galacturonic acid with minor proportions of D-galactose, D-glucose and L-arabinose; fraction II gave mainly D-galactose and L-arabinose with a trace of D-galacturonic acid and D-glucose, and both fraction III and IV gave D-galactose, D-glucose, L-arabinose and D-galacturonic acid. Fraction II was again dissolved in water and reprecipitated with ethanol. This gave pure arabinogalactan. It had $[\alpha]_D^{30} +146^\circ$ (c, 0.1 in N-Potassium hydroxide) and on hydrolysis it gave L-arabinose and D-galactose in the molar ratio of 1:1.1.

The polysaccharide on methylation followed by methanolysis and Gas-liquid chromatographic analysis indicated the presence of 1,5-linked L-arabinose, endgroup L-arabinose, and 1,4- and 1,3-linked D-galactose, and end group D-galactose units.

The present work was started with a view to finding the structural patterns of the neutral as well as the acidic polysaccharide present in the chalta fruit.

Partial hydrolysis studies on both the polysaccharides have been carried out and a number of neutral and acidic oligosaccharides separated and characterized. Information on the overall structure of the macromolecules has also been obtained by the application of methylation, periodate oxidation and Smith degradation.

EXPERIMENTAL SECTION

GENERAL PROCEDURES (G.P.)

G.P. 1.

Dialyses of polysaccharide solutions were effected in cellophane tubes against running water. Toluene was added to prevent bacterial action.

G.P. 2.

All evaporations were carried out at reduced pressure.

G.P. 3.

Optical rotations were measured in a 1 dm. polarimeter tube at room temperature. Water was used as solvent unless otherwise stated.

G.P. 4.

Melting points were recorded on Kofler's hot stage microscope.

G.P. 5.

Ash contents were determined by ignition of samples (ca. 50 mg) in a platinum crucible on a muffle furnace until constant weight was attained.

G.P. 6.

All reductions were carried out with sodium borohydride in aqueous solution in an atmosphere of nitrogen. The alkaline solution was neutralised with acetic acid and boric acid was removed by repeated evaporation with methanol.

G.P. 7. Determination of Uronic anhydride

Uronic anhydride was determined by the decarboxylation¹⁵⁵ method of Johansson et.al.

G.P. 8. Acid hydrolysis of polysaccharide and oligosaccharide

The samples (10-20 mg) were heated in a tube on a boiling water-bath ^{with} 1N sulphuric acid (1-2 ml.) for 5-8 hours. The hydrolysates were neutralised with solid barium carbonate, filtered and the residue thoroughly washed with water. Filtrate and washings were combined, deionised with Amberlite IR 120(H⁺) resin and then with Amberlite IR-45(OH⁻) resin (in case of neutral sugars only) and concentrated.

G.P. 9. Estimation of reducing sugars

(a) Phenol-sulphuric acid method:¹⁵⁶ Carbohydrate solution (1 ml.) containing 20-80 μ g sugar was pipetted into a test-tube followed by addition of 4% solution of phenol in water (1 ml.). Analar concentrated sulphuric acid (5 ml.) was then added rapidly, the stream of acid being directed against the liquid surface,

rather than the side of the test tube, in order to obtain good mixing. After standing for 30 minutes, the optical density of the solution was read in Erma photoelectric colorimeter at 530 m μ against a blank, prepared by using water in place of sugar solution.

Sugar concentration were then calculated by reference to a standard curve for known solutions of the sugar.

(b) Hypoiodite method:¹⁵⁷ A 0.1N solution of iodine (1ml) was added from an Agla micrometer syringe to the sugar solution (5 ml.) (which did not contain more than 2.5mg of sugar; otherwise oxidation would have been incomplete) contained in a boiling tube (22 x 3 cm) fitted with B-24 ground glass stopper. A solution (2 ml.) containing 0.2M sodium bicarbonate and 0.2M sodium carbonate (pH 10.6) was then pipetted into the solution and the tube stoppered in order to prevent loss of iodine due to evaporation, the stopper was moistened with little potassium iodide solution (10%). A controlled determination was carried out on a 5 ml. of water contained in a similar boiling tube. After 2-2½ hours the stopper was washed and the reaction mixture diluted to 25 ml. and then acidified carefully by running 2N sulphuric acid down the side of the tube, avoiding unduly vigorous evolution of carbon dioxide. After acidification the tube was stoppered pending titration of the liberated iodine with 0.01N sodium thiosulphate solution.

1 ml. 0.01N $\text{Na}_2\text{S}_2\text{O}_3$ solution $\equiv$ 0.0009 gm. of hexose
 $\equiv$ 0.00075 gm. of pentose.

G.P. 10. Estimation of Relative Molar Proportions of Sugars

The polysaccharide samples (30-40 mg.) were heated with 1N sulphuric acid on the boiling water-bath for 5-8 hours. After ^{lisa}neutration and deionisation, the hydrolysate was concentrated to a syrup and separated into individual sugars on chromatographic papers by elution with a suitable solvent and then extracted with water. The solution was made upto a definite volume and the sugars were estimated by either G.P. 9(a) or 9 (b). Pieces of paper of equal size, devoid of sugar, were eluted and constituted the blanks.

G.P. 11. Degree of polymerisation.

Degree of polymerisation was determined according to the Timell modification ¹⁵⁸ of the method of Peat, Whelan and Roberts ¹⁵⁹ as follows:

To 1 ml. of a solution of a carbohydrate containing 30 - 60 μ g of sugar, in a test tube, was added 1 ml. of a solution of 1% sodium borohydride in water. To another ml. of a similar sugar solution, in a second test tube, was added 1 ml. of a solution of 1% sodium borohydride in 1N sulphuric acid ("inactive borohydride"). After 1 hour 1 ml. of a 4% solution of phenol in water followed by 5 ml. of analar

concentrated sulphuric acid was added to each of these tubes. Optical densities of these solutions were then measured after 30 minutes, in Erma Photoelectric Colorimeter at 530 m μ against a blank. DP is given by the ratio $\frac{Q}{Q-1}$, where Q is the ratio of the optical densities of the nonreduced to the reduced samples.

G.P. 12.

(a) Paper chromatography: Unless otherwise stated the paper chromatograms were carried out on Whatman No. 1 chromatography paper in descending technique.

(b) Principal solvents (v/v proportions) used for chromatography were:

- A. Ethyl acetate; acetic acid; formic acid; water
(18:3:1:4)
- B. Ethyl acetate; pyridine; water (10:4:3)¹⁶⁰
- C. 1-Butanol; ethanol; water (40:11:19)¹⁶¹
- D. 1-Butanol; acetic acid; water (2:1:1)
- E. 1-Butanol; pyridine; Water (6:4:3)¹⁶²
- F. Ethyl acetate; pyridine; water (8:2:1)
- G. 1-Butanol; ethanol; water (upper layer) (4:1:5)¹⁶³

(c) Developing agents: The following reagents were used to develop the chromatograms either as dip or as sprays:

(1) A saturated aqueous solution of aniline oxalate (spray), followed by heating at 120°C for ca. 5 minutes.

(2) A 10% solution of silver nitrate (1 ml.) diluted with acetone (100 ml.) followed by 0.5N sodium hydroxide in ethanol, followed by 2% sodium thiosulphate solution. The chromatograms were dipped in each solution and dried at room temperature between each operation.¹⁶⁴

(3) Bromocresol green (spray) (0.1% solution in ethanol) made slightly alkaline to give a blue green solution.

(4) Tetrazolium spray: The spray was freshly prepared by mixing 2% triphenyl tetrazolium hydrochloride and 1N sodium hydroxide solutions (1:1 v/v). The paper was heated at 40°C and on a water-saturated atmosphere for 10 minutes after it was sprayed. Intense red colour indicates the absence of 1, 2 - linkage.

G.P. 13. Ionophoresis.

Ionophoresis was carried out on Whatman No. 1 paper.

The following buffers were used:

(a) Phosphate (pH 7) at 2000 for ca. 2 hours.

(b) Borate (pH 10) at 2000 for ca. 1 hour.¹⁶⁵

(c) Molybdate (pH 5) at 2000 for ca. 1 hour.¹⁶⁶

Sugars were located on the ionophoretograms with developing agent (2) (dip) [G.P. 12(c)] .

The following abbreviations were used to describe the distances travelled by sugars on chromatograms or ionophoretograms:

$$R_g = \frac{\text{distance travelled by a sugar}}{\text{distance travelled by glucose}}$$

$$R_G = \frac{\text{distance travelled by a sugar}}{\text{distance travelled by 2,3,4,6 - tetra-O-methyl glucose.}}$$

$$R_{\text{Gala}} = \frac{\text{distance travelled by a sugar}}{\text{distance travelled by galacturonic acid}}$$

$$M_G = \frac{\text{distance between a sugar and 2,3,4,6-tetra-O-methylglucose}}{\text{distance between glucose and 2,3,4,6-tetra-O-methylglucose.}}$$

$$M_{\text{Gala}} = \frac{\text{distance between a sugar and 2,3,4,6-tetra-O-methylglucose}}{\text{distance between galacturonic acid and 2,3,4,6-tetra-O-methylglucose}}$$

G.P. 14. Preparation of a cellulose column¹⁶⁷

(a) Diethylaminoethylcellulose (DEAE-50) was suspended in 0.5N hydrochloric acid (50 g. in 1 litre HCl solution) in a suction flask. This was then kept under vacuum for 20 minutes to deaerate, after 20 minutes standing the supernatant was decanted, then the swelled mass was filtered on a Buchner funnel and washed with water to neutrality.

(b) The wet material was treated in the same way as in

(a) with 0.5N sodium hydroxide solution. The operations (a) and (b) were repeated thrice. In the last alkali treatment the washings were not carried out on a Buchner funnel. The material was transferred to a column (30 x 4.5 cm.) and washed with water to neutrality.

(c) The column was equilibrated with 0.5N potassium chloride solution (2l.) and washed with water till free from chloride.

G.P. 15. Preparation of charcoal-celite column.¹⁶⁸

400 ml. charcoal (activated, May and Baker) and 200 ml. celite (B.D.H. Grade) was thoroughly mixed together. The mixture was covered with concentrated hydrochloric acid (1l.) and stirred overnight (this treatment was necessary for deactivation of the charcoal and for the removal of traces of iron and alkaline ash). The mixture was repeatedly washed with large volumes of water to neutrality. The neutral mass was then stirred with distilled water in a beaker and poured into a column (40 x 4 cm.). Distilled water was then slowly passed through the column for 2 days for proper setting of the column.

G.P. 16. Methylation

Methylation was carried out by Haworth method and Hakomori method.

(A) Haworth method.^{169,170}

A polysaccharide (5 g.) was dissolved in water (200 ml.). Sodium borohydride (200 mg.) dissolved in 10 ml. of water was added dropwise into the solution with stirring under nitrogen atmosphere. 30% aqueous sodium hydroxide solution (80 ml.) and dimethyl sulphate (40 ml) were added dropwise and simultaneously with vigorous stirring over a period of four hours. Similar four additions were made on successive days. After complete neutralisation of the methyl sulphate with 2N sulphuric acid, the product was freed from salt by dialysis (2 days) and concentrated under reduced pressure to a syrup. Four such methylations were carried out in this manner and the methylated product was extracted finally by shaking the dialysed concentrated liquor with chloroform. After washing and drying (anhydrous sodium sulphate) the chloroform extract was concentrated and dropped slowly into excess rapidly stirred light petroleum ether, when the methylated sugar was precipitated as a white powder. The methylated sugar was separated by centrifugation, dried in vacuum and weighed. The methylated product was further purified by dissolution in chloroform and reprecipitation with petroleum ether.

(B) Hakomori method.¹⁷¹

Methylation by this method was carried out in two ways:

- (i) with the help of base, methyl sulphinyI.carbanion,
- (ii) by direct use of the reagents.

(i) With the help of base.

(a) Preparation of base, methyl sulphinyI carbanion. 172-174

Into a three-necked flask containing a glass magnetic stirrer bar was weighed sodium hydride (2.4 g.). The hydride was washed with n-pentane (30 ml.) and the supernatant was removed by decantation. After three washes the flask was fitted with a rubber serum cap at one side neck, with a thermometer at another side neck and with a stoppered condenser at the middle neck. The residual n-pentane was removed by successive evacuation through a 17 gauge needle (G.T.V.) inserted into the serum cap. After each evacuation the flask was regassed with nitrogen and finally nitrogen was passed through the flask via the needle. Redistilled dimethyl sulphoxide (60 ml.) was poured into the flask through the open end of the up-right condenser. The flask was heated on an oil-bath at 70°C for about $2\frac{1}{2}$ hours with constant stirring until the solution became clear greenish coloured and evolution of hydrogen gas ceased.

Concentration of the anion in dimethyl sulphoxide was determined by withdrawing an aliquot (1 ml.) and titrating it with 0.1N hydrochloric acid using phenolphthelein indicator. The anion solution was preserved in a serum bottle in an atmosphere of *nitrogen* in the cold and was stable for 12-15 weeks.

(b) Methylation.

A polysaccharide (10 mg.) in a 20 ml. serum bottle sealed with a rubber cap, was dissolved (wherever necessary by stirring or shaking overnight, in some cases it was necessary to start with a good suspension) in dimethyl sulphoxide (3 ml.). The bottle was evacuated, flashed with nitrogen and the base prepared as in G.P. 16 (B)(1), methyl sulphiny l carbanion (3 ml.) was added dropwise with a syringe. The resulting gelatinous solution was shaken in a shaker for 2 hours and allowed to stand overnight at room temperature. Excess of gas formed inside the bottle was released with the help of the needle. Methyl iodide (0.5 ml.) was then added dropwise with the syringe with external cooling by tap water and the resulting turbid solution was agitated in the shaker for 1 hour. The process was repeated twice. In the last operation excess of methyl iodide (1 ml.) was added. The solution was then poured into water (50 ml.) and nitrogen was passed through it until excess methyl iodide was removed. The methylated product was extracted by vigorous shaking with chloroform (2 x 100 ml). The chloroform solution was washed repeatedly (7 times) with an excess of water (ca 200 ml.) to remove dimethyl sulphoxide, dried over anhydrous sodium sulphate and concentrated under reduced pressure to a syrup.

(ii) Methylation by direct use of reagents.¹⁷⁵

A polysaccharide (200 mg) was dissolved in dimethyl

sulphoxide (20 ml.) by stirring under nitrogen atmosphere (heating at 70°C for several hours was done wherever necessary). Sodium hydride (500 mg.) was added in four equal instalments with gentle stirring during 1 hour. The mixture was stirred until evolution of hydrogen ceased (2 hour). Methyl iodide (2 ml.) was added dropwise in four equal instalments with stirring during 2 hours. The solution was stirred overnight. A further three additions of sodium hydride and methyl iodide were made to the reaction mixture on successive days, as described for the first addition. The mixture was then poured into water (100 ml.) and extracted with chloroform (2 x 200 ml.). The chloroform solution was washed repeatedly (7 times) with an excess of water (ca 250 ml.) to remove dimethyl sulphoxide, dried over anhydrous sodium sulphate and evaporated to a syrup under reduced pressure (rotary evaporator at less than 40°C).

G.P. 17. Glycosidation and methanolysis.

Glycosidation and methanolysis were carried out by refluxing the carbohydrate with 3% methanolic hydrogen chloride for 10 hours. The acidic solutions were neutralised with dry silver carbonate and filtered. The precipitated silver salts were extracted with methanol under reflux. The filtrate and methanolic extracts were combined and concentrated to a syrup under reduced pressure.

G.P. 18. Hydrolysis of methylated polysaccharides.

Hydrolysis of methylated polysaccharides was carried out

either (a) by heating it with formic acid (90%) in a sealed tube in an atmosphere of carbon dioxide for 6 hours at 100°C, followed by dilution of the formyl esters with water (5 vols.) and further heating for 2 hours at 100°C.

or (b) by heating methanolysed methylated polysaccharide (prepared according to G.P. 17) with 1N sulphuric acid according to G.P. 8.

G.P. 19. Demethylation.

Demethylations of methylated sugars were carried out by the method of Hough, Jones and Wadman.¹⁷⁶ The methylated sugar (5-10 mg.) in hydrobromic acid (48% w/w; 1 ml.) contained in a sealed tube was heated in a boiling water-bath for 5 minutes. The contents were then immediately diluted with water (10 ml.). Portions of silver carbonate were added until the solution was neutral, whereafter a small quantity of decolourising charcoal was stirred in and the solution filtered. The last traces of silver were removed by the passage of hydrogen sulphide and filtration through a charcoal bed. The filtrate was concentrated to a syrup and was examined on the paper chromatogram.

G.P. 20. Gas-liquid partition chromatography.

Gas-liquid chromatography (GLC) was carried out by a modification of the method of Aspinall¹⁷⁷ by courtesy of Dr. C.W. Ford, Cunningham Laboratory, C.S.I.R.O., Australia.

Two columns were used with the following conditions:

(a) 15% Butane-diol-succinate on chromosorb W. (80/100 mesh) at 175°C column 122 cm. x 3 mm. O.D. stainless steel

(b) 10% Polyphenylether m-bis(m-phenoxy-phenoxy) benzene on chromosorb W (80/100 mesh) at 190°C. Column 147 cm. x 3 mm. O.D. stainless steel .

Injector, 225°C, detector (FID) 290°C. Carrier gas-nitrogen 25 ml./minute, hydrogen 25 ml./minute, air 280 ml./minute.

Retention times (I) were quoted relative to methyl 2,3,4,6-tetra-O-methyl- β -D-galactopyranoside as an internal standard.

G.P. 21. Estimation of Methoxyl content.

This was done according to a modification of Zeisel's method.¹⁷⁸

G.P. 22. Periodate oxidation.¹⁷⁹

A 0.03M solution of the polysaccharide material was oxidised with a 0.05M solution of sodium metaperiodate in the presence and absence of acetate buffer. The solution was kept at 0°C in the dark throughout the oxidation to minimize side reactions. A blank experiment was carried out at the same time under the same conditions.

Aliquots of the sample and the blank were removed at

regular intervals and analysed for periodate consumption and liberation of formic acid. Estimation of periodate consumption was carried out by titrimetric procedure and the liberation of formic acid by direct titration with standard alkali¹⁸⁰ as described below:

(a) Estimation of periodate consumption.

The reaction mixture (5 ml.) was taken out in a conical flask (50 ml.), neutralised with a saturated solution of sodium bicarbonate and to it was added immediately a standard (0.1N) sodium arsenite solution (20 ml.) and 20% potassium iodide solution (1 ml.). This was allowed to stand for 10-15 minutes. Then it was titrated with standard 0.1N iodine solution using starch as the indicator.

A blank determination was carried out simultaneously using sodium metaperiodate solution (0.05M, 5 ml.), 0.1N sodium arsenite solution (20 ml.) and 20% potassium iodide solution (1 ml.). Amount of iodine solution needed in the blank was less than that needed in the reaction product.

Calculation.

1 ml. 0.1N sodium arsenite $\equiv$ 0.010696 g. sodium metaperiodate.

Moles of periodate consumed per hexose unit were plotted against time and the primary oxidation determined by extrapolation to zero time.

(b) Estimation of formic acid.

1. The reaction mixture (10 ml.) was taken in a conical flask (100 ml). The reaction was stopped by the addition of ethylene glycol (2-3 drops). The solution was then titrated against standard (0.1N) sodium hydroxide solution using phenolphthalein as the indicator.

extraction was hydrolysed with 1N sulphuric acid on the boiling water-bath for 8 hours. The cooled hydrolysate was neutralised with solid barium carbonate, deionized with Amberlite IR-120(H⁺) resin and concentrated to a syrup. Chromatographic examination of the syrup in solvent A and E (using spray I) revealed spots corresponding to L-arabinose, D-galactose and D-mannose.

Expt.3. Examination of the Supernatant from Ethanolic Extract.

The concentrated supernatant from ethanolic extraction was examined by paper chromatography in solvents A and E (using spray I). This revealed the presence of D-glucose only.

Expt.4. Extraction of mucilaginous core with cold and hot water.

The mucilaginous inner core (500 g.) was extracted with water at room temperature for 24 hours with occasional stirring. The residue was separated by filtering through muslin and extracted twice in this way. The combined filtrate was centrifuged, the supernatant concentrated to a suitable volume and poured into ethanol. The resulting precipitate was removed at the centrifuge and dried in a desiccator over anhydrous calcium chloride. This was called polysaccharide sample I.

The residual material was then extracted with hot water twice. The residue (mainly seeds) was removed by filtering through muslin. The combined filtrate was centrifuged, the supernatant concentrated to a suitable volume and poured into excess

rapidly stirred ethanol. The resulting precipitate was removed at the centrifuge and dried in a desiccator over anhydrous calcium chloride. This afforded polysaccharide sample II.

Expt.5. Examination of the water-extracts.

Small portions (100 mg.) of each of the polysaccharide samples I and II (Expt. 4) were separately hydrolysed with 1N sulphuric acid on a boiling water-bath for 8 hours. The cooled hydrolysates were neutralised with solid barium carbonate, and filtered. The filtrates were separately deionised with Amberlite resin IR 120 (H^+), filtered, and the filtrates concentrated to syrups. On paper chromatographic examination in solvents A and E (using spray I) both the syrups revealed spots corresponding to L-arabinose, D-galactose and D-mannose. The polysaccharide samples I and II were, therefore, combined (22.0 g.) and the combined mass was designated as Polysaccharide A.

Expt.6. Examination of the supernatant from water-extract.

A small portion of the supernatant from water-extract of mucilaginous inner core of the fruits (Expt. 4) was treated with Amberlite IR 120(H^+) resin and concentrated to a syrup. Paper chromatographic analysis of the syrup in solvents A and E (using spray I) revealed only one spot corresponding to D-glucose.

III. Extraction of pulp.

Expt.7. Extraction with Petroleum Ether (b.p.60°-80°C).

The powdered chalta pulp (ca 1000 g.) was kept immersed for 5 days under petroleum ether (b.p., 60°-80°C; 3l.) in an aspirator bottle. The petroleum ether was then separated and evaporated to dryness when a yellow waxy substance (4.1 g.) was obtained.

Expt.8. Extraction of the Residual Pulp with Ethanol (95%).

The pulp left after petroleum ether extraction, was dried and exhaustively extracted with ethanol (95%). Evaporation of the ethanolic extract gave a light yellow solid (1.1 g.).

Expt.9. Examination of the Yellow Solid.

The yellow solid (Expt.8), after decolourisation with activated charcoal and purification by repeated crystallization from ethanol, afforded a white substance (0.6 g.). A small quantity of the substance was dissolved in chloroform and acetic anhydride and to the solution was added a few drops of concentrated sulphuric acid. The formation of red colour indicated that the substance was a steroidal compound. Elemental analysis suggested an empirical formula, $C_{27}H_{46}O$ for the compound. The substance charred at 135°C and melted at 275°-276°C. Hydrolysis of the substance with 1N sulphuric acid followed by chro-

matographic analysis revealed only one spot corresponding to glucose. This result in conjunction with the positive test for a steroidal compound suggested that the substance was a glycoside of a steroidal compound. Since the detailed study of this compound was beyond our scope, further investigation in this line was, therefore, given up.

Expt.10. Extraction of Pulp with cold and hot water.

A portion of the residual pulp (50 g.) after ethanolic extraction was exhaustively extracted first with cold water (3 x 400 ml.) and then with hot water (5 x 400 ml.). Each of the eight extracts was separately centrifuged, concentrated to a suitable volume and was poured into rapidly stirred ethanol (1.5 l. in each case). The resulting precipitate in each case was separated at the centrifuge, dried in a desiccator over anhydrous calcium chloride. The dried substance was separately dissolved in water, dialysed extensively (3 days), concentrated to a suitable volume and then poured into ethanol. The resulting precipitate was separated at the centrifuge, washed with ethanol and dried in a desiccator over anhydrous calcium chloride, and weighed. The yields of various fractions are given below:

1st cold water extract = 1.7 g.

