STUDIES ON POLYSACCHARIDES FROM THE SEEDS OF BORASSUS FLABELLIFER LINN.

A thesis presented

by

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To my parents

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C O N T E N T S

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LIST OF ABBREVIATIONS

Man_p	= Mannopyranosyl residue
Gal_p	= Galactopyranosyl residue
DEAE	= Diethylaminoethyl
EDTA	= Ethylenediaminotetra-acetic acid disodium salt
DMSO	= Dimethyl sulphoxide
TMS	= Tetramethylsilane
n.m.r.	= Nuclear magnetic resonance
g.l.c.	= Gas liquid chromatography
g.l.c.-m.s.	= Gas liquid chromatography-mass spectroscopy
I.R.	= Infra-red
R_t	= Retention time
TMG	= 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol
DMF	= N,N-dimethyl formamide
TFA	= Trifluoroacetic acid
D.P.	= Degree of polymerization

SUMMARY

Seeds of Borassus flabellifer Linn. (Bengali: Tal)were collected from different areas of Bangladesh in different seasons. Their inner hard endosperms were carefully separated, sun-dried and powdered. The powdered kernels were successively extracted with pertroleum ether (b.p. 40-60^{o}C), ethanol (80%), cold and hot water, 3% and 10% aqueous sodium hydroxide at room temperature. The variations of the yields of different extracts of different samples were insignificant. Hydrolysis of each fraction of the different samples afforded the same sugars showing the similar nature of components in each fractions.

1. Polysaccharide from 10% alkali extract

Crude polysaccharide, obtained from 10% aqueous sodium hydroxide extract (44.1%) was subjected to gel chromatography on sepharose CL-4B and sepharose CL-2B gel columns. This purified homogeneous polysaccharide (designated as M_1) gave, on hydrolysis, 97% and 3% D-mannose and D-galactose respectively.

The specific rotation of polysaccharide M_1 was - 7.2^{o}. The negative specific rotation of the polymer indicated that the monosaccharide units were glycosidically linked in the β-form.

The purified polysaccharide M_1 was completely methylated by Hakomari method, hydrolysed, converted into alditol acetates which were analysed by g.l.c. and g.l.c.-m.s. 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-galactitol (3.18%), 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-mannitol (3.09%), 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-D-mannitol (87.54%) and 1,4,5,6-tetra-O-acetyl-2,3-di-O-methyl-D-mannitol (6.18%) were identified from g.l.c. analysis.

The straight chain nature of polysaccharide M_1 having a β-(1→4) linked mannopyranose residues was evidenced by the presence of 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-D-mannitol in large proportion. 1,4,5,6-tetra-O-acetyl-2,3-di-O-methyl-D-mannitol showed a similar involvement of C_5 in pyranose ring having branching at its C_1, C_4 and C_6 positions. 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-mannitol indicated the substituted non-reducing sugar in the pyranose ring with branching at its C_1 position with C_6 position of mannose unit of the backbone. Similarly 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-galactitol indicated that these residues were also in the pyranose ring having branching at its C_1 position with C_6 position of the mannose unit of the backbone.

Further support for the structural nature of polysaccharide M_1 consisting largely of 1,4 linked β-D-mannose units

as its backbone having branching of D-galactose and D-mannose residues came from g.l.c.-m.s. analysis. The $m/_e$ values of different fragment of alditol acetates obtained from the polymer as obtained by g.l.c.-m.s. studies corresponded to those of 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-galactitol, 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-D-mannitol and 1,4,5,6-tetra-O-acetyl-2,3-di-O-methyl-D-mannitol.

Identification of these fragments in the hydrolytic products of the fully methylated polysaccharide M_1 provided evidence for the presence of the following structural units in its molecule.

$$Man_p1- \quad , \quad -4\ Man_p1- \quad , \quad \overset{6}{-4\ Man_p1-} \quad \text{and} \quad Gal_p1-$$

Where

Man_p = D-mannopyranose

Gal_p = D-galactopyranose

Periodate oxidation of the polysaccharide M_1 revealed also the presence of 1,4 linkages in its molecule. Controlled acid hydrolysis of the polymer gave three oligosaccharides which were identified as O-D-mannopyranosyl-(1→4)-D-

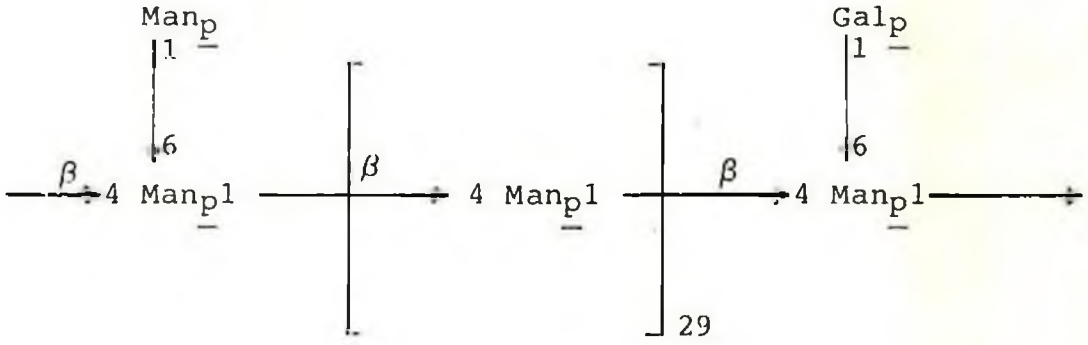
mannopyranose, O-D-mannopyranosyl (1→4)-D-mannopyranosyl(1→4)-D-mannopyranose and O-D-mannopyranosyl (1→4)-D-mannopyranosyl (1→4)-D-mannopyranosyl (1→4)-D-mannopyranose. This lent further support to its 1,4 linked mannose units.

^{13}C-n.m.r. studies of polysaccharide M_1 gave coupling constant $J\ ^{13}C\text{-}^{1}H$= 160.5 Hz which confirmed the β-configuration for 1→4-linked D-mannopyranose residue of the polysaccharide M_1.

^{13}C-n.m.r. studies of the polysaccharide M_1 in noise-decoupled spectrum gave six main peaks (C_1= δ 101.7 p.p.m., C_2= δ 72.0 p.p.m., C_3= δ 73.3 p.p.m., C_4= δ 77.5 p.p.m., C_5= δ 77.0 p.p.m. and C_6= δ 62.2 p.p.m.) together with a weak one ($-CH_2-$ = 67.4 p.p.m.). The main six peaks corresponded to those of a β-(1→4) linked mannan. The weak peak indicated the presence of 1,6 linked hexose in the polysaccharide, M_1, in addition to 1,4 linked mannose units.

From the ratio of the amourts of tetra-, tri- and di-O-methyl sugars, the length of the repeating units of the polysaccharide M_1 came to 32 hexose units.

From the studies mentioned above it is concluded that the polysaccharide M_1 is a galactomannomannan having the following structure of a repeating unit :



2. Polysaccharide from hot water extract

The crude polysaccharide (2.5%) obtained by hot water extraction was purified by gel filtration using sepharose CL-2B gel column. This purified homogeneous product was designated as polysaccharide M_2. On hydrolysis it gave, 80.12% and 19.87% D-mannose and D-galactose respectively.

The specific rotation of the polysaccharide M_2 was -2.25^{o}. This negative specific retation indicated β-linkage in the main mannose chain of the polymer.

The purified polysaccharide M_2 was completely methylated, hydrolysed, converted into alditol acetates and analysed by g.l.c. and g.l.c.-m.s. These alditol acetates were identified as 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-galactitol (18.08%), 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-D-mannitol (64.01%) and 1,4,5,6-tetra-O-acetyl-2,3-di-O-methyl-D-mannitol (17.95%) from their respective retention times as obtained from g.l.c. analysis.

$m/_e$ values obtained from m.s. analysis of the fragmants corresponded to those of 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-galactitol, 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-D-mannitol and 1,4,5,6-tetra -O-acetyl-2,3-di-O-methyl-D-mannitol, supporting further their identification.

The presence of 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-D-mannitol in higher proportion revealed the straight chain nature of polysaccharide M_2 having a β-(1→4) linked mannopyranose residues like the polysaccharide M_1. The appearance of 1,4,5,6-tetra-O-acetyl-2,3-di-O-methyl-D-mannitol showed branching at C_1, C_4 and C_6 positions of D-mannopyranose units. The existance of 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-galactitol also indicated that D-galactose unit in the pyranose form constituted the branching between its C_1 position and C_6 position of D-mannose unit of the backbone.

The polysaccharide M_2, therefore, contained the following structural units in its molecule.

$$\text{-4 Man}_p\text{1-} \quad , \quad \overset{6}{\text{-4 Man}_p\text{1-}} \quad \text{and} \quad \text{gal}_p\text{1-}$$

Where

Man_p = D-mannopyranose

Gal_p = D-galactopyranose

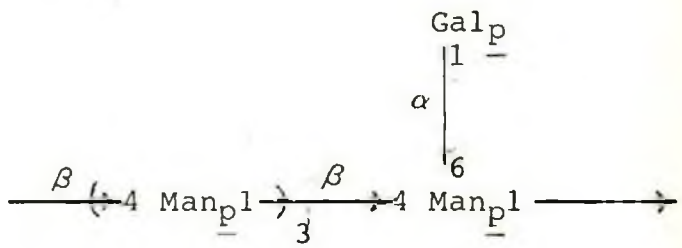
In the ^{1}H-n.m.r. spectrum the anomeric proton signals at δ 4.90 p.p.m., δ 5.12 p.p.m. and δ 5.19 p.p.m. supported the presence of β-linked mannose units and the attachment of a galactose unit by α-linkage to C_6 position of a mannose unit of the main chain.

In the ^{13}C-n.m.r. spectrum the signals at δ 101.2 p.p.m., δ 72.9 p.p.m. δ 73.3 p.p.m., δ 77.7 p.p.m., δ 75.0 p.p.m. and δ 63.4 p.p.m. again affirmed the presence of mannose unit in the main chain in regular repeating structure and galactose unit at C_6 position of the mannose.

The presence of mannose and galactose in the ratio of 4:1 suggested that for every four mannose units there existed one galactose unit . From the ratio of the amounts of tetra-,

tri- and di-O-methyl sugars, the length of the repeating units of the polysaccharide M_2 comes to 5 hexose units.

From all these sugar and methylation analyses, ^1H and ^{13}C-n.m.r. spectral data as well as the value of specific rotation it was concluded that the polysaccharide M_2 was essentially (1→4)- β-linked mannose that had a galactose unit at its C_6 position by α-linkage. Accordingly the structure of repeating unit of polysaccharide M_2 may be as follows :



3. Polysaccharide from 3% alkali extract

3% aqueous sodium hydroxide extraction of the residual kernel remained after hot water extraction yielded a polysaccharide (3.5%). This crude polysaccharide was purified by passing

through sepharose CL-2B gel column. This purified homogeneous product was designated as polysaccharide M_3. Sugar analysis of this polysaccharide M_3 revealed the presence of D-mannose (85.7%) and D-galactose (14.37%).

The polysaccharide M_3 had a specific rotation of -1.56° which indicated β-linkage in the main mannose chain.

The chemical shifts at δ 4.73 p.p.m., δ 4.95 p.p.m. and δ 5.26 p.p.m. in ^{1}H-n.m.r. supported the presence of β-linked mannose units of the main chain and of α-linked galactose to mannose unit, like the polysaccharide M_2.

^{13}C-n.m.r. spectrum of the polysaccharide M_3 was similar to that of the polymer M_2, indicating similar structure of M_3, that is, M_3 contained predominently β-linked mannose units and a galactose unit attached to C_6 position of mannose unit.

I. REVIEW

1.1. POLYSACCHARIDES

1.1.1. Introduction

Polysaccharides are naturally occuring polymeric substances, the building blocks of which are monosaccharides joined by glycosidic linkages[1]. They form the skeletal structure 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[2]. They are abundant in fungi and in the oxoskeletons 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. They have wide spread distribution in nature in a variety of roles like reserve food, sizing material, medicinal and structural framework of the living being. Polysaccharides are classified in many ways. However, they are usually classified, based on (a) sources (Page 3) 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. The most common practice is to take the units of sugars as the basis of names with ending of "an". A polysaccharide comprised of pentoses is called a pentosan. Similarly, a polysaccharide consisting of mannose is mannan,

of galactose is galactan, of xylose is xylan etc. Sometimes, presence of certain constituents form the basis of names, for instance, those containing uronic acid residues are called uronides and those containing amino-sugar residues are called mucopolysaccharides.

Homoglycans or polysaccharides containing only one kind of polymerized sugar units are more abundant than heteroglycans. Similarly, cellulose, laminaran, amylose and amylopectin belong to this class. Next in abundance is xylan, D-xylose constitute xylan, whereas D-galactose makes up the galactans of pectin and certain seaweed polymers.

Polysaccharides, like cellulose, are almost always linear molecules whereas polysaccharides which serve primarily as reserve foods are commonly branched, a mixture of linear and branched polysaccharides with the branched type predominating. Linear molecules 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. Branched polysaccharides, on the other hand, are easily soluble in water and have immense thickening powers.

Polysaccharides play 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 macromolecules[3]. Thus, cellulose hemicelluloses and chitin serve obviously as structural molecules and as links that help to control cell wall permeability. The 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 thickening agents they serve in physical or mechanical roles in animals and as specific substance, they are of importance in blood group specificity and in other immunological reactions. Polysaccharides are important components of normal connective tissue, in granular tissue of healing animal wounds and probably in calcification processes.

1.1.2. Sources of polysaccharides

1.1.2.1. Plant kingdom

A. Higher plants

a. Cellulose

b. Lichenan and isolichenan

c. Pustulan

d. Pectic substances

e. Xylan

f. Ivory nut mannan, wood mannan and sapel mannan

g. Uronic polysaccharide of *Phorunium 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

1. Cell constituents :

a. Cellulose

b. Chitin

c. Glycogens

d. Glucans of Penicillum 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. Dextrin of Betabacterium vermiforuni

c. Dextran of Leuconostoc mesenteroides

d. Levans

e. Galactocarolose

f. Mannocarolose

g. Rhizobium radicicolum polysaccharide

h. Varian

i. Rugulose

j. Capreolan

k. Luteic acid

l. Sclerotiose

m. Yeast glucan

n. Torula polysaccharide

1.1.2.2 Animal Kingdom

A. Non-Mammals

1. Chitin

2. Glycogens

3. Hyaluronic acid (Fowl Sarcoma Fluid)

4. Ovalbumin and ovomucoid

5. Galactan of Helix pomatia

6. Heparin, chondroitin sulphuric acid and Mucoitin sulphuric acid

7. 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 globogycoid.

1.1.3. Plant polysaccharides

Plant polysaccharides are present in greatest quantity in the higher order of plants where they constitute approximately three-quarters of the dry weight[4]. The majority of plant polysaccharides are components of the cells in a typical plant tissue consists of three morphologically distinct layers,namely the intercellular cement or middle lamella, the primary walls and the secondary walls[5]. The middle lamella or intercellular 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.The middle lamella contains cellulose, xylans, uronic acid containing polysaccharides and

sometimes a mannan. Polysaccharides composed of pentose sugar units are the most prevalent but still constitute less than 15% of the layer. The most abundant middle lamellar substance is lignin which constitutes more than 70% of the layer[5]. In the mature tissue the tenuous intercellular layer contains a large amount of lignin approximately 71% in the case of micro-dissected middle lamella of Douglars fir[6]. There is also present about 14% pentosan, probably xylan, uronic acid containing material and a small amount of pectin.

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 consists 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 layers is small. The primary walls and middle lamella of soft tissues like apple and other fruits devoid of secondary walls, 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 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. Plant gums are composed of dissolved or partially dissolved polysaccharides. Economic gums of commerce encompass a broad group of substances[7], 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, sugar and quince seed gums. Fructans are important food reserves in grasses and accumulate in the healthy mature plant.

The plant gums are practically always highly branched structures composed of two to five types of monosaccharides[5]. Almost all of the exudates contain uronic acid units in more or less abundance.

1.1.4. Plant mucilages

The plant mucilages are polysaccharides which form colloidal solutions in water from which they can often be precipitated with ammonium sulphate, sodium chloride and the usual protein precipitants[8]. 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. The mucilages

may arise from starch[9,10] and in certain plants atleast, they seem to act, as do the polysaccharides in succulent plants (Aloe, Euphorpiaceae), as agents for holding water[11].

An excellent source of mucilages composed of neutral sugar residues are the seeds of many "Liguminoseae" such as the locust bean or carob bean (ceratonia siliqua L.), guar (cyamopsis tetragonolobus), Kentucky coffee bean(Gymnocladus diocia) and many other palm seed also provide a neutral mucilaginous polysaccharide[12].

Recently a large number of polysaccharides have been extracted and characterised from the roots[13,14], stem[15], bark[16,16a] leaves[17], fruits[18,19] and seeds[20] of certain plants of Bangladesh. They are products of normal plant metabolism and may serve as food reserves in much the same process as starch in many plants and glycogen in animals. The mucilage is sometimes a food reserve (Linum, Cruciferae) and sometimes a water reservoir. Resins and oils are also the function of a mucilaginous membrane .The hydrolysis products of mucilages from orchids and from cell wall thickening material of numerous endosperms (Palm, strychnos, Leguminosae) susually contain D-mannose and D-galactose. These mucilages are reserve polysaccharides and are split by the enzyme seminase and by the enzymes occuring in aspergillus niger, a. fucus and in barley malt. Mannan occurs in the root of conophalus konjaku and the mucilage in Hydrangea paniculata contains D-galactose, D-mannose and L-arabinose.

1.1.5. Root mucilages

The roots of certain plants like Iles mannan (Amorphophallus oncophyllus) and Konjak mannan (A Konjak) consists of a polysaccharide which can be extracted with hot water or dilute alkali to yield mucilages. These excellent mucilages are useful in the paper or rubber latex Industries.

1.1.6. Polysaccharides of seaweeds and other algae

Most of the polysaccharides isolated from seaweeds are 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 1-20%. 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.

Various commercial materials have been extracted from seaweeds of which alginate, agar and carrageenin are the most important. About 80% or more of most seaweeds are extractable by hot water or dilute alkaline solutions. Most seaweed polysaccharides are homoglycans; i.e. polysaccharides composed of only a single sugar as the repeating polymer unit. These polysaccharides are applicable in the food, cosmetic, pharmaceutical, textile and paper industries.

Japan and China possess flourishing seaweed industries of great antiquity and a large part of the annual harvest is devoted to human consumption.

1.1.7. Polysaccharides of fungi

A great variety of polysaccharides are produced by fungi. Although some of these polysaccharides serve as reserve foods, many are fabricated into the structural framework of the fungi.

Fungi do not have a characteristic composition in respect to industrial constituents or types of compounds. 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. In many fungi, chitin acts as an important frame substance in the cell wall. Often chitin content increases progressively with age of the fungi.Cellulose is the principal cell wall component in some fungi[21]. Pentosans seem to be widely distributed among the lower fungi.Glycogen has also been found in some fungi.

Closer examination[22] has revealed that the D-galactofuranosyl end groups present in the glycopeptides (Glycoproteins of animal polysaccharide group) play no direct part in the "immediate activity" and the major activity arises from the highly branched D-mannopyranose residues. Other mannans, which are devoid of protein, cross-react with sensitized Guineapigs and always give only the "immediate" type of reactivity.

Correlation of structure using, immunological cross reactions seems to be feasible.

When the glycoproteins are separated on 0-(diethylamino)-ethyl-sephadex, it appears that each species of fungi produces two or three galacto-mannan peptides having variable proportions of constituents.

1.1.8. Bacterial polysaccharides

Bacteria produces a great variety of polysaccharides. Many of these polysaccharides are fabricated into the structural framework of the capsular sheath of bacteria. Polysaccharides which have particularly engrossed the biochemist are those of immunological importance which occur in capsules of the pneumococci and tubercle bacilli.

The capsular polysaccharides of pneumococcus (type III) are shown to be a linear molecule of D-glucose and D-glucuronic acid units in equal amounts. They are linked alternately so that the molecule may be regarded as a chain of aldobiuronic acid units (Fig. 1)[23].

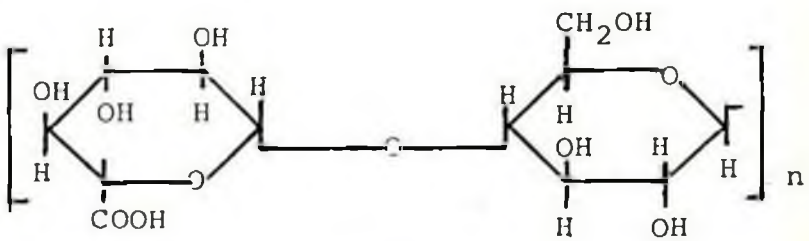


Fig. 1.

While the compositions of other pneumococcal polysaccharides have not been fully clarified, the compositions of several have beendetermined as follows:-

Type I..... D-Galacturonic acid (28%), aminosugar and acetic acid.

Type II..... D-Glucose, L-rhamnose (40%) and D-Glucuronic acid.

Type IV.... D-Glucose and N-acetyl hexosamine.

Type VIII.. D-Glucose and D-Glucuronic acid in 7:2 ratio.

Type XIV... D-Galactose and N-acetyl-D-Glucosamine.

Aspergillus polysaccharide is a linear chain of glucopyranose units joined alternately by α -1→4 and α- 1→5 bonds[24].

Many organisms produce polysaccharides with antigenic properties. Most polysaccharides synthesized by micro-organisms 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[12].

1.1.9. Animal polysaccharides

The polysaccharides of animal tissues include a wide range of compounds of interesting and varied distribution[4]. Many of the animal polysaccharides exist in the living animal and in isolated products as loose salt complexes or chemically bonded with proteins (or with lipids or both). The polysaccharides produced by animals play an important role in the physiology of the organism[2].

Glycogens are found generally throughout the animal kingdom and are the principal reserve polysaccharides of the animals. Structurally glycogens consist of chains of 1→4 linked α-D-gluco-pyranose units with α-1→6 links at the branch points[25,26].

Hyaluronic acid in animals serves as a lubricant and shock absorbant in the joints. Structurally hyaluronic acid has been characterised as 3-O-(β-D-glucopyranosyl uronic acid)- 2 - amino - 2 - deoxy - D - glucose[27].

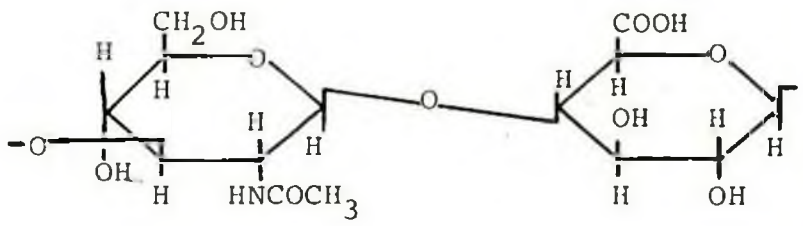


Fig.2. Repeating unit of Hyaluronic acid.

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 polysaccharides play an important role in synovial fluids and in certain cancers, for example, mesothelioma. Moreover, chondroitin sulphuric acid has a structural importance in animal cartilage and connective tissues. Lower animals use chitin as their principal frame substance.

Heparin is a polymer of D-glucuronic acid and D-glucosamine.

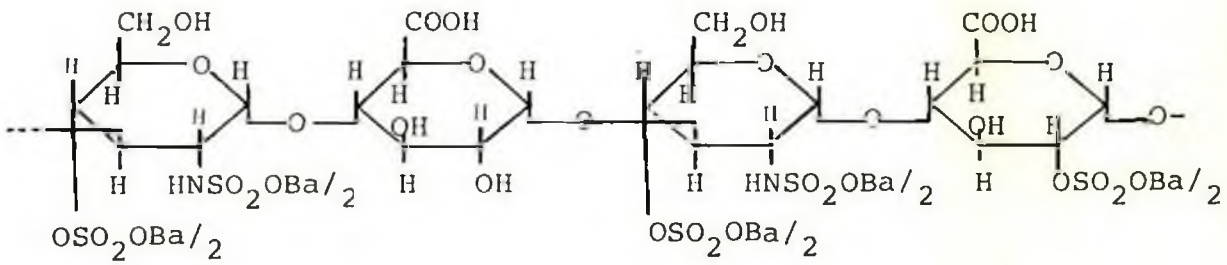


Fig. 3. Repeating unit of heparin.

Heparin, a blood anticoagulant, is found in liver,lung, thymus, spleen and blood[28]. Heparin may be applicable in the treatment of frostbile, acute rheumatic fever and atherosclerosis.

The blood-group polysaccharides[29], found in the red blood cells, gastric mucin, saliva, ovarian cyst fluid and other body secretions are similar to each other in respect to the component simple sugar parts. If red cells of a specific type are brought in contact with their antibodies, agglutination of the red cells occur.

1.2. STRUCTURAL INVESTIGATIONS OF POLYSACCHARIDES

Structural investigations of polysaccharides have been attended with many problems[30]. Some of them are as follows:

1.2.1. Seasonal variation

The proportions in which the main constituents are present are affected by seasonal variation[31]. Therefore, it is necessary to ascertain the source and time of collection of the raw material before starting an investigation of the structure of polysaccharides.

1.2.2. Purification

Purification of polysaccharides from colouring matter and protein contents is very difficult. Complete removal of

nitrogen-containing substances from the water-soluble extracts of algae is very difficult.

Heterogeneity is also an obstacle in the determination of structures of polysaccharides.

The difficulties of structural studies are greatly increased in many polysaccharides containing a large proportion of uronic acid residues. 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.

A polysaccharide may be regarded as free from other polysaccharides if it may be separated by at least two suitable procedures into fractions each of which has the same properties.

1.2.3. Methods of purification of polysaccharides

Heterogeneity in polysaccharides is more common than the Homogeneity of its structure[32]. It is, therefore, the utmost importance to consider the methods available for purification of polysaccharides.

1.2.3.1.Fractional precipitation of polysaccharides

1.2.3.1.1. With alcohol or other organic liquids

Some polysaccharides, as well as their acetates, nitrates and methylated derivatives has been purified by using mainly this method of precipitation, This technique consists in dissolving the polysaccharide in water and adding ethanol gradually to effect precipitation.

The crude glucofructan may be purified by precipitation from pyridine with acetone [33]. The resulting precipitate can be filtered off and dried. The partially purified polysaccharides may be reprecipitated in the usual manner. Dimethyl sulphoxide has shown promise as a solvent for preferential extraction and precipitation[34] of polysaccharides. The arabans[35] and fructans[36] have found to respond particularly well to this type of fractionation. By careful fractionation araban was obtained practically free from other polysaccharides and by fractionation of the methylated derivative prepared from the crude polysaccharides, separation of a pure methylated araban was effected.

1.2.3.1.2. With salts

Polysaccharides can be fractionated by precipitation from aqueous solution with salts. Acidic polysaccharides such as alginic acid and pectic acid can be separated almost quantitatively from neutral polysaccharides as their insoluble

calcium[37] and barium[38] salts. The technique has been determination of alginic acid in seaweed gums[39]. 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.

1.2.3.1.3. With Copper salts

Polysaccharides containing a high proportion of mannopyranose units joined by 1⟶4-β-D-glycosidic bonds[40] have been purified widely be employing Fehlings solution technique. Upon fractionation of Iles mannan meal from the tubers of the Amorphophallus variabilis plants by adding Fehling solution B and Fehling solution A, the polysaccharide precipitates and does not give a blue colour with iodine and is found to be insoluble both in water and 0.5 N Potassium hydroxide. Most of 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.

1.2.3.1.4. With borates

The reaction of polysaccharides with sodium tetraborate (borax) is used for classification and identification purposes, it does not appear to have been used for the purification of polysaccharides in general, inspite of the fact that almost all galactomannans form gelatinous complexes with borates.

1.2.3.1.5. With aluminium hydroxide

Potato starch has been fractionated and purified[41] by means of Aluminium hydroxide.

Aluminium hydroxide, precipitated in situ, almost completely adsorb potato starch from aqueous solution. When the precipitate is boiled with water the amylose fraction passes into solution but little or none of the amylopectin dissolves. If less aluminium hydroxide is used the amylopectin is preferentially adsorbed, and an amylose fraction high blue-value remains in the superanatant liquid. Thus the linear and branched chain components of starch have been separated by using this technique.

Moreover, other polyvalent inorganic substances may be considered to be separated and purified in connection with the resolution of mixtures of polysaccharides.

1.2.3.1.6. With organic molecules

A new technique for fractionation of starch by selective precipitation with polar organic molecule as 1-butanol[42] has been developed which yield markedly purer products. The specific application of these methods is considered to corn starch, potato starch and waxy maize.

A wide variety of other agents besides alcohols may be used for the purpose including fatty acids, nitroparaffins, ketones, esters, phenols and aromatic nitrocompounds[39].

1.2.3.1.7. With other polysaccharides

Since polysaccharides having similar structures are difficult to separate, it is suggested that one polysaccharide can be used to purify another provided that the new polysaccharide complex formed can be split either by chemical or enzymic means. For instance, the use of cellulose to form a complex with amylose is reported[43] whereby it is separated from amylopectin.

1.2.3.1.8. With proteins

This is perhaps the most selective method at present available and is a considerable promise for the preparation of pure polysaccharides.

Specific polysaccharides like gum arabic, containing uronic acid groups, form complexes with proteins such as gelatin[44]. 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 purification of polysaccharides.

1.2.3.2. Separation by means of enzymes

Mixtures of polysaccharides can be resolved by destroying the undesirable components with specific enzymes. Thus the glucomannan in Iles mannan can be freed from the amylose like impurity by preferential decomposition of the latter with α or β amylose. Similarly, the wheat pentosan gum has been freed from starch by amylolysis[45].

Adams et al.[46] studied the behaviour of several yeast invertase preparations toward a series of substrates containing the β -D-fructofuranoside linkage which occurs in sucrose and also toward a number of substrates containing other types of glycosidic linkages. They have separated yeast gum from glycogen in a similar manner by autolysis.

1.2.3.3. Separation by Gel-filtration[47]

Gel chromatography is based on the decreasing permeability of the three-dimensional net work of a swollen gel to molecules of increasing size. If a solution containing a mixture of solutes of different molecular sizes is passed through a column packed with a suitable gel, the smaller molecules penetrate further into the gel pores than do the larger, and they are therefore retained for a longer time on the column. The solutes are thus eluted in the order of decreasing molecular size. Thus, this procedure affords a rapid way of separating

substances that differ in molecular size, or for fractionating polymers, such as polysaccharides, having broad molecular-weight distributions. The technique provides a means of determining molecular weights of polymers, particularly for labile biological materials.

Fractionation ranges for separation of polysaccharides by gel chromatography are conventionally given in terms of molecular weights. The fractionation ranges of sephadex gels both for proteins[48,49] and dextrans[50] are shown in the table 1.

Table 1. Fractionation Ranges of Sephadex Gels

Type of gel	Fractionation Range (mol.wt.)	
	Globular proteins	Dextrans
G-10	$\leqslant$700	$\leqslant$700
G-15	$\leqslant$1,500	$\leqslant$1,500
G-25	1,000-5,000	100-5,000
G-50	1,500-30,000	500-10,000
G-75	3,000-70,000	1,000-50,000
G-100	4,000-150,000	1,000-100,000
G-150	5,000-400,000	1,000-150,000
G-200	5,000-800,000	1,000-200,000

Theoretically, Gel-filtration should be applicable in fractionation of all water-soluble polymers.

Gel Chromatographic technique is applicable for the fractionation of dextrans[50,51], galactans, arabinogalactans[52,53] and other polysaccharides like glucan, synthetic polysucrose[54], pneumococcal polysaccharides etc.

The range of molecular weight of different kinds of polysaccharides obtained by eluting through different types of sepharose are shown in Table 2.

