THE STRUCTURE ~~A STRUCTURAL INVESTIGATION~~ OF THE EXTRACELLULAR POLYSACCHARIDE ~~ELABORATED~~ OF ~~BY~~ *Beijerinckia mobilis*

by

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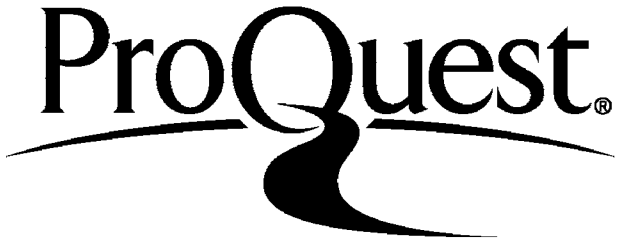

To my husband and parents

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A B S T R A C T

Structural studies on the extracellular polysaccharides elaborated by Beijerinckia mobilis, a tropical soil species of the group Azotobacteriacae, have been made and the component monosaccharides characterised. Fractionation of the total extracellular mucilage, after removal of contaminating protein, was effected by the precipitation of an acid fraction with quaternary ammonium salts. The uncomplexed neutral fraction (Mw = 2,000) was further subdivided into a neutral polysaccharide (N_2) and a low molecular weight fraction (N_1) consisting of glucose, mannose and related oligosaccharides. The origin of this fraction is unknown but it is thought to arise either from incomplete metabolism by the organism at the time of harvesting, or from the presence of an enzyme in the medium which breaks down the polysaccharide

The neutral polysaccharide (N_2) is composed of glucose, mannose, and arabinose (1:1:1), and was found to have a low molecular weight and to be highly branched.

The acid fraction is a high molecular weight polymer ($Mw = 20 \times 10^6$) composed of L-guluronic acid (55%), D-glucose (15%) and D-glycero-D-manno-heptose (20%), together with acetic and pyruvic acids. From the results of methylation, periodate oxidation and partial hydrolysis experiments the following repeating unit is proposed as a backbone for the polysaccharide:

—→3)L-GulUA(1→3)L-GulUA(1→2)D-gly-D-Manhept(1-

The remaining glucose and uronic acid residues were also found to be 1,3-linked and are thought to form separate side chains linked to C-4 of the uronic acid residues in the backbone. Combined pyruvic acid was found to be in ketal linkage to C-4 and C-7 of about 25% of the D-glycero-D-manno-heptose units.

The similarities between this polysaccharide and that from the related species Azobacter indicum are discussed.

In order to establish the linkages of the guluronic acid units it was necessary to prepare and characterise the methylated derivatives of gulose. These experiments are detailed in a reprint to be found at the end of the thesis.

Also included are reports on two six week projects carried out at Alginate Industries, Girvan:

Appendix IV. Studies on gel formation with Alginates.

Appendix V. Studies on the viscosity of solutions of the extracellular polysaccharide of B. mobilis under different conditions.

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C O N T E N T S

GENERAL INTRODUCTION

Bacteria

Bacteria are unicellular organisms which generally multiply by elongation and transverse binary fission. Some bacteria can also form an 'endospore' or resting cell in its own vegetative cell.[1] After germination a new vegetative cell is formed and liberated by lysis of the surrounding mother cell. Endospores are resistant to heat and other treatments lethal to vegetative cells and can remain dormant for long periods. Members of the genus Azotobacter can form a different type of resting cell known as a 'cyst'.[2] This is formed by the shortening of a normal cell accompanied by the secretion of a thick enclosing wall. Cysts, unlike endospores, are not significantly more resistant to heat than the cells from which they are derived.

Only a minority of bacteria can form endospores or cysts. Generally survival is essentially dependent on the presence of unmodified vegetative cells. Bacteria occur as spheres of about 1 μ in diameter, or rods or helices which are 1 to 10 μ long and 0.2 to 1 μ in width.

The majority of bacteria are divided into Gram-negative and Gram-positive organisms according to their behaviour in the 'Gram staining test'.[3] The cells are treated with methyl violet and then with dilute iodine solution. Gram-positive bacteria retain the stain after washing with alcohol, but Gram-negative bacteria do not. This results from consistent and major differences in composition of the cell walls (see later).[4, 5] It is essential to perform the stain on cells derived from a young culture since certain bacteria, particularly spore-formers, are Gram-positive only during the course of active growth, and older cultures, become Gram-variable or Gram-negative. After the major

division into Gram-negative and Gram-positive bacteria, classification is based to a large extent on functional attributes. The main divisions are:-

photosynthetic or non-photosynthetic

aerobic or anaerobic

spore forming or non-spore forming.

The largest single group is the 'enteric group' these are non-photosynthetic Gram-negative bacteria which can grow anaerobically on carbohydrates, but also possess a respiratory electron transport system, which enables them to grow aerobically using a wide range of organic substrates.

Organisation of the bacterial cell

The main features are:-

(i) The nucleus

The cells of bacteria are termed 'Procaryotic' while those of most higher plants and animals are 'Eucaryotic'. The difference between these two types is that the Eucaryotic cell has a membrane bound nucleus whereas the nuclear material of the Procaryotic cell is not bound by a membrane and is a single circular molecule of double-stranded DNA which replicates by the unwinding of the DNA double helix and synthesis of each complimentary strand.

(ii) The Cytoplasm

An aqueous solution of soluble protein and low molecular weight metabolites lying inside the cell membrane. Also present are small deposits of lipids, energy reserve materials, ribosomes, and high energy inorganic polyphosphates. No structures resembling mitochondria have been detected.

(iii) The cell membrane[6]

A mosaic of proteins and lipids, the cell membrane is a thin barrier 75-80Å thick through which all materials from the external environment must pass. In some bacteria the membrane is simple in shape, but often the shape is complex as a result of intrusions into the cytoplasm. One common intrusion is a 'mesosome', a deep invagination of the cytoplasm whose function is not known. In the genus Azotobacter which has the highest known respiratory rate of any bacteria, vesicular membrane intrusions are abundant and are thought to have a respiratory function.

The cell membrane consists of a continuous lipid layer with polar groups at the outer edge and non-polar groups directed inwards. There may be protein sub-units floating in this pool of lipids. The synthesising enzymes, which are found in other organisms in mitochondria, have been isolated from bacterial cells and are thought to be a part of the cell membrane.

(iv) The Cell Wall[7, 8]

This is a rigid structure consisting partly of polysaccharides which differ from the polysaccharides found in multicellular organisms. A universal component of the cell wall is murein, a glycoprotein which comprises about 50% of the cell wall of a Gram-positive bacteria but less than 10% of a Gram-negative wall.[8] The murein layer of a Gram-positive bacteria is covalently linked to teichoic acids,[9] carbohydrates which will be described later. The only Gram-negative bacteria found to contain a teichoic acid is Escherichia coli.[10]

The outer layers of the Gram-negative wall, which may account for up to 80% of the dry weight of the cell, consist of lipopolysaccharides and

and lipoprotein. In contrast the cell walls of Gram-positive bacteria do not contain any discrete layers of lipopolysaccharide or lipoprotein. Differences also occur in the amino acid composition, all amino acids found in proteins have been found in the cell walls of Gram-negative bacteria[11] but the Gram-positive wall is characterised by the simplicity of its amino acid composition.

The polysaccharides found in the wall will be dealt with in a later section.

(v) The capsule

This consists generally of a polysaccharide or protein. Most of the capsules of Gram-negative bacteria are polysaccharides and protein-containing capsules are rare, Pasturella pestis[12 - 14] for example has a protein capsule, whereas protein capsules are frequently encountered with Gram-positive bacteria. Occasionally capsules may be found to be a mixture of the two types of polymer, for example Bacillus megatherium.[15]

Bacterial Polysaccharides

These can be divided into three main classes according to their position in the cell.

(i) Intracellular polysaccharides, inside the cytoplasmic membrane.

(ii) Cell wall polysaccharides.

(iii) Extracellular surface polysaccharides often called capsules, slimes or microcapsules.

(i) Intracellular Polysaccharides

These are energy reserve materials of the starch-type or glycogen-type.[16] Generally a bacterium can synthesise only one type of storage material which is not necessarily carbohydrate. In active growing cells, the amount of such reserve material is small, but when

nitrogen supply is limited and carbon is still freely available, large quantities can be quickly accumulated. Glycogen and Starch can be extracted from the cells, which have first been ruptured by grinding or by ultrasonic disintegration, by extraction with aqueous chloral hydrate.[17]

(ii) Cell Wall Polysaccharides

Murein, a peptidoglycan, is a universal component of bacterial cell walls. It consists of linear chains of amino sugars with amino acid side chains which are cross-linked by peptide bridges. The linear chains are composed of alternating units of β-1,4-linked N-acetyl-D-glucosamine and 3-O-(2-D-carboxy ethyl)-N-acetyl-D-glucosamine (muramic acid) linked through the carboxyl group of the muramic acid to the amino group of an amino acid. Other constant components are D- and L-alanine, D-glutamic acid and L-lysine or D,L-diamino pimelic acid. But additional amino acids have been isolated from different genera. The structure of the peptide chains varies considerably in different species. Fig. 1 shows a typical example from Staphylococcus aureus.[18] Here the amino acid side chains are linked by a penta-glycine bridge. In some mureins the L-alanine may provide the bridge between the peptide chains[19] and in others large sections of the linear polysaccharide may be devoid of amino acid side chains.[20] The murein can be isolated from the disintegrated cells[21, 22] by extracting the insoluble fraction of the cell walls with a phosphate-ethylene glycol two phase system.[23] It can be further purified by extracting the lipids with chloroform and removing other proteins with enzymes which do not attack the peptidoglycan.

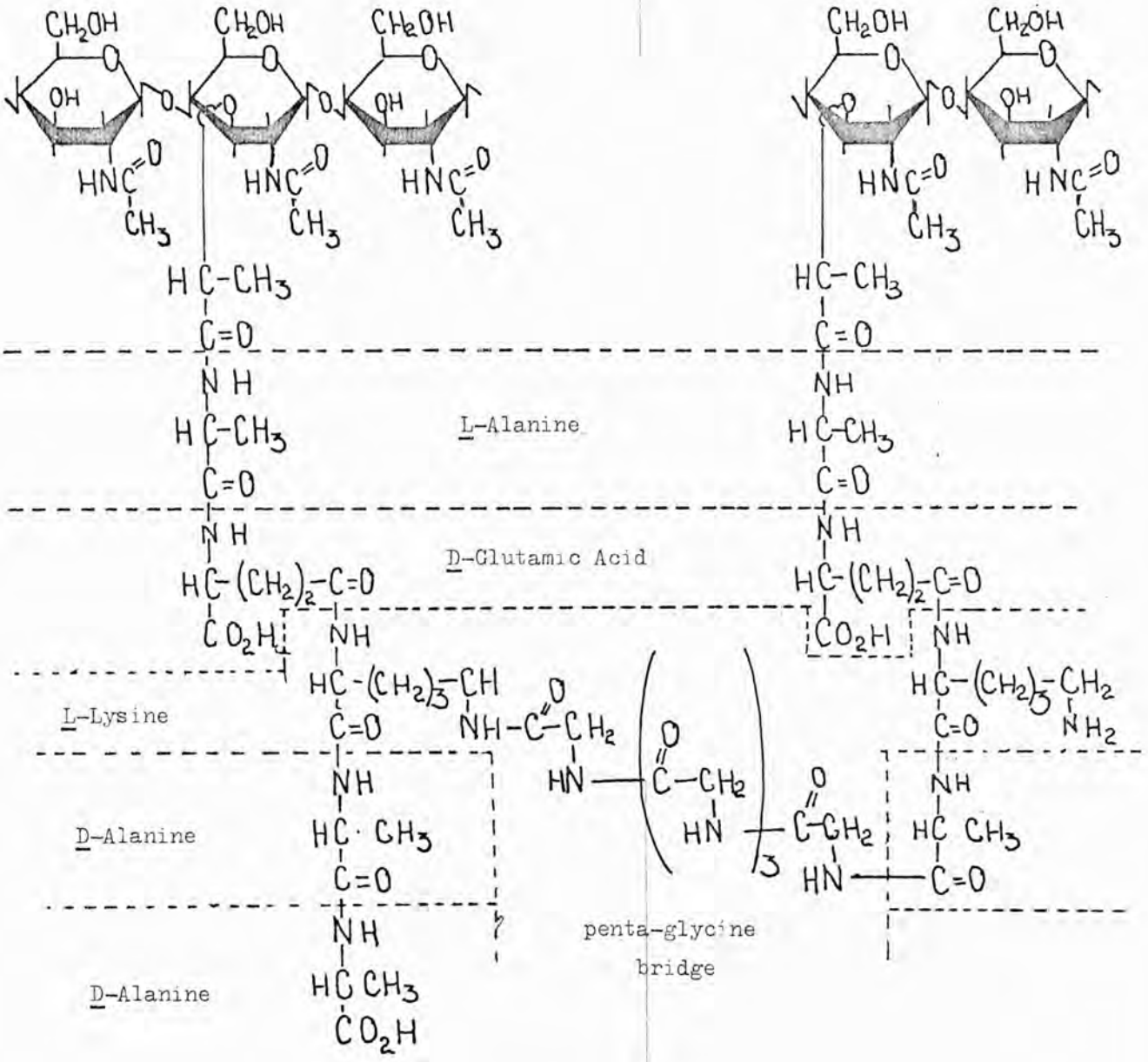


Fig. 1 Murein from Staphylococcus aureus

Teichoic Acids are linear polymers composed of alternating polyalcohol - either ribitol or glycerol-and phosphate groups. Both types of polymer contain adjacent polyol residues linked through phospodiester linkages and carry D-alanine residues in ester linkages to some of the hydroxyl groups. Glycosidically linked sugar residues are present in all the ribitol teichoic acids but in only some glycerol

teichoic acids. Fig. 2 shows a typical example of a ribitol teichoic acid.

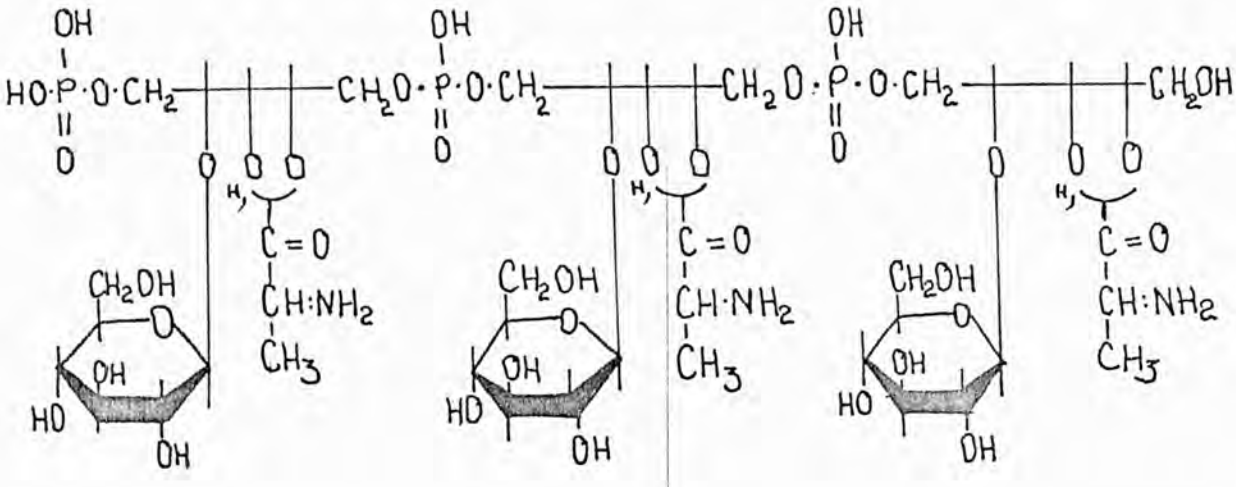


Fig. 2 Teichoic Acid from Cell Wall of Bacillus subtilis (24)

The teichoic acids may be isolated from the cells by treatment with cold 10% trichloroacetic acid from which they are then precipitated with alcohol.[25] The lipid portion of the wall must first be removed.

Lipopolysaccharides of Gram-negative walls[26]

This fraction of the cell wall is responsible for the somatic 'O' antigenicity of Gram-negative bacteria, that is the ability to react with antibodies and to induce antibody formation. The lipopolysaccharide is also responsible for the symptoms that occur when Gram-negative bacteria are introduced into the blood-stream - called the endotoxic activity. The lipopolysaccharide can be hydrolysed and separated into a lipid moiety called 'Lipid A' and a polysaccharide moiety. Neither part alone is responsible for the endotoxic activity but the polysaccharide

moiety carries the antigenic determinant or the site of reaction with the antibody.

The lipopolysaccharides characteristic of the enteric bacteria[27, 28] may contain as many as twelve different sugars. The structure appears to comprise a backbone with up to five different sugars which is common to a given species - see Fig. 3. The side chains known as 'O' side chains vary in different strains of the same species and give the serological specificity.

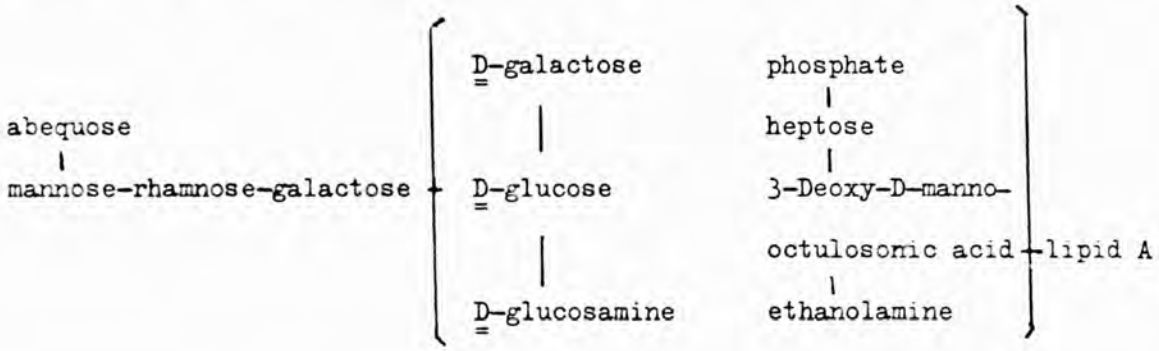


Fig. 3

General structure for the lipopolysaccharide from Salmonella typhimurium[27] this can be further generalised to

LIPID A — POLYSACCHARIDE CORE — POLYSACCHARIDE SIDE CHAINS (TYPE SPECIFIC SUGARS)

This type of structure is a common feature of all Gram-negative walls. The lipopolysaccharides are not indispensable structural constituents and their structure can be modified by mutation without affecting the viability of the cell. The loss of side chains is accompanied by the loss of antigenic specificity. This often leads to

change in appearance of the culture and the concept of 'Rough' and 'Smooth' forms of the colony. The 'rough' and 'smooth' forms are designated 'R' and 'O' antigens respectively. A species of bacteria may be classified into different strains depending on the serological specificity. In this way the genus Salmonella has been subdivided into over 1000 strains.[29, 30, 31]

The lipopolysaccharides may be isolated from the cells by extraction with phenol-water. This extract contains the ribonucleic acids as contaminants. These react preferentially with cetyltrimethyl-ammonium bromide ('Cetavlon') and precipitate, leaving the purified lipopolysaccharide in solution.[32] The polysaccharide moiety is then obtained by hydrolysing with aqueous acetic acid and the lipid is extracted from solution with hexane.[33]

(iii) Extracellular or Capsular Polysaccharides

A capsule is a layer outside the cell wall which is visible under the light microscope.[34] However some bacteria release their capsules into the culture medium and the enzymes which synthesise them may be isolated from the medium in the form of highly pure proteins.[35] The presence of a capsule may often be reflected in the moist, glistening, gelatinous appearance of the bacterial colony and can be demonstrated by special staining techniques[36, 37] and also by a specific immunochemical reaction.[15] The capsular polysaccharide is often termed the 'K-antigen'. If this is present it will interfere with the reactions of the 'O'-antigen with 'O' antibody. Capsulated cells agglutinate in homologous 'OK'-serum but not in 'O'-serum. Where the capsule is easy to remove, the 'O'-antigen is revealed and will react

with the 'O'-antibody. However if the capsule is hard to remove, as in the genus Klebsiella, serological classification is based on the reactions of the 'K'-Antigen.[38]

Functions of the capsular polysaccharide

The capsule has no apparent structural function but, unless it is solely an excretory product, it must in some way help to maintain a bacterium in its environment, and a number of uses have been suggested.[34]

(i) Resistance to phagocytosis (engulfment by leucocytes) This has been shown to be an important factor with species of Salmonella,[39, 40] E. coli[41] and Pasturella pestis.[42, 43, 44] It is thought that resistance to phagocytosis is due to the negative surface charge on the capsule.

(ii) Resistance to bacteriophage (bacterial virus).[38]

(iii) Protection against amoeba

(iv) Protection against desiccation.[45]

Soil bacteria in particular are often exposed to prolonged periods of exposure to water followed by periods of drought. A too rapid gain or loss of water by the cell might result in cell death.

(v) A storage material.[46]

(vi) In special cases eg. Azotobacter capsular polysaccharides play a role in formation of the cyst.[47]

Cultivation

A variety of liquid media are used for cultivation, usually consisting of a meat broth to which sugars and certain salts are added.

For convenience of storage the liquid medium can be set to a gel by the use of such materials as agar or gelatine. The cultivation of colonies on a gel is the normal means of isolating pure strains of bacteria since each colony grows from a single cell. A mixture of bacteria can thus be separated by selecting individual colonies.

Conditions for growing the bacteria vary and should be chosen to suit the particular genus. For example, bubbling in sterile air for an aerobic bacterium such as Bacillus subtilis or B. megatherium.[48] The medium in which the bacteria are grown frequently influences the composition of the polysaccharide produced and polysaccharides grown in different media should be regarded as potentially different. Azotobacterindicum produces a polysaccharide containing 30% uronic acid and 70% glucose when grown on glucose as carbon source but when grown on sucrose produces a fructose containing polymer.[49] The period of growth is also an important factor, the optimum time for producing polysaccharide in large quantities may differ from that required for producing polysaccharide of high molecular weight. It has been found that it is essential to maintain a high ratio of carbohydrate to nitrogen[34, 50] and that the optimum temperature is frequently between 15° and 20°C.[51, 52] Also the presence of sulphate in the medium has a stimulating or depressing effect on polysaccharide production depending on the genus involved.[53, 54] Optimum conditions for the production of polysaccharide can thus be established experimentally. The bacteria are separated from the medium by centrifugation and the capsular polysaccharide is frequently left in the medium from which it can be removed by precipitation.[55, 56] In some cases it is necessary to solubilize the polysaccharide or remove it from the cell surface, so that on centrifugation the polysaccharide will be separated from the cells, this is effected by stirring with alkali[57, 58] or physical

methods may be employed, such as forcing suspensions of encapsulated bacteria through chromatogram sprayers[59] or through a hypodermic needle.[60] Using more than one of the above methods it is possible to compare the extracellular polysaccharide produced in the medium with that more closely attached to the cell wall.[61, 51]

Purification

The most likely contaminants are residual medium, - especially the sugars - whole bacterial cells, protein and lipopolysaccharides. All these may be trapped in the polysaccharide which frequently has a viscous and gelatinous consistency. Purification is mainly effected by repeated dissolution, centrifugation and reprecipitation. There are chemical methods of removing proteins such as the Sevag method,[62] which consists of shaking an aqueous solution with chloroform. This denatures the protein which is then separated, after centrifugation, at the chloroform-water interface. Lipopolysaccharides would dissolve in the chloroform and would be removed by this method.

Structure of Extracellular polysaccharides

Extracellular polysaccharides have complex and varied structures and the following brief introduction is confined to those polysaccharides derived from bacteria isolated from the soil, the original environment of the genus Azotobacter. However reviews and articles given in references 26, 34, 63 were found to be of interest concerning the polysaccharides of other bacteria.

In the soil the genera Arthrobacter and Corynebacterium (both of the Coryneform family) account for more than 50% of the bacterial inhabitants.[64] The genera Bacillus and Clostridium (family Bacillacae) account for 25% of the bacterial flora[65] whereas genera accounting for no

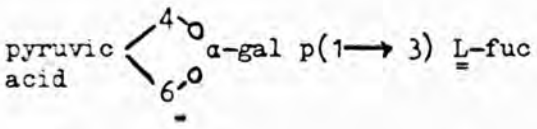
more than 10% are Achromobacter, Nitrosomonas, Proteus, Nitrobacter, Agrobacterium, Azobacter, Aerobacter (or Klebsiella), Pseudomonas, Rhizobium and some species of Serratia. Of these genera, there is little reported detailed work on the extracellular polysaccharides of the soil species of Achromobacter, Nitrosomonas, Proteus, Arthrobacter, Nitrobacter and Clostridium. The genus Azotobacter will be dealt with more fully in a later section.

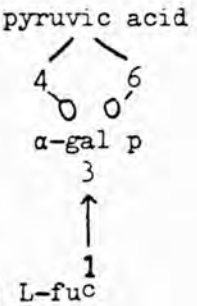
Agrobacterium and Bacillus

A summary of the constituents and types of linkage, where determined, found in the polysaccharides of Agrobacteria and Bacilli species will be found in tables A and B in Appendix 1. Those of the Agrobacteria comprise mainly β-1,2-linked glucans[66 - 69] whereas the Bacilli species metabolise β-2,6-linked fructans[70 - 76] and glucomannans.[70, 77 - 79] In contrast B. macerans synthesises Schardinger dextrins.[80] These are cyclodamyloses in which 6-8 glucose units are joined by α-1,4-linkages in a continuous loop.

Corynebacterium

Nearly all the members of this genus are pathogenic or phytopathogenic. They seem to fall into two groups, uronic acid containing species and those which contain acids which are not uronic acid (probably pyruvic acid) their constituents are summarised in Appendix 1 table C.[81] The major sugars are glucose, galactose, mannose, fucose and rhamnose. The only one to have been analysed structurally is that from Corynebacterium insidiosum[82] which contains the following structural units.





gal = galactose
fuc = fucose
glc = glucose

Aerobacter or Klebsiella

These two names are thought to be synonymous for the same genus. According to Kauffman[38] Aerobacter should not be used. However this name is preferred in general microbiological texts. Members of this genus produce large capsules and slimes. These were investigated as early as 1900 and found to be carbohydrate in nature[83 - 86] and the first chemical studies appeared in 1925.[87 - 88] It has been shown that the optimum production of capsules and slime polysaccharide occurs with a low nitrogen level in the medium[50] and that capsules and slime are identical in composition, so that slime can be regarded as excreted capsule.[51, 61] Klebsiella are differentiated into serotypes on the basis of their capsular polysaccharides.[89] The major sugars are glucose, galactose, mannose and rhamnose with fewer types containing fucose. 78 of the 80 types known contain uronic acid which is usually glucuronic although some contain galacturonic. In addition many contain acetyl groups and pyruvic acid ketals. The composition of the polysaccharides of the 80 types and some additional untyped species is given in Appendix 1 table D and structures or structural units, where these have been determined, are given in table E (also in Appendix 1).[87-126] Klebsiella K-type 1 on partial hydrolysis gave an aldobiouronic acid which was found to contain glucuronic acid and lyxose. The origin of the lyxose was not determined

since it was not present in a total hydrolysate.

After further purification it has been shown [127] that the acid polysaccharides of types 1 - 5 are contaminated with a neutral galactose containing polysaccharide. On more detailed examination the neutral polysaccharide fraction was found to consist of more than one antigen. All were different from the type-specific polysaccharides and seemed to be the same for all the strains of a given species investigated. Thus K. pneumoniae types 1, 2 and 3 shared an identical neutral antigen which was different from the antigen shared by K. ozaenae types 3, 4 and 5.

Pseudomonas

Members of this genus are normally found in soil and water but may be phytopathogenic and one (Ps. aeruginosa) is phytopathogenic. Many of the species produce levans, β-2,6-linked fructans. Some of them are linear but others contain branching linkages of the inulin type (β-2,1), the details of these and the composition of some of the other polysaccharides is listed in Appendix 1 table F. [128-134]

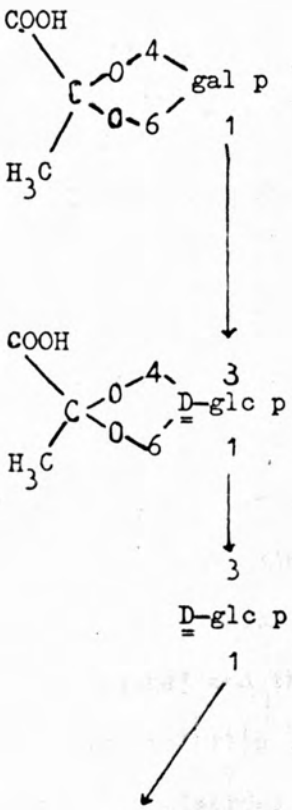
Serratia

Of the polysaccharides produced by the genus Serratia the capsule polysaccharides of S. marcescens have been most widely studied. They consist of a spectrum of similar polymers (see table G, Appendix 1). [135-138] A prominent feature is the presence of heptose units, which were characterised as D-glycero-D-mannoheptose and L-glycero-D-mannoheptose. The only structural features reported were 1,3-linked mannose in strain 1 capsule "a" and predominantly 1,4-linked glucose in strain 1 capsule "b". Capsule "a" was the major component of strain 1.

Rhizobium

Members of the genus Rhizobium are found in the 'root region' or rhizosphere of the soil. They are nitrogen-fixing organisms which infect plant roots causing nodule formation. A species is often named according to its host e.g. R. trifolii from clover. Table H (Appendix 1) gives a summary of the composition of extracellular polysaccharides of Rhizobium. The subject is confused due to lack of correlation between the various workers concerning the taxonomy of the genus and its differentiation into strains. However the results[139-151] can be summarised in that R. trifolii polysaccharides contains galactose, glucose, pyruvic acid, acetic acid, and glucuronic acid or its 4-O-methyl derivative and R. meliloti polysaccharides contain mainly glucose, with traces of galactose and glucuronic acid (or 4-O-methyl glucuronic acid) and probably pyruvic and acetic acids. Other species are more variable in composition but nearly all contain glucose and glucuronic acid (or its 4-O-methyl derivative). Also galactose, mannose, fucose and rhamnose may be present. Fructose was also reported in one strain of R. leguminosarum,[147] however since fructose has not been found previously in a hetero acid polysaccharide, it is thought that it arises here as an artefact.

Structural studies have been carried out on some of the strains.[140,150,143] However only in one case has a repeating unit been proposed[140] viz. for R. trifolii T.A1 and this, anoctasaccharide, is shown below.



→ 4)D-glc p (1→ 4)-D-glc p(1→ 4)D-glc p(1→ 4)D-glc p(1→ 4)D-glc p(1→

glc = glucose

gal = galactose

The Azotobacteriacae

The members of this group are the principal agents of aerobic nitrogen fixation in soil and water. They are plump rods or cocci and occur characteristically in pairs. Most Azotobacter organisms produce gummy extracellular polysaccharides giving the colonies a mucoid appearance. As previously mentioned, members of this group can form cysts. During the formation of the cyst[47] the vegetative cell changes from a rod to an oval or spherical shape and a loose envelope is deposited round the periphery of the cell which thickens as the cyst matures. Between the envelope or 'exine' and the cell is an area called the 'intine'.[154] In the mature cyst this is very large and is composed of multiple layers differing in electron density. In a suitable environment the cyst will germinate. During this period the overall size of the cyst remains the same but the cell body becomes larger at the expense of the intine. A new vegetative cell is liberated by rupture of the exine frequently leaving the latter in a characteristic horseshoe shape. It is thought that the intine and exine are polysaccharides containing the same sugar units: glucose and rhamnose in the case of Azotobacter vinelandii, and that the intine is used as a reserve storage material to facilitate germination. It has been shown that Azotobacter vinelandii can utilise extracellular polysaccharide as a carbon and energy source.[46]

The group Azotobacteriacae consists of two principal genera Azotobacter and Beijerinckia. Azotobacter species grow under neutral or alkaline conditions (above pH 6.5) in both soil and water. Beijerinckia species have a much wider range (pH 3-9) and occur principally in tropical soils. In general Azotobacter species form much the larger cells which form cysts which are resistant to desiccation[153],

whereas Beijerinckia species are smaller and contain large polar fat inclusions. Growth of Beijerinckia colonies is slower although there is copious production of thick slime. They are generally motile by means of lateral flagella[154] but form no cysts.

Bergey's Manual of Determinative Bacteriology (1957 edition) describes only three species of Azotobacter (A. chroococcum, A. agilis and A. indicum). However the latter is often[155 - 157] placed in the genus Beijerinckia with B. mobilis. In the literature, the names Azotobacter and Beijerinckia are used interchangeably. Also A. vinelandii, according to Bergey, synonymous with A. agilis, is thought by some to be a distinguishable species.[158]

Extracellular polysaccharides of Azotobacter and Beijerinckia Species

Azotobacter vinelandii under most cultural conditions synthesises large quantities of extracellular polysaccharide which occurs morphologically in two distinct forms (i) discrete capsules adhering to the cell walls (ii) an amorphous slime found loosely attached to the capsules and in the culture medium free from the cells.