2nd " " " = 0.7 g.

3rd " " " = 0.4 g.

= 2.8 g.

1st hot water extract	=	2.9 g.
2nd " " "	=	1.5 g.
3rd " " "	=	0.7 g.
4th " " "	=	0.6 g.
5th " " "	=	0.5 g.
		= 6.2 g.

Total water extract = $2.8 + 6.2 = 9.0$ g. (18%).

Expt.11. Examination of the Extracts.

Solutions of each fraction of aqueous extracts in water gave a blue colour with iodine indicating the presence of starch in them. Each of the extracts was hydrolysed with 1N sulphuric acid on a boiling water-bath for 14 hours and examined chromatographically in solvents A and E (using spray 1 and 3). In each case spots corresponding to L-arabinose, D-galactose, D-mannose (trace), D-glucose (trace) and D-galacturonic acid were obtained.

Because of similarity of composition all fractions were combined together and collectively treated to make them free of starch.

Expt.12. Removal of Starch from above water extracts.

The collective extracts (8.5 g.) was mixed with celite (9.0 g.) and then dispersed in aqueous ammonium carbonate

solution (0.3% ; 500 ml.). The mixture was heated on an oil-bath for 30 minutes at 117° - 120° C under nitrogen with occasional shaking, cooled to room temperature and to it an aqueous solution of sodium chloride (20%; 150 ml.) together with a solution of iodine (12%) in potassium iodide (20%; 15 ml.) was added. It was diluted with water (600 ml.) and vigorously shaken when starch-iodine complex appeared as precipitate which was removed by centrifugation. The starch-free supernatant was treated first with sodium thiosulphate solution (0.5N) to destroy the excess iodine, dialysed, concentrated and finally poured into excess rapidly stirred ethanol. The resulting precipitate was recovered by centrifugation and dried in a desiccator over anhydrous calcium chloride (yield, 7.5 g.). This starch free polysaccharide was called Polysaccharide B.

The derived precipitate of starch-iodine complex was shaken with 20% aqueous sodium chloride (300 ml.) and treated with 0.5N aqueous sodium thiosulphate (25 ml.). Celite was removed by centrifugation. 1N hydrochloric acid (80 ml.) together with iodine-potassium iodide (15 ml.) was added to the supernatant. After standing for 15 minutes the starch-iodine complex was, again centrifuged, suspended in ethanol (400 ml.) and treated with sodium thiosulphate. Water was added to make the concentration of alcohol approximately 70%. Starch was isolated after dialysis and concentration.¹⁸¹ The yield was 80 mg. Therefore amount of starch present in water-extracts was 1% (approx.).

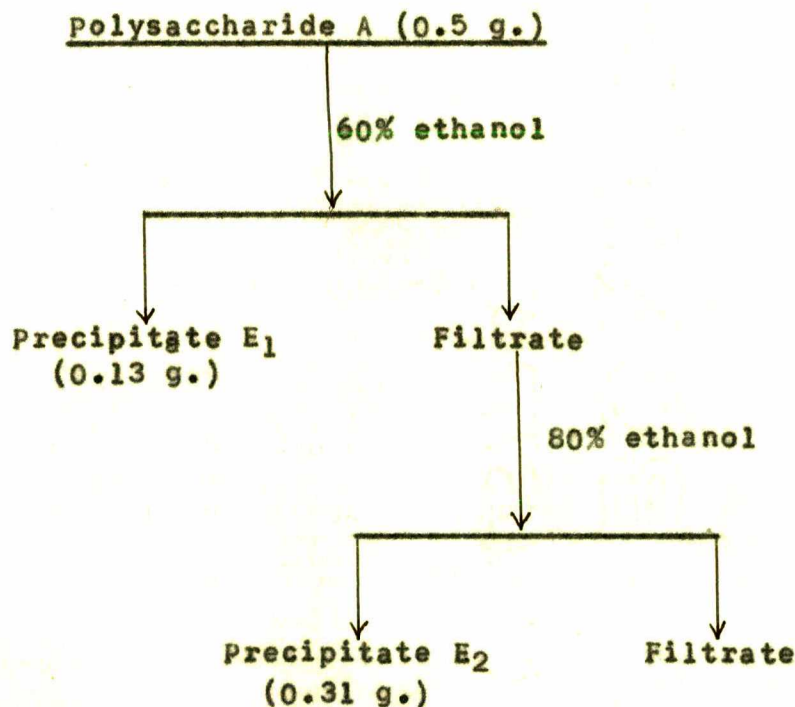
STUDY OF POLYSACCHARIDE A

Expt.13. Attempted fractionation of Polysaccharide A.

A portion of the polysaccharide A was subjected to the following fractionation techniques:

(a) Selective precipitation with Ethanol.¹⁸²

Polysaccharide A (0.5 g.) was dissolved in water (100 ml.) and centrifuged. The supernatant was treated with ethanol as in the scheme below:

Examination of E₁ and E₂ .

Each of these materials was hydrolysed with 1N sulphuric acid on a boiling water-bath for 8 hours. The cooled hydrolysates were neutralised with barium carbonate, deionised with

Amberlite resin IR 120(H⁺), concentrated to syrups and examined on a paper chromatogram in solvents A and E (using spray I). The presence of L-arabinose, D-galactose and D-mannose was revealed in each case.

(b) By precipitation with Fehling solution.¹⁸³

Polysaccharide A (0.5 g.) was dissolved in water (100 ml.). A small quantity of an insoluble material was removed at the centrifuge. Freshly prepared Fehling solution was added to the polysaccharide solution until precipitation of the "copper complex" was just complete. The blue gelatinous precipitate was removed by centrifugation, washed with water and again centrifuged. The precipitate was then suspended in water, cooled with iced water and treated with 1N hydrochloric acid when a clear light green solution was obtained. The solution was added to ethanol (4 vols) with stirring. The derived precipitate was collected at the centrifuge, dissolved in water and dialysed (2 days). The dialysed solution was concentrated to a suitable volume and poured into excess rapidly stirred ethanol. The precipitate was collected and dried in a desiccator over anhydrous calcium chloride.

Examination of the precipitate.

The precipitate was hydrolysed with 1N sulphuric acid on a boiling water-bath for 8 hours (G.P.8) and examined by paper chromatography in solvents A and E (using spray I). This indi-

cated the presence of L-arabinose, D-galactose and D-mannose.

(c) By precipitation with Ammonium sulphate solution.

The polysaccharide A (1.0 g.) was dissolved in water (200 ml.). A small portion of undissolved material was removed at the centrifuge. To the supernatant was added solid ammonium sulphate gradually with stirring till the solution became saturated with respect to ammonium sulphate. No precipitate was formed at this stage. The polysaccharide was recovered by dialysis (2 days) and subsequent concentration to a suitable volume and precipitation with ethanol.

(d) By precipitation with Barium hydroxide solution.¹⁸⁴

The polysaccharide A (1.0 g.) was dissolved in water (200 ml.). A small portion of undissolved material was removed at the centrifuge. To the supernatant was added saturated barium hydroxide solution (100 ml.) gradually with stirring. No precipitate was formed.

From all the above results it appeared that the polysaccharide A was essentially homogeneous.

Expt.14 Methylation of Polysaccharide A.

The polysaccharide A (5.0 g.) was dissolved in aqueous sodium hydroxide solution (30%, 200 ml.) and methylated following the procedure of Haworth [G.P.16(A)] which gave a methyla-

ted product having a methoxyl content of 37.5%.

Expt.15 Further attempts to increase the methoxyl content of the methylated polysaccharide.

The following methods were employed:

(i) Purdie Method.¹⁸⁵

The methylated polysaccharide (200 mg.) (OMe, 37.5%) was dissolved in methyl iodide (10 ml.) and treated with dry silver oxide (3 g.) in one-eighth portion every 30 minutes. The mixture ^{was} allowed to reflux overnight after which the methylated polysaccharide was recovered from the filtrate and chloroform washings of the silver salts by evaporation to dryness. Three more methylations were carried out and the recovered product (160 mg.) had a methoxyl content of 38.9%.

(ii) Modified Kuhn Method.¹⁸⁶

The methylated polysaccharide (200 mg.) (OMe, 38.9%) was dissolved in freshly distilled dimethyl formamide (5 ml.) and the solution was cooled to 0°C. Methyl iodide (5 ml.) and silver oxide (2.5 g.) were then added to the cooled solution. The mixture was shaken in a stoppered flask covered with black cloth for 3 hours in an ice-bath and then for 20 hours at room temperature. The product was filtered and the residue washed with chloroform. The filtrate and the washings were combined, dried over anhydrous sodium sulphate and concentrated to dryness under high vacuum to remove the last traces of dimethyl

formamide. The residue was dissolved in dry chloroform and concentrated to a syrup. The product was added to an excess of light petroleum ether (b.p. 40°- 60°C) and the derived precipitate of methylated polysaccharide was separated and dried (150 mg.). It was found to have a methoxyl content of 38.9% .

(iii) Hakomori Method.

The methylated polysaccharide (100 mg.) was subjected to further methylation by Hakomori Method [G.P.16(B) (ii)] . This gave a product with a methoxyl content of 38.9%.

Expt.16. Formic acid hydrolysis of the methylated Polysaccharide.

The methylated polysaccharide (50 mg., OMe 38.9%) was heated in a sealed tube on a boiling water-bath with 90% formic acid (2 ml.) in an inert atmosphere of carbon dioxide. After 4 hours the tube was opened and heated for a further period of an hour with addition of water (5 ml.). The hydrolysate was then deionized with Amberlite IR 120 (H⁺) and IR-45(OH⁻) resins and filtered. The filtrate was evaporated to a syrup under reduced pressure and examined by paper chromatography in solvent G (using spray I). It showed the presence of

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

2,6-di-O-methyl-D-mannose

2,4-di-O-methyl-D-galactose

2-O-methyl-D-galactose

Expt.17. Methanolysis and Gas-liquid chromatographic analysis of the methylated polysaccharide.

The methylated polysaccharide (50 mg.) was converted into a mixture of methyl glycosides of methylated sugars by boiling with methanolic 3% hydrogen chloride for 10 hours. The cooled solution was neutralised with silver carbonate, filtered, deionised with Amberlite resin IR 120 (H^+) and concentrated to a syrup under reduced pressure. Gas-liquid chromatographic analysis of the syrup on column a and b (G.P.20) showed the presence of methyl glycosides of the sugars as shown in Table II below:

Table II

Relative retention times (I) of methyl glycosides.¹⁸⁷

	<u>column a</u>	<u>column b</u>
2,3,5-tri-O-methyl-L-arabinose	0.53,0.70	0.48,0.61
2,3-di-O-methyl-L-arabinose	1.44,(1.75)	0.68
2,3,4,6-tetra-O-methyl-D-galactose	(1.75)	1.53
2,3,6-tri-O-methyl-D-galactose	3.10,3.94	(1.53),2.01
2,4,6-tri-O-methyl-D-galactose	(3.94),4.47	2.31
2,3,4-tri-O-methyl-D-galactose	6.96	2.83

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

Expt.18. Hydrolysis of the methylated polysaccharide and separation of methylated sugars on filter sheets.

The purified methylated polysaccharide (600 mg.) was methanolysed by boiling with methanolic 3% hydrogen chloride (50 ml.) for 10 hours. The cooled solution was neutralised with silver carbonate, deionised with Amberlite IR 120 (H^+) and Amberlite IR-45(OH^-) resins and concentrated to a syrup under reduced pressure. The syrup was then hydrolysed with 1N sulphuric acid (10 ml.) on a boiling water-bath for 7 hours. The cooled solution was neutralised with barium carbonate, deionised with Amberlite IR 120 (H^+) and IR-45(OH^-) resins and concentrated under reduced pressure to a syrup (520 mg.).

The syrup was separated on filter sheets (using solvent G) to give eight fractions. The Table III summarises the results of preliminary examination of the various fractions.

Fraction 1. A chromatographically pure syrup (20 mg.) identical in mobility and colour with 2,3,5-tri-O-methyl-L-arabinose (R_G 0.95; lit R_G 0.95). It had $[\alpha]_D^{20} = 41^\circ$ (c 1.2); lit - 40° for 2,3,5-tri-O-methyl-L-arabinose.¹⁸⁸ On oxidation with bromine water this fraction gave in good yield crystalline 2,3,5-tri-O-methyl-L-arabonolactone, m.p. $29^\circ C$. On reaction with liquid ammonia the lactone was converted into 2,3,5-tri-O-methyl-L-arabonamide, m.p. $137-138^\circ$. On demethylation it gave L-arabinose.

Table IIIAnalysis of hydrolysate of methylated polysaccharide.

Frac- tion	wt.(mg.)	$[\alpha]_D^{20}$	<u>Paper chromatography</u>		Sugars given on demethylation
			R_G (solvent G)	Sugar	
1	20	-40°	0.95	2,3,5-tri-O-methyl -L-arabinose	L-arabinose
2	41	+112°	0.88	2,3,4,6-tetra-O- methyl-D-galactose	D-galactose
3	26	+95°	0.71	2,3,6-tri-O-methyl -D-galactose	D-galactose
4	55	+92°	0.67	2,4,6-tri-O-methyl -D-galactose	D-galactose
5	25	+100°	0.63	2,3-di-O-methyl -L-arabinose	L-arabinose
6	53	-	0.52	2,6-di-O-methyl -D-mannose	D-mannose
7	45	+84°	0.42	2,4-di-O-methyl -D-galactose	D-galactose
8	30	+85°	0.23	2-O-methyl -D-galactose	D-galactose

Fraction 2. A chromatographically pure syrup (41 mg.) which had $[\alpha]_D^{20} + 114^\circ$ (c 0.9); lit. $[\alpha]_D^{20} + 116^\circ$ for 2,3,4,6-tetra-O-methyl-D-galactose. It had R_G 0.88; lit 0.88 for 2,3,4,6-tetra-O-methyl-D-galactose. The aniline derivative had m.p. 196°C; lit¹⁸⁹ m.p. 198°. On demethylation it gave D-galactose.

Fraction 3. A chromatographically pure syrup (26 mg.). It had

R_G 0.71; lit 0.71 for 2,3,6-tri-O-methyl-D-galactose; $[\alpha]_D^{20} +94^\circ$ (c 1.8); lit $+96^\circ$ for 2,3,6-tri-O-methyl-D-galactose.¹⁸⁹ The derived lactone had m.p. $95 - 97^\circ$. On demethylation it gave D-galactose.

Fraction 4. A chromatographically pure syrup (55 mg.). It had R_G 0.67; lit¹⁹⁰ 0.67 for 2,4,6-tri-O-methyl-D-galactose; $[\alpha]_D^{20} +92^\circ$ (c 1.2); lit $+90^\circ$. It was converted into the aniline derivative, m.p. $169-171^\circ$; lit¹⁸⁹ m.p. $165-166^\circ$. On demethylation it gave D-galactose.

Fraction 5. The sugar (25 mg.) was shown by paper chromatography to be a single compound having the chromatographic and ionophoretic mobility of 2,3-di-O-methyl-L-arabinose (R_G 0.63; lit 0.64 for 2,3-di-O-methyl-L-arabinose.¹⁹⁰ It had $[\alpha]_D^{20} +100^\circ$ (c 0.5); lit $+101^\circ$. The derived 2,3-di-O-methyl-L-arabonamide had m.p. $162-163^\circ$; lit m.p. $160-161^\circ$. On demethylation it gave L-arabinose only.

Fraction 6. The syrup (53 mg.), on demethylation gave D-mannose. Although attempts to prepare crystalline derivatives of the sugar failed, it was chromatographically indistinguishable from the authentic 2,6-di-O-methyl-D-mannose.

Fraction 7. The sugar (45 mg.) was shown by paper chromatography to be a single compound having the chromatographic and ionopho-

retic mobility of 2,4-di-O-methyl-D-galactose, (R_G 0.42; lit 0.42 for 2,4-di-O-methyl-D-galactose.¹⁹⁰ It had $[\alpha]_D^{20} + 84^\circ$ (c 1.1); lit $+ 85^\circ$ for 2,4-di-O-methyl-D-galactose. The aniline derivative had m.p. $216 - 218^\circ$. On demethylation it gave D-galactose only.

Fraction 8. A chromatographically pure syrup (30 mg.) which had R_G 0.23; lit 0.23 for 2-O-methyl-D-galactose.¹⁹⁰ The product crystallised from ethyl alcohol. The crystals melted at $149-150^\circ$; lit¹⁹¹ m.p. $147 - 148^\circ$. It had $[\alpha]_D^{20} + 52 \rightarrow + 85^\circ$ (equil) (c 1.0).

Expt. 19. Graded hydrolysis of Polysaccharide A.

The polysaccharide A (600 mg.) was heated with 0.1N oxalic acid (40 ml.) on a boiling water-bath. Fractions (5 ml.) were withdrawn at intervals (1 hour). Degraded polysaccharides in each fraction were precipitated by the addition of ethanol (4 vols). The supernatants left after removal of degraded polysaccharide by centrifugation were each neutralised with calcium carbonate, deionised with Amberlite IR 120(H^+) and Amberlite IR-45(OH^-) resins and concentrated to a syrup.

The degraded samples of polysaccharides were separately hydrolysed with 1N sulphuric acid on a boiling water-bath for 8 hours. The cooled hydrolysates were each neutralised with barium carbonate, deionised with Amberlite resin IR 120 (H^+)

and concentrated to a syrup.

The syrups obtained from the supernatants as well as from the hydrolysates of the degraded polysaccharide samples were each examined by paper chromatography in solvents A and F (using spray 1 and 3) separately. The results showed that the L-arabinose units were rapidly released together with smaller amounts of D-galactose and D-mannose.

It also showed that when the polysaccharide A was hydrolysed with 0.1N oxalic acid on a boiling water-bath for 4 hours, it afforded a good yield of oligosaccharides, but the degraded polysaccharide obtained by precipitation with ethanol from the cooled hydrolysate, on hydrolysis with mineral acid, gave D-galactose and D-mannose only but no L-arabinose.

Expt. 20. Partial hydrolysis of Polysaccharide A.

After a series of trial hydrolyses on small quantities of polysaccharide A with different concentrations of sulphuric acid and oxalic acid, it was found that 0.1N oxalic acid at 100°C for 4 hours gave the highest yield of oligosaccharides.

The polysaccharide A (10.0 g.) was, accordingly, refluxed with 0.1N oxalic acid (700 ml.) on a boiling water-bath for 4 hours. An insoluble residue was filtered off, dried and analysed. It showed the absence of carbohydrate.

The filtrate was treated with ethanol (5 vols) and left

overnight. The precipitated degraded polysaccharide (D_1) was removed at the centrifuge, washed with ethanol, dried in a vacuum desiccator over anhydrous calcium chloride and weighed (4.9 g.). The supernatant was concentrated to about 150 ml. under reduced pressure, neutralised with calcium carbonate and filtered. The calcium salts were washed with water and the combined filtrate and washings were deionised with Amberlite IR 120 (H^+) and IR-45(OH^-) resins and concentrated to a syrup, S_1 (2.35 g.) after drying in vacuo. Chromatographic examination of the syrup in solvents A and E (using spray I and 3) revealed spots corresponding to L-arabinose, D-galactose, D-mannose (trace) and three oligosaccharides.

Expt. 21. Fractionation of syrupy hydrolysate (S_1) on charcoal-celite column.

The syrup (S_1) obtained from the 0.1N oxalic acid hydrolysis of the polysaccharide A ($100^\circ C$, 4 hours) (Expt. 20) was dissolved in the minimum volume of water and was allowed to soak into the charcoal-celite column, (40 x 4 cm.) prepared as described in G.P. 15.

The monosaccharides (L-arabinose, D-galactose and D-mannose) were eluted with water (2 l) and discarded. The oligosaccharides were successively eluted with different concentrations of ethanol (2½%, 5%, 10% and 20%). The elution patterns were followed by testing an aliquot (1 ml.) with phenol-sulphuric acid.

Elution with water containing 2½% ethanol (2½ l.) gave two fractions (peak No. 1 & 2, graph No. 1 page 86a) which were isolated by evaporation to dryness at reduced pressure. Elution with water containing 5% ethanol (2 l.) gave only one fraction (graph No. 2 page 86b). The fractions collected in an automatic fraction collector are shown in Table IV. Elution with water containing 10% and 20% ethanol gave negligible amount of sugars and were discarded.

Table IV

Fractionation of syrupy hydrolysate (S₁).

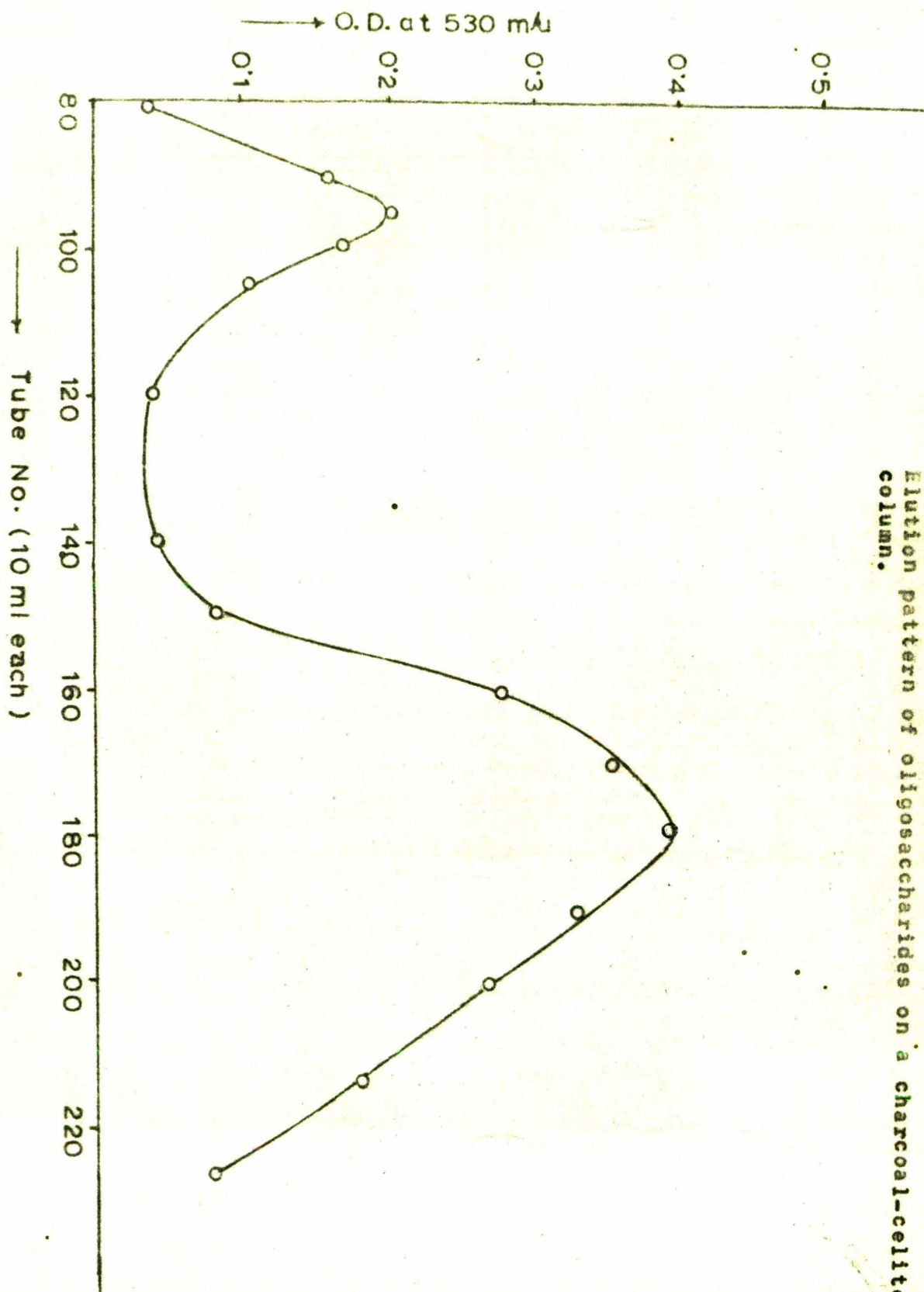
	<u>Tubes</u> (10 ml. each).	<u>Frac-</u> <u>tion.</u>	<u>Examination</u> <u>by paper</u> <u>chromato-</u> <u>graphy.</u>	<u>R_GGalactose</u> <u>(Solvent B).</u>	<u>Weight</u> <u>(mg.).</u>
Elution with water con- taining 2½% ethanol	1-79	-	-	-	-
	80-120	1	single compound	0.55	95
	140-220	2	single compound	0.47	386
Elution with water con- taining 5% ethanol	1-95	-	-	-	-
	100-180	3	single compound	0.29	82
					Total = 563 mg.