Table 2. Determination of molecular weight[55]

Sepharose	Approximate agarose concentration (%)	Range of mol. wt.
2B	2	10^5 —20×10^6
4B	4	3×10^4—5×10^6
6B	6	10^4 —1×10^6

1.2.3.4. Chromatographic separation

Oligosaccharides of smaller molecular weight 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 column[56] may sometimes be used.An advantage of the technique

is that the effectiveness of the separation is not affected by small variations in the composition of the developer, by the degree of dilution of the sugar solution, or by the presence of inorganic salts. An added advantage is that a mixture of substantially large quantities of sugars can be separated by the use of a single small column.

The polysaccharide associated purified invertase has been shown[57] to be identical with the mannan & yeast gum obtained by autolysis of yeast. Yeast mannan can be purified by this technique using 15 to 20% alcohol as the eluting solvent. Hyaluronic acid may be similarly purified.

1.2.3.5. 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.

Montgomery et al.[58] studied the constitution of a water-soluble hemicellulose of the endosperm of wheat. They showed that cereal gum pentosan can readily be seperated from contaminating glucans such as starch, since the pentosan acetates

are much less soluble in water than the hexosan acetates.After resolution of the polysaccharides in this way, the acetyl groups can readily be saponified and the polysaccharide recovered unchanged.

1.2.3.6. Separation by ultrafiltration

Zsigmondy[59] has developed the method of ultrafiltration using membranes or filters of controlled porous size. The method has successfully been used by Jones and Coworkers[60] for the fractionation of dextrans.

Mould and synge have reported the small scale fractionation of polysaccharides on strips of collodion membrane by electrokinetic ultrafiltration, in which the movement of polysaccharide solution was within the thickness of the membrane. The degree of fractionation achieved depends upon both the molecular weight and the shape of the polysaccharide macromolecules.

Duclauz et al.[61] explained the methods for fractionating cellulose ester. Fractional precipitation of com. nitrocellulose yields several fractions, whose solutions differ in viscosity. The low viscocity fractions may be separated by ultrafiltration or coagulation while those of high viscosity precipitates spontaneously. The technique is applicable to such cellulose derivatives as the nitrate[62] and the acetate,

but because of the coiling of linear chains it has been suggested that the method is not suitable for linear polymer[63].

1.2.3.7. Separation by Electrophoresis and Ionophoresis:

Northcote[64] studied separations of neutral polysaccharides by Zone Electrophoresis. Qualitative and quantitative microanalysis of the mixtures of neutral polysaccharides can be achieved by electrophoresis of these substances in borate buffer[65,66]. The electrophoretic separations may also be done on a macroscale for individual polysaccharides from a mixture[67] by the use of a column of the type described by Porath[68].

A partial success on the separation of polysaccharides has been done by using the Tiseleus apparatus. A pentosan constituent of pear cell walls has been purified in this process but application of this method is limited to other groups of polysaccharides[69]. However, some progress has been made in the separation of glycogen, starch, yeast mannan[70] and gum arabic by using a borate buffer.

Some polysaccharides can be separated and distinguished by the technique of Electrophoresis (Ionophoresis, Zone electrophoresis)[71] using filter paper and a borate buffer.The borate buffer and polysaccharide form a complex which is expected to move towards the anode as in the Tiseleus apparatus,but on strip supports and on the glass powder column at pH 9.3 electroendosmosis is great and results in a net movement towards the cathode.

1.2.4. Chemical and enzymic methods of structural elucidations.

The structural study of polysaccharides involves chemical[72] and enzymic methods. Some chemical methods are given below :

1.2.4.1. Hydrolysis

The usual way of determining the sugar residues present in a polysaccharide is to hydrolyse 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. Several different methods[72] are used for the hydrolysis of methylated polysaccharides. As these ethers are generally insoluble in hot water (and sometimes, even in cold water), it is usually necessary to use a non-aqueous or only partially aqueous medium for the initial hydrolysis. Treatment with methanolic hydrogen chloride and subsequent hydrolysis of the methyl glycosides formed is widely employed. Another method involves heating with concentrated or aqueous formic acid (formolysis), followed by hydrolysis of the resultant formate esters in aqueous mineral acid. Another method involves prehydrolysis in fairly concentrated sulphuric acid (about 70%) at a low temperature and then completion of the hydrolysis by dilution and warming.

1.2.4.2. Methylation

The aim of methylation is to achieve an etherification of all of the free hydroxyl groups in the polysaccharide. 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 substitutions 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 summerized 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.

Methylated polysaccharides are often purified by fractional precipitation, usually using mixtures of chloroform and light petroleum , or ether. Hirst and Jones[73] separated a

methylated arabinan from a less reactive and only partially methylated galactan, simply by acetone extraction.

1.2.4.3. 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, analogues to those used for polysaccharides, the only difference being that, within oligosaccharide, it is possible to obtain a definite solution.

Partial hydrolysis of polysaccharides produced,sometimes offers the only unambiguous method of proving the presence of some linkages, for example, the α – (1→ 6) linkages in glycogen and amylopectin.

Hamilton and Thomson, N.S.[74], suggested that, in the xylan polyuronide, the glycosiduronic linkage at "c" and the

glycosidic linkage at "a" show an increased stability toward hydrolysis, whereas the glycosidic linkage at "b" is apparently hydrolysed as a normal rate.

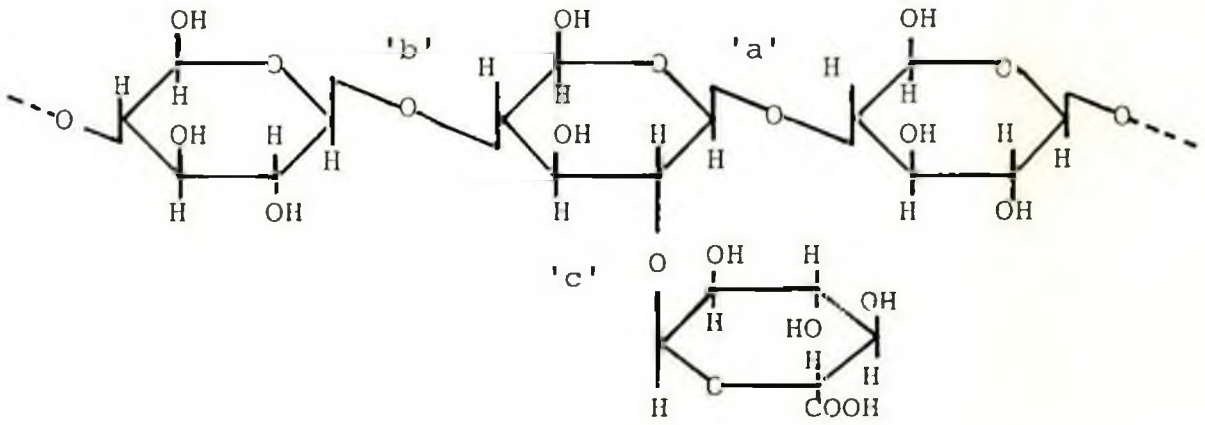


Fig. 4. Xylan polyuronide.

Aspinall and Cowokers[75] described that the primary alcoholic groups in a polysaccharide are transformed into carboxyl groups by catalytic oxidation and the aldobiouronic acids obtained on acid hydrolysis are isolated and characterized.

1.2.4.4. Periodate Oxidation

Glycol-splitting reagents, in particular periodate and lead tetraacetate are valuable tools in carbohydrate chemistry[72].

Oxidation with periodate ion, resulting in 1,2 - glycol scission, is one of the most widely used reactions in structural investigations of polymer chemistry[76]. 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 condition of the reaction is especially well adapted for application to the sensitive carbohydrate structures.

Hay et al.[77] reported that 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.

In the case of $\alpha\beta\gamma$ – triols, a double cleavage of the carbon chain occurs 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[78]. Consequently, non-reducing terminal units in a polysaccharide or (1 ⟶ 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⟶ 2) or (1⟶ 4) bonds undergo cleavage by one molecular proportion of periodate, but no formic acid is generated. Units which don't contain adjacent hydroxyl groups like non-terminal units joined by (1⟶ 3) bonds or units consisted in branching at C-2 and C-4 are unaffected 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[79,80].

Normally the polysaccharide is oxidized by a dilute resolution 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 procedures[81] or by spectrophotometric method on a micro scale[82].

Formic acid is determined by direct titration with standard alkali[83], indirectly by liberation of iodine from a solution of potassium iodide and iodate[83,84] or by a manometric procedure[85] involving liberation of carbon dioxide from a hydrogen carbonate buffer.

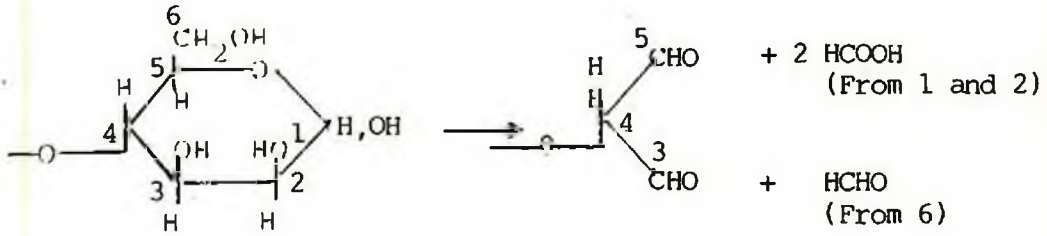
Production of formic acid may be used to calculate the degree of polymerization of a linear (1 $\longrightarrow$ 4) - linked polymer since three molecular proportions of formic acid are liberated per linear chain, one from the non-reducing end and two from the reducing end. Similarly in the case of branched polysaccharide 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$\longrightarrow$6) - linked residues within the chain invalidates this method since they too will give rise to formic acid.

The periodate oxidation of glycosides and polysaccharides is almost always accompanied by relatively slow and continuing reduction of periodate after the primary oxidation, that of α - glycol cleavage, is over. This phenomenon is most often referred to as overoxidation.[85a]

The procedure by which overoxidation occurs is most probably a combination of the hydrolysis of O-formyl ester groups formed by periodate oxidation of α - glycols and the oxidation of active methylene (or methine) groups produced in the reaction.

However, overoxidation must be avoided maintaining carefully the reaction conditions. Extremes of pH must be avoided and the rate of oxidation controlled either by using the less soluble potassium periodate or by carrying out the reaction at 0°C and in the absence of sunlight.

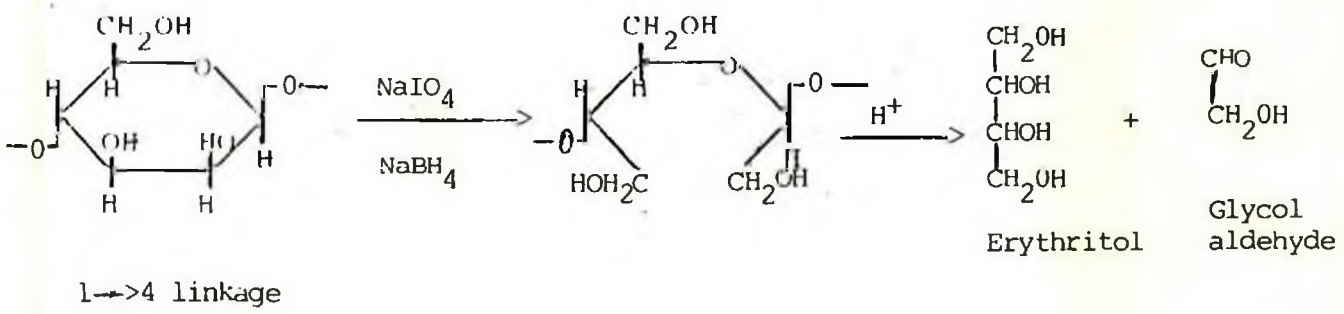
Reducing end group of a polysaccharide is attacked by periodate with the liberation (in the first instance) of a mole of formic acid and the formation of a formyl ester grouping on C_1. On hydrolysis of this, a mole of formic acid together with a mole of formaldehyde[85b] is liberated by further cleavage of the carbon carbon links (Fig. 4a). These two moles of formic acid is taken into account in the calculation of average chain-length unless very large highly branched polysaccharides are being considered when their effect is negligible.



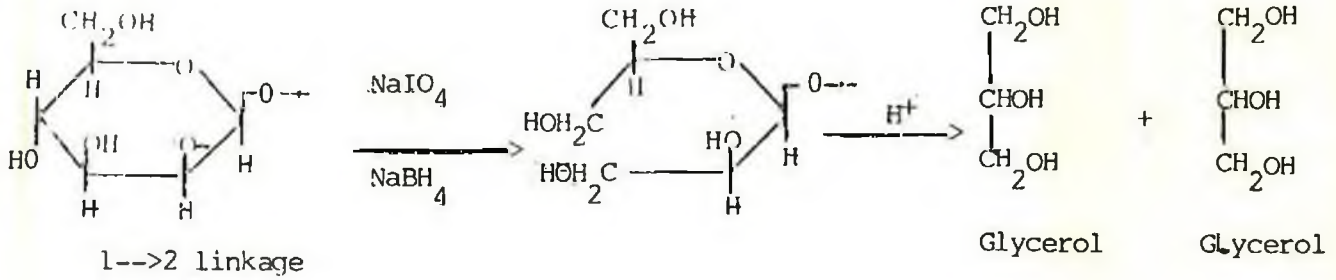
Reducing end group

Fig.4a.

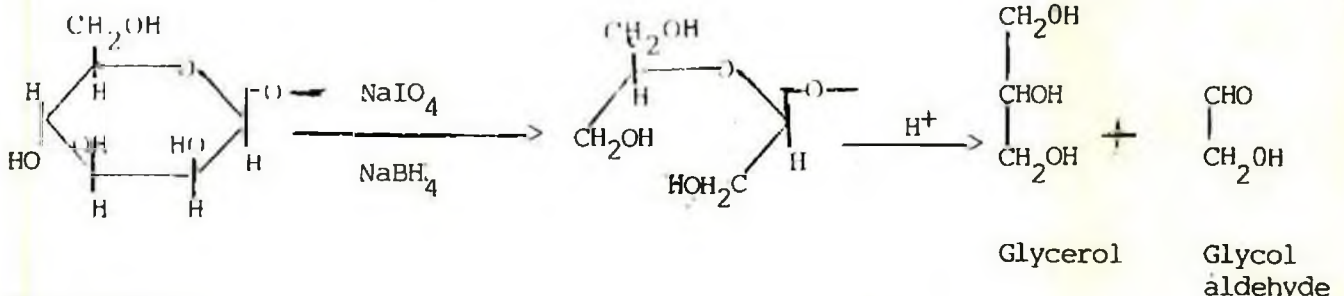
Reduction with sodium borohydride of a periodate oxidized polysaccharide followed by acid hydrolysis gives fragments[85c] that show the mode of linkage present in the original polysaccharide. Examples are shown in Fig. 4b. for some of the possibilites.



1—>4 linkage



1-->2 linkage



Nonreducing end group

Fig. 4b.

1.2.4.5. Enzymatic method

Enzymes are proving increasingly valuable in the structural elucidation of polysaccharides. The chemical methods like methylation and periodate oxidation, for the determination of the structures of polysaccharides have some apparent limitations. It is, for instance, difficult to effect complete methylation of a highly branched polysaccharide[86] and the interpretation of the results of periodate oxidation of a polysaccharide is frequently complicated by under or over oxidation[87].

Very extensive investigations have been carried out on the enzymes which synthesize and degrade both the starch components, and the general out line of their action pattern is established[88].

The complete acid hydrolysis of a polysaccharide is usually a satisfactory method for determining the component monosaccharides. For example, the presence of L-galactose is 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 inulinase 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, for instance, glucose is the sole product of "amyloglucosidase" a polysaccharase from (Aspergillus niger) action on amylase[89]. 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 example, 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. 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. The most common type of polysaccharase action involves random hydrolysis of the substrate thereby producing a series of oligosaccharides; for example, pectic acid (a polymer of gatacturonic acid) is degraded by a purified fungal polygalacturonase to give a mixture of mono -, di -, tri - and tetra - galacturonic acids.

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. For instance, amylo-pectin 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. For instance, salivary α - amylase can not 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 α - 1:6 linkage, and the products of amylolysis therefore, include isomaltose.

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[90]. If the substrate is insoluble the last two methods can be used.

Before a polysaccharase can be used in structural investigations a thorough knowledge of both its synthetic activity and synergic reactions is necessary. At the same time care must be exercised to ensure the homogeneity and purity of all enzyme preparations before utilizing them in the elucidation of the structure of polysaccharide. Moreover, precaution must be taken to avoid inadvertent degradation during isolation.

1.2.5. Spectroscopic methods of structural elucidation.

The following techniques were used for the structural determinations of polysaccharides.

a) Infrared spectroscopy
b) ^{1}H-n.m.r. spectrometry
c) ^{13}C-n.m.r. spectrometry
d) Mass spectroscopy

1.2.5.1. Infrared spectroscopy :

Infrared analysis has made useful contributions to the study of carbohydrate chemistry and is applied with due consideration for alternative interpretations of the results and that proper comparison is made with appropriate reference substances[91].

The intensity of an infrared band depends on the magnitude of the change in dipole moment associated with the molecular vibration, and accordingly strong features in infrared spectra usually originated in the vibrational motions of polar linkages such as O-H, N-H, C=O, C-N, C-Cl, etc.

Besides identification of chemical compounds, infrared spectra are capable of distinguishing polymer types and of indicating the predominant type of chain linking and the existance of chain branching[92].

In a strict comparison[93] of derivatives of D-galactopyranose and D-mannopyranose with corresponding ones of D-glucopyranose, it was found that the first two groups displayed an extra absorption peak at Ca 875 cm^{-1}, which appeared to be independent of O-substituents and of anomeric character. This absorption is due to deformations of the equatorial C-H bonds at positions 4 and 2 in galactopyranose and mannopyranose respectively. A rise of frequency from Ca 840 cm^{-1} for an equatorial $C_{(1)}$-H deformation to ca. 875 cm^{-1} for equatorial $C_{(2)}$-H or $C_{(4)}$-H deformations is consistent with the subjection of the equatorial hydrogen atoms on $C_{(2)}$ and $C_{(4)}$ to van der Waals foreces from neighbouring groups which are stronger than those affecting the equatorial hydrogen on $C_{(1)}$.

Ring vibration of α-polyglucosans are valuable particularly for identification of the point of attachment of the glycosidic linkages[92,94]. For example, α-polyglucosans of the starch class (1:4 linkages) absorb at 930 $\pm$4and 758 $\pm$ 2 cm^{-1}, while those of the dextran class (1:6 linkages) absorb at 917 $\pm$2

and 768 ± 1 cm^{-1}. Again the peak at 793 ± 3 cm^{-1} given by 1:3 branched dextrans and by the nigerian series of saccharides (α - 1:3- and α - 1:4 - linkages arranged alternately in unbranched chains) seems to arise from α - 1:3 - linkages.

1.2.5.2. ^{1}H-n.m.r. spectrometry

Nuclear Magnetic Resonance (nmr) gives information about the number of each type of hydrogen in a molecule. It is also concerned regarding the nature of the immediate environment of each of these types of hydrogen atoms[95].

There is an environmental influence on the resonance characteristics of a proton in the presence of other protons in its neighbourhood. These can have their spins aligned with or against the applied field, and consequently enhance or diminish it causing multiplicity in the resonance patterns observed. Valuable stereochemical information can come from this since the splitting is directly dependent on a known way on the angle subtended by two C-H bonds. Splitting is at a maximum of 6-10 H_Z when the dihedral angle is 180°. If coupling constants are found within this range then the anomeric proton is axial and C-1 has β — configuration if the C-2 proton is axial. This is true for glucose, xylose, galactose, arabinose and the common sugars found in polysaccharides.

The ^{1}H-nmr. spectrum can not only distinguish how many different types of protons a molecule has, but it can also reveal how many of each different types are contained within the molecule.

1.2.5.3. ^{13}C-nmr. Spectrometry

In ^{13}C-nmr. spectroscopy, we observe the carbon skeleton directly and thus we find peaks arising from all of the carbon atoms, whether they bear hydrogen or not. One of its great advantage is the wide range of chemical shifts over which C-13 nuclei absorb. Here, signals from organic compounds spread over a chemical shift range of 200 ppm, compared with a range less than 20 ppm, in proton spectra.

^{13}C-nmr. spectroscopy has proven to be one of the most efficient methods for structural, configurational and conformational investigations in carbohydrate chemistry.

^{13}C spectra allow one to determine the number of different carbons in a molecule and a recognition of equivalences or symmetry elements[95]. For instance, cyclooctatetraene readily dimerizes to a solid. Two possible structures can be proposed for this dimer :

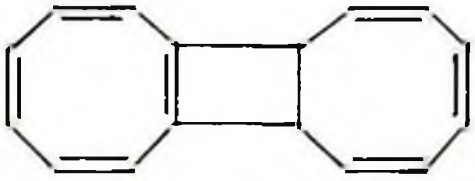


Fig. 5.

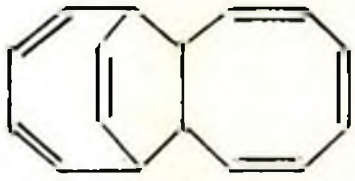


Fig. 6.

The proton decoupled ^{13}C spectrum of the dimer shows only four sharp lines. This effectively proves structure 1 (Fig.5) which having two planes of symmetry, fits rather well.

Structure 1 (Fig.6) could fit only under the conditions in which several different types of carbons accidentally had the same chemical shift.

Applications of ^{13}C-nmr spectroscopy also involve isotopic tracer or labeling studies, the study of reaction mechanisms and conformational analysis.

1.2.3.4. Mass spectroscopy

Mass spectrometry measures the mass to charge ratio of organic ions created by electron bombardment. It gives structural information from the moderately predictable fragmentation of organic molecules; the masses of the fragment ions can often be related to likely structures. The technique helps to record the molecular weight by measurement of the mass to charge ratio (m/z value). The molecular ion breakes down into charged fragments whose structures can be related to the structures of the intact molecule. The technique is quite importtant in structure elucidation studies.

1.3. STRUCTURES OF SOME POLYSACCHARIDES

1.3.1. Mannans

The polysaccharides which yield only mannose on hydrolysis are mannans and these are found in ivory nut, seaweeds, yeast, etc.

1.3.1.1. Mannan of vegetable Ivory

Mannan from vegetable ivory, commonly known as ivory nut mannan[96], was isolated first by extraction with cold sulphuric acid[97] in 1989.

Two types of mannan are isolated from ivory nuts (phyletephas macrocarps) and termed as mannan A and mannan B. Mannan A is extracted with alkali and mannan B which can not, however, be extracted directly, is separated from cellulose by precipitation from cuprammonium solution[98]. Meyer and Mark proposed [99] a structure consisting of a linear chain of D-mannose units linked in β-1, 4 fashion.

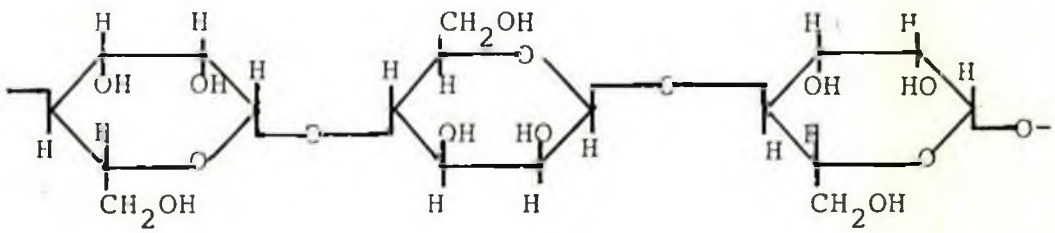


Fig. 7.

A flexible, tough paper can be formed by the addition to cellulose pulp of mannan meal previously treated with ammonia[100].

1.3.1.2. Salep mannan

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. Pringsheim et al.[101] have first prepared salep mannan in 1924. Husemann[102] recommends preparing the compound by boiling the bulbs with alcohol followed by water extraction and precipitation with methanol.

Acid hydrolysis of the methyl mannan yields 2,3,6 trimethyl-D-mannose (84%) and 2,3,4, 6 - tetramethyl-D-mannose (1.7%), indicating a 1,4 union of the D-mannose residues.

1.3.1.3. Mannans of wood

The mannan is a true polymer of D – mannose that exists in wood. Wise and Ratliff [103] have studied the composition of slash pine and black spruce (Table 3) and found that those hemicellulose fractions which are least soluble in alkali should offer the best starting material for its isolation.

Table 3 Mannan content of the various individuals components of slash pine and black spruce

Fraction	Slash pine %	Black spruce %
Hemicellulose – A	11.5	9.3
Hemicellulose – B	27.2	26.4
Hemicellulose – C	45.2	44.9
α – Cellulose	12.2	11.9

It was suggested[104] that the greater-swelling properties of soft wood pulps as contrasted to hard wood pulps may be due to the large mannan content (11-12%) of the former. In general, it may be said that the hemicellulose, when present in reasonable amounts, has a beneficial effect on pulp strength[105].

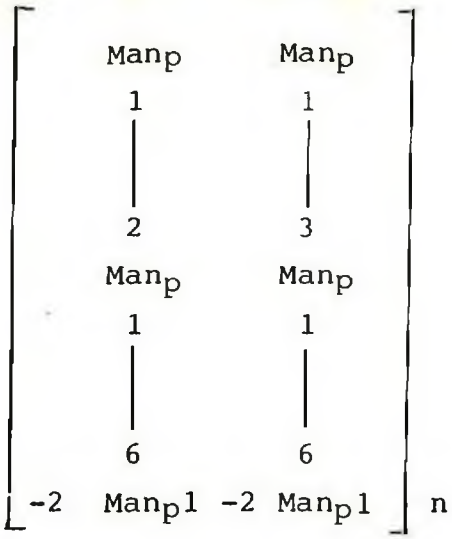
1.3.1.4. Mannan of Porphyra umbilicalis

A polysaccharide isolated[106] from the red algae Porphyra umbilicalis is composed of D-mannose units united by β-1→4 linkages. This is the first reported occurance of a mannan in seaweeds, which is of the branched chain type with about one branch for each 12 D-mannose residues.

1.3.1.5. Yeast mannan

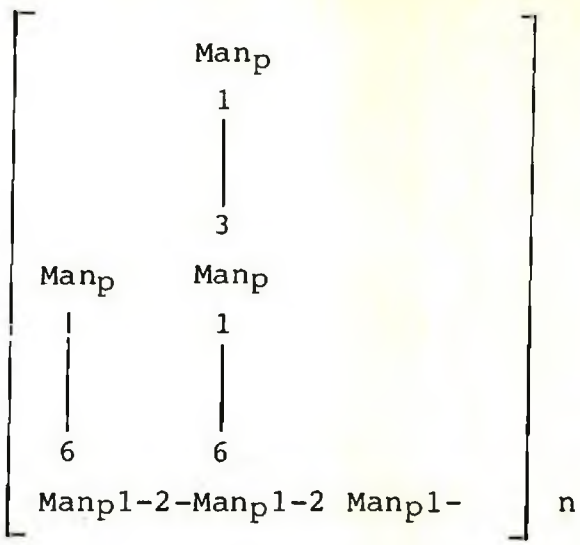
Yeast mannan, commonly called yeast gum, was first isolated[107] in 1894. Yeast mannan and glycogen are the polysaccharide constituents of the yeast cell. It may be extracted from the cell membrane of baker's yeast by boiling with 6% sodium hydroxide solution for 8 hours.

Haworth, Heath and Peat proposed[108] the following three possible formulae in which Man P represents a D-mannopyranose residue and n is equal to 30-60.



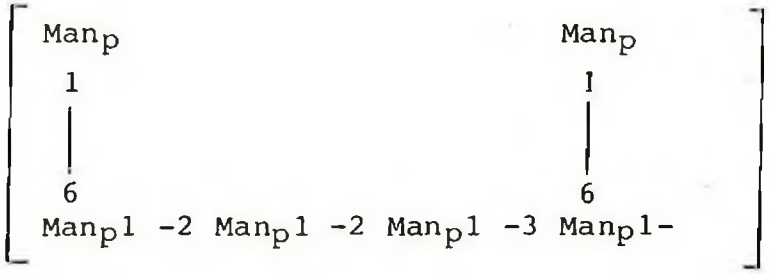
(I)

Fig. 8.



(II)

Fig. 9.



(III)

Fig. 10.

Methylation and hydrolysis of these polysaccharides yield 2, 3, 6-tri-O-methyl-D-mannose as the main product[109], while partial acetolysis followed by deacetylation yield a β-D- (1→4) - linked disaccharide (mannobiose), mannotriose, and higher homologs[110]. Thus the D-mannans must be linear chains of D-mannopyranose residues linked by β-(1→4)-glycosidic bonds (Fig. 11).

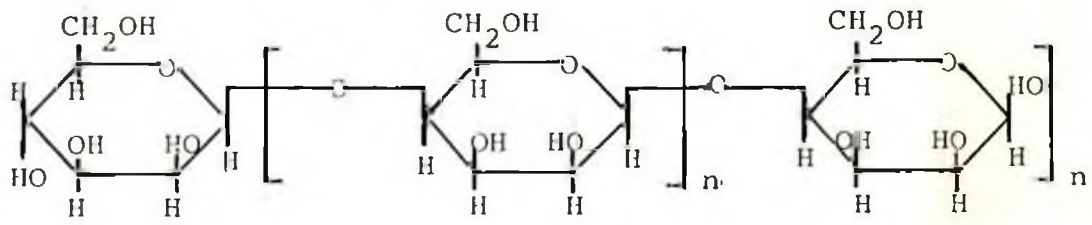


Fig. 11.

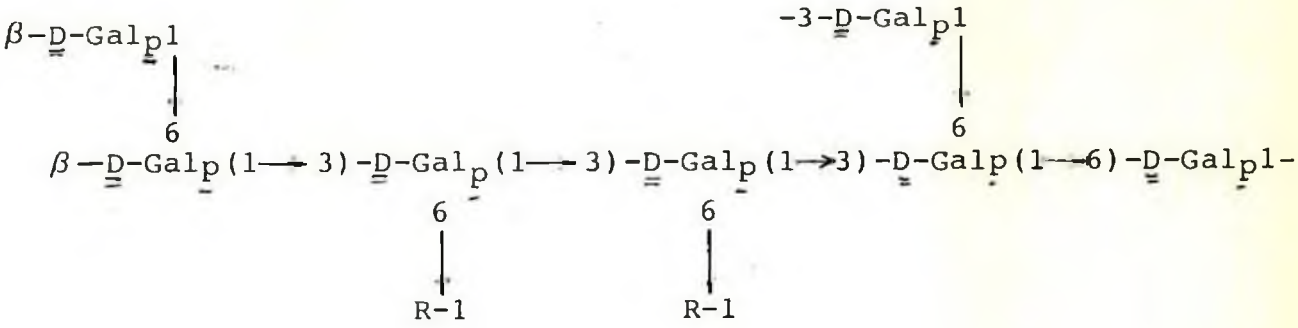
D- Mannans have been defined[111] as polysaccharides containing 95% or more of D - mannose residues. D - Mannans isolated from different sources have the same general structure[112] but they appear to differ in chain length.

1.3.2. Galactans

The polysaccharides which yield only galactose on hydrolysis are galactans and these are found mainly in the plant world. The true galactans known in land plants, namely, those from Lupinus allus pectin and from strychnos nux vomica seeds contain linear chains of β (1 —→4) - linked D - galactopyranose units[113,114].

" A branched galactan was isolated[114a] from garlic (Allium sativa) bulbs after extensive fractionation procedure. Methylation analysis indicated a considerable degree of branching of the (1 —→4) - linked chains of galactosyl residues at position 6.

White [115] suggested that the arabinogalactan from larch wood contains (1 —> 3) - linked, (1—> 6) - linked D - galactose and the L - arabinose units attached to C-6 of a (1 —> 3) - linked D - galactose.



Where R = D-Gal p or L - Ara f.

Fig. 12.

Aspinall and Wood[116] reported that an arabinogalactan present in the Scots pine wood is 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.

Hirst and Co-workers[117] isolated an arabinogalactan from European larch, but concluded that the product was not homogeneous. The polysaccharide is a highly branched polymer, containing a framework 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. Aspinall et al.[118] have shown an additional evidence for the attachment of the D-galactopyranose and 3-O-β L- arabopyranosyl - L - arabofuranose side chains obtained by catalytic oxidation of the arabinogalactan[118].