Early reports indicated that the chemical composition of A. vinelandii capsules consisted of glucose and rhamnose[159] whereas the slime consisted of glucose and a trace of uronic acid.[160] Subsequent workers[56, 161, 162] found that the slime and the capsule had the same constituent sugars, namely galacturonic acid, glucose and rhamnose in the ratio 43:2:1 and in addition small amounts of mannuronolactone. Of the three strains investigated, only slight differences in composition were found to occur, only two strains contained O-acetyl groups, and one of these strains also contained a component thought to be sialic acid. Further work on the

capsular polysaccharide by Claus[163] of A. vinelandii indicated that this acid component was 2-keto-3-deoxygalactonic acid. However in this study the sugar composition of the polysaccharide was different, rhamnose was the main constituent (64%) and no uronic acids were detected. When Azotobacter vinelandii was grown on Burks nitrogen free medium[164] the extracellular material was found to consist of large amounts of hexuronic acid and smaller amounts of glucose, arabinose, ribose and rhamnose, detected after hydrolysis, by paper chromatography.[165] The authors considered that this probably comprised a polysaccharide consisting entirely of uronic acid residues together with another polysaccharide containing the neutral sugars. They succeeded in separating the polyuronide by acidifying the culture medium with hydrochloric acid when it precipitated out. The uronic acid was shown to be a mixture of L-guluronic and D-mannuronic acids, linked through positions 4 or 5, thus making the polymer similar structurally to the alginic acid found in brown algae. A similar extracellular polysaccharide has been isolated from Pseudomonas aeruginosa, a bacterium causing respiratory diseases.[167, 166]

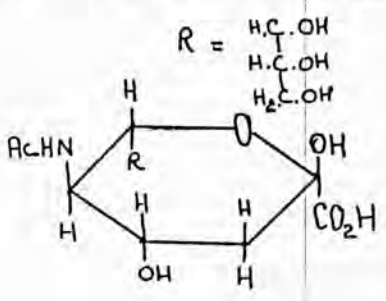
Alginic acid is composed of linear chains with sections comprising alternating guluronic and mannuronic acid residues, interspersed by blocks composed of a single acid.[168, 169] The proportions of the two monomers may vary from 25-75% of mannuronic acid.[170, 171]

Results obtained by Haug and Larsen[172, 173] indicated that the alginate produced by A. vinelandii is of a similar nature to the algal alginic acid. It has also been shown that the variation of the calcium level in the initial growth medium leads to differences in the ratio of mannuronic to guluronic acids. With a low calcium content only a small amount of guluronic acid is produced, and maximum guluronic acid is

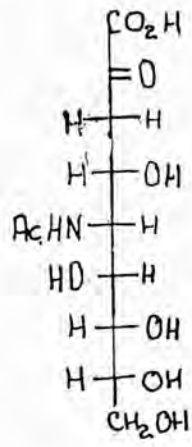
produced at intermediate calcium concentration. High calcium concentrations again lead to a predominantly mannuronic acid polymer. Even after the cells have been removed by centrifugation, an increase in the calcium concentration of the medium leads to a change in the composition of the alginate. This suggested the presence of an enzyme in the culture medium capable of epimerising D-mannuronic residues into L-guluronic residues in the alginate molecule, and this enzyme has indeed been isolated.[174]

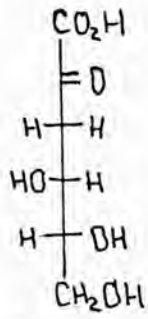
It was thought by Haug and Larsen[173] that the methods used by Cohen and Johnstone[56] for identification of the galacturonic acid would not exclude the possibility of both mannuronic and guluronic acids being present. It is also possible that the positive thiobarbituric acid reaction found.by Claus[163] may be due to degradation products of uronic acids. However, the possibility that different polysaccharides are produced by different strains of Azotobacter vinelandii cannot be ruled out.

A. agilis forms a well-defined capsule and no slime in the medium.[175] The polymer contains galactose and rhamnose in the ratio 1.0:0.7. An acid component similar to the acid found in A. vinelandii was also found. This gave the positive thiobarbituric acid test[176] similar to that given by the acid found in A. vinelandii.[56]



N-Acetylneuraminic acid, a sialic acid





2-keto-3-deoxy-galactonic acid

The polysaccharide of A. chroococcum when first investigated by Stacey[177] was found to contain 87% glucose and 4% uronic acid. Further investigation[178] revealed in addition the presence of galactose and a trace of mannose, together with an unidentified component thought to be a dideoxy sugar. Methylation analysis indicated the presence of 1,3- and 1,4-linked glucose, 1,3-linked galactose and 1,4-linked glucuronic acid. Glucose in significant yield was also found linked at positions 1,4 and 6. The authors interpreted this as evidence that the polymer was a highly branched molecule, however no corresponding fully methylated end group residues were detected.

Azotobacter indicum was investigated by Martin in 1945[49] and found to produce a fructan containing small amounts of uronic acid when grown on sucrose and a glucan containing 30% uronic acid when grown on glucose An early study on 'A. indicum'[179] indicated that it was a 'levan'-forming organism and this was in agreement with later work by Fuchs[180] who also found that the polysaccharide was broken down after a few days. These results were obtained when the species was grown on Agar slopes containing sucrose, but no 'levan' could be detected when it was grown in a liquid medium. It is thought that the organism possesses a levan degrading enzyme system. This has been found previously to occur with A. chroococcum.[179] B. mobilis was grown under the same conditions (both on liquid and on slopes) and no slime formation was detected. Work by Lopez and Backing[161, 162] indicated the presence of glucose, galactose, mannose, and glucuronic and galacturonic acids in both the slime and capsular polysaccharides of A. indicum.

Quinnell and co-workers[181] found that A. indicum when grown on glucose as carbon source produced a polysaccharide containing glucose

uronic acid and an aldoheptose in the ratio of 3:2:1. The aldoheptose was characterised by Sowa, Perry, Jones[182] as D-glycero-D-mannoheptose. In contrast to Quinnell however the uronic acid content was found to be 19% and in addition there was 25% mannose and 7% of other unidentified components (see Table 1, p26) Also the presence of a second neutral polysaccharide was reported. This was a minor fraction, about 12% of the total and consisted of glucose, mannose, rhamnose and arabinose. Previous workers had not reported this second polysaccharide or even the presence of trace amounts of its component sugars in the hydrolysates. The uronic acid was identified as glucuronic acid with traces of galacturonic and guluronic acids, by esterification, reduction, hydrolysis, and paper chromatography of the neutral sugars. The uronic acid content was found by the carbazole[183] colorimetric method and by titration to give the equivalent weight. Results from both methods put the uronic acid content at 17-19%.

A further study of this polysaccharide was undertaken by Parikh and Jones.[184] However, the results did not agree with those of the previous work in that only a trace of mannose was found and the other constituents (glucose, heptose and uronic acid) were present in equal amounts. The only uronic acid found was glucuronic and the unfractionated polysaccharide was obtained in higher yield, 25% compared to 4.5% by Sowa and Jones,[182] both based on the quantity of glucose in the culture medium. These fundamental differences were ascribed to the absence of buffer from an otherwise similar growth medium. Also there were differences in the procedure for harvesting and purifying the polysaccharide. After centrifuging off the cells, Sowa obtained the polysaccharide by repeated precipitation with acetone whereas Parikh and Jones filtered the

supernatant after centrifuging through a Celite bed and then precipitated the polysaccharide with 0.5% ethanolic hydrogen chloride and this might well account for the differences. Jones and Parikh found partial hydrolysis of the polysaccharide difficult and resorted to partial degradation of the methylated polymer to obtain oligosaccharides. The polysaccharide was methylated three times by the Haworth method[185] and then by the Kuhn method.[186] The polysaccharide was considerably degraded on hydrolysis (70% recovery). Two methylated oligosaccharides were obtained and 3 methylated monosaccharides. Two additional fractions thought to contain mannose were lost during chromatography. Uptake of sodium metaperiodate by the polysaccharide was equivalent to 1 mole per mole of anhydroaldose unit and 0.95 moles of formaldehyde were released per anhydro heptose unit indicating two free hydroxyls on C_6 and C_7 of 95% of the heptose units. Smith-type degradation gave glycolaldehyde, D-glucose, glycerol and glycerose. A trace of arabinose, was also reported which the authors were unable to account for. The absence of tetronic acid in the degradation products required for the proposed structure was thought to be due to loss by absorption on ion-exchange resin. On the basis of these results the polymer was thought to be a linear molecule having the following trisaccharide as repeating unit;-

→ 4) D-glucuronic acid (1→ 3) D-glucose (1→ 2) D-glycero-D-mannoheptose (1→

Norwegian workers[187] subsequently examining the same organism did not find any neutral polysaccharide. They found the polymer to contain glucose, an aldoheptose thought to be D-glycero-D-mannoheptose and a uronic acid. Also present were acetyl groups. These workers

characterised the uronic acid as guluronic (with no trace of other uronic acids) by comparison of the carbazole reaction at different temperatures, with and without borate ions[188] and also by its mobility on ionophoresis in a special buffer[189] and by comparing the chromatographic mobility of the lactones. Its optical rotation was $[\alpha]_D = +36.3^\circ$ c.f. L-guluronic acid from alginic acid $[\alpha]_D = +37^\circ$. This evidence, repeated on several batches of polysaccharide produced by using different media seems to indicate that the uronic acid in A. indicum is indeed L-guluronic acid. Table 1 gives a summary of these results and those of the previous workers.

Table 1. Composition of polysaccharides produced by A. indicum

	Quinnell[181]	Jones, Perry & Sowa[182]	Jones & Parikh[184]	Haug & Larsen[187]
Neutral polysaccharide	-	12	12	-
Acid polysaccharide				
glucose	45%	33%	30%	+ n.d.
heptose	15%	16%	30%	+ n.d.
mannose	-	25%	tr.	tr.
glucuronic acid	30%	)+	30%	-
galacturonic acid	-	)+ 17%	-	-
guluronic acid	-	)+	-	30%
acetyl groups	n.d.	n.d.	n.d.	20%

Beijerinckia mobilis (NC1B 9879)

B. mobilis is a species of Beijerinckia which is not officially recognised by Bergey's Manual of Determinative Bacteriology. It is considered by some to be a variant of Beijerinckia indica or Azotobacter indicum. It is however a very actively motile species which produces an amber-yellow pigment.[190] It appears to be confined to tropical regions and has so far been found only in India and Burma[191] Java[192] tropical Africa,[193] Northern Australia and South America.[194] Quite special soil conditions seem to be required for the establishment of a population in the soil. In common with the other members of the group, it produces poly-β-hydroxybutyrate (P.H.B.) as an intracellular carbon and energy reserve material.[195] It was noted during attempts to measure the amount of P.H.B. produced[195] that B. mobilis produced an extracellular slime which gave rise to a turbid solution.

Fuchs[180] reported that B. mobilis was not a slime producing organism and did not produce an emulsion when grown on agar slopes. Emulsion formation is considered to be indicative of a polysaccharide-containing capsule. However, when this organism was grown by Alginate Industries Ltd., on Burks nitrogen-free medium[196] supplemented with sucrose and potassium nitrate, copious slime production was noted.

Culture Conditions

The culture was obtained from the National Collection of Industrial Bacteria, Aberdeen, as a lyophilised sample [strain number NC1B 9879]. After hydration with distilled water, the sample, in suspension was transferred to Burk's agar slopes. Large scale growth was then achieved in a liquid medium, details of which can be found in Appendix II, table 1.

After 1 - 7 days growth at 26°C and 220 rpm on a shaker the medium was centrifuged using an MSE continuous head centrifuge and the crude polysaccharide was obtained by precipitation with acetone.

We were supplied with the following batches.

Sample No.	Quantity	Experiment No.	Additional purification before precipitation.
S11A	10g	11/15 ~~or Expt~~. 111/4	
S11B	5g	11/15	centrifuged twice.
S11C	3g	11/15	protein coagulated by autoclaving.
S11D	15g	11/21	
S11E	20g	11/22	
S11F	10g	11/23	
S11G	40g	11/24	
S11H	-	11/15	dehydrated cell debris.
S11J	-	11/15	dehydrated cell debris.

The average properties of the crude off-white polysaccharides as a milled dried powder were:-

Yield, 15 g/litre of culture medium.

% yield 30% based on quantity of sucrose used.

10% protein

12-20% moisture.

The viscosity of a 1% solution at pH 4.5 was 850 cps and at pH 2.6 was 1600 cps.

The individual properties of the different batches are given in Appendix II table 2.

This thesis describes the structural investigation of this polysaccharide.

G.L.C. Mass Spectrometry in the Identification of methylated sugar derivatives

G.L.C. can be used to identify mixtures of partially methylated sugar derivatives from hydrolysates of methylated polysaccharides. This is generally achieved by comparison of retention times with those of standard compounds both as the methyl glycosides and as alditol acetates. However these relative retention times are not completely conclusive, due to the overlapping of peaks and the frequent presence of peaks in the G.L.C. chromatogram originating from the methylating reagents. Thus once the components of the hydrolysate of a methylated polysaccharide have been identified by G.L.C. confirmation of their identity can be effected by mass spectrometry of the methylated alditol acetates. The eluent from the G.L.C. machine passes directly into the mass spectrometer where it is bombarded with high energy electrons. These remove electrons from the molecules leaving positively charged ions. The largest ion usually produced is the molecular ion, M^+. This ion may be broken down, either due to bombardment by electrons, or because the excess energy resulting from bombardment is lost resulting in rearrangement and fragmentation. The ions produced are separated by an electric field according to their mass/charge (m/e) ratio. Ions of charge greater than one are rare. With partially methylated alditol acetates the molecular ion (M^+) is too unstable to be detected and it has been found that the fragmentation occurs by two major pathways.

a) Primary fragmentation

This usually occurs once in each ion and is due to fragmentation between the carbon - carbon bonds of the sugar chain. The fragment bearing the positive charge is the only one to be detected. According to the

deductions of Lindberg[236] et al, primary fragmentation is most likely between two carbon atoms bearing methoxyl groups (figure 4) and the probability decreases with a decrease in the number of methoxyl groups in the carbon chain

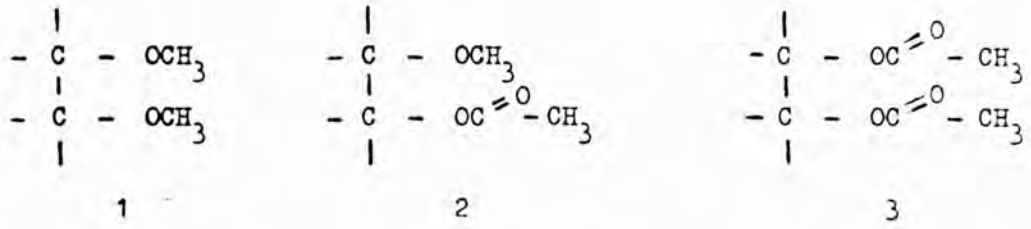


Figure 4 Probability of fragmentation occuring 1 > 2 > 3

In general after fragmentation has occurred, the positive charge resides on the oxygen of the methoxyl group. In addition fragmentation between two methoxyl bearing carbons seems to produce ions of high abundance.

b) Secondary fragmentation

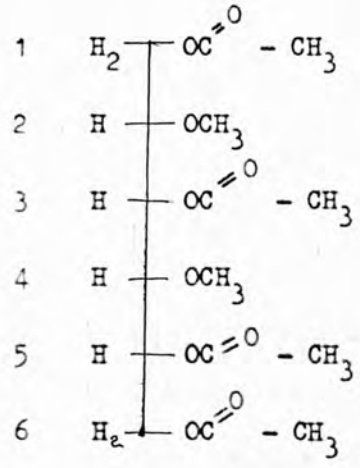
This can occur once or more than once from either the molecular ion or a primary fragment and is due to rearrangement and loss of methanol (32), ketene (40), formic acid (42), and acetic acid (60). Usually the most abundant peak present in the mass spectrum of a partially methylated alditol acetate is m/e 43 and is the acetylium ion $CH_3\overset{\oplus}{C}=O$.

It has been found that alditols with different distribution of methoxyl and acetyl derivatives can be easily distinguished with the following exceptions:

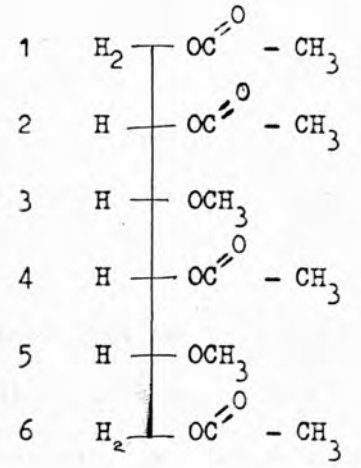
1. The mass spectrum will not differentiate between two partially methylated hexitols which differ only in the spatial arrangement of hydroxyl groups. i.e. the same methylated derivative of glucitol and mannitol will give the same mass spectrum. Here the G.L.C. retention time is vital in the identification.

2. Since no information about spatial arrangement can be gained, any derivative will give the same mass spectrum as a derivative which has the acetyl and methoxyl groups in the reverse order.

e.g.



2,4-di-O-methyl hexitol acetate



3,5-di-O-methyl hexitol acetate

These two molecules will give identical mass spectra.

Differentiation between these two can however be achieved by the use of sodium borodeuteride to reduce the sugar to the alcohol. This would result in an alditol acetate containing D_2 at position C_1 and thus C_1 of the 2,4-di-O-methyl hexitol could be distinguished from C_6 of the 3,5-di-O-methyl derivative.

GENERAL METHODS

I. Physical Techniques

(i) Evaporations were carried out under reduced pressure between 30 and 45°C.

(ii) The water used in all experiments was distilled or deionised.

(iii) Melting points were determined on a Gallenkamp micro melting point apparatus.

(iv) Dialysis of solutions was performed in Visking cellophane tubing against distilled water with toluene added as a bacteriostat.

(v) Specific rotations were measured in a 1 dm polarimeter tube using a Perkin-Elmer 141 polarimeter. All measurements were made in aqueous solution using the sodium D-line.

(vi) Unless otherwise stated all resin used was Amberlite

II. Acid Hydrolysis

(i) Formic Acid method

The sample was dissolved in 90% formic acid and solid carbon dioxide added to give an inert atmosphere. The tube was sealed and heated for 6 hours at 100°C. The hydrolysate was diluted with water (5 vol.) and heated at 100°C for a further two hours, to hydrolyse the formyl esters. The solution was evaporated to dryness and residual formic acid removed by codistillation with methanol.

(ii) Sulphuric acid (1N or 0.5N) method

The sample was heated in the acid solution with stirring for 2 to 8 hours at 100°C. After cooling, the acid was neutralised with solid barium carbonate.

(iii) 72% Sulphuric Acid[197]

Cold 72% sulphuric acid was added to the sample and the mixture left at room temperature for one hour. Water (10 vols) was added with cooling and the solution heated at 100°C for 4 hours. After cooling the acid was neutralised with barium carbonate.

III. Chromatography

(i) Paper Chromatography using the following solvent systems for descending chromatography (v/v).

(a) Ethyl Acetate : acetic acid : formic acid : water (18:3:1:4).

(b) n-butanol : pyridine : water (6:4:3).

(c) n-butanol : ethanol : water (40:11:19).

(d) ethyl acetate : pyridine : acetic acid : water (5:5:1:3).[198]

(e) n-butanol : ethanol : water (3:1:1).[182]

(f) methylethylketone : acetic acid : water (9:1:1) saturated with boric acid.

Whatman No. 1 paper was used for qualitative work. For preparative paper chromatography Whatman No. 3 MM or No. 17 paper was used.

(ii) Electrophoresis. The Shandon high voltage electrophoresis apparatus L24 was used with Whatman No. 3 MM paper and the following electrolytes.

(a) Borate[199]

0.2M-Sodium borate in water adjusted to pH 10 with sodium hydroxide. Electrophoresis was carried out for 1.5 h. at 2.5 kv. The non-migrating marker was 2,3,4,6-tetra-O-methyl-D-glucose.

(b) Molybdate[199]

0.1M-Sodium molybdate dihydrate in water, adjusted to pH 5.0 with concentrated sulphuric acid. Electrophoresis was carried out at 1.5 kv for 2.5 h. The non-migrating marker was glycerol.

(c) Pyridine/Acetic acid

Pyridine (1-litre) adjusted to pH (i) 4.0[200]

(ii) 6.8

with 5% acetic acid in water. The electrophoresis was carried out for 2.0 h at 3.0 kv. Glucose was the non-migrating marker.

(d) Ammonium carbonate[200]

0.1M-Ammonium carbonate (pH 8.9). Electrophoresis was carried out for 2 h at 3.0 kv.

(e) Borate with Calcium ions[189]

0.01M-Sodium tetraborate (borax) in water containing 0.005M-calcium chloride (pH 9.2). Electrophoresis was carried out for 2.0 h at 0.5 mA/cm.

(iii) Thin Layer Chromatography was carried out on pre-coated Kodak cellulose plates in

(a) Ethyl acetate, acetic acid, formic acid, water (18:3:1:4).

(b) Ethyl acetate.[201]

All qualitative chromatography papers and TLC plates were dried for 5 mins at 100°C before location of spots.

IV. Staining Reagents

(i) Silver nitrate dip.[202]

Three solutions through which the paper was sequentially dipped.

(a) Saturated aqueous silver nitrate solution (2.5 ml) and water (10 ml) in acetone 500 ml.

(b) Sodium hydroxide (20 g) in water (40 ml) and ethanol (960 ml).

(c) 10% aqueous sodium thiosulphate.

(ii) (a) Aniline oxalate spray.[203]

Aniline oxalate (25 g) in 50% aqueous ethanol (1 L).

(b) Aniline oxalate spray.[204]

Aniline oxalate (25 g) in glacial acetic acid (1 L).

(iii) p-Anisidine hydrochloride spray.[203]

p-Anisidine hydrochloride (0.5 g) in ethanol (10 ml) with n-butanol (40 ml). After spraying with reagents (ii) and (iii) the papers were heated for 5 min at 100° to develop the colour.

(iv) Glucose oxidase.[205]

'Glucostat' kit (Worthington Biochemical Company) made up as directed. A pink colour indicating β-D-glucose develops after 5 min at room temperature.

(v) Heptose Spray.[182]

The paper is sprayed successively with the following

a 0.03M sodium periodate in pH 3.6 0.2M acetate buffer.

b after 1 min, 1% aqueous inositol.

c ammonium acetate (15 g) acetic acid (0.2 ml)
acetylacetone (1 ml)
in methanol (50 ml).

A yellow colour develops after 30 minutes at room temperature.

(vi) Bromophenol Blue Spray.[200]

0.1% Bromophenol blue in 96% aqueous ethanol saturated with sodium carbonate. A blue colour on a yellow background develops in 5-30 min. This fades on further standing.

(vii) O-phenylene diamine spray for keto acids.[206]

0.2% ethanolic O-phenylene diamine (5 mls) and 20% aqueous trichlofoacetic acid (5 mls).

A white fluorescent spot visible under ultra violet light develops after 10 minutes at room temperature.

(viii) Galactose oxidase Spray.[205]

Galactose oxidase (2-3 mg) + horse radish peroxidase (2-3 mg) + O-dianisidine (10 mg) in 0.1M-phosphate buffer pH 6.9 (10 ml). Colour develops at room temperature.

(ix) Urea hydrochloride.[207]

Urea (10 g) in ethanol (200 ml) and concentrated hydrochloric acid (8 ml) in water (32 ml). A blue colour specific for ketoses develops after 5 minutes at 100°C.

V. Gas Liquid Chromatography (G.L.C.)

A. Instrumentation

(i) A Pye Argon gas chromatograph with an Argon ionisation detector, and dry argon as carrier gas was used for the methylated methylglycosides. Glass columns (1 m x 5 mm) (1) and (2).

(ii) A Pye 104 gas chromatograph with nitrogen carrier gas and flame ionisation detector with glass columns (3 m x 5 mm). Column (3) for the TMS derivatives of the sugars and alditols. Column (4) for the methylated alditol acetates.

(iii) A Perkin-Elmer F11 gas chromatograph with flame ionisation detector and helium gas with a glass column (4 m x 1.6 mm) (Column 5).

B The columns were packed with the following materials

(1) Butane 1,4 diolsuccinate polyester, 15% on silane treated Celite.

(2) Polyphenyl ether (P.P.E.) m-bis-(m-phenoxy)-benzene 10% on silane-treated Celite.

(3) Apiezon K, 7.5% on silane treated chromosorb W.

(4) OV 225, 3% on Gaschrom Q.

(5) OV 225, 3% on Gaschrom Q.

C. Gas Chromatography linked to Mass spectrometry.

The F11 gas chromatograph was coupled via an all glass system through a Watson-Biemann separator to a Hitachi RMS-4 mass spectrometer. Mass spectra of partially methylated alditol acetates were obtained by operating the 'ion source' at 230°C, 50 eV and 80 μa target current.

VI. Assays and Analyses

(i) Carbohydrate Content was assayed by the phenol sulphuric acid method.[208] Water (1 ml) containing 10-100 μg sugar was added to 4% phenol (1 ml) and concentrated sulphuric acid (5 ml) added rapidly. The colour developed was read at 487 mμ on a Unicam SP500. Standard graphs were prepared for different sugars and mixtures of sugars in the ratios corresponding to those of the particular polysaccharide.

(ii) Uronic acid estimation was by the modified carbazole reaction.[209] A saturated aqueous solution (1 ml) of benzoic acid, containing 4-40 μg uronic acid was layered onto concentrated sulphuric acid (5 ml) containing 0.025 M-sodium tetraborate in an ice bath. Keeping the temperature below 4°C the two layers were mixed. The mixture was warmed to room temperature and then heated in a boiling water bath for 20 minutes. After cooling 0.125% carbazole in analar methanol (200 μl) was added and the tubes heated at 100°C for a further 15 minutes. The colour which developed was read at 530 mμ on Unicam SP500. Standard graphs for different acids were prepared.

(iii) Glucose Content was estimated using a Boehringer Blood Sugar kit. The polysaccharide (10 mg) was hydrolysed with 2N-HCl (3 ml)

for 8 hours at 100°C. After neutralisation with 2N-NaOH (3 ml) the solution was made up to 100 ml.[210] This solution (1 ml)(containing 0-50 µg glucose) was added to the enzyme solution (5 ml) and left to stand for 1 h in the dark. The absorbance was read at 430 mµ on the Unicam SP500. A standard graph for glucose was used.

(iv) Degree of polymerisation of oligosaccharides was measured by the Timell modification,[211] of the Peat method.[212] Two tubes are required for each sample.

A 2% KBH_4 (0.5 ml) + water (0.5 ml) containing 60-80 ug oligosaccharide.

B 2% KBH_4 (0.5 ml) + 2N-H_2SO_4 (0.5 ml) containing same amount of oligosaccharide as tube A.

The tubes are left overnight and then 4% phenol (1 ml) and concentrated sulphuric acid (5 ml) added. The colours formed are read at 487 mµ.

$$\text{For a homo oligosaccharide D.P.} = \frac{\text{Absorbance B}}{\text{Absorbance B} - \text{Absorbance A}}$$

The equation is modified for hetero oligosaccharides to allow for the difference in response to the phenol-sulphuric acid test of the respective sugars.

(v) Nitrogen and Protein content. Nitrogen content was measured by A.Bernhardt (W. Germany) and the protein content calculated by multiplying by 6.25.[213]

(vi) Molar proportions of sugars were estimated from the peak areas on G.L.C. of the T.M.S. derivatives of derived alditols or methylated alditol acetates. Standard graphs were prepared to check the response of different sugars to the G.L.C. detection system.

VII. General Reactions and Preparations

(i) Preparation of IR $120H^+$ dry form in methanol. The resin washed with water until free of colour. It was then stirred with methanol for 18 h and then filtered. This was repeated three times, the third time with dry methanol. The resin was then stored under dry methanol.

(ii) Preparation of methanolic hydrogen chloride. Hydrogen chloride gas was passed into dry methanol until saturation was reached. The solution was titrated with N-NaOH and diluted with dry methanol as required.

(iii) Preparation of dimethyl sulphinyl carbanion.[214] Sodium hydride (1.5 g , 55% coated with mineral oil) was washed three times with n-pentane (30 ml) which was removed by successive evacuations of the vessel. After each evacuation, dry nitrogen was passed into the vessel. Dry distilled D.M.S.O. (15 ml) was added and the contents of the vessel heated at 55°C until evolution of hydrogen ceased. The carbanion was transferred to serum bottles and stored at 0°C under an atmosphere of nitrogen. Its normality (~ 2N) was determined by titration with 0.1N-HCl.

(iv) Preparation of diazomethane.[215] Potassium hydroxide (6 g) in water (10 ml) was added to methyl digol* (35 ml) and dichloromethane (10 ml). This solution was heated to 70°C. A solution of 0.1M-p-tolyl sulphonyl methyl nitrosamide in dichloromethane (125 ml) was added dropwise. The diazomethane in dichloromethane distilled over as a yellow solution. When all the nitrosamide had been added, further dichloromethane was added until the distillate was colourless. The diazomethane was stored at -5°C.

*methyl digol = diethylene glycol monomethyl ether

(v) Preparation of chromotropic acid reagent.[216] Di-sodium-4,5-dihydroxy-2,7-naphthalene disulphonate (chromotropic acid) (2g) in water (200 ml) was diluted to 1 L with 66% sulphuric acid.

(vi) Methyl glycosides of sugars. The sugar was dried in a desiccator over concentrated sulphuric acid and then dissolved in dry methanol and a small amount of IR 120H^+/(dry form) resin added as catalyst. The mixture was refluxed for 18 h, the resin filtered off and the methanol removed by evaporation

(vii) Esterification and glycosidation of uronic acid. The dried acid oligo- or polysaccharide was refluxed with 2% methanolic hydrogen chloride for 18 h. The solution was neutralised with silver carbonate, filtered and the silver salts washed with dry methanol. The filtrate and washings were evaporated to dryness.

(viii) Reduction of Sugar to Alditol. The sugar was dissolved in aqueous 0.01M-boric acid (5 ml) and potassium borohydride added to give a 2% solution. After 18 hours, if the solution was alkaline, the residual borohydride was neutralised with glacial acetic acid and the potassium removed with IR 120(H^+) resin. Acetic and boric acids were respectively removed by evaporation and codistillation with methanol. If the solution was not alkaline, more borohydride was added and left for 2 h. This was repeated until the solution remained alkaline.

(ix) Reduction of uronic acid to sugar. The acid was first esterified by method VII(vii) and then reduced by method VII (viii) the methyl ester of the acid being reduced to a sugar while the aldehydo group was not reduced since C-1 was protected as the methyl glycoside. The free sugar was then recovered by hydrolysis with formic acid.

(x) Trimethyl silyl derivatives.[217] The material (10-15 mg), dried by co-distillation with methanol and dry benzene, was dissolved in dry pyridine (1 ml) and trimethyl chlorosilane (0.1 ml) added followed by hexamethyldisilazane (0.2 ml). After shaking for 5 mins, the precipitate of ammonium chloride was removed by centrifugation and the sample evaporated to dryness. The residue was dissolved in dry hexane and analysed on G.L.C. (column 1).

(xi) Alditol acetates.[218] A sample of mixed, partially methylated alditols, dried in a desiccator, was dissolved in a pyridine: acetic anhydride (1:1 v/v) mixture and heated for 10 mins at 100°C. After dilution with water the sample was evaporated to dryness. The residue was dissolved in chloroform and analysed by G.L.C. or GLC-MS [columns (4) and (5)].

(xii) Methylation

(a) Hakomori Method[219] (modified by Bjorndal and Lindberg[220]): The polysaccharide (50-35 mg) was dissolved in dry DMSO (1 ml) in a serum bottle under an atmosphere of nitrogen. Dimethylsulphinyl carbanion (1 ml) was injected into the serum bottle and the mixture shaken for 8 h. Freshly distilled methyl iodide (0.2 ml) was added with cooling and the mixture again shaken for 8 h. This procedure was repeated, except an excess of methyl iodide (1.0 ml) was added. After the final period of shaking the solution was poured into water (25 ml) and the total volume dialysed for three days. For methylation of oligosaccharides, after pouring the solution into water, the methylated derivatives were extracted from aqueous solution with chloroform.

(b) Modified[221] Kuhn Method.[186] The oligo- or polysaccharide was dissolved in methyl iodide (5 ml) to which was added silver oxide (20 mg). After shaking in ice for 30 minutes, the mixture was refluxed for 1 - 18 h. The silver oxide was filtered off (the silver salts washed with methanol) and the methyl iodide and methanol removed by evaporation.

(c) Diazomethane.[222] The oligosaccharide was suspended in methanol to which was added 1,2 dimethoxy ethane (2 ml) containing 0.1% boron trifluoride etherate at -5°C. To this was added aliquots of diazomethane until the yellow colour was stable. The mixture was kept at 0° for 18 h after which the white precipitate (polymethylene) was filtered off and the filtrate evaporated to dryness.

(xiii) Periodate Oxidation. Estimation of the extent of oxidation was measured by the spectrophotometric method of Aspinall and Ferrier.[223] An aqueous solution of polysaccharide was added to an equal volume of 0.03N-sodium metaperiodate. Aliquots (0.1 - 1 ml) were withdrawn at intervals and diluted 250 times and the absorbance read at 223 mμ. The initial absorbance of the periodate before the addition of polysaccharide and the absorbance of an equimolar solution of sodium iodate were measured and thus the number of moles of periodate reduced at any time was measured. After 24 h the reaction was terminated by the addition of ethylene glycol.

(xiv) Determination of moles of formaldehyde liberated during periodate oxidation.[216]

The periodate oxidation mixture (1 ml) which should liberate 0-100 μg formaldehyde was added to 26.5% sodium sulphite (1 ml). An aliquot (1 ml)

was mixed with chromotropic acid (10 ml). The mixture was heated for 30 min at 100°C. On cooling the absorbance was read at 570 mμ on the Unicam SP 500. A standard graph was prepared using erythritol.

(xv) Hinton's Indicator

The indicator is prepared by mixing solution 1, (1 vol.), solution 2 (3 vol.) and solution 3 (1 vol.) with water (1 vol.).