Graph No.1.

Fractionation of 0.1N oxalic acid hydrolysate of polysaccharide A.

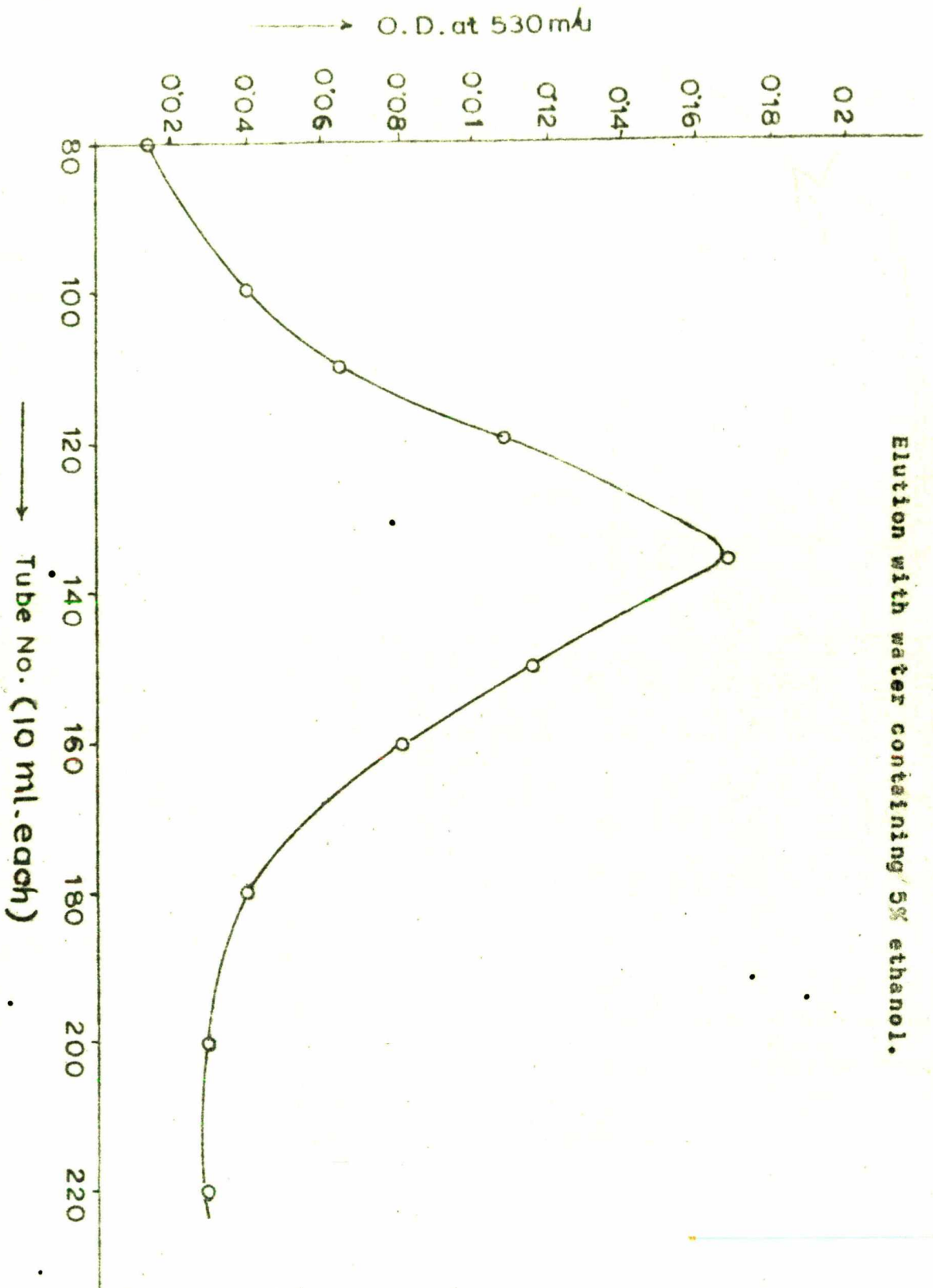
Elution with water containing 2% ethanol.

Elution pattern of oligosaccharides on a charcoal-cellulose column.



Graph No. 2. Fractionation of 0.1N oxalic acid hydrolysate of polysaccharide A.

Elution with water containing 5% ethanol.



Investigation of the individual fractions.

Fraction 1. A chromatographically pure syrup (95 mg.) which had DP2; $R_{\text{Galactose}}$ 0.55 in solvent B. On hydrolysis it gave D-galactose only. Methylation followed by methanolysis, hydrolysis and paper chromatographic analysis (solvent G) revealed spots corresponding to 2,3,4,6-tetra-O-methyl-D-galactose and 2,3,6-tri-O-methyl-D-galactose. Gas-liquid chromatographic examination of the methanolysate also confirmed the presence of the above sugars. All these facts indicated that this fraction was

O-D-galactopyranosyl (1→4)-D-galactopyranose.

Fraction 2. A chromatographically pure sample (386 mg.) which had DP 2; $R_{\text{Galactose}}$ 0.47 in solvent B; lit 0.49 for O-D-galactopyranosyl (1→3)-D-galactopyranose.¹⁹² On hydrolysis it gave D-galactose only. Methylation of the material followed by methanolysis, hydrolysis and paper chromatographic analysis (solvent G) revealed the presence of 2,3,4,6-tetra-O-methyl-D-galactose and 2,4,6-tri-O-methyl-D-galactose. Gas-liquid chromatographic analysis also confirmed the presence of the above sugars. All these facts revealed that the fraction was

O-D-galactopyranosyl (1→3)-D-galactopyranose.

Fraction 3. A chromatographically pure syrup (82 mg.) which had DP 2; $R_{\text{Galactose}}$ 0.29 in solvent B; lit 0.30 for O-D-galactopyranosyl (1→6)-D-galactopyranose.¹⁹² On hydrolysis it gave D-galactose only. Methylation followed by methanolysis, hydroly-

sis and paper chromatographic analysis (solvent G) revealed spots corresponding to 2,3,4,6-tetra-O-methyl-D-galactose and 2,3,4-tri-O-methyl-D-galactose. Gas-liquid chromatographic examination also revealed the presence of the above sugars. All these facts indicated that this fraction was
O-D-galactopyranosyl (1→6)-D-galactopyranose.

Expt. 22. Examination of the degraded polysaccharide, D₁ .

The degraded polysaccharide, D₁, [Expt. 20] (100 mg.) obtained from partial hydrolysis of polysaccharide A with 0.1N oxalic acid (100°C, 4 hours), was hydrolysed with 1N sulphuric acid (5 ml.) on a boiling water-bath for 8 hours. The cooled solution was neutralised with barium carbonate, filtered, the filtrate deionised with Amberlite IR 120 (H⁺) and IR-45(OH⁻) resins and concentrated to a syrup. Chromatographic examination of the syrup in solvent A (spray I) revealed spots corresponding to D-galactose and D-mannose.

Expt. 23. Partial hydrolysis of the degraded polysaccharide, D₁.

The degraded polysaccharide, D₁ (600 mg.) was refluxed with 0.1N oxalic acid (50 ml.) on a boiling water-bath for 4 hours. The cooled solution was treated with ethanol (5 vols) and left overnight. The precipitated degraded polysaccharide, D₂, was removed at the centrifuge, washed with ethanol, dried in a vacuum desiccator over anhydrous calcium chloride and

weighed (2.100)

weighed (260 mg.). The supernatant was neutralised with calcium carbonate, deionised with Amberlite IR 120 (H^+) and IR-45(OH^-) resins and concentrated to a syrup (230 mg.). Chromatographic analysis of the syrup in solvent A (using spray I) revealed the presence of D-galactose, D-mannose and an oligosaccharide.

The oligosaccharide was isolated as a pure entity by repeated separation of the syrup on filter sheets by using solvent A.

Examination of the oligosaccharide.

The oligosaccharide (66 mg.) had DP 2; $R_{Galactose}$ 0.48. On hydrolysis it gave D-galactose only. Methylation followed by methanolysis, hydrolysis and paper chromatographic examination in solvent G revealed spots corresponding to 2,3,4,6-tetra-O-methyl-D-galactose and 2,4,6-tri-O-methyl-D-galactose. All these facts indicated that the oligosaccharide was

O-D-galactopyranosyl (1 $\rightarrow$ 3)-D-galactopyranose.

Expt. 24. Periodate oxidation of the degraded polysaccharide, D₁.

The degraded polysaccharide, D₁, (300 mg.) was oxidised with 0.05M sodium metaperiodate solution (200 ml.) for 48 hours at room temperature until the periodate consumption was constant. The amount of periodate consumed was measured by withdrawing aliquots (5 ml.) at regular intervals and examination by Arsenite method [G.P. 22(a)] and is shown in Table V.(page 90).

Table V.

	<u>Periodate consumption</u>				
Time in hour	4	12	18	30	48
Moles of periodate reduced per C ₆ anhydro-unit	0.04	0.07	0.15	0.22	0.22

Expt. 25. Methylation of degraded polysaccharide, D₁.

The degraded polysaccharide, D₁, (600 mg.) was dissolved in dimethyl sulphoxide (50 ml.) by stirring under nitrogen atmosphere at 70°C. To the cooled solution sodium hydride (1.50 g.) was added in four instalments with stirring in one hour. The mixture was stirred until evolution of hydrogen ceased (3 hours). Methyl iodide (6 ml.) was added dropwise with stirring in four equal instalments during 2 hours. The solution was stirred overnight. A further three additions of sodium hydride and methyl iodide were made to the reaction mixture on successive days, as described for the first addition. The mixture was then poured into water (300 ml.) and extracted with chloroform (2 x 500 ml.). The chloroform solution was washed repeatedly (7 times) with an excess of water (ca 500 ml.) to remove dimethyl sulphoxide, dried over anhydrous sodium sulphate and evaporated to a syrup (430 mg.) under reduced pressure (rotary evaporator at less than 40°C.).

Expt. 26. Hydrolysis of the methylated degraded polysaccharide and separation of methylated sugars on filter sheets.

The methylated degraded polysaccharide (430 mg.) was methanolysed by boiling with methanolic 3% hydrogen chloride (30 ml.) for 10 hours. The cooled solution was neutralised, deionised and concentrated to a syrup. The syrup was then hydrolysed with 1N sulphuric acid (8 ml.) on a boiling water-bath for 7 hours. The cooled solution was neutralised, deionised and concentrated to a syrup (380 mg.).

The syrup was separated on filter sheets (using solvent G) to give five fractions. The Table VI summarises the results of preliminary examination of the various fractions.

Table VI.

Analysis of hydrolysate of methylated degraded polysaccharide.

Frac- tion	wt(mg.)	$[\alpha]_D^{20}$	Paper chromatography R _G (solvent G) Sugar	Sugars given on demethylation
1	62	+116°	0.90 2,3,4,6-tetra-O-methyl-D-galactose	D-galactose
2	37	-8°	0.85 2,3,6-tri-O-methyl-D-mannose	D-mannose
3	59	+91°	0.68 2,4,6-tri-O-methyl-D-galactose	D-galactose
4	33	+85°	0.48 2,6-di-O-methyl-D-galactose	D-galactose
5	20	+83°	0.43 2,4-di-O-methyl-D-galactose	D-galactose

Fraction 1. A chromatographically pure syrup (62 mg.) which had R_G 0.90; lit 0.88 for 2,3,4,6-tetra-O-methyl-D-galactose; $[\alpha]_D^{20} + 118^\circ$ (c 0.9); lit $[\alpha]_D^{20} + 116^\circ$ for 2,3,4,6-tetra-O-methyl-D-galactose. On demethylation it gave D-galactose.

Fraction 2. The sugar (37 mg.) was shown by paper chromatography to be a single compound having the mobility of 2,3,6-tri-O-methyl-D-mannose. It had R_G 0.85; lit 0.86; $[\alpha]_D^{20} - 8^\circ$ (c 1.8); lit -10° for 2,3,6-tri-O-methyl-D-mannose.¹⁸⁹ On demethylation it gave D-mannose.

Fraction 3. A chromatographically pure syrup (59 mg.) which had R_G 0.68; lit 0.67 for 2,4,6-tri-O-methyl-D-galactose; $[\alpha]_D^{190} + 91^\circ$ (c 1.2); lit $+92^\circ$ for 2,4,6-tri-O-methyl-D-galactose. On demethylation it gave D-galactose.

Fraction 4. The sugar (33 mg.) was shown by paper chromatography to be a single compound having the chromatographic and ionophoretic mobility of 2,6-di-O-methyl-D-galactose. It had R_G 0.48; lit¹⁹⁰ 0.47; $[\alpha]_D^{20} + 85^\circ$ (c 1.1); lit $+86^\circ$ for 2,6-di-O-methyl-D-galactose.¹⁸⁹ On demethylation it gave D-galactose.

Fraction 5. It was also a chromatographically pure syrup (20 mg.), which had R_G 0.43; lit¹⁹⁰ 0.42 for 2,4-di-O-methyl-D-galactose; $[\alpha]_D^{20} + 83^\circ$ (c 1.1); lit $+85^\circ$ for 2,4-di-O-methyl-D-galactose.¹⁹¹ On demethylation it gave D-galactose.

SMITH DEGRADATION STUDIESExpt. 27. Periodate oxidation of polysaccharide A in (buffered solution).¹⁹³

The polysaccharide A (600 mg.) was oxidised with 0.05M sodium metaperiodate solution in 0.2M acetate buffer (150 ml., pH 3.75) at room temperature for 30 hours. Aliquots (5 ml.) of this reaction mixture were removed at regular intervals and examined for periodate consumption by Arsenite method [G.P. 22(a)].

Moles of periodate consumed per hexose unit were plotted against time and the primary oxidation determined by extrapolation to zero time.

The amount of periodate reduced is shown in Table VII (graph No. 3; page 99a).

Table VII.

	<u>Periodate consumption</u>						
Time in hour	2	4	8	14	20	30	400014
• Moles of periodate reduced per C ₆ anhydro-unit	0.04	0.09	0.15	0.22	0.27	0.27	

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গ্রন্থাগার

After 30 hours the unreduced periodate was destroyed with ethylene glycol. The solution was then neutralised with aqueous N sodium hydroxide and dialysed against distilled water in a closed system. Examination of the dialysate indicated the presence of carbohydrate.

The solution from the dialysis sac was concentrated (25 ml.) and reduced with sodium borohydride (25 mg.) in an atmosphere of nitrogen for 24 hours. The solution was then neutralised with acetic acid to destroy the excess borohydride and concentrated to a suitable volume. The concentrated solution was co-distilled with methanol. Four additions of methanol were made to facilitate the removal of volatile methyl borate. The residue was finally dried in a vacuum desiccator over anhydrous calcium chloride. As the yield of polyalcohol (130 mg.) was low, mild hydrolysis was not carried out.

Expt. 28. Periodate oxidation of polysaccharide A
(without buffer solution).

100 ml. of aqueous solution of the polysaccharide A (600 mg.) was mixed with 100 ml. of 0.1M sodium metaperiodate solution. Aliquots (5 ml.) of this mixture were taken out at regular intervals and examined for periodate consumption by Arsenite method as before.

The amount of periodate reduced is shown in Table VIII (graph No. 3; page 99a).

Table VIII

	<u>Periodate consumption</u>				
Time in hour	2	4	8	20	30
Moles of periodate consumed per C ₆ anhydro-unit.	0.10	0.16	0.23	0.35	0.35

After 30 hours the unreduced periodate was destroyed with ethylene glycol and the solution was dialysed extensively (72 hours). The solution from the dialysis sac was concentrated (25 ml.) and reduced with sodium borohydride (250 mg.). The polyalcohol (A_1) was recovered in the usual way as before and weighed 540 mg.

Mild hydrolysis of polyalcohol A_1 .

The polyalcohol (500 mg.) was treated with 1N sulphuric acid (15 ml.) at room temperature for 24 hours. After removing a small quantity of insoluble residue by centrifugation, the supernatant was treated with ethanol (6 vols) and left overnight at 0°C. The precipitated degraded polyalcohol (P_1) was removed at the centrifuge, dried in a desiccator and weighed (240 mg.). The supernatant was concentrated (15 ml.), neutralised with barium carbonate, deionised with Amberlite IR 120 (H^+) and IR-45 (OH^-) resins, concentrated to a syrup and examined by paper chromatography in solvent A (using spray I and dip 2). This indicated the presence of glycerol, threitol, glycollic aldehyde, D-galactose, D-mannose and a reducing sugar having $R_{\text{galactose}}$ 0.58 in solvent A.

Hot hydrolysis of polyalcohol (A_1).

The polyalcohol A_1 (40 mg.) was hydrolysed with 1N sulphuric acid (2 ml.) on a boiling water-bath for 12 hours. The cooled solution was neutralised, deionised and concentrated to a syrup

Chromatographic examination of the syrup in solvent A (using spray 1 and dip 2) revealed spots corresponding to glycerol, threitol, glycollic aldehyde, D-galactose and D-mannose.

Expt. 29. Large scale Smith degradation of polysaccharide A.

(i) Periodate oxidation - To an aqueous solution of the polysaccharide A (1.5 g. in 250 ml.) was added aqueous sodium metaperiodate solution (0.1M, 250 ml.) and the oxidation was allowed to continue for 30 hours. Aliquots (5 ml.) were taken out at intervals and examined for periodate consumption.

The quantity of periodate reduced is shown in Table IX.

Table IX.

Periodate consumption

Time in hour	2	8	20	30
Moles of periodate reduced per C ₆ anhydro-unit	0.11	0.23	0.35	0.35

After 30 hours the excess periodate was destroyed with ethylene glycol and the solution dialysed extensively (72 hours).

(ii) Reduction - The dialysed solution was concentrated to a suitable volume. To the cooled solution was added sodium borohydride (1 g.) slowly with stirring under nitrogen atmosphere and the reaction was allowed to continue for 20 hours. The polyalcohol (A₁) was recovered as before, dried and weighed (1.4 g.)

(iii) Mild hydrolysis - The polyalcohol A₁ (1.4 g.) was treated with 1N sulphuric acid (50 ml.) at room temperature for 24 hours. After removing a small quantity of an insoluble residue (110 mg.) at the centrifuge, the supernatant was treated with ethanol (6 vols.) and allowed to stand overnight at 0°C. The precipitated degraded polyalcohol P₁ was removed at the centrifuge, dried and weighed (900 mg.). The supernatant was concentrated (ca 50 ml.), neutralised, deionised and concentrated to a syrup (300 mg.) and examined chromatographically in solvent A (using spray I and dip 2). This indicated the presence of glycerol, threitol, glycollic aldehyde, D-galactose, D-mannose and a reducing sugar(R) having $R_{\text{Galactose}}$ 0.58 in solvent A.

Expt. 30. Examination of the degraded polyalcohol P₁ .

The degraded polyalcohol P₁ (100 mg.) was heated with 1N sulphuric acid (3 ml.) on a boiling water-bath for 12 hours. The cooled solution was neutralised with barium carbonate, deionised with Amberlite IR 120 (H⁺) and IR-45 (OH⁻) resins and concentrated to a syrup. Paper chromatographic examination of the syrup revealed the presence of D-galactose and D-mannose but no L-arabinose.

Expt. 31. Smith degradation of the degraded polymer, P₁ .

(i) Periodate oxidation - The degraded polyalcohol, P₁ (800 mg.) was dissolved in water (100 ml.). 0.1M aqueous sodium metaperiodate solution (100 ml.) was added to the sugar solution. Aliquots

(5 ml.) of the reaction mixture were taken out at regular intervals and examined for periodate consumption.

The quantity of periodate reduced is shown in Table X.
(graph No. 3; page 99a)

Table X.

	<u>Periodate consumption</u>				
Time in hour	4	12	18	30	40
Moles of periodate reduced per C ₆ anhydro-unit.	0.06	0.12	0.17	0.22	0.22

(ii) Reduction - After 40 hours the excess periodate was destroyed by ethylene glycol, and the solution dialysed extensively (72 hours), concentrated to a suitable volume, cooled and reduced with sodium borohydride (500 mg.). The resulting polyalcohol, A₂, was recovered in the usual way, dried and weighed (750 mg.).

(iii) Mild hydrolysis - The polyalcohol A₂ (700 mg.) was treated with 1N sulphuric acid (30 ml.) at room temperature for 24 hours. After removing a small quantity of insoluble residue by centrifugation, the supernatant was treated with ethanol (6 vols.) and left overnight at 0°C. The precipitated degraded polyalcohol (P₂) was removed at the centrifuge, dried and weighed (500 mg.). The supernatant was concentrated (ca 30 ml.), cooled, neutralised, ^{concentrated} deionised to a syrup and then examined chromatographically in solvent A. This indicated the presence of glycerol, erythritol, threitol (trace), glycollic aldehyde, D-galactose and two other

unidentified non-reducing components having $R_{\text{Galactose}}$ 0.61 and 0.20 respectively in solvent A.

Hot hydrolysis of polyalcohol A₂ .

The polyalcohol A₂ (100 mg.) was hydrolysed with 1N sulphuric acid (3 ml.) on a boiling water-bath for 12 hours. The cooled solution was neutralised, deionised, concentrated to a syrup and examined by paper chromatography in solvent A (spray 1 & dip 2). This indicated the presence of D-galactose, glycerol, erythritol, threitol (trace) and glycollic aldehyde.