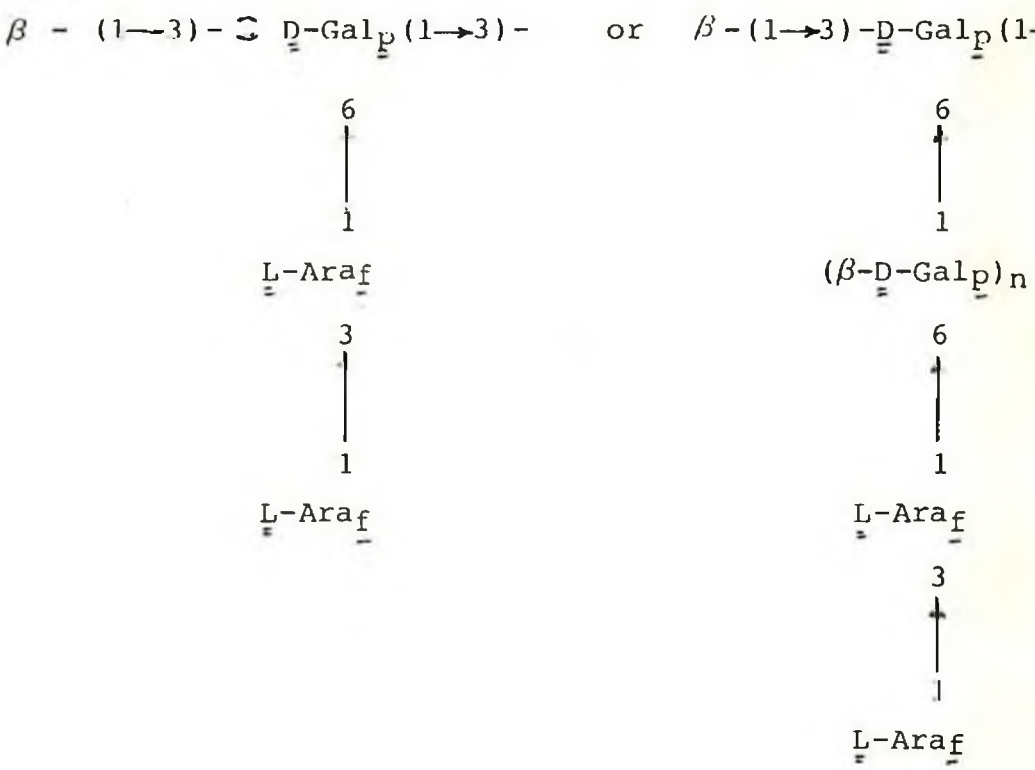


Fig. 13.

Adams and Co-workers[119,120] studied the arabinogalactan present in the wood of tamarak (Larix laricina) and found that this polysaccharide contains more L-arabinose residues than most arabinogalactans from larch wood[119]. 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.

1.3.3. Galactomannans

Galactomannans are branched polysaccharides composed of D- galactose and D - mannose and are commonly found in the endosperms of the Leguminosae where they serve as reserve foods. They are water-soluble and form thick, highly viscous solutions and classified as plant mucilages.

The most promising industrial source of galactomannans is the annual crop guar[121]. Pure galactomannan can be prepared from the crude commercial flours by fractionation from water[122]. Galactomannan from alfalfa seed can be prepared [122,123]by extraction of the ground seed with 10% potassium hydroxide solution and ethanol precipitation of the dissolved gum.

Sedimentation experiments indicate a molecular weight of 310,000 for a locust bean galactomannan[124] and osmotic pressure measurements a molecular weight of 220,000 for guaran triacetate[125]. Galactomannans have many properties which make them useful in a wide variety of applications.They are used both as highly viscous and transparent films (strong and pliable). Locust bean gum is widely used in the textile industry as a size and as a finishing agent. It is also important in the preparation of printing and dying mixtures. Guaran is found to be important[126] as a tub size for paper.

In the food industry, locust bean gum is used in the production of ice cream powders, pudding powders, preserves, jams, salad dressings and in many other applications where a thickening agent is desirable.

Other uses include the preparation of pharmaceutical and cosmetic jellies and solutions. Treatment of the galactomannan in 20% sodium hydroxide solution with acrylonitrile introduces 0.9 - 1.8 cyanoethyl ether groups per anhydrosugar unit and produces a product of interest as a thickener and film former[127].

1.3.3.1. Galactomannan from guaran

Heyne et al.[122] reported that the purified polysaccharide isolated from guar seed endosperm contains 64% D-mannose anhydride and 36% D-Galactose anhydride. Methylation[128] and periodate oxidation[129] revealed the structure that best fits the data is one in which all D- mannose units in the chain are connected by β - glycosidic linkages (Fig. 14).

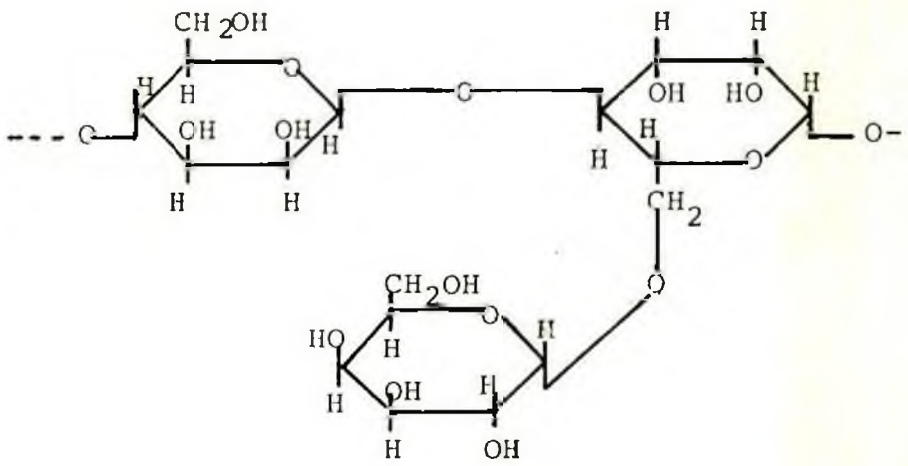
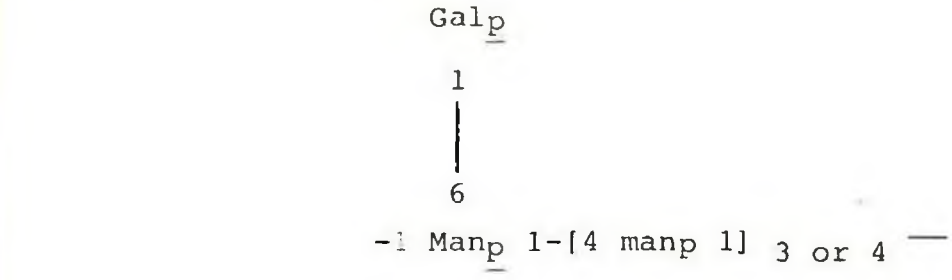


Fig. 14.

It is interesting to note that extensive acetolysis of guaran leads to the production of aldehydo - D - galactose heptaacetate which may be isolated[130] in a yield of about 3%.

1.3.3.2. Galactomannan from carob or locust bean gum

Locust bean gum seemingly differs from guaran only in the number of D - galactose units attached as side chains.The most probable molecular structure of the polysaccharide is one which resembles that of guaran except that there are fewer unit side chains[131]



(Fig. 15)

1.3.3.3. Galactomannan from alfalfa seed gum

The diheteroglycan of alfalfa (Lucerne) seed is apparently unusual among known galactomannans by containing D-galactose and D-mannose units in the ratio[132] of 2:1. Nevertheless, the polysaccharide differs, from the other galactomannans by having only half of the D-galactose as single unit side chains or chain ends while the other half appears to be joined in the molecular chain through hydroxyls on carbon atoms C_1 and C_3. The D-mannose units seemingly constitute branch points with linkages through hydroxyls on carbon atoms C_1, C_2 and C_6.

1.3.3.4. Galactomannan from coconut[133]

Rao et al.isolated a water soluble galactomannan from coconut . It was found to contain D-galactose and D-mannose in the molar ratio of 1:2.

1.3.3.4a. Galactomannan from the seeds of Cassia.

Gupta et al.[133a] isolated a water-soluble galactomannan from the seeds of Cassia alata Linn. The polysaccharide was found to be composed of heptasaccharide units joined by β -(1—> 4) linkages.

1.3.4. Glucans

The water soluble cereal gums[134,135,136] from barley and oats contain β - glucans as major components. Hydrolysis of methylated barley β-glucan yields approximately equal proportions of 2,3,6 and 2,4,6 - tri - O - methyl -D-glucose, thus indicating the presence of linear chains of β-D-glucopyranose residues with (1→4) and (1→3) linkage in equal proportions[137] Oat β-glucan is constituted similarly, but contains (1→4) and (1→3)-linkages in the proportions of 2:1[138] or 3:1[139].

Partial hydrolysis of the polysaccharides yielded cellobiose, laminaribiose, cellotriose and two trisaccharides shown in figures 16 and 17 containing both (1→3) and (1→4)-linkages[139]. On the basis of these results, the following partial structure (Fig. 18) has been proposed for the oat β-glucan.

β - D-Gp-(1→3)-β-D-Gp(1→4)-D-Gp

(Fig. 16)

β-D-Gp-(1→4)-β-D-Gp-(1→3)-D-Gp

(Fig. 17)

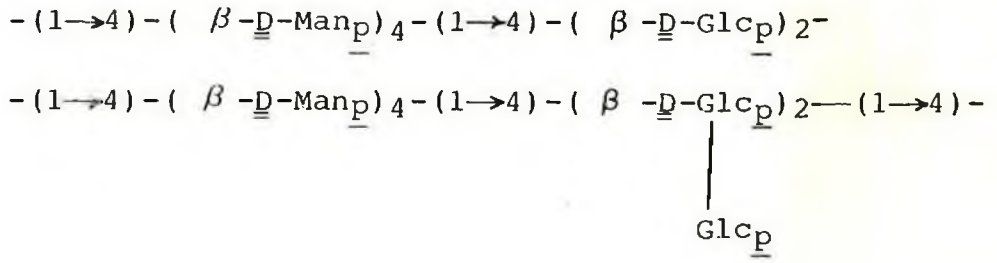
..... 3-β-D-Gp-(1→4)-β-D-Gp(1→4) β-D-Gp-(1→3)-

β-D-Gp-(1→4)-β-D-Gp.....

(Fig. 18)

1.3.5. Glucomannans

Wood of the gymnosperms contains a larger proportion of D-gluco-D-mannans than that of angiosperms. The D-gluco-D-mannan, which can be separated as its insoluble copper complex, has been shown by methylation analysis[140] to contain linear chains of (1→4) -linked β-D-mannopyranose (70%) and β-D-glucopyranose (30%) residues. A partial structure of the polysaccharide is shown as follows (Fig. 19).



(Fig. 19)

Similar D-gluco-D-mannans are present in iris seeds[141], lily bulbs and bluebell seeds[142].

1.3.6. Galactoglucomannans

The wood of all gymnosperms contain a low-molecular weight water-soluble polysaccharide composed of D-galactose, D-glucose, and D-mannose residues in the ratio of 1:1:3. The water-soluble galactoglucomannan in soft wood was first

isolated by Hamilton and co-workers[143] in 1958. The galacto-glucomannan obtained in a yield of 0.5% of the original wood had a specific rotation of $+5^{o}$ to $+12^{o}$ in water.

The structures of the oligosaccharides obtained on partial hydrolysis (Table 3)[144,145], show that the linkages in the backbone are β -D-(1→4) and that D-galactose is linked to D-mannose and also to D-glucose residues, by way of α -D-(1→6)-glycosidic bonds.

Table 4. Oligosaccharides obtained from D-galacto-D-gluco-D-mannan

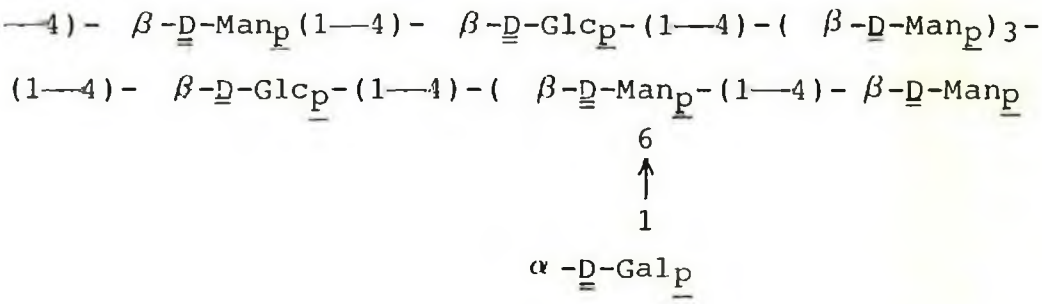
Disaccharides	Trisaccharides	Higher oligosaccharides
M→M	M→M→M	M→M→M→M
M→G	M→M→G	G→M→M→M
G→M	M→G→M	Gal→M→M→M
G→M	M→G→M	Gal→M→M→M→M
Gal→M	G→G→M	
	Gal→M→M	

Key : G→ : β - D-glucopyranosyl-(1→4) -;

M→ : β - D-mannopyranosyl-(1→4)-;

Gal→ : α -D-galactopyranosyl-(1→6)-.

The D-galacto-D-gluco-D-mannans are subjected to mild bydrolysis [146] and also partially O-acetylated. The general structure of these polysaccharides is similar to the following structure (Fig. 20) for the soft wood D-gluco-D-mannans.



(Fig. 20).

They differ, however, in being of lower molecular weight and in having a relatively large number of D-galactoside chains.

1.3.7. Starches

Carbohydrates serve as reserve foods stored 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; [147] one a linear, thread-like molecule termed amylose and the other branched or bush-shaped structure termed amylopectin.Starch granules vary in size and shape depending on the source.

Evidences regarding the manner in which D-glucose units are joined in the starch components has been obtained largely from

hydrolysis, methylation and periodate experiments.

Complete hydrolysis of starch yields D-glucose quantitatively in the form of α-glucosidic type. Optical rotatory considerations and comparisons of the rotations of methylated starches to the rotations of methylated D-glucose, maltose, maltotriose and maltotetrose also suggest the presence of α-1, 4-glucosidic linkages.

Studies of methylation, periodate oxidation and partial hydrolysis conclude that the amylose contains 1-4, linked α-glucopyranose units and has a degree of polymerization of atleast 250 units (Fig. 21) 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 for each 25-27 units (Fig.22) [148].

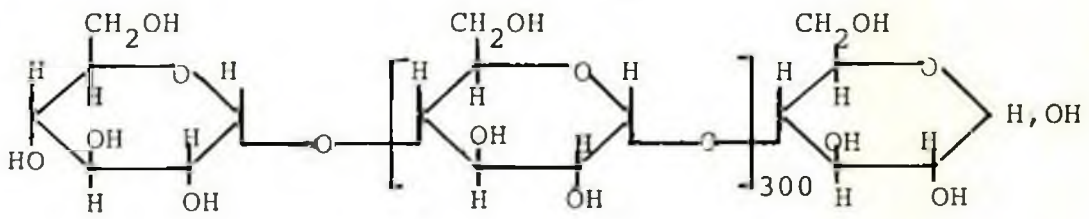


Fig. 21.

The isolation of 2,3-di-O-methyl -D-glucose from amylopectin indicates that the branch chain is linked through C-6 of a glucose unit. Hydrolysis of amylopectin with diastase (β-amylose) gives a 55% yield of maltose.

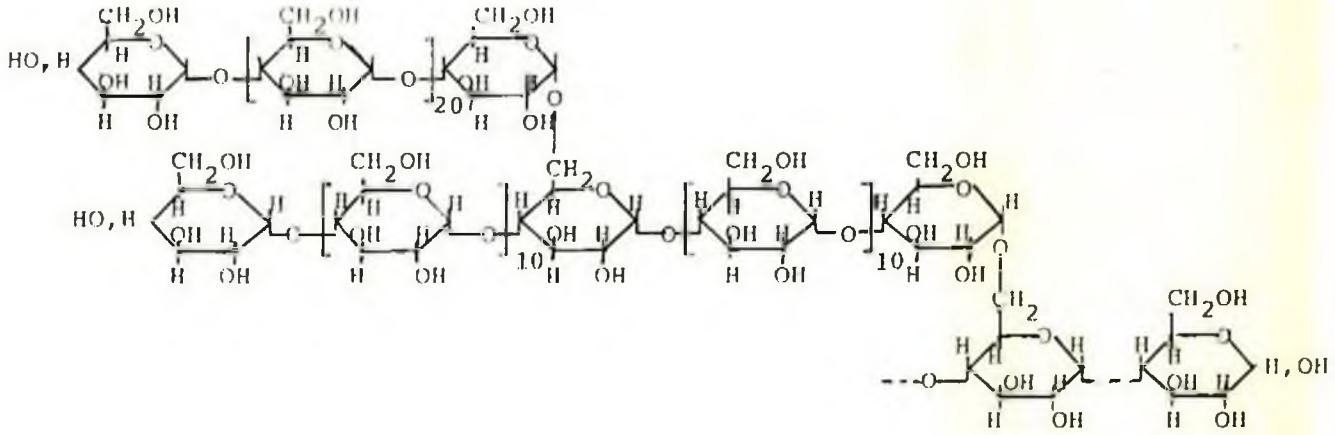


Fig. 22.

1.3.8. Pectic substances

The pectic triad of polysaccharides or in otherwords, pectic substances consists of galacturonan, araban and galactan of which the galacturonan is a linear polymer of α-1,4-linked D-galacturonic acid units either fully or partially esterified.

Pectic substances have been detected mainly in beech wood [149], box wood [150], black locust [151], lemon, mesquite, white pine [152], cotton wood [153] and in all higher plants with a few exception.

Acid hydrolysis of pectin leads to D-galacturonic acid, D-galactose and L-arabinose and in some cases minor proportions of L-rhamnose [154-6], D-xylose, L-fucose [157], D-glucose [156], 2-O-methyl-L-fucose [157] and 2-O-methyl-D-xylose [157].

In some pectins galactose occurs as a β-1, 4-linked galactan [158] and arabinose as the highly branched arabinan [159-61].

1.3.9. Cellulose

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

Acid hydrolysis gives a quantitative yield of D-glucose. Hence cellulose is a homoglucan.

Acetylation, nitration and methylation show that the $C_6H_{10}O_5$ unit possesses three free hydroxyl groups.

Hydrolysis of fully methylated cellulose gives 2,3, 6-tri-O-methyl glucose (86%) and 2,3,4,6-tetra-O-methyl 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 of cellulose with acetic anhydride and sulphuric acid gives a 50% yield of octa-O-acetyl cellobiose (Fig. 23). Thus it is possible that cellulose is made up of a chain of cellobiose units, but these could be linked to each other by α-or β-glycosidic links, giving rise to products of structures shown in Fig. 24 and 25.

Haworth showed that when fully methylated cellulose is subjected to acetolysis at $15^{o}C$ for 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.

So it is clear that cellulose consists of D-glucopyranose units linked through C_1 and C_4 by β-links (Fig.24) and that chain is linear and atleast 100-200 units in length.

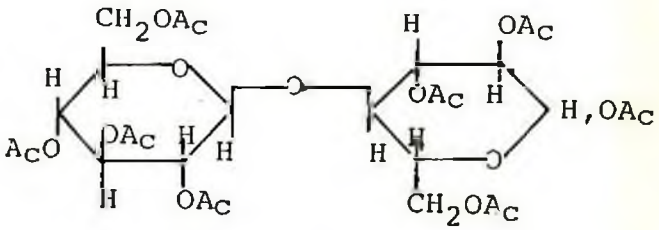


Fig. 23.

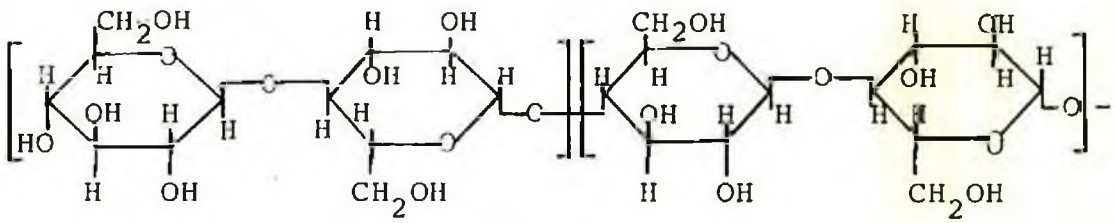


Fig. 24.

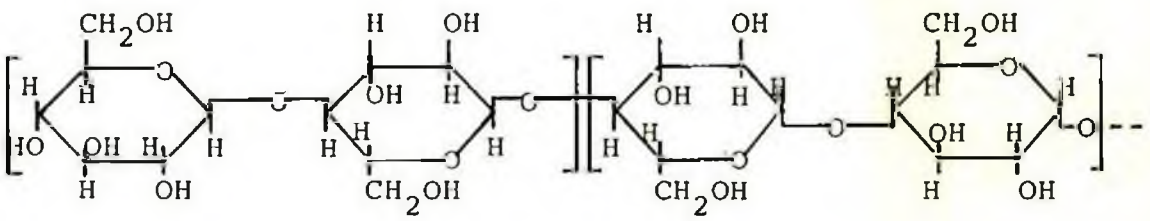


Fig. 25.

1.3.10. Hemicellulose

Hemicelluloses are found in association with the cellulose of cell walls and can be extracted with dilute aqueous alkali.

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.

In general, hemicellulose fraction can contain a number of different polysaccharides; the most commonly found sugars in these are D-xylose, D-glucose, D-galactose, D-mannose and D-fructose, L-arabinose, D-glucuronic acid, D-galacturonic acid and D-mannuronic acid.

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.

1.3.11. Xylans

Xylans are pentosans composed either entirely or almost entirely of D-xylose units. Some are linear and some have a branched structure; in others L-arabinose or D-glucuronic acid are linked as side chains.

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 [162]. The pure xylan, obtained by alkaline extraction of C. filimormis[162], 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. filimormis consists essentially a linear chain of 1,3-linked xylose units. Enzymic studies [163] on H. Cuneata xylan confirmed the presence of 1,3-linkage and the absence of 1,4-linkage.

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 may be attached as side chains [164]. Algal xylans contain β-1,3-D-xylopyranose units in addition to the usual 1,4-linked residues [165].

Studies of 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.

Extensive studies [166-9] of xylan from Rhodymenia palmata, a species of red algae confirmed to have a linear structure [170] the chain containing β-1,4-linked and β-1,3-linked D-xylose units.

$$-4X(1\longrightarrow 4)X(1\longrightarrow 4)X(1\longrightarrow 3)X(1\longrightarrow 4)X(1\ -\ )$$

X = β-xylopyranosyl

Fig. 26

On the otherhand, xylans from Porphyra umbilicalis and Bangia fuscopurpurea are said to be built up entirely of 1,3-linked xylose units [171].

1.3.12. Arabans

Araban is a pure polymer of L-arabinose and is widely distributed throughout the plant world. It is a member of the pectic triad and is found in nature wherever pectic substances occur, very likely it 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.

Ehrlich and co-workers [172] have examined araban prepared from pectin or plant tissue but the most extensive investigation of the isolation and characterization of araban has been made by Hirst and Jones [173-7].

Structural investigations by acid hydrolysis of araban leads only to L-arabinose [178] which may be obtained crystalline in yields [179] of 96%. Methanolysis of the methylated product gives equimolar quantities of methyl 2,3,5-tri-O-methyl-L-arabofuranoside, methyl 2,3-di-O-methyl-L-arabinoside and methyl 2-O-methyl-L-arabofuranoside. From these observations along with specific rotation, the general type of structure in the araban was suggested [177] as follows :

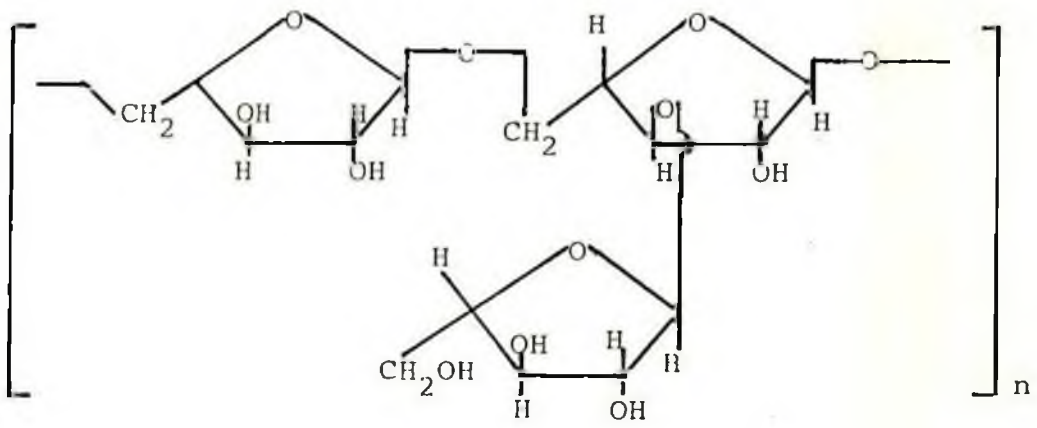


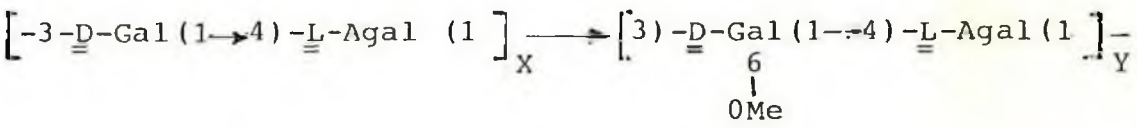
Fig.27. Possible structure of araban.

1.3.13. Agar

Agar is a polysaccharide of seaweed and is widely used as a gel-forming agent in media for culturing microorganisms.

The agar-type of polysaccharide is composed of 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. Structual investigations like partial hydrolysis[180], methylation[181-2] and enzymic studies[180] depicted the following general formula :



Gal = galactopyranose

Agal = 3,6-anhydro galactopyranose

Me = Methyl.

Fig. 28.

Agaropectin is possibly a mixture of polysaccharides containing mainly 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 agaropectin from G. amansii and G.subcostatum [183] Ahnfeltia plicala also contains L-arabinose units [184].

1.3.14. Alginic acid

Alginic acid is a polyuronide of the brown seaweed.

Fischer and Dorfel [185] reported that guluronic acid in addition to mannuronic acid is present in alginic acid from various species of brown algae. They were able to separate two crystalline lactones [186] 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.

Hirst et al.[187] have isolated 4-O- β-mannosylgulose and β-1, 4-mannobiose from reduced sample of alginic acid and characterized them. These results showed that atleast some of the polymeric molecules contain both mannuronic and guluronic acid residues and also that mannuronic acid residues are adjacent.

2. AIMS OF THE PRESENT STUDY

Borassus flabellifer Linn.[188] (Bengali:Tal) is a very tall dioecious palm, trunk stout and unarmed. It is cultivated throughout the plains of Bangladesh, India, Burma, Sree Lanka and Malayasia. It is also distributed in the tropical Africa. The palm tree attains a height of 40-60 ft. (sometimes even 100 ft.) and a girth of 3½-7 ft. The stem is black, erect, rarely branched and consists of hard outer portion which contains stiff longitudinal fibres. The central portion of the stem is called pith which is soft and starchy. The leaves are 6-10 ft. in diameter, palmately fan-shaped and rigidly coriaceons segments of 2-4 ft. The spedices are very large, simply branched and very stout. The fruit is a kind of drupe which is large and fibrous containing usually 3 to 5 nut like portions each of which encloses a seed when the fruits are tender, the seeds consist of a soft sweet gelatinous pulp with a little liquid in them. These are much relished in summer season. The tender pulp gradually hardens into a bony kernel and develops a fibrous coat Fig.(29 and 30).

The wood of the trunk is very hard and durable and resists salt water; it is also used for rafters, well-sweeps etc.[189]. Leaves are used as thatch and made into Olas or writing 'paper' sheets. Palmyra fibre collected from the base of the leaf is used for making brushes etc. The split leaves are woven into mats, baskets etc. The fruit is eaten roasted

and the inflorescence is tapped for toddy from which sugar or jaggery is made as well as vinegar etc. The young seedlings are also eaten and yield a good flour when ground, and there are many other uses.

Almost all parts of the palm are used medicinally[190]. The young plant is considered a cure for gonorrhoea, dysentery and bilious condition. The juice of the leaf stalk and of the young root is administered for checking hiccuh and for relief of gastric catarrh. The extract of the green leaves is taken internally in secondary syphilis. The light brown, downy bloom covering the outside of the base of the leaves is applied as a styptic over superficial wounds. The root is considered cooling and restorative a good diuretic and anthelmintic; it is given in gonorrhoea, a decoction of the young root is used by some in diseases of the respiratory tract. The ash obtained by burning the dry flowering stalk is useful as an antacid in heartburn; it is also prescribed as an antipyretic and in bilious affections. It is considered also useful in enlarged spleen and liver. Some consider that the flowering stalk is useful as an antithelmintic, diuretic and antithermic; it is also used in certain abdominal affections. A decoction of the bark with the addition of a little salt is used as mouth wash for strengthening gums and teeth; the charcoal made out of the bark is used as a dentrifice.

Fig.29. Palm fruit.

Fig.30. Palm seed kernel.

Palm sugar prepared from the juice of the flower stalk is considered an antidote in case of poisoning; it is antibilious and alterative; it is administered in disorders of the liver. Sugar candy prepared from this sugar is used in cough and pulmonary diseases.

A survey of literature revealed that in 1956 Subrahmanyan et al.[191] reported the nature of free sugars and of polysaccharides present in the soft kernel of the palm seeds. The tender kernels contained free sugars : namely sucrose (0.38%), fructose (1.46%) and glucose (3.21%). The purified polysaccharide-a galactomannan, extracted with 10% sodium hydroxide and precipitated with alcohol from soft kernel gave, on hydrolysis D-galactose (27.69%) and D-mannose (78.32%). However, no further investigations were carried out on its structure.

In 1961 Mukherjee et al.[192] prepared a water-soluble polysaccharide by precipitating the aqueous extract of the soft kernel of the green palmyra palm nut (Borassus flabellifer Linn.). It was shown to be a galactomannan containing galactose and mannose in the molar ratio of 1:2:4. They proposed the following structure for the galactomannan.

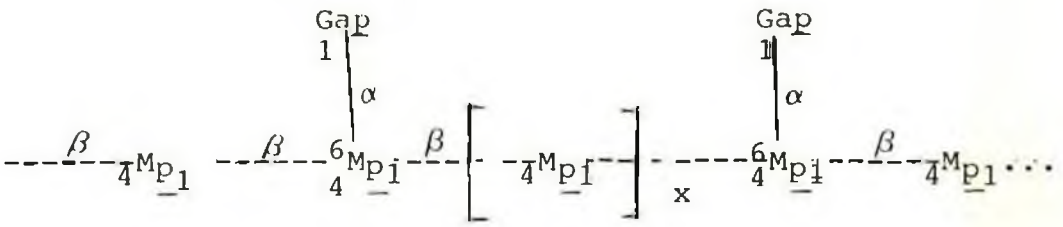


Fig. 31.

Where x has the value of 1 in one segment and 2 in the next, Gap = galactopyranose residue and M_p=mannopyranose residue.

Subsequently Rao and Mukherjee[193] extracted from the kernel of palmyra palm nut a galactomannan with 4% alkali and a mannan with 18% alkali.It was reported that the percentage of the mannan increased with the age of nut. From different study the mannan was shown to be considerably branched and the non-reducing ends of the molecule consisted of mannopyranose residue, the backbone of the molecule was made up of 1→4 linked mannopyranose residue and mannose residues were linked through C_1,C_4 and C_6.

Mukherjee et al.[192] further reported that the galactomannan obtained from palm kernel was similar in the essential structure of those obtained from fenugreek seed[194], carob seed[195,196] and guar seed[197,198] but differed from that obtained from the kernel of coconut[133].