Solution 1

Bromothymol blue	0.25 g
Ethanol	300 ml
Water	198 ml
0.5M-NaOH	0.75 ml

Solution 2

Phenol red	0.25 g
Ethanol	300 ml
Water	198 ml
0.5M-NaOH	0.75 ml

Solution 3

Cresol red	0.25 g
Ethanol	100 ml
Water	398 ml
0.5M-NaOH	0.75 ml

EXPERIMENTAL

Experiment I Routine Analysis of polysaccharide samples

Each polysaccharide sample was hydrolysed with formic acid [GM II (i)] and the hydrolysate analysed by paper chromatography in solvent systems (i), a, b and c, and by G.L.C. of the T.M.S. derivatives [GM V (3)] [GM VII (x)] prepared from the hydrolysate and from a sample of the hydrolysate which had been reduced with sodium borohydride [GM VII (viii)].

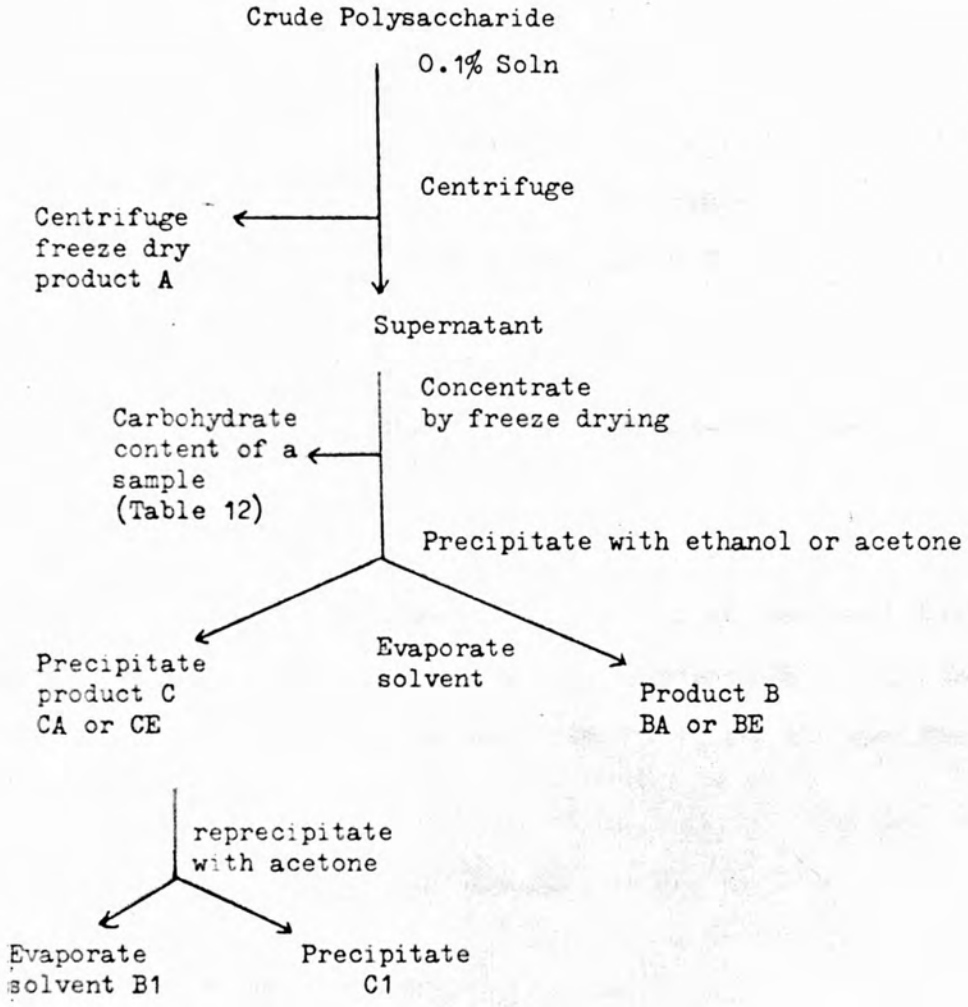
Experiment II Quantitative Analysis of the polysaccharide samples

The polysaccharides were dried in a vacuum desiccator at 60°C for 18 hours and then analysed for nitrogen [GM VI (v)] and the carbohydrate content determined [GM VI (i)] from a glucose graph. (Table 1 p.85)

Experiment III Purification

The polysaccharide as 0.1% solution was suspended in water using a Stilson high speed stirrer and then centrifuged in a Beckman J.21 centrifuge with a JA 10 rotor at 10,000 r.p.m. for 60 minutes. The centrifugate was freeze-dried (product A) the supernatant was found to be too dilute to precipitate with either ethanol or acetone and it was therefore necessary to concentrate it. However, it was found to froth when evaporated under reduced pressure, and was therefore concentrated by partial freeze-drying, which proved to be an effective method. To the concentrated aqueous solution was added acetone or ethanol (5 vol) and the precipitate (product C) was recovered by centrifugation, the acetone and ethanol were removed from the supernatant by evaporation, and the aqueous solution freeze-dried (product B).

Purification Flow Chart



Product (A), after freeze-drying was a light flocculent cream to pink solid, (B) was a sticky yellow substance and (C) was a light white solid. It was found that acetone gave a higher yield of precipitate (product C_A) with a higher carbohydrate content when compared to the ethanolic precipitate (product C_E).

For the first batch (S11A) of polysaccharide, the dissolution and precipitation with acetone was repeated producing products B_1 and C_1 which proved to be no purer than C. Each of the products A, B and C were analysed for nitrogen, and carbohydrate as in Experiment II (see results table II p.86.

Experiment IV Physical properties of purified polysaccharide

(a) Specific Rotation

The polysaccharide (10 mg) was dissolved in water (10 ml) and the cloudy solution filtered through a millipore filter (mesh size 0.45). The resulting solution was assayed for carbohydrate [GM VI (i)], The rotation of this solution was measured [GM I (v)] and the specific rotation calculated from the relation

$$[\alpha]_D = \frac{\alpha \times 100}{d \times c}$$

d = length of cell in decimeter

c = concentration in g/100 ml

$$[\alpha]_D \text{ polysaccharide} = +69^\circ$$

(b) Infra red of the polysaccharide

The polysaccharide was dissolved in water to form a smooth paste, which was spread on a microscope slide and allowed to dry. The thin film was peeled from the slide and mounted in a cardboard photographic slide mount. An infra red spectrum was taken using the Perkin Elmer 257 grating spectrometer. See P. 109

Experiment V Monosaccharide composition

The polysaccharide (product C) (100 mg) was hydrolysed with formic acid [GM II (i)] and analysed by paper chromatography [solvents (i) a, b and c, and spray reagents IV (i), (ii) a, (iv)]. Hydrolysis was accompanied by considerable degradation and charring and the production of large quantities of slow-moving acidic oligosaccharides which held back the monosaccharide spots on paper chromatograms. Attempts were thus made to remove the acid oligosaccharides from the neutral components.

Experiment VI Fractionation into acidic and neutral constituents

1. Preparative paper chromatography.

(a) The hydrolysate (from 1 g polysaccharide) was applied to 4 washed Whatman No. 3MM preparative papers which were eluted for 60 hours with solvent system III (i) c. The stained side strips indicated the presence of six components. The portions of the paper thus indicated were eluted with water and the eluent concentrated and analysed by paper chromatography.

(b) The hydrolysate (from 1 g of polysaccharide) was applied to 4 washed preparative Whatman No. 3MM papers and eluted for 48 hours [solvent system III (i) b]. This solvent was found to give a very good separation between the acid fraction (very slow moving) and the neutral monosaccharides, but the individual components of each fraction were not well resolved. Accordingly two fractions, acid and neutral, were obtained by this method.

2. Resin column[224]

A column containing Permutit DeaciditeFFIP(SRA67) (chloride form, 200 g) was prepared; the resin was washed with 2N-sodium hydroxide (~3L) until no further chloride ions were detected and then excess alkali removed

by washing with water until a neutral pH was obtained. The resin was converted into the formate form by elution with 2N formic acid (~ 5L) and then with water until the eluent was neutral.

The hydrolysate, obtained as above (from 1 g in water 10 ml), was applied to the column. The neutral components were eluted with water until no further carbohydrate was present (~ 2L). The acid components were obtained by gradient elution of 0-2N-formic acid (2L). Fractions (2 ml) were collected and monitored by the phenol sulphuric acid method [GM VI (i)]. The neutral components were obtained free from acid by this method.

3. Partial hydrolysis[182]

The polysaccharide (10 g) was hydrolysed by GM II (ii) (0.5N-H_2SO_4 for 8 hours). The neutralised hydrolysate ($BaCO_3$) was concentrated to a small volume and ethanol added (10 vol). The uronic acid components, precipitated as their barium salts, were removed by centrifugation. The supernatent (neutral fraction) was deionised by passage down columns of IR 120 (H^+) and IR45B(OH^-) resins. The uronic acids were converted into their ammonium salts by removing the barium ions with IR120(H^+) resin and then neutralising with ammonium hydroxide solution.

Experiment VII Characterisation of the uronic acid

The acid fraction from experiment VI1b was applied to a 3MM preparative paper and eluted in solvent system III(i)a and was found to consist of four components, the fastest of which was separated and found to be monouronic acid. Only trace amounts of other components were obtained and these were not investigated further.

The monouronic acid component was treated as follows:-

1. Thin layer chromatography in solvent system III (iii)a.
   The plates were double developed and stained with spray IV (iii)

2. Paper electrophoresis in buffer III (ii)e, stained with spray IV (ii)b.

Both these techniques give a good separation of the four common uronic acids, and, when the ionophoretogram was run with authentic standards, the mobility of the uronic acid was identical with that of guluronic acid. However due to the streaking effect often observed with electrophoresis, the presence of a small amount of glucuronic acid could not be ruled out.

3. Reduction of the uronic acid to a neutral sugar [GM VII (ix)]
   An aliquot was reduced and the derived neutral sugar was analysed by

(a) Paper electrophoresis in molybdate buffer III(ii)b.

Mobile and stationary components were observed, the former indicates the presence of gulose. However gulose is very easily partly converted into the 1,6-anhydrosugar which does not have the correct conformation[199] for mobility in molybdate buffer and like galactose and glucose remains on the starting line and this could account for the stationary component.

(b) Paper chromatography [solvent systems III(i)a-c,e and spray reagents IV(i), (iv) and (viii].

The enzymic sprays [(iv and (viii)] eliminated the possible presence of glucose and galactose and solvent III(i)e gives a good separation between gulose and mannose. The mobility of the unknown sugar was identical with that of the gulose standard. A T.M.S. derivative was prepared [GM VII (x)] and this proved to have the same retention time as an authentic sample of L-gulose TMS and there was no further resolution of peaks when the two samples were mixed and injected onto G.L.C. [GM V,(ii)3].

Experiment VIII Uronic acid content of the polysaccharide

1. Carbazole determination [GM VI(ii)]

The uronic acid content when calculated from a guluronic standard graph was 58% and from a glucuronic standard graph was 29%.

2. Titration of the polysaccharide

The polysaccharide (0.1 g) in water (50 ml) was converted into the free acid form by passage three times down a column of IR 120(H^+) resin. The resin was washed to remove any residual polysaccharide and the solution and washings combined. The solution was divided into 4 parts (a–d) and the volume adjusted to 100 ml. The amount of carbohydrate was assayed [GM VI(i)]. Excess 0.1N-sodium hydroxide was added and the 4 parts titrated against 0.1NHCl in the following ways:-

(a) titrated immediately using phenolphthalein indicator.

(b) titrated immediately using Hintons indicator, see GM VII (xv).

(c) titrated after 2 hours using phenolphthalein indicator.

(d) titrated after 2 hours using Hintons indicator.

The equivalent weight of the polysaccharide was then calculated; and from this the uronic acid content and also the amount of low molecular weight acids present as esters, which would be liberated by the sodium hydroxide on standing - see table 2. The uronic acid content was found to be about 50%.

Table 2 Calculation of Equivalent weight

	Methods of titration			
	a	b	c	d
Amount 0.01N-NaOH(ml)	11	10	10	10
titre 0.01N-HCl (ml)	5.05	4.35	2.91	3.3
∴ vol. NaOH used (ml)	5.95	5.65	7.09	6.7
Normality polysaccharide	5.95×10^{-4}	5.65×10^{-4}	7.09×10^{-4}	6.7×10^{-4}
Amount of polysaccharide used		21.5 mg in 100 ml		
∴ Equivalent	359	378		
% uronic acid	49	47		
Normality of polysaccharide due to low molecular weight acids				
Normality = c–a or d–b	1.14×10^{-4}		1.05×10^{-4}	
If acids are acetic acid Equil wt.	1885		2047	
% acetic acid	3.2		2.9	

Experiment IX Measurement of the degree of degradation caused by hydrolysis

The polysaccharide (100 mg) of known carbohydrate content and uronic acid content was hydrolysed by formic acid (10 ml)[GM II(i)] and the hydrolysate freeze-dried and weighed. A sample of the hydrolysate was assayed for carbohydrate content and uronic acid content. This indicated a loss of 11% of neutral components (read as glucose) and 30% uronic acid (read as guluronic acid).

Experiment X Identification of the Neutral Monosaccharides

The neutral fraction from the resin column hydrolysate [Expt. VI(2)] was analysed by paper chromatography[solvents III(i)a-c and sprays IV(i), (ii)a, (iii), (iv), (viii), (ix)] and found to consist of glucose, mannose, arabinose, rhamnose and an unknown with a similar mobility to galactose.

Galactose was not present, this was confirmed by the enzymic galactose oxidase spray.

The neutral fraction from the partial hydrolysis [Expt. VI3] was applied to a cellulose column (Whatman CF11, 500 g) and eluted with n-propanol; ethyl acetate; water (7:2:1).[182] Fractions (5 ml) were collected and analysed by paper chromatography [solvent III (i)a] after partial evaporation. This method is less sensitive than the phenol-sulphuric acid method [GM VI (i)] but propanol was found to interfere with the colour reaction of the latter method. The fractions were combined as appropriate into 10 components and analysed by paper chromatography [solvent III (i) a-c].

1. oligosaccharide (trace, mixture)
2. oligosaccharide (trace, mixture)
3. oligosaccharide (trace, mixture)
4. glucose + unknown
5. glucose
6. glucose + mannose
7. mannose + arabinose
8. arabinose
9. rhamnose
10. glycerol.

Experiment XI Characterisation of the neutral components

The impure components were reseparated by preparative paper chromatography [solvent system III (i)a]. By this method, four monosaccharides and glycerol were obtained pure. The solutions of the

monosaccharides were filtered through a millipore (mesh size 0.45μ). The volume was adjusted to 10 ml and the carbohydrate content measured and calculated from an appropriate standard graph. The specific rotation of each sugar was measured. An aliquot of each solution was converted into the T.M.S. derivative [GM VII (x)] and analysed by G.L.C. A second aliquot was reduced with sodium borohydride and similarly examined.[GMV, ii(3)].

A crystalline derivative was prepared from each of the monosaccharides, and from an authentic sample. Melting points and mixed melting points were taken.

1. Glycerol p-nitrobenzoate.[225]

Glycerol (10 mg) was dissolved in pyridine (0.5 ml) and a 10% molar excess of p-nitrobenzoyl chloride added. The mixture was heated at 70°C for 40 minutes. The crystals formed were recrystallised from petroleum ether (60/40)/ethyl acetate (1:1) and dried in a desiccator. Melting point and mixed melting point = 187°C. Standard glycerol-p-nitrobenzoate = 188°.

2. Rhamnose 2,4-dinitrophenylhydrazone.[226]

Rhamnose (5 mg) was dissolved in water (0.1 ml) and 2,4-dinitrophenylhydrazine (10 mg) in glacial acetic acid (0.5 ml) added. The mixture was heated at 50°C for twenty minutes and then kept at 5°C for 18 hours. The deep yellow crystals were washed with cold alcohol and dried in a desiccator. Melting point and mixed melting point 170°. Literature value = 179°C.

3. Arabinose benzoylhydrazone.[227]

Benzoylhydrazine (10 mg) in 95% alcohol (1 ml) was added to arabinose (5 mg) in water (0.1 ml). The mixture was kept at 20°C for 24 hours and at -5°C for 48 hours. The white crystals were washed with ice cold

ethanol and dried in a vacuum desiccator for 48 hours. Melting point and mixed melting point = 184° (d). Literature value = 186°.

4. Mannose phenylhydrazone.[228]

5 % phenylhydrazine in 95% ethanol (0.5 ml) was added to mannose (10 mg). The mixture was warmed in a beaker of hot water for 30 minutes and then kept at -5°C for 18 hours. The white crystals after filtration were washed successively with a few drops of water, ethanol and ether. Melting point and mixed melting point 199°. Literature value 199°.

5. Glucose.

The syrup, when kept for 18 hours at 5°C, crystallised as the β anomer. The yellowy crystals were washed with ice cold ethanol until colourless. Melting point 147°. Literature value 148–150°. A single crystal was weighed and dissolved in water and the optical rotation followed until equilibrium was reached. $[\alpha]_D$ = +22° ⟶ +53°. Literature value +19 ⟶ +53.

Experiment XII Identification of the unknown monosaccharide

The fourth monosaccharide fraction (Expt. X) was found to consist of glucose and a monosaccharide thought to be galactose. When this mixture was analysed by paper chromatography [solvent systems III (i) a–c. Sprays IV (i), (ii)a, (iv), (v), (viii)] (see table 3). The presence of galactose was ruled out and a heptose was indicated. An aliquot of the mixture was converted into the TMS derivative [GM VII (x)] and a further aliquot was reduced [GM VII (viii)], and the TMS derivative made. Comparison of the G.L.C. chromatograms with that of the T.M.S. derivative of the authentic sample of D-glycero-D-mannoheptose indicated that the latter was the same as the unknown.

A sample of D-glycero-D-mannoheptose was kindly supplied by Dr M.B. Perry. The R_G values in column 4 are taken from the paper by Jones Perry and Sowa.[182]

Table 3. R_{Glc} values of unknown monosaccharide

Solvent system	R_{Glc} galactose	D-Glycero-D-mannoheptose	unknown	J.P. & S[182]
(i) a	0.97	0.875	0.875	0.90
(i) b	0.88	0.94	0.94	
(i) c	0.93	0.94	0.92	
(i) e		0.82	0.82	0.82

The mixture was applied to a preparative 3MM paper chromatogram and eluted in solvent system [III (i) a] for 48 hours, and the two components separated. This was repeated three times, the heptose eventually being obtained chromatographically pure. Its carbohydrate content was measured and a specific rotation taken. $[\alpha]_D$ = + 17°. Literature value + 21°.[229] Insufficient material was obtained to prepare a crystalline derivative.

To obtain more of the heptose, the neutral eluent from the resin column (experiment VI2) was applied to a 3MM preparative paper chromatogram and eluted in solvent system III(i)a, for 24 hours. The fraction corresponding to the glucose/heptose mixture was eluted. The glucose was converted into gluconic acid by the enzyme glucose oxidase, and the gluconic acid removed by 'Biodemineralit' resin (carbonate form). The enzyme was removed by boiling for 5 minutes with methanol, when the protein coagulated and precipitated, and was then removed by filtration. The solution of the heptose was filtered through millipore and concentrated. Paper

chromatographic examination of the syrup indicated that it was chromatographically pure.

Three attempts were made to make crystalline derivatives:

1. Acetate[229]

The heptose (5 mg) was dissolved in acetic anhydride (0.5 ml) containing concentrated sulphuric acid (1 drop) and the solution kept at room temperature for 48 hours. The mixture was then poured onto ice. A creamy syrup was produced, which did not crystallise.

2. Diethyl dithio acetal.[229]

The heptose (5 mg) was dissolved in ice cold concentrated hydrochloric acid and shaken with ethanethiol (0.2 ml) at 0°C for 30 minutes and then set aside at -5°C for 18 hours. The reaction mixture was neutralised with lead carbonate and concentrated. The deep yellow syrup was kept at 0°C for 24 hours. No crystals appeared.

3. p-nitro phenylhydrazone.[229]

The heptose (10 mg) was dissolved in methanol (0.2 ml) and refluxed for 2 hours with p-nitrophenylhydrazine (10 mg). The solution was concentrated and dissolved in ethyl acetate. On cooling no crystals appeared.

The dark yellow syrup was spotted onto thin layer chromatograms [solvent III (iii)b]. The starting material (p-nitrophenylhydrazine) moved with the solvent front but there appeared to be no starting material left in the reaction mixture. The syrup was diluted with water and filtered through a millipore and concentrated. The syrup still failed to crystallise.

Experiment XIII. Molar proportions of neutral sugars in the hydrolysate

The neutral fraction from the resin column (Expt. VI2] was used to determine the molar proportions. The following experiments did not take into account the neutral sugars still present as oligosaccharides. No result was obtained for the heptose from method 1 as no standard graph of the T.M.S. derivative was available. The result for the heptose for method 2 was obtained by reading from a standard graph of D-glycero-D-mannoheptose. See table 4.

These were calculated by two methods:

1. as GM VI(vi).
2. microgram quantities of the sugars were eluted from preparative paper chromatograms developed in solvent III(i)c. Where these resulted in mixtures, the carbohydrate content of the total was determined and the mixture reseparated by preparative paper chromatography in solvent III(i)a. The individual carbohydrate contents of each of the fractions was determined by the phenol sulphuric acid method [GM VI(i)] and calculated from individual standard graphs.

Table 4. Molar proportions of neutral sugars in polysaccharide

Sugar	Proportions	
	Method 1	Method 2
mannose	1.0	1.0
glucose	5.6	4.9
arabinose	1.05	1.1
rhamnose	0.9	0.95
heptose	-	1.25

Experiment XIV - Identification of Neutral Oligosaccharides

The neutral oligosaccharides obtained in experiment X (fractions 1-3) were combined and separated on 2 preparative paper chromatograms in solvent III(i)a. Unfortunately the separation appeared to be different on the two papers and so two series of oligosaccharide fractions A and B were obtained called A1-7 and B1-8. Each fraction was analysed by paper chromatography [solvents III(i)a and b and sprays IV(i),(ii) a, and b, (iv)]. The carbohydrate content [GM VI(i)] and the degree of polymerisation [GM VI (iv)] were measured. The remainder of each fraction was hydrolysed and analysed by G.L.C. of the T.M.S. derivatives [GM V(3),VII(x)].The values for degree of polymerisation were corrected according to the composition found on G.L.C. It appeared that many of the fractions were still mixtures, however insufficient material was available for reseparation. Table 5 gives a summary of the results obtained.

Table 5. Neutral oligosaccharides

fraction	mg	Dp	heptose	glucose	mannose	arabinose
OA1	0.175	15			+	+
OA2	0.135	11.3			+	+
OA3	0.162	8.5		+	+	+
OA4	0.198	6.6			+	+
OA5	0.198	6	+	+	+	+
OA6	0.518	5	+		+	
OA7	0.346	3	+	+		+
OB1	1.1	3.2			+	+
OB2	3.8	5		+	+	+
OB3	1.14	3.7	+		+	+
OB4	0.86	3.5	+	+	+	+
OB5	0.52	4		+	+	
OB6	0.42	3.4	+	+	+	
OB7	0.76	4.3		+	+	
OB8	0.46	2	+			

Experiment XV. Separation of Acidic oligosaccharides

The acid fraction from experiment VI,3 was applied to 4 preparative papers which were eluted in solvent III(i)a. Side strips indicated the presence of 7 components. After separation, the solutions were filtered through a millipore filter (mesh size 0.45μ) and the total volume of water adjusted to 50 ml. The fractions were assayed for carbohydrate [GM VI(i)] and the degree of polymerisation measured [GM VI(iv)]. The mobilities of the fractions were measured on paper chromatography [solvents III(i) a, b, c, d] and on paper electrophoresis [buffers III(ii) c and e]. Samples of each were hydrolysed with formic acid [GM III(i)] and analysed by paper chromatography [solvents III(i) a, b and d]. A further aliquot was esterified, reduced, and hydrolysed [GM VII (ix)] and analysed by paper chromatography [solvents III(i) a, b and e] and by G.L.C. of the T.M.S. derivatives [GM V(3), VII(x)]. The results obtained are summarised in table 6.

Table 6 Acid oligosaccharides

fraction	mg	D.P.	Composition heptose	glucose	uronic acid
1	10.0	15	+	+	+
2	5.2	5	+		+
3	12	3	+		+
4	7	2			+
5	1.65	1.22*	+		+
6	5.8	1.24*	+		+
7	4.8	1			+

* Chromatographic analysis of hydrolysates indicated that these two fractions were identical, but still contained mixtures of oligosaccharides.

No glucose was found in the sm ller oligos ccharides, either after hydrolysis or after reduction and hydrolysis indicating that neither glucose nor glucuronic acid were present. Fraction 1 was thought to be partially degraded polysaccharide.

Experiment XVI Periodate oxidation of the polysaccharide

The polysaccharide (1.5 g) was dissolved in water (250 ml) 0.03M sodium metaperiodate (250 ml) was added and the reaction followed spectrophotometrically [GM VII(iii)]. After 24 hours the reaction was stopped by the addition of ethylene glycol (5 ml) and the mixture allowed to stand for 1 hour. It was then reduced with potassium borohydride [GM VII, (viii)]. After neutralisation of the excess borohydride, the solution was dialysed against distilled water for 3 days and the solution freeze-dried.

Recovery of polyalcohol; 0.8 g at 65% carbohydrate. Moles of periodate consumed per anhydroaldose unit = 0.825.

The freeze-dried polyalcohol (20 mg) was hydrolysed with formic acid [GM II(i)] and analysed by paper chromatography [solvent systems III(i) a, b, c and sprays IV(i), (ii) a, (iv)]. In addition to oxidised fragments which were not identified, only guluronic acid, glucose and a trace of heptose were present.

Experiment XVII Autohydrolysis

The polysaccharide (2 g) dissolved in water (500 ml) was shaken with IR 120(H^+) resin to give pH 2.6 and the mixture placed in a dialysis sac and heated with stirring in a water bath at 80°C. The water surrounding the dialysis sac was changed every 12 hours and concentrated. The resin was renewed every 24 hours. Although it was washed thoroughly each time,

this resulted in some loss of polysaccharide adhering to the resin. Each concentrated sample of dialysate constituted a separate fraction and the experiment was continued until 11 fractions had been obtained. The residual polysaccharide inside the dialysis sac was freeze-dried (100 mg). An aliquot (20 mg) was hydrolysed with formic acid [GM II (i)]. Each of the eleven dialysate fractions and the hydrolysate of the residual polymer were examined by paper chromatography [solvents III(i) a-c and sprays IV (i), (ii)a,(iv)]. A summary of the chromatographic results on the fractions is given below.

Table 7 Composition of fractions from Autohydrolysis

fraction	composition					
	oligosaccharides	heptose	glucose	mannose	arabinose	acid
1	tr.		+		+	
2	tr.		+		+	
3	tr.		+		+	
4	tr.		+	+	+	
5	tr.		+	+	+	tr.
6	tr.		+		+	tr.
7	tr.		+	+	+	+
8		tr.	+	+	tr.	tr.
9		tr.	+	+	tr.	tr.
10		+	+	+	tr.	tr.
11		+	+	+		

The hydrolysate of the polymer contained only uronic acid and heptose.

The degraded polymer in aqueous solution would not precipitate from solution with ethanol (5 vol.).

The degraded polymer (5 mg) in 1% sodium chloride (0.5 ml) was applied to a Whatman column of Sephadex G.100 and the column eluted with 1% sodium chloride. 3 Mg were eluted with the void volume, indicating a chain length of greater than 750.

A further sample (5 mg) in 1% sodium chloride (0.5 ml) was applied to a Whatman column of Sephadex G.200. About 2 mg were eluted with the void volume indicating a chain length of greater than 1000.

Experiment XVIII Periodate oxidation of the degraded polymer

The degraded polymer (60 mg at 78% carbohydrate) in water (10 ml) was added to 0.03M sodium metaperiodate (10 ml) and the reaction followed spectrophotometrically [GM VII (xiii)]. After 24 hours the reaction was stopped by the addition of ethylene glycol (1 ml). After 1 hour the solution was reduced with potassium borohydride [GM VII (viii)]. After 24 hours the solution gave a negative reaction to Fehling's solution and, after neutralisation of the excess borohydride, was dialysed for 3 days, and freeze-dried (yield 36 mg; 72% carbohydrate). Moles of periodate consumed per anhydroaldose unit = 0.90.

The freeze-dried polyalcohol (10 mg) was hydrolysed with formic acid [GM II(i)] and analysed by paper chromatography [solvent systems III(i) a, b, c and sprays IV (i), (ii)a, (iv)]. In addition to oxidised fragments which were not identified, the only monosaccharide was guluronic acid.

Experiment XIX Fractionation of the Initial Polysaccharide

Three methods of fractionation were attempted:

1. Resin column.

The column was prepared as in experiment VI, 2. A very dilute solution of polysaccharide was prepared (100 mg in 1L H_2O). The

polysaccharide solution was applied to the column under the influence of a peristaltic pump. The column was washed with water until no further carbohydrate was eluted (2 L). The column was washed with 2N-formic acid until no further carbohydrate was eluted. Examination of the two fractions by paper chromatography after hydrolysis with formic acid [GM II (i)] indicated that both fractions contained uronic acid.

2. Diethyl Amino Ethyl (D.E.A.E.) Cellulose.

The cellulose used was Whatman grade D.E.50 (preswollen, containing 75% water). The cellulose (150 g) was stirred in 0.5N-hydrochloric acid (2 L) and deaerated for 20 minutes under reduced pressure with stirring. The acid was removed by filtration and the cellulose washed with water until the filtrate was neutral. The cellulose was then suspended in 0.5N-potassium hydroxide (2L) and treated as above. This procedure was repeated twice. The cellulose slurry was then transferred to a column and eluted with 2M-potassium chloride (3 L) and then with water until the eluant was free from chloride ions.

The polysaccharide (500mg at 85% carbohydrate content) in water (250 ml) was applied to the column and eluted with water until no further carbohydrate was obtained. It was then eluted sequentially with 0.3M-, 0.5M- and 1.0M-potassium chloride and then with 2M-potassium acetate and finally with 1M-sodium carbonate. Each fraction was concentrated, and in the case of salt solutions dialysed against distilled water for 3 days, and freeze-dried. The carbohydrate content was measured [GM VI(i)] and a sample hydrolysed with formic acid [GM II(i)] and analysed by paper chromatography [solvents III (i) a, b, c].

Table 8 Fractions obtained from DEAE-Cellulose column.

Eluent	Quantity used (L)	freeze-dried wt. (mg)	wt. as carbohydrate (mg)
H_2O	4	77	21.5
0.3M KCl	3	135.7	96
0.5M KCl	2	28	6
1.0M KCl	2.5	23.3	8
2M K_2Ac	2.5	806*	40
1M Na_2CO_3	2	88	37.6
			209 mg

* incompletely dialysed.

(Total recovery of carbohydrate 209 mg from 425 mg).

Analysis of hydrolysates of fractions indicated that the aqueous fraction was devoid of uronic acid, but the other fractions which contained uronic acid appeared to be identical and still contained all the other sugars.

3. Precipitation with quaternary ammonium salts.[230]

(a) the polysaccharide (1 g as a 1% aqueous solution) was treated with 10% w/v cetyltrimethylammonium bromide (Cetavlon British Drug Houses) 8 ml. A complex was formed which was removed by centrifugation. The complex was stirred with 50% acetic acid (50 ml) as indicated by Jones, Perry and Sowa.[182] The complex did not dissolve, the supernatant was found to be devoid of carbohydrate(phenol-sulphuric acid)method [GM VI(i)].

(b) The polysaccharide (1 g m 1L H_2O) was treated with 10% w/v Cetavlon (20 ml; supplied by British Drug Houses). The complex formed was removed by filtration through nylon cloth and washed with water to remove excess Cetavlon. The complex was then stirred with 2M-sodium chloride (1 L) for about 48 hours. About 50% of the complex dissolved (fraction A1), the undissolved complex was removed by centrifugation (complex A2). The fraction (A1) was precipitated from solution with ethanol. To remove the final traces of Cetavlon the polysaccharide was dissolved in water and reprecipitated with ethanol several times. Complex (A2) was dissolved with stirring in 60% ethanol at 30 - 37°C. The polysaccharide (A2) was precipitated from solution by the addition of 5M-sodium acetate and removed by centrifugation, and purified by repeated dissolution in water and reprecipitation with ethanol.

To the supernatant from the Cetavlon precipitation was added Cetavlon (5 mls, 10% w/v). No further precipitation occurred. The solution was tested for carbohydrate [CM VI (i)] and was found to contain 100 mg. It was concentrated by partial freeze-drying (100 ml). The carbohydrate was precipitated with ethanol (5 vol). This fraction (N_2) was freeze-dried. It was found that not all the carbohydrate was precipitated with ethanol. The supernat t (N_1) was dialysed to remove the Cetavlon, but the carbohydrate (N_1) also passed through the dialysis sac. The Cetavlon was then removed with Biodemineralit (carbonate form) resin.