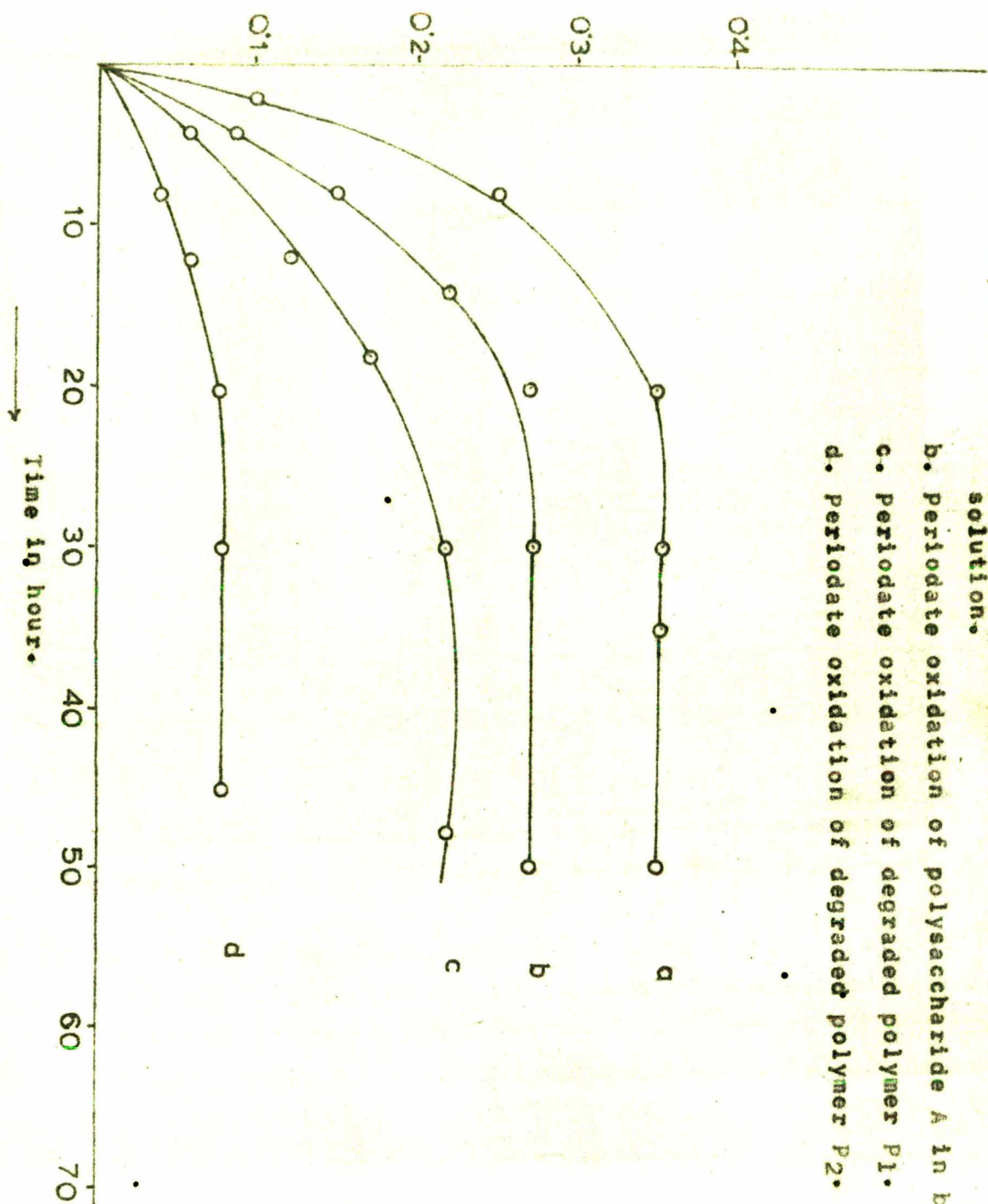
Expt. 32. Examination of the degraded polymer, P₂ .

The degraded polyalcohol P₂ (100 mg.) was hydrolysed with 1N sulphuric acid (3 ml.) on a boiling water-bath for 10 hours. The cooled solution was neutralised with barium carbonate, deionised with Amberlite IR 120 (H⁺) and IR-45 (OH⁻) resins and concentrated to a syrup. Chromatographic examination of the syrup in solvent A (spray 1 and ^{dip} 2) revealed spot for D-galactose only.

Expt. 33. Smith degradation of polyalcohol P₂ .

(i) Periodate oxidation - The degraded polymer P₂ (400 mg.) was dissolved in water (100 ml.). 0.1M aqueous sodium metaperiodate solution (100 ml.) was then added to the sugar solution. Aliquots (5 ml.) of this reaction mixture were taken out at regular intervals and examined for periodate consumption.

Moles of periodate reduced per C_6 anhydro-unit.



The quantity of periodate reduced is shown in Table XI (graph No. 3; page 99a).

Table XI.

	<u>Periodate consumption</u>				
Time in hour	2	8	12	20	30
Moles of periodate reduced per C ₆ anhydro-unit	0	0.04	0.06	0.08	0.08

After 30 hours the excess periodate was destroyed with ethylene glycol, dialysed extensively (72 hours).

(ii) Reduction - The dialysed solution was concentrated to a suitable volume and reduced with sodium borohydride (400 mg.) and the resulting polyalcohol (A₃) was recovered in the usual way, dried and weighed (350 mg.).

(iii) Mild hydrolysis - The polyalcohol A₃ (300 mg.) was treated with 1N sulphuric acid (20 ml.) at room temperature for 24 hours. The solution was then treated with ethanol (6 vols.) and left overnight at 0°C. The degraded polymer P₃ was removed at the centrifuge, dried and weighed (260 mg.). The supernatant was neutralised, deionised and concentrated to a syrup. Chromatographic examination of the syrup in solvent A revealed the presence of only D-galactose (trace).

Hot hydrolysis of polyalcohol A₃ .

The polyalcohol A₃ (50 mg.) was hydrolysed with 1N sulphu-

ric acid on a boiling water-bath for 10 hours. The cooled hydrolysate was neutralised with barium carbonate, deionised with Amberlite IR 120 (H^+) and IR-45(OH^-) resins, concentrated to a syrup and examined by paper chromatography. It indicated the presence of D-galactose only.

Expt. 34. Examination of the degraded polymer P₃ .

The degraded polymer P₃ (50 mg.) was hydrolysed with 1N sulphuric acid (3 ml.) on a boiling water-bath for 10 hours. The cooled solution was neutralised, deionised, and concentrated to a syrup. Chromatographic examination of the syrup in solvent A (using spray I and dip 2) revealed the presence of D-galactose only.

Expt. 35. Fractionation of the supernatant obtained from mild hydrolysis of polyalcohol A₁.

The supernatant (260 mg.) obtained from the mild hydrolysis of polyalcohol A₁ [Expt. 29 (iii)] was fractionated by repeated separation on filter sheets and the following chromatographically and ionophoretically pure fractions were obtained. .

Fraction I - (20 mg.) had the chromatographic mobility of glycollic aldehyde (R_f 0.71 in solvent A).

Fraction 2 (50 mg.) had the chromatographic mobility of glycerol in solvent A, R_f 0.43 in solvent A ; lit¹⁹⁴ R_f 0.44. This was characterised as the crystalline tri-p-nitrobenzoate which had m.p. 187 - 189°.

Fraction 3 (20 mg.) had the chromatographic mobility of D-threitol, R_f 0.30 in solvent A; lit ¹⁹⁴ 0.30 .

Fraction 4 (20 mg.) had the chromatographic mobility of D-mannose.

Fraction 5 (30 mg.) had the chromatographic mobility of D-galactose.

Fraction 6 (60 mg.) was a chromatographically pure compound which had DP 2; $R_{Galactose}$ 0.58 in solvent A; on hydrolysis it gave D-galactose and D-mannose. Hydrolysis after reduction gave galactose and mannitol. Methylation followed by methanolysis, hydrolysis and paper chromatographic analysis in solvent G (spray I) revealed spots corresponding to 2,3,4,6-tetra-O-methyl-D-galactose and 2,3,6-tri-O-methyl-D-mannose. All these facts indicated that the fraction was

O-D-galactopyranosyl (1→4)-D-mannopyranose.

DISCUSSION

Previously¹⁵⁴ a water-soluble polysaccharide is reported to be isolated from the aqueous extract of ethanol (95%)-extractive-free fruits of Dillenia indica. It gave L-arabinose, D-galactose and D-galacturonic acid on hydrolysis.

In the present investigation a neutral water-soluble polysaccharide, called polysaccharide A, was isolated from the ~~ethanol-extracted~~ mucilaginous inner core of the fruits by aqueous extraction (Expt. 4). The polysaccharide A was subjected to several fractionation processes (Expt. 13) which demonstrated that it was homogeneous in character. It was hydrolysed. The hydrolysate was separated on filter sheets and the constituent sugars were estimated following G.P. 9. Their percentage proportion is shown below:

L-arabinose -	20%
D-galactose -	55%
D-mannose -	18%

The polysaccharide A was initially methylated by Haworth method. After five consecutive methylations (Expt. 14) the methylated product was found to have a methoxyl content of 37.5%. This was then subjected to further methylation by Purdie method which afforded a product having a methoxyl content of 38.9%. Further methylation of the product by modified Kuhn method and Hakomori method failed to increase the methoxyl content (Expt. 15).

The purified methylated polysaccharide (methoxyl content, 38.9%) was methanolysed, hydrolysed and the hydrolysate was repeatedly separated on filter sheets. (Expt. 18). The methylated sugars obtained are listed in Table XII showing their respective percentage proportion.

Table XII

Hydrolytic cleavage fragments from the methylated polysaccharide A.

2,3,5-tri-O-methyl-L-arabinose	(<u>ca</u> 7%)
2,3,4,6-tetra-O-methyl-D-galactose	(<u>ca</u> 13%)
2,3,6-tri-O-methyl-D-galactose	(<u>ca</u> 9%)
2,4,6-tri-O-methyl-D-galactose	(<u>ca</u> 19%)
2,3-di-O-methyl-L-arabinose	(<u>ca</u> 9%)
2,6-di-O-methyl-D-mannose	(<u>ca</u> 16%)
2,4-di-O-methyl-D-galactose	(<u>ca</u> 14%)
2-O-methyl-D-galactose	(<u>ca</u> 10%)

The quantitative analysis of the mixture of sugars was carried out by the chromatographic separation on filter sheets, followed by extraction of the fractions which were freed from solvent and weighed. Such a process is extremely difficult when applied to a mixture of sugars. So the percentage given should be considered as approximations. However the percentage proportions of sugars, namely 2,3,4,6-tetra-O-methyl-D-galactose; 2,3,6-tri-O-methyl-D-galactose and 2,4,6-tri-O-methyl-D-galactose, as calculated from the peak areas of these sugars in the

Gas-liquid chromatogram of the methanolysed methylated polysaccharide A, lay close to the values as calculated from extraction processes.

The sugars in Table XII were characterised by their chromatographic mobilities, the formation of crystalline derivatives, specific rotations and by demethylation.

The methanolysis products of the methylated polysaccharide showed, when examined by Gas-liquid partition chromatography, the presence of the methyl glycosides of

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

On the assumption that the methylation of the polysaccharide A was complete, the location of the hydroxyl groups in the eight cleavage fragments of its methylated derivative indicates the positions through which the monosaccharide units were engaged in union with other residues.

The existence of 2,3,5-tri-O-methyl-L-arabinose (7%) in the hydrolytic products and of the methyl glycosides of this sugar in the methanolysis products (identified by GLC) of the methylated polysaccharide A demonstrates that these L-arabinose

residues exist in the furanose form and are linked at C-1 position. Similarly the identification of 2,3,4,6-tetra-O-methyl-D-galactose indicates that these residues are in the pyranose form and linked at C-1 position. In other words, these L-arbofuranose and D-galactopyranose residues constitute the end groups in the average repeating units of the molecular complex. The ease with which the L-arabinose residues were eliminated from the basic nucleus of the polysaccharide A during the course of different hydrolyses supports strongly the furanose character of this sugar moiety. Therefore, identification of 2,3-di-O-methyl-L-arabinose in the hydrolytic products shows that this unit is interposed through its C-1 and C-5 between two other units in the molecule. Similarly, the existence of 2,3,4-tri-O-methyl-D-galactose in the methanolysis products shows that this unit is linked to two other units through C-1 and C-6. The characterisation of 2,4-di-O-methyl-D-galactose and 2-O-methyl-D-galactose shows in the same way the involvement of C-1, C-3, C-6 of the former and C-1, C-3, C-4, C-6 of the latter in union with other units in the molecule.

The identification of the cleavage fragments in the hydrolytic products of the methylated polysaccharide A has provided evidence for the presence of the structural units as shown in Table XIII, (page 107).

Table XIII

The identity

Structural units of the polysaccharide A.

Gal _p I-;	-4Gal _p I-;	-3Gal _p I-;	-6Gal _p I-;
	1 6		
-3Gal _p I-;	-4Gal _p I-;		
6 1	3 1		
Ara _f I-;	-5Ara _f I-;		
-4Man _p I-			
3 1			
Gal _p = D-galactopyranose			
Ara _f = L-arabofuranose			
Man _p = D-mannopyranose			

The hydrolytic cleavage fragments of the methylated polysaccharide A as shown in Table XII shows that all the D-galactose and D-mannose residues have the pyranose structure and L-arabinose residues have the furanose structure.

The existence of 2-O-methyl-D-galactose, 2,4-di-O-methyl-D-galactose, 2,6-di-O-methyl-D-mannose reveals the highly branched nature of the polysaccharide A. It is no exception, rather it forms the general pattern of water-soluble polysaccharides which are reported to be highly branched. It may be seen that some of the D-galactopyranose residues and all the D-mannopyranose residues are highly linked; the L-arabofuranose and D-galacto-

pyranose residues constitute end groups and the D-galactopyranose residues occur in a variety of combinations, namely, 1,3 - , 1,4 - and 1,6 - linkages, while the D-mannopyranose residues are present as 1,3,4-linked units.

Further evidence concerning the structure of the polysaccharide A came from its partial hydrolysis studies. Polysaccharide A, when refluxed with 0.1N oxalic acid for 4 hours underwent graded hydrolysis and yielded a degraded polymeric material D₁, L-arabinose, D-galactose, D-mannose (trace) and three disaccharides (Expt. 20).

The polymeric material D₁ was completely methylated and hydrolysed with 90% formic acid. The cleavage fragments of the methylated degraded polysaccharide D₁ were identified and characterised (Expt. 26). They are shown in Table XIV. For convenience of comparison, the hydrolytic cleavage fragments of the methylated polysaccharide A are also given in the same table, (page 109)

By comparison, it may be seen that while 2,3,5-tri-O-methyl-L-arabinose, 2,3-di-O-methyl-L-arabinose, 2-O-methyl-D-galactose, and 2,6-di-O-methyl-D-mannose did not occur amongst the cleavage products of the methylated polymer D₁, two new products, 2,3,6-tri-O-methyl-D-mannose and 2,6-di-O-methyl-D-galactose, appeared and these two sugars did not occur amongst the cleavage products of the methylated original polysaccharide A. The formation of these two new products took place due to the methylation of the

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Table XIVHydrolytic cleavage fragments from methylated polysaccharide A and methylated degraded polysaccharide D₁

Methylated Polysaccharide A		Methylated degraded Polysaccharide D ₁
(1) 2,3,5-tri-O-methyl-L-arabinose	:	
(2) 2,3-di-O-methyl-L-arabinose	:	
(3) 2,3,4,6-tetra-O-methyl-D-galactose	:	(1) 2,3,4,6-tetra-O-methyl-D-galactose
(4) 2,3,6-tri-O-methyl-D-galactose	:	(2) 2,3,6-tri-O-methyl-D-mannose
(5) 2,4,6-tri-O-methyl-D-galactose	:	(3) 2,4,6-tri-O-methyl-D-galactose
(6) 2,6-di-O-methyl-D-mannose	:	(4) 2,6-di-O-methyl-D-galactose
(7) 2,4-di-O-methyl-D-galactose	:	(5) 2,4-di-O-methyl-D-galactose
(8) 2-O-methyl-D-galactose	:	

newly formed hydroxyl groups at C-3 of 2,6-di-O-methyl-D-mannopyranose residues and at C-6 of the 2-O-methyl-D-galactopyranose residues. Remembering that the locations of the hydroxyl groups in the cleavage fragments of the methylated degraded polymer D₁ indicated the positions through which these units were involved in the union with other units, it may be inferred that the hydroxyl groups at C-3 of the D-mannopyranose units and at C-6 of the galactopyranose units arose due to the cleavage of the side

chains attached to these positions, i.e. the side chains were attached to the C-3 position of the D-mannopyranose units and to the C-6 position of the galactopyranose units.

During the partial hydrolysis of polysaccharide A, polymeric material D_1 , alongwith L-arabinose, D-galactose, formed amongst others, (Expt. 20) in the accompanying hydrolysate. This indicates that L-arabinose units and D-galactose units were being removed while the original polysaccharide A was gradually converted into the degraded polymer D_1 . In other words, it may be logically inferred that the L-arabofuranose residues or the D-galactopyranose residues or the side chains comprised of either or both of these monosaccharide units were linked at the C-3 of the D-mannopyranose units and/or at the C-6 of the D-galactopyranose units in the original polysaccharide A.

Furthermore the presence of 2,6-di-O-methyl-D-galactose and 2,4-di-O-methyl-D-galactose in the cleavage fragments of the methylated degraded polysaccharide D_1 reveals that this degraded polysaccharide is still branched, but to a much smaller extent than the original polysaccharide.

The polymeric material D_1 , on acid hydrolysis, gave D-galactose and D-mannose only, lending further support to the fact that the L-arabinose residues were removed during the hydrolytic cleavage with 0.1N oxalic acid.

During partial hydrolysis of polysaccharide A with 0.1N

oxalic acid (Expt. 20) three disaccharides (1-3) alongwith the polymeric material D₁, L-arabinose and D-galactose formed.

The disaccharide which had the highest chromatographic mobility in solvent B, on hydrolysis, gave D-galactose only. Methylation followed by methanolysis, hydrolysis and paper chromatographic analysis, showed the presence of 2,3,4,6-tetra-O-methyl-D-galactose and 2,3,6-tri-O-methyl-D-galactose. These results indicated that the disaccharide was

O-D-galactopyranosyl (1→4)-D-galactopyranose

The formation of this disaccharide confirms that some of D-galactopyranose units occur as 1,4-linked residues.

The second disaccharide, on hydrolysis, also gave D-galactose only. Methylation followed by methanolysis; hydrolysis and paper chromatographic analysis revealed spots corresponding to 2,3,4,6-tetra-O-methyl-D-galactose and 2,4,6-tri-O-methyl-D-galactose. From all these results it was evident that the disaccharide was

O-D-galactopyranosyl (1→3)-D-galactopyranose.

The formation of high proportion of this sugar (Expt. 21) signifies that 1,3-linked D-galactopyranose residues constitute the main structural feature of the macromolecule.

The third disaccharide which had the lowest chromatographic mobility in solvent B, on hydrolysis, also gave D-galactose only. This was subjected to methylation, methanolysis and hydrolysis.

Paper chromatographic examination of the hydrolysate revealed the presence of 2,3,4,6-tetra-O-methyl-D-galactose and 2,3,4-tri-O-methyl-D-galactose. Thus it was indicated that the disaccharide was

O-D-galactopyranosyl (1→6)-D-galactopyranose.

The formation of this disaccharide reveals that D-galactopyranose residues are also involved in 1,6-linkages.

The identification of the above three disaccharides thus proves conclusively the existence of 1,3-; 1,4- and 1,6-linked D-galactopyranose residues in the polysaccharide A.

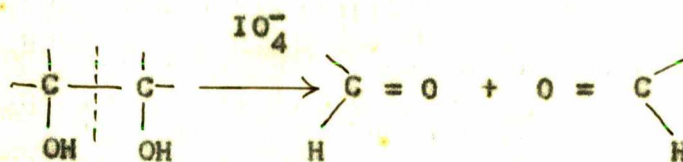
Further hydrolysis of degraded polysaccharide D₁ with 0.1N oxalic acid for additional four hours afforded a second degraded polysaccharide D₂ and a disaccharide, characterised as O-D-galactopyranosyl (1→3)-D-galactopyranose. The complete hydrolysis of degraded polysaccharide D₂ with 1N sulphuric acid gave D-galactose only.

The existence of only D-galactose in the last degraded polymer D₂ reveals that the inner relatively acid-resistant core of the macromolecule consist of D-galactopyranose units to which the acid-labile arabofuranose and other residues are attached.

Although the results of methylation and partial hydrolysis give the major linkages of the individual sugars, they give little idea, apart from those which occur as end groups, of the

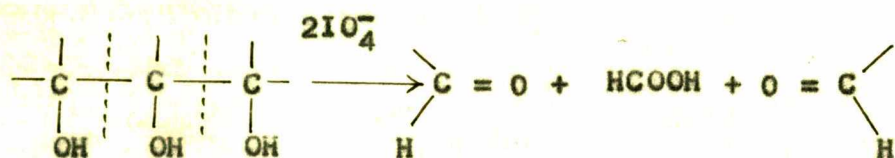
overall structure of the macromolecule. Further evidence concerning the highly ramified outer-chains of L-arabofuranose and D-galactopyranose residues in the polysaccharide and the evidence of outer-chains of D-mannopyranose units in the degraded polymer D₁ and an indication of the way in which some of these chains are attached to the inner core of D-galactopyranose residues was obtained by degrading the polysaccharide A in a step-wise manner by removing those sugar residues which were attacked by periodate in the procedure of Smith and his co-workers.¹⁹⁵

Smith degradation is carried out in three stages: Oxidation with periodate, reduction and mild hydrolysis. When oxidation is carried out in carefully controlled conditions, measurement of the periodate uptake and identification of the cleavage fragments gives valuable structural information on polysaccharides. Whenever $\alpha\beta$ -diol systems occur in constituent sugar residues, oxidation with sodium metaperiodate causes cleavage of the carbon-carbon bond with resultant conversion to two dialdehyde groups and consumption of one molecular proportion of periodate.



In the case of $\alpha\beta\gamma$ -triols, a double cleavage of the carbon chain occurs with the formation of two aldehydic groups and liberation of one molecular proportion of formic acid, the process involving the consumption of two molecular proportion of

periodate. Consequently non-reducing terminal units in a polysaccharide or (1→6)-linked nonterminal units having three adjacent hydroxyl groups will be cleaved by two molecular proportions of periodate to give one molecular proportion of formic acid



Nonterminal units joined by (1→2) or (1→4) bonds undergo cleavage by one molecular proportion of periodate, but no formic acid is generated. Units which do not possess adjacent hydroxyl groups such as nonterminal units joined by (1→3) bonds or units involved in branching at C-2 and C-4 are not affected by periodate. The residue at the reducing end of a polysaccharide having hexose residues solely linked through C-1 and C-6 is attacked by periodate with the liberation in the first instance of a mole of formic acid and the formation of a formyl ester grouping on C-1. This is hydrolysed if the reaction is allowed to proceed and a mole of formic acid together with a mole of formaldehyde is liberated by further cleavage of the carbon-carbon links.

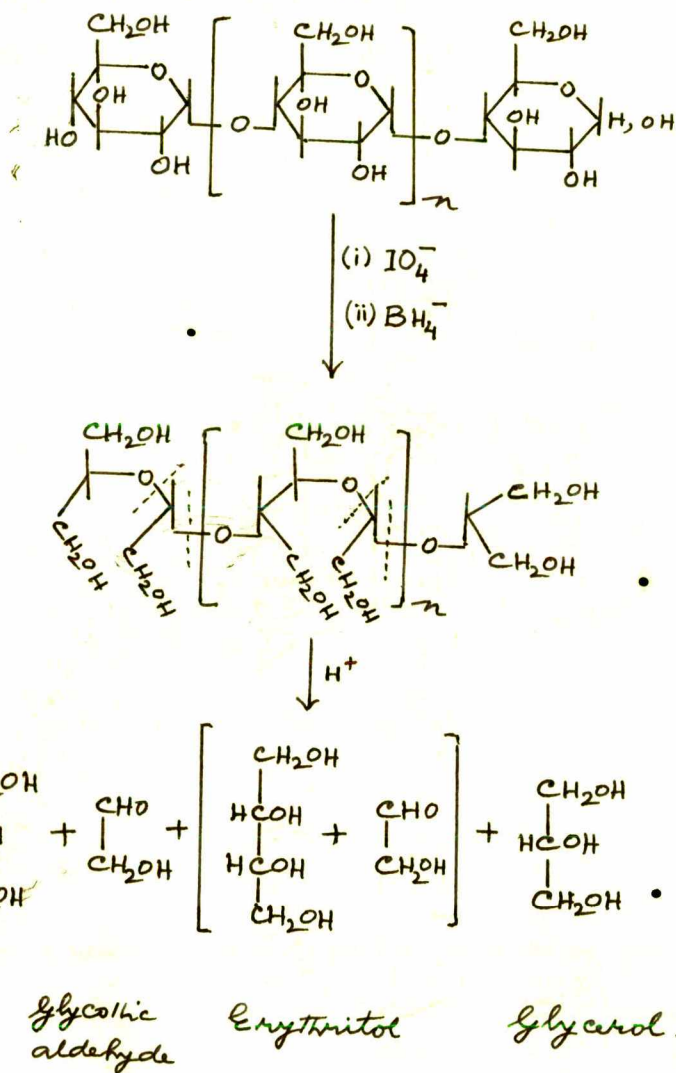
The presence of branch points in 1,2 - or 1,4-linked linear polysaccharides is also revealed by the action of periodate. The residues involved in linkage to the branches, if devoid of α-glycol groups, will be immune to periodate attack and will appear as nonosaccharide units in an acid hydrolysate of the completely oxidised polysaccharide (oxo-polysaccharide).