The aims of the present investigation were to study further the polysaccharides of the matured palmyra palm kernel using convensional and spectroscopic methods. For this purpose purified homogeneous polysaccharides were prepared from water, 3% alkali and 10% alkali extracts of the kernel and studied. The results of the investigation are presented in this thesis.

3. EXPERIMENTAL

3.1. GENERAL PROCEDURES

3.1.1. Determination of ash contents

Ash contents were determined by ignition of samples (Ca. 50-100 mg.) using a platinum crucible in a muffle furnace until a constant weight was obtained.

3.1.2. Centrifugation

All centrifugations (LABOFUGE-A, West Germany) were carried out at 4000 r.p.m.

3.1.3. Determination of moisture contents

Moisture contents were determined by heating of samples in an electric oven at 105 - 110^{o}C until constant weight was obtained.

3.1.4. Measurement of optical rotation

Optical ratations were measured in a Perkin Elmer-141 instrument having a polarimeter tube (lenghth 1 dm) at room temperature. Water was used as solvent unless otherwise stated.

3.1.5. Evaporations and concentrations

Evaporations and concentrations were carried out under reduced pressure using rotary vacuum evaporator at bath temperature not exceeding $40^{o}C$.

3.1.6. Test and determination of uronic anhydride

Carbazole test

Reagents: (a) Sulphuric acid reagent - Sodium metaborate (0.95 g) was dissolved in concentrated sulphuric acid (100 ml) and (b) Carbazole reagent - Carbazole (62.5 mg) was dissolved in ethanol (50 ml).

Sulphuric acid reagent (3 ml) was added to 500 μL of sample solution. The mixture was shaken and heated in a boiling water bath for 10 min. It was cooled and carbazole reagent (100 μL) was added to it. The mixture was again shaken and heated in a boiling water bath for 15 min.

Uronic anhydride was determined by the decarboxylation method of Johansson et al.[199], which is as follows :

The sample (10 - 75 mg) was placed in a flask together with 12% hydrochloric acid (25 ml) and some porous substance. The flask was connected to a condenser which was connected

with the scrubber (filled with water) and finally with an absorption tube. A stream of nitrogen washed with alkaline pyrogallol solution (5%) was passed through the apparatus at a speed of about 10 ml/minute. After 20 minutes at room temperature the flask was heated to 80°C by means of an electrically heated paraffin oil bath and kept there for 10 minutes. Then 25 ml of barium hydroxide solution was introduced into the absorption tube. During this process the tip of the burette was introduced through the socket in the top of the tube which was then fitted with the glass capillary stopper.

The temperature of the bath was then raised to 145°C and kept for three hours. Then the heating bath was removed and ammonium chloride (10 ml) was added to the absorption tube. The contents were mixed by bubbling for 2 minutes after which the tube was disconnected and the precipitate was filtered through a glass funnel with a sinctered disc. The tube and the precipitate were washed with 50% aqueous ethanol. The precipitate remaining in the tube and on the filter was dissolved in 5 ml of 0.5 N hydrochloric acid and the solution was sucked down into a 100 ml filtering flask. The tube and the funnels were washed three times with in all 10 ml of water. The contents of the flask were mixed and 10 ml of 0.17 M iodic acid was added to it. After exactly three minutes the solution was sucked off by means of an immersion filter with a sinctered disc of porosity M and the barium iodate was washed five times with 5 ml portions

of 96% ethanol. The last washed liquid was sucked off. The precipitate was disintigrated in 2 ml ethanol, 20 ml of 10% potassium iodide solution and 5 ml of 2 N hydrochloric acid were added and the solution was titrated with standard 0.1 N thiosulphate solution. When the liquid was colourless, barium iodate remaining in the filter was dissolved by sucking some of the solution up and down through the filter. Starch indicator was added and titration was continued until the liquid was colourless and again the suction was repeated until all iodine was consumed.

Calculation

$$\% \text{ of uronic anhydride} = \frac{\text{ml thio X N thio X } 194{\cdot}1 \text{ X } 10}{\text{mg sample X } 12 \text{ X } 1{\cdot}019}$$

3.1.7. Dialyses

Dialyses of polysaccharide solutions were effected in seamless cellulose tubing (Union Carbide, molecular weight cut off 10,000) against running water for 3 days. Toluene was added to prevent bacterial action.

3.1.8. Freeze - drying

All freeze-drying were carried with the help of a HETOSICC CD 52 (HETO Lab Equipments, Denmark) freeze-dryer.The samples were prefrozen in round bottomed flasks in a methanol freeze (HETOFRIG cooling bath, Type CB 5, HETO BIRKERD,Denmark) at -30^{o} to-35^{o}C.

3.1.9. Acid hydrolysis of polysaccharide and oligosaccharide

The samples (10-20 mg.) were heated in a tube on a boiling water bath with 1 N sulphuric acid (1-2 ml) for 5-9 hours. The hydrolysates were neutralised with solid barium carbonate, filtered and the residue thoroughly washed with water. The 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 to a syrupy liquid.

3.1.10. Reduction

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

3.1.11. Detection of reducing sugars

Phenol-sulphuric acid method[200]: Carbohydrate solution (1 ml.) containing 20-80 μg 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 490 mμ against a blank.

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

3.1.12. Estimation of relative molar proportions of sugars

The polysaccharide samples (30-40 mg) were heated with 1 N sulphuric acid on the boiling water bath for 5-8 hours. After neutralisation and deionisations, the hydrolysate was concentrated in vacuum to a syrup and separated into individual sugar 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 according to sec. 3.1.11. Pieces of paper of equal size, devoid of sugar, were eluted and constituted the blanks.

3.1.13. Degree of polymerisation (DP)

Degree of polymerisation was determined according to the Timell modification[201] of the method of Peat, Whelan and Roberts[202] as follows :

To 1 ml. of a solution of a carbohydrate containing 30-60 mμ 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 1 N solphuric 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 490 mμ 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.

3.1.14. Paper chromatography[203-7]

Unless otherwise stated the paper chromatograms were carried out on Whatman No.1 chromatography paper in descending technique.

The following solvents were used (V/V proportions) for chromatography :

A. Ethyl acetate : acetic acid : formic acid : water (18:3:1:4).[204]

B. Ethyl acetate : pyridine : water (10:4:3 and 8:2:1).[203]

C. I-Butanol :ethanol :water (40:11:19).[204]

D. I-Butanol : acetic acid : water (2:1:1).[205]

E. I-Butanol : pyridine : water (6:4:3).[205]

F. I-Butanol : ethanol : water (upper layer) (4:1:5).[206]

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 0.5 N sodium hydroxide in ethanol and then by 2% sodium thiosulphate solution. The chromatograms were dipped in each solution and dried at room temperature between each operation.[207]

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

3.1.15. Gas liquid chromatography (g.l.c.)

Gas liquid chromatography was carried out with a Varian 2700 gas chromatograph fitted with a flame ionization detector, and capillary column (2800 cm X 0.2 cm. i.d.)Separations were performed on (a) OV-275 at 140 -200^{o}C, 4^{o}C min^{-1} (alditol acetates) and (b) OV-225 at 170^{o}C, isotherm (partially methylated alditol acetates).

Separations were also performed on a SE-54 fused-silica capillary column, using a temperature programme 150^{o}, 2 min; 150-220^{o} at 3^{o}/min. Peak areas were measured with an Autolab Minigrator.

For qualitative analysis a PYE UNICUM GCD gas chromatograph with flame ionization detector with a nitrogen flow of 30 ml/min. was used. Glass column (150 cm X 0.2 cm i.d.) packed with OV-225 on gas chrome Q 100/120 mesh at 200^{o}C was used.

3.1.16. Ionophoresis

Ionophoresis was carried out on Whatman No.1 paper.

The following buffers were used :

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

(b) Borate (pH 10) at 2000 V for Ca. 1 hour.[208]

(c) Molybdate (pH 5) at 2000 V for Ca. 1 hour.[209]

Sugars were located on the ionophoretograms with developing reagent [Sec. 3.1.14(2)].

Abbreviations

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

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

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

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

3.1.17. Preparation of a cellulose column[210]

(a) Diethylaminoethyl cellulose (DEAE-50) was suspended in 0.5 N hydrochloric acid (50 g. in 1 litre HCl solution) in a suction flask. This was kept standing under vacuum for 20 minutes, 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 0.5 N 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 equilibriated with 0.5 N potassium chloride solution (2 L.) and washed with water till free from chloride.

3.1.18. Preparation of charcoal-celite column[211]

500 ml. of charcoal (activated, May and Baker) and 250 ml. of celite (B.D.H. Grade) were thoroughly mixed together. The mixture was covered with concentrated hydrochloric acid (1.3 L.) 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 50 hours for proper setting of the column.

3.1.19. Preparation of phosphate buffer

31.2 g of sodium dihydrogen phosphate ($NaH_2PO_4, 2H_2O$) was dissolved in water to make one litre of solution. 71.6 g of disodium hydrogen phosphate (Na_2HPO_4, $12H_2O$) was dissolved in water to make one litre of solution. 685 ml of sodium dihydrogen phosphate solution (0.2 M) and 315 ml of disodium hydrogen phosphate solution (0.2 M) were mixed together and was diluted to two litre with water to make the solution overall 0.1 M. The buffer had a pH of 6.5. To prevent any microbial growth 0.4 g of sodium azide was added to make the buffer 0.02% with respect to sodium azide.

3.1.20. Preparation of gel column

Sepharose CL-4B and 2B (Pharmacia Fine Chemicals, Sweden) were available as a suspension in water containing 0.01% merthiolate as preservative. The gel suspension was washed with 0.1 M phosphate buffer (pH 6.5) containing 0.02% sodium azide as preservative. Excess stirring was avoided. It was allowed to settle and the excess liquid was decanted off until a fairly thick slurry was formed. The suspension of gel was then deaerated with the help of a suction pump to remove any trapped air bubbles in the gel slurry.

A glass column (50 X 1.6 cm; C type column from Pharmacia AB, Sweden) was mounted vertically on a stable laboratory stand. The dead space under the net and in the tubing

was filled with the eluent (0.1 M phosphate buffer). This was done by injecting the eluent into the outlet tubing. When the dead space was properly filled and no air bubbles remained in it the outlet tubing was closed.

The washed gel suspension was poured into a reservoir which was fitted at the top of the column. After settling the gel, the reservoir was removed and the top piece of the column was fitted. This was followed by the connection of an eluent reservoir to the column through plastic tubing. The last traces of air was removed through air vent in the top piece. The flow of the eluent was started as soon as possible after filling the column in order to obtain an even sedimentation. Three column volumes of eluent was passed through the column in order to stabilize and to equilibrate the gel bed. Then the column became ready for application of the sample.

3.1.21. Fractionation by gel-filtration

100 mg. of the sample was dissolved in dilute alkali solution to a suitable volume.

The column outlet was opened and the eluent was drained away until it almost touched top of the gel bed. After closing the outlet the sample was carefully added on the top

of the gel bed. The column outlet was then opened. After the sample solution had drained into the bed the gel surface and the column wall in contact with the sample were washed with a small amount of eluent. Under no circumstances the bed was allowed to run dry during this procedure. The column was then filled with eluent, the top pieces were replaced and connected to an eluent reservoir flask.

The first 100 ml of the collected solution was rejected. Then fractions were collected at 15 minute interval using an automatic fraction collector (REDIRAC FRACTION COLLECTOR LKB, Sweden) and constant flow rate was regulated by using a peristaltic pump (Peristaltic pump, Pharmacia Fine Chemicals AB, Sweden) in such a way that 7 ml fractions were collected in each 15 min.

The carbobydrates in the collected fractions were monitored by the phenol - sulphuric acid method as described in Sec. 3.1.11.

3.1.22. Purification of solvents and chemicals

Analytical or reagent grade (Sigma, E. Merck or BDH) solvents and chemicals were used in all experiments, unless otherwise stated.

(a) Pyridine

Pyridine was shaken with potassium hydroxide pellets, decanted and distilled twice from phosphorus pentoxide and was finally collected over potassium hydroxide pellets in a glass bottle.

(b) Ether

Ether referasto diethyl ether.

(c) Light petroleum

Light petroleum refers to petroleum ether, boiling range of temperature 40-60^{o}C.

(d) N,N-Dimethylformamide (DMF)[212]

N,N-Dimethylformamide (DMF) was stirred with potassium hydroxide and then distilled from calcium oxide.

(e) Methanol[213]

Methanol (50 ml.), magnesium turnings (5 g.) and sublimed iodine (0.5 g.) were heated under reflux until the

iodine colour was discharged. Further methanol (1 L.) was then added and the solution boiled for 30 minutes, followed by distillation at atmospheric pressure.

(f) Acetic anhydride

Acetic anhydride (analytical grade) was distilled in a quick fit distilling apparatus and the fraction boiling at 139^{o} was collected. Freshly distilled acetic anhydride was always used during acetylation.

3.1.23. Preparation of alditol acetates for quantitative sugar analysis 214,215

About 5 mg. of polysaccharide sample and 1 mg. myo-inositol was treated with 0.5 M trifluoroacetic acid (TFA,5 ml.) and refluxed on boiling water bath for 18 hours.

The acid was removed by rotavapor with added water (3X3 ml.) and the residue was again dissolved in water (2 ml.). It was reduced with sodium borohydride (5 mg) for 3 hours, acidified with Dowex H^{+} resin and filtered. The filtrate was evaporated to dryness with added methanol (3X3 ml.) and acetylated with a mixture of pyridine and acetic anhydride (1:1; 1 ml.) by heating on a boiling water bath for 20 minutes. The acetylated reagent was removed by evaporation with added

rectified spirit (3X5 ml.). The reduced and acetylated hydrolysate was dissolved in chloroform (1 ml.) and analysed by g.l.c.

3.1.24. Methylation

Methylation was carried out by Haworth method and Hakomori method.

(A) Haworth method[216,217]

Polysaccharide sample (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. Aqueous sodium hydroxide solution (30%, 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 2 N 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 over anhydrous sodium sulphate the

chloroform extract was concentrated and dropped slowly into excess rapidly stirred light petroleum ether, where upon 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[218]

Methylation by this method was carried out in two ways :

(i) with the help of base, methyl sulphinyl carbanion.

(ii) by direct use of the reagents.

(i) With the help of base

(a) Preparation of base, methyl sulphinyl carbanion[219,220]

Sodium hydride (20% suspension in oil, 2.4 g) was weighed in a three-necked flask containing a glass magnetic stirrer bar. 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 2½ 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 0.1 N 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 one month.

(b) Methylation analysis

Polysaccharide sample (5 mg) was dissolved in dry dimethyl sulphoxide (DMSO,5 ml) contained in a serum vial (20 ml) which was sealed with a rubber septum. The vial was flushed with dry nitrogen via two injection needles. The dissolution of the sample was facilitated by stirring or shaking.

Dimsylsodium base in DMSO (2 M, 3 ml) was added dropwise through an injection needle using a syringe. The resulting gelatinous solution was shaken in a shaker for 2 hours and allowed to stand overnight at room temperature.

The vial was cooled in an ice-water bath and methyl iodide (3 ml) was added dropwise using a syringe. The resulting turbid solution was agitated in the shaker for 1 hour at room temperature. The process was repeated twice. The excess methyl iodide was removed by flushing a stream of nitrogen. The reaction mixture was poured into a dialysis tubing and dialysed against running distilled water for 24-48 hours. The dialysate was evaporated to dryness under reduced pressure. Alternatively the reaction mixture was poured into water (50 ml). 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 to remove dimethylsulphoxide, dried over anhydrous sodium sulphate and evaporated to dryness under reduced pressure.

(ii) Methylation by direct use of reagents[221]

Polysaccharide sample (200 mg) was dissolved in dimethylsulphoxide (20 ml) by stirring under nitrogen atmosphere (heating at $70^{o}C$ 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 hours). 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 (2X200 ml). The chloroform solution was washed repeatedly (7 times) with an excess of water (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^{o}C).

3.1.25. 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.

3.1.26. Hydrolysis of methylated polysaccharides[222]

Hydrolysis of methylated plysaccharides was carried

out either (a) by heating it with formic acid (90%) in a sealed tube in an atmosphere of carbondioxide 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 methylated polysaccharide with 1 N sulphuric acid according to Sec. 3.1.9.

3.1.27. Acid hydrolysis of methylated polysaccharide and transformation into alditol acetates[214,215,223]

(a) Hydrolysis by formic acid and trifluoroacetic acid (TFA) to methylated monosaccharides:

The methylated material (5 mg) was treated with 90% (V/V) aqueous formic acid (2 ml) and heated on boiling water bath for one hour. The reaction was carried out in a pear shaped flask with a glass stopper. The acid was removed in a rotary evaporator. The residue was treated with 0.5 M trifluoroacetic acid (5 ml) along with myo-inositol (1 mg) and refluxed on boiling water bath for 18 hours. The acid was removed by rotavapor with added water (3 X 5 ml).

(b) Reduction and acetylation:

The hydrolysate was reduced with sodium borohydride and acetylated with a mixture of pyridine and acetic anhydride

according to the procedure described in Sec. 3.1.23. The resulting alditol acetates of methylated product were dissolved in chloroform and analysed by g.l.c.

3.1.28. Periodate oxidation[224]

A 0.03 M solution of the polysaccharide material was oxidised with a 0.05 M solution of sodium meta-periodate in the presence and absence of acetate buffer. The solution was kept at $0^{\circ}C$ 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. Estimation of periodate consumption was carried out by titrimetric procedure as described below :

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 (0.1 N) 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 0.1 N iodine solution using starch as the indicator.

A blank determination was carried out simultaneously using sodium metaperiodate solution (0.05 M, 5 ml), 0.1 N sodium arsenite solution (20 ml) and 20% potassium iodide solution (1 ml).

Calculation:

1 ml 0.1 N sodium arsenite = 0.010696 g sodium metaperiodate.

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

3.1.29. Infra-red spectrometry

Infra-red spectra were measured for potassium bromide discs or as a film using a Perkin-Elmer 257 or 157G spectrophotometer.

3.1.30. Proton nuclear magnetic resonance (^{1}H-n.m.r.) spectroscopy[223,225-6]

^{1}H-n.m.r. spectra were recorded at 400 MH_z in D_2O (3 mg PS / ml) at 85^oC on a varian VXR 400.

3.1.31. C-13 Nuclear magnetic resonance (^{13}C-n.m.r.) spectroscopy[223,225-6]

(a) The spectra were recorded on a 250 MH_z Bruker Spectrometer (Brand 010) for which ^{13}C frequency was 63 MH_z.

(b) The ^{13}C-n.m.r. spectra were also recorded at 89.55 MH_z on JEOL FX 90 Q.

(c) 13-n.m.r. were also recorded at 101 MH_z in D_2O (10 mg PS/ml) at room temperature (on a Varian VXR 400)using an inverse gated decoupling technique (45^o r.f.pulse,4.5 Sec. pulse delay) to supress n.o.e.

3.1.32. Gas liquid chromatography-mass spectrometry (g.l.c.-m.s.)[223,225-6]

For g.l.c.-m.s, a Finingan 4021 gas chromatograph-

mass spectrometer instrument was used. The spectra were obtained at an ionization potential of 70 eV, ion source temperature 160°C and the helium flow rate was 25 ml/min.

3.2. EXPERIMENTS

3.2.1. Materials

Ripe palm fruits (Borassus flabellifer Linn.)were collected from the districts of Dhaka (Sample S_1) and Laksmipur (sample S_2) in August and December respectively. The inner hard seeds were separated from the outer fibrous coat. The solid part of the seed was peeled off into pieces, dried in the sun, powdered and used for the extraction of polysaccharides.

Each of the samples was separately extracted successively with petroleum ether, 80% ethanol, water (cold and hot) and aqueous sodium hydroxide (3 and 10%).

3.2.2. Extraction of palm seed kernel (sample S_1)

3.2.2.1. Extraction with petroleum ether

The powdered dried palm seed kernel (Ca 400 g) of

sample S_1 was kept immersed for 3 days under 500 ml. petroleum ether (b.p. 40-60^{o}C) in an aspirator bottle. The bottle was occassionally shaken and the solvent was separated from the mixture. The process of adding petroleum ether, immersing, shaking and separating the solvent was repeated twice. The total volume of the extract was evaporated to dryness by using rotavapour at reduced pressure when an yellow oily substance was obtained and weighed. Paper chromatographic investigation of the petroleum ether extract in solvent A (using spray 1) showed no sugar in it.

3.2.2.2. Extraction with ethanol (80%)

The residue of kernel left after petroleum ether extraction was dried and extracted with ethanol (80%) at room temperature for 2 days. The residue was separated by filtering through muslin, washed with ethanol and extracted further 3 times with ethanol (80%). The filtrates and the washings were combined, centrifuged at 4000 r.p.m. and concentrated by using rotavapour at reduced pressure to a syrup.

A small portion of this alcoholic extract was dissoved in water and examined by paper chromatography in solvents A and E (using spray 1). This revealed the presence of sucrose, D-glucose and D-fructose.

3.2.2.3. Extraction with cold water

200 g of the residual kernel left after etherial and alcoholic extraction was extracted with water (600 ml) with vigorous stirring for 24 hours at room temperature. The residue was separated by filtering through nylon cloth and extracted thrice in the same manner. The combined filtrate (1800 ml) was centrifuged and dialysed for three days in running water and distilled water (using magnetic stirrer) respectively. The supernatant was again centrifuged, concentrated (500 ml) and precipitated by adding ethanol with stirring. The precipitate was separated by centrifugation and dried in a desiccator over anhydrous calcium chloride and weighed.

A small portion of this cold water extract was dissolved in water and examined by paper chromatography in solvents A and E (using spray 1). No free sugar was found in it.

Another small portion of this cold water extract was hydrolysed with 1 N sulphuric acid on a boiling water bath for 9 hours. The sample dissolved completely during hydrolysis. The cooled hydrolysate was neutratised with barium carbonate, filtered, passed through Amberlite IR-120 (H^+)

and Amberlite IR-45(OH^-). The elute was concentrated to a syrup and examined on paper chromatography in solvents A and E (using spray 1). The syrup revealed spots corresponding to D-galactose, D-mannose and L-arabinose (trace).

3.2.2.4. Extraction with hot water

The residual part of the sample obtained after cold water extraction was extracted with water (600 ml) in boiling water bath with occassional shaking for 24 hours. The mixture was cooled and the residue was separated by filtration through nylon cloth. The hot water extraction of the residue was repeated twice in the same manner. The combined filtrate (1800 ml) was centrifuged and dialysed for three days in running water and distilled water (using magnetic stirrer) respectively. The supernatant was again centrifuged (4000 r.p.m.), concentrated (500 ml) and precipitated by adding alcohol. The precipitate was separated by centrifugation, dried in a vacuum desiccator over anhydrous calcium chloride and weighed.

A small portion of this hot water extract was dissolved in water and examined by paper chromatography in solvents A and E (using spray 1). No free sugar was found in it.

50 mg of this hot water extract was hydrolysed with 1 N sulphuric acid on a boiling water bath for 9 hours. The sample was found to undergo a complete solution during hydrolysis. The cooled hydrolysate was neutralised with barium carbonate, filtered, passed through Amberlite IR-120 (H^+) and Amberlite IR-45(OH^-). The elute was concentrated to a syrup. On paper chromatographic examination in solvents A and E (using spray 1). The syrup revealed spots corresponding to D-galactose, D-mannose and L-arabinose(trace).

3.2.2.5. Extraction with 3% alkaline solution

The residue of the sample left after hot water extraction was extracted with 3% sodium hydroxide solution (500 ml) for 24 hours with vigorous shaking at room temperature in presence of nitrogen gas. The resulting mass was filtered through a piece of nylon cloth and centrifuged (4000 r.p.m.). The process of 3% alkaline extraction was repeated twice (500 ml,each time) in a similar manner and the clear filtrate was collected from each treatment. The combined filtrate was acidified with glacial acetic acid to pH 5-6 in the cold ($0.5^{o}C$). The whole mass was treated with a few drops of toluene and dialysed for 3 days in running water and then distilled water (using magnetic stirrer) respectively to remove the inorganic salts. The dialysate was

centrifuged at 4000 r.p.m. and the clear supernatant was concentrated (500 ml) and precipitated by alcohol. The precipitate was separated by centrifugation at 4000 r.p.m., dried in a vacuum desiccator over anhydrous calcium chloride and weighed.

A small portion of 3% alkali extract was dissolved in dilute aqueous sodium hydroxide and examined by paper chromatography in solvents A and E (using spray 1). No free sugar was observed in it.

Another small portion of this extract was hydrolysed with 1 N sulphuric acid on a boiling water bath for 9 hours. The sample was found to undergo a complete solution during hydrolysis with sulphuric acid. The cooled hydrolysate was neutralised with barium carbonate, filtered, passed through Amberlite IR-120 (H^+) and Amberlite IR-45 (OH^-). The elute was concentrated tc a syrup. On paper chromatographic examination in solvents A and E (using spray 1) the syrup revealed spots corresponding to D-galactose and D-mannose.

3.2.2.6. Extraction with 10% alkaline solution

The residue of the sample after extraction with 3% alkaline solution was extracted exhaustively with 10% sodium

hydroxide solution (700 ml) for 24 hours with vigorous shaking in presence of nitrogen gas at room temperature. The resulting mass was filtered through a piece of nylon cloth and centrifuged (4000 r.p.m.). The process of 10% alkaline extraction was repeated three times (500 ml, each time) in a similar manner and the clear filtrate was collected from each treatment. The combined filtrate was acidified with glacial acetic acid to pH 5-6 in the cold ($0.5^{o}C$). The whole mass was treated with a few drops of totuene and dialysed for 3 days in running water and distilled water (using magnetic stirrer) respectively to remove inorganic salts.The dialysate was again centrifuged at 4000 r.p.m., concentrated (500 ml) and precipitated by ethanol with stirring. The precipitate was separated by centrifugation and dried in a vacuum desiccator using anhydrous calcium chloride and weighed.

50 mg of this 10% alkali extract was hydrolysed with 1 N sulphuric acid on a boiling water bath for 9 hours. The sample was found to undergo a complete solution during hydrolysis. The cooled hydrolysate was neutralised with barium carbonate, filtered, passed through Amberlite 1R-120 (H^{+}) and Amberlite 1R-45 (OH^{-}). The elute was concentrated to a syrup. On paper chromatographic examination in solvents A and E(spray 1) the syrup showed spots corresponding to D-mannose and D-galactose (trace).

3.2.3. Extraction of palm seed kernel (Sample-S_2)

The powdered palm seed kernel (sample-S_2) was extracted exhaustively with petroleum ether, 80% ethanol, water (cold and hot) and aqueous sodium hydroxide (3 and 10%) according to the procedure adopted for the extraction of sample S_1.

Table-5: A comperative data on percentage yield of the different extracts of the samples S_1 and S_2.

Sl. No.	Extracts	Yield	
		Sample S_1	Sample S_2
1.	Petroleum ether extract	0.24	0.20
2.	Alcoholic extract	1.02	1.00
3.	Cold water extract	2.10	2.00
4.	Hot water extract	2.52	2.20
5.	3% Alkali extract	3.50	3.30
6.	10% Alkali extract	44.10	43.80

Table-6. Hydrolysed products of different extracts of sample S_1 and S_2

Sl. No.	Different extracts	Paper chromatographic identification of hydrolysates of different extracts	
		Sample S_1	Sample S_2
1.	Cold water extract	ara, gal, man	ara, gal, man
2.	Hot water extract	ara, gal, man	ara, gal, man
3.	3% alkali extract	gal, man	gal, man
4.	10% alkali extract	gal, man	gal, man

Where

ara = L-arabinose

gal = D-galactose

man = D-mannose

3.2.4. Purification of 10% alkali extract

3.2.4.1. Precipitation by Fehling's solution

10% alkali extract(crude polysaccharide,1 g) was again dissolved in 10% sodium hydroxide solution (50 ml). Small quantities of insoluble materials were removed by centrifugation at 4000 r.p.m. for 20 minutes. The supernatant was neutralised by glacial acetic acid and dialysed for 48 hours. Freshly prepared Fehling's solution was added to the crude polysaccharide solution until precipitation of the "copper complex" was just completed. The blue gelatinous precipitate was removed by centrifugation, washed with water and again centrifuged. The precipitate was suspended in water, cooled in ice water and treated with 1 M hydrochloric acid when a clear light green solution was obtained. 4 volumes of ethanol were added to this solution with constant stirring. The precipitate thus formed was collected by centrifugation, dissolved in 10% sodium hydroxide solution, neutralised by glacial acetic acid and dialysed in running water for 48 hours. The dialysed solution was concentrated in a rotary vacuum evaporator under reduced pressure to a suitable volume. Ethanol was added to this concentrated solution with rapid stirring when precipitate appeared.

This was collected by centrifugation, dried in a desiccator over anhydrous calcium chloride and weighed (400 mg).

A portion of this polysaccharide was hydrolysed with 1 N sulphuric acid on a boiling water bath for 9 hours (Sec. 3.1.9). The process of neutralisation by barium carbonate, deionization by Ambertite resin and concentration of the hydrolysate was carried out when a sirupy liquid was obtained . Paper chromatographic examination of the sample using solvents A and E (spray 1) showed the presence of mainly D-mannose and a trace of D-galactose.

3.2.4.2. Precipitation by ammonium sulphate.

10% alkali extract (crude polysaccharide, 0.5 g) was dissolved in a minimum quantity of 10% sodium hydroxide solution (20 ml). The sample solution was centrifuged at 4000 r.p.m. for 20 minutes. Solid ammonium sulphate was gradually added to the supernatant with stirring till the solution became saturated with respect to ammonium sulphate. The precipitate formed at this stage was separated by centrifugation, dissolved in dilute sodium hydroxide solution, neutralised by glacial acetic acid and dialysed for 48 hours in water. The dialysate was concentrated in a rotary

vacuum evaporator under reduced pressure to a suitable volume. Sufficient ethanol was added to this concentrated solution with rapid stirring . The precipitate thus formed was collected by centrifugation, dried in a desiccator over anhydrous calcium chloride and weighed (190 mg).

20 mg of this precipitate was hydrolysed with 1 N sulphuric acid on a boiling water bath for 9 hours. The process of neutralisation, deionization and concentration of the hydrolysate was carried out according to Section 3.1.9. Examination of the sample by paper chromatography using solvents A and E (spray 1) indicated the presence of D-mannose and D-galactose (trace).

3.2.4. 3. Fractionation by gel-filtration

(i) By using sepharose CL-4B

The gel column was prepared according to Sec.3.1.20. 100 mg of 10% alkali extract (crude polysaccharide) was dissolved in 2 ml of dilute sodium hydroxide solution. The sample solution was carefully layered on the top of the gel bed and fractionated as per Sec. 3.1.21. The carbohydrates present in the collected fractions were monitored by the phenol sulphuric acid method (Sec. 3.1.11).