(c) The polysaccharide (1 g 1L H_2O) was treated with 10% w/v Cetavlon B.D.H. (20 ml). The complex was filtered through nylon cloth and washed with water to remove the excess Cetavlon. The complex was dissolved in 60%

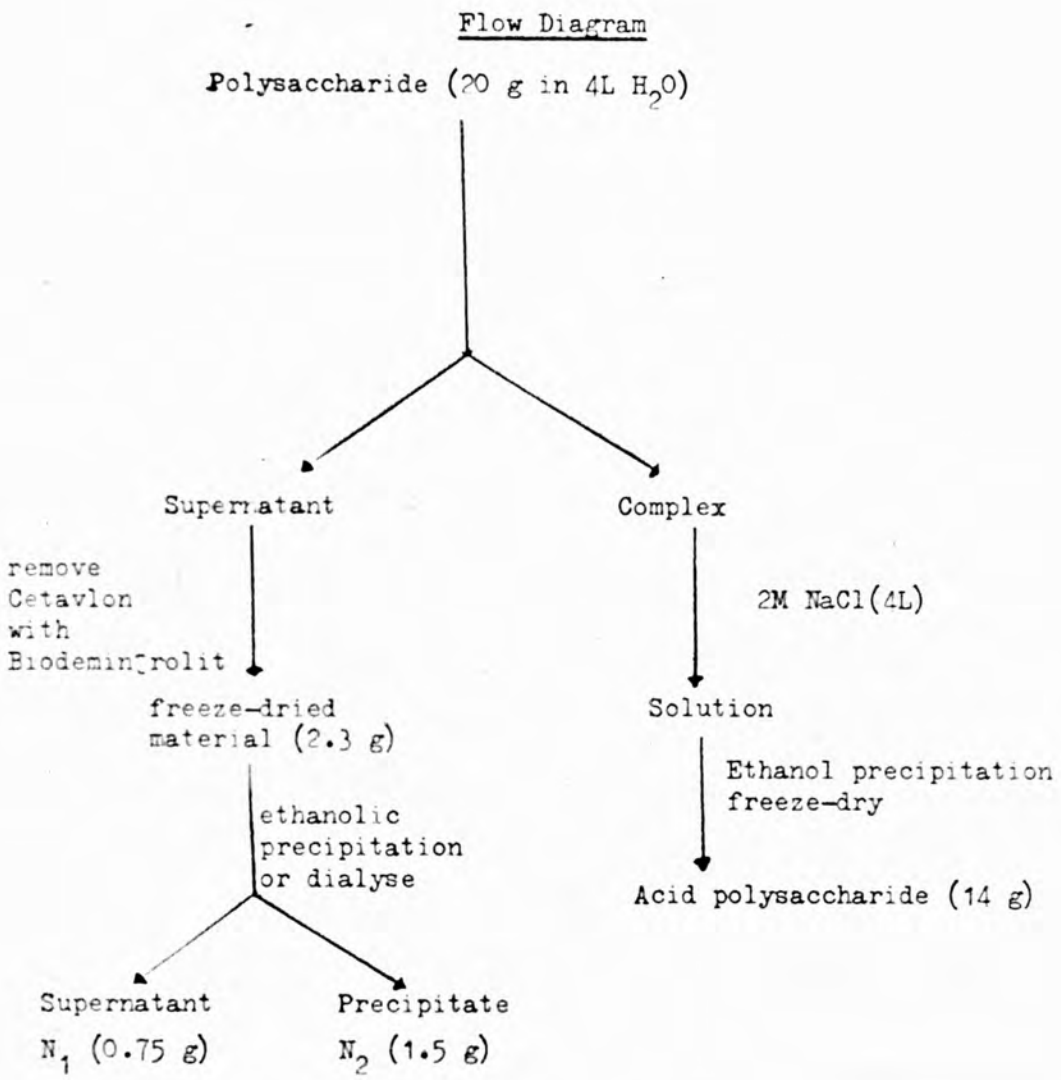


Fig. 4 Fractionation with Quaternary Ammonium Salts

isopropylalcohol (200 ml) with stirring. The polysaccharide was precipitated with sodium acetate (25 g) and purified by repeated dissolution in water and reprecipitation with isopropyl alcohol. The polysaccharide was hardened by washing with acetone.

(d) The polysaccharide (20 g in 4L H_2O) was treated with 10% w/v Cetavlon (hexadecyltrimethylammonium bromide, Eastman Kodak) (100 ml) and the complex removed by filtration through a nylon cloth and the excess Cetavlon removed by washing with water. The complex was stirred with 2M-sodium chloride (2 L) for 48 hours. All the precipitate dissolved and the polysaccharide was recovered from solution by precipitation with ethanol. Purification was effected by repeated dissolution in water and reprecipitation with ethanol.

The supernatant from the Cetavlon precipitation in experiments (c) and (d) was retreated with Cetavlon. No further complex formation occurred. The Cetavlon was removed from the solution with Biodeminrolit resin (carbonate form) and the solution was freeze-dried; the yellow freeze-dried material was treated as in experiment 3b (see flow diagram fig. 5).

Experiment XX Physical properties of the neutral polysaccharide

(a) Specific rotation

The neutral polysaccharide (10 mg) was dissolved in water (10 ml). The solution was assayed for carbohydrate [G.M. VI (i) as glucose] and then used for taking a rotation as in experiment IV.

$$[\alpha]_D = +16^\circ$$

(b) Infrared

The polysaccharide was dried in a vacuum desiccator for 18 hours at 60°C. The polysaccharide was ground with potassium bromide and a potassium bromide disc made. The infrared spectrum was obtained using a Perkin Elmer 257 grating spectrometer.

Experiment XXI Neutral 'Polysaccharide' Analysis

The polysaccharide from the fractionation, not divided into fractions (N_1) and (N_2) was assayed for carbohydrate content [GM VI(i)], uronic acid content [GM VI (ii)], nitrogen content [GM VI (v)] and glucose content [GM VI (iii)]. It was found to contain no nitrogen, (and thus no protein) and no uronic acid. Of the total carbohydrate (63% read as glucose), glucose constituted 55%. The proportions of the other constituents, mannose, arabinose, and rhamnose were obtained by method [VI (vi)].

As indicated by the flow chart the neutral fraction could be separated into two parts, (N_2), which precipitated with ethanol and (N_1) which would not precipitate. Paper chromatographic analysis [solvents III (i), a, b and c, sprays (i), (ii)a, and (iv)] indicated that the latter consisted mainly of free glucose with smaller amounts of mannose and oligosaccharides. No arabinose was detected. Fraction (N_2), the neutral polysaccharide, was analysed as above and found to contain 33% of glucose.

Experiment XXII Methylation Analysis of the Neutral Polysaccharide (N_2)

The polysaccharide (50 mg) was methylated by the Hakomori method [GM VII (xii)a]. After freeze-drying the methylated polysaccharide (38 mg) was hydrolysed with formic acid [GM II (i)]. The hydrolysate was divided into two parts. One part was converted into the methyl glycosides; the other part was reduced with sodium borohydride [GM VII (viii)] and acetylated [GM VII (xi)]. G.L.C. of the methyl glycosides were performed on columns (1) and (2) (GMV). The alditol acetates were analysed by G.L.C. on column (4) and by G.L.C. linked to Mass Spectrometry [GMV5] and the results are summarised in Table 16, p.104.

Experiment XXIII Periodate Oxidation of the Neutral Polysaccharide

The polysaccharide (0.05g=31.75 mg carbohydrate) was dissolved in water (50 ml) and added to 0.3M-sodium metaperiodate (50 ml) and the reaction followed spectrophotometrically [GM VII (iii)]. Aliquots (1 ml) were removed after 3 hours and 18 hours and used for formaldehyde determination [GM VII (xiv)]. After 24 hours the reaction was stopped by the addition of ethylene glycol (1 ml) and the mixture stirred for 1 hour. The solution was reduced with potassium borohydride [GM VII(viii)] and the derived polyalcohol was freeze-dried.

Moles of periodate consumed per anhydroaldose unit = 1.8. Moles of formaldehyde released per mole carbohydrate after 3 and after 18 hr = 0.26. Recovery of polyalcohol = 0.014g at 50.2% carbohydrate as glucose.

The polyalcohol (20 mg) was hydrolysed with formic acid [GM II (i)] and analysed by paper chromatography [solvents III (i), a, b and c]. In addition the glucose content was measured quantitatively [GM VI (iii)] and found to be 2.25%.

Experiment XXIV Acid polysaccharide analysis

The acid polysaccharide from Experiment XIX 3c was assayed for carbohydrate content [GM VI(i)], uronic acid content [GM VI (ii)], nitrogen content [GM VI (v)] and glucose content [GM VI (iii)]. The proportion of heptose was obtained by method [GM VI (vi). The results are shown below:

	%
nitrogen	0
uronic acid	65
glucose	15
heptose	20

Experiment XXV Physical properties of the acid polysaccharide

(a) Specific rotation.

The polysaccharide (10 mg) was dissolved in water (10 ml) and after filtration through millipore assayed for carbohydrate and the rotation measured. It had $[\alpha]_D$ = 25.6

(b) Infra-red.

The polysaccharide was prepared as a thin film as in Experiment IVb and the infra-red spectrum obtained as before.

Experiment XXVI Periodate oxidation of the acid polysaccharide

The polysaccharide (2.0, 1.54 carbohydrate) was dissolved in water (250 ml) and 0.03M-sodium metaperiodate added (250 ml). The reaction was followed spectrophotometrically [GM VII (xiii)]. After 3 hours and after 18 hours, aliquots were withdrawn and used to determine the amount of formaldehyde liberated [GM VII (xiv)]. After 24 hours the reaction was stopped by the addition of ethylene glycol (5 ml). After a further 1 hour the solution was reduced with potassium borohydride [GM VII (viii)]. 24 hours later the solut on was found to be non-reducing to Fehling's reagent and after neutralisation of the excess borohydride was dialysed for 3 days. The polyalcohol was freeze-dried. (Yield 1.3903 g corresponding to 0.8467 g carbohydrate, (Std. Graph 6).

Moles of periodate consumed per anhydroaldose unit = 0.59

Moles of formaldehyde released per mole of carbohydrate after 3 hours and after 18 hours = 0.15.

Experiment XXVII Analysis of the derived polyalcohol

An aliquot of the polyalcohol was assayed for glucose [GM VI (iii)] and found to contain 16.9%. A second sample (20 mg) was hydrolysed with formic acid [GM II (i)] and the hydrolysate analysed by paper chromatography [solvents III (i) a, b and c; sprays IV (i), (ii)a, (iv) and (v)]. This indicated that the polyalcohol contained, in addition to small fragments uncleaved glucose, heptose and guluronic acid.

Experiment XXVIII Partial hydrolysis of the derived polyalcohol

The polyalcohol (100 mg) was dissolved in 0.5N-sulphuric acid (50 ml) and allowed to stand at room temperature. Aliquots (5 ml) were withdrawn after 1, 2, 4, 6 and 24 hours. The residual material was set aside at 0°. The aliquots were neutralised ($Ba\ CO_3$), concentrated, and analysed by paper chromatography [solvent system III (i)c, sprays IV (i) and (ii)a]. No hydrolysis had apparently occurred. The residual solution was warmed at 50° for 1 hour, an aliquot withdrawn and neutralised and analysed as above. In addition to some small fragments glucose was present.

A second sample of polyalcohol (100 mg) was dissolved in 1N-sulphuric acid and allowed to stand at room temperature. Aliquots (5 ml) were withdrawn after 1, 2, 4 and 8 hours. The residual solution was set aside at $0^{\circ}C$ to arrest the hydrolysis. The aliquots were neutralised ($BaCO_3$) and analysed by paper chromatography [solvent III (i)c and spray IV (i)]. Hydrolysis for 1 hour had no effect but the other three aliquots contained some small fragments but no monosaccharides. The residual hydrolysis solution was neutralised and the small fragments separated by preparative paper chromatography on a Whatman No.3 MM paper

[solvent III (i)c]. Three fractions were obtained:

(a) material which remained on the starting line

(b) glycerol

(c) glyceric acid.

Fraction (a) was then applied to paper chromatograms and eluted in solvents IIIa, and c for several days. No oligosaccharide fractions were present indicating that the polyalcohol was still present as one long chain.

Fractions b and c were identified by their chromatographic mobility in several solvents, and comparison with standard materials.

Experiment XXIX N.M.R. Analysis of the polysaccharide

This was carried out on the following polysaccharide samples on a Varian EM360. The polysaccharides were dissolved in the solvent by shaking for 18 hours to form a 2% solution. The solvent was removed by the method indicated and the polysaccharide redissolved. The process was repeated 3 or 4 times. The solution was transferred to an N.M.R. tube and the spectrum taken.

1. Acid polysaccharide [Expt. XIX 3d] in D_2O, dried by freeze-drying.
2. Acid polyalcohol [Expt. XXVI] in D_2O, dried by freeze-drying.
3. Methylated acid polysaccharide [Expt. XXXIX 3] in D_6-DMSO, dried by evaporation under high vacuum.
4. Methylated reduced polysaccharide [Expt. XXXIX 2] in $CDCl_3$, dried by evaporation.
5. Acid polysaccharide; methylated, reduced, remethylated [Expt. XXXIX] in $CDCl_3$, dried by evaporation.

In addition, the following samples of standards were used:

6. Sodium acetate in D_2O
7. Glucose pentaacetate in $CDCl_3$
8. Sodium pyruvate in D_2O
9. Pyruvic acid (freshly distilled) in D_2O
10. Methyl ester of pyruvic acid in $CDCl_3$.

These results indicated the presence of $-CH_3$ at 2.1 relative to TMS in the polysaccharide. This is similar to the $-CH_3$ of pyruvic acid. The peak was still present after treatment with alkali, indicating a ketal linkage rather than an ester.

Experiment XXX Removal of pyruvic acid from the acid polysaccharide

The acid polysaccharide (1.5 g) was dissolved in water (500 ml) and shaken with IR120(H^+) three times. The resin was removed by filtration and washed. The solution and washings (at pH 2.5) were refluxed for 6 hours. After cooling, the solution was extracted 3 times with ether in a liquid-liquid extractor. The aqueous layer was freeze-dried and weighed (0.8 g).

Experiment XXXI Analysis of the depyruvylated polysaccharide

The polysaccharide was assayed for carbohydrate (82%) [GM VI (i)] uronic acid [GM VI (ii)] (65%), glucose content 16% [GM VI (iii)] and nitrogen (0%).

The polysaccharide was hydrolysed with formic acid [GM II(i)] and analysed by paper chromatography [solvents III (i)a, b and c, and sprays IV (i), (ii)a, (iv) and (v)]. The only other component was heptose.

Experiment XXXII Periodate oxidation of the depyruvylated polysaccharide

The polysaccharide (131 mg ≡ 102 mg of carbohydrate) was dissolved in water (50 ml) and the solution oxidised and the polyalcohol recovered as in Expt. XXVI. Yield of freeze-dried polyalcohol 36.2 mg ≡ 30.8 mg. of carbohydrate.

Moles of periodate consumed per anhydroaldose unit = 0.6[illegible]
Moles of formaldehyde released per anhydroaldose unit after 3 hours and 18 hours = 0.19.

Experiment XXXIII Analysis of polyalcohol from depyruvylated polysaccharide

The polyalcohol was analysed for carbohydrate, (82%) [GM VI (i)] uronic acid (60%) [GM VI (ii)] and glucose content (19%) [GM VI (iii)]. The polyalcohol was hydrolysed with formic acid [GM II (i)] and analysed as in Expt. XXVII. In addition to small fragments, the only uncleaved sugars were glucose and uronic acid.

The polyalcohol was partially hydrolysed under the same conditions as the polyalcohol from the acid polysaccharide (Expt. XXVIII). The results indicated the formation of glycerol and glyceric acid and no other fragments.

Experiment XXXIV Acetyl content of the acid polysaccharide[231]

The acetate, to be determined, is dissolved in sodium acetate buffer (5 ml) at pH 4.5 to give a 0.001M-solution. The following are then added:

(a) 2M-hydroxylamine hydrochloride (5 ml)

(b) 3.5N-NaOH, 5 ml.
After 1 minute the pH is adjusted to 1.2 ± 0.2 with

(c) concentrated hydrochloric acid diluted with 2 parts by volume of water.

(d) 0.37M-$FeCl_3$ in 0.1N-HCl (5 ml) is then added.

The absorbance of the resulting solution is then read at 540 nm. A standard graph using glucose pentaacetate was prepared. The acetyl content of the polysaccharide was found to be about 12%. This corresponds to a degree of acetylation of 0.3 or one acetate ester group for every three monosaccharide units.

Experiment XXXV Identification of pyruvic acid

The ethereal extract from Expt. XXX was concentrated to dryness and dissolved in water. This solution was analysed by paper chromatography [solvent III (i)c, sprays IV (vii) and (viii)] and paper electrophoresis [buffers III(i)c and d and sprays IV (vii) and (viii)]. An aliquot was extracted and dried by evaporation with methanol and benzene. It was then refluxed with methanolic hydrogen chloride to prepare the methyl ester [GM VII (vii)]. After neutralisation (Ag_2CO_3), and washing the silver salts, the filtered solution was evaporated, dissolved in ether and analysed by G.L.C. [VB (iv) and (v)]. The results indicated that pyruvic acid was present in the polysaccharide.

Experiment XXXVI Preparation of a crystalline derivative of pyruvic acid.[232]

An aliquot of the aqueous syrup from the ether extract was added to an ethanolic solution (0.5 ml) of 2,4-dinitrophenylhydrazine.

The solution was heated at 100°C for a few minutes and then set aside at 0°C for 18 hours. The yellow orange crystals (dried in a desiccator) had m.p. ~216°C. A derivative was prepared in the same way from a sample of authentic freshly distilled pyruvic acid. This had melting point 218°C and mixed melting point with the extracted sample 216°C.

The orange yellow crystals were dissolved in methanol and examined by thin layer chromatography [solvent system III (iii)b]. No starting material remained in the crystals. The crystals were also examined by I.R. as potassium bromide discs. The spectra of the derivative of the authentic pyruvic acid and that of the derivative of the ether extract were found to be identical and quite dissimilar from that of the starting material, 2,4 dinitrophenylhydrazine.

Experiment XXXVII <u>Attempted isolation of a disaccharide unit carrying the pyruvic acid ketal.</u>[233]

The polysaccharide (5 g) was suspended in 0.5% methanolic hydrogen chloride (50 ml) and heated under reflux for 2 hours. The undissolved polysaccharide was removed by filtration (~1 g). The filtrate was neutralised with silver carbonate. The silver salts were removed by filtration and washed thoroughly with methanol. The filtrate and washings were combined and concentrated by evaporation. The resulting syrup was saponified by heating in 0.2N-barium hydroxide solution (50 ml) for 2 hours at 60°. Excess barium hydroxide was removed by neutralisation with solid carbon dioxide and the precipitate removed by filtration. The filtrate was passed successively down columns of IR120(H^+) and IR45 B(OH^-) resins. The columns were washed thoroughly with

water and the neutral effluent was concentrated to a syrup. (53 mg) consisting of heptose and glucose. The IR-45B(OH^-)$_{resin}$ was stirred with 1N-sulphuric acid (30 ml) at 0°C for 10 minutes. The resin was filtered and washed with additional aliquots (10 ml) of ice cold 1N sulphuric acid and then with water (200 ml). All solutions were immediately neutralised by filtration into a suspension of barium carbonate. The barium sulphate and residual barium carbonate were removed by filtration and the filtrate concentrated. The syrup was dissolved in methanol (1 ml) under reflux. No crystals formed on cooling. The syrup was examined chromatographically [solvents III (i) a, b and c and sprays IV (i) and (ii)a] and by N.M.R. spectroscopy in $CDCl_3$. The results indicate that although pyruvic acid was present in the mixture it was not attached to the sugar components.

Experiment XXXVIII Quantitative estimation of pyruvic acid

Two methods were attempted:

1. complexing of pyruvic acid with 2,4-dinitrophenylhydrazine after hydrolysis from the polysaccharide.[234]

The pyruvate containing polysaccharide (100 mg) was dissolved in 2NHCl (40 mls). The solution was set aside at room temperature for 3 hours. An aliquot (2 ml) was removed and to it the 2,4-dinitrophenylhydrazine reagent (0.5% in 2N-HCl 1 ml) was added. After 5 minutes the solution was extracted with ethyl acetate (5 mls). The aqueous phase was discarded. The ethyl acetate layer was then extracted successively with aliquots of 10% sodium carbonate (3 x 5 ml). The sodium carbonate extracts were combined and the volume adjusted to 250 ml. The colour developed was read at 375 nm. A standard graph was prepared using sodium pyruvate.

This indicated a pyruvic acid content of 3%, equivalent to 0.55 moles per mole carbohydrate, or 1 unit in 20 carrying pyruvic acid.

2. Enzymic method (Duckworth and Yaphe[206], modification of the Hadjivassilou and Rieder method[235]).

The polysaccharide (10 mg) was hydrolysed for 4 hours at 100°C with 0.04N-Oxalic acid (3 ml). After cooling the hydrolysate was neutralised ($CaCO_3$) and filtered. The filtrate was diluted to 10 ml. An aliquot (2 ml) was added to 1M-triethanolamine (1 ml), 1% NADH in 0.1% sodium bicarbonate (0.5 ml) was added and the absorbance read at 340 nm. Lactic dehydrogenase ('Sigma' type II from rabbit muscle, 0.1 ml) was then added, and the absorbance noted until a constant value was obtained.

When the above was attempted on the polysaccharide and on a freshly distilled sample of pyruvic acid, no change in absorbance occurred on the addition of lactic dehydrogenase.

The following variations were attempted:

a) The triethanolamine was made 0.1M and adjusted to pH 6.7 with 0.1N-HCl. The method then gave reasonable results with standard pyruvic acid, but not with the polysaccharide sample.

b) Hydrolysis of the polysaccharide was carried out with 0.1N-HCl and the triethanolamine used as in (a).

c) After hydrolysis the pyruvic acid was extracted from the hydrolysate with ether, the ether evaporated and the extract diluted to 10 ml and the assay performed on this solution, using the triethanolamine as in variation (a). This modification gave a value of less than 1% for the pyruvic acid content, probably due to incomplete extraction with ether.

$$\% \text{ pyruvic acid} = \frac{5 \times \text{M.Wt.}_{\text{pyac}} \times 100 \; (A_{\text{initial}} \times V_{\text{initial}} - A_{\text{final}} \times V_{\text{final}})}{W_{\text{polys(mg)}} \times 6.22 \times 1000}$$

Experiment XXXIX Methylation Analysis of the Acid Polysaccharide

The polysaccharide was methylated in the following ways:

1. The polysaccharide (20 mg) was methylated by the Hakomori method [GM VII (xii)a]. After dialysis and freeze-drying, the polysaccharide was dissolved in water and reduced with potassium borohydride [GM VII(viii)]. The solution was again freeze-dried and then remethylated by the Hakomori method. An aliquot of the derived methylated neutral polysaccharide was hydrolysed with formic acid [GM II (i)] and the derived sugars reduced to the alditols [GM VII (viii)] and acetylated [GM VII (xi)].

2. The polysaccharide (20 mg) was methylated by the Hakomori method [GM VII (xii)a]. After dialysis and freeze-drying the polysaccharide was dissolved in water and reduced with potassium borohydride [GM VII (viii)]. The solution was again freeze-dried. A sample was hydrolysed with formic acid [GM III (i)] and the hydrolysate was reduced to the alditols [GM VII (viii)] and acetylated [GM VII (xi)].

3. The polysaccharide (20 mg) was methylated by the Hakomori method [GM VII (xii)a]. After dialysis and freeze-drying a sample of the methylated polysaccharide was hydrolysed with formic acid [GM II (i)] and the hydrolysate reduced with potassium borohydride [GM VII (viii)] and the alditol acetates prepared [GM VII (xi)].

4. The polysaccharide (20 mg) was methylated as in Experiment XXXIX(2), but the potassium borohydride was replaced by potassium borodeuteride for the polysaccharide reduction. Reduction of the hydrolysate was carried out with potassium borohydride as before.

5. The polysaccharide (50 mg) was methylated by the Hakomori method (double quantities) [GM VII (xii)a]. After dialysis and freeze-drying the polysaccharide was dissolved in D_2O and reduced with potassium borodeuteride [GM VII (viii)]. The solution was again freeze-dried and then remethylated by the diazomethane method [GM VII (xii)c]. An aliquot of the methylated polysaccharide was hydrolysed reduced and acetylated as in the previous experiments.

6. Three samples of the polysaccharide (20 mg each) were treated as in method 1 but the reduction of the three methylated polysaccharides was carried out as follows:

1. dissolved in H_2O, reduced with potassium borohydride
2. dissolved in H_2O, reduced with potassium borodeuteride
3. dissolved in D_2O, reduced with potassium borodeuteride

Each of the three samples was treated as in method 1. All of the methylated alditol acetates were analysed separately by G.L.C. linked to mass spectrometry.

Experiment XL Methylation Analysis of a) Acid polyalcohol
b) Depyruvated polysaccharide
c) Depyruvated polyalcohol.

The 3 polysaccharides (20 mg) were methylated as in Experiment XXXIX(1). The results are shown in Table 10.

Experiment XL a Acid polyalcohol; methylated, reduced, remethylated

b Depyruvylated polysaccharide; methylated, reduced, remethylated

c Depyruvylated polyalcohol; methylated, reduced, remethylated

Peak No.	T+	corresponding to	mixtures present in
4	3.1	heptose 3,4,6,7-tetra-O- methyl	XL(a), XL(b) (tr.)
3	1.75	glucose 2,4,6-tetra-O-methyl	All three samples.
1	1.00	guluronic acid 2,3,4,6-tetra-O-methyl	All three samples.
2	1.68	2,4,6-tri-O-methyl	All three samples.
5	3.8	2,6-di-O-methyl	All three samples.

+ T is relative to 2,3,4,6-tetra-O-methylglucitol 1,5 acetate.

Experiment XLI Isolation of oligosaccharides from the acid polysaccharide

The polysaccharide (10 g) was hydrolysed with 0.5 N-sulphuric acid for 4 hours [GM II (ii)]. After neutralisation ($BaCO_3$) the filtered solution was concentrated and poured into ethanol (5 vol.). The precipitate (barium salts of uronic acids) was removed by centrifugation. The solution was shaken with IR120(H^+) resin to remove the barium ions and the resin removed by filtration. The solution was neutralised with dilute ammonium hydroxide solution and the solution concentrated. The resulting syrup was applied to 8 preparative papers (Whatman No. 3MM) and eluted with solvent III (i)a for 48 hours. The fractions, denoted by stained side strips, were eluted with water and concentrated. Five fractions were obtained. Each fraction was filtered through a millipore(0.45μ

Experiment XLII Analysis of the oligosaccharides

Each oligosaccharide fraction was assayed for carbohydrate [GM VI (i)] uronic acid [GM VI (iii)] and glucose [GM VI (iii)] and the degree of polymerisation measured [GM VI (iv)]. An aliquot of each solution was hydrolysed with formic acid [GM II (i)] and analysed by paper chromatography [solvents III (i)a and b, sprays IV (i), (ii)a, (iv) and (v)]. A second aliquot was glycosided reduced and hydrolysed [GM VII (ix)] and analysed by paper chromatography as above. A third aliquot of oligosaccharide (1), (2) and (4) was reduced [GM VII (viii)] followed by hydrolysis with formic acid and analysis by paper chromatography as above. Heptitol was detected in each of the hydrolysates. The results are summarised in table 11.

Experiment XLI Methylation of oligosaccharides from acid polysaccharide

The oligosaccharide fractions (1-4) were thoroughly dried and then moistened with dry methanol and methylated by the diazomethane method [GM VII (xii)c]. The methylating reagents were removed by distillation and the yellow syrup was dissolved in a methanol and a water mixture, and the solution reduced with potassium borohydride [GM VII (viii)]. The potassium ions were removed with IR120(H^+) resin and the solutions concentrated by evaporation. The partially methylated reduced oligosaccharides were remethylated by the modified Kuhn method [GM VII (xii)b]. Each oligosaccharide fraction was then hydrolysed with formic acid [GM II (i)] and the hydrolysate reduced [GM VII (viii)] and acetylated [GM VII (xi)]. The alditol acetates were analysed by G.L.C. linked to mass spectrometry. The results are summarised in table 12.

Experiment XLIV Partial Hydrolysis of Hexasaccharide(1)

An aliquot of oligosaccharide (1) was hydrolysed with N-sulphuric acid for 4 hours at 100°. After neutralisation ($BaCO_3$), the hydrolysate was analysed by paper chromatography. [solvents IIIa, b and c, and spray IV (i)]. In addition to guluronic acid and heptose there were two spots, one corresponding to unhydrolysed hexasaccharide and the other to the trisaccharide (2).

Results and Discussion

Attempted Fractionation

Initial investigation of the total extracellular material indicated a very complex mixture of carbohydrate and protein, the former comprised uronic acid and at least 5 neutral sugars, and it seemed probable that it was a mixture of more than one polysaccharide. Accordingly, the first attempts were directed towards fractionation. However this proved to be a difficult and initially unsuccessful procedure. This was largely due to the high viscosity of the solution (1000 cps* for a 1% solution) and the fact that the solution gelled when salt was added. For this latter reason, gel filtration was not attempted as a means of fractionation, but other columns of, for example D.E.A.E.-cellulose, and resin [see Expt. XIX 1 and 2, p.62] were used. The resin retained much of the carbohydrate (Yield 0.32 g from 1.0 g carbohydrate, as glucose) and did not produce any fractionation as both the 'acid' and 'neutral' fractions contained both neutral sugars and uronic acid. The D.E.A.E.-cellulose column also gave a very low recovery of about 50% of the carbohydrate applied, although a small fraction (ca. 10% of that recovered) containing no uronic was obtained by this method, the other fractions contained mixtures of all the neutral monosaccharides and uronic acid. In addition, fractionation with barium hydroxide[237] and precipitation with ethanol[238] was attempted but both procedures resulted in all the carbohydrate being removed from solution, whereas addition of Fehling's solution[239] caused no precipitation. Following the work by Jones *et al*.[182], precipitation with quaternary ammonium salts (Cetavlon) was attempted. Again it appeared that all the polysaccharide complexed with the Cetavlon, and the supernatant was devoid of carbohydrate. Furthermore the complex did not dissolve in 50% acetic acid, although it was reported to do so by the above authors. In spite of these unsuccessful attempts, the fractionation experiments were continued

*cps = centipoises

alongside examination of the total polysaccharide material. Consequently the first eighteen experiments were on the total polysaccharides. Thereafter a neutral and an acidic polysaccharide were separated and examined.

Purification and Examination of the Partially Purified Polysaccharide

The crude polysaccharide, as supplied by Arginate Industries Ltd., was an off-white, milled powder with a slightly fishy smell. Individual batches varied in their carbohydrate and protein contents (see Table 10 below), depending upon the effectiveness of the initial centrifugation of the gelatinous medium to remove the bacterial cells. This is probably because different batches came from media which differed in viscosity and

Table 10

Sample	% Protein	% Carbohydrate (as glucose)
S11 A	20	38
S11 B	7.0	63.5
S11 C	7.0	58
S11 D	9.36	69
S11 E	20	57
S11 F	21	58
S11 G	19	58
S11 H	43	4.5 (cell debris , p.28)
S11 J	50	6 (cell debris, p. 28)

thus some of the more viscous broths would centrifuge less effectively. However when a 0.1% solution was centrifuged (Expt. III p.44), it was found that all the nitrogen, and thus all the protein in the centrifugate (A) (Flow diagram p.45) had been removed.

The carbohydrate content of the extracellular material (Table 10) and of the different fractions of polysaccharide (Table 11) was determined by the phenol-sulphuric acid method, which is dependent on the standard graph from which the absorbance is read. Initially the standard graph used was glucose, as the exact composition of the polysaccharide was unknown. However these carbohydrate contents were corrected as a more accurate graph became available and an average value of 70% was obtained. The residues from the purification were also analysed for carbohydrate. (see Flow diagram p.45 and Table 11 below).

Table 11

Yield and Carbohydrate Contents of Purification for S11 A

Product	From 1 g sample Yield (g)	Carbohydrate content % as glucose
S11 A	1 g	38
Product (A)	.3 g	11.2
A sample of freeze-dried supernatant	-	49
Product BA	.2 g	18
Product CA	.46 g	64
Product BE	.225 g	60
Product CE	.42 g	24
Product B1	.05 g	25
Product C1	.4 g	65

Paper chromatography of a hydrolysate of the partially purified polysaccharide (C) (Expt. V, p.47) indicated that the uronic acid was hindering the mobility of the neutral sugars. However the use of colour spray reagents such as aniline oxalate showed that at least one pentose was present (pink colour) and probably 4 hexoses, one of which was confirmed

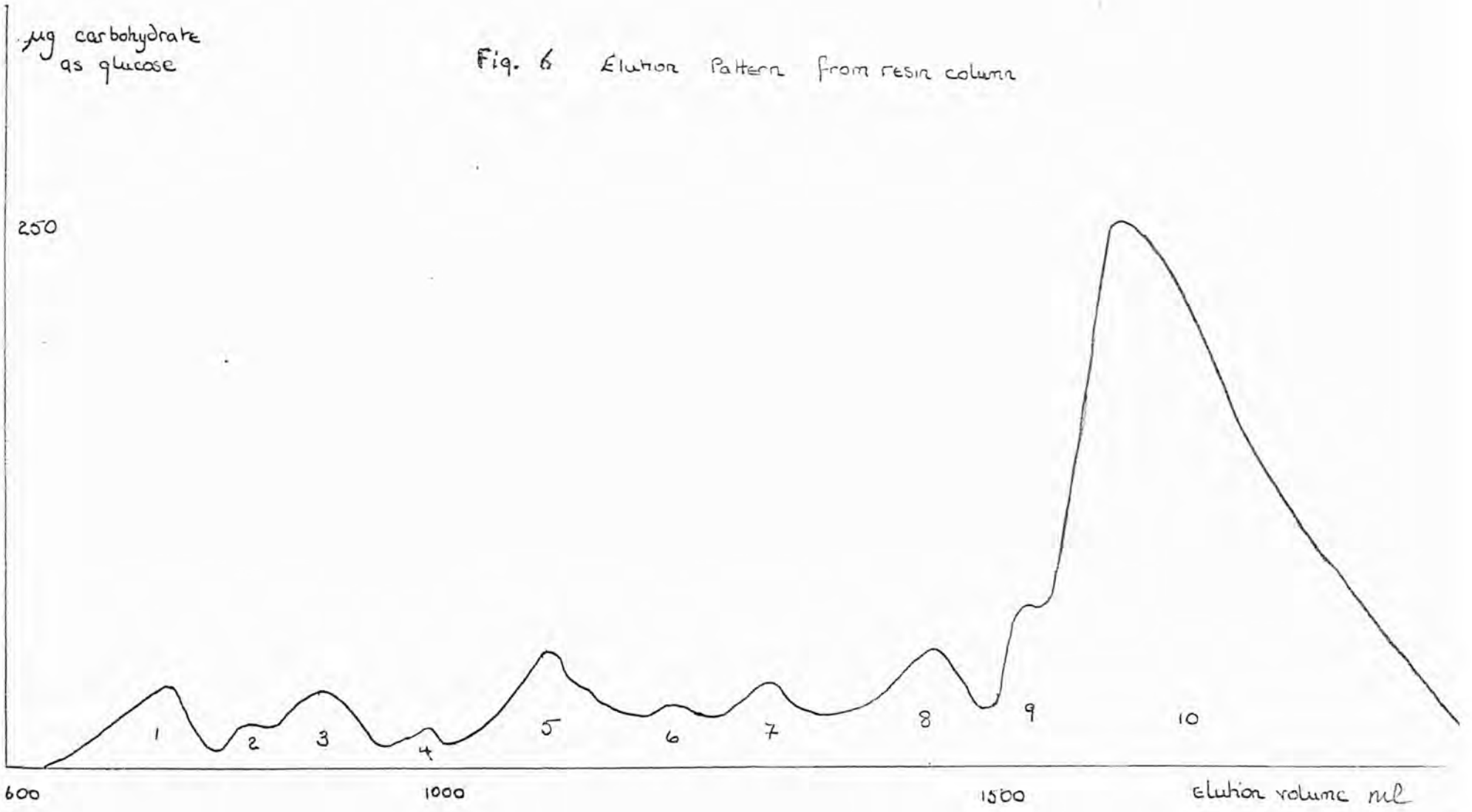
as glucose by the enzymic glucose oxidase spray. In order to get accurate R_{glc} values, the uronic acid was removed.