Acid hydrolysis of the polyaldehyde, obtained from periodate oxidation of a polysaccharide, gives cleavage fragments diagnostic of the type of units and linkage present in the polymer molecule. The acid hydrolysis of a polyaldehyde, however, has some drawbacks. First, some of the products are unstable in the acidic conditions used during hydrolysis and secondly, in some cases, intra- and intermolecular acetal formation between free aldehydic and hydroxyl groups may occur which hinders complete oxidation. To overcome these problems the polyaldehyde is converted in situ to the polyalcohol by reduction.¹⁹⁶ Mild hydrolysis of the polyalcohol may furnish further information about the structure. The mild hydrolysis will cause hydrolysis of the acetal linkages leaving the glycosidic linkages intact.¹⁹⁵

Thus in the case of oxo-polysaccharides derived from α -1,4-linked glucans the products from the non-reducing end are glycerol and glycollic aldehyde, from the reducing end glycerol only, and intermediate units give glycollic aldehyde and erythritol, (chart I; page 116).

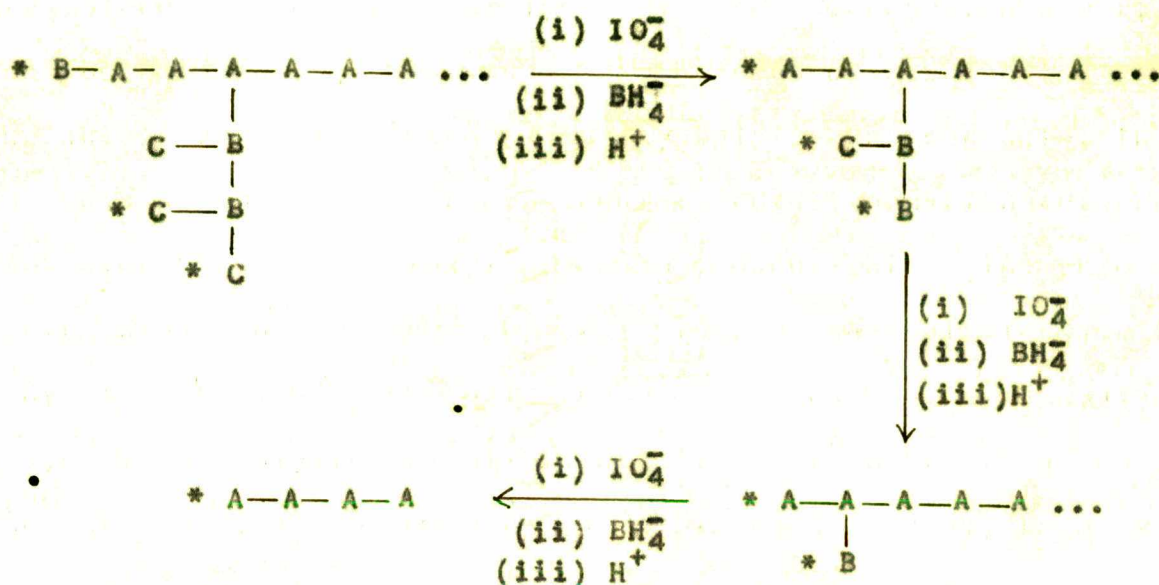
The sequential periodate oxidation, reduction and mild hydrolysis (Smith degradation) of a polysaccharide removes the residues in the molecule which contain α -glycol groups and new vicinal hydroxyl groups are exposed in the residual polymer. Repeated Smith degradation of this material will gradually erode the molecule down to a core which is immune to periodate

CHART I



or products where no surviving sugar residues are present,
(chart 2).

Chart 2.



*Unless linked at C-3 these residues are vulnerable to periodate.

The system above shows that it is possible under the conditions which avoid over oxidation, for all the A core to be revealed as long as 'in chain' A residues are not attacked by periodate.

In the present investigation the polysaccharide A was oxidised with periodate in the presence and in the absence of acetate buffer, (Expts. 27 & 28). The periodate uptake was 0.26 moles per C₆ anhydro-unit with buffer and it was 0.35 moles per C₆ anhydro-unit without buffer, and the yield of polyalcohol was 22% with the buffer and 90% without buffer. There appeared to be no over-oxidation as evident from the periodate oxidation graph, (graph No. 3; page 99a). Because of this high yield of

polyalcohol, the periodate oxidation was carried out without buffer.

The periodate oxidation of polysaccharide A, followed by reduction of the resulting polyaldehyde with sodium borohydride afforded polyalcohol A₁ which, on mild hydrolysis, gave a degraded polymer P₁ and glycerol (major), threitol, glycollic aldehyde, D-galactose D-mannose and a disaccharide identified as O-D-galactopyranosyl (1→4)-D-mannopyranose, (Expt. No. 35).

Glycerol and glycollic aldehyde may be regarded to have been derived from terminal L-arabofuranose residues and terminal D-galactopyranose and reducing D-galactopyranose residues, threitol and glycollic aldehyde from nonreducing, non-terminal D-galactopyranose units. D-galactose and D-mannose arose from the units that resisted the periodate oxidation, but cleaved during hydrolysis. That means these units came from such D-mannose and D-galactose units which were immune to periodate attack. Such structural units would be -3Galp1- , -3Galp1- , -4Galp1- and -4Manp1- where there exists no vicinal hydroxyl groups. It may be seen that the presence of these groups were suggested from the examination of the hydrolysis products of methylated polysaccharide A (Table XIII). The Smith degradation studies thus offered further support to the existence of these groups in the polymer.

The identification of the disaccharide provides unequivocal evidence for the presence of D-galactopyranose units linked gly-

cosidically to the C-4 of D-mannopyranose residues in the parent polysaccharide. Further the degraded polymer P₁ on hydrolysis, gave D-galactose, D-mannose and no L-arabinose demonstrating that L-arabinose residues were removed during the Smith degradation of polysaccharide A.

The degraded polymer P₁, on further periodate oxidation followed by reduction, gave another polyalcohol A₂ in 93% yield. This, on mild hydrolysis, afforded another degraded polymer P₂ and glycerol, threitol, erythritol, glycollic aldehyde, D-galactose and smaller amounts of two unidentified components having R_D Galactose 0.61 and 0.20 respectively in solvent A (Expt. 31). The formation of these products resulted from further degradation of the outer layers of sugar residues viz D-galactose and D-mannose. The degraded polymer P₂, on hydrolysis, gave D-galactose only (Expt. 32) showing that the D-mannose residues were removed during the Smith degradation of polymer P₁.

The degraded polymer P₂, on further periodate treatment, reduced negligible amount of periodate (0.08 moles of periodate per anhydro hexose unit). The periodate oxidised product (oxopolysaccharide) was reduced and the polyalcohol A₃ was obtained in 96% yield. The polyalcohol A₃, on mild hydrolysis, yielded another degraded polymer P₃ and no fragments of smaller molecular weight (Expt. 33). This shows that the outer layers of sugar residues were removed by previous treatments and the degraded polymer P₂ contained no or negligible sugar moiety as side chains

which are vulnerable to periodate attack.

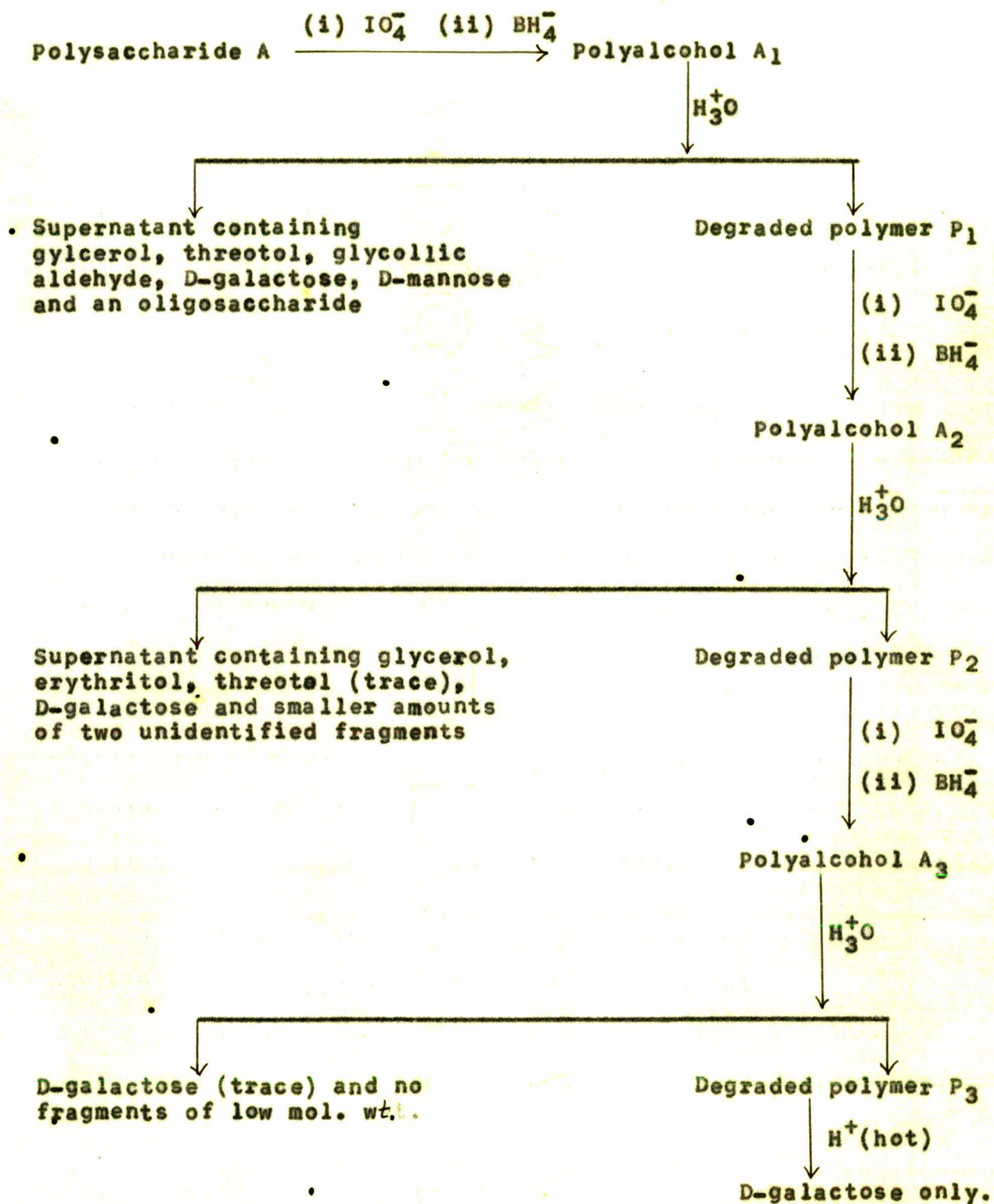
The degraded polymer P_3 , on hydrolysis, gave D-galactose only. This means that the polymer P_3 is composed of only D-galactose units which are immune to periodate attack. The scheme of Smith degradations can be found in chart 3 (page 121).

From the methylation studies of polysaccharide A it appeared that $-4\text{Man}_{\text{pl}}-$ residues were present and its positions C-1, C-3 and C-4 were linked to side-chains (Table XIII). The isolation of O-D-galactopyranosyl (1 $\rightarrow$ 4)-D-mannopyranose during Smith degradation of polysaccharide A (Expt. 28) confirmed the involvement of C-4 of $-4\text{Man}_{\text{pl}}-$ residues in linkage with D-galactose units. The polymeric material P_1 , obtained by Smith degradation of polysaccharide A (Expt. 28), on hydrolysis, gave D-galactose and D-mannose. That means the D-mannose residues were not attacked by periodate in the Smith degradation of polysaccharide A. In other words the C-1 and C-3 positions of D-mannose units were initially linked to other units or chains in the original polysaccharide. Had it not been so, the -OH groups would arise at C-1 and/or C-3 positions and the resulting 1,2-glycols and/or 1,2,3-glycols would have been oxidised. Thus the periodate oxidation lent further support to the existence of $-4\text{Man}_{\text{pl}}-$ residues in the polysaccharide A.

It is interesting to examine the results of Smith degradations. In the first Smith degradation all the L-arabinose units

Chart - 3

Smith Degradations



and some D-galactose units were removed. In the second Smith degradation all the D-mannose residues and still some more D-galactose residues were released. This shows that the erosion of the polysaccharide molecule took place gradually, exposing more new vicinal hydroxyl groups to be attacked by periodate. The repeated formation of fragments of smaller molecular weight during the mild hydrolysis of polyalcohol A₁ and A₂ further lent support to this phenomenon. This process of gradual erosion continued till a skeleton immune to periodate was reached. The degraded polymer P₂, being almost immune to periodate attack, appears to have such a structure. Such a periodate resistant structure would be possible only if it is composed of 1,3-linked D-galactopyranose residues and therefore, the skeleton structure of the polysaccharide A appears to be



On the basis of all the above results, the following structural character of the polysaccharide A may be suggested.

- (1) In view of the homogeneity of the polysaccharide A as evidenced from fractionation studies, it appears that the polysaccharide is composed of L-arabinose, D-galactose and D-mannose residues.
- (2) The polysaccharide contains main chains of 1,3-linked D-galactopyranose residues to which other residues are attached.
- (3) L-arabofuranose and D-galactopyranose residues constitute

end groups. 1,5-linked L-arabofuranose, 1,4-linked D-galactopyranose, 1,6-linked galactopyranose and 1,3,4-linked D-mannopyranose residues exist as side chains.

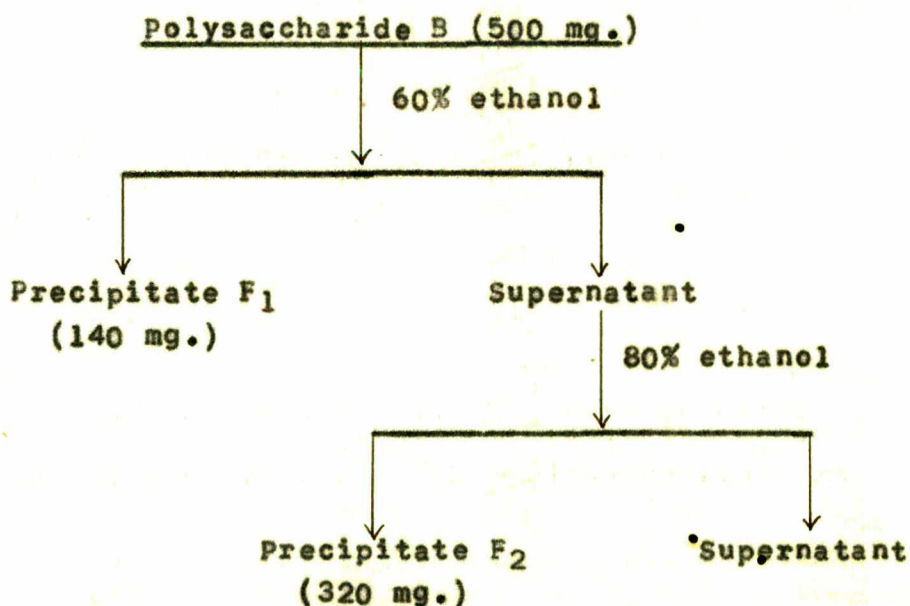
STUDY OF POLYSACCHARIDE B

Expt. 36. Attempted fractionation of polysaccharide B.

Fractionation of the polysaccharide B was attempted with the following conventional techniques.

(a) Fractionation by precipitation with ethanol.

The polysaccharide B (500 mg.) was dissolved in water (100 ml.). A small proportion of undissolved material was removed at the centrifuge. The supernatant was treated with ethanol with stirring as in the scheme below:

Examination of precipitate F₁ and F₂ .

Each of the fractions F₁ and F₂ was hydrolysed with 1N sulphuric acid on a boiling water-bath for 8 hours (G.P.8) and examined chromatographically in solvent A and E (using spray 1 and 3). Spots corresponding to L-arabinose, D-galactose,

D-galacturonic acid and D-mannose (trace) appeared in each case.

(b) By precipitation with Fehling's solution.

This was carried out by following exactly the same procedure as for polysaccharide A [Expt. 15(B)]. The precipitate was dried in a desiccator over anhydrous calcium chloride.

Examination of the precipitate.

The precipitate was hydrolysed with 1N sulphuric acid on a boiling water-bath for 8 hours (G.P.8) and examined chromatographically in solvents A and E (using spray 1 and 3) which revealed spots corresponding to L-arabinose, D-galactose, D-galacturonic acid and D-mannose (trace).

(c) By precipitation with ammonium sulphate.

Attempts to effect fractionation of polysaccharide B by using solid ammonium sulphate [Expt. 15(C)] proved futile.

(d) Fractionation by precipitation with quaternary ammonium salts (cetyl pyridinium chloride). 197

The polysaccharide B (500 mg.) was dissolved in 0.02M solution of sodium sulphate in water (100 ml.). Sodium sulphate was used to increase the rate of aggregation of the precipitate. A 5% solution of cetylpyridinium chloride (CPC) (30 ml.) was added to the polysaccharide solution slowly with stirring,

when a precipitate was formed. The precipitate was allowed to aggregate on standing for 1 hour with slight warming at 40°C , collected by centrifugation and dissolved in 4M potassium chloride solution (25 ml.) with stirring at 40°C . The polysaccharide was precipitated directly as the salt of the cation of the solvent electrolyte, by adding 4 volumes of ethanol. The quaternary ammonium salt (QN^{+}) was left in the supernatant. The process was repeated by dissolving the precipitate in electrolyte solution and precipitating with ethanol to remove last traces of the QN^{+} . This gave a product T_1 (250 mg.).

The supernatant was dialysed extensively (3 days) in running water to remove the inorganic salts, concentrated to a suitable volume and poured into excess ethanol (4 vols) with stirring when a precipitate was obtained. The precipitate was removed at the centrifuge and dried in a desiccator over anhydrous calcium chloride. This gave a product T_2 (200 mg.).

Examination of products T_1 and T_2 .

Each of the substances T_1 and T_2 was hydrolysed with 1N sulphuric acid on a boiling water-bath for 8 hours. The cooled hydrolysates were separately neutralised with barium carbonate, deionised with Amberlite IR 120 (H^{+}) resin, concentrated and examined by paper chromatography in solvent A and E (using spray 1 and 3). Both revealed the presence of L-arabinose, D-galactose, D-galacturonic acid and D-mannose (trace).

(e) Fractionation by DEAE-cellulose column.¹⁹⁸

A column (30 x 4.5 cm.) was made as outlined in G.P. 14.

Polysaccharide B (500 mg.) was dissolved in the minimum quantity of water (50 ml.) and the solution was put on the top of the column very slowly and carefully so that the column remained undisturbed. The column was eluted first with water (1 l.) and then with 0.3M KCl solution (1 l.), 0.5M KCl solution (1 l.) and 1M KCl solution (1 l.) in water successively. Four fractions were collected which were separately dialysed (2 days), concentrated (ca 50 ml.) and poured into ethanol (4 vols) with stirring. No precipitate was obtained in the case of water eluate. The precipitates formed in the three other cases were separated at the centrifuge and dried in a desiccator.

The yields of polysaccharides are given below:

<u>Eluent</u>	<u>Amount of polysaccharide</u>	<u>% uronic anhydride</u>	<u>Polysaccharides designated as</u>
0.3M KCl solution	390 mg.	36.8	L ₁
0.5M KCl "	45 "	37.5	L ₂
1M KCl "	20 "	38.3	L ₃
Total = 455 mg.			

Uronic anhydride in unfractionated polysaccharide B was 34% which was calculated following G.P. 7.

Examination of the samples L₁, L₂ and L₃ .

Each of the samples L₁, L₂ and L₃ was hydrolysed with 1N sulphuric acid on a boiling water-bath for 8 hours (G.P.8). The cooled hydrolysates were separately neutralised, deionised, concentrated and examined by paper chromatography in solvents A and E (using spray I and 3). Each showed the same chromatographic pattern indicating the presence of L-arabinose, D-galactose, D-galacturonic acid and D-mannose (trace).

From all these facts it ^{was} evident that the polysaccharide B was essentially homogeneous.

Expt. 37. Methylation of polysaccharide B.

The polysaccharide B did neither dissolve nor swell in dimethyl sulphoxide and hence could not be methylated by Hakomori method directly. It was, therefore, initially methylated to a partially methylated product by Haworth method and the partially methylated ^{product} was then completely methylated by the Hakomori method.

(a) Partial Methylation by Haworth Method.

The polysaccharide B (15 g.) was dissolved in water (500 ml.). Dimethyl sulphate (200 ml.) and 30% aqueous sodium hydroxide (400 ml.) were then added slowly with stirring during 4 hours to the solution in an atmosphere of nitrogen and stirred overnight. Similar four additions of dimethyl sulphate and 30%

sodium hydroxide were made to the reaction mixture on successive days. The reaction mixture was taken out, excess alkali was neutralised by the addition of 50% aqueous sulphuric acid, dialysed extensively (3 days), concentrated under reduced pressure, dried in a vacuum desiccator over anhydrous calcium chloride and weighed (10.5 g.; Found, methoxylcontent, 22%).

(b) Complete Methylation by modified Hakomori Method.

The partially methylated polysaccharide (5 g.) was dissolved in dimethyl sulphoxide (250 ml.) by stirring overnight under nitrogen atmosphere. Powdered sodium hydride (2 g.) was added in small portions with gentle stirring during 2 hours. The stirring was continued till no more hydrogen was evolved. Methyl iodide (5 ml.) was then added dropwise with stirring during 2 hours. The solution was stirred overnight. A further three additions of sodium hydride and methyl iodide were made to the reaction mixture on successive days in the same way as described for the first addition. The mixture was then poured into water (1.5 l.) to precipitate the methylated product and excess of methyl iodide was removed by aeration with nitrogen. The precipitate was collected at the centrifuge and dissolved in chloroform. The supernatant solution was extracted with chloroform and the extract was combined with the solution of precipitated methylated polysaccharide in chloroform. The chloroform solution was washed repeatedly (7 times) with water to remove dimethyl sulphoxide, dried over anhydrous sodium sulphate and filtered. The filtrate

was concentrated under reduced pressure and then added to light petroleum ether (b.p. 60-80°) when the methylated polysaccharide precipitated. It was removed by centrifugation, dried in a vacuum desiccator over anhydrous calcium chloride and weighed (4.3 g.; Found, methoxyl content, 36.5%).

Expt. 38. Formic acid hydrolysis of the methylated polysaccharide B.

The methylated polysaccharide B (50 mg.) was heated in a sealed tube with 90% formic acid (1 ml.) in an inert atmosphere of carbon dioxide on a boiling water-bath for 5 hours. The tube was then opened and heated for a further hour with addition of water (5 ml.). The hydrolysate was treated with Amberlite IR 120 (H^+) resin and filtered. The filtrate was evaporated to a syrup. Chromatographic examination of the syrup in solvent G (using spray I) indicated the presence of

- (i) 2,3,5-tri-O-methyl-L-arabinose,
- (ii) 2,3,4,6-tetra-O-methyl-D-galactose,
- (iii) 2,5-di-O-methyl-L-arabinose,
- (iv) 2,3,6-tri-O-methyl-D-galactose,
- (v) 2,4,6-tri-O-methyl-D-galactose,
- (vi) 2,4-di-O-methyl-D-galactose,
- (vii) 2,3,4-tri-O-methyl-D-galacturonic acid, and
- (viii) 2-O-methyl-D-galactose.

Expt. 39. Methanolysis and Gas-liquid chromatographic analysis of the methylated polysaccharide B.