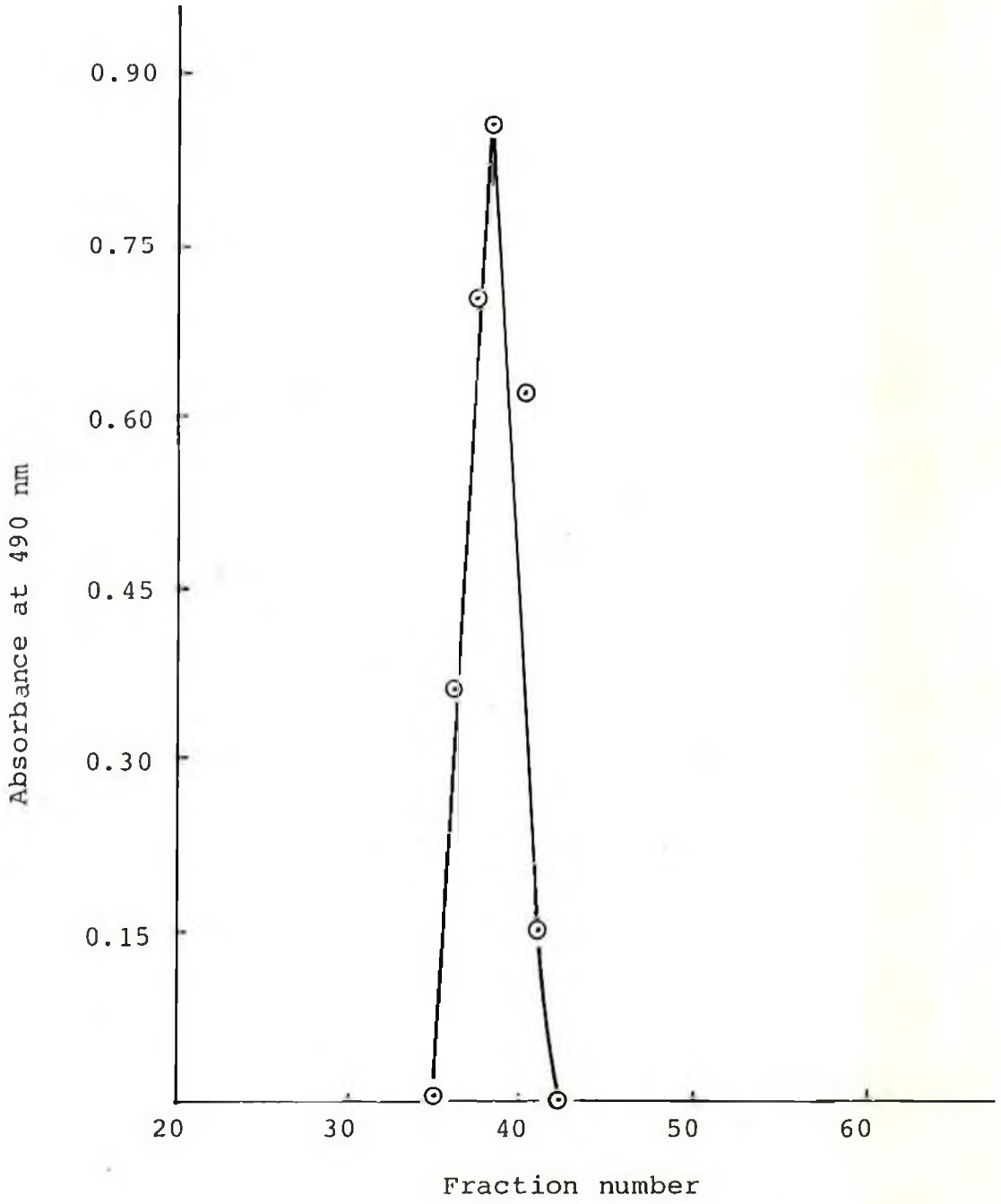
The absorbances of the fractions were measured by a colorimeter at 470 nm (Table-7).

A plot of absorbances against fraction numbers was shown in fig.32.

Fraction numbers 35 to 43 were combined, dialysed for 50 hours against distilled water, freeze dried and weighed.

Table-7. Absorbances of different fractions of polysaccharide M1 as collected from gel column using sepharose CL-4B

Fraction Number	Absorbance	Fraction Number	Absorbance
1-34	00	42	0.001
35	0.01	43	00
36	0.37	44-100	00
37	0.725		
38	0.855		
40	0.625		
41	0.15		



Fiq. 32. Purification of polysaccharide M_1 on sepharose CL-4B qel column.

20 mg of this homogeneous product was hydrolysed with 1 N sulphuric acid on a boiling water bath for 9 hours. The process of neutralisation, deionization and concentration of the hydrolysate was carried out according to Sec. 3.1.9. Examination of the sample by paper chromatography using solvents A and E (spray 1) revealed the presence of D-mannose and a trace of D-galactose.

(ii) By using sepharose CL-2B

100 mg of 10% alkali extract (crude polysaccharide) was dissolved in 2 ml of dilute sodium hydroxide solution. The gel column was prepared according to Sec. 3.1.20. The sample solution was poured carefully on the top of the gel bed and fractionated by following the procedure of Sec. 3.1.21. The carbohydrates present in the collected fractions were monitored by the phenol sulphuric acid method (Sec. 3.1.11.)

The absorbances of the fractions were measured by a colorimeter at 470 nm and presented in Table 8.

A plot of absorbances against fraction numbers was given in Fig. 33.

Table-8. Absorbances of different fractions of polysaccharide M_1 as collected from column using sepharose CL-2B

Fraction Number	Absorbance at 470 nm.	Fraction Number	Absorbance at 470 nm.
1-15	00	21	0.285
16	00	22	00
17	0.28	23	00
18	2.00	24	00
19	7.00	25	00
20	6.00	26-100	00

Fraction numbers 16 to 22 were combined, dialysed for 50 hours against distilled water, freeze dried and weighed.

A portion of this homogeneous product (designated as polysaccharide M_1) was hydrolysed with 1 N sulphuric acid on a boiling water bath for 9 hours. The hydrolysate was neutralised, deionized and concentrated according to Sec.3.1.9. Paper chromatographic examination of the sample using solvents A and E (spray 1) showed the presence of D-mannose and D-galactose (trace).

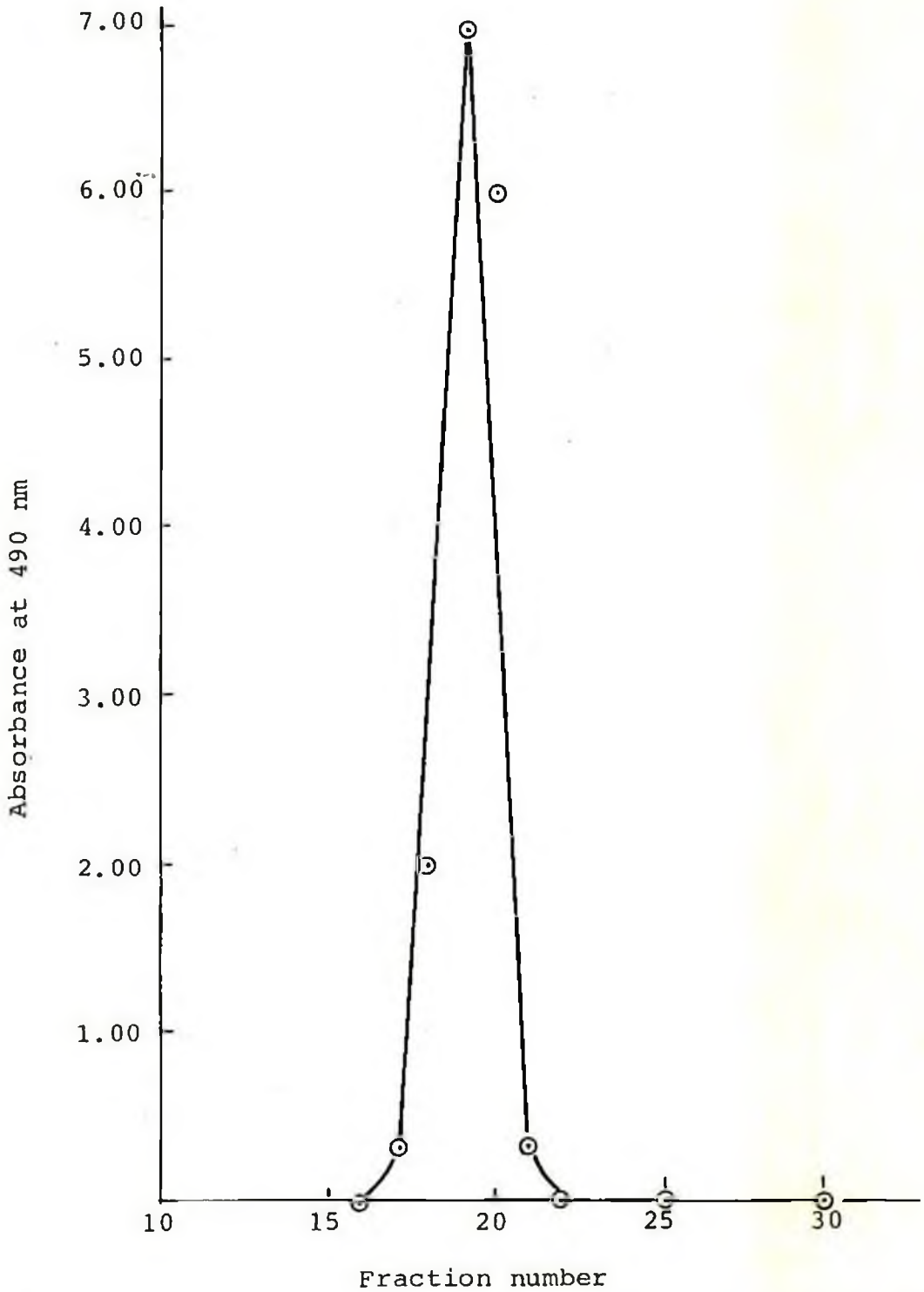


Fig.33. Purification of polysaccharide M_1 on sepharose CL-2B gel column.

3.2. 5. Purification of hot water extract through fractionation by Gel-filtration using sepharose CL-2B

100 mg of hot water extract (crude polysaccharide) was dissolved in 2 ml of water. The gel column was prepared according to Sec. 3.1.20. The sample solution was poured carefully on the top of the gel bed and fractionated according to the procedure described in section 3.1.21. The carbohydrates present in the collected fractions were monitored by the phenol sulphuric acid method (Sec. 3.1.11).

The absorbances of the fractions were measured by a colorimeter at 470 nm (Table9).

A plot of absorbances against fraction numbers was shown in Fig. 34.

Fraction numbers 15 to 28 were combined, dialysed for 50 hours against distilled water, freeze dried and weighed.

A portion of this homogeneous product (designated as polysaccharide M_2) was hydrolysed with 1 N sulphuric acid on a boiling water bath for 9 hours. The hydrolysate was neutralised, deionized and concentrated according to Sec.3.1.9. Examination of the sample on paper chromatography using

solvents A and E (spray 1) gave D-mannose, D-galactose and L-arabinose (trace).

Table 9. Absorbances of different fractions of polysaccharide M_2 as collected from gel column using sepharose CL-2B

Fraction Number	Absorbance at 470 nm.	Fraction Number	Absorbance at 470 nm.
1-13	00	20	0.51
14	0.05	22	0.66
15	0.11	23	0.85
16	0.175	24	0.93
17	0.22	26	0.75
18	0.28	27	0.065
19	0.40	28	0.028
		29-100	0.00

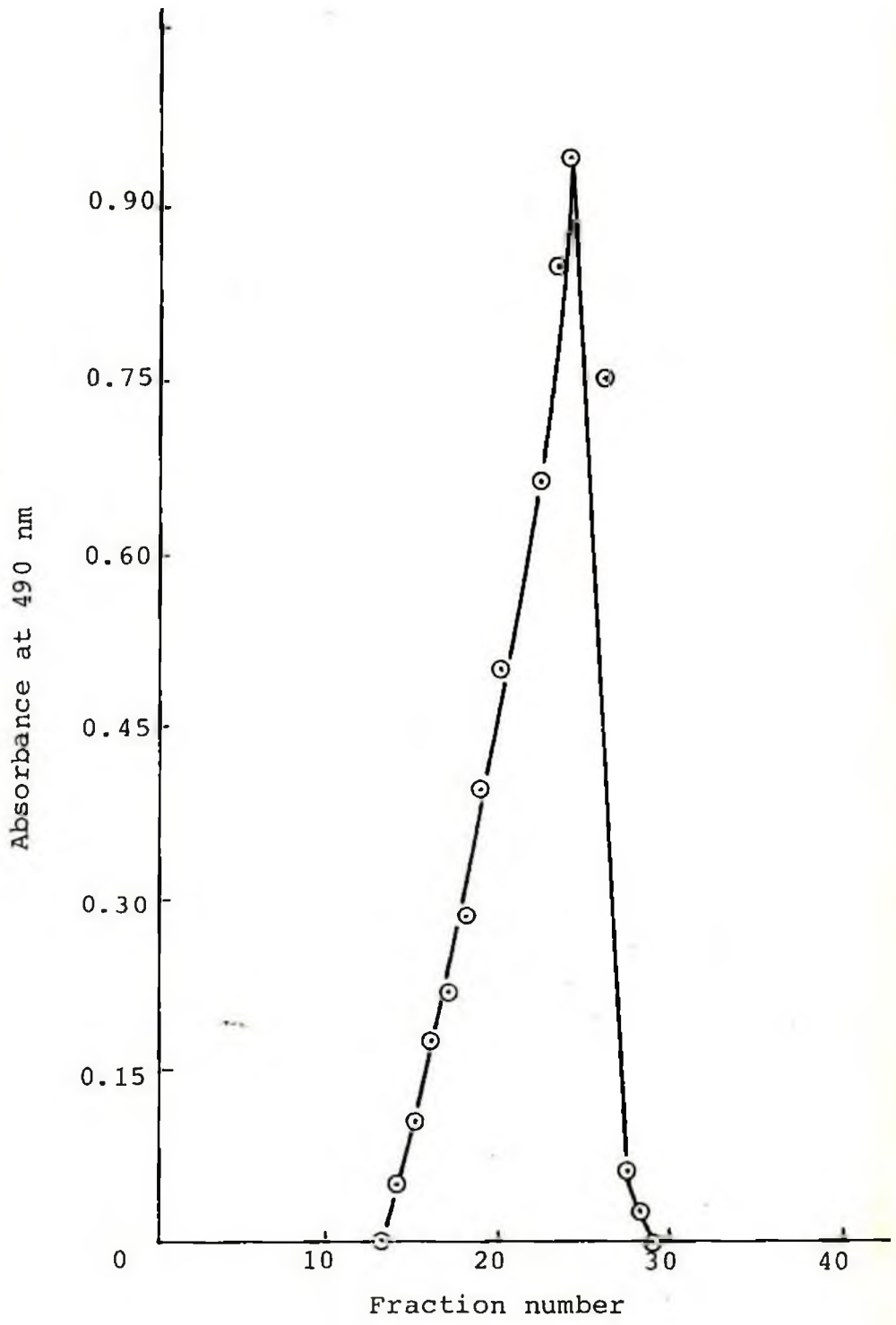


Fig.34. Purification of polysaccharide M_2 on sepharose CL-2B gel column.

3.2. 6. Purification of 3% alkali extract through fractionation by Gel-filtration using sepharose CL-2B.

100 mg of 3% alkali extract (crude polysaccharide) was dissolved in 2 ml of dilute sodium hydroxide solution. The gel column was prepared according to Sec.3.1.20. The sample solution was poured carefully on the top of the gel bed and fractionated as per Sec.3.1.21. The carbohydrates present in the collected fractions were monitored by the phenol sulphuric acid method (Sec. 3.1.11.).

Absorbances of the fractions were measured by a colorimeter at 470 nm. The results were presented in Table 10 and Fig. 35.

Table 10. Absorbances of different fractions of polysaccharide M_3 as collected from gel column using sepharose CL-2B

Fraction Number	Absorbance at 470 nm.	Fraction Number	Absorbance at 470 nm.
1-12	00	19	0.52
13	00	20	0.596
14	0.02	21	0.86
15	0.15	22	6.00
16	0.20	23	5.00
17	0.32	24	0.55
18	0.38	25-100	00

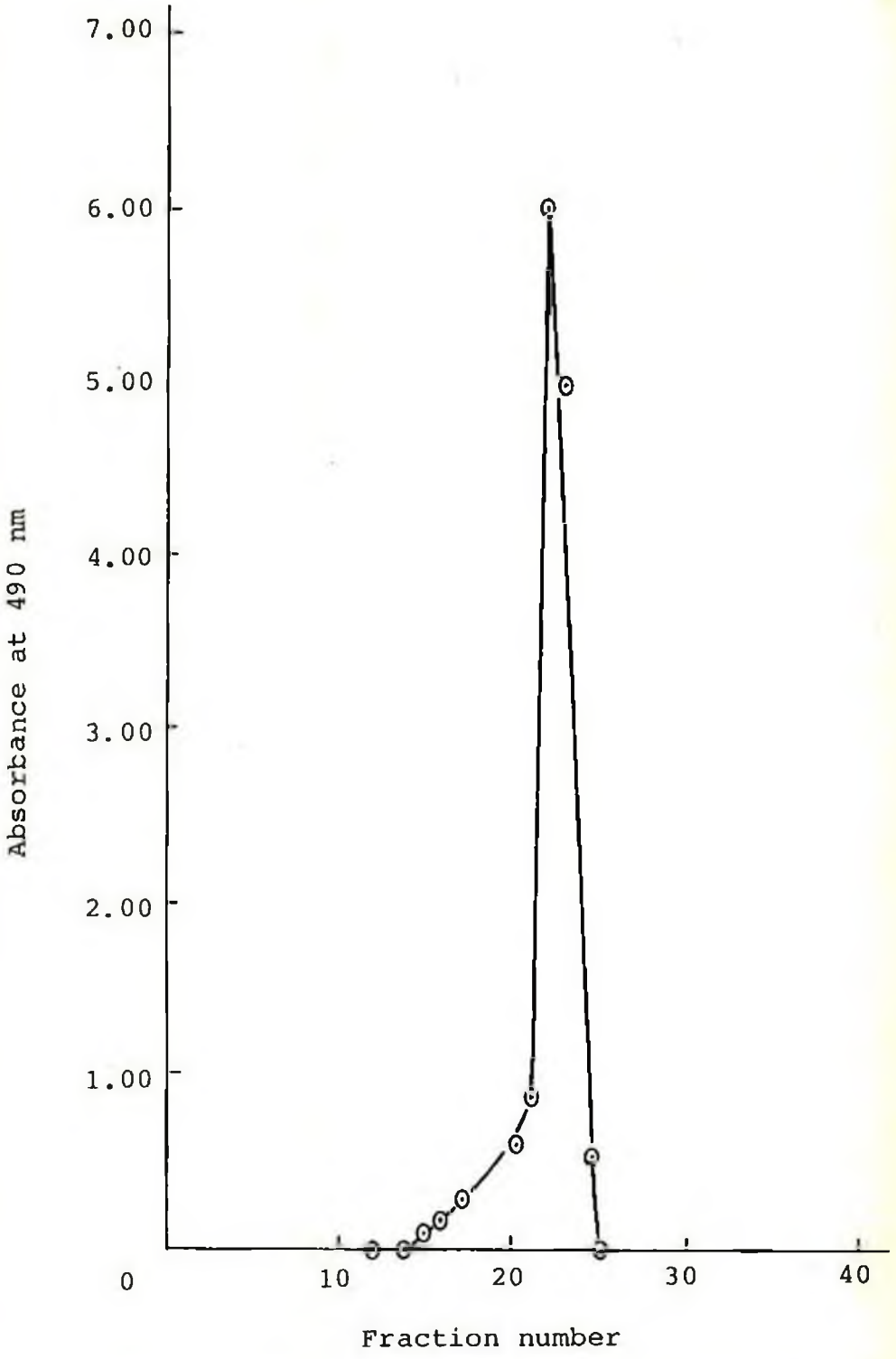


Fig. 35. Purification of polysaccharide M_3 on sepharose CL-2B gel column.

Fraction numbers 14 to 25 were combined, dialysed for 50 hours against distilled water, freeze dried and weighed.

20 mg of this homogeneous 3% alkali extract(designated as polysaccharide M_3) was hydrolysed with 1 N sulphuric acid on a boiling water bath for 9 hours. The hydrolysate was neutralised, deionized and concentrated according to Sec. 3.1.9. Paper chromatographic examination of the sample using solvents A and E (spray 1) showed the presence of D-mannose and D-galactose.

3.2.7. Structural studies of polysaccharide M_1

3.2.7.1. Measurement of optical rotation of the polysaccharide M_1

The sample (50 mg) was dissolved in 100 ml of 10% aqueous sodium hydroxide solution. The optical rotation of this solution was measured as per procedure described under Sec. 3.1.4. The result was calculated by the relation

$$\left[\alpha\right]_n^t = \frac{100\ \alpha}{l.c}$$

Where

α = Observed angle of rotation (in degree)

l = Length of the polarimeter tube

c = Concentration of the solution (mg/dl)

n = Wave length of the light used (589 sodium D-line)

t = Temperature at which readings were taken ($30^{o}C$)

The optical rotation of polysaccharide M_1 obtained by using sepharose CL-2B and 4B were found to be - 7.2° (in 10% aqueous sodium hydroxide, c 0.05).

3.2.7.2. Sugar analysis of polysaccharide M_1 by gas liquid chromatography

The polysaccharide M_1 (10 mg) was hydrolysed by trifluoroacetic acid, reduced with sodium borohydride and acetylated with a mixture of pyridine and acetic anhydride according to Sec. 3.1.23. The resulting alditol acetates of sugars were analysed by g.l.c. which indicated the presence of 97 and 3% mannose and galactose respectively.

3.2.7.3. Methylation of polysaccharide M_1 by Haworth method

The purified polysaccharide M_1 (100 mg) was dissolved

in minimum quantity of 10% alkaline solution (10 ml) and methylated following the procedure of Haworth(Sec. 3.1.24 A.). This gave a product with a methoxyl content of 41.5%.

3.2.7.4. Methylation of polysaccharide M_1 by Hakomori method

The polysaccharide M_1 (20 mg) was dissolved in dimethyl sulphoxide (3 ml) and methylated by using the procedure of Hakomori method (Sec. 3.1.24 B.). This gave a methylated product which showed no hydroxyl absorption band in the I.R. spectrum.

3.2.7.5. Gas-liquid chromatographic analysis of methylated polysaccharide M_1

The methylated polysaccharide M_1 (5 mg) was hydrolysed by formic acid (90%) for one hour followed by 0.5 M trifluoroacetic acid (Sec. 3.1.27). The hydrolysate was reduced with sodium borohydride and acetylated with a mixture of pyridine and acetic anhydride according to the procedure described in Sec. 3.1.23. The resulting alditol acetates of the methylated sugars were analysed by g.l.c. and mole% of various components were calculated from the peak areas and recorded in Table 11.

Table 11. Methylation analysis of the polysaccharide M_1

Sugar and location of methoxyl groups	R_t of Sugars	T^a of Sugars	Mole %
1,5-di-O-acetyl-2,3,4, 6-tetra-O-methyl-D-mannitol.	2.45	0.98	3.09
1,5-di-O-acetyl-2,3,4, 6-tetra-O-methyl-D-galactitol.	2.9	1.16	3.18
1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-D-mannitol	5.1	2.04	87.54
1,4,5,6-tetra-O- acetyl-2,3 -di-O-methyl-D-mannitol	9.2	3.68	6.18

Where

R_t = retention time

T^a = retention time of the corresponding alditol acetate relative to that of 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol on a OV-225 glass capillary column at 170°C.

3.2.7.6. Gas-liquid chromatography-mass spectrometry studies of methylated polysaccharide M_1

Methylated polysaccharide M_1 (5 mg) was converted into alditol acetates according to Sec. 3.1.27. The resulting alditol acetates were studied by g.l.c.-m.s. as per procedure described under Sec. 3.1.32. The following $m/_e$ values obtained from Fig. 36, 37 and 38 indicated the presence of 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-galactitol ; 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-D-mannitol and 1,4,5,6-tetra-O-acetyl-2,3-di-O- methyl-D-mannitol respectively.

$m/_e$ 43, 45, 60, 73, 86, 103, 115, 127 and 145;
$m/_e$ 43, 45, 87, 101, 113, 117, 129, 131, 161, 173 and 233 and
$m/_e$ 43, 85, 97, 103, 115, 127, 145 and 157.

3.2.7.7. ^{13}C-n.m.r. studies of polysaccharide M_1

A small quantity of polysaccharide M_1 was dissolved in D_2O containing NaOD and used for ^{13}C-n.m.r. studies.Sodium 2,2-dimethyl-2-silapentane-5-sulphonate (TMS) was used as the referance compound. The spectra were recorded using the instrument as mentioned in Sec. 3.1.31.

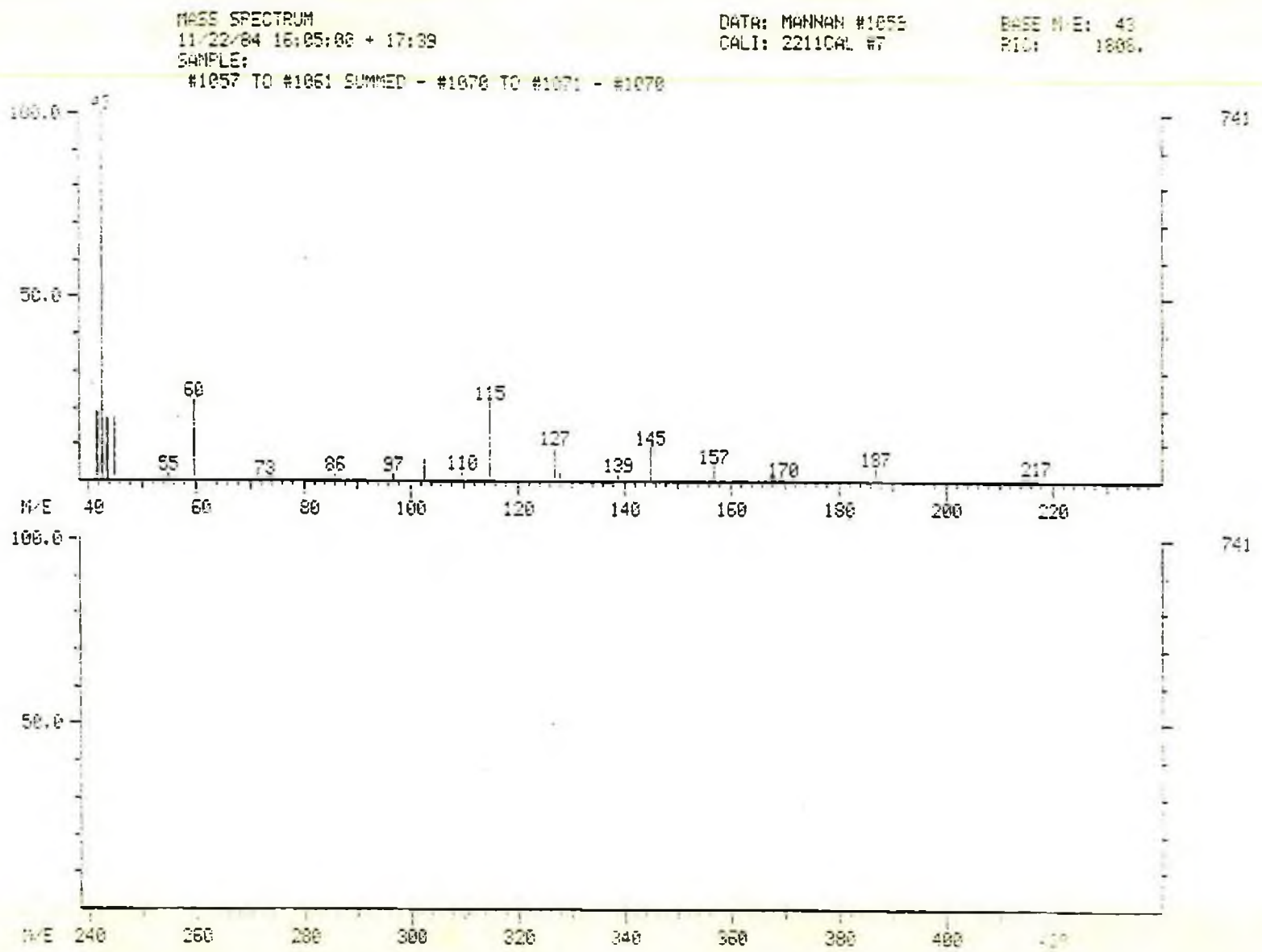


Fig.36. Mass spectrum of polysaccharide M_1 showing $m/_e$ values of 1,5-di-O-acetyl-2,3,4,6- tetra-O-methyl-D-galactitol.

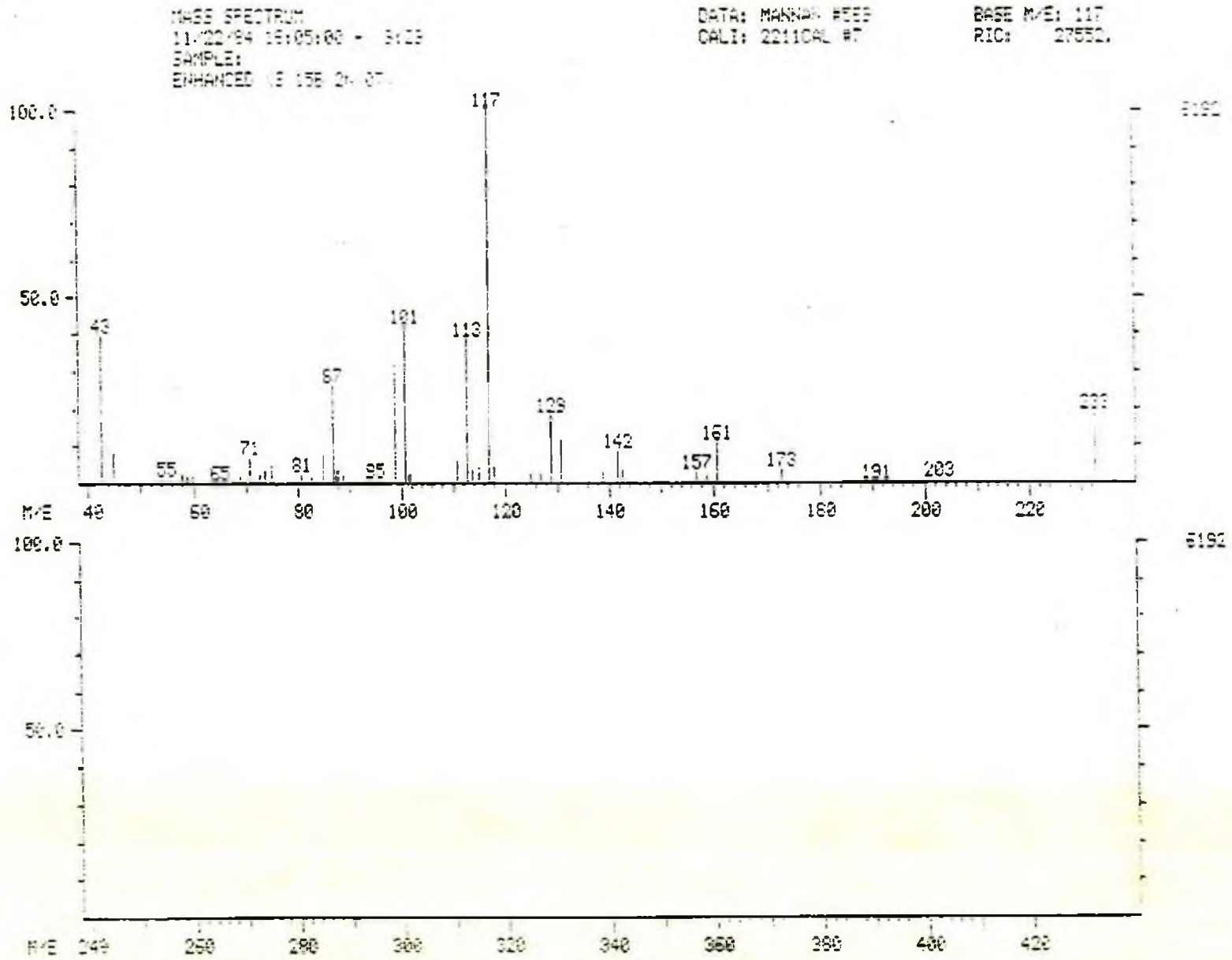


Fig. 37. Mass spectrum of polysaccharide M_1 showing $m/_e$ values of 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-D-mannitol.