The first attempt at paper chromatographic separation of the total hydrolysate (Expt. VI 1a, p.47) into individual sugars and acids was not entirely successful. The solvent used did not completely separate the monosaccharides from each other or from the uronic acid. However with a basic solvent (Expt. VI 1b p.47) separation of the acid and neutral fractions was achieved, although further separation of the two fractions into their individual components was then necessary.

The acid and neutral fractions were also separated by the application of a hydrolysate to a resin column (Expt. VI 2 p.47). A mixture of the neutral components was separated by elution with water and the individual components of the acid fraction by gradient elution with increasing acid concentration. (see Fig. 6 overleaf). However although quite large quantities of the neutral fraction (yield 0.175 g) free from uronic acid were obtained by this method, the acid components which should have been separated into individual acids, were found to be mixtures (total yield 0.25 g) the low recovery yield about 40% is possibly due to degradation during hydrolysis. The lack of resolution is probably due to different oligouronic acids having the same ratio of uronic acid to neutral sugar. These would be removed from the resin by the same concentration of acid. In contrast such oligo uronic acids separate on paper chromatography according to molecular size.

Separation of a partial hydrolysate was also effected by precipitation of the barium salts of uronic acids with ethanol (Expt. VI 3, p.48). This produced 2.2 g of neutral sugars and about 2.5 g of oligo uronic acids as ammonium salts from 10 g of polysaccharide.

Fig. 6 Elution Pattern from resin column



Identification of the uronic acid

The identification of the uronic acid was of great interest, because of the conflict between Jones et al[182] in Canada and Haug and Larsen[187] in Norway, as to the identity of the uronic acid present in the extracellular polysaccharide from Azotobacter indicum. According to Jones, paper chromatography of the uronic acid fraction showed three spots corresponding to glucuronic acid (major) and galacturonic acid (minor) and also a large spot corresponding to glucurono lactone. No solvent system or R_{glc} values were given for this separation. Reduction of the uronic acid, after esterification, gave three neutral sugars corresponding in mobility to glucose (major) galactose and gulose. The latter two were present in only minor amounts.

Measurement of uronic acid content by these workers by both the carbazole[183] (read off a glucuronic acid graph) and the titration method gave values of 17% and 19% respectively. The former is higher if read off a guluronic acid standard graph (see Appendix III) in fact this doubles the uronic acid content, compared with the value from a glucuronic acid graph. Although good agreement was obtained by these workers between the two methods, the neutralisation equivalent may be very high (giving too low an acid content) due to incomplete conversion into the free acid form of the polysaccharide, particularly where the polysaccharide forms a viscous solution. For these reasons it is possible that both methods have given too low a result.

Haug and Larsen[187] titrated the polysaccharide with cetyl pyridinium chloride and obtained an equivalent weight of 560, - or 30% uronic acid. Although they studied the uronic acid content with and without borate[188] and they state that the results confirmed that guluronic acid was present, no figures were given for the uronic acid content by this method. In addition these workers separated the uronic acid from the neutral sugars and examined it by paper chromatography, paper electrophoresis and

lactonisation and concluded that only guluronic acid was present.

It can be seen from the above summaries that the two sets of workers used different methods of characterisation and achieved conflicting results. Thus it was decided that the acid present in B. mobilis should be examined by the methods used by both groups of workers. The acid form of the polysaccharide was titrated with sodium hydroxide under nitrogen and was found to contain about 50% uronic acid (Expt. VIII (2) p. 50). When assayed by the modified carbazole reaction and read from a guluronic acid graph, the uronic acid content was found to be 58%, but from a glucuronic acid graph 29%. This was thought to indicate a polysaccharide containing predominantly guluronic acid, as the equivalent is likely to give a somewhat lower figure because of the afore mentioned difficulties of complete conversion into the free acid form.

The monouronic acid in the hydrolysate, after separation (Expt. VII p.48) was examined chromatographically in many solvents, but it was not possible to clearly differentiate all 4 common uronic acids (glucuronic, galacturonic, mannuronic and guluronic) and the possibility of more than guluronic acid being present could not be eliminated. The only chromatographic method found to give a clear separation of the uronic acids was paper electrophoresis in borate buffer containing calcium ions,[189] but the technique of paper electrophoresis often leads to streaking and although guluronic acid was clearly indicated (see table 12 below). The presence of a second uronic acid again could not be eliminated.

Table 12. Mobilities of Uronic acids in borate with calcium[189]

Acid	Reported[189] M_{glc}	Found M_{glc}
mannuronic acid	0.88	0.86
glucuronic	1.26	1.22
galacturonic	1.03	0.99
guluronic	0.75	0.71
Unknown acid (from polysaccharide)		0.70 (m) slight streak to 1.1

Lactonisation of the unknown acid was attempted using the methods of Blake and Richards[240] but no lactone was detected. This was also the case for authentic guluronic acid.

After conversion of the uronic acid into the corresponding sugar, analysis by paper chromatography indicated a major component with the mobility of gulose and a fast moving component (Table 13 below).

Table 13 Mobilities of gulose and mannose

Solvents C.M. III(i)	a	R_{glc} b	f
gulose	1.2; 3.7	1.15; 2.9	1.84; 2.5
mannose	1.3	1.12	1.33
galactose	0.95	0.84	1.13
gulose-1,6-anhydride	3.7	2.9	2.5

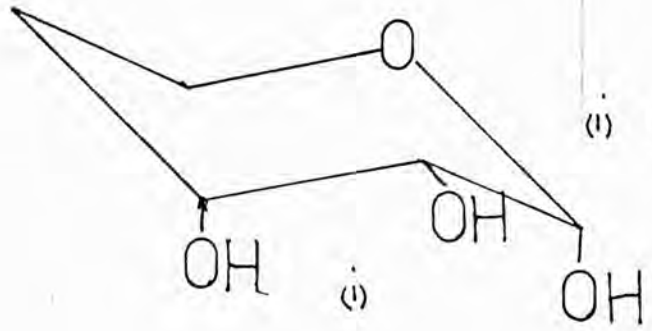
It is well known that gulose readily forms a 1,6-anhydro sugar on evaporation and comparison with authentic gulose, which had been similarly treated, confirmed that the fast spot had the same mobility as the 1,6-anhydride. The reduced uronic acid was also examined by paper

Table 14 Mobilities in Molybdate Buffer

	$M_{sorbitol}$
glucose	0
mannose	0.99
gulose	1.18; 0
unknown sugar	1.18; 0

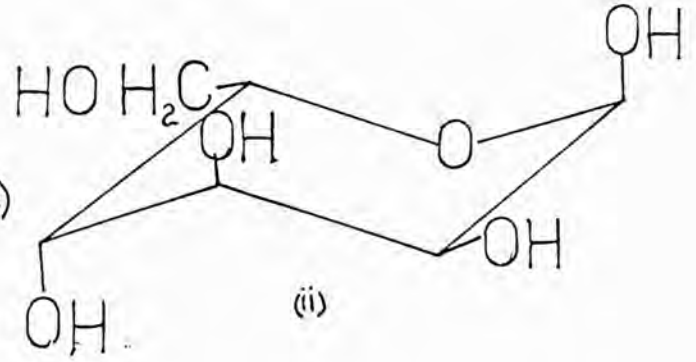
electrophoresis in molybdate buffer (Table 14 above). A sugar will complex with molybdate only if it possesses the configuration 1,2,3 cis,cis of its hydroxyl groups (see fig. 7 overleaf). Of the sugars derived from the common uronic acids, gulose and mannose possess this configuration

Fig 7 Mobility in Molybdate Buffer



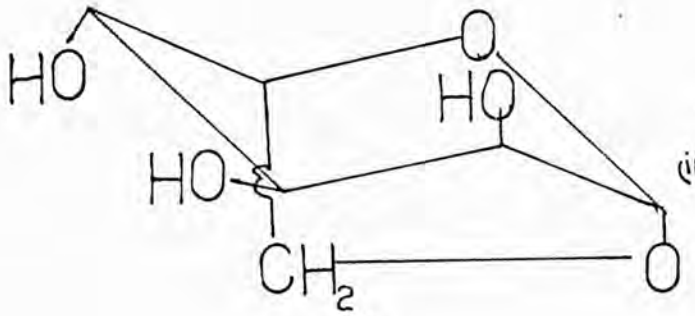
(i) 1,2,3-cis-cis-

(i)



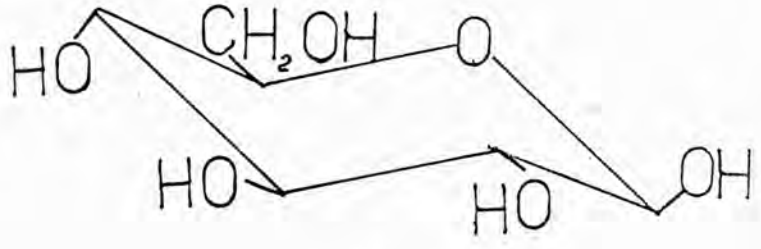
(ii) β-L-gulopyranose (1C form)

(ii)



(iii) 1,6 Anhydro-d-L-gulopyranose

(iii)



(iv) β-D-glucopyranose

(iv)

but glucose, galactose and 1,6-anhydrogulose do not. Ability to complex leads to mobility under the influence of a potential. It was found that the unknown sugar was mobile under these conditions, but a smaller non-mobile fraction was also present. While this might be due to glucose and/or galactose, it it more likely to be the gulose-1,6-anhydride. Differentiation between gulose and mannose is best achieved chromatographically in a solvent saturated with borate ions (see Table 13f). This confirmed the absence of mannose.

G.l.c. of the T.M.S. derivatives of the reduced acid and standard gulose gave identical chromatograms which were easily distinguished from that of glucose. The absence of the latter and hence of glucuronic acid was also confirmed with glucose oxidase spray on a paper chromatogram of the reduced acid.

In addition the rotation of a dilute solution of the uronic acid was found to be $[\alpha]_D = 42^\circ$ c.f. L-guluronic acid from alginate $[\alpha]_D = 37^\circ$. The small difference is probably due to the low concentration of the solution and the fact that the quantity present was measured by the phenol-sulphuric acid method.

Thus it appears that the uronic acid present in B. mobilis is L-guluronic acid and is the same as that found by the Norwegian workers in A. indicum.

Characterisation of the neutral components

The neutral components from the resin column (Expt. X p.51) were tentatively identified as galactose, glucose, mannose, arabinose, and rhamnose. These components were then separated on a large scale and purified. Each sample, with the exception of galactose, was shown to be chromatographically pure and its rotation was measured (see table 15 overleaf) Crystalline derivatives of these sugars were prepared (Expt. XI p.52) confirming their identity.

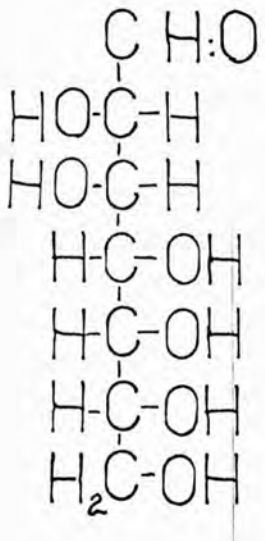
Table 15 Identification of the monosaccharides

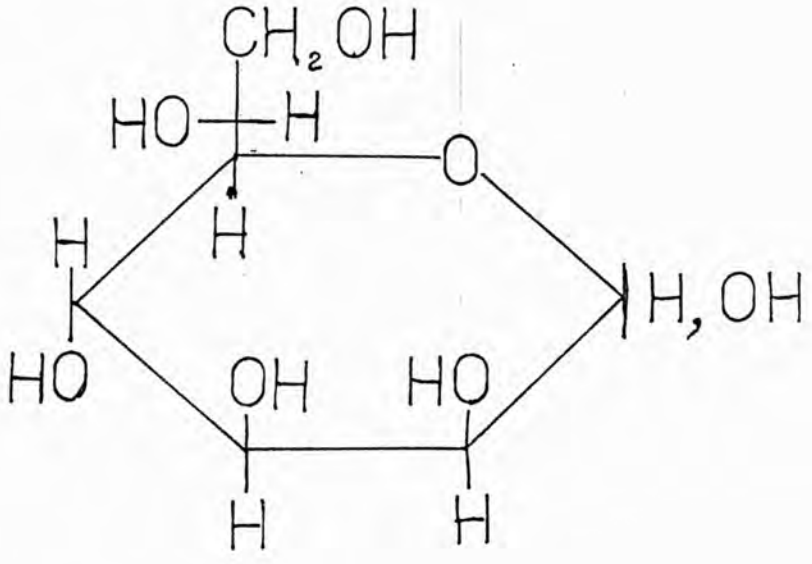
Sugar	Amount separated mg	$[\alpha]_D$	Literature value $[\alpha]_D$	GMIII(1) R_{glc} a	b	c
α-D-glucose	200	98 → 53°	111 → 53°	1.00	1.00	1.00
D-mannose	20	14°	14.4°	1.21	1.12	1.17
L-arabinose	15	-105°	-105°	1.59	1.12	1.17
L-rhamnose	8	8°	8.5°	2.86	1.49	1.83
'galactose'	100	42°	81°			

It was noted that the 'galactose' rotation was not in agreement with the literature value. When the sample was examined further chromatographically it was found to be contaminated with glucose (expt. XII p.54). It was extremely difficult to remove all the glucose. However after repeated chromatographic separation a small amount (~ 2 mg) of pure material was obtained. This had the same chromatographic mobility, both on paper chromatographs (see Experimental Table 3, p.55) and on GLC with that of a sample of D-glycero-D-mannoheptose, kindly supplied by Dr M.B. Perry and in addition the rotation $[\alpha]_D$ 17°, was found to be the same for both materials. The dif ference of 4° from that of the literature value $[\alpha]_D$ 21° is probably due to the fact that the above rotations were taken on syrups. These results provide strong evidence that the unknown sugar is D-glycero-D-mannoheptose (Fig. 8 overleaf).

Jones, Perry and Sowa[182] also found this heptose in the acid polysaccharide from A. indicum and they developed a spray specific for heptoses. This spray, which is dependent upon the rapid release of formaldehyde from a heptose, by the action of sodium metaperiodate, was used and proved positive. Other compounds, such as ketoses and alcohols,

Fig. 8 D-Glycero-D-mannoheptose





and, if the periodate is left on the paper too long, all the other sugars present in the hydrolysate, also give formaldehyde. However it was found that if the periodate was destroyed quickly enough, (in less than the one minute indicated by the original authors) the spray reagents could be used effectively.

Insufficient heptose was obtained initially to prepare a crystalline derivative, and as it seemed that chromatographic removal of the glucose would produce only small amounts of pure heptose, the glucose in a large scale hydrolysate was removed with glucose oxidase. This enzyme converts the glucose into gluconic acid, which was subsequently removed with resin. The enzyme was then removed from solution by coagulation. However, in spite of repeated attempts to prepare a crystalline derivative (Expt. XII p.56) and preparative purification of the derivative mixture, the syrups failed to crystallise. It is thought that this is due to traces of residual protein, left after the removal of the glucose, which would not be detected by paper chromatography. Another difficulty was that of obtaining any quantity of the free heptose. This was mainly due to the strength of the glycosidic bonds linking the heptose, since it was found that complete hydrolysis of the polysaccharide required very strong acid conditions and this resulted in considerable degradation (Expt. IX, p.51). This suggests that the heptose is linked to uronic acid residues and this indeed was found to be the case on partial hydrolysis (see Table 6 p. 59). Although the preparation of a crystalline derivative would have been conclusive proof, it seems reasonable to conclude that the heptose is D-glycero-D-mannoheptose (Fig. 8, p.95) because of the identical chromatographic mobilities and rotations, and since the same heptose has been found in a polysaccharide from a bacterium of the same genus.

Composition of the Extracellular Polysaccharide

The proportions of sugars in the neutral fraction were calculated using two methods (Expt. XIII, p.57) and were in reasonable agreement (see Table 4, p.57). Taking into account the uronic acid content, the approximate composition of the total extracellular polysaccharide (measured on a hydrolysate) is guluronic acid 58%, glucose 22%, heptose 6%, arabinose 5%, mannose 4.5% and rhamnose 4.25%. These figures do not take into account any uronic acid or neutral sugars present as oligouronic acids or of those lost by degradation during hydrolysis and for reasons it is thought that the percentage of heptose is probably higher.

When the neutral sugars were separated, some glycerol (100 mg by weight) was obtained. The origin of this glycerol is unknown as it was not found in subsequent hydrolysates of the polysaccharide.

Neutral Oligosaccharides from a Partial Hydrolysate

A small quantity of neutral oligosaccharides was obtained (Expt. XIV, p.58) (see table 5, p.58). These were found to be mixtures, even after reseparation by paper chromatography. The quantities were not sufficient to enable further purification, but the high incidence (OA1, OA2, OA4 and OB1 Table 5) of oligosaccharides containing solely mannose and arabinose is strong presumptive evidence that these two sugars are mutually linked in the macromolecule, similarly with glucose and mannose (OA3, OB5, OB7).

The oligouronic acids (Expt. XV, p.59) which were obtained in higher yield were examined more fully. However it will be more appropriate to discuss these at a later stage, except to note that they did not contain any mannose, arabinose or rhamnose.

Periodate Oxidation Studies

The total polysaccharide reduced 0.825 moles of sodium metaperiodate per anhydroaldose unit (Expt. XVI, p.60) (see fig. 9 overleaf) and gave a polyalcohol (yield 55%) containing only guluronic acid and much reduced proportions of glucose and heptose. It would seem that the uronic acid is not vulnerable to attack by periodate and that some of the glucose and heptose is also resistant. Since the uronic uronic acid constitutes about 58% of the molecule, the uptake of periodate by the remainder of the constituents is about 2 moles per sugar unit. The oxidised sugar fragments were a complex mixture and were not identified.

The polysaccharide was autohydrolysed (Expt. XVII, p.60) giving a degraded polymer and eleven dialysed fractions (table 7, p.61). Examination of the fractions revealed that some of the glucose is removed very easily, but glucose continued to be liberated during the 132 hr of the experiment. Mannose and arabinose were also released by the hydrolysis, but very little heptose and uronic acid were liberated. On examination of the degraded polymer, it was found to comprise only the latter two sugars. This is in agreement with earlier evidence that the heptose and uronic acid are mutually linked. When the degraded polymer was applied to columns of Sephadex gel, it was partly excluded from both G.100 and G.200 and therefore at least some of it retained a high average degree of polymerisation.

Periodate oxidation of the degraded polymer gave a polyalcohol (yield 48%) containing only uncleaved guluronic acid. The uptake of periodate was 0.9 moles per sugar unit (see fig. 10, p. 100). This is a high figure considering that most of the susceptible units were removed by autohydrolysis and corresponds to more than 2 moles/anhydroheptose unit. It is however thought to be due to the presence of additional end group due to cleavage of the chains. Another important point is that autohydrolysis rendered all the heptose units susceptible to attack by periodate. This evidence indicates

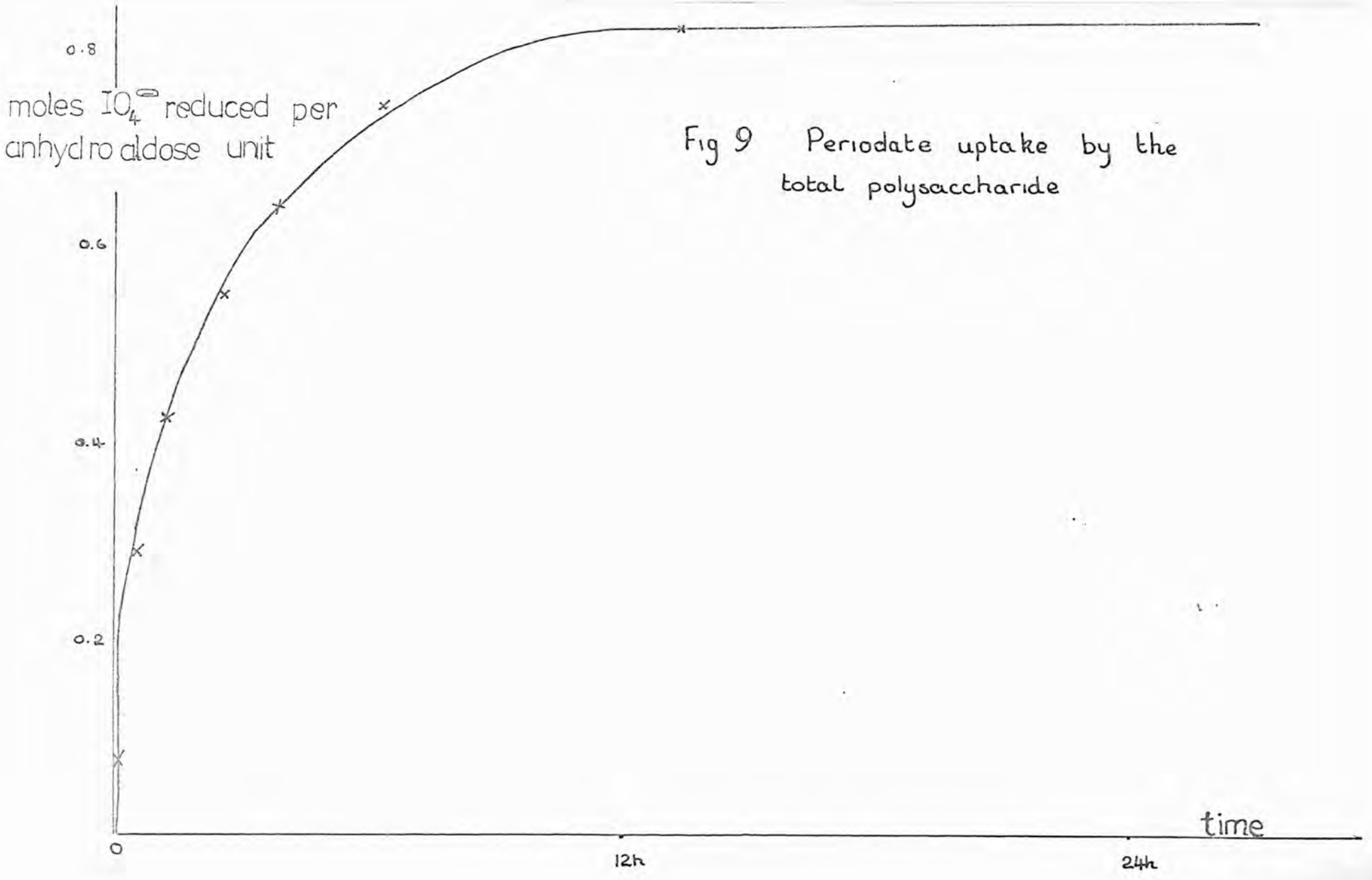


Fig 9 Periodate uptake by the total polysaccharide

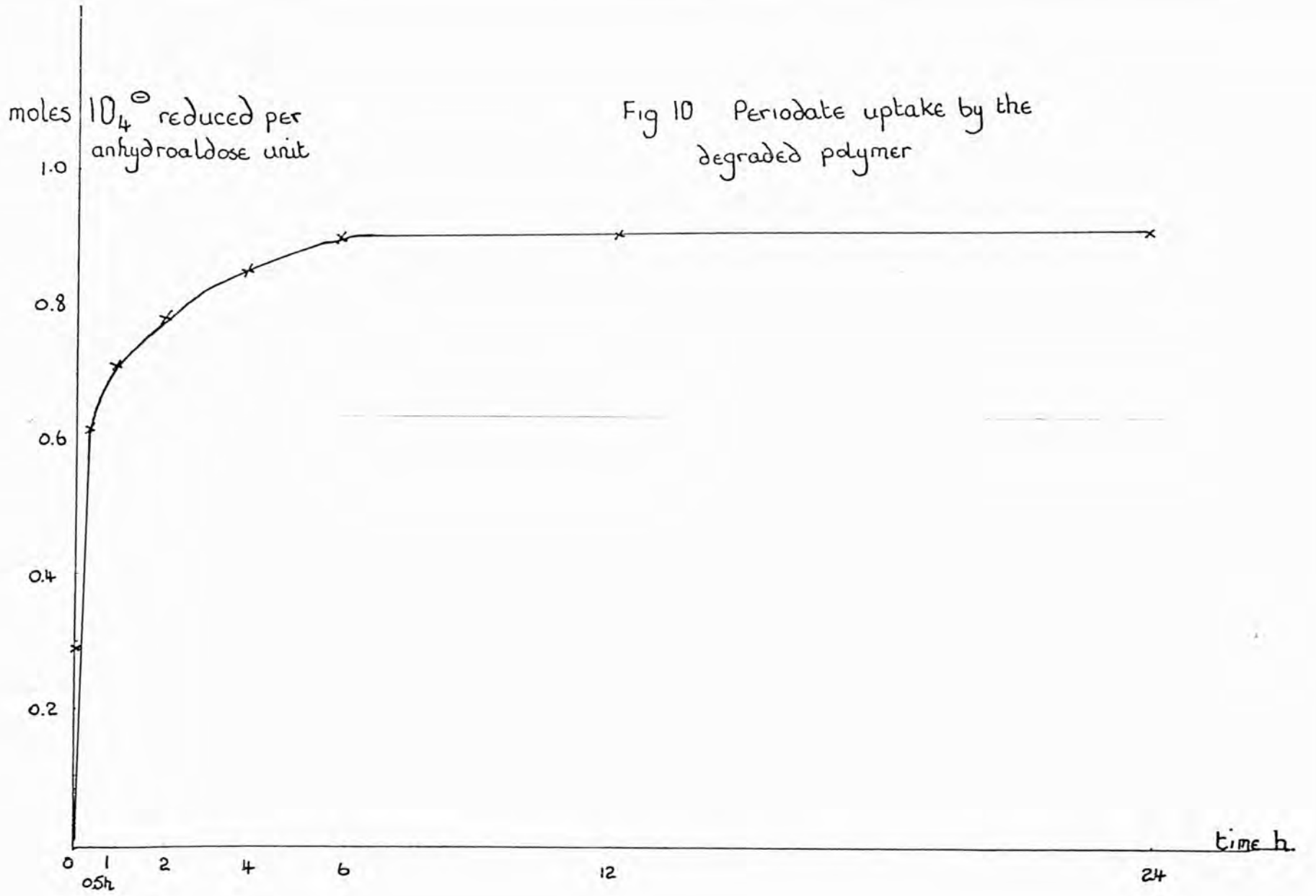


Fig 10 Periodate uptake by the degraded polymer

a polysaccharide with long chains of heptose and uronic acid, possibly as a backbone with the other sugar components present in side chains, or as a separate polysaccharide, linked to the other sugar units only by the gel structure. For this reason the fractionation experiments were continued.

Fractionation into Neutral and Acidic Polysaccharides

It was found that addition of 'Cetavlon' to a very dilute solution of the polysaccharide only complexed with about 85% of the carbohydrate present and left some carbohydrate (Expt. XIX, 3b, p.65) in solution. The complex was only partly broken down by dissolution in sodium chloride, and the remainder was dissolved in ethanol. At first it was thought that this represented fractionation into two acidic components, (A_1) and (A_2) but on examination of the two fractions, they were found to have the same composition and it was concluded that the fractionation was due solely to molecular weight differences. After consultation with Dr. J. Scott, the experiment was repeated using a purified form of 'Cetavlon' 'Hexadecyltrimethylammonium bromide' (Eastman Kodak). Only a single acid fraction was obtained by this method. A further addition of 'Cetavlon' to the supernatant failed to yield a precipitate.

The supernatant from the initial complex was dialysed to remove the excess 'Cetavlon', and it was found that some of the carbohydrate passed through the dialysis sac. The neutral polysaccharide was thus separated into two fractions N_1, which was dialysable, and N_2, which was of higher molecular weight. In subsequent experiments the 'Cetavlon' was removed from the supernatant by Biodeminrolit resin.

Three fractions were thus obtained: (A) a white fluffy freeze-dried material; (N_1) a sticky yellow substance which would not freeze-dry properly, and (N_2), a crisp yellow freeze-dried material. In the following discussion,

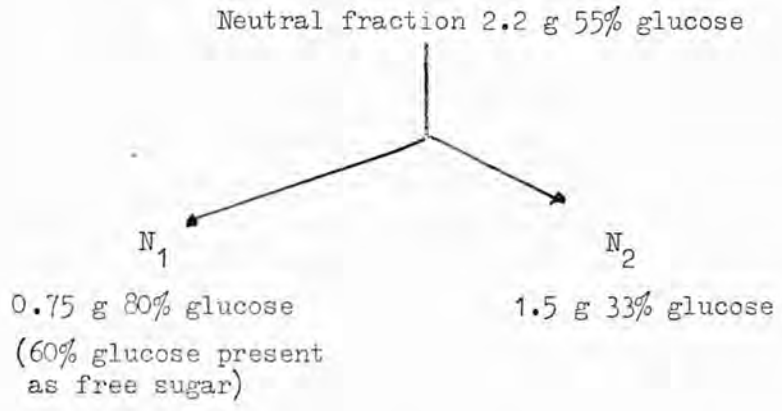
the fractions (N_1) and (N_2) will be considered as the neutral polysaccharide.

Examination of the Neutral Polysaccharide

The material recovered from the 'Cetavlon' supernatant ($N_1 + N_2$) has $[\alpha]_D = 16^\circ$ and comprises about 14% of the total extracellular material. It was found to be devoid of protein and uronic acid and to contain 55% glucose (Fig. 11 below).

The dialysable fraction (N_1) was examined chromatographically and found to consist mainly of glucose, mannose and oligosaccharides. After hydrolysis arabinose was also found to be present. No heptose was detected. The amount of free glucose present in this fraction (N_1) was measured before hydrolysis and found to be 60% of the total and after hydrolysis to be about 80%.

Fig. 11 Neutral fractions and proportions of glucose



It was thought at first that these small fragments resulted from the presence of an enzyme which caused cleavage of these sugars, while the polysaccharide was in solution, during purification and fractionation. A sample of the crude polysaccharide in the dried milled form was therefore taken and suspended in boiling water for 20 minutes to denature any enzymes, but the same small oligo- and mono- saccharides were removed on dialysis.

A parallel dialysis experiment carried out on polysaccharide which had not been boiled, contained the same amount of small fragments (judged visually from paper chromatograms) thus it would appear that these low molecular weight fragments are present in the medium during the growth of the bacteria, and are produced, either because the polysaccharide is incompletely metabolised, or are due to the presence of an enzyme in the medium which partially hydrolyses the polysaccharide leaving the mono- and oligo-saccharides trapped inside the gel.

The neutral polysaccharide fraction (N_2) consisted of glucose, mannose and arabinose in approximately equal proportions.

The very large number of methyl glycosides derived from the methylated neutral polysaccharide (N_2) gave an extremely complex pattern of peaks on G.L.C. It was therefore decided to rely on characterisation of the methylated alditol acetates. Analysis of the latter (Table 16 overleaf) revealed a large amount of end group, mainly arabinofuranose, but with some mannopyranose indicating a highly branched polymer, probably with fairly short chains. The remainder of the arabinose was 1,2- and 1,3-linked furanose and 1,4 and/or 1,5-linked. In addition it occurred at branch points linked 1,2,4 and/or 1,2,5. The latter two derivatives give no information as to the ring size of the units, (see fig. 12, p.105). However since the majority of the arabinose was furanose, it seems safe to assume that this is also furanose.

The mannose in addition to end group, was 1,3-linked and 1,3,6-linked. The glucose was 1,6- or 1,4-linked in the chains and a very small proportion also occurred at branch points, 1,2,4- and 1,2,6-linked. It is possible that these latter derivatives result from undermethylation, but the periodate oxidation studies do not support this conclusion. Further discussion of the techniques and general results of methylation analysis can be found in the discussion on the acid polysaccharide.