The methylated polysaccharide B (50 mg.) was converted into a mixture of methyl glycosides of methylated sugars by boiling with methanolic 3% hydrogen chloride for 10 hours. The cooled solution was neutralised with barium carbonate, deionised with Amberlite IR 120 (H^+) and concentrated to a syrup under reduced pressure. Gas-liquid partition chromatography of the syrup on column a and b (G.P. 20) showed the presence of methyl glycosides of the sugars as shown in Table XV.

Table XV

Relative retention times (T) of methyl glycosides.¹⁸⁷

	<u>Column a</u>	<u>Column b</u>
2,3,5-tri-O-methyl-L-arabinose	0.54, 0.71	0.48, 0.60
2,5-di-O-methyl-L-arabinose	(1.75)	0.70
2,3,4,6-tetra-O-methyl-D-galactose	(1.75)	1.53
2,3,6-tri-O-methyl-D-galactose	3.15, (3.95)	(1.53), 2.03
2,4,6-tri-O-methyl-D-galactose	(3.95), 4.55	(2.03), 2.32
2,3,4-tri-O-methyl-D-galacturonic acid	6.98	(3.95)

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

Table XVIAnalysis of hydrolysate of methylated polysaccharide B.

Frac- tion	wt.(mg.)	$[\alpha]_D^{20}$	<u>Paper chromatography</u>		Sugars given on demethylation
			R_G (solvent G)	Sugar	
1	22	-42°	0.96	2,3,5-tri-0-methyl L-arabinose	L-arabinose
2	43	+118	0.89	2,3,4,6-tetra-0- methyl-D-galactose	D-galactose
3	18	-4°	0.83	2,5-di-0-methyl -L-arabinose	L-arabinose
4	63	+95°	0.72	2,3,6-tri-0-methyl -D-galactose	D-galactose
5	58	+91°	0.68	2,4,6-tri-0-methyl -D-galactose	D-galactose
6	35	+87	0.42	2,4-di-0-methyl -D-galactose	D-galactose
7	40	-	0.30	2,3,4-tri-0-methyl -D-galacturonic acid	D-galacturonic acid
8	23	+84°	0.24	2-0-methyl -D-galactose	D-galactose

Fraction 3. A chromatographically pure syrup (18.mg.) which had the chromatographic mobility of 2,5-di-0-methyl-L-arabinose. (R_G 0.83; lit¹⁸⁹ 0.85). It had $[\alpha]_D^{20} = -4^\circ$ (c 1.7); lit - 2° for 2,5-di-0-methyl-L-arabinose.¹⁸⁹ The derived 2,5-di-0-methyl-L-arabonamide had m.p. 128-130°; lit¹⁸⁹ 126 - 128°. On demethylation it gave L-arabinose.

Fraction 4. A chromatographically pure syrup (63 mg.) which had

the chromatographic mobility of 2,3,6-tri-O-methyl-D-galactose. (R_G 0.72; lit 0.71). It had $[\alpha]_D^{20} + 95^\circ$ (c 1.8); lit $+ 96^\circ$ for 2,3,6-tri-O-methyl-D-galactose. The derived lactone had m.p. 96-98°. On demethylation it gave D-galactose.

Fraction 5. A chromatographically pure syrup (58 mg.) which had R_G 0.68; lit¹⁹⁰ 0.67 for 2,4,6-tri-O-methyl-D-galactose; $[\alpha]_D^{20} + 91$ (c 1.2); lit¹⁸⁸ $+ 90^\circ$. On demethylation it gave D-galactose. The aniline derivative had m.p. 169 - 170°; lit¹⁸⁹ 165 - 166°.

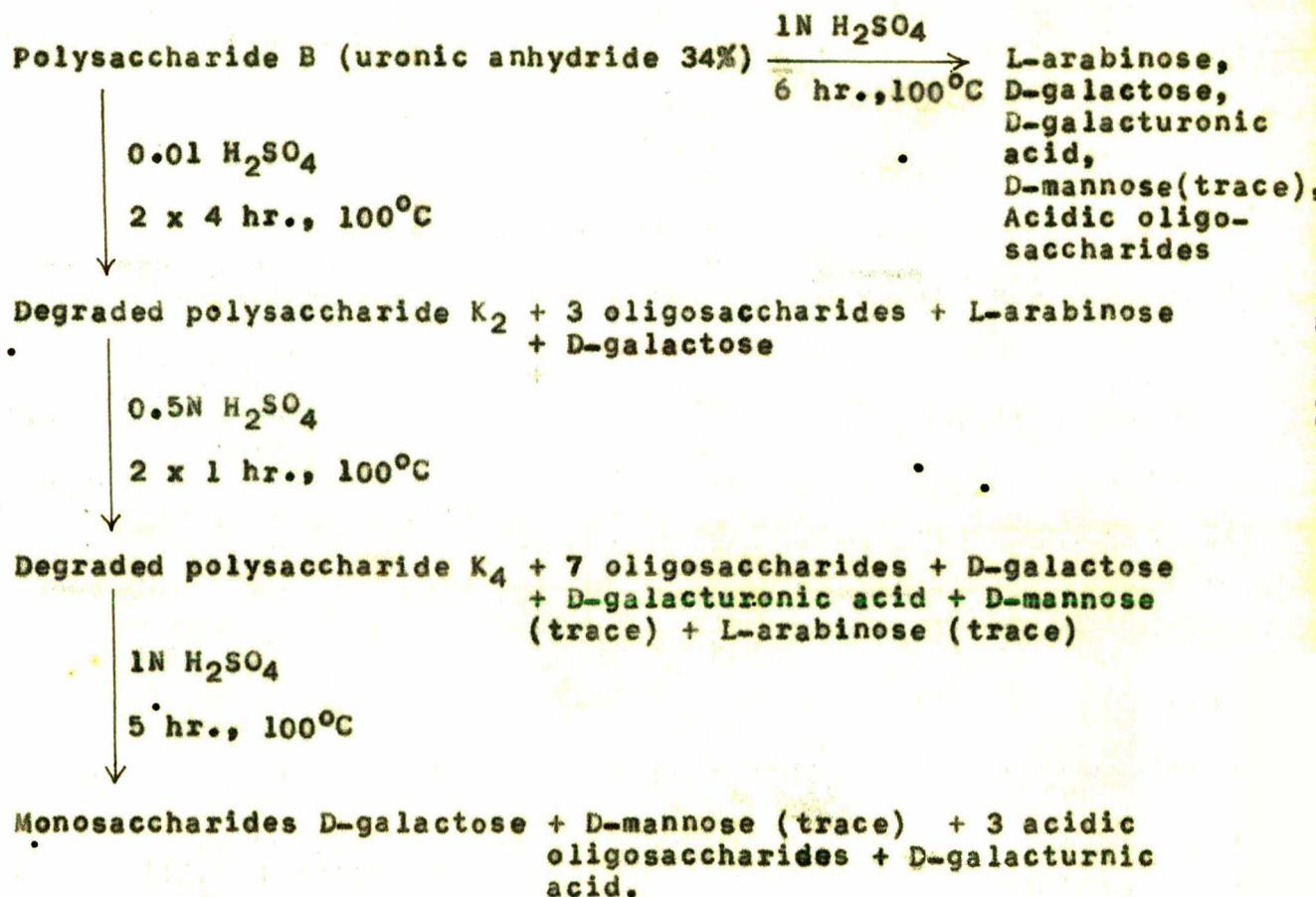
Fraction 6. The sugar (35 mg.) was shown by paper chromatography to be a single compound. It had R_G 0.42; lit¹⁹⁰ 0.41 for 2,4-di-O-methyl-D-galactose. It had $[\alpha]_D^{20} + 124 \rightarrow +87$ (c 1.1); lit $+ 123 \rightarrow +85^\circ$ for 2,4-di-O-methyl-D-galactose. The aniline derivative had m.p. 218 - 220°; lit¹⁹¹ 216 - 217°. On demethylation it gave D-galactose.

Fraction 7. The sugar (40 mg.) was shown by paper chromatography to be a single compound. It had R_G 0.30; lit¹⁸⁸ 0.31 for 2,3,4-tri-O-methyl-D-galacturonic acid. It was converted into methyl ester methyl glycosides, reduced with lithium aluminium hydride in tetrahydrofuran and hydrolysed to furnish a syrup. Chromatographic examination of this syrup showed it to be 2,3,4-tri-O-methyl-D-galactose.

Fraction 8. It was also a chromatographically pure syrup (23 mg.) which had R_G 0.24; lit¹⁹⁰ 0.23 for 2-O-methyl-D-galactose; $[\alpha]_D^{20} + 85^\circ$ (c 1.0); lit¹⁸⁸ $+ 84^\circ$ for 2-O-methyl-D-galactose. On demethylation it gave D-galactose.

Partial hydrolysis of polysaccharide B

In preliminary experiments on partial hydrolysis with sulphuric acid of different strengths under varying conditions many oligosaccharides, both neutral and acidic, were detected as products. Accordingly, graded hydrolysis was carried out in three stages as shown in the flow sheet I and degraded polysaccharides K_2 and K_4 were separated from the soluble sugars after the first and the second stages. At each stage the various oligosaccharides were fractionated by adsorption and/or paper partition chromatography.

Flow Sheet I

Expt. 41. Partial hydrolysis of polysaccharide B with 0.01N sulphuric acid

The polysaccharide B (30 g.) was heated in 0.01N sulphuric acid (1500 ml.) on a boiling water-bath for 4 hours. Insoluble residue (1.2 g.) was removed at the centrifuge. The cooled solution was neutralised with Amberlite IR-45(OH⁻) resin and filtered. The filtrate was concentrated to a suitable volume and poured into ethanol (5 vols.). The precipitated degraded polysaccharide K₁ (22 g.) was separated and the supernatant liquid was concentrated to a syrup (3.5 g.). The degraded polysaccharide K₁, on further hydrolysis under the same conditions, yielded another degraded polysaccharide K₂ (16.5 g.) and a syrup (2 g.). Both the syrups showed the same chromatographic pattern in solvent A. Each showed spots corresponding to L-arabinose, D-galactose, and three oligosaccharides and, therefore, both the syrups were combined (5.5 g.).

Expt. 42. Fractionation of the combined syrup on charcoal-celite column

The combined syrup (5.5 g.) as obtained from Expt. 41 was dissolved in the minimum quantity of water and adsorbed on charcoal-celite column (1:1 ; 40 x 4 cm.) (G.P. 15).

The column was then eluted with water which gave fraction I (4.9 g.) containing L-arabinose and D-galactose.

Elution with water containing 2½% ethanol gave fraction 2

which, on chromatographic analysis in solvent A, showed the presence of three oligosaccharides (1—3) which were isolated as pure entities by repeated separation on filter sheets using solvent A. The elution pattern was followed by testing with phenol-sulphuric acid. [Graph No.4, page 137a]

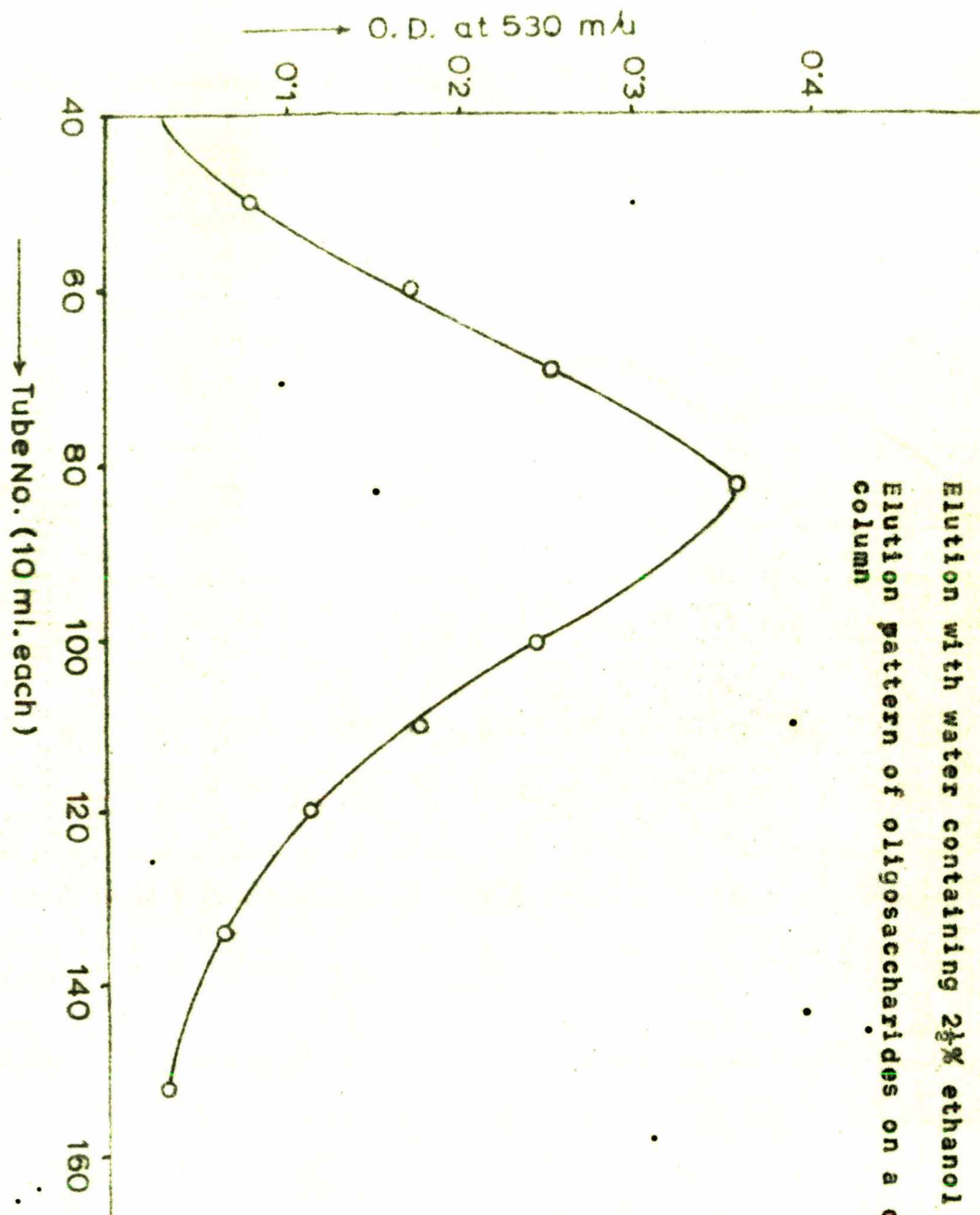
Oligosaccharide 1 (80 mg.) which had DP 2; $R_{\text{Galactose}}$ 1.20 in solvent A. On hydrolysis it gave L-arabinose only. Methylation followed by methanolysis, hydrolysis and paper chromatographic examination in solvent G revealed spots corresponding to 2,3,5-tri-O-methyl-L-arabinose and 2,5-di-O-methyl-L-arabinose. All these facts revealed that the oligosaccharide was
O-L-arabofuranosyl (1→3)-L-arabofuranose.

Oligosaccharide 2 (100 mg.) which had DP 2; $R_{\text{Galactose}}$ 0.40 in solvent A. On hydrolysis it gave D-galactose and L-arabinose. Hydrolysis after reduction gave D-galactose and arabitol indicating that the reducing moiety was L-arabinose. It was subjected to methylation, methanolysis and hydrolysis. Paper chromatographic analysis of the hydrolysate in solvent G showed the presence of 2,3,4,6-tetra-O-methyl-D-galactose and 2,5-di-O-methyl-L-arabinose. All these facts showed that the oligosaccharide was

O-D-galactopyranosyl (1→3)-L-arabofuranose.

Oligosaccharide 3 (160 mg.) which had DP 2; $R_{\text{Galactose}}$ 0.35 in solvent A. On hydrolysis it gave galactose only. Methylation

- 137a -



followed by methanolysis, hydrolysis and paper chromatographic analysis in solvent G revealed spots corresponding to 2,3,4,6-tetra-O-methyl-D-galactose and 2,3,6-tri-O-methyl-D-galactose. From all these facts it was evident that the oligosaccharide was

O-D-galactopyranosyl (1 4)-D-galactopyranose.

Expt. 43 Partial hydrolysis of degraded polysaccharide K₂ with 0.5N sulphuric acid

The degraded polysaccharide K₂ (16 g.), obtained from Expt. 41, was heated with 0.5N sulphuric acid (500 ml.) on a boiling water-bath for 1 hour and the cooled solution was neutralised with barium hydroxide and subsequently with barium carbonate. Inorganic material was removed at the centrifuge; the supernatant was concentrated to a suitable volume and poured into ethanol (6 vols.). The precipitated degraded polysaccharide K₃ was separated by centrifugation and the supernatant liquid was concentrated to a syrup (4.1 g.). The degraded polysaccharide K₃ (8.2 g.) was again hydrolysed under the same conditions and furnished another degraded polysaccharide K₄ (3.6 g.) and a syrup (0.8 g.).

Both the syrups, on examination by paper chromatography in solvent A, showed the same chromatographic pattern and were, therefore, combined. The combined syrups (4.9 g.) dissolved in minimum water were then adsorbed on a charcoal-celite column (1:1, 40 x 4 cm.) [G.P. 15] and were eluted first with water and

then with water containing different proportions of ethanol (2½%, 5% and 10%) successively (stepwise process). The elution patterns were followed by testing with phenol-sulphuric acid.

(1) Elution with water (2 l.) gave fraction I (3.4 g.) containing D-galactose, D-galacturonic acid and traces of D-mannose and L-arabinose.

(2) Elution with water containing 2½% ethanol (1.5 l.) gave fraction 2 (550 mg.) containing oligosaccharide 1, oligosaccharide 2, and oligosaccharide 3. [Graph No.5, page 139a]

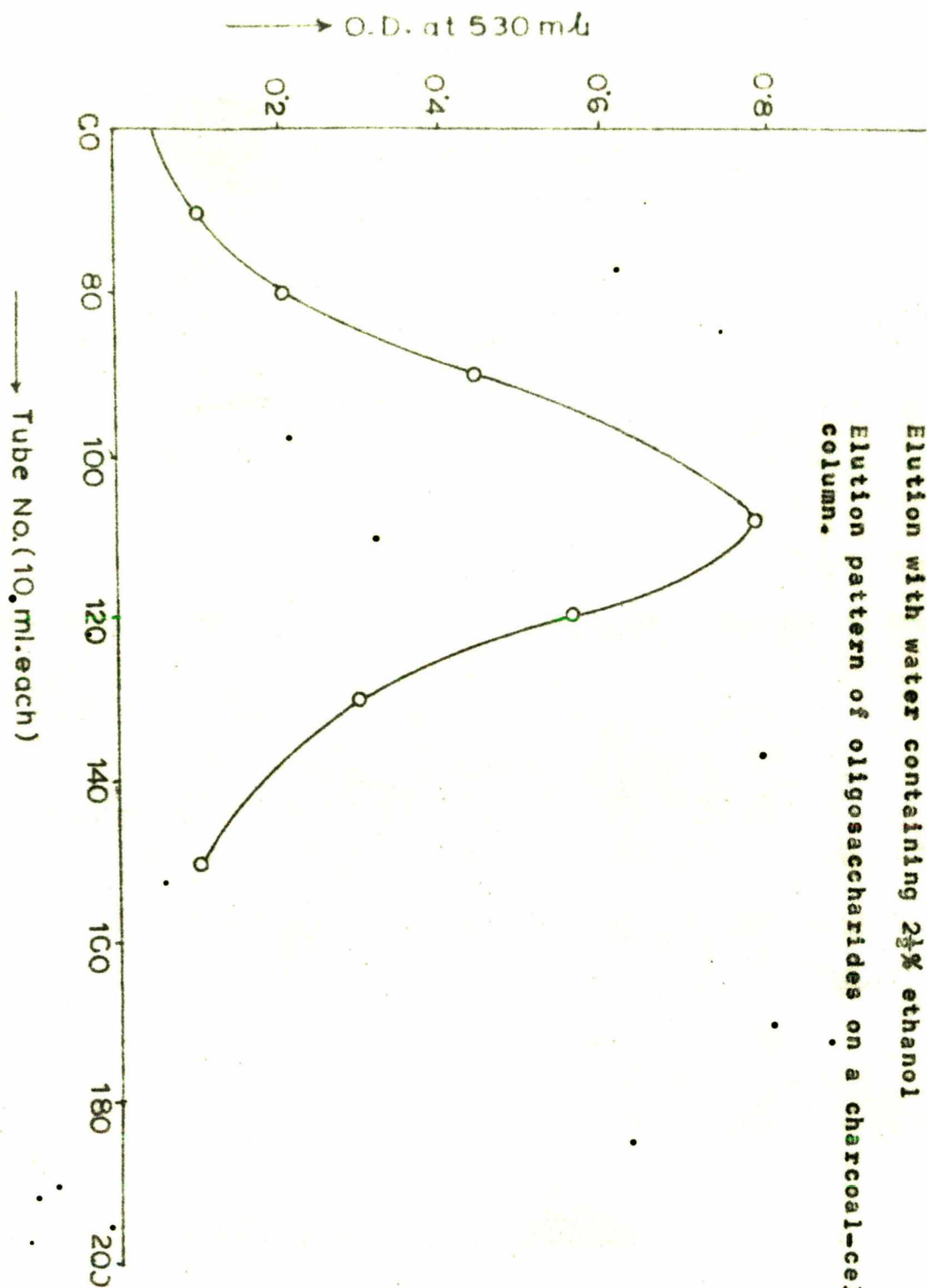
(3) Elution with water containing 5% ethanol (1.5 l.) gave fraction 3 (280 mg.) containing acidic oligosaccharides a and b and neutral oligosaccharide 4. [Graph No.6, page 139b]

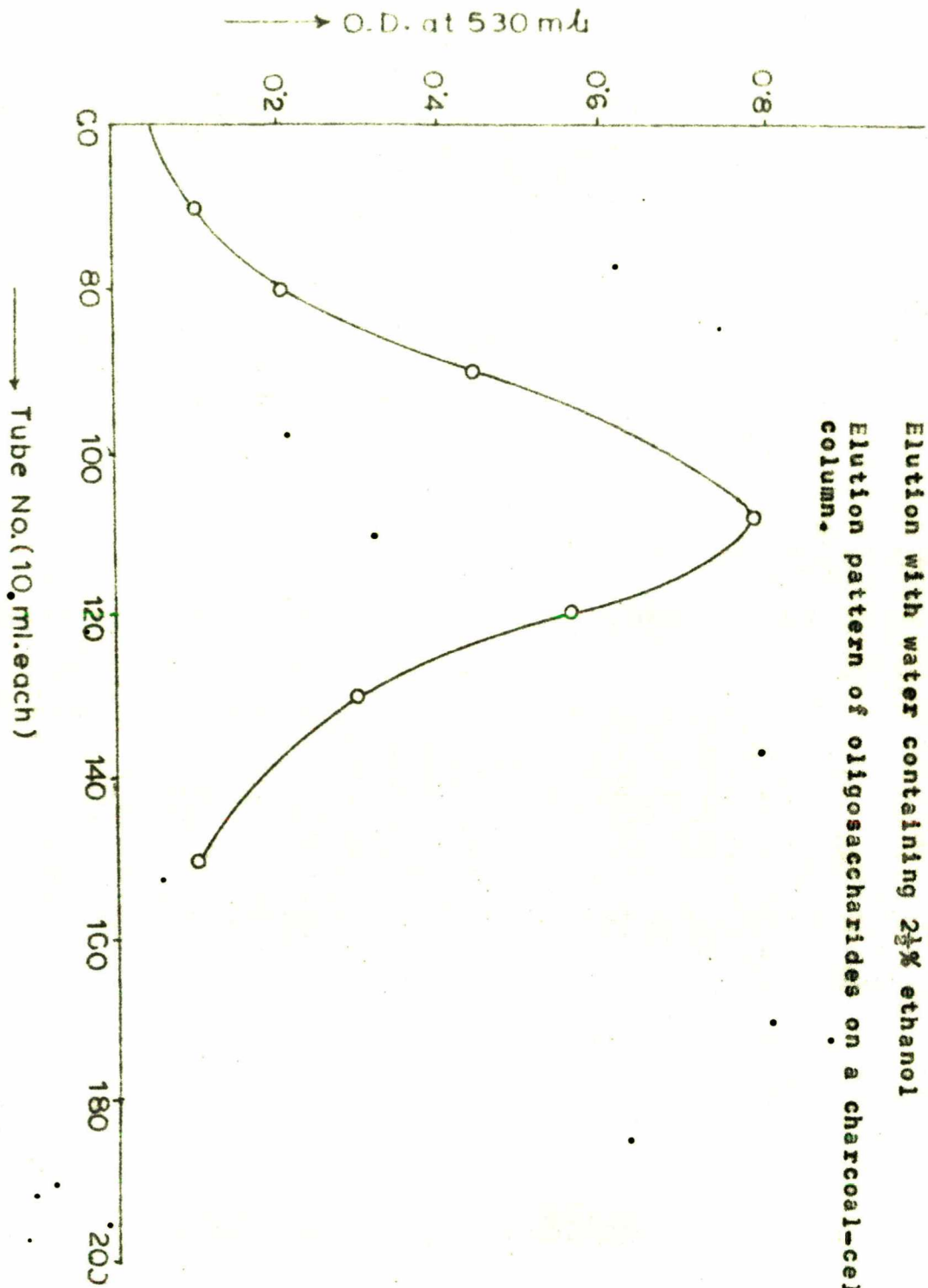
The individual sugars present in fractions 2 and 3 were separated on filter sheets using solvent B.