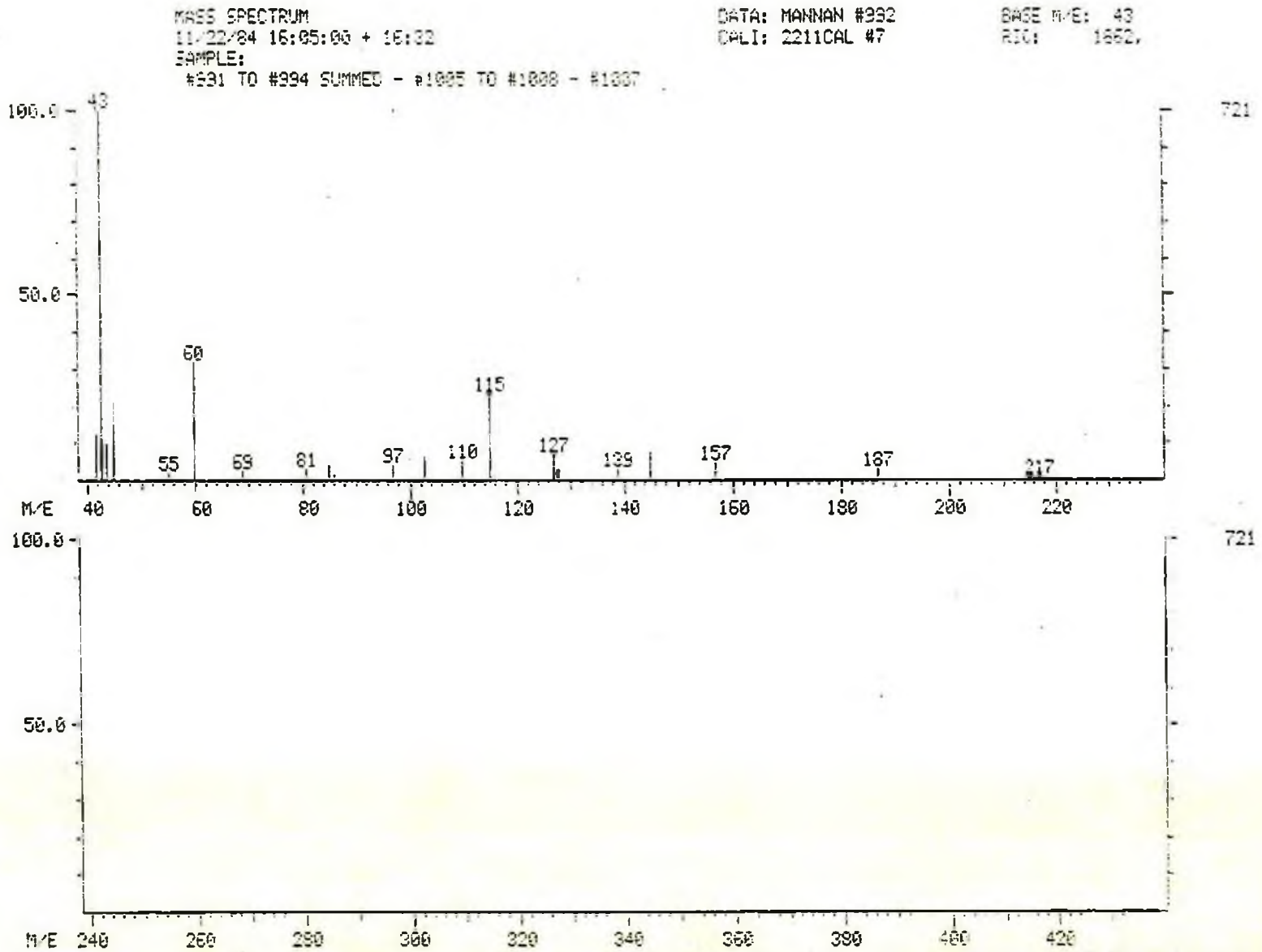


Fig.38. Mass spectrum of polysaccharide M_1 showing $m/_e$ values of 1,4,5,6-tetra-O-acetyl-2,3-di-O-methyl-D-mannitol.

The value of coupling constant $J^{13}C-^{1}H$ was found to be $J^{13}C-^{1}H$ = 160.5 Hz (Fig. 39).

In the noise-decoupled spectrum, ^{13}C-n.m.r. showed six main peaks (Fig 40). The chemical shifts, as determined from the peaks, were presented in the tabular form (Table 12).

Table 12. ^{13}C-n.m.r.spectral data of polysaccharide M_1

δ	Assignment
101.7 p.p.m.	C_1
77.0 "	C_5
77.5 "	C_4
72.0 "	C_2
73.3 "	C_3
62.2 "	C_6

3.2.7.8. Smith degradation[226a] studies of polysaccharide M_1

(i) Periodate oxidation of polysaccharide M_1

The polysaccharide M_1 (300 mg) was oxidised with 0.05 M sodium metaperiodate solution (200 ml) in water for

^{13}C-NMR

with coupling ^{13}C-^{1}H

Mannan

2 mal

10% alk ext

D_2O solvent

external TMS reference

5000 Hz spectral width

gated decoupling used

83080 pulses

45° pulse

16 s data period

3 sec. pulse delay

30 Experiment

double precision

C-1 $J^{13}C$-^{1}H = 160,5 Hz ⇒ β-D-mannan

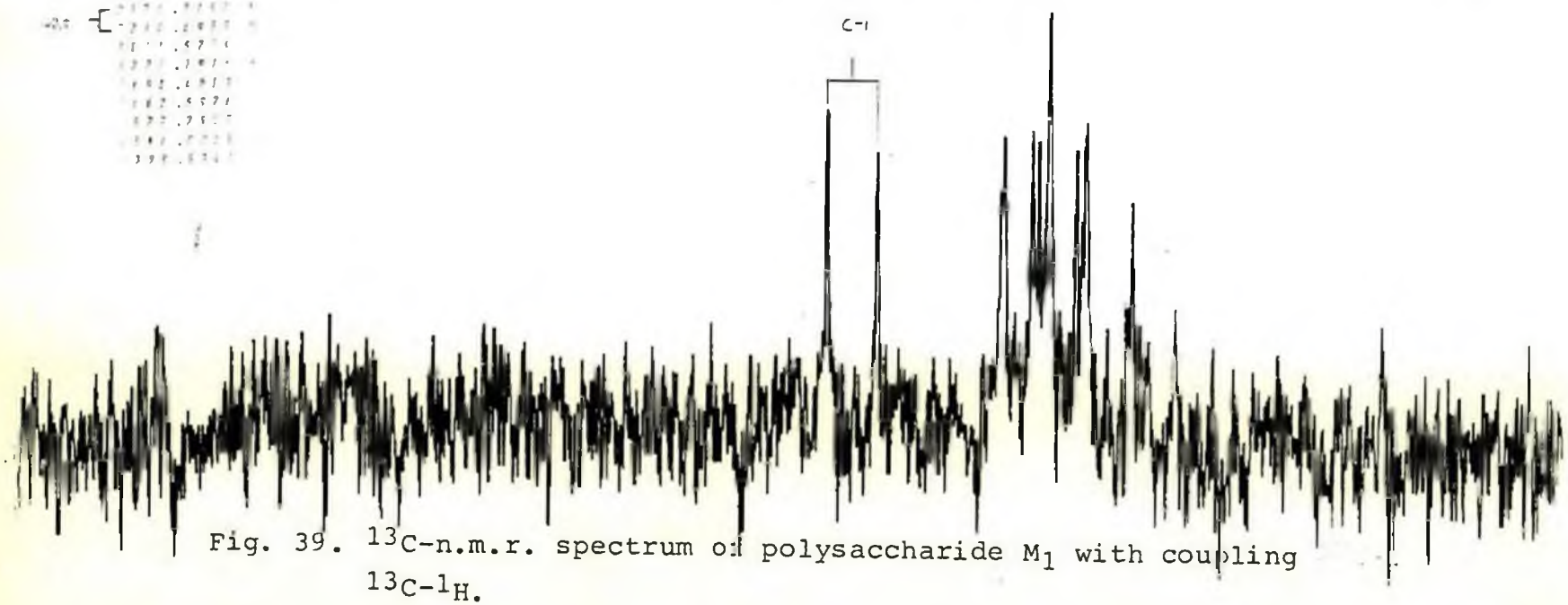


Fig. 39. ^{13}C-n.m.r. spectrum of polysaccharide M_1 with coupling ^{13}C-^{1}H.

^{13}C - NMR

no coupling ^{13}C - ^{1}H

JEOL FX 90 Q

55 MHz

- β-D-(1-4)mannan

TMS (tetramethylsilan) reference

C-1	101,7 ppm
C-2	72,0 "
C-3	73,3 "
C-4	77,5 "
C-5	77,0 "
C-6	62,2 "

Lit. ref. P.A.J. Gorin Advances in carbohydrate Chemistry and Biochemistry. 38 (1981) 13-104

45° puls

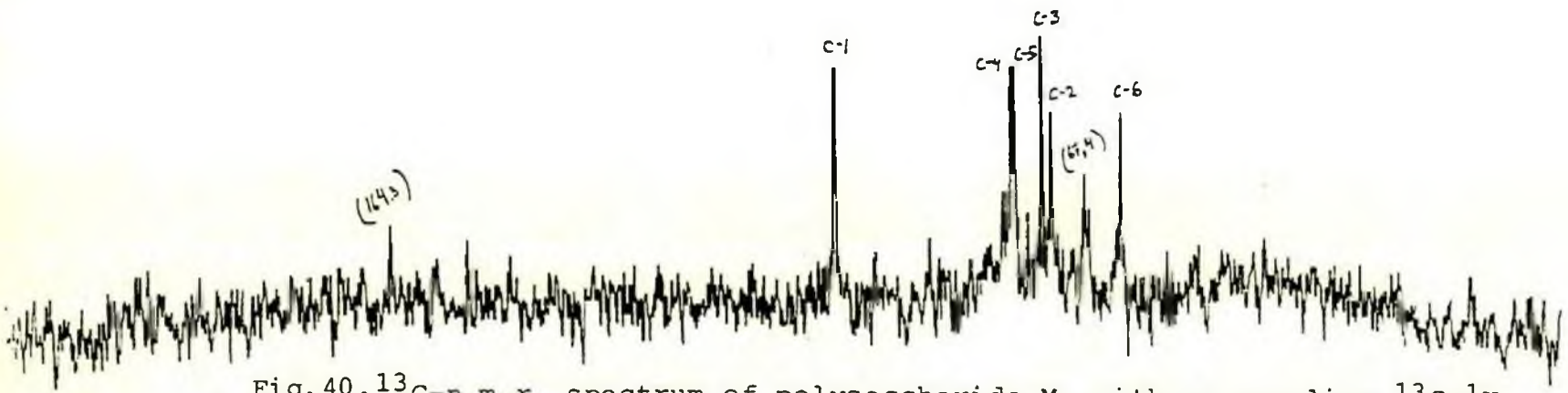


Fig. 40. ^{13}C-n.m.r. spectrum of polysaccharide M_1 with no coupling ^{13}C-^{1}H.

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 examined by arsenite method (Sec. 3.1.28). Consumption of periodate by polysaccharide M_1 with time was presented in Table 13 and Fig. 41.

Table 13. Periodate consumption by polysaccharide M_1

Time in hour	Moles of periodate reduced per C_6 anhydro units
6	0.18
12	0.41
18	0.66
24	0.98
36	0.98
48	0.98

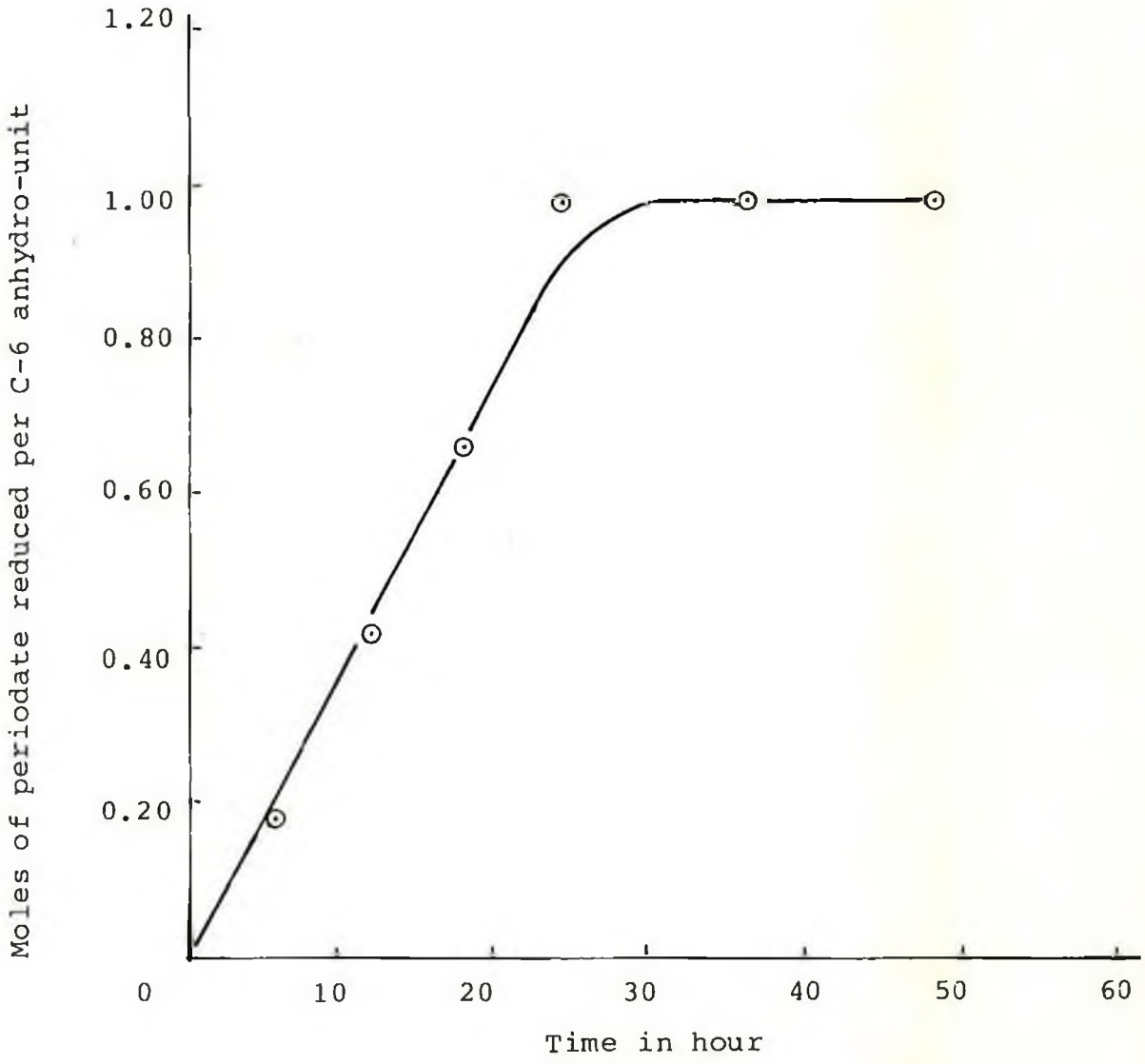


Fig.41. Periodate oxidation of polysaccharide M_1.

After periodate oxidation (48 hours) the excess periodate was destroyed with ethylene glycol. The solution was dialysed extensively (72 hours).

(ii) Reduction - The dialysed solution was concentrated to a suitable volume. Sodium borohydride (.5 mg) was added slowly to the cooled solution of the dialysate with stirring under nitrogen atmosphere and the reaction was allowed to continue for 20 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 and subjected to the following hydrolytic processes:

(iii) a) Mild hydrolysis - The resulting polyalcohol(50 mg.) was treated with 1 N Sulphuric acid (10 ml) at room temperature for 24 hours. The supernatant was concentrated, neutralised with barium carbonate, deionised with Amberlite IR 120 (H^+) and IR45 (OH^-) resins, concentrated to a syrup and examined by paper chromatography in solvent A (using spray 1 and dip 2). This indicated the presence of erythritol, glycol aldehyde and glycerol (trace).

b) Hot hydrolysis- The polyalcohol (50 mg.) was hydrolysed with 1 N sulphuric acid (5 ml.) on a boiling water-bath for 12 hours. The hydrolysate was cooled, neutralised, deionised and concentrated to a syrup. Chromatographic examination of the syrup in solvent A (using spray 1 and 2) revealed spots corresponding to erythritol, glycol aldehyde and glycerol (trace).

3.2.7.9. Partial hydrolysis of polysaccharide M_1

Several attempts were made using different concentrations of sulphuric acid and oxalic acid separately to obtain an optimum condition for hydrolysis of polysaccharide M_1 in order to get a maximum yield of oligosaccharides. However, the highest yield of oligosaccharide was obtained when the polysaccharide was hydrolysed with 0.1 N sulphuric acid at 100°C for 4 hours. Accordingly hydrolysis of the polysaccharide M_1 was conducted as per procedure given below :

The polysaccharide M_1 (10 g) were refluxed with 0.1 N sulphuric acid (700 ml) on a boiling water bath for 4 hours. The reaction mixture was filtered and the filtrate was mixed with ethanol (5 volumes) and left overnight. The precipitate (D_1) was separated by centrifugation (4000 r.p.m.), washed with 95% ethanol, dried in a vacuum desiccator over anhydrous calcium chloride and weighd(6.9 g). The degraded polysaccharide (D_1), on further hydrolysis under the same conditions, yielded another degraded polysaccharide (D_2) and a syrup.

Hydrolysis of the degraded polysaccharide (D_2) gave only D-mannose which was identified by paper chromatography.

Both the supernatants (after separation of D_1 and D_2) were concentrated under reduced pressure, neutratised with

calcium carbonate and filtered. The calcium salts thus obtained were also washed with water (3 times). The combined filtrate and washings were deionised with Amberlite IR-120 (H^+) and IR-45 (OH^-) resins and concentrated to a syrup in vacuo and dehydrated in a desiccator. The syrups showed the same chromatographic pattern in solvent A. Both the syrups were combined, concentrated in vacuo and dehydrated in a desiccator (2.35 g).

Paper chromatographic studies of this syrup in solvents A and E using spray 1 and 2 (Sec. 3.1.14)showed spots which corresponded to D-galactose, D-mannose and three oligosaccharides.

3.2.7.10.Study of oligosaccharides present in polysaccharide M_1

(a) Fractionation of syrupy hydrolysate on charcoal celite column

The syrup obtained from partial hydrolysis of polysaccharide M_1 (Sec. 3.2.7.9.) was dissolved in a minimum volume of water and allowed to soak into the charcoal celite column (40 x 4 cm) as prepared according to the procedure described in Sec. 3.1.18.

The column was eluted separately with water and aqueous ethanol solutions of different concentrations and

the fractions were collected in an automatic fraction collector. Each fraction was analysed by paper chromatography. Aqueous fraction contained only monosaccharides. Fractions eluted by different concentrations of ethanol contained different kinds of oligosaccharides as shown in table 14.

Table 14. Fractionation of partially hydrolysed products of polysaccharide M_1

Elution with	Tubes (10 ml each)	Fraction	Sugar components
Distilled water	1 -600	N_1	discarded
2½% ethanol	100-260	F_1	3 oligos.
5% ethanol	50-410	F_2	4 oligos.
10% ethanol	80-700	F_3	2 oligos.
15% ethanol	100-480	F_4	1 oligos (very small quantity)
20% ethanol	60-390	F_5	1 oligos. "
30% ethanol	270-330	F_6	1 oligos. "
95% ethanol	41-120	F_7	1 oligos. "

N.B.:-oligos. stands for oligosaccharide.

The elution patterns were followed by testing an aliquot (1 ml) with phenol sulphuric acid.

(i) Fraction F_1 contained oligosaccharide 1, oligosaccharide 2 and oligosaccharide 3.

(ii) Fraction F_2 contained oligosaccharide 1, oligosaccharide 2, oligosaccharide 3 and oligosaccharide 4.

The major three oligosaccharides (viz No. 1,2 and 3) were isolated as pure entities after repeated separation on filter sheets using solvent B (Sec. 3.1.14.).

(b) Quantitative estimation of oligosaccharides

All the fractions of oligosaccharides were individually concentrated to syrupy liquid and diluted to a suitable volume . Quantitative estimation of these oligosaccharides were conducted by using standard graph prepared from D-(+)-galactose (Sec. 3.1.11). The yields of oligosaccharides (1,2 and 3) were found to be 2.75, 9.25 and 7.15 mg respectively.

(c) Structural analysis of oligosaccharides 1,2, and 3.

Oligosaccharides 1,2 and 3 were analysed for their DP

(Sec.3.1.13), $R_{galactose}$, structural components and linkages. These are as follows :

Oligosaccharide 1 (R_{gal} = 0.49)

This fraction was found to have DP2 and $R_{galactose}$ 0.49 in solvent B. On hydrolysis it gave L-mannose only. Methylation followed by methanolysis, hydrolysis and paper chromatographic analysis (solvent B) revealed spots corresponding to 2,3,4,6 tetra-O-methyl-D-mannose and 2,3,6-tri-O-methyl-D-mannose in the molar ratio of 1:1.

All these facts indicated that this oligosaccharide might be O-D-mannopyranosyl (1—4)-D-mannopyranose.

Oligosaccharide 2 (R_{gal} = 0.35)

This fraction was found to have DP3 and $R_{galactose}$ 0.35 in solvent B. On hydrolysis it gave D-mannose only. Methylation followed by methanolysis, hydrolysis and paper chromatogrophic analysis (solvent B) revealed spots corresponding to 2,3,4,6-tetra-O-methyl-D-mannose and 2,3,6-tri-O-methyl-D-mannose in the molar ratio of 1:2. The observations revealed that this oligosaccharide might be O-D-mannopyranosyl (1—4)-D-mannopyranosyl(1—4)-D-mannopyranose.

Oligosaccharide 3 (R_{gal} = 0.14)

This fraction was found to have DP4 and $R_{galactose}$ 0.14 in solvent B. On hydrolysis it gave D-mannose only. Methylation followed by methanolysis, hydrolysis and paper chromatographic analysis (solvent B)revealed spots corresponding to 2,3,4,6-tetra-O-methyl-D-mannose and 2,3,6-tri-O-methyl-D-mannose in the molar ratio of 1:3. These findings indicated that this oligosaccharide might be O-D-mannopyranosyl-(1→4)-D-mannopyranosyl (1→4)-D-mannopyranosyl(1→4)-D-mannopyranose.

3.2.8. Structural studies of polysaccharide M_2

3.2.8.1. Measurement of optical rotation of polysaccharide M_2

The sample (50 mg) was dissolved in 100 ml water.The optical rotation of this solution was measured as per procedure described under Sec. 3.1.4. The result was calculated according to Sec. 3.2.7.1. and it was found to be -2.25^{o} (in water, c 0.05).

3.2.8.2. Sugar analysis of polysaccharide M_2 by gas liquid chromatography

The polysaccharide M_2 (5 mg) was hydrolysed by trifluoracetic acid, reduced with sodium borolydride and acetylated with a mixture of pyridine and acetic anhydride according to Sec. 3.1.23. The resulting alditol acetates of sugars were analysed by g.l.c. The results as calculated manually from the peak areas showed the presence of mannose and galactose in the ratio of 4.03 and 1.0 respectively.

3.2.8.3. Methylation of polysaccharide M_2 by Hakomori method

The polysaccharide (20 mg) was dissolved in dimethyl sulphoxide (3 ml) and methylated by using Hakomori method (Sec. 3.1.24.B.). The product showed no hydroxyl absorption band in the I.R. spectrum.

3.2.8.4. Gas-liquid chromatographic analysis of methylated polysaccharide M_2

The methylated polysaccharide M_2 (5 mg) was hydrolysed initially by formic acid (90%) (Sec. 3.1.27) and finally by 0.5 M trifluoroacetic acid (Sec. 3.1.23). The hydrohysate

was reduced with sodium borohydride and acetylated with a mixture of pyridine and acetic anhydride according to the procedure described in Sec. 3.1.23. On analysis of the resulting alditol acetates of the methylated sugars by g.l.c.,the following components were revealed (Table 15).

Table 15. Methylation analysis of the polysaccharide M_2

Sugar and location of methoxyl groups	R_t of Sugars	T^a of Sugars	Mole %
1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-galactitol	11.9	1.11	18.08
1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-D-mannitol	22.3	2.08	64.01
1,4,5,6-tetra-O-acetyl-2,3-di-O-methyl-D-mannitol	39.4	3.68	17.9

T^a=Retention time of the corresponding alditol acetate relative to that of 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol on a OV-225 glass capillary column at 170°C.

3.2.8.5. Gas-liquid chromatography-mass spectrometric studies of methylated polysaccharide M_2

Methylated polysaccharide M_2 was transformed into alditol acetates according to Sec. 3.1.27. Sodium borodeuterite was used as reducing agent. The resulting alditol acetates were studied by g.l.c.-m.s. according to the procedure described under Sec. 3.1.32. The following $m/_e$ values obtained from Fig. 42,43 and 44, indicated the presence of 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-galactitol; 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-D-mannitol and 1,4,5,6-tetra-O-acetyl-2,3-di-O-methyl-D-mannitol respectively.

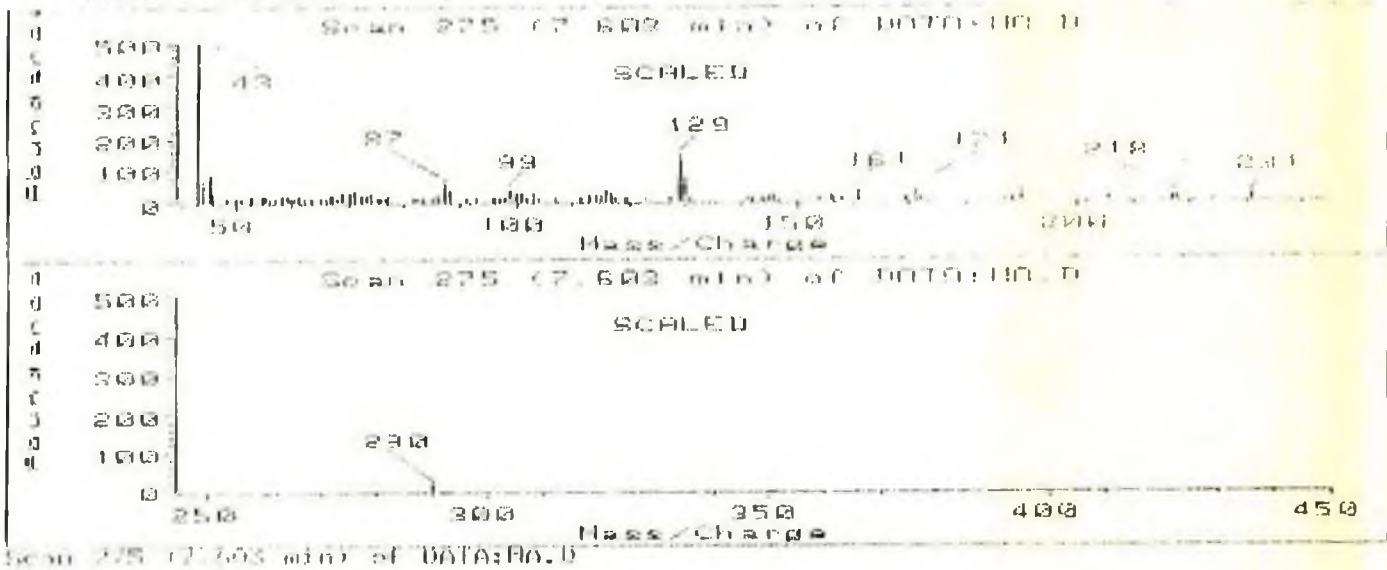
$m/_e$ 43, 45, 87, 102, 118, 129, 145, 161 and 205.

$m/_e$ 43, 45, 87, 102, 113, 118, 162,173 and 233.

$m/_e$ 43, 87, 101,118, 129, 160, 189 and 194.

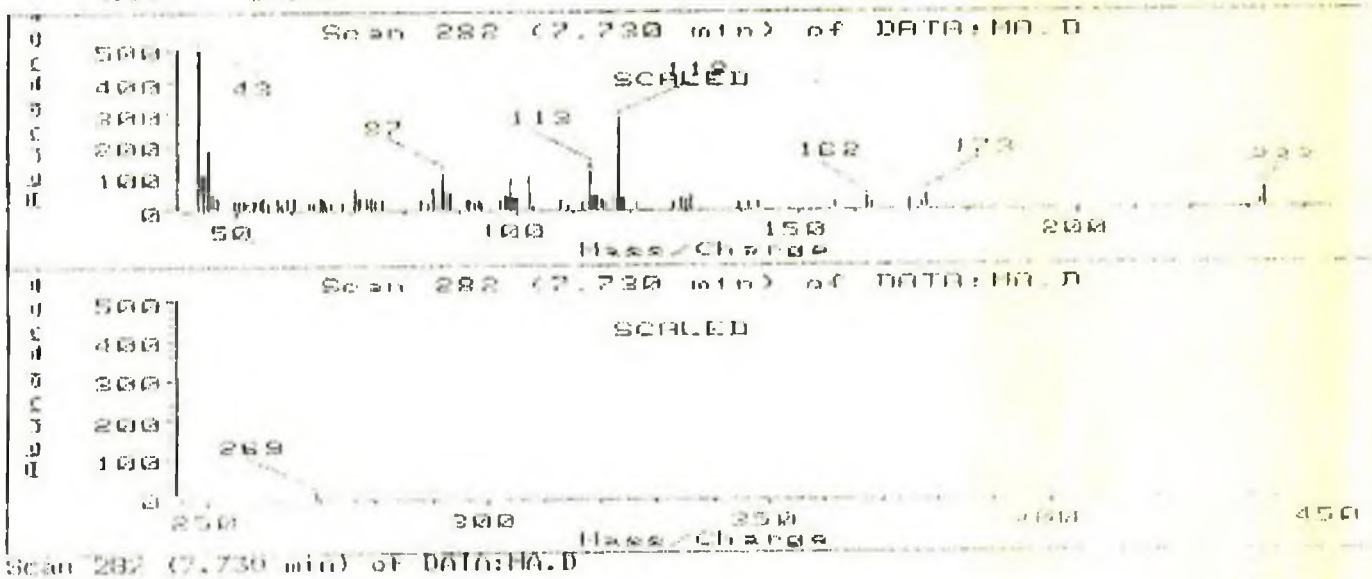
3.2.8.6. ^{1}H-n.m.r. study of polysaccharide M_2

^{1}H-n.m.r. spectrum was studied using the instrument as mentioned in Sec. 3.1.30. ^{1}H-n.m.r. spectrum (Fig. 45) of polysaccharide M_2 was found to contain signals at δ 4.90, δ 5.12 and δ 5.19 p.p.m. These signals were assigned to anomeric protons of 1,4, linked mannose residues, 1,4,6 linked mannose residues and terminal galactose residues respectively.



m/z	abund.	m/z	abund.	m/z	abund.	m/z	abund.
43.00	1000	68.75	27	99.00	34	144.10	7
43.70	72	69.05	27	100.00	28	144.90	15
45.10	82	70.15	25	100.80	23	145.70	17
45.80	23	71.15	31	102.00	11	146.90	6
48.50	11	72.15	16	103.00	22	153.90	9
50.20	11	73.05	18	103.90	10	156.00	15
50.70	13	73.95	25	106.90	7	156.70	15
52.80	16	75.35	12	111.05	15	157.95	11
54.80	17	75.85	10	111.85	7	158.95	6
55.10	17	76.65	7	113.05	14	161.05	30
56.00	13	77.15	14	114.15	14	169.35	8
56.90	12	81.55	10	115.05	21	171.05	30
58.00	18	82.05	12	116.05	31	172.05	10
59.10	17	82.65	9	116.95	25	186.80	6
59.60	11	83.35	15	117.85	10	188.10	9
59.90	11	84.95	12	119.45	12	188.80	8
60.20	10	86.15	15	119.95	8	189.20	8
61.10	13	87.10	35	120.15	9	190.00	31
62.05	9	88.10	31	127.45	9	215.85	8
63.35	12	91.00	8	127.95	144	215.95	8
65.15	8	92.90	10	130.05	30	217.35	11
65.95	15	95.70	12	130.85	12	221.05	6
66.75	21	96.10	11	141.00	7	230.00	15
67.25	15	96.90	11	142.00	10	230.10	10
68.05	11	98.00	11	143.60	7		

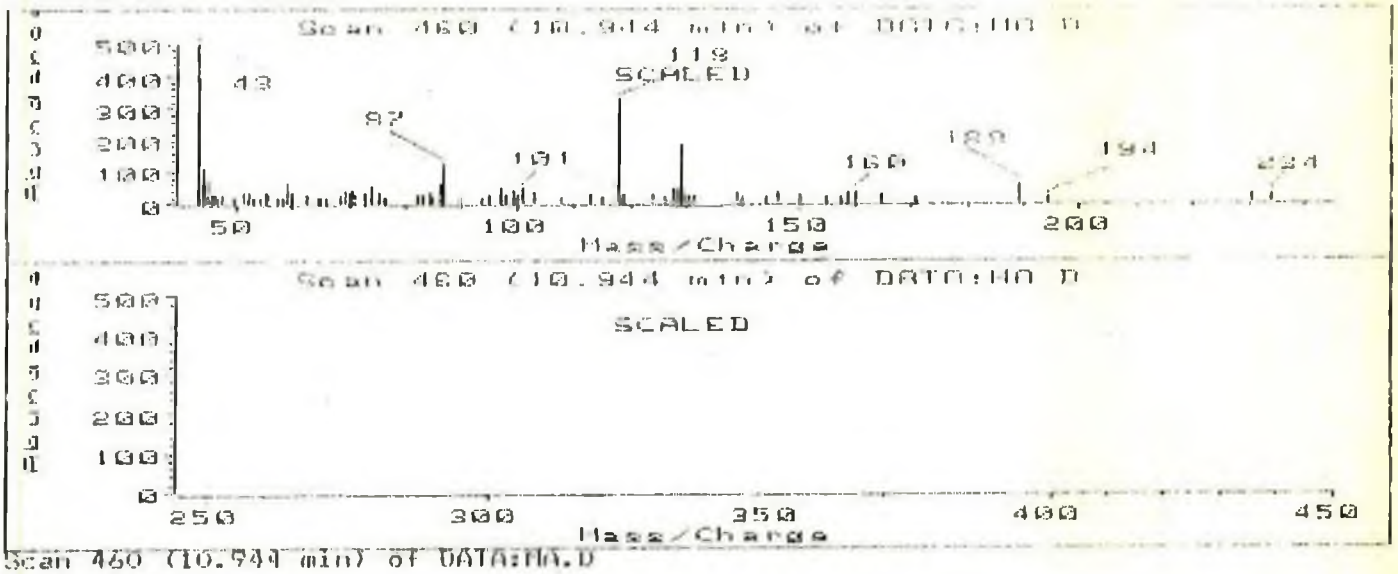
Fig. 42. Mass spectrum of polysaccharide M_2 showing $m/_e$ values of 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-galactitol.