Table 16

Methylation Analysis of the Neutral polysaccharide

b) F.11 linked to Mass spectrometer (OV.225)

Peak No.	Retention time*		Corresponding to
1	0.42(m)	Arabinose	2,3,5-tri-O-methyl
2	0.66		3,5-di-O-methyl
3	0.8		2,5-di-O-methyl
5	1.09(tr.)		2,3-di-O-methyl
6	1.83		3-mono-O-methyl
4	0.95(tr.)	Mannose	2,3,4,6-tetra-O-methyl
7	1.91		2,4,6-tri-O-methyl
12	4.6		2,4-di-O-methyl
8	2.3(m)	Glucose	2,3,4-tri-O-methyl
9	2.4		2,3,6-tri-O-methyl
10	3.69		3,6-di-O-methyl
11	4.5		3,4-di-O-methyl

a) Pye 104 OV.225

Peak No.	Retention time*		Corresponding to
1	0.45(m)	Arabinose	2,3,5-tri-O-methyl
2	0.70		3,5-di-O-methyl
3	0.85		2,5-di-O-methyl
5	1.07(tr.)		2,3-di-O-methyl
6	1.85		3-mono-O-methyl
4	0.99(tr.)	Mannose	2,3,4,6-tetra-O-methyl
6	1.85		2,4,6-tri-O-methyl
11	4.5		2,4-di-O-methyl
7	2.2(m)	Glucose	2,3,4-tri-O-methyl
8	2.3		2,3,6-tri-O-methyl
9	3.7		3,6-di-O-methyl
10	4.2		3,4-di-O-methyl

* relative to 1,5-di-O-acetyl, 2,3,4,6-tetra-O-methyl glucitol

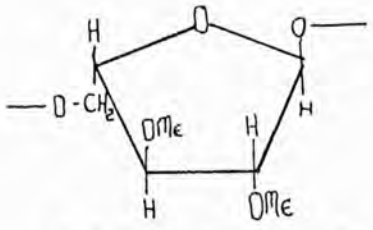
m = major tr. = trace

Figure 12

Peak 5

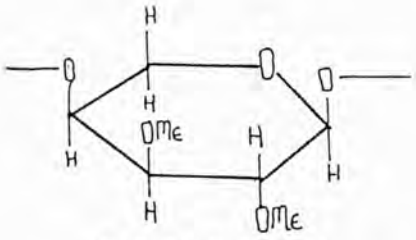
2,3 di-O-methyl L-Arabinitol could arise from

a



2,3 di-O-methyl α-Arabinofuranose linked 1-5

b

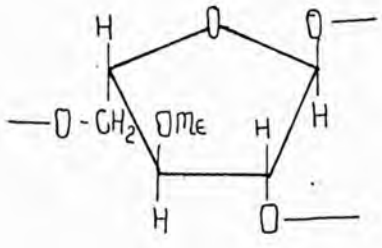


2,3 di-O-methyl α-Arabino pyranose linked 1-4

Peak 6

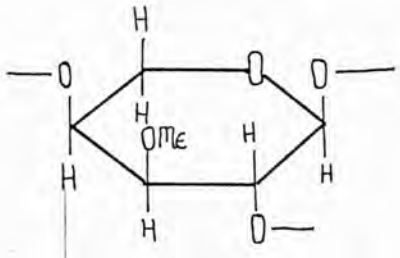
3 Mono methyl Arabinitol could arise from

a



3 Mono methyl α-Arabinofuranose linked 1-2-5

b



3 Mono methyl α-Arabinopyranose linked 1-2-4

Periodate Oxidation Studies

Periodate oxidation of the polymer (N_2) [Expt. XXXIII, p.69] with a periodate uptake of 1.3 moles of periodate per anhydro unit, yielded a polyalcohol which contained mannose (major) and arabinose and some glucose (2%). This is in agreement with the methylation results, since the only mannose units which are vulnerable to attack are those present as non-reducing end units. In contrast the only glucose units which would not be attacked are those at branch points - this seems to rule out the possibility of under methylation.

The yield of polyalcohol (29.4%) was very low, possibly due to the lability of the arabinofuranose units. Although fraction (N_2) was used for the periodate oxidation experiments, even this appeared to consist of fairly low molecular weight material. When spotted, unhydrolysed, onto paper chromatograms oligosaccharides of D.P. about 8-15 were found. It would seem probable, therefore, that after periodate oxidation and reduction, some of these were lost during dialysis.

The neutral polysaccharide (N_2) was subjected to gel permeation chromatography by Pharmacea Ltd., using columns of Sephadex G50/G75, and this indicated that the molecular weight was $\bar{M}_W = 5,400$

$\bar{M}_N = 1,900$

An attempt was made to determine the molecular weight of the total polysaccharide by ultracentrifugation, but the large difference in molecular weight between the neutral and acid polysaccharides (see p. 133 discussion on acid polysaccharide) resulted in the neutral polysaccharide remaining immmobile, when the acid polysaccharide had sedimented, and so no photographic plates of the neutral polysaccharide were obtained.

All the results on this neutral polysaccharide indicate a highly branched molecule with fairly short chains, little more than oligosaccharides in nature. It is concluded that it is held in the matrix of the gel possibly by hydrogen bonding to the acid polysaccharide.

The Acid Polysaccharide

The acid polysaccharide, $[\alpha]_D = 25.6^\circ$, comprises about 85% of the total extracellular carbohydrate and consists of guluronic acid (65%) heptose (20%) and glucose (15%). Infra red analysis (Fig. 13) on a thin film gave a spectrum which was very similar to that of the total polysaccharide (Fig. 14) and quite distinct from that of the neutral polysaccharide which was in general broad and featureless (Fig. 15). The absorption bands at 1420 cm^{-1} (carboxylate) and at 1735 cm^{-1} (C^{O} stretching) found in the spectra of the acid and total, but not in the neutral is indicative of uronic acid. The latter is relatively smaller (cf. the band at 1600 cm^{-1}) in the total than in the spectrum of the acid polysaccharide. Also shown (Fig. 16) is the infra red of 'Cetavlon' as a KBr disc. The complex spectrum shows no similarity with either the acid or neutral spectra, indicating the freedom of the two polysaccharides from contamination with this reagent. The absorption band at 1550-1650 cm^{-1} in the total polysaccharide, reduced to a smaller band at 1620 cm^{-1} in the acid polysaccharide, is thought to be due to secondary amide and could be explained by the presence of protein.

Occurrence of Pyruvic acid

Although Jones et al[182] and Haug[187] in their studies on A. indicum had not reported the presence of pyruvic acid in this polysaccharide, its frequent occurrence in extracellular bacterial polysaccharides [not only in the Klebsiella species (Appendix I table D) and corynebacterium species[241] (Appendix I table C) but also in Xanthamonus species,[242] Pseudomonas species[243] and in Microsporum guinckeanum[244] a yeast, and in addition in the seaweed polysaccharides agaropectin[245] and -carrageenan[246]] led us to look for it in this polysaccharide. Following the work of Choy and Dutton[247] the polysaccharide was analysed by N.M.R. in solution in D_2O (see fig. 17).

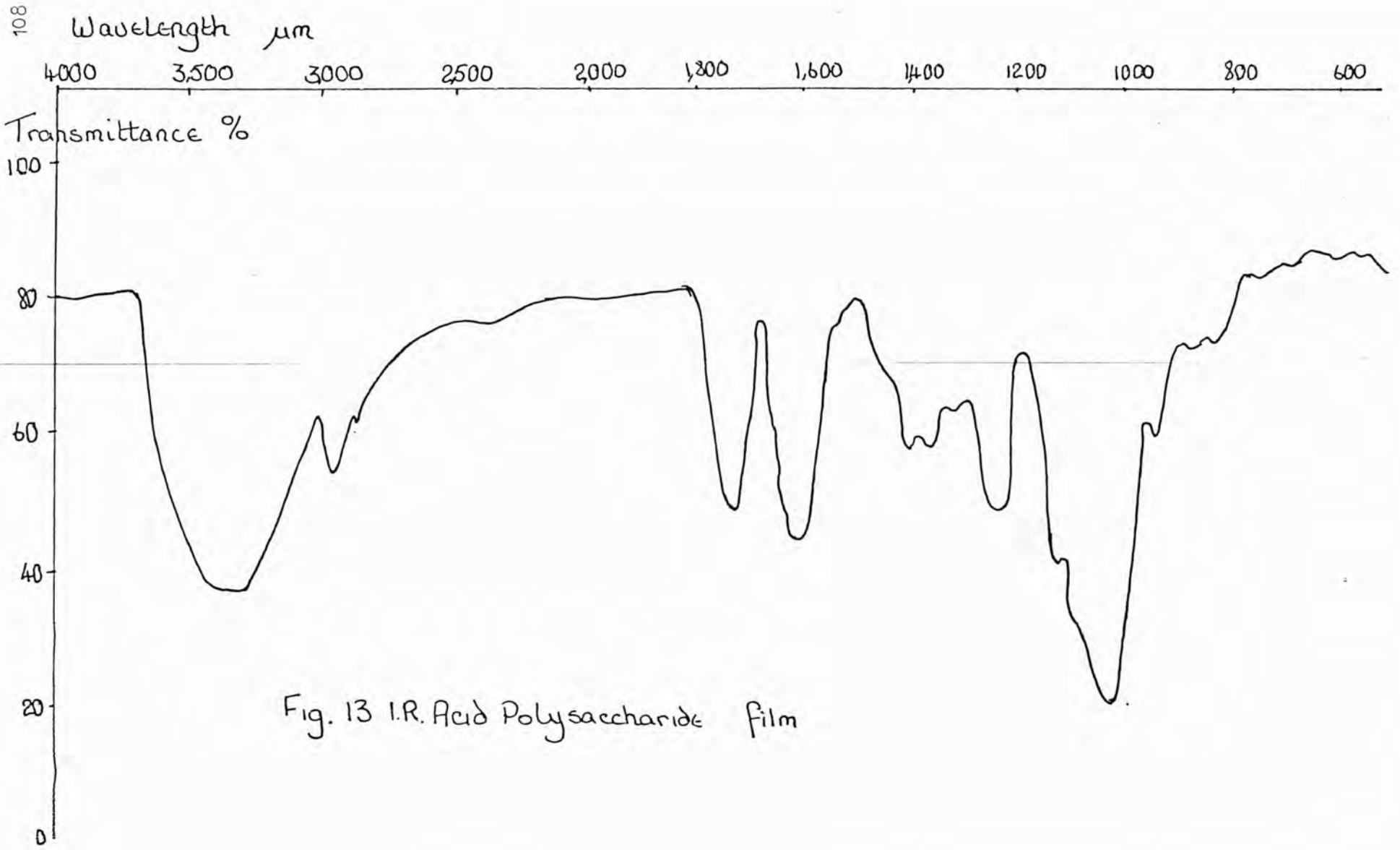


Fig. 13 I.R. Acid Polysaccharide film

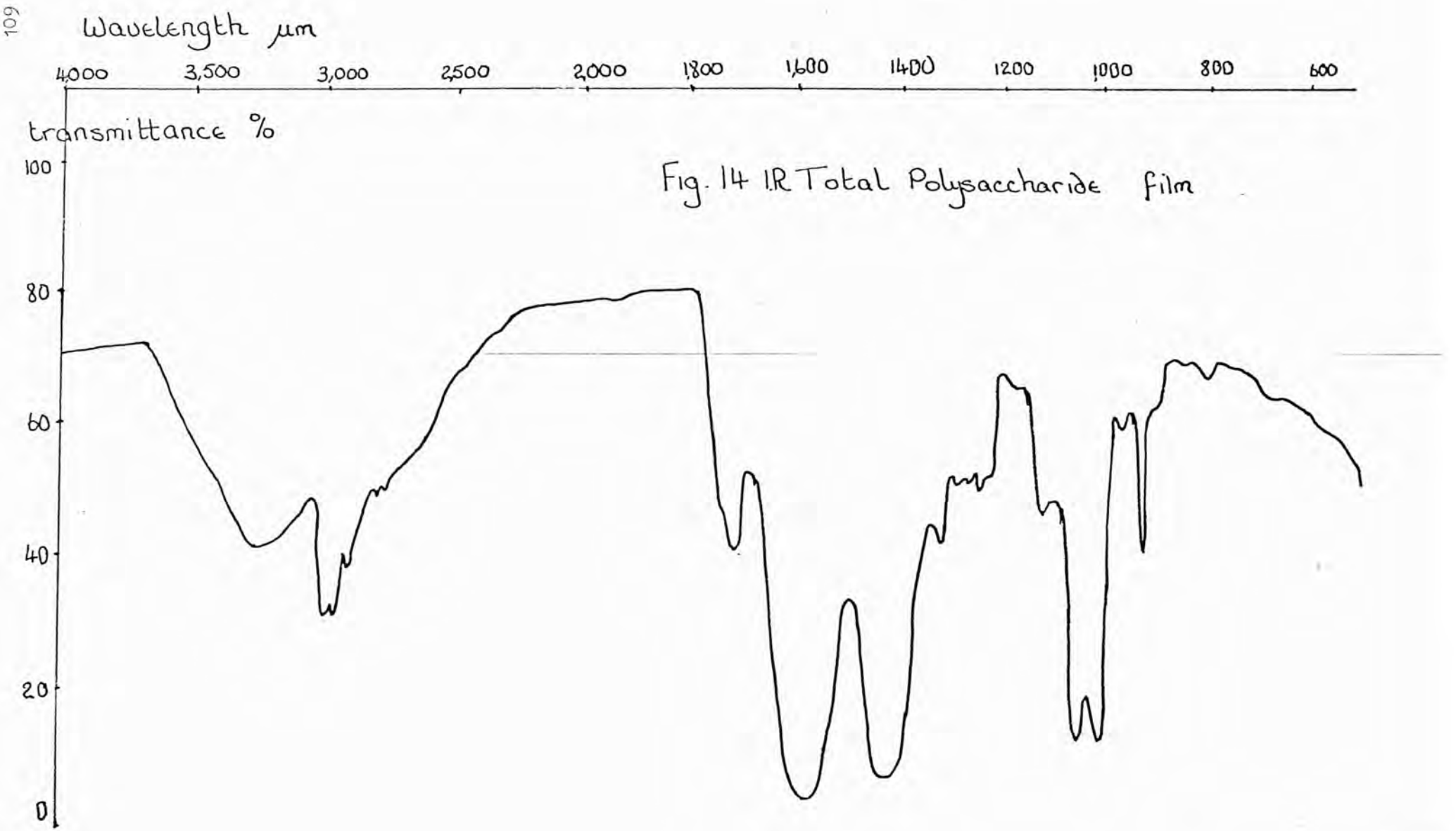


Fig. 14 I.R Total Polysaccharide film

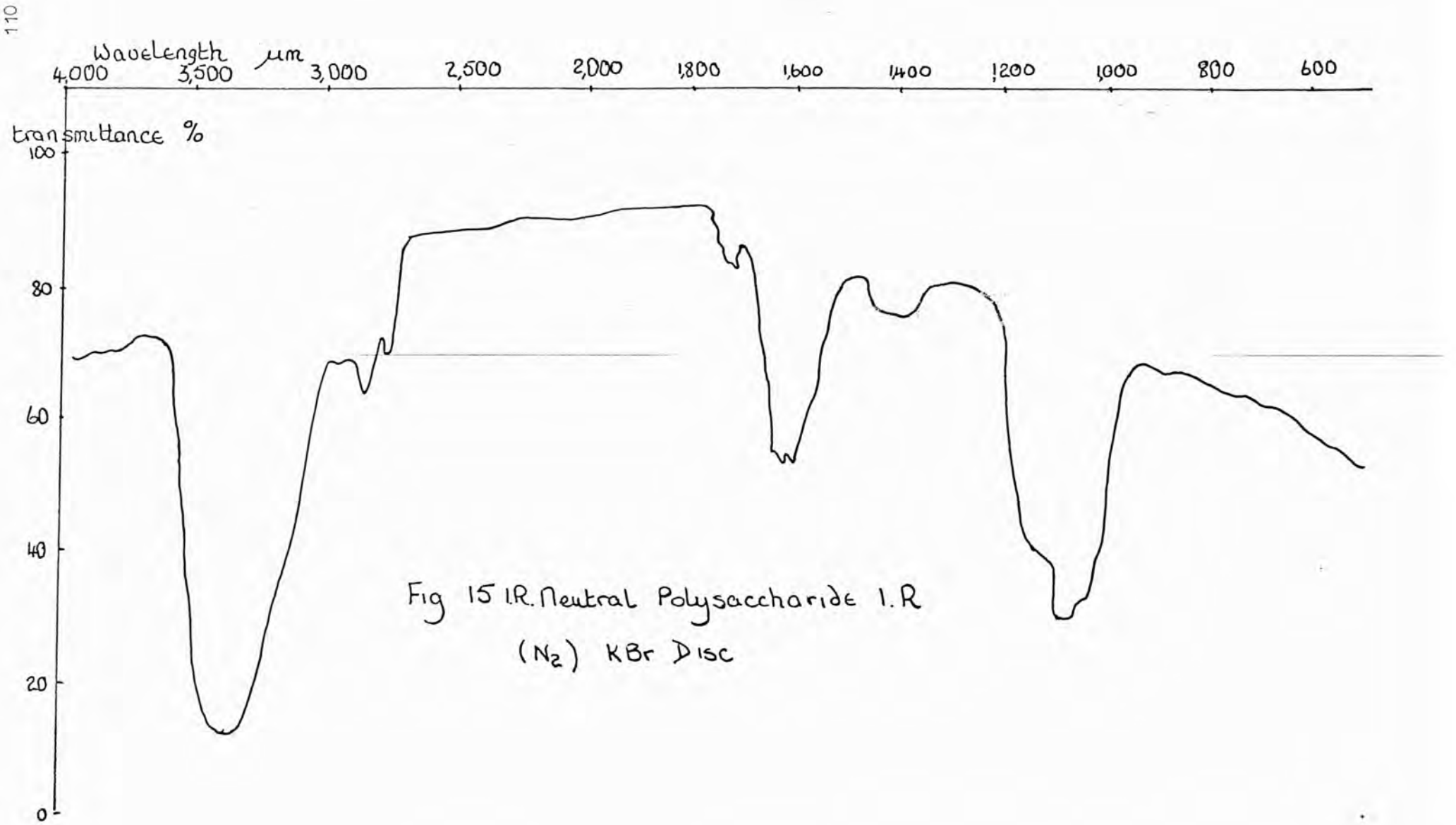


Fig 15 I.R. Neutral Polysaccharide I.R
(N_2) KBr Disc

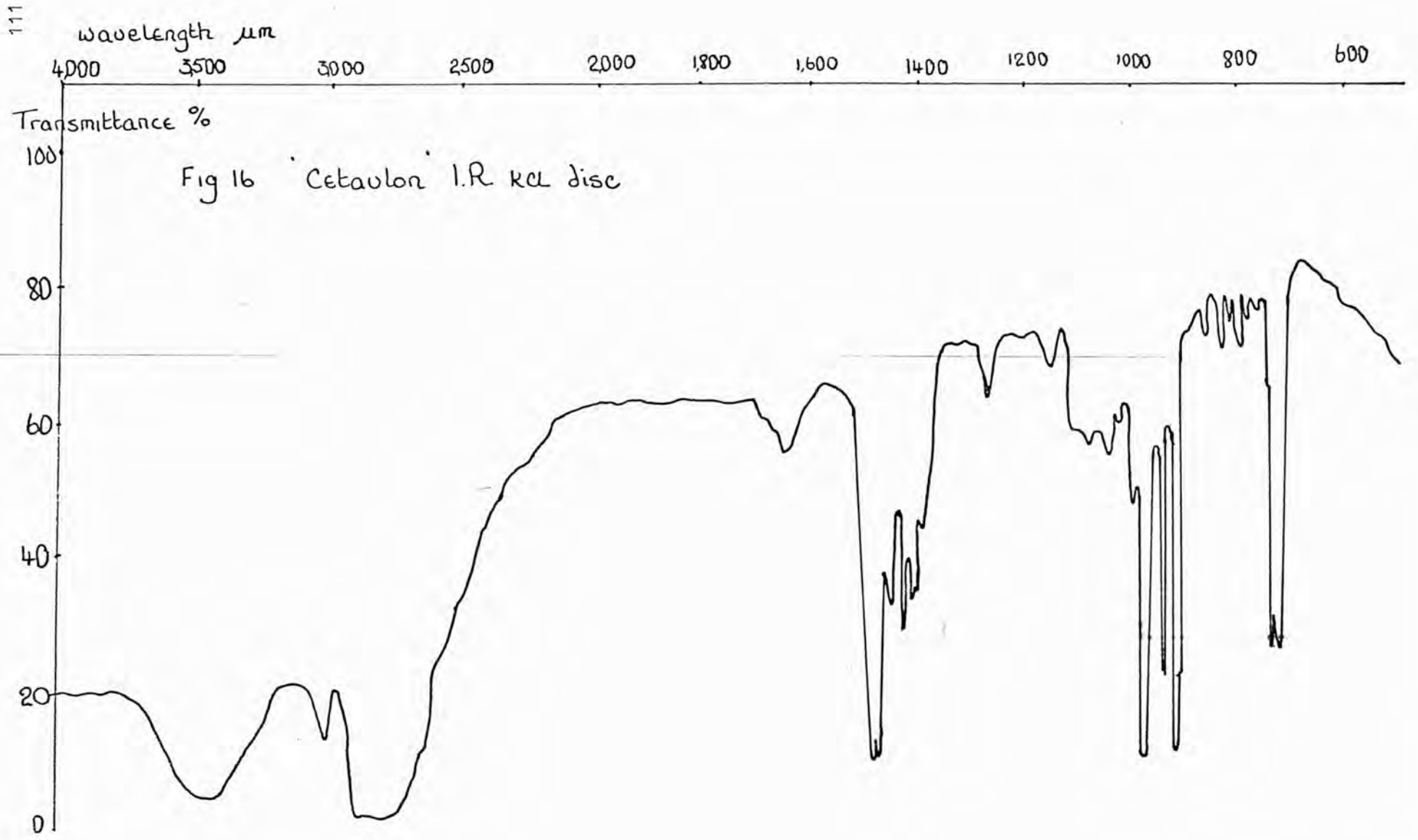
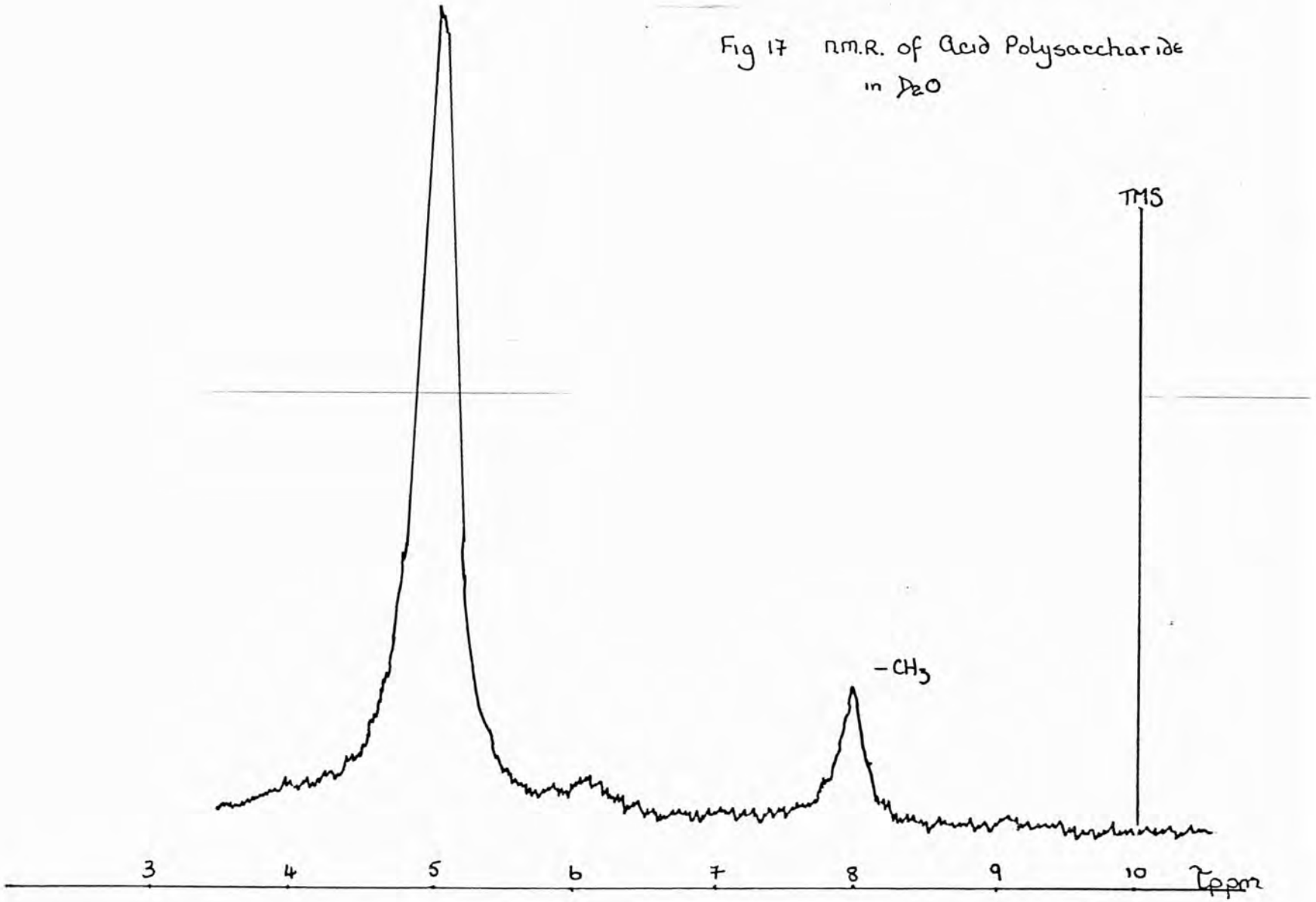


Fig 16 Cetavlon I.R KCl disc

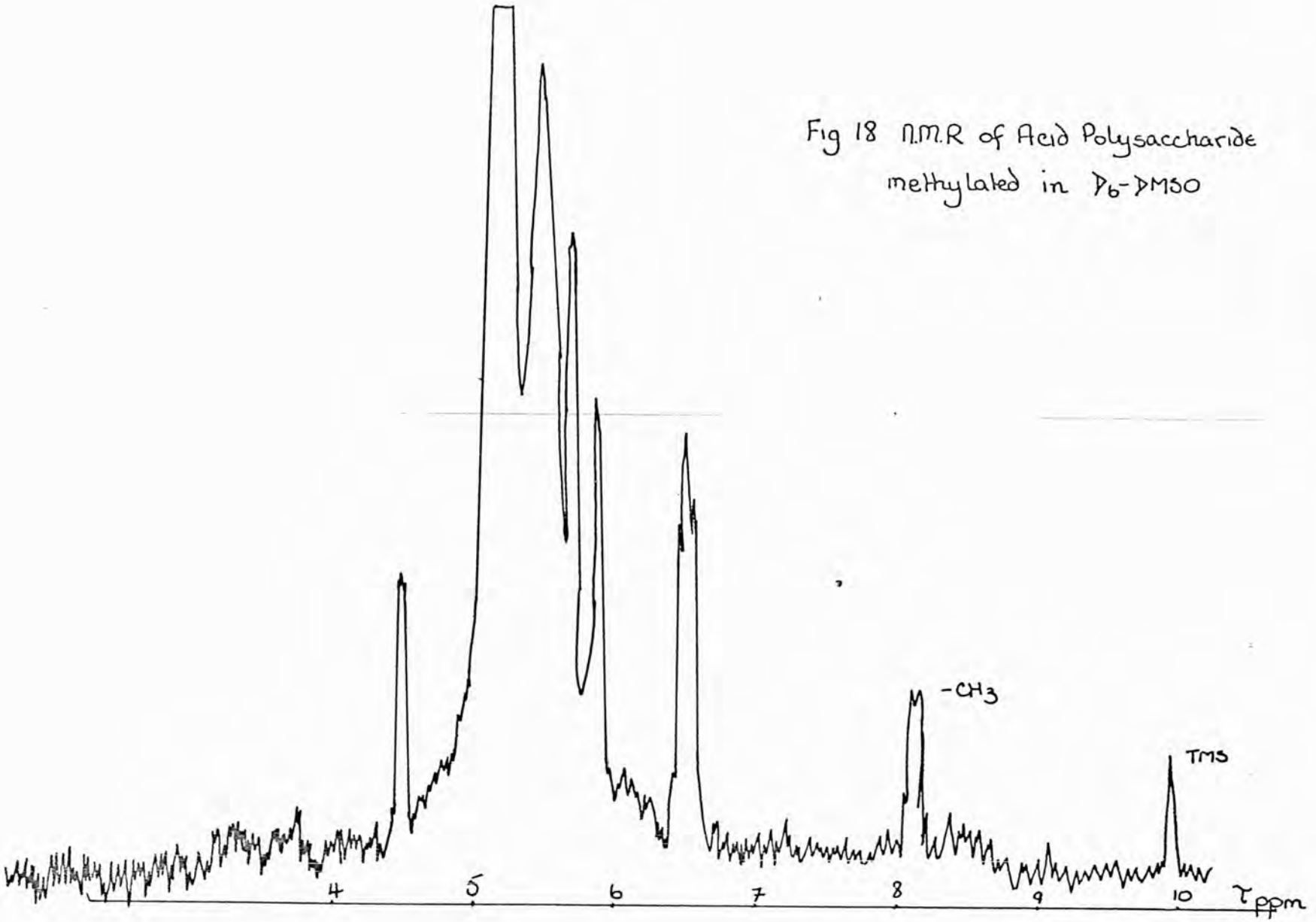
Fig 17 n.m.r. of Acid Polysaccharide in D_2O



The N.M.R. spectrum can be used to show the presence of uncoupled methyl protons. The above authors state that the $-CH_3$ in the N.M.R. spectrum of Klebsiella polysaccharide at 8.5 relative to T.M.S. arises from a pyruvic acid ketal linked to positions 4 and 6 of a D-galactose residue [see fig. 20a]. Other authors (Gorin and Spencer[242]) found a similar peak in the N.M.R. spectrum corresponding to uncoupled methyl protons at positions relative to T.M.S. from 8.35 to 8.45. However Gorin and Ishikawa[248] synthesised the 4,6-carboxyethylidene-D-galactose (pyruvic acid linked to positions 4 and 6 of D-galactose) and found that it existed in two conformations. N.M.R. spectra of the two anomers had peaks corresponding to uncoupled methyl protons at 8.09 and 8.18. These anomers correspond to the $-CH_3$ being equatorial or axial to the sugar ring.

Although Choy and Dutton[247] state that the N.M.R. peak at 8.5 relative to T.M.S. is proof of pyruvic acid ketals, results obtained by us (spectra fig. 17, p. 112) show that while the N.M.R. peak at 8.5 - 7.8 relative to TMS indicates the presence of pyruvic acid linked to the molecule, it does not conclusively prove whether the pyruvic acid is linked as a ketal or as an ester. This is due to the imprecise nature of the N.M.R. measurements, and also to the variation of the position of the peak depending on conditions, such as the pH of the solution, nature of solvent and temperature. The 2% 'solution' of polysaccharide, necessary for these measurements, was very viscous and almost opaque, thus it seems likely that it was not a true solution. However it was considered that the introduction of other solvents or alkali to assist the solubilisation of the polysaccharide would lead to a shift in the position of the peak, and thus were not attempted, except with the methylated polysaccharide which was insoluble in water (fig. 18, overleaf). In addition the fact that the solution was so dilute increased the complication due to the HOD peak (= 5 relative TMS) which was very large in comparison to the $-CH_3$ peak.

Fig 18 N.M.R of Acid Polysaccharide methylated in D_6-DMSO



In a later paper[249] Bobault et al use trifluoroacetic acid to move the HOD peak down field, however because of the lability of the pyruvic acid to acid conditions (see later) this was not attempted. Nevertheless since the N.M.R. of the acid polysaccharide did show the presence of uncoupled methyl groups, it was investigated for the presence of pyruvic acid.

Removal of Pyruvic Acid from the Polysaccharide and its Quantitative Estimation

De-pyruvylation (Expt. XXX, p.73) and ether extraction yielded a syrup which was characterised as pyruvic acid. Measurement of the quantity of pyruvic acid (found to be 2.6% by weight, equivalent to 1 unit in 20 carrying pyruvic acid) was effected by the large scale method of Sloneker and Orentas[234] by coupling the liberated pyruvic acid with 2,4-dinitrophenylhydrazine and measuring the intensity of colour of the resulting solution of hydrazine (Expt. XXXVIII(1) p.77]. This method requires relatively large quantities of material and involves a number of extractions. Consequently it had to be performed several times before repeatable results and straight line graphs were obtained. Thus another more accurate method was sought. The Duckworth and Yaphe modification[206] of the enzymic method of determining the pyruvic acid in blood[235] appeared to be ideal. However after much experimentation and variation of the stated experimental technique (Expt. XXXVIII (2), p.78] it appeared only to be feasible for the ether extract of the polysaccharide containing pyruvic acid and not for solutions which also contained the polysaccharide. The result obtained by this method (less than 1%) was considered to be too low, when compared with the amount obtained quantitatively from 1 g of polysaccharide (20-30 mg) and used for the preparation of the derivative. Later results, which inferred the presence of pyruvic acid on one sugar unit in twenty of the polysaccharide were consistent with the results

from method 1. These later results, together with the linkage of the pyruvic acid to the polysaccharide chain will be discussed with the methylation studies.