(4) Elution with water containing 10% ethanol (1.5 l.) gave fraction 4 (70 mg.) containing acidic oligosaccharide c.

Examination of the individual oligosaccharides.

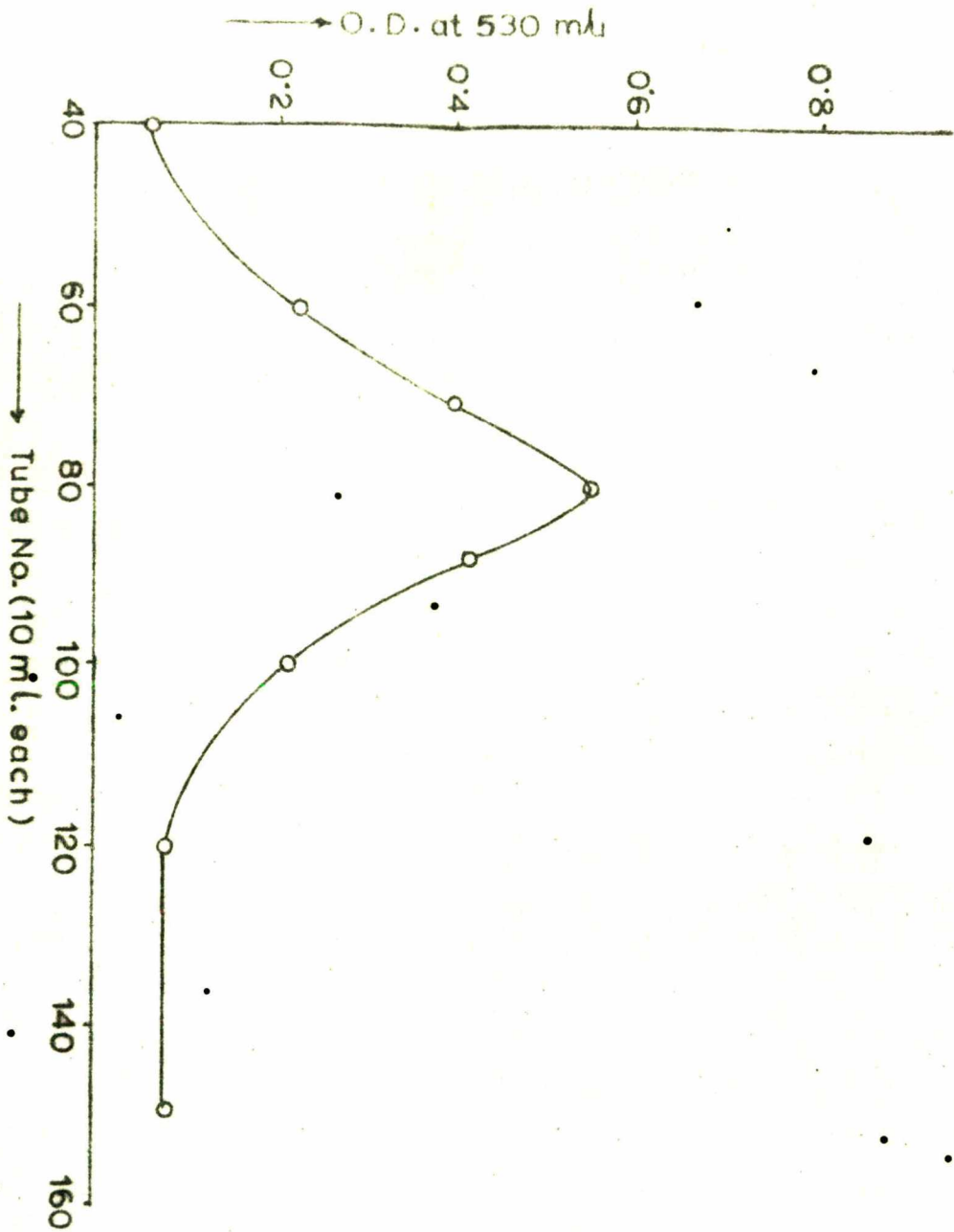
Oligosaccharide I (40 mg.). It had DP 2; R_{Galactose} 0.61 in solvent B. On hydrolysis it gave D-galactose and L-arabinose. Hydrolysis after reduction gave D-galactose and L-arabitol indicating that the reducing moiety of this disaccharide was L-arabinose. It was subjected to methylation, methanolysis and hydrolysis. Paper chromatographic examination of the hydrolysate





Graph No. 6. Fractionation of 0.5N H₂SO₄ hydrolysate of degraded polysaccharide K₂.

Elution with water containing 5% ethanol.



revealed spots corresponding to 2,3,4,6-tetra-O-methyl-D-galactose and 2,5-di-O-methyl-L-arabinose. These facts revealed that the oligosaccharide was O-D-galactopyranosyl (1→3)-L-arabinose.

Oligosaccharide 2 (120 mg.). It had DP 2; $R_{\text{Galactose}}$ 0.55 in solvent B. On hydrolysis it gave D-galactose only. Methylation followed by methanolysis, hydrolysis and paper chromatographic analysis in solvent G revealed spots corresponding to 2,3,4,6-tetra-O-methyl-D-galactose and 2,3,6-tri-O-methyl-D-galactose, indicating that the oligosaccharide was

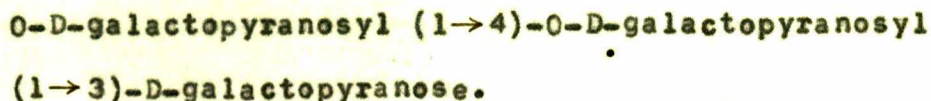
O-D-galactopyranosyl (1→4)-D-galactopyranose.

Oligosaccharide 3 (155 mg.). It had DP 2; $R_{\text{Galactose}}$ 0.47 in solvent B. On hydrolysis it gave D-galactose only. Methylation followed by methanolysis, hydrolysis and paper chromatographic analysis showed the presence of 2,3,4,6-tetra-O-methyl-D-galactose and 2,4,6-tri-O-methyl-D-galactose. These facts revealed that the oligosaccharide was

O-D-galactopyranosyl (1→3)-D-galactopyranose.

Oligosaccharide 4 (40 mg.). It had DP 3, $R_{\text{Galactose}}$ 0.31. On hydrolysis it gave D-galactose only. Partial acid hydrolysis gave D-galactose and O-D-galactopyranosyl (1→3)-D-galactopyranose. It was subjected to methylation, methanolysis and hydrolysis. Paper chromatographic analysis in solvent G revealed spots corresponding to 2,3,4,6-tetra-O-methyl-D-galactose, 2,3,6-tri-

-O-methyl-D-galactose and 2,4,6-tri-O-methyl-D-galactose. Gas-liquid chromatographic analysis also supported the presence of the methyl glycosides of these methylated sugars. All these facts indicated that the oligosaccharide was



Acidic oligosaccharide a (30 mg.). It had DP 2; $R_{\text{Galactose}}$ 0.34, $R_{\text{Gal A}}$ 0.31 in solvent A. On hydrolysis it gave D-mannose and D-galacturonic acid. Reduction of the derived methyl ester methyl glycosides followed by hydrolysis gave D-galactose and D-mannose. Methylation followed by methanolysis, hydrolysis and paper chromatographic examination in solvent G revealed spots corresponding to 2,3,4-tri-O-methyl-D-galacturonic acid and 2,4,6-tri-O-methyl-D-mannose. All these facts revealed that the oligosaccharide was



Acidic oligosaccharide b (75 mg.). It had DP 2; $R_{\text{Galactose}}$ 0.19, $R_{\text{Gal A}}$ 0.16 in solvent A. On hydrolysis it gave D-galactose and D-galacturonic acid. Reduction of the methyl ester methyl glycosides followed by hydrolysis gave D-galactose only. It was subjected methylation, methanolysis and hydrolysis. Paper chromatographic analysis of the hydrolysate in solvent G indicated the presence of 2,3,4-tri-O-methyl-D-galacturonic acid and 2,3,4-tri-O-methyl-D-galactose. Gas-liquid chromatographic analysis also supported the presence of the methyl glycosides of these

methylated sugars. All these facts indicated that the oligosaccharide was

O-D-galactopyranosyluronic acid (1→6)-D-galactopyranose.

Acidic oligosaccharide c (70 mg.). It had DP 4; $R_{\text{Galactose}}$ 0.03 and $R_{\text{Gal A}}$ 0.024 in solvent A. On hydrolysis it gave D-mannose, D-galactose and D-galacturonic acid. Reduction of the methyl ester methyl glycosides followed by hydrolysis gave D-galactose and D-mannose. Methylation followed by methanolysis, hydrolysis and paper chromatographic analysis in solvent G revealed spots corresponding to 2,3,4-tri-O-methyl-D-galacturonic acid, 2,4,6-tri-O-methyl-D-mannose, 2,4,6-tri-O-methyl-D-galactose and 2,3,6-tri-O-methyl-D-galactose. On the basis of these results it was suggested that the oligosaccharide was

O-D-galactopyranosyluronic acid (1→3)-O-D-mannopyranosyl (1→4)-O-D-galactopyranosyl (1→3)-D-galactopyranose.

Expt. 44. Hydrolysis of degraded polysaccharide K₄ with 1N sulphuric acid

The degraded polysaccharide K₄ (3.6 g.), obtained from Expt. 43, was heated with 1N sulphuric acid on a boiling water-bath for 5 hours. The cooled hydrolysate was neutralised with barium hydroxide and subsequently with barium carbonate, deionised with Amberlite resin IR 120 (H⁺) and concentrated to a syrup (2.5 g.). The syrup dissolved in minimum volume of water was adsorbed on a column (35 x 4.5 cm.) of Amberlite resin IR-45(OH⁻) to absorb acids.

Elution with water gave a fraction (1.6 g.) containing monosaccharides, viz, D-galactose, D-galacturonic acid and D-mannose (trace), while elution with water containing 10% formic acid afforded a fraction containing a mixture of ^{acidic} oligosaccharides (0.4 g.). [Graph No. 7, Page 143a]. Chromatographically pure acidic oligosaccharides (1-3) were obtained after repeated separation on filter sheets using solvent A.

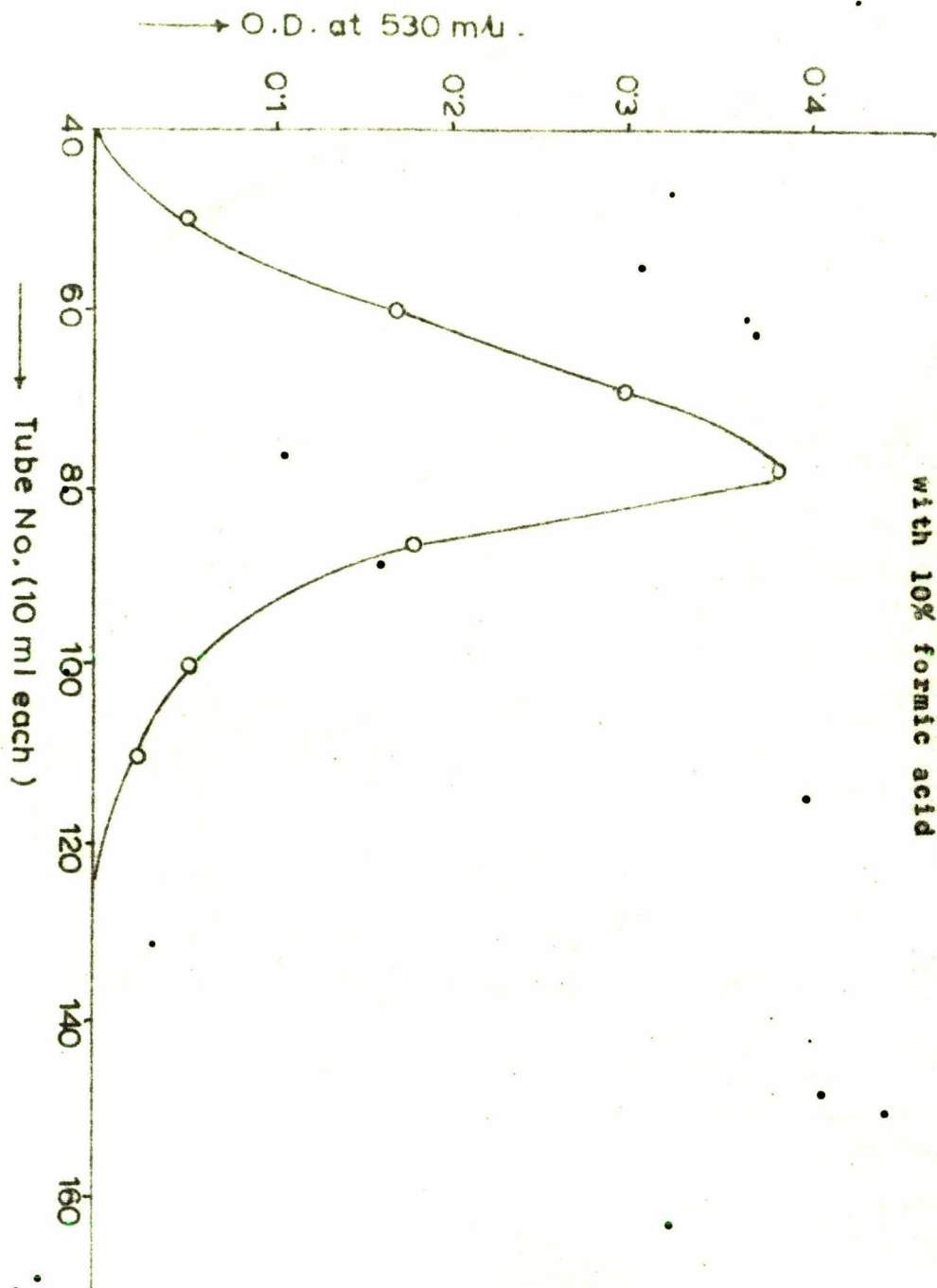
Oligosaccharide 1 (50 mg.). It had DP 2; $R_{\text{Galactose}}$ 0.33 in solvent A. On hydrolysis it gave D-mannose and D-galacturonic acid. Reduction of the methyl ester methyl glycosides followed by hydrolysis gave D-mannose and D-galactose. Methylation followed by methanolysis, hydrolysis and paper chromatographic analysis revealed spots corresponding to 2,4,6-tri-O-methyl-D-mannose and 2,3,4-tri-O-methyl-D-galacturonic acid. All these facts indicated that the oligosaccharide was

O-D-galactopyranosyluronic acid (1 $\rightarrow$ 3)-D-mannopyranose.

Oligosaccharide 2 (180 mg.). It had DP 2; $R_{\text{Galactose}}$ 0.18 in solvent A. On hydrolysis it gave D-galactose and D-galacturonic acid. Reduction of the methyl ester methyl glycosides followed by hydrolysis gave D-galactose only. It was subjected to methylation, methanolysis and hydrolysis. Paper chromatographic examination of the hydrolysate revealed the presence of 2,3,4-tri-O-methyl-D-galactose and 2,3,4-tri-O-methyl-D-galacturonic acid. All these facts indicated that the oligosaccharide was

Graph No. 7. Fraction of 1N sulphuric acid hydrolysate of degraded polysaccharide K₄.

Elution of acidic oligosaccharides from a resin column with 10% formic acid



O-D-galactopyranosyluronic acid (1→6)-D-galactopyranose.

Oligosaccharide 3 (70 mg.). It had DP 3, $R_{\text{Galactose}}$ 0.09 in solvent A. On hydrolysis it gave D-galactose and D-galacturonic acid. Reduction of the methyl ester methyl glycosides followed by hydrolysis gave galactose only. Methylation followed by methanolysis, hydrolysis and paper chromatographic examination revealed the presence of 2,3,4-tri-O-methyl-D-galacturonic acid, 2,3,4-tri-O-methyl-D-galactose and 2,4,6-tri-O-methyl-D-galactose. All these facts indicated that the oligosaccharide was O-D-galactopyranosyluronic acid (1→6)-O-D-galactopyranosyl (1→3)-D-galactopyranose.

Expt. 45. Smith degradation of polysaccharide B.

(1) Periodate oxidation - To an aqueous solution of the polysaccharide B (6.0 g. in 500 ml.) was added an aqueous solution of sodium meta-periodate (0.1M, 500 ml.) and the oxidation was allowed to continue for 40 hours. Aliquots (5 ml.) were taken out at regular intervals and examined for periodate consumption.

The quantity of periodate reduced is shown in Table XVII (graph No. 8 ; page 148a).

Table XVII

	<u>Periodate consumption</u>					
Time in hour	2	5	10	20	30	40
Moles of periodate reduced per C_6 anhydro-unit	0.16	0.4	0.72	1.08	1.2	1.2

After 30 hours the excess periodate was destroyed by the addition of ethylene glycol and the solution dialysed extensively (72 hours).

(ii) Reduction - The dialysed solution was concentrated to a suitable volume and reduced with sodium borohydride (3.0 g.) and the resulting polyalcohol B₁ was recovered, dried and weighed (5.8 g.).

(iii) Mild hydrolysis - The polyalcohol (5.8 g.) was hydrolysed with 1N sulphuric acid (100 ml.) at room temperature for 24 hours. The solution was treated with ethanol (6 vols.) and left overnight at 0°C. The precipitated degraded polymer M₁ was removed at the centrifuge, dried and weighed (2.2 g.). The supernatant was concentrated (100 ml.), neutralised with barium carbonate, deionised with Amberlite IR 120 (H⁺) resin, concentrated to a syrup (2.3 g.) and examined by paper chromatography which indicated the presence of glycerol, glycollic aldehyde, threitol, glyceric acid, D-galactose, L-arabinose (trace) D-mannose (trace) and an oligosaccharide having $R_{\text{Galactose}} = 0.48$ in solvent B.

(iv) Hot hydrolysis - The polyalcohol B₁ (100 mg.) was hydrolysed with 1N sulphuric acid (3 ml.) in a boiling water-bath for 14 hours. The hydrolysate was neutralised, deionised and concentrated to a syrup. Paper chromatographic analysis of the syrup (solvent A) revealed spots corresponding to glycerol, glycollic aldehyde, threitol, glyceric acid, D-galactose,

D-mannose (trace) and L-arabinose (trace).

Expt. 46. Examination of the degraded polymer M₁ .

The degraded polymer M₁ (100 mg.), on hydrolysis with 1N sulphuric acid on a boiling water-bath for 14 hours, followed by chromatographic analysis revealed spots corresponding to D-galactose, D-mannose (trace) and L-arabinose (trace).

Expt. 47. Smith degradation of degraded polymer M₁ .

(1) Periodate oxidation - The degraded polymer M₁ (2.0 g.) was oxidised with aqueous 0.05 M sodium metaperiodate solution (500 ml.) for 40 hours. The amount of periodate reduced is shown in Table XVIII, (graph No. 8 , page 148a)

Table XVIII

Periodate consumption

Time in hour	3	10	20	30	40
Moles of periodate reduced per C ₆ anhydro-unit	0.08	0.2	0.28	0.36	0.36

(ii) Reduction - The unreduced periodate was destroyed as before and the solution dialysed (72 hours). The solution from the dialysis sac was concentrated and reduced with sodium borohydride (1.0 g.). The resulting polyalcohol B₂ was recovered, dried and weighed (1.8 g.).

(iii) Mild hydrolysis - The polyalcohol B₂ (1.7 g.) was hydrolysed

with 1N sulphuric acid (30 ml.) at room temperature. Degraded polymer M_2 (1.3g.) was recovered from the hydrolysate by precipitation with ethanol as before. The supernatant liquid was neutralised, deionised and concentrated to a syrup. Paper chromatographic examination of the syrup in solvent A revealed the presence of glycerol, glycollic aldehyde, erythritol (trace) and D-galactose (trace).

(1v) Hot hydrolysis - The polyalcohol B_2 (100 mg.), on hydrolysis with 1N sulphuric acid (2 ml.) on a boiling water-bath for 12 hours and subsequent chromatographic analysis in solvent A indicated the presence of glycerol, glycollic aldehyde, erythritol (trace), and D-galactose.

Expt. 48. Examination of the degraded polymer M_2 .

The degraded polymer M_2 (100 mg.) was hydrolysed with 1N sulphuric acid (3 ml.) on a boiling water-bath for 12 hours. The hydrolysate was neutralised, deionised, concentrated to a syrup and examined by paper chromatography in solvent A. This revealed the presence of D-galactose only.

Expt. 49. Smith degradation of degraded polymer M_2 .

(i) Periodate oxidation - The degraded polymer M_2 (1.0 g.) was oxidised with aqueous 0.05M sodium metaperiodate solution (250 ml.). Aliquots (5 ml.) were taken out at regular intervals to examine the periodate consumption.

The periodate consumption is shown in Table. XIX
(Graph No. 8 ; page 148a).