Scan 282 (7.730 min) of DATA:HA.D

m/z	abund.	m/z	abund.	m/z	abund.	m/z	abund.
43.00	1000	60.50	30	91.20	24	118.05	284
44.10	107	63.05	20	91.40	22	119.05	31
45.00	181	63.75	18	92.30	19	121.35	16
45.70	45	64.85	30	93.20	24	126.95	15
46.80	29	65.35	22	93.60	15	129.15	34
49.50	22	65.95	17	97.20	28	130.25	25
50.40	27	66.75	20	98.10	40	130.95	43
51.40	26	68.85	19	99.00	95	139.40	17
51.90	18	70.95	60	100.30	27	141.10	20
52.20	20	71.95	30	102.10	101	142.20	17
53.00	23	73.05	30	103.00	20	143.10	20
53.50	26	74.05	29	107.60	24	156.20	15
54.10	23	75.05	28	108.90	12	161.35	29
54.80	34	75.75	24	111.55	21	162.85	16
55.70	21	82.95	28	113.15	134	169.85	25
57.20	31	84.35	17	114.15	39	172.05	15
57.80	21	85.05	59	115.05	26	172.95	41
58.30	17	86.10	19	115.55	19	232.30	20
59.00	30	87.00	109	116.05	15	233.10	56
60.30	30	87.50	51	117.05	52	269.35	12

Fig.43. Mass spectrum of polysaccharide M_2 showing m/e valued of 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-D-mannitol.



Scan 460 (10.944 min) of DATA:HA.D
HA AA BCSIR

m/z	abund.	m/z	abund.	m/z	abund.	m/z	abund.
43.00	1000	64.45	19	90.00	18	129.05	184
44.10	107	65.05	20	93.80	16	130.35	26
45.00	691	66.05	21	94.90	26	131.15	28
45.60	24	68.35	27	97.00	45	139.10	34
46.10	25	69.15	40	97.80	23	139.90	19
46.70	21	70.05	36	99.10	38	144.20	20
47.40	22	70.55	37	100.00	23	146.30	29
49.40	21	71.05	28	100.90	46	150.30	23
51.30	35	72.55	33	102.90	29	154.80	16
52.30	33	74.05	52	107.70	16	155.40	17
53.10	19	75.25	31	113.05	28	158.85	29
54.30	19	76.05	15	115.15	16	160.05	33
55.30	31	76.35	15	117.05	59	164.15	26
55.60	26	81.95	22	118.05	325	170.35	61
57.10	21	82.95	25	119.05	23	187.80	54
58.10	21	84.05	32	123.95	27	194.20	19
59.00	62	84.65	20	126.05	21	230.30	24
59.80	34	86.20	59	127.95	48	231.10	28
62.25	26	87.00	120				

Fig.44. Mass spectrum of polysaccharid M_2 showing $m/_e$ values of 1,4,5,6-tetra-O-acetyl-2,3-di-O-methyl-D-mannitol.

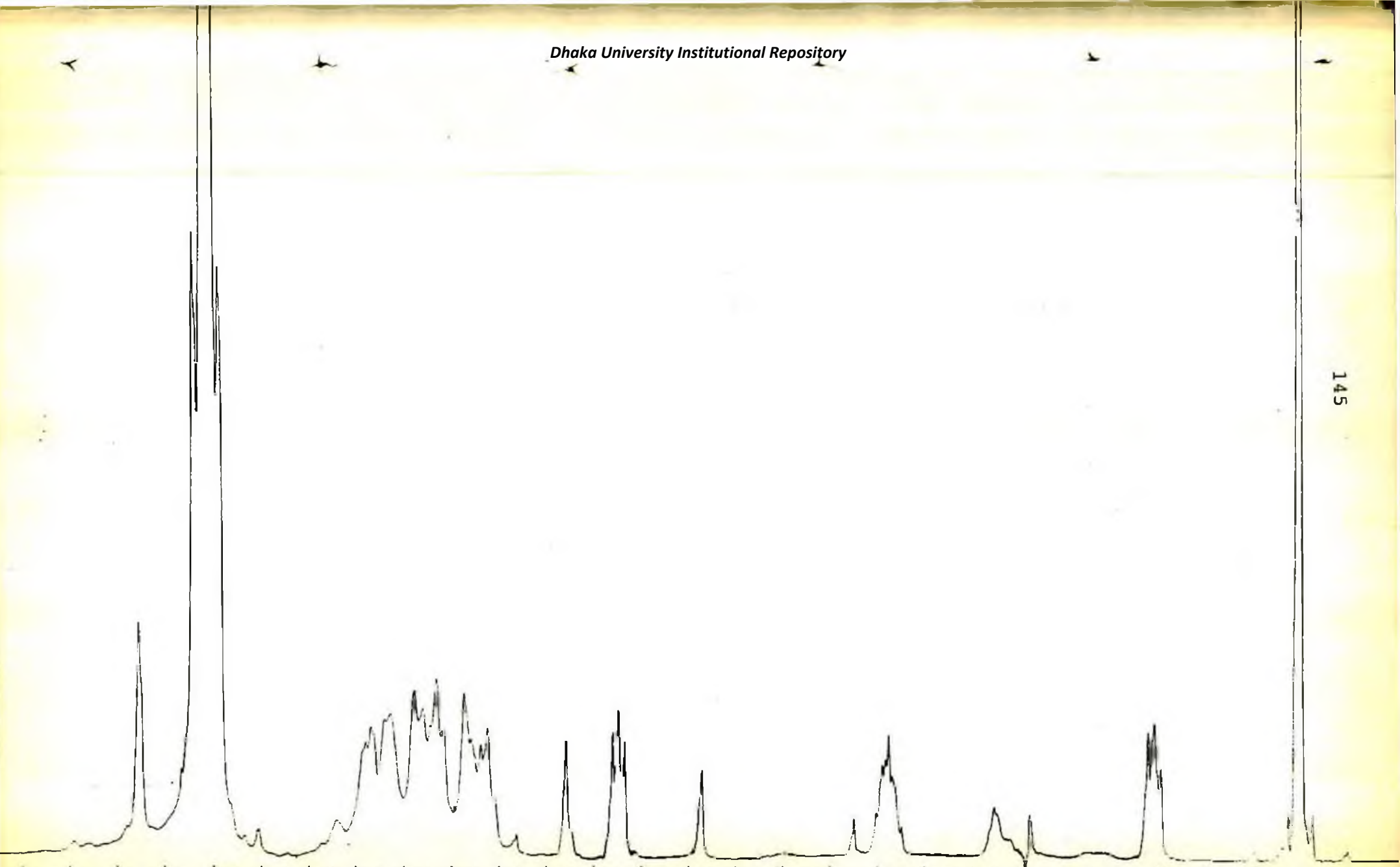


Fig. 45. ^{1}H-n.m.r. spectrum of polysaccharide M_2.

3.2.8.7. ^{13}C-n.m.r. study of polysaccharide M_2

^{13}C-n.m.r. spectrum was studied using the instrument as mentioned in Sec. 3.1.31. The sample was dissolved in D_2O and external tetra methyl silane was used as reference.

In the nose-decoupled spectrum, ^{13}C-n.m.r. showed six main peaks and some weaker ones. The chemical shifts, as determined from the peaks, were presented in Table 16.

Table 16. ^{13}C-n.m.r. spectral data of polysaccharide M_2

δ	Assignment
101.2 p.p.m	C_1
72.9 "	C_2
73.3 "	C_3
74.7 "	C_4
75.0 "	C_5
63.4 "	C_6

3.2.9. Structural studies of polysaccharide M_3

3.2.9.1. Measurement of optical rotation of polysaccharide M_3

50 mg of the sample was dissolved in 100 ml of 3% aqueous sodium hydroxide solution. The optical rotation of this solution, measured as per procedure described under 3.1.4., was calculated according to 3.2.7.1. and found to be -1.56° (in water, c 0.05).

3.2.9.2. Sugar analysis of polysaccharide M_3 by gas liquid chromatography.

The polysaccharide M_3 (10 mg) was hydrolysed by trifluoroacetic acid, reduced with sodium borohydride and acetylated with a mixture of pyridine and acetic anhydride according to Sec. 3.1.23. The resulting alditol acetates of sugars were analysed by gas liquid chromatography. The results were calculated from the peak areas of the spectrum which indicated the presence of 85.7% mannose and 14.3% galactose.

3.2.9.3. ^{1}H-n.m.r. study of polysaccharide M_3

^{1}H-n.m.r. spectrum was measured using the instrument as mentioned in Sec. 3.1.30. ^{1}H-n.m.r. spectrum (Fig. 46) of polysaccharide M_3 contained signals at δ 4.74, δ 4.95 and δ 5.26 p.p.m. which were assigned to anomeric protons of 1,4 linked mannose recidues, 1,4,6- linked mannose residues and galactose residues respectively.

3.2.9.4. ^{13}C-n.m.r. study of polysaccharide M_3

^{13}C-n.m.r. spectra were measured using the instrument as mentioned in Sec. 3.1.31.

In the noise-decoupled spectrum, ^{13}C-n.m.r. showed six main peaks and some weaker ones (Fig. 47). The chemical shifts of these main peaks were assigned as shown in Table 17.

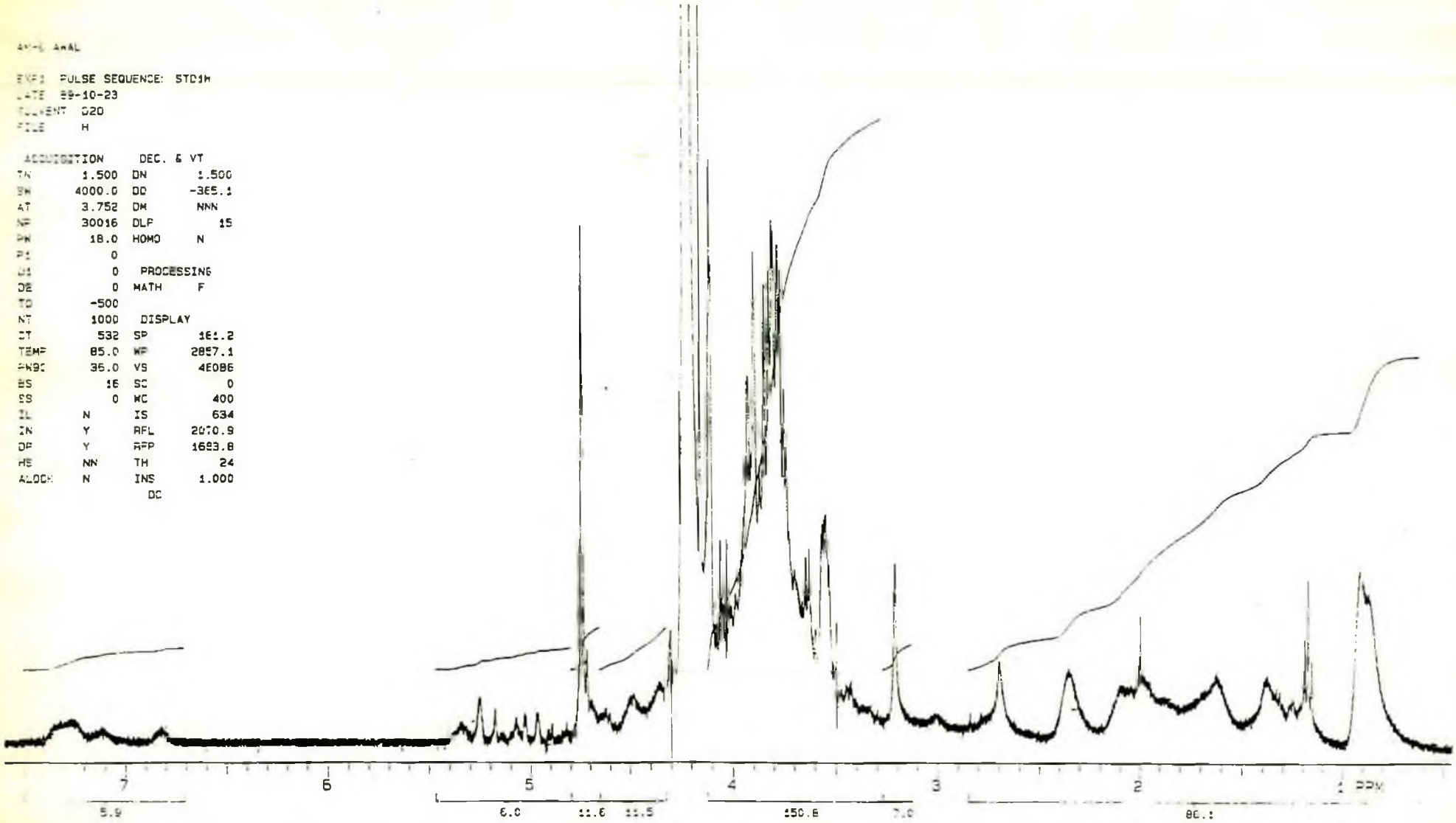


Fig.46. ^{1}H-n.m.r. spectrum of polysaccharide M_3.

Table 17. ^{13}C-n.m.r. spectral data of polysaccharide M_3

δ	Assignment
103.4 p.p.m.	C_1
73.4 "	C_2
74.8 "	C_3
79.8 "	C_4
78.4 "	C_5
64.0 "	C_6

AM-E AWAL

EXPS PULSE SEQUENCE: STD13C
DATE 89-10-23
SOLVENT D2O
FILE C

ACQUISITION		DEC. & VT	
TN	13.500	DN	1.500
SW	20000.0	DO	-365.1
AT	1.501	DM	YYY
NP	60032	DMM	S
PW	6.0	DMF	9600
P1	0	DHP	72.0
D1	0	DLP	0
D2	0	HOMO	N
TO	-300		
NT	100000	PROCESSING	
CT	36501	SE	0.159
TEMP	85.0	LB	2.000
PWBO	16.0	MATH	F
BS	100		
SS	0	DISPLAY	
IL	N	SP	-61.7
IN	Y	WP	20000.0
DP	Y	VS	77
HS	NN	SC	0
ALOCK	S	WC	400
		IS	500
		RFL	61.7
		RFP	0
		TH	31
		INS	1.000

INDEX	HERTZ	PPM	INTENSITY
01	14408.1	103.485	74.367
02	9520.4	79.033	83.142
03	9447.9	78.495	76.902
04	9435.0	78.398	82.636
05	7351.5	73.481	71.394
06	6441.2	64.842	60.704
07	3646.1	30.308	41.120

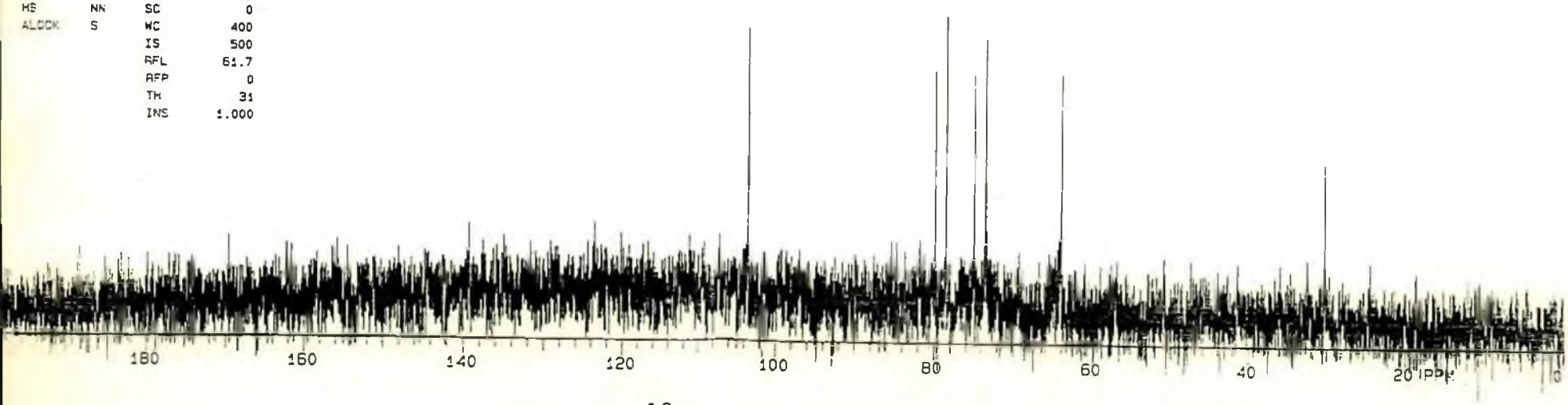


Fig. 47. ^{13}C-n.m.r. spectrum of polysaccharide M_3.

4. RESULTS AND DISCUSSION

Ripe palm fruits of Borassus flabelifer Linn. collected from Dhaka (August) and Laksmipur (about 200 Km south of Dhaka, December) were designated as samples S_1 and S_2 respectively. The inner hard endosperm of both the samples were separated, sun-dried and powdered separately. Each sample was extracted successively with petroleum ether (b.p. 40 - 60°C), ethanol (80%), cold water (room temperature), hot water (on boiling water bath), 3% and 10% aqueous sodium hydroxide at room temperature. Results are given in table 5. The variations of yields of different extracts are not of much significance.

Hydrolysis of each of the different extracts afforded the same sugar (Table 6) showing the similar nature of sugars in each fractions. The alcoholic extract gave sucrose, D-glucose and L-fructose while cold water extract contained L-arabinose (very small), D-mannose and D-galactose. Water and alkali extracts (3% and 10%) did not contain any free sugar.

Since the yield of the polysaccharide from the kernel was maximum (44.1%) when 10% aqueous sodium hydroxide was used, it seems reasonable to consider this fraction as the major polysaccharide of the kernel. As such study on the structural pattern of this fraction was undertaken first.

4.1. Study of polysaccharide M_1

10% alkali extract was purified separately by precipitating with Fehling's solution (Sec. 3.2.4.1), with ammonium sulphate (Sec. 3.2.4.2) and by gel filtration with sepharose CL-4B (Sec. 3.2.4.3.) and 2B (Sec. 3.2.4.4). Each of the purified polysaccharide fractions gave same sugars : mainly D-mannose and small quantity of D-galactose, indicating that they were identical. Therefore the sample purified by sepharose CL-2B was used for further study. This homogeneous component is designated as a pure polysaccharide M_1 [ash 2.2%, moisture 10%, $[\alpha]_D^{30} = -7.2^o$ (in 10% aqueous sodium hydroxide, c 0.05), uronic acid nil].

The low specific rotation of the polysaccharide M_1 indicated that the monosaccharide units were glycosidically linked in the β-form.

4.1.1. Methylation and g.l.c. analysis

The methylation of polysaccharide M_1 was initially carried out by Haworth method. Five consecutive methylations as described in Sec. 3.2.7.3. afforded a product with methoxyl

content 41.5% [For trimethoxyhexose, ($C_9H_{16}O_5$), 45%]. This was further subjected to methylation by modified Hakomori method. The methylated product showed no hydroxyl band in the I.R. spectrum.

The methylated sugar M_1 was hydrolysed with formic acid and trifluoroacetic acid, reduced with sodium borohydride and acetylated with acetic anhydride in pyridine (Sec. 3.1.27). The resulting alditol acetates were indentified by g.l.c.from their retention times as 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-mannitol; 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-galactitol; 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-D-mannitol and 1,4,5,6-tetra-O-acetyl-2,3,-di-O-methyl-D-mannitol and their relative proportions of 3:3:88:6 were calculated from the peak areas of the chromatograms. The result was recorded in table 11.

The location of the hydroxyl groups in the above four cleaved methylated alditol acetates indicated the positions through which the monosaccharide units were engaged in union in the polysaccharide. The presence of 1,4,5-tri-O-acetyl-2,3, 6-tri-O-methyl-D-mannitol indicated that these residues were in the pyranose forms (C_5-linked) and this unit is linked to two other units through C_1 and C_4 (scheme 1). The presence of 1,4,5,6-tetra-O-acetyl-2,3-di-O-methyl-D-mannitol showed a similar involvement of C_5 in pyranose ring and C_1, C_4 and C_6

in the union with other units (sheme 1). The presence of 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-mannitol indicated that these residues were also in the pyranose form and linked at its C_1 position to the D-mannose backbone. The appearance of 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-galactitol indicated that these residues were also in the pyranose form and linked at C_1 position of D-galactose.

The identification of these fragments in the hydrolytic products of the methylated polysaccharide M_1 provided evidence for the presence of the structural units as shown in Fig.48.

Fig. 48. Structural units of the polysaccharide M_1

Man_p1- , $-4\ Man_p1-$

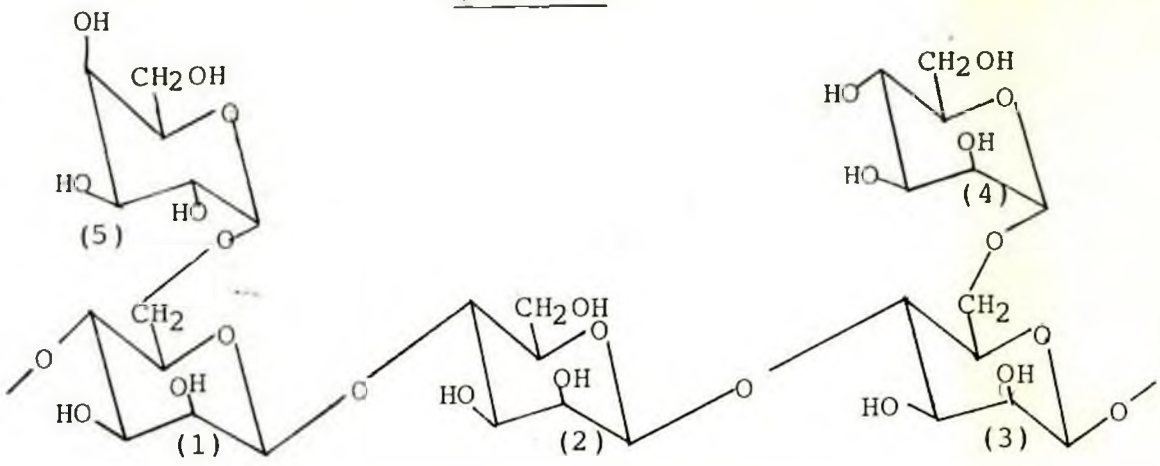
$-4\ Man_p1-$ and Gal_p1-

Where

Man_p = D-mannopyranose

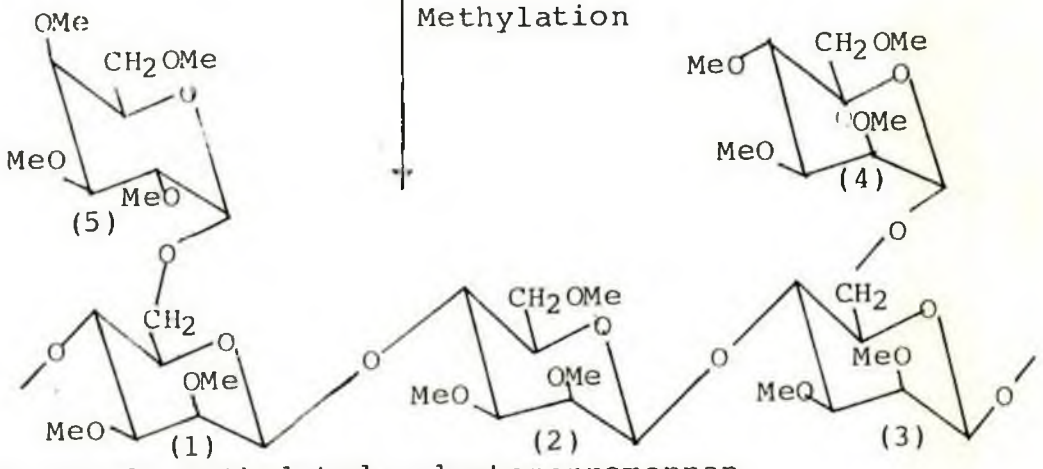
Gal_p = D-galactopyranose

Scheme 1



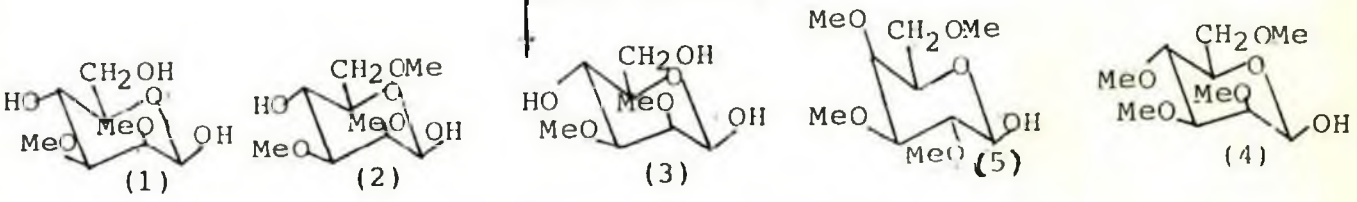
A hypothetical galactomannomannan

Methylation



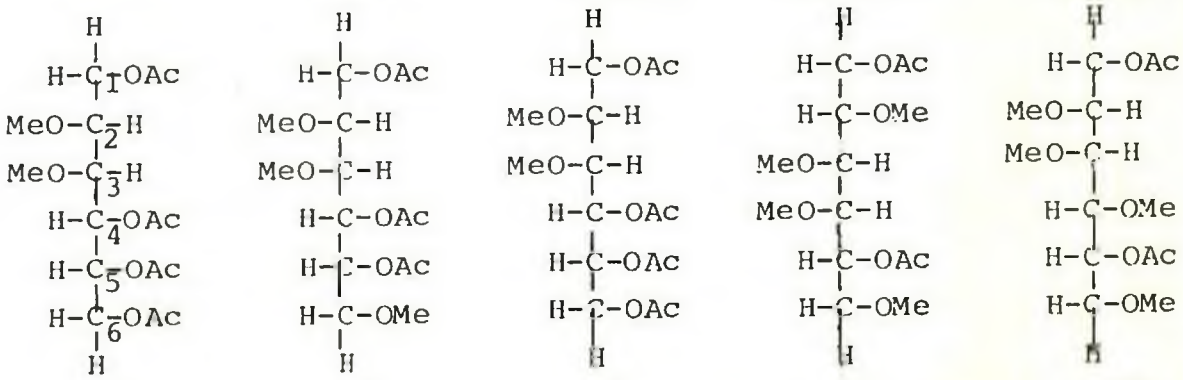
Completely methylated galactomannomannan

Acid hydrolysis



After reduction and acetylation

from (1) from (2) from (3) from (4) from (5).



6 7 8 9 10

Methylated alditol acetates of M_1.

The presence of 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-D-mannitol mainly as a major product (87.54%) revealed a linear chain of D-mannose units linked at 1,4, fashion in the polysaccharide M_1. This is similar to the general structural pattern of alkali soluble mannans[98]. A.K. Mukherjee et al.[192] also showed that the back bone of the molecule of the mannan extracted from the palm seed kernel with 18% NaOH was made up of 1-->4 linked mannopyranose residues.However, 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl D-mannitol might arise from 1,5-linked mannofuranose units. Such possibility was not considered for the following reasons:

(a) Such linkage is not common.

(b) Furanosidic sugars are highly susceptible to acid hydrolysis[223] but M_1 took 4-8 hours to hydrolyse in O.1 N sulphuric acid and

(c) O-D- mannopyranosyl (1—>4)-D- mannopyranose, O-D-mannopyranosyl (1-->4)-D-mannopyranosyl (1—>4)-D-mannopyranose and O-D-mannopyranosyl (1-->4)-D-mannopyranosyl (1—>4)-D-mannopyranosyl (1-->4)-D-mannopyranose (p.137 and 138) were identified in partial hydrolysis of M_1.

The presence of 1,4,5,6-tetra-O-acetyl-2,3-di-O-methyl -D-mannitol (6.18%) revealed further a branching at C_6 position of the D-mannose units. The presence of 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-galactitol (3.18%) indicated that the C_6 position

of D-mannose was linked with C_1 position of D-galactose. Similarly, the appearance of 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-mannitol (3.09%) further indicated a branching at its C_1 position with C_6 position of the mannose units of the back bone.

Further supports for such a 1,6-linked galactomannan where C_6 position of D-mannose of the back bone was linked with C_1 position of D-galactose came from ^{13}C-n.m.r.(p.165).

The appearance of 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl derivatives indicated that all the positions except C_1 and C_5 were free and not substituted. Having C_1 and C_5 already linked there remained no position to contain any more monosaccharide unit. Thus presence of more than one monosaccharide units in the side chain was not considered.

From the ratio of the amounts of tetra-, tri-and di-O-methyl sugars, the length of the repeating units of the polysaccharide M_1 comes to 32 hexose units and the molecular weight of the repeating units was found to be about 5200 as shown in Table 18.

Table 18. Determination of length and molecular weight of repeating units

Sugars	Mole %	No. of units	Mol.wt. of the unmethylated monosaccharide
1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-mannose.	3.09	1.00	179X1 =179
1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-galactose.	3.18	1.02	179X1 =179
1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-D-mannose.	87.54	28.33	162X28=4536
1,4,5,6-tetra-O-acetyl-2,3-di-O-methyl-D-mannose.	6.18	2.00	162X2 =324
			5218

Further supports that the polysaccharide M_1 was a β-D-(1→4)-galactomannomannan came from gas liquid chromatography - mass spectrometric analysis of alditol acetates, periodate oxidation studies and ^{13}C-n.m.r. of the polysaccharide.