The Occurrence of Acetic Acid

Although the NMR results did not indicate the presence of acetate esters, these have often been found attached to polysaccharides which also contained pyruvic acid. Also acetate had been found on the polysaccharide of A. indicum[187] and was found to correspond to one acetate group to every two sugar units.

Accordingly the degree of acetylation was measured (Expt. XXXIV, p.74) using a method which did not involve steam distillation as it was thought that this would also remove the pyruvic acid and lead to a high result. The degree of acetylation was found to be 8.5% which corresponds to 1 unit in 4 carrying the acetate group. A parallel experiment carried out on A. indicum indicated an acetate content of 15% (16% acetate would correspond to 1 unit in 2 carrying acetate groups). The very low acetic acid content found from the titration results (p.51) is probably due to the difficulty of converting the polysaccharide completely to the free acid form.

Only the Norwegian workers[187] reported the presence of acetate in A. indicum, the Canadian workers[182,184] reported no experiments to ascertain the presence of either pyruvate or acetate. An experiment to locate the position of the acetate groups on the molecule was attempted using the method of DeBelder and Norrman.[250] This method involves condensing methyl vinyl ether onto the polysaccharide followed by methylation which replaces the acetate groups with methoxyl, and thus in the G.L.C. analysis of the hydrolysate (converted to alditol acetates) the

positions of the methoxyl groups mark the position of the acetate groups in the original polymer. However due to the insolubility of the polysaccharide in p-toluenesulphonic acid in dimethylsulphoxide the method was not successful. In addition no evidence of or the location of the acetate groups could be obtained from the periodate oxidation results because of the resistance of the most of the units in the chain to periodate oxidation either before or after deacylation.

Analysis of the Acid Polysaccharide and Depyruvylated Acid Polysaccharide

These two polysaccharides should have identical composition and primary structure except for the absence of acetate and pyruvate in the latter. (The acetate would also be removed from the polysaccharide under the conditions of depyruvylation, but would be lost by evaporation). The compositions are given in table 17 below. However it is probable that some degradation of the chains has occurred during the depyruvylation process.

Table 17 Composition of Acid and Depyruvylated Acid Polysaccharides

	Acid Polysaccharide%	Depyruvylated Acid Polysaccharide %
Nitrogen	0	0
Carbohydrate	85	82
consisting of		
Uronic acid	65	65
heptose	20	19
glucose	15	16

Methylation Studies on the Acid Polysaccharide

The linkages occurring in the acid polysaccharide were determined by methylation analysis. The standard procedure used was the Lindberg[220] modification of the Hakomori method.[219] It was found [Expt. XXXIX(3), p.79] that when the methylated polysaccharide was hydrolysed much degradation occurred and the resulting G.L.C. trace had a broad solvent peak containing degraded material, and the base line was also high and not smooth. No uronic acid derivatives were identified (Tables 18, 19 p.119,121] but since the G.L.C. retention times are not known and the fragmentation of acetates of methylated uronic acids has not been fully established, it is possible that complete degradation had not occurred, but that the peaks were not recognisable by mass spectrometry. In view of this, uronic acid units in the partially methylated polysaccharide were reduced to the corresponding sugar. Since the retention times of methylated guloses had not been established it was necessary to determine these. Gulose was therefore partially methylated and the retention times of the resulting methylated guloses were determined (see reprint attached).

The different methylated sugars characterised from the hydrolysates of the variously methylated polysaccharide are given overleaf in Tables 18 and 19. It can be seen that the glucose is mainly 1,3-linked. The presence of 2,3,4,6-tetra-O-methyl glucose shows that glucopyranose is also present as end group. Although no methylated heptoses were available for comparable experiments, two peaks were identified from their mass spectra as 3,4,6,7-tetra-O-methyl and 3,6-di-O-methyl heptose (their respective retention times are given in Tables 18, 19) showing 1,2- and 1,2,4,7-linked or substituted

Table 18. Methylated methyl glycosides from acid polysaccharide

(1) Acid polysaccharide, methylated, reduced, re-methylated and hydrolysed [Expt. XXXIX(1)]

Column (1)	*T Column (2)	Corresponding to:	
4.3; 4.7	3.3	Heptose	2,4,6,7-tetra-O-methyl
1.0; 1.36	1.0; 1.32	Glucose	2,3,4,6-tetra-O-methyl
3.0; 3.7	1.65; 2.3		2,4,6-tri-O-methyl
1.0; 1.36	1.0; 1.32	Guluronic acid	2,3,4,6-tetra-O-methyl
2.1; 3.7	1.65; 2.0		2,4,6-tri-O-methyl
4.7; 6.3	2.3; 3.3		2,6-di-O-methyl

(2) Acid polysaccharide, methylated, reduced and hydrolysed [Expt. XXXIX (2)]

Column (1)	*T Column (2)	Corresponding to:	
4.4; 4.7	-	Heptose	3,4,6,7-tetra-O-methyl
1.0(tr); 1.38	1.0; 1.3	Glucose	2,3,4,6-tetra-O-methyl
3.0; 3.7	1.65; 2.3		2,4,6-tri-O-methyl
3.2; 4.5	1.3; 1.85	Guluronic acid	2,3,4-tri-O-methyl
	2.3; 3.3		2,4-di-O-methyl

*T is the retention time relative to that of methyl 2,3,4,6-tetra-O-methyl-β-D-glucopyranoside.

heptose. (see fig. 19 page 120 for the mass spectra breakdown pattern).

In view of the impossibility, as mentioned in the introduction, of distinguishing different hexitols, with methoxyl groups in the same positions, by their mass spectra, it was not possible in the hydrolysates of the methylated reduced polysaccharide, to be certain which arose from glucose and which from reduced uronic acid. The incorporation of deuterium at C-6 of the uronic acid by reduction with potassium borodeuteride [Expt. XXXIX (5)] made this distinction possible.

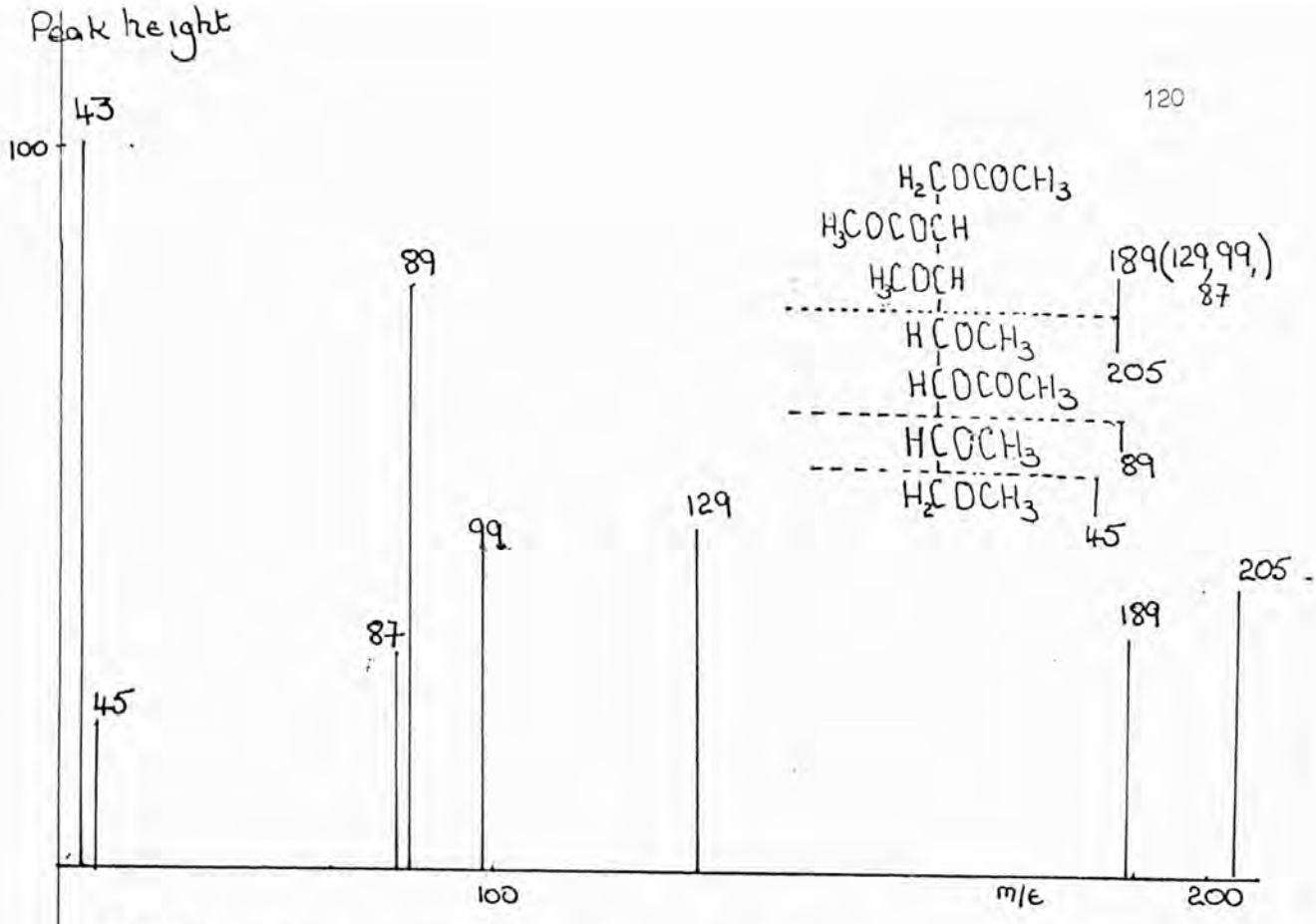


Fig 19a M.S. Fragmentation pattern of 1,2,5-tri-O-acetyl-3,4,6-7-tetra-O-methyl-D-glycero-D-mannoheptitol

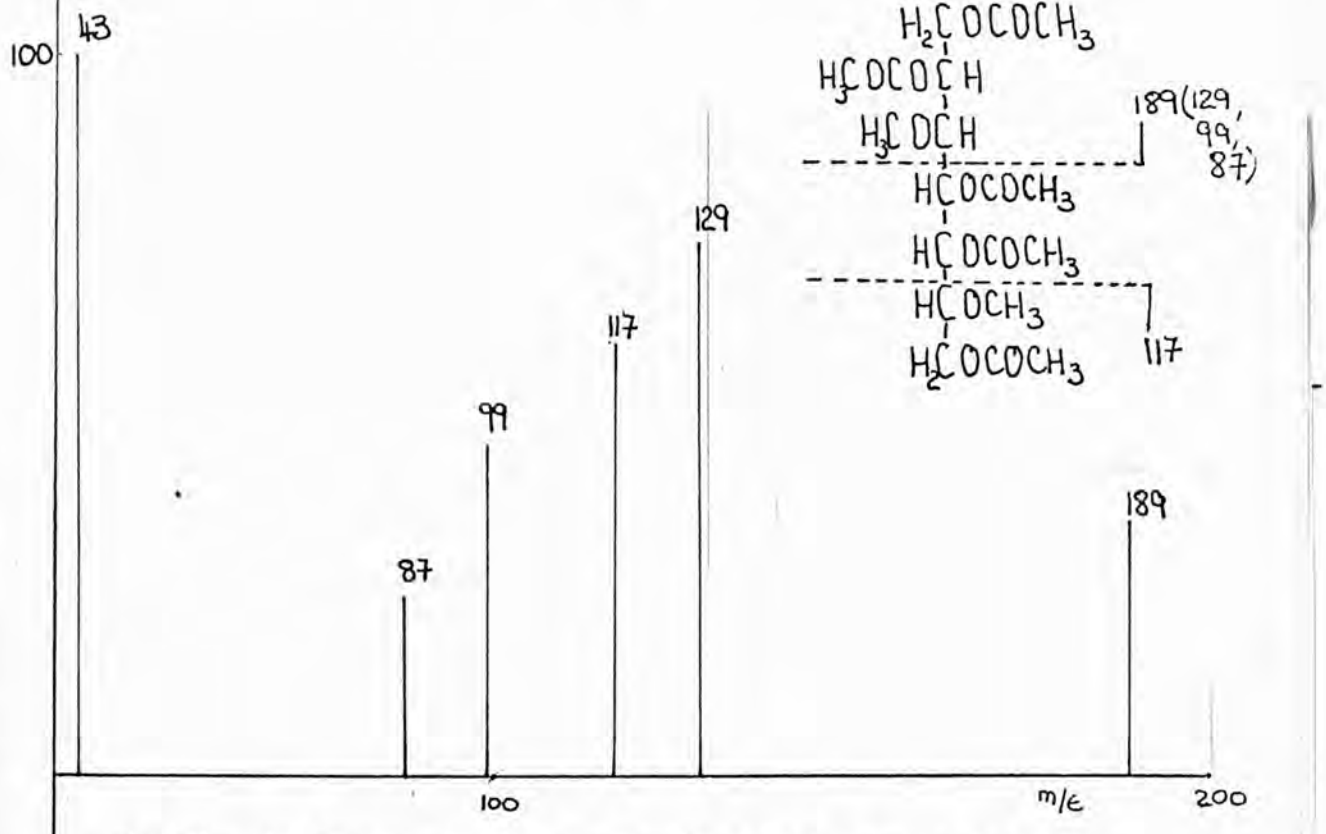


Fig 19b M.S. fragmentation pattern of 1,2,4,5,7-penta-O acetyl-3,6-di-O-methyl-D-glycero-D-mannoheptitol

Table 19 Analysis of the Methylated Alditol acetates from the Acid Polysaccharide

1. Acid Polysaccharide; methylated, reduced and remethylated.

Peak No.	T ≠	corresponding to:	
4	3.1	heptose	3,4,6,7-tetra-O-methyl
3	1.75	glucose	2,4,6-tri-O-methyl
1	1.03	guluronic acid	2,3,4,6-tetra-O-methyl *
2	1.68(M)		2,4,6-tri-O-methyl
5	3.8		2,6-di-O-methyl

* may contain 2,3,4,6-tetra-O-methyl glucose

2. Acid Polysaccharide; methylated and reduced.

4	3.1	heptose	3,4,6,7-tetra-O-methyl
7	7.5		3,6-di-O-methyl
1	1.0	glucose	2,3,4,6-tetra-O-methyl
2	1.75		2,4,6-tri-O-methyl
3	1.89	guluronic acid	2,3,4-tri-O-methyl
5	4.0(M)		2,4-di-O-methyl
6	6.0		2-mono-O-methyl

3. Acid Polysaccharide; methylated.

1	1.0	glucose	2,3,4,6-tetra-O-methyl
2	1.75		2,4,6-tri-O-methyl
3	3.1	heptose	3,4,6,7-tetra-O-methyl
4	7.3(tr)		3,6-di-O-methyl

≠ T is relative to 2,3,4,6-tetra-O-methylglucitol 1,5-diacetate = 1.0

m = major tr = trace

4 and 5 Incorporation of deuterium occurred only in the methylated derivatives of guluronic acid, not in glucose; indicating that no glucuronic acid was present. The results were otherwise identical to those obtained from methods (2) and (1) respectively.

6 All results were identical to those obtained from method(1). No incorporation of dueterium was observed.

However once the deuterium is introduced no alkaline conditions can be used, since exchange would then occur. Thus Experiment XXXIX (6) showed no incorporation of deuterium, and only confirmed the results obtained previously. However methylation with diazomethane, which is slightly acidic, enables methylation at C-6 of the reduced uronic acid, without loss of deuterium.

Garegg et al[251] give fragmentation patterns for partially methylated alditol acetates containing deuterium. This enabled the characterisation of 2,3,4,6-tetra-O-methyl, 2,4,6-tri-O-methyl and 2,6-di-O-methyl guloses as their glycosides and alditol acetates confirming that the guluronic acid is mainly 1,3-linked (see Tables 18, 19) with some end group, and some 1,3,4-linked units.

Since no deuterium was found in the 2,4,6-tri-O-methyl glucose (1,3,5-tri-O-acetyl 2,4,6-tri-O-methyl hexitol) peak after reduction with borodeuteride and remethylation [Expt. XXXIX (5)] it was concluded that no glucuronic acid is present in the polysaccharide.

The Site of the Pyruvic Acid

The presence of 1,2,4,7-linked or substituted heptose suggests that it is these units which carry the pyruvic acid in C-4 and C-7 linkage (see fig. 20 below).

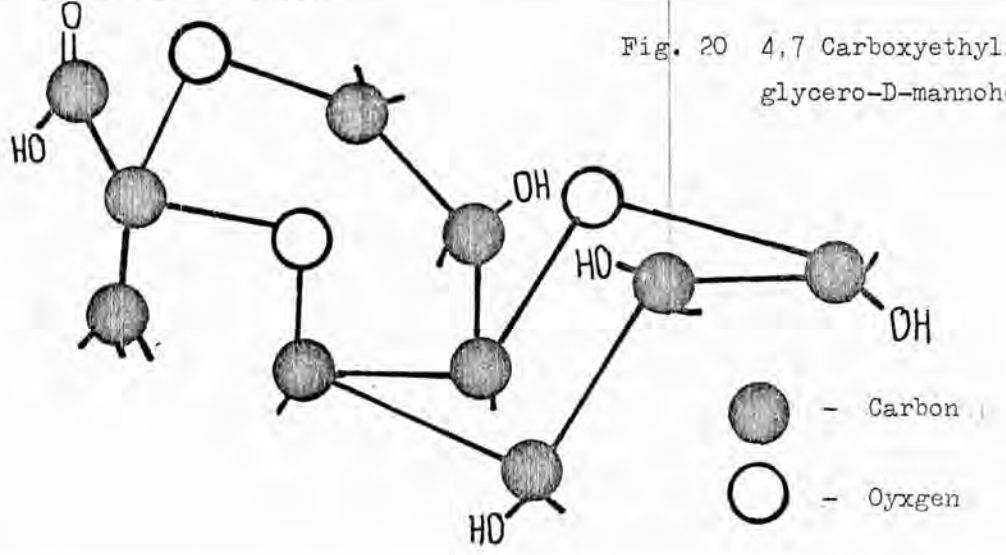


Fig. 20 4,7 Carboxyethylidene-D-glycero-D-mannoheptose

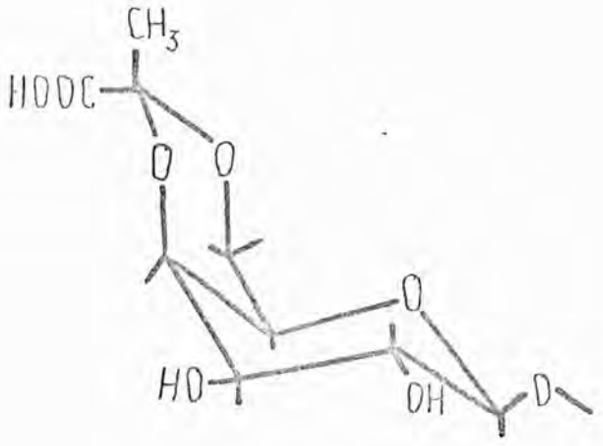
The pyruvic acid in other polysaccharides is linked as a ketal to positions C-4 and C-6 of either glucose, galactose or mannose, thus forming a 6-membered ring [Fig. 21(a)]. However it is thought to be linked to positions 3 and 4 of galactose [fig. 21(b)] in the polysaccharide, colonic acid[251] forming a 5-membered ring. However, a model of the heptose with pyruvic acid linked in ketal linkage to C-4 and C-7, as shown in fig. 21 (c) was found to be free from strain.

The ketal linkage should be acid labile and base stable. Thus the pyruvic acid should not be removed from the polysaccharide by methylation or periodate oxidation. Nevertheless the di-O-methyl heptose derivative disappears during reduction and remethylation, indicating that the pyruvic acid is lost during these experiments. In order to establish when this occurred an N.M.R. analysis was carried out after each stage of the procedure. It was found that it was lost during dialysis, after the reduction of the methylated polysaccharide. It was concluded that the pyruvic acid was cleaved when shaking with IR120(H^+) resin to remove the inorganic cations.

The total amount of end group was found to be about 12%. This was calculated by measuring the total area of guluronic acid from the G.L.C. and the area of the end groups and taking the amount of uronic acid as 65%.

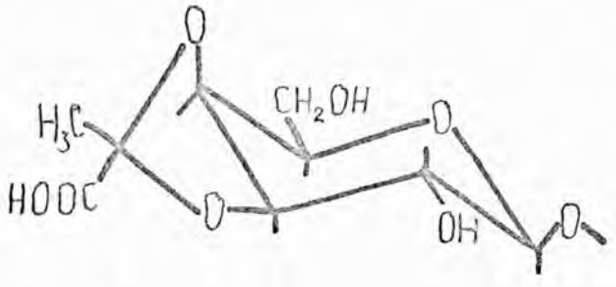
Periodate Oxidation of the Acid Polysaccharide

Periodate oxidation of the acid polysaccharide yielded a polyalcohol (yield 73%) and 0.15 moles of formaldehyde per anhydro unit. (Expt. XXVI, p.70). The low uptake of periodate (0.59 moles per anhydro sugar unit) (see fig. 22, p. 125) can be explained by the high proportion of the 1,3-linked units in the molecule. Formaldehyde is only released by oxidation of the heptose and thus release of formaldehyde mole for mole



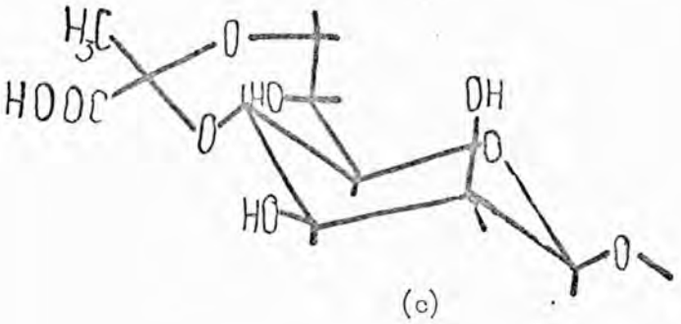
(a)

4,6-O-Carboxyethylidene-D-galactopyranose[251]



(b)

3,4-O-Carboxyethylidene-D-galactopyranose[251,253]

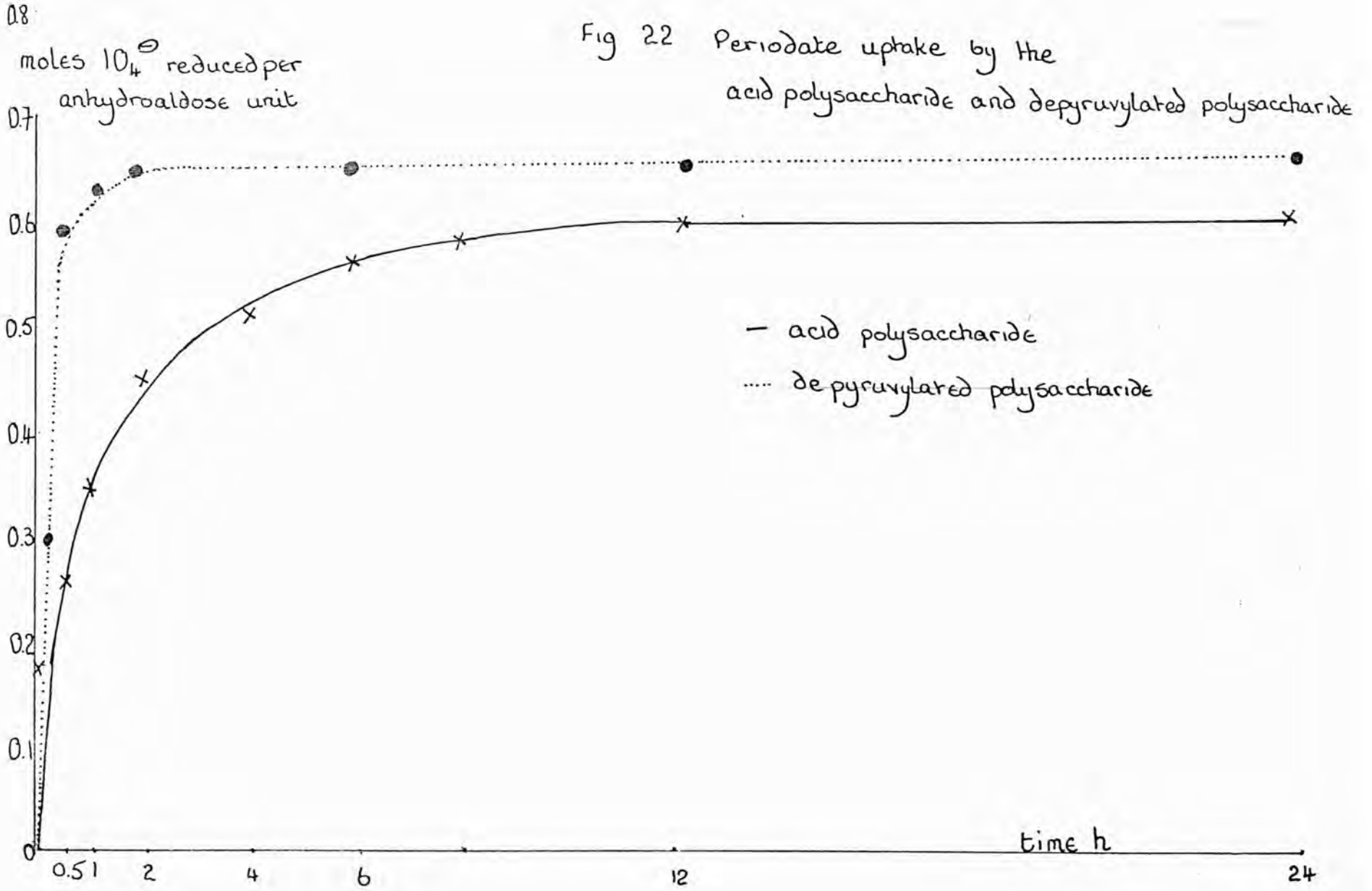


(c)

4,7-O-Carboxyethylidene-D-glycero-D-mannoheptopyranose

Figure 21

Fig 22 Periodate uptake by the acid polysaccharide and depyruvylated polysaccharide



gives an assay of 15% of heptose. However this did not quite agree with the figure (20%) obtained by previous methods (see p.) and indeed, on hydrolysis of the derived polyalcohol, it was found that not all the heptose had been oxidised. This could be due to those units which carried the pyruvic acid and was further confirmation that it was indeed the heptose which carried these units.

Periodate oxidation of the depyruvylated polysaccharide yielded a polyalcohol (yield 63%) and 0.19 moles of formaldehyde. The latter results give an assay of heptose of 19% which is in good agreement with the 20% estimated from G.L.C. when this polyalcohol was hydrolysed no heptose was found. These results also confirm that the pyruvic acid is linked to the heptose.

Each available unit of heptose consumes two moles of periodate, and thus in the acid polysaccharide accounts for the reduction of 0.30 moles of periodate. Thus the remaining 0.29 moles of periodate were presumably consumed by end group units, each of which takes up 2 moles of periodate. This corresponds to a degree of branching of 14%. This is in reasonable agreement with the proportion of end group (12%) found from methylation studies. Measurement of the quantity of uronic acid and glucose uncleaved in the polyalcohol indicated a loss of 11% of uronic acid and 3% of glucose, confirming a 14% degree of branching. Thus the acid polysaccharide would appear to be a branched polymer with L-guluronic acid at branch points and D-glucose and L-guluronic acid at the non-reducing ends of the chains. The remainder of the D-glucose, L-guluronic acid and D-heptose form the chains with the heptose and uronic acid mutually linked.

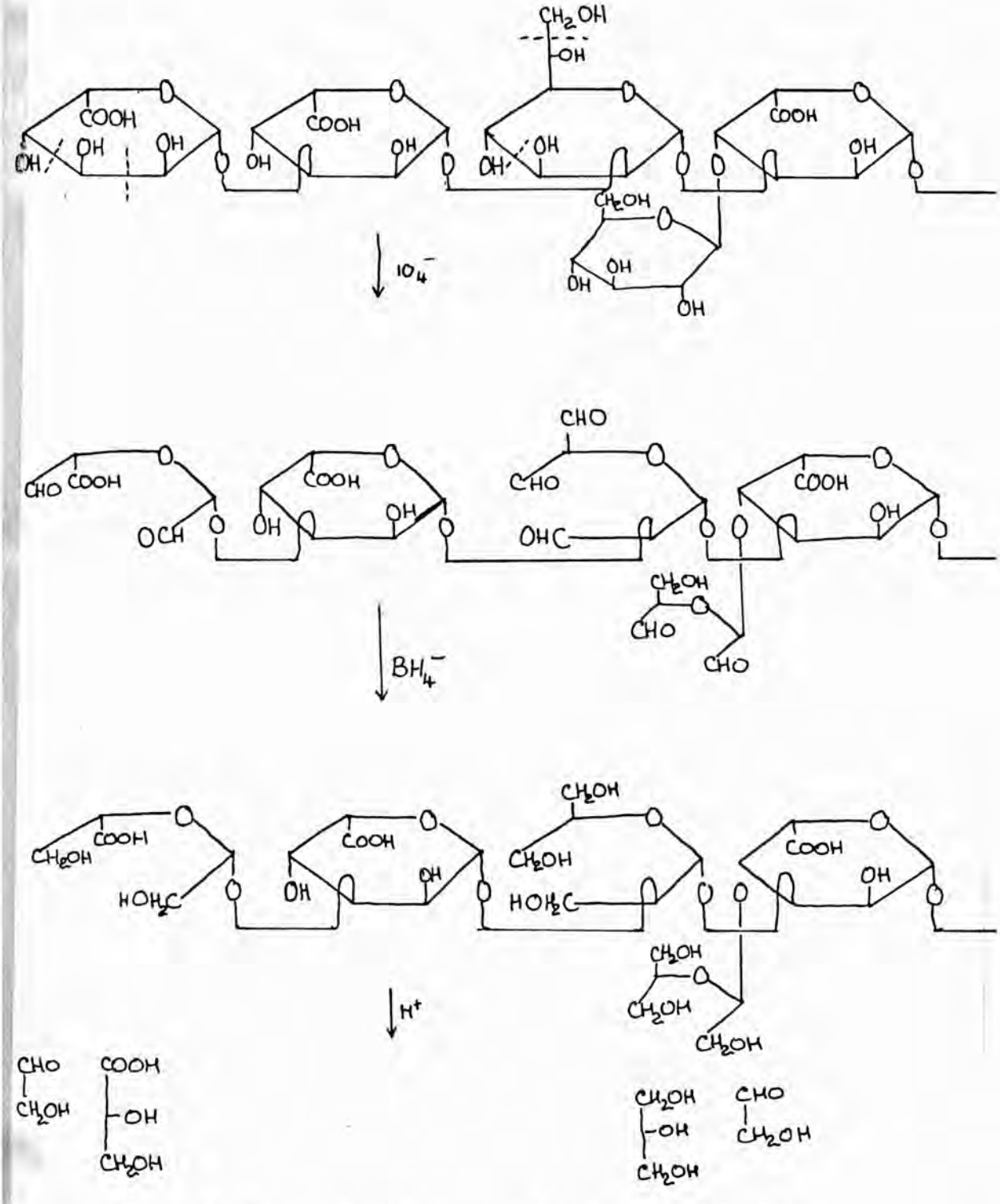
Similar measurements of amount of glucose uncleaved in the polyalcohol derived from the depyruvylated polysaccharide indicated a loss of glucose of 4%, compared to 3% from the original polysaccharide. This might indicate that the polysaccharide had been degraded by the depyruvylation leaving more of the glucose available for attack by periodate.

In contrast the heptose in the depyruvylated polysaccharide accounts for the reduction of 0.38 moles of periodate and thus the remaining 0.27 moles of periodate is presumably used to oxidise the non-reducing end groups corresponding to 13.5% branching. Thus it would appear that very little degradation has occurred during the depyruvylation.

Smith Degradation of the Acid Polysaccharide

Mild acid hydrolysis of the polyalcohol revealed the expected fragments of glycerol and glyceric acid. It was found that the degraded polyalcohol retained a high average degree of polymerisation as no oligosaccharides were detected by paper chromatography. This indicates that the partial hydrolysis was only cleaving the acyclic acetal linkages at end groups and not those of degraded heptose, probably due to a steric effect. Stronger hydrolysis conditions resulted in the liberation of free monosaccharides (see Fig 23,p.128). This was also found to be the case for the depyruvylated polyalcohol.

The methylation results from the acid polyalcohol (Expt. XL p.80) were substantially identical to those obtained from the acid polysaccharide (Expt. XXXIX1, p.79) except that the proportion of 3,4,6,7-tetra-O-methyl heptose was much reduced.



NOTE Chain Lengths and α-Linkages shown are arbitrary

Glycolaldehyde, authentic and from hydrolysates was not detected chromatographically

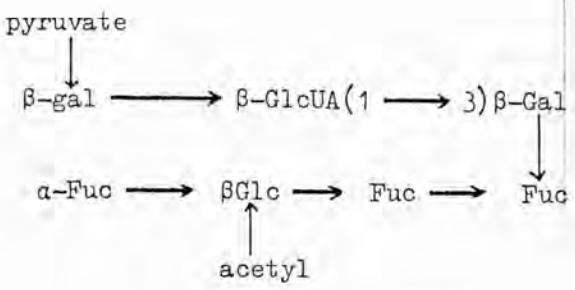
Fig 23

The results from the methylation of the depyruvylated polysaccharide confirmed the theory that very little degradation of the polysaccharide had occurred; the measurement of peak areas gave a degree of branching of 13%, a value which is not significantly different from the value (12%) obtained from methylation of the original polysaccharide.