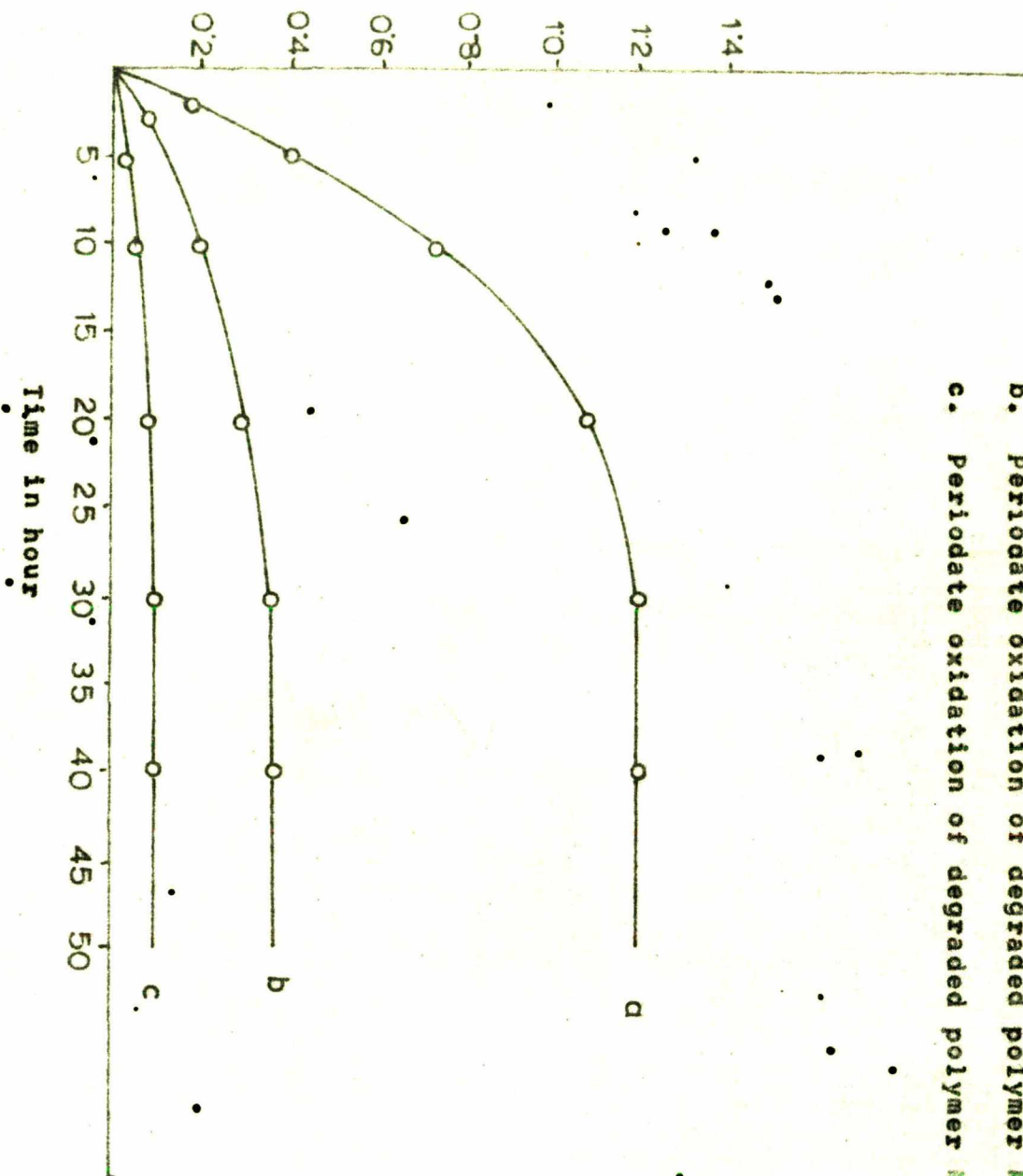
Table XIX

	<u>Periodate consumption</u>				
Time in hour	5	10	20	30	40
Moles of periodate reduced per C ₆ anhydro-unit	0.03	0.06	0.08	0.09	0.09

(ii) Reduction - After 40 hours the unreduced periodate was destroyed as before and the solution dialysed (72 hours). The solution from the dialysis sac was concentrated and reduced with sodium borohydride (600 mg.). The resulting polyalcohol B₃ was recovered, dried and weighed (900 mg.).

(iii) Mild hydrolysis - The polyalcohol B₃ (800 mg.) was hydrolysed with 1N sulphuric acid (20 ml.) at room temperature for 24 hours. The degraded polymer M₃ (750 mg.) was recovered from the hydrolysate by precipitation with ethanol and subsequent centrifugation. The supernatant liquid was neutralised, deionised, concentrated to a syrup and examined by paper chromatography. This indicated the presence of D-galactose (trace).

(iv) Hot hydrolysis - The polyalcohol B₃ (100 mg.), on hydrolysis with 1N sulphuric acid (3 ml.) on a boiling water-bath for 12 hours, followed by chromatographic analysis revealed the presence of D-galactose only.

Moles of periodate reduced per C_6 anhydro-unit

Expt. 50. Examination of the degraded polymer M₃.

The degraded polymer (100 mg.) was hydrolysed with 1N sulphuric acid (3 ml.) on a boiling water-bath for 14 hours. The cooled hydrolysate was neutralised, deionised and concentrated to a syrup. Paper chromatographic analysis of the syrup in solvent A indicated the presence of D-galactose only.

Expt. 51. Fractionation of the supernatant obtained from mild hydrolysis of polyalcohol B₁.

The supernatant syrup (2.3 g.) obtained from the mild hydrolysis of polyalcohol B₁ (Expt. 45) was repeatedly separated on filter sheets using solvent B. The following chromatographically pure fractions were obtained.

Fraction 1 (70 mg.) had the chromatographic mobility of glycollic aldehyde.

Fraction 2 (150 mg.) had the chromatographic mobility of glycerol. This was characterised as the tri-p-nitrobenzoate, m.p. 187 - 189°.

Fraction 3 (80 mg.) had the chromatographic mobility of threitol.

Fraction 4 (90 mg.) had the chromatographic mobility of glyceric acid.

Fraction 5 (120 mg.) had the chromatographic mobility of D-galactose.

Fraction 6 (10 mg.) had the chromatographic mobility of D-mannose.

Fraction 7 (40 mg.) had $R_{\text{Galactose}}$ 0.48 in solvent B; DP 2.

On hydrolysis it gave D-galactose only. Methylation followed by methanolysis, hydrolysis and paper chromatographic examination in solvent G revealed spots corresponding to 2,3,4,6-tetra-O-methyl-D-galactose and 2,4,6-tri-O-methyl-D-galactose.

All these facts indicated that the oligosaccharide was

O-D-galactopyranosyl (1 $\rightarrow$ 3)-D-galactopyranose.

DISCUSSION

The outer pulp of Dillenia indica was successively extracted with petroleum ether, ethanol (95%) and water. A glycosidic steroidal compound was separated from the alcoholic extract. The water-extract afforded an acidic polysaccharide (uronic anhydride 34%). It was associated with starch (1%) which was completely removed and the starch-free polysaccharide, called polysaccharide B, on hydrolysis, gave L-arabinose (15%), D-galactose (45%), D-galacturonic acid together with smaller proportions of D-mannose.

The polysaccharide B was initially methylated by Haworth method [Expt. 37 (a)] to a partially methylated product (methoxyl content, 22%; yield, 64.5%), which, on further methylation by recent versatile Hakomori method gave a product (methoxyl content, 36%). This was methanolysed and hydrolysed and the hydrolysate was repeatedly separated on filter sheets (Expt. 40). The methylated sugars were obtained as listed in Table XX showing their respective percentage proportions, (page 152).

The sugars in Table XX were characterised by their chromatographic mobilities, the formation of crystalline derivatives, specific rotations and demethylation.

The methanolysis products of the methylated polysaccharide showed, when examined by Gas-liquid partition chromatography,

Table XXHydrolytic cleavage fragments from the methylated polysaccharide B.

2,3,5-tri-O-methyl-L-arabinose	(ca 7%)
2,3,4,6-tetra-O-methyl-D-galactose	(ca 14%)
2,5-di-O-methyl-L-arabinose	(ca 6%)
2,3,6-tri-O-methyl-D-galactose	(ca 21%)
2,4,6-tri-O-methyl-D-galactose	(ca 19%)
2,4-di-O-methyl-D-galactose	(ca 12%)
2,3,4-tri-O-methyl-D-galacturonic acid	(ca 13%)
2-O-methyl-D-galactose	(ca 8%)

the presence of the methyl glycosides of

2,3,5-tri-O-methyl-L-arabinose
 2,5-di-O-methyl-L-arabinose
 2,3,4,6-tetra-O-methyl-D-galactose
 2,3,6-tri-O-methyl-D-galactose
 2,4,6-tri-O-methyl-D-galactose
 2,3,4-tri-O-methyl-D-galacturonic acid

While the polysaccharide B gave a small amount of D-mannose, on hydrolysis, no methylated derivative of this sugar moiety could be detected by paper chromatography or by GLC. However, subsequently in the degraded hydrolysis of polysaccharide B, a disaccharide, O-D-galactopyranosyluronic acid (1→3)-D-mannose was detected (Expt.43 and 44) which demonstrates

the presence of D-mannopyranose residues in the polymer. While dealing with separation of such a complex mixture of variety of methylated sugars, it is not unlikely to miss a sugar of smaller amount due to overlappings or poor fractionation.

The formation of high proportion of 2,3,5-tri-O-methyl-L-arabinose, 2,3,4,6-tetra-O-methyl-D-galactose and 2,3,4-tri-O-methyl-D-galacturonic acid demonstrates the highly branched nature of the polysaccharide.

On the assumption that the methylation of the polysaccharide B was complete, the location of the hydroxyl groups in the cleavage fragments of its methylated derivative is regarded as the positions through which the monosaccharide units were engaged in union with other residues.

The existence of 2,3,5-tri-O-methyl-L-arabinose (being linked at C-1 only) and 2,5-di-O-methyl-L-arabinose (being linked at C-1 and C-3) both in the hydrolysis products and in the methanolysis products of the methylated polysaccharide B reveals that these units exist in the polymer in the furanose form.

The identification of 2,3,4,6-tetra-O-methyl-D-galactose demonstrates that these residues occur in the pyranose form and are linked at C-1. Similarly the identification of 2,3,4-tri-O-methyl-D-galacturonic acid reveals that these units are

also linked at C-1 only. In other words, those L-arabinose, D-galactose and D-galacturonic acid residues which gave the methylated sugars as mentioned above constitute end groups in the macromolecule.

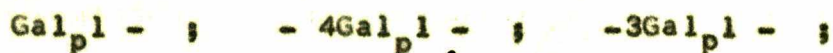
A portion of the L-arabinose residues of the polysaccharide is isolated from the methylated polymer as 2,5-di-O-methyl-L-arabinose and this must represent those L-arabinose residues which are interposed between the terminal groups of the side chain and the nucleus of the macromolecule. The isolation of O-L-arabofuranosyl (1→3)-L-arabofuranose and O-D-galactopyranosyl (1→3)-L-arabofuranose as the partial hydrolysis products of the polysaccharide B (Expt. 42) is in agreement with this view and the presence of 2,5-di-O-methyl-L-arabinose residues in the methylated polysaccharide strongly suggests that in the polysaccharide the above disaccharides are present in the form of O-L-arabofuranosyl (1→3)-L-arabofuranose and O-D-galactopyranosyl (1→3)-L-arabofuranose respectively. The existence of 2,4,6-tri-O-methyl-D-galactose shows that this unit is linked to two other units through C-1 and C-3. Similarly the characterisation of 2,4-di-O-methyl-D-galactose and 2-O-methyl-D-galactose shows in the same way the involvement of C-1, C-3, C-6 of the former and C-1, C-3, C-4, C-6 of the latter in union with other units in the molecule.

The identification of the cleavage fragments in the hydrolytic products of the methylated polysaccharide B has,

hydrolytic products of polysaccharide B (Expt. 43 & 44).

Table XXI

Structural units of the polysaccharide B



Gal_p = D-galactopyranose

Ara_f = L-arabofuranose

$\text{Man}_D = \text{D-mannopyranose}$

Gal_pA = D-galactopyranosyluronic acid

It may be seen that some L-arabofuranose residues, some D-galactopyranose residues and all the D-galacturonic acid residues constitute the end groups. L-arabofuranose residues also occur as 1,3-linked residues and D-galactopyranose residues in

1,4-and 1,3-linkages.

Further evidence concerning the structure of the polysaccharide B came from its partial hydrolysis studies. The partial hydrolysis of the polysaccharide B was carried out in three stages (flowsheet at page 135).

In the first stage of the hydrolysis the polysaccharide B was hydrolysed with 0.01N sulphuric acid on a boiling water-bath for 4 hours which afforded a degraded polysaccharide K_1 and a syrup. The degraded polysaccharide K_1 after hydrolysis with 0.01N sulphuric acid under the same conditions afforded another degraded polymer K_2 and a syrup. Both the syrups were found to contain L-arabinose, D-galactose and three disaccharides (1 - 3), showing that similar sugar moieties were eroded during both the degradation.

The disaccharide 1, which had the highest chromatographic mobility, gave L-arabinose only on hydrolysis. Methylation, methanolysis and then hydrolysis of the disaccharide gave 2,3,5-tri-O-methyl-L-arabinose and 2,5-di-O-methyl-L-arabinose, thus indicating that the disaccharide was

0-L-arabofuranosyl(1 $\rightarrow$ 3)-L-arabofuranose(Expt.42)

The formation of this disaccharide supports further that some of L-arabofuranose residues occur as non-terminal 1,3-linked residues.

The second disaccharide 2, on hydrolysis, gave D-galactose and L-arabinose. Hydrolysis after reduction of the disaccharide gave D-galactose and L-arabitol indicating that L-arabinose unit constitutes the reducing moiety. Methylation followed by methanolysis and hydrolysis of the disaccharide gave 2,3,4,6-tetra-O-methyl-D-galactose and 2,5-di-O-methyl-L-arabinose. These facts revealed that the disaccharide was

O-D-galactopyranosyl(1→3)-L-arabofuranose

The formation of the above two disaccharides reveals that those L-arabinose residues which give rise to 2,5-di-O-methyl derivative in the methylated polysaccharide B, are linked to terminal L-arabofuranose residues in one case and to terminal D-galactopyranose residues in the other.

The disaccharide 3, on hydrolysis, gave D-galactose only. Methylation, methanolysis and hydrolysis of the disaccharide gave 2,3,4,6-tetra-O-methyl-D-galactose and 2,3,6-tri-O-methyl-D-galactose, thus indicating that the disaccharide was

O-D-galactopyranosyl(1→4)-D-galactopyranose.

The formation of this disaccharide reveals that D-galactopyranose residues are also involved in 1,4- linkage.

The degraded polysaccharide K₂ was further hydrolysed (second stage) with 0.5N sulphuric acid on a boiling water-bath for 1 hour which afforded a degraded polysaccharide K₃ and a syrup. The degraded polysaccharide K₃, on hydrolysis, under

the same conditions gave another degraded polymer K₄ and a syrup (Expt. 43.). Both the syrups, on chromatographic examination revealed the presence of D-galactose, D-galacturonic acid, L-arabinose (trace), D-mannose (trace) and four neutral oligosaccharides together with three acidic oligosaccharides.

The neutral oligosaccharide I was identified as O-D-galactopyranosyl (1→3)-L-arabofuranose.

The neutral oligosaccharide 2 was characterised as O-D-galactopyranosyl (1→4)-D-galactopyranose.

The neutral oligosaccharide 3 had DP 2. On hydrolysis it gave D-galactose only. Methylation and then hydrolysis of the sugar gave 2,3,4,6-tetra-O-methyl-D-galactose and 2,4,6-tri-O-methyl-D-galactose thus indicating that the oligosaccharide was

O-D-galactopyranosyl (1→3)-D-galactopyranose.

The neutral oligosaccharide 4 had DP 3. On hydrolysis it gave D-galactose only. Hydrolysis of the methylated derivative afforded 2,3,4,6-tetra-O-methyl-D-galactose, 2,3,6-tri-O-methyl-D-galactose and 2,4,6-tri-O-methyl-D-galactose. All these facts revealed that the oligosaccharide was

O-D-galactopyranosyl (1→4)-^O-D-galactopyranosyl (1→3)-D-galactopyranose.

The first acidic oligosaccharide had DP 2. On hydrolysis

it gave D-galacturonic acid and D-mannose. Reduction of the methyl ester methyl glycoside followed by hydrolysis gave D-galactose and D-mannose. Hydrolysis of the methylated derivative gave 2,3,4-tri-O-methyl-D-galacturonic acid and 2,4,6-tri-O-methyl-D-mannose. These facts revealed that the oligosaccharide was

O-D-galactopyranosyluronic acid (1→3)-D-mannopyranose.

The identification this disaccharide reveals for the first time that D-mannopyranose residues are also an integral constituent of the polysaccharide B and that they are linked at C - 1 and C - 3 (vide, Table XXI).

The second acidic oligosaccharide also had DP 2. On hydrolysis it gave D-galactose and D-galacturonic acid. Reduction of the methyl ester methyl glycoside followed by hydrolysis gave D-galactose. Methylation followed by hydrolysis gave 2,3,4-tri-O-methyl-D-galacturonic acid and 2,3,4-tri-O-methyl-D-galactose, thus indicating that the oligosaccharide was

O-D-galactopyranosyluronic acid (1→6)-D-galactopyranose.

The formation of this disaccharide reveals that some of D-galacturonic acid residues are attached to C-6 of the D-galactopyranose residues.

The third acidic oligosaccharide had DP 4. On hydrolysis it gave D-galacturonic acid, D-mannose and D-galactose. Reduction of the methyl ester methyl glycoside followed by hydrolysis

afforded D-galactose and D-mannose. Hydrolysis of the methylated derivative gave 2,3,4-tri-O-methyl-D-galacturonic acid, 2,4,6-tri-O-methyl-D-mannose, 2,3,6-tri-O-methyl-D-galactose and 2,4,6-tri-O-methyl-D-galactose. These facts suggested that the oligosaccharide was

O-D-galactopyranosyluronic acid (1→3)-O-D-mannopyranosyl (1→4)-O-D-galactopyranosyl (1→3)-D-galactopyranose.

The formation of this oligosaccharide indicates that D-mannopyranose residues are linked to C-4 of 1,4-linked D-galactopyranose units.

The degraded polysaccharide K₄ was again hydrolysed (3rd stage) with 1N sulphuric acid for 5 hours on a boiling water-bath, which afforded three acidic oligosaccharides.

The first acidic oligosaccharide was identified as the aldobiuronic acid O-D-galactopyranosyluronic acid (1→3)-D-mannopyranose.

The second oligosaccharide was characterised as O-D-galactopyranosyluronic acid (1→6)-D-galactopyranose.

The third acidic oligosaccharide had DP 3. On hydrolysis it gave D-galacturonic acid and D-galactose. Reduction of the methyl ester methyl glycosides followed by hydrolysis gave D-galactose only. Hydrolysis of the methylated derivative gave 2,3,4-tri-O-methyl-D-galacturonic acid, 2,3,4-tri-O-methyl-D-galactose and 2,4,6-tri-O-methyl-D-galactose. All these facts

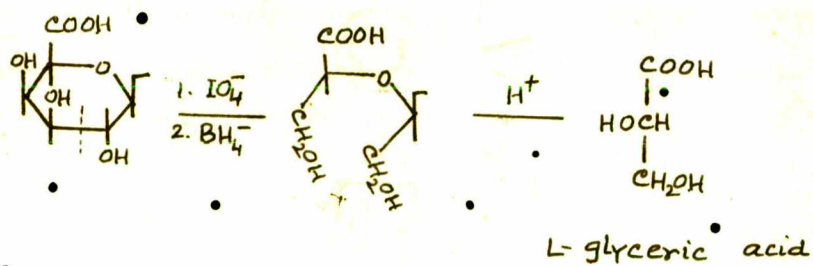
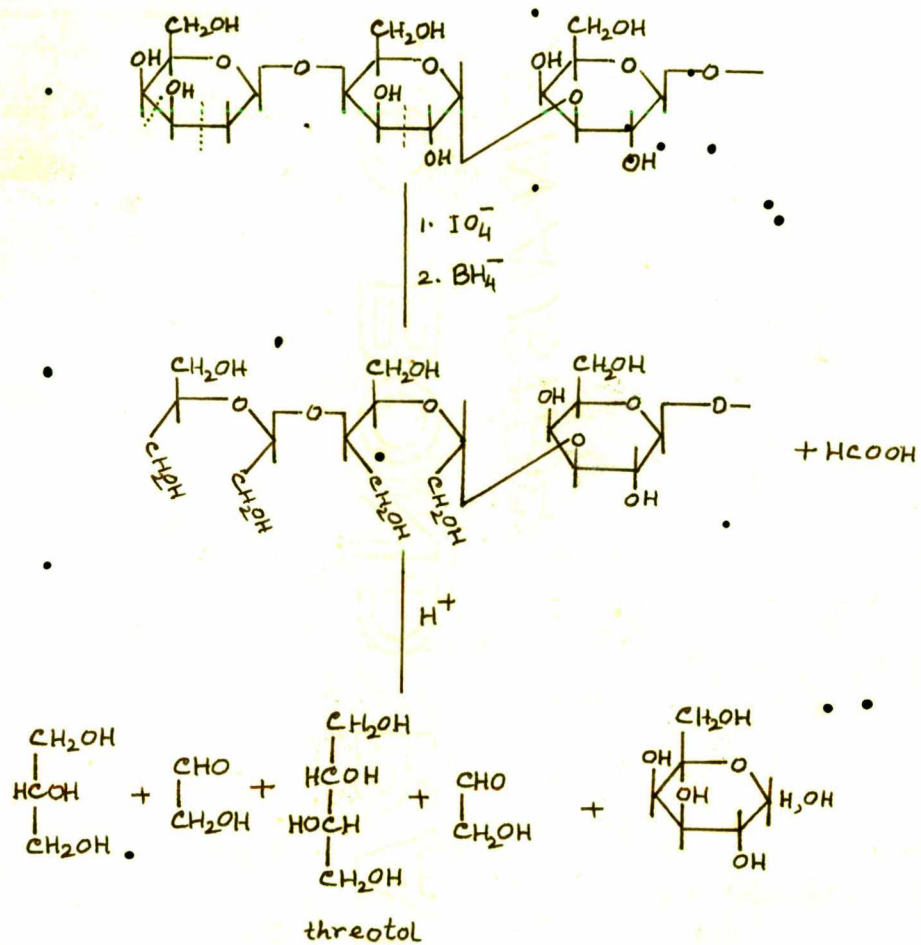
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CHART 2



and the formation of a higher proportion of fragments of low molecular weight during the mild hydrolysis of polyalcohol B₁ indicates that the polysaccharide B contains a higher proportion of periodate vulnerable residues ^{as} side chains.

The degraded polymer M₁, on hydrolysis, gave L-arabinose (trace), D-galactose and D-mannose (trace). This fact reveals that some of the L-arabinose residues resist periodate oxidation in the first treatment, thus confirming the presence of a part of L-arabinose residues as 1,3-linked units.

The degraded polymer M₁, on further periodate oxidation followed by reduction, gave another polyalcohol B₂. This, on mild hydrolysis, afforded another degraded polymer M₂ and glycerol, glycollic aldehyde, erythritol (trace) and D-galactose.

The formation of these products resulted from further degradation of the outer layers of sugar residues viz D-galactose, L-arabinose and D-mannose. The degraded polymer M₂, on hydrolysis, gave D-galactose only (Expt. 48) thus showing that the L-arabinose and D-mannose residues were removed during Smith degradation of polymer M₁.

The degraded polymer M₂ on further periodate treatment, reduced negligible amount of periodate (0.09 moles per anhydrohexose unit). The periodate oxidised product on reduction, afforded polyalcohol B₃. This, on mild hydrolysis, gave another

degraded polymer M_3 and no or undetectable amount of fragments of low molecular weight (Expt. 49). This shows that the outer layers of sugar residues were removed by previous treatments and the degraded polymer M_3 contained no or negligible sugar moiety as side chains which are vulnerable to periodate oxidation.

The degraded polymer M_3 , on hydrolysis, gave D-galactose only. This means that the polymer M_3 is composed of only D-galactose residues which are immune to periodate attack. Such a periodate resistant structure would be one which is composed of 1,3-linked D-galactopyranose residues and, therefore, the skeleton structure of the polysaccharide B appears to be



Thus the skeleton structures of both polysaccharide A and polysaccharide B are similar.

On the basis of all the above results the following structural character of the polysaccharide B may be suggested.

(1) In view of the homogeneity of the polysaccharide B, as evidenced from fractionation studies, it appears that the polysaccharide is composed of L-arabinose, D-galactose, D-galacturonic acid, and trace of D-mannose.

(2) The polysaccharide contains main chains of 1,3-linked D-galactopyranose residues to which other residues are attached as side chains.

(3) D-galacturonic acid, L-arabofuranose and D-galactopyranose residues constitute end groups. 1,3-linked L-arabofuranose residues, 1,4-linked D-galactopyranose residues and 1,3-linked D-mannopyranose residues exist as side chains.

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