4.1.2. Mass spectrometric study

Methylated alditol acetate fragments obtained from polysaccharide M_1 viz. (a) 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-mannital, (b) 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-galactitol, (c) 1,4,5-tri-Oacetyl-2,3,6-tri-O-methyl-D-mannitol and (d) 1,4,5,6 tetra-O-acetyl-2,3,-di-O-methyl-D-mannitol were subjected to mass spectrometric analysis. The results are given as follow :

(i) 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-galactitol

$m/_e$ 43, 45, 73, 86, 103, 115, 127 and 145 (Fig. 36) [recorded [223] $m/_e$ values are 43, 45, 71, 87, 101, 117, 129, 145, 161 and 205 (Fig. 49)].

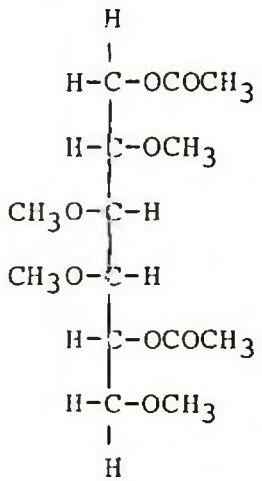


Fig.49.1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-galactitol.

(ii) 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-D-mannitol

$m/_e$ 43, 45, 87, 101, 113, 117, 129, 131, 161,173 and 233 (Fig. 37) [recorded[223] $m/_e$ values are 43, 45, 87, 99, 101, 113, 117, 129, 161, 173 and 233 (Fig.50)]

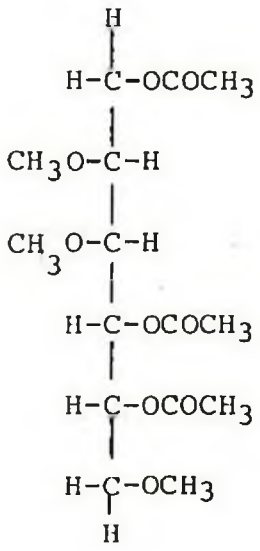


Fig. 50. 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-D-mannitol.

The fragmentation pattern of the different methylated alditol acetates are illustrated with 1,4,5-tri-O-acetyl-2,3, 6-tri-O-methyl-D-mannitol with respect to their individual $m/_e$ values (Fig. 51) in support of the mass spectrum (Fig.37).

Two primary fragments at $m/_e$ 233 (F_I) and $m/_e$ 117(F_E) were obtained through fission between C_2 and C_3 of 1,4,5- tri-O-acetyl-2,3,6-tri-O-methyl D-mannitol as shown in Fig.51. The fragment having $m/_e$ 101 (F_D) was obtained due to the loss of one molecule of acetic acid from a fragment of $m/_e$ 161 (F_C) which also arose from 1,4,5-tri-O-acetyl 2,3,6-tri-O-methyl D-mannitol. The fragment at $m/_e$ 161 (F_C) was further fragmented to give a fragment at $m/_e$ 129 (F_H) through the loss of one molecule of methanol. A minor peak at $m/_e$ 173 (F_F) was derived from $m/_e$ 233 (F_I) possibly by loss of one molecule of acetic acid and subsequent further loss of another molecule of acetic acid to give a prominent peak at $m/_e$ 113 (F_G). It is also possible that this secondary fragment ($m/_e$ 113; F_G) was formed by loss of two molecules of acetic acid from $m/_e$ 233 (F_I). The fragment at $m/_e$ 43 (F_A) was indicative of an acetyl group and another fragment $m/_e$ 45 (F_B) of low abundance was exhibitive of a terminal unit bearing a methoxy group.

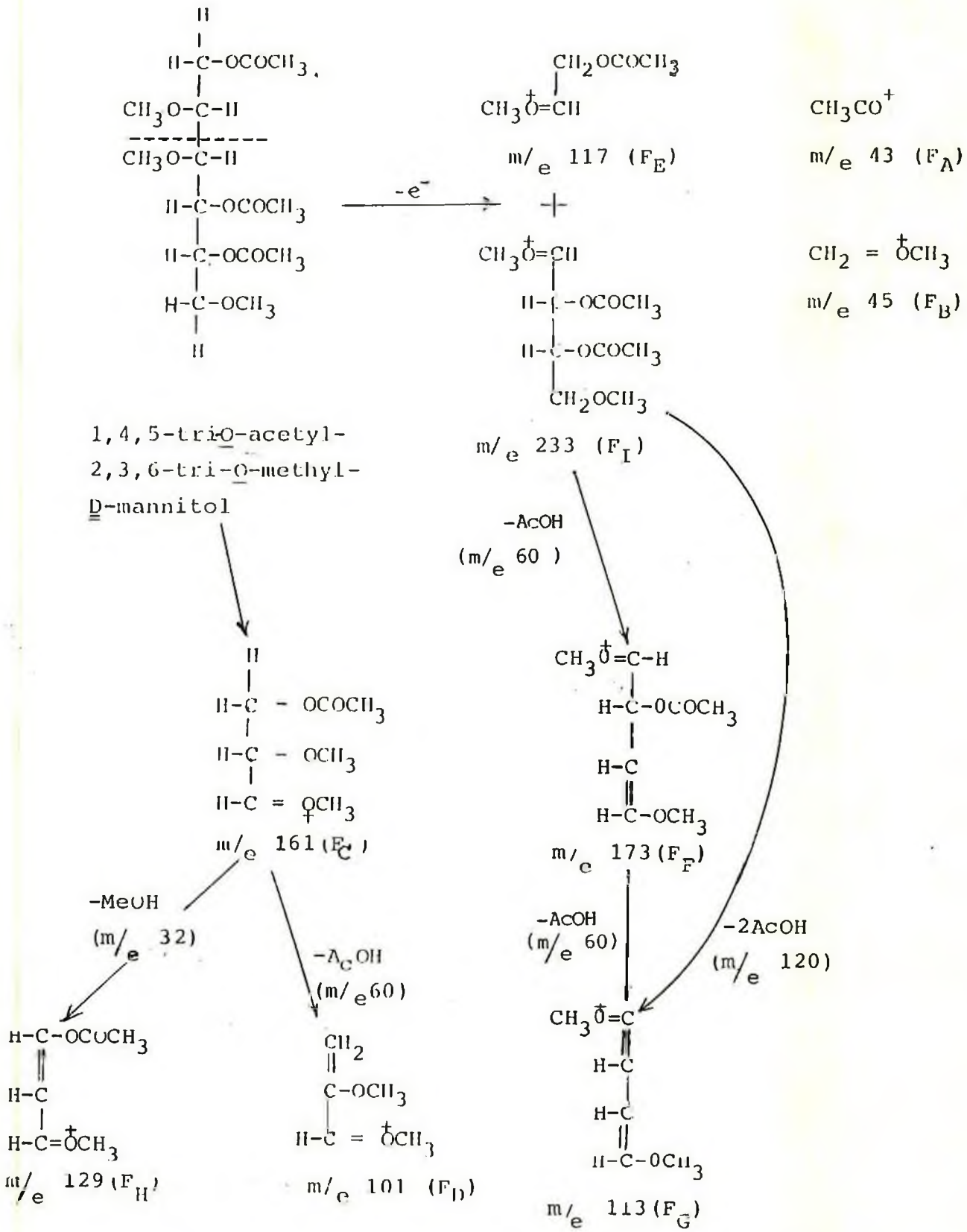


Fig. 51. Fragmentation pattern of 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-D-mannitol.

(iii) 1,4,5,6-tetra-O-acetyl-2,3-di-O-methyl-D-mannitol

$m/_e$ 43, 87, 97, 103, 115, 127, 145, and 157 (Fig. 38) [reported [223] $m/_e$ values are 43, 87, 99,101, 117, 127, 159 and 201 (Fig. 52)]

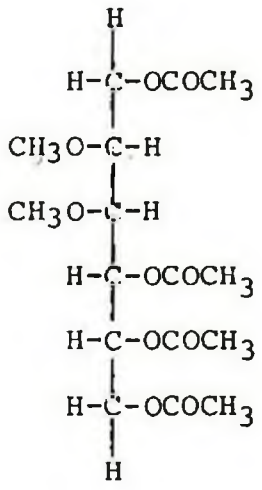


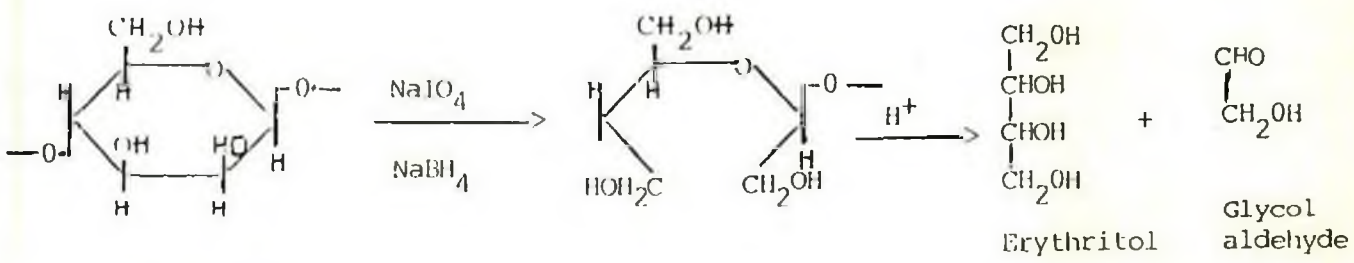
Fig. 52. 1,4,5,6-tetra-O-acetyl-2,3-di-O-methyl-D-mannitol.

4.1.3. Smith degradation study

The polysaccharide M_1 was subjected to periodate oxidation as described in Sec. 3.2.7.8. The consumption of periodate was found to be 0.98 moles (Fig. 41) per anhydro sugar residues. This indicated that polysaccharide M_1 was a straight chained polymer having linkage either in 1—>2 or 1—>4 positions.

It is true that a 1—>2 linked hexopyranose unit would consume the same amount of periodate but methylation studies showed the presence of 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-hexopyranose (p.156) unit indicating 1—>4 linked hexopyranose unit. Had there been 1—>2 linked hexopyranose units, there would have 1,2,5-tri-O-acetyl-3,4,6-tri-O-methylhexopyranose derivatives. Therefore, periodate oxidation coupled with methylation studies showed M_1 to have 1—>4 linkages.

Moreover, the periodate oxidised product of M_1 gave mainly erythritol and glycol aldehyde by Smith degradation (p.132 a).



1—>4 linkage

The formation of these products (p.33b, Fig 4b) suggested 1—>4 linkage in the back bone of M_1.

4.1.4. ^{13}C-n.m.r. study

^{13}C-n.m.r. studies of the polysaccharide M_1 gave the coupling constant $J^{13}C-^{1}H$=160.5 Hz (Fig.39, recorded[227] value, $J^{13}C-^{1}H$=160 Hz). This value of coupling constant confirmed the β-configuration for 1→4-linked D-mannopyranose residues of the polysaccharide M_1.

In ^{13}C-n.m.r. studies (Sec. 3.2.7.7) of the polysaccharide M_1 in the noise-decoupled spectrum there are six main peaks together with some weaker ones (Fig. 40). These six peaks corresponded to the D-mannose residue in regular repeating structure. Comparative data of chemical shifts (δ) of the polysaccharide M_1 with those of a β-D-(1→4)-linked D-mannopyranan and the corresponding assigments are shown in Table 19.

Table 19. A comparative ^{13}C-n.m.r. spectral data of polysaccharide M_1

Polysaccharide M_1 δ	β-D-(1→4)-linked-D-mannopyranan[22] δ	Assignment
101.7 p.p.m.	101.7 p.p.m.	C_1
72.0 "	72.0 "	C_2
73.3 "	73.8 "	C_3
77.5 "	78.8 "	C_4
77.0 "	78.8 "	C_5
62.2 "	62.1 "	C_6

In the undecoupled spectrum, two $-CH_2-$carbons can be identified from the centre lines of the triplets. In addition to a $-CH_2-$carbon at δ 62.2 p.p.m., there is also a $-CH_2-$carbon at δ 67.4 p.p.m. (Fig. 40). δ 67.4 p.p.m. stands for substituted C_6 and δ 62.2 p.p.m. for unsubstituted C_6 of the chain. This suggested that a 1,6-linked hexose was present in polysaccharide M_1 in addition to 1,4-linked mannose.

4.1.5. Partial hydrolysis

Further evidence regarding the structure of the polysaccharide M_1 came from its partial hydrolysis studies. Initially the polysaccharide was hydrolysed with 0.1 N sulphuric acid on a boiling water bath for 4 hours (Sec. 3.2.7.9.) which afforded a degraded polysaccharide named D_1 and a syrup. Hydrolysis of D_1 with 0.1 N sulphuric acid under the same conditions gave another degraded polysaccharide named D_2 and a syrup. Hydrolysis of D_2 gave only D-mannose which was identified by paper chromatography. Both these syrups were found to contain D-galactose, D-mannose and three oligosaccharides as revealed from paper chromatographic studies.

The syrupy hydrolysate was fractionated by a chracoal celite column (Sec. 3.2.7.10). Monosaccharides were removed by elution with water. Gradient elution of the column with increasing concentration of ethanol gave three oligosaccharides from polysaccharide M_1.

The oligosaccharide-1 having DP2, appeared to be a disaccharide comprising two molecules of mannose units. On hydrolysis, it gave D-mannose only. After methylation followed by methanolysis and hydrolysis, paper chromatographic studies of this hydrolysed product showed two spots corresponding to

2,3,4,6-tetra-O-methyl-D-mannose and 2,3,6-tri-O-methyl-D-mannose in the molar ratio of 1:1. Above observations indicated that this oligosaccharide had the following structure.

O-D-mannopyranosyl (1—4)-D-mannopyranose.

The oligosaccharide - 2 having DP3, appeared to be a trisaccharide comprising three molecules of mannose units. On hydrolysis, it gave D-mannose only. After methylation followed by methanolysis and hydrolysis, paper chromatographic studies of this hydrolysed product showed two spots corresponding to 2,3,4,6-tetra-O-methyl-D-mannose and 2,3,6-tri-O-methyl-D-mannose in the moler ratio of 1:2. Above observations indicated that this oligosaccharide had the following structure.

O-D-mannopyranosyl (1—4)-D-mannopyranosyl (1—4)-D-mannopyranose.

The oligosaccharide - 3 having DP4, appeared to be tetra saccharide comprising four molecules of mannose units. On hydrolysis it gave D-mannose only. After methylation followed by methanolysis and hydrolysis, paper chromatographic studies of this hydrolysed product showed two spots corresponding to 2,3,4,6-tetra-O-methyl-D-mannose and 2,3,6-tri-O-methyl-D-mannose in the molar ratio of 1:3. Above observations indicated that the oligosaccharide had the following structure.

O-D-mannopyranosyl (1-->4)-D- mannopyranosyl (1-->4)-D-mannopyranosyl (1-->4)-D-mannopyranose.

The structural pattern of these oligosaccharides as shown above indicated that D-mannopyranose residues were linked to one another through C_1 and C_4 having a 1,4 linked straight chain.

However, the presence of D-mannose and D-galactose in both the syrups, in addition to the oligosaccharides, revealed that these groups were present as side chains.

Conclusion

Thirty two units mentioned on p.157 and 158 came from manual calculations of methylation analysis. Thirty three units arose from sugar analysis (p.121). Thirty three units were preferred to 32 as it (33) was determined by integration.

Thus from the studies of specific rotation, methylation and g.l.c. analysis, ^{13}C n.m.r., mass spectroscopy, periodate oxidation and Smith degradation it is concluded that the polysaccharide M_1 has the following structure of a repeating unit.

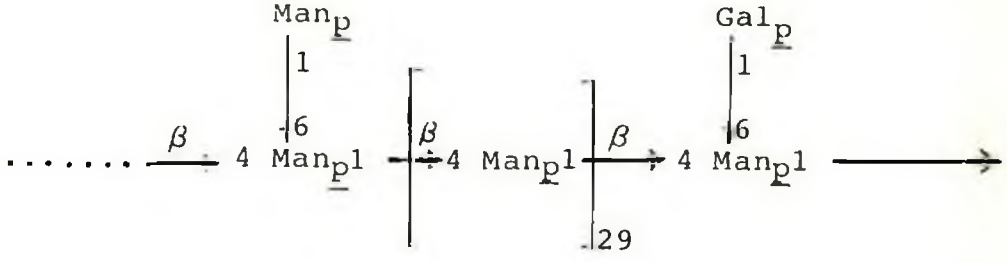


Fig.53. A repeating unit of polysaccharide M_1.

4.2. Study of polysaccharide M_2

Having estabished the structure of polysaccharide M_1, attempts were made to determine the structure of polysaccharide M_2 (extracted with water).

Both cold and hot water extracts were found to contain the same sugar components (Table 5) and as such hot water extract was used to study the structural pattern. It was purified by gel filtration with sepharose CL-2B and designated as a pure polysaccharide M_2 $[\alpha]_D^{30} = -2.25^o$ (in water, c 0.05).

The negative value of specific rotation of the polysaccharide M_2 indicated that the monosaccharide units were glycosidically linked in the β-form.

On sugar analysis it showed the presence of D- galactose and D-mannose in the molar ratio of (1:4.03).along with a very small quantity of L-arabinose (Sec. 3.2.8.2.) As the amount of L-arabinose in the polysaccharide was very small, its contribution towards the structure was considered insignificant. It is, therefore, referred as a galactomannan.

4.2.1. Methylation and g.l.c. analysis

The methylation of polysaccharide M_2 was carried out by modified Hakomori method (Sec. 3.2.8.3.) . The methylated product showed no hydroxyl band in the I.R. Spectrum.

The methylated polysaccharide M_2 was hydrolysed with formic acid and trifluoroacetic acid, reduced with sodium borohydride and acetylated with acetic anhydride in pyridine (Sec. 3.1.27). The resulting alditol acetates were identified from their respective retention times as obtained from g.l.c. These were as follows :

(a) 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-galactitol, (b) 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-D-mannitol and (c) 1,4,5,6-tetra-O-acetyl-2,3-di-O-methyl-D-mannitol.

The relative proportions of the alditol acetates were determined by measuring the peak areas of the chromatograms. The results were presented in table 15.

As previously described the location of hydroxyl groups in these products indicated the positions of linkage of monosaccharide units. The presence of 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-D-mannitol indicated that these residues were in the

pyranose forms (C_5 linked) and linked to two other units through C_1 and C_4 (scheme 2). 1,4,5,6-tetra-O-acetyl-2,3-di-O-methyl-D-mannitol showed a similar involvement of C_5 in pyranose ring and C_1,C_4 and C_6 in the union with other units and 1,5-di-O-acetyl-2,3,4,6- tetra-O-methyl-D-galactitol indicated that these residues were also in the pyranose form and linked at C_1 position of D-galactose. Thus the polysaccharide M_2 contained the following units :

$$\text{-4 Man}_p\text{1-} \quad , \quad \overset{6}{\text{-4 Man}_p\text{1-}} \quad \text{and} \quad \text{Gal}_p\text{1-}$$

Where

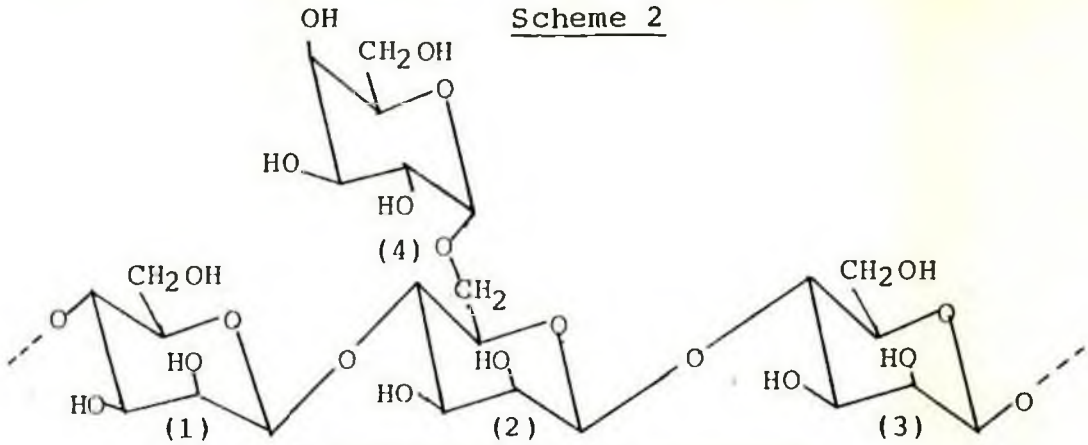
Man_p = D-mannopyranose

Gal_p = D-galactopyranose

Fig. 54. Structural units of the polysaccharide M_2

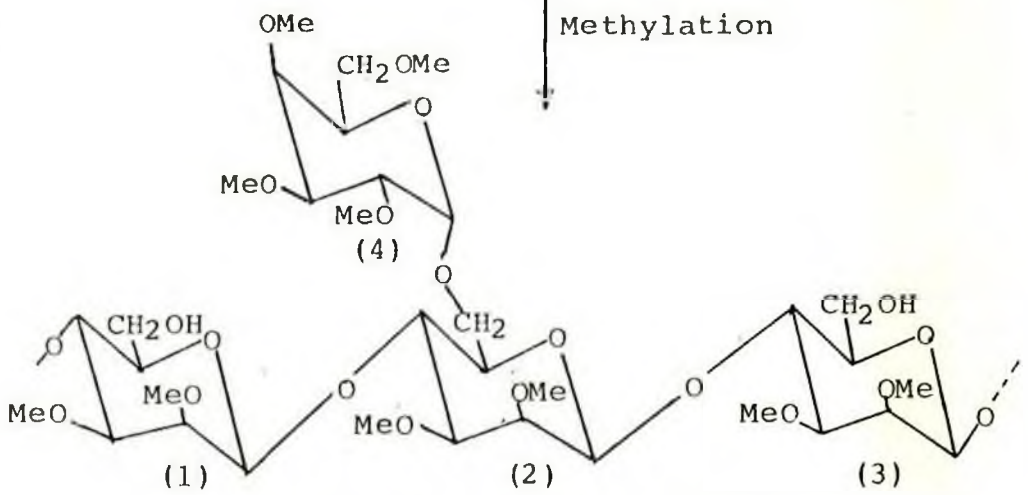
The presence of 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-D-mannitol in large quantity (64.01%) showed that the polysaccharide M_2 is largely a straight chain one, made up of 1→4 linked mannopyranose units and partly similar to the polysaccharide M_1 having branching at C_6 position of D-mannose with C_1 position of D-galactose.

Scheme 2



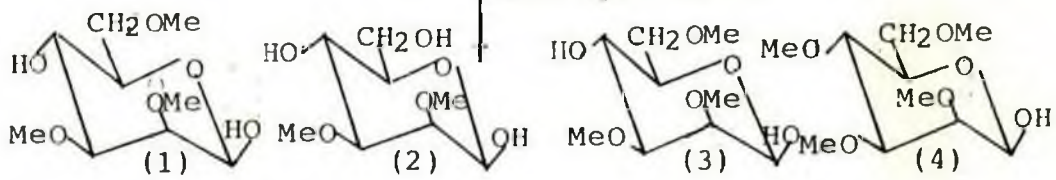
A hypothetical galactomannan

Methylation

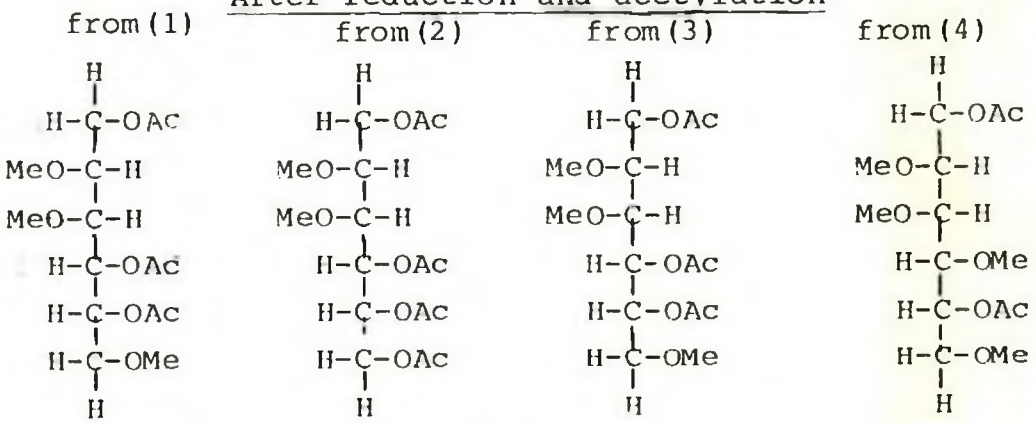


Completely methylated galactomannan

Acid hydrolysis



After reduction and acetylation



Methylated alditol acetates of M_2

The fact that the polysaccharide M_2 was a $\beta(1\rightarrow4)$-D-galactomannan was further supported by combined g.l.c.-m.s. analysis in the form of alditol acetate, ^{1}H-n.m.r. and ^{13}C-n.m.r. studies.

4.2.2. G.l.c.-m.s. analysis

(a) G.l.c. study

Polysaccharide M_2 was completely methylated by using Hakomori method (Sec. 3.1.24 B) followed by hydrolysis and transformation into alditol acetate (Sec. 3.2.8.5). Gas chromatographic study of this acetylated product gave peaks relative R_t of 1.11, 2.08 and 3.68 minutes respectively (Table 15).These revealed that the polysaccharide M_2 contained 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-galactitol, 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-D-mannitol and 1,4,5,6-tetra-O-acetyl-2,3-di-O-methyl-D-mannitol.

(b) Mass spectrometric study

Mass spectrometric study of methylated alditol acetate fragments obtained from polysaccharide M_2 showed the following considerable $m/_e$ values.

(i) For 1,5-di-O-acetyl-2,3,4,6-tetra-Omethyl-D-galactitol

$m/_e$ 43, 45, 87, 102, 118, 129, 145, 161 and 205 (Fig. 42) [reported[223] $m/_e$ values 43, 45, 71, 87, 101, 117, 129, 145, 161 and 205].

(ii) For 1,4,5-tri-O-acetyl-2,36-tri-O-methyl-D-mannitol

$m/_e$ 43, 45, 87, 102, 113, 118, 162, 173 and 233 (Fig. 43), [reported[223] $m/_e$ values 43, 45, 87, 99, 101, 113, 117, 129, 161, 173 and 233].

(iii) For 1,4,5,6-tetra-O-acetyl-2,3-di-O-methyl-D-mannitol

$m/_e$ 43, 87, 101, 118, 129, 160, 189 and 194 (Fig. 44) [reported[223] $m/_e$ values 43, 87, 99, 101, 117, 159 and 201,].

4.2.3. ^{1}H-n.m.r. study

^{1}H-n.m.r. spectrum of polysaccharide M_2 had an axial anomeric proton signal at δ 4.90 p.p.m. (Fig. 45) reported [227a] value δ 4.89 p.p.m.). This supported β -linkage of mannose residues of the main chain. A weak signal at δ 5.12 p.p.m. was due to an axial anomeric proton of the mannose unit having a linked galactose. Another weak anomeric signal at δ 5.19 p.p.m. was due to the α -linked galactose.

4.2.4. ^{13}C-n.m.r. study

In ^{13}C-n.m.r. studies (Sec. 3.2.8.7.) of the polysaccharide M_2 in the noise-decoupled spectrum there were six main peaks together with some weaker ones. These peaks corresponded to D-mannose residues in a regular repeating structure. These peaks further corresponded closely in chemical shifts to the values of a β-(1→4) linked mannan. A comparative data of chemical shifts (δ) of the polysaccharide M_2 with those of a reference compound (β-D-1→4-linked-D-mannopyranan) and the corresponding assigments are shown in TAble 20.

Table 20. A comparative ^{13}C-n.m.r. spectral data of polysaccharide M_2

Polysaccharide M_2 δ	β-D-(1→4)-linked-D-mannopyranan[27] δ	Assignment
101.2 p.p.m.	101.7 p.p.m.	C_1
72.9 "	72.2 "	C_2
73.3 "	73.8 "	C_3
74.7 "	78.8 "	C_4
75.0 "	78.8 "	C_5
63.4 "	62.1 "	C_6

From the ratio of the amount of tetra-, tri- and di-O-methyl sugars, the length of the repeating units of the polysaccharide M_2 came to 5 hexose units.

From the observations mentioned above the structural arrangements of repeating unit of polysaccharide M_2 may be as follows :

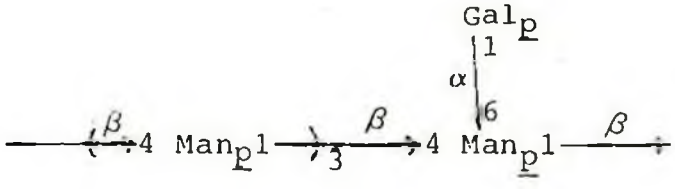


Fig. 55. A repeating unit of polysaccharide M_2.

4.3. Study of polysaccharide M_3

3% alkali extract was purified by gel filtration using sepharose CL-2B. Studies of the polysaccharide was conducted on this purified sample. This homogeneous polysaccharide was designated as polysaccharide M_3, $[\alpha]_D^{28} = -1.56^{o}$ (in 3% aqueous sodium hydroxide solution, c 0.05). The negative value of specific rotation of the polysaccharide M_3 indicated that the monosaccharide units were glycosidically linked in the β-form.

On sugar analysis the polysaccharide M_3 was found to contain D-galactose and D-mannose in the molar ratio of 1:6 This indicated that the polysaccharide is a galactomannan.

4.3.1. ^{1}H-n.m.r. study

^{1}H-n.m.r. spectrum of polysaccharide M_3 had an axial anomeric proton signal at δ 4.74 p.p.m. (Fig. 46)[reported[227a] value δ 4.89 p.p.m. , D_2O]. The small difference is due possibly to solvent effect. This supported the β-linkage of D-mannose residues of the main chain. A signal at δ 4.95 p.p.m. assigned an axial anomeric proton of D-mannose residues having D-galactose unit linked to it. Another signal at δ 5.26 p.p.m. was due to an equitorial anomeric proton of the linked D-galactose. This indicated an α-linked substituted D-galactose.

The ^{1}H n.m.r. data of M_3 were also compared with those of M_2(p.175) and it was found that their chemical shifts differ slightly. This small difference in chemical shift might have arisen from solvent effect [227b]. The ^{1}H n.m.r. spectrum of M_2 was taken in D_2O and that of M_3 in D_2O/NaOD.

4.3.2. ^{13}C-n.m.r. study

In the noise-decoupled spectrum six main peaks together with some weaker ones (Fig. 47) were obtained through ^{13}C-n.m.r. study of the polysaccharide M_3. These peaks corresponded to D-mannose residues in a regular repeating structure. These

peaks further corresponded closely in chemical shifts to the values of a β -D-(1→4) - linked - D - mannopyranan. A comparative data of chemical shifts (δ) of the polysaccharide M_3 assignments are shown below:

Table 21. A comparative ^{13}C-n.m.r. spectral data of polysaccharide M_3

Polysaccharide M_3 δ	β-D-(1→4)-linked -D-mannopyranan[227] δ	Assignment
103.4 p.p.m.	101.7 p.p.m.	C_1
73.4 "	72.2 "	C_2
74.8 "	73.8 "	C_3
79.8 "	78.8 "	C_4
78.4 "	78.8 "	C_5
64.0 "	62.2 "	C_6

It appears that the ^{13}C-n.m.r. spectrum of the polysaccharide M_3 was in agreement with that of the polysaccharide M_2 with the exception of C_1. This inconformity may be due to alkali induced shift as $D_2O/NaOD$ [227c] were used. When δ 1.7 p.p.m. is subtracted from the value of chemical shift for C_1 of M_3 the resulting values come close to that of M_2 and thus they relate to one another.

Based on these observations it may be suggested that the structural pattern of the polysaccharide M_3 is also similar to that of the polysaccharide M_2 having predominantly a β -(1—>4) linked mannopyranose chain in the molecule and an α -linked galactopyranose at C_6 position of a mannopyranose residue of the repeating unit.

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