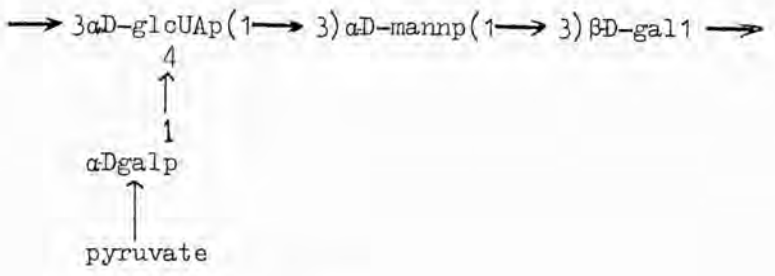
The depyruvylated polyalochol gave a similar methylation pattern, but no methylated heptose was detected as expected.

Oligouronic acids from the Acid Polysaccharide

Several workers[252] have reported the isolation of a monosaccharide or disaccharide carrying the pyruvic acid ketal by partial hydrolysis, or methylation and partial hydrolysis, of the polysaccharide. The work of Hirase[233] was followed to isolate such a disaccharide, but this proved unsuccessful (Expt. XXXVII, p. 76). It is thought that the acid conditions used hydrolysed the ketal. Most of the ketal bearing residues isolated by the various workers have been isolated from neutral polysaccharides, where hyrolysis conditions would be less severe. Choy and Dutton[247] considered that the close proximity of the pyruvic acid to a glucuronic acid residue in Klebsiella K.21 polysaccharide was responsible for the inability to isolate such a disaccharide. However Sutherland[252] isolated several oligosaccharides, still bearing the pyruvic acid moiety, from the colanic acid polysaccharide in which the sugar carrying the pyruvic acid is directly linked to a uronic acid residue (see fig. 24 (overleaf). However both the galactose residues in the polysaccharides shown form pseudoaldobiuronic acid residues. If, however, in the B. mobilis polysaccharide the pyruvate bearing moiety, i.e. the heptose, is 'sandwiched' between uronic acid residues, it seems likely that the weak ketal links would be broken in less strong acid conditions than those of the glycosidic linkages.



Colanic acid structure[252]



Klebsiella K.21[247]

Fig. 24 Repeating units of two polysaccharides showing justaposition of uronic acid and pyruvate residues

Returning to the oligouronic acids isolated by partial hydrolysis of the total unfractionated polysaccharide (Expt. XV, p.59) these must arise solely from the acid polysaccharide.

Only one oligosaccharide was found which contained all three constituents of the acid polysaccharide (see Table 6, p.59) and this had a degree of polymerisation greater than 15.

It is thought that this was simply degraded polysaccharide.Of the remaining oligouronic acids, one, a disaccharide, was found to contain only guluronic acid and the remainder contained both guluronic acid and heptose. The quantities obtained were relatively small and thus the experiment was repeated using the acid polysaccharide (Expt. XLI, p.81) (Table 20). These oligosaccharides appear to be basically similar to

Table 20 Analysis of oligosaccharides from Acid polysaccharide

No.	Wt. obtained as carbohyd (mg)	D.P.	% uronic acid	% glucose	Others constits.	reducing end	rotation $[\alpha]^{o}$
1	167	6	63%	0	heptose	heptose	50
2	107	3	67%	0	heptose	heptose	47
3	116	2	98%	0	-	uronic acid	64
4	165	2	50%	0	heptose	heptose	75
5*	25	1.1	50%	0	heptose	-	-

* this fraction was thought to be a mixture of heptose and monouronic acid which were not separated.

those obtained previously, except that no glucose was found. In addition, no oligosaccharide containing only neutral sugars, either mixtures, or singly was obtained, indicating that the heptose is not mutually linked but only to uronic acid residues.

Where the oligouronic acids contained heptose (Nos. 1, 2 and 4) this was found as follows to be the reducing unit. A sample of hydrolysed oligouronic acid was applied to a paper chromatogram, together with a sample of reduced hydrolysed oligouronic acid. Thus it is discovered which component is no longer present. In Nos 2 and 4 the heptose spot disappeared and a non-reducing spot of different mobility appeared. In No 1 the heptose was reduced and the new spot appeared together with unreduced heptose. Methylation analysis of the oligouronic acids (table 21, overleaf) confirmed that uronic acid formed the non-reducing end group in all cases, and that all the oligouronic acids were straight chains with no branch-points.

Table 21. Methylation Analysis of reduced oligosaccharides from Acid polysaccharide

oligosaccharide	Components		
	3,4,6,7-tetra-O-methyl heptitol	2,3,4,6-tetra-O-methyl gulitol	2,4,6-tri-O-Me-gulitol
1	+	+	(m)* +
2	+	+	+
3		+	+
4	+	+	

*m = major

In addition partial hydrolysis of the hexasaccharide (Expt. XLIV, p.83) produced heptose, uronic acid and a component with the same chromatographic mobility as the trisaccharide (No. 2) guluronosyl(1— 3)guluronosyl(1—2) heptose.

These results thus lead us to postulate a repeating unit structure of two uronic acid units linked to a heptose (see fig. 25 below).

—→ 3)guluronic acid(1—→ 3)guluronic acid(1—→ 2)heptose(1—→

Fig. 25 Repeating unit occurring in Acid Polysaccharide.

The structural position of the glucose is as yet undetermined. No glucose units linked to uronic acid units were obtained, neither were any mutually linked glucose units. However since these are probably 1,3-linked, they may be sufficiently acid labile to be hydrolysed even under the conditions of partial hydrolysis. It therefore seems likely

that the glucose forms chains of 1,3-linked units which are attached to the main polysaccharide through uronic acid residues, since this is the only unit which occurs at branch points and methylation analysis shows that some end group glucose is present. However it is possible that the glucose is not covalently linked to the uronic acid containing chain but is merely connected by such forces as hydrogen bonding, but is sufficiently interwoven with the acid polysaccharide not to be separated on fractionation.

Molecular Weight Determination of the Acid Polysaccharide

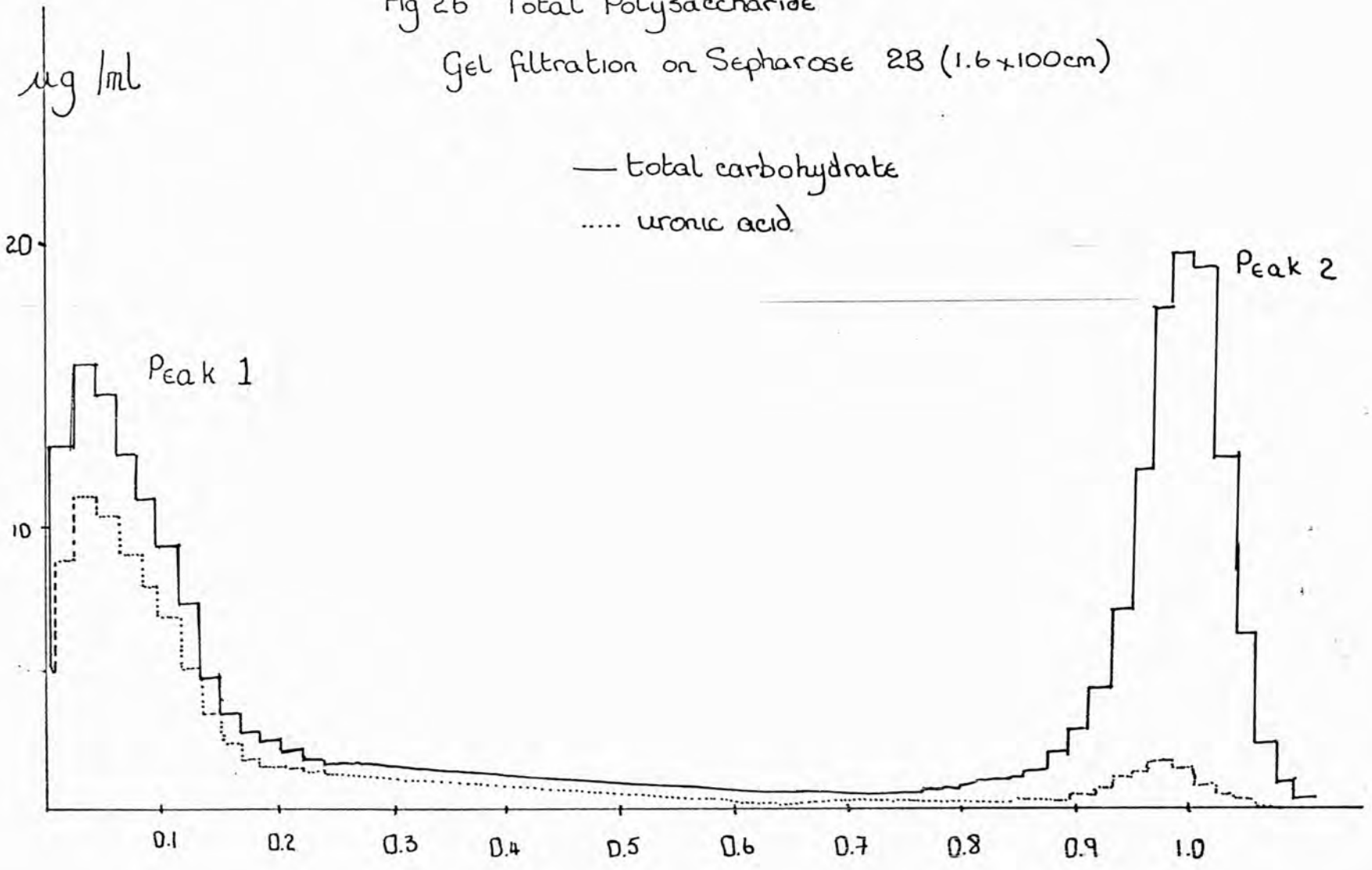
This was found to be very difficult due to the high viscosity of the aqueous solution. However very dilute solutions of the polysaccharides were examined under the influence of the ultracentrifuge. Both the total and the acid polysaccharide were found to separate into two peaks and these could possibly be the two materials fractionated in Expt. XIX3 (b) p.65.

Dr A.N. deBelder (Pharmacia) kindly applied both the total and acid polysaccharides to columns of Sepharose 2B and 6B (see figs. 26, 27 and 28). The total polysaccharides on Sepharose 2B (fig. 26) gave an elution pattern with (1) a high molecular weight fraction comprising mainly uronic acid, and (2) a very low molecular weight fraction which contained a trace of uronic acid. This latter peak probably represents the neutral polysaccharide. On this column it is impossible to differentiate this from monosaccharides.

The acid polysaccharide was totally excluded from the Sepharose 6B column (fig. 27) indicating a molecular weight greater than five million. The same polysaccharide on Sepharose 2B (fig. 28) gave a broader distribution, however a considerable amount was still excluded from the gel. This indicates a very high molecular weight, probably greater than 20 million.

Nevertheless these figures cannot be taken as exact due to the possibility of molecular aggregation under gel-like conditions. Thus the 'apparent' molecular weight could be a multiple of the actual molecular weight. However the results still indicate a very large polymer.

Fig 26 Total Polysaccharide
Gel filtration on Sepharose 2B (1.6×100cm)



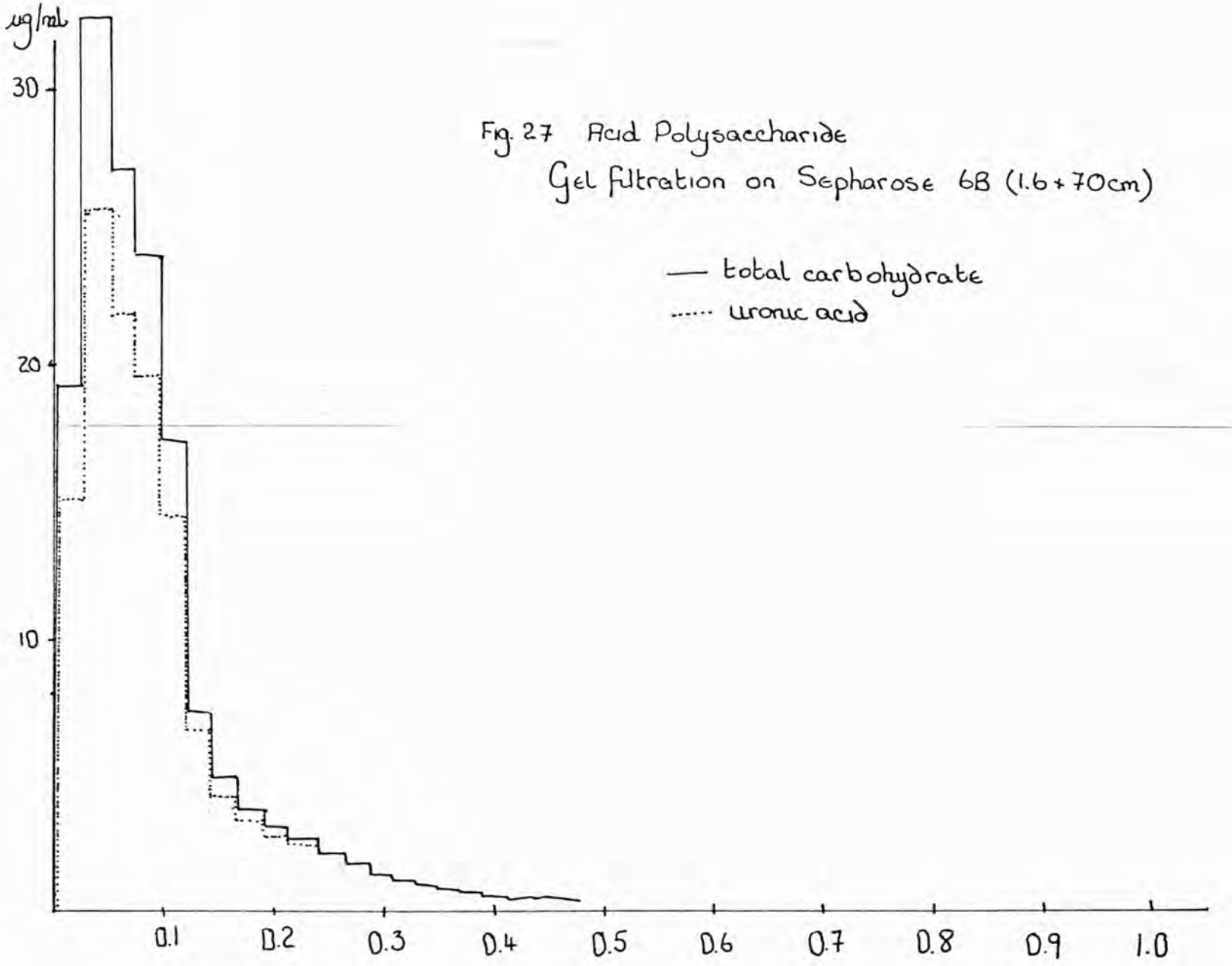


Fig. 27 Acid Polysaccharide
Gel filtration on Sepharose 6B (1.6 × 70cm)

— total carbohydrate
····· uronic acid

Fig 28 Acid Polysaccharide
Gel filtration on Sepharose 2B (1.6 x 100 cm)

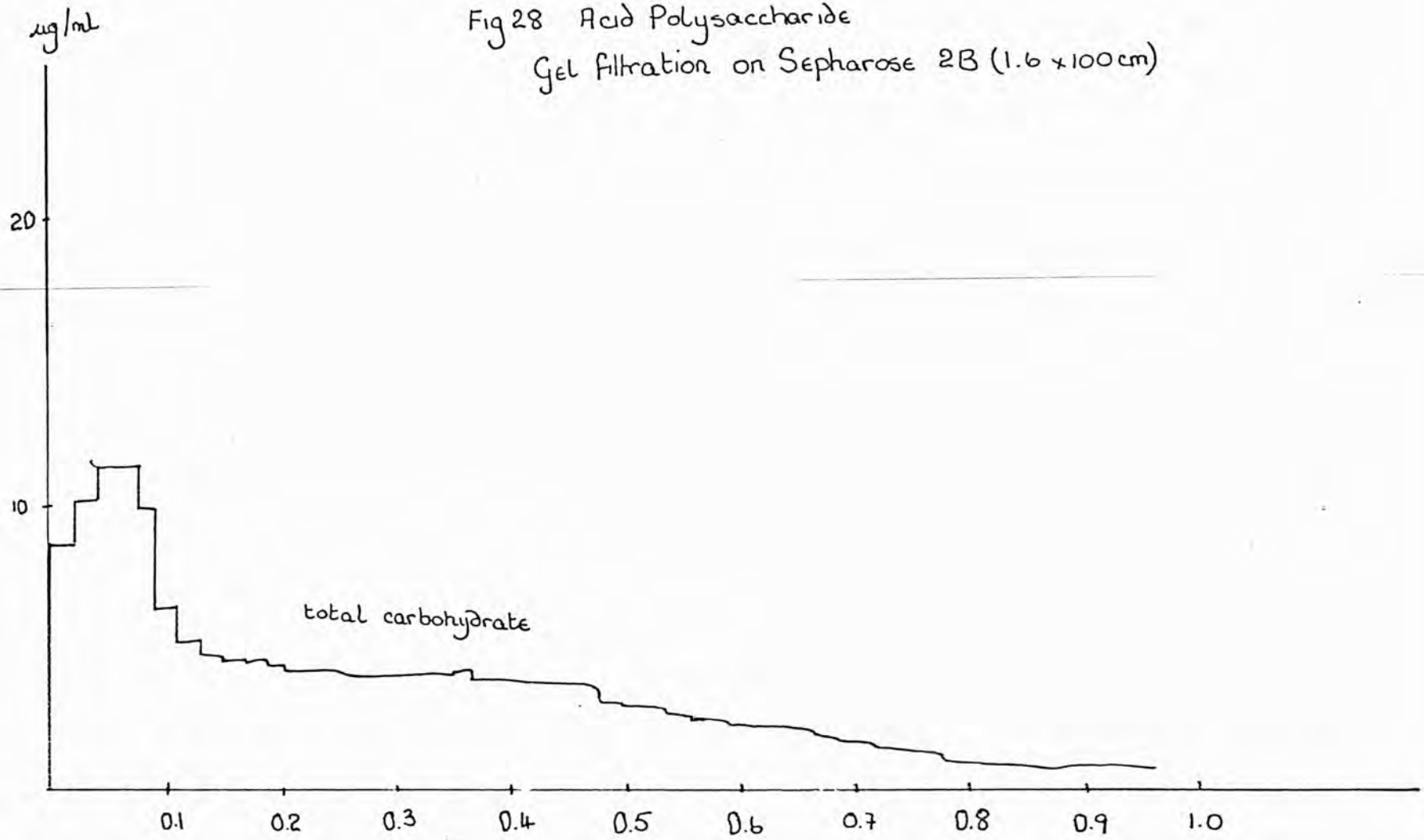


Fig 29 N.M.R of Acid Polyalcohol in D_2O

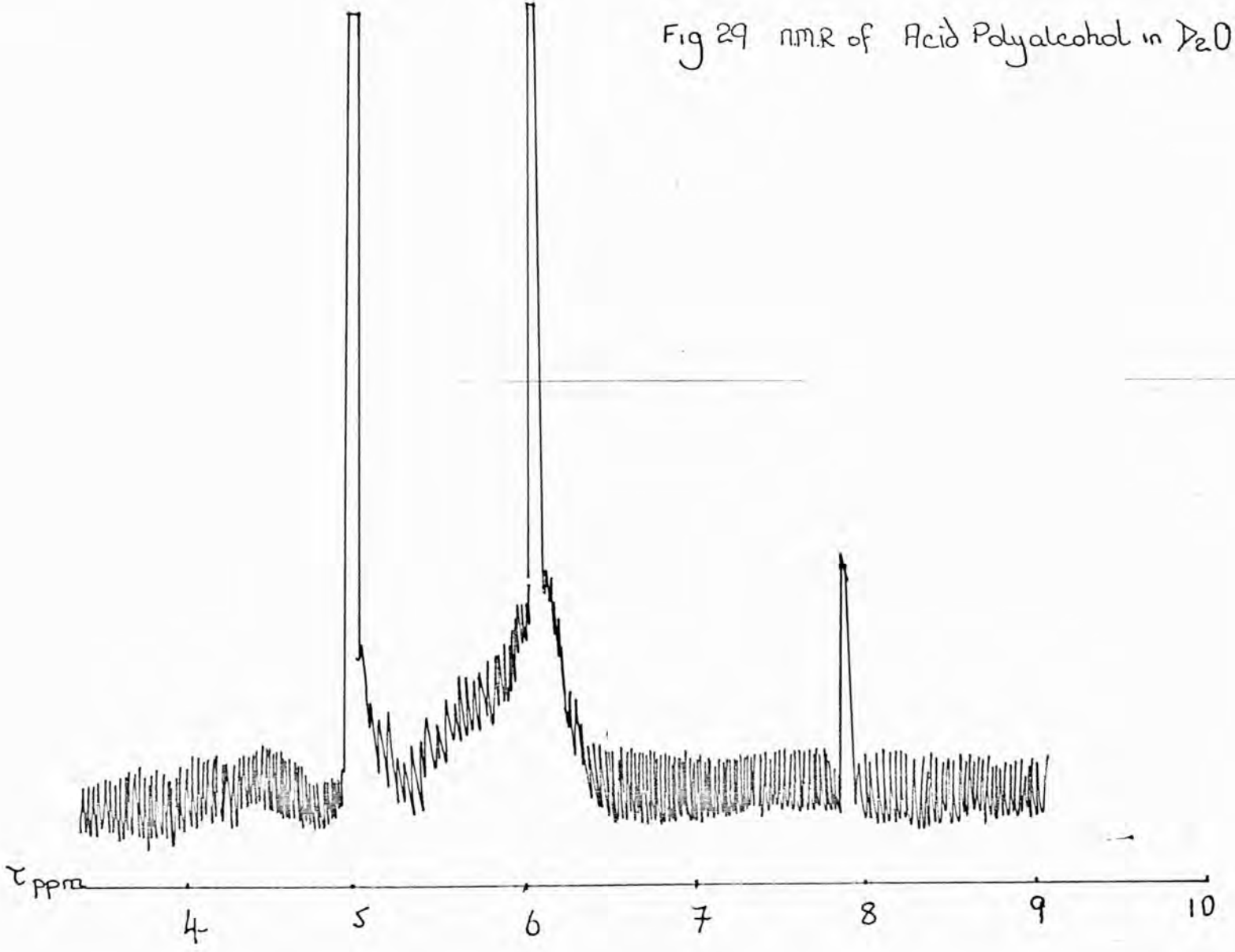


Fig 30 N.M.R of Acid Polysaccharide methylated and reduced in $CDCl_3$

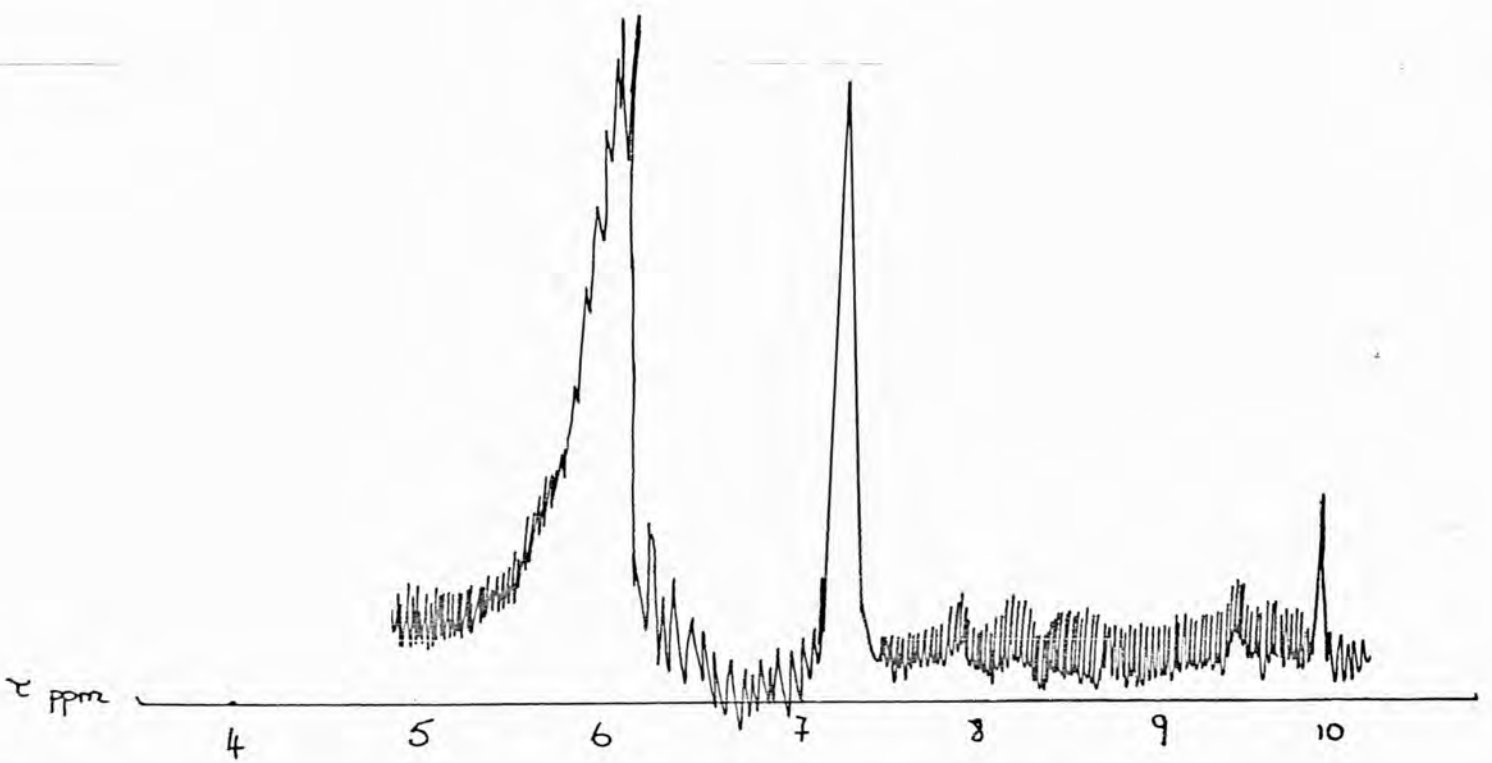


Fig 31 N.M.R of Acid Polysaccharide
methylated, reduced and remethylated in $CDCl_3$

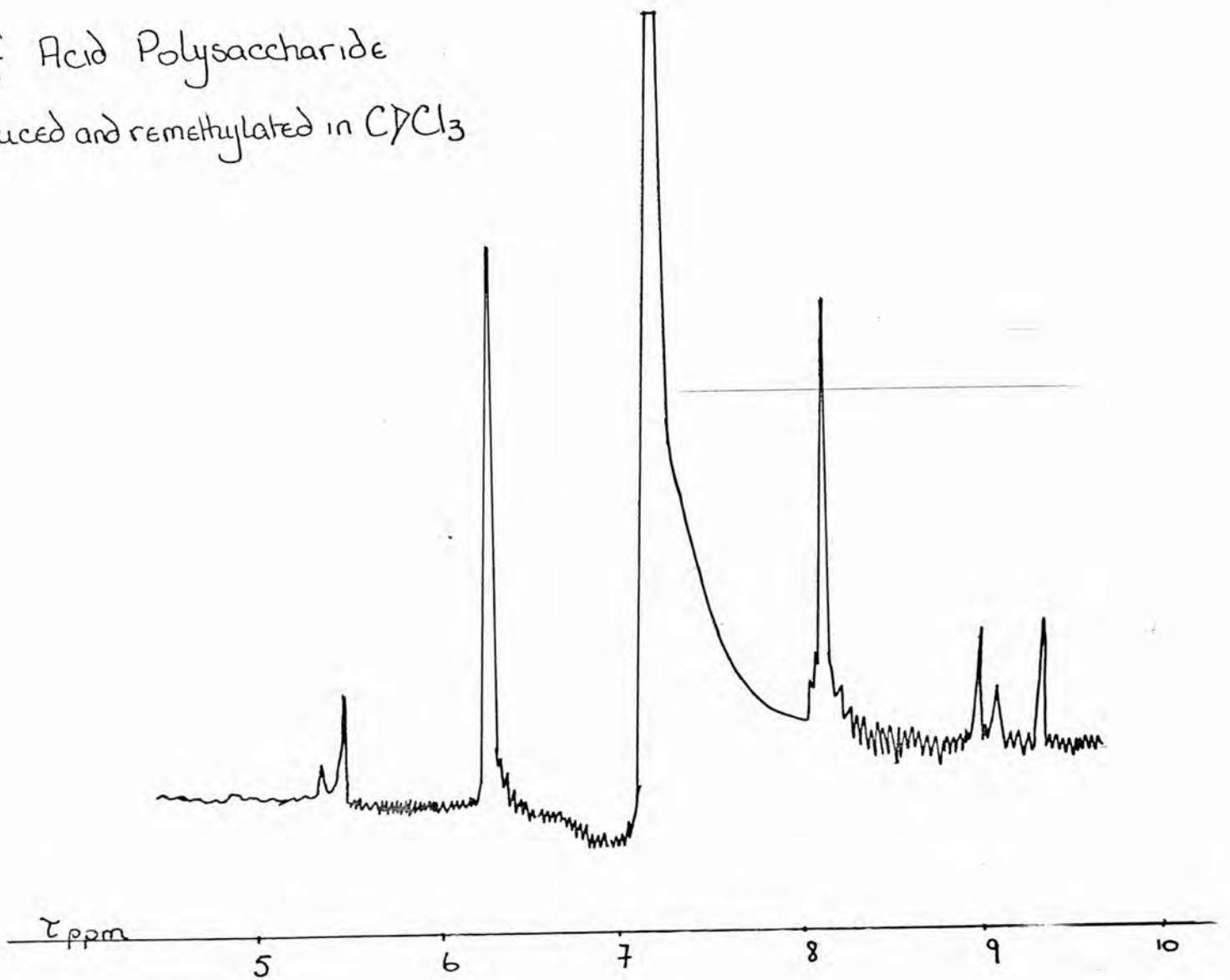


Fig 32 N.M.R of Sodium Acetate in D_2O

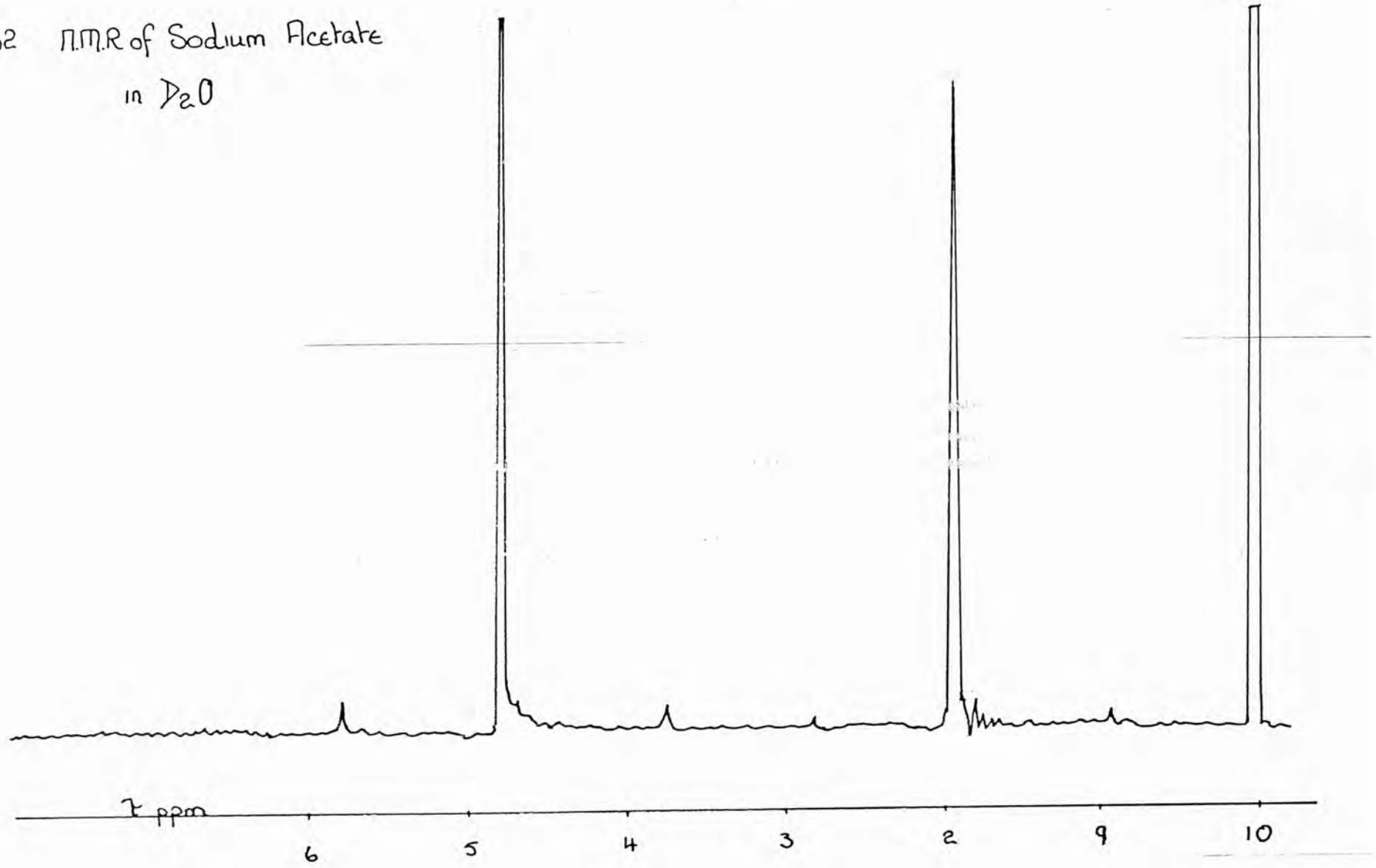


Fig. 33 N.M.R. Sodium Pyruvate in D_2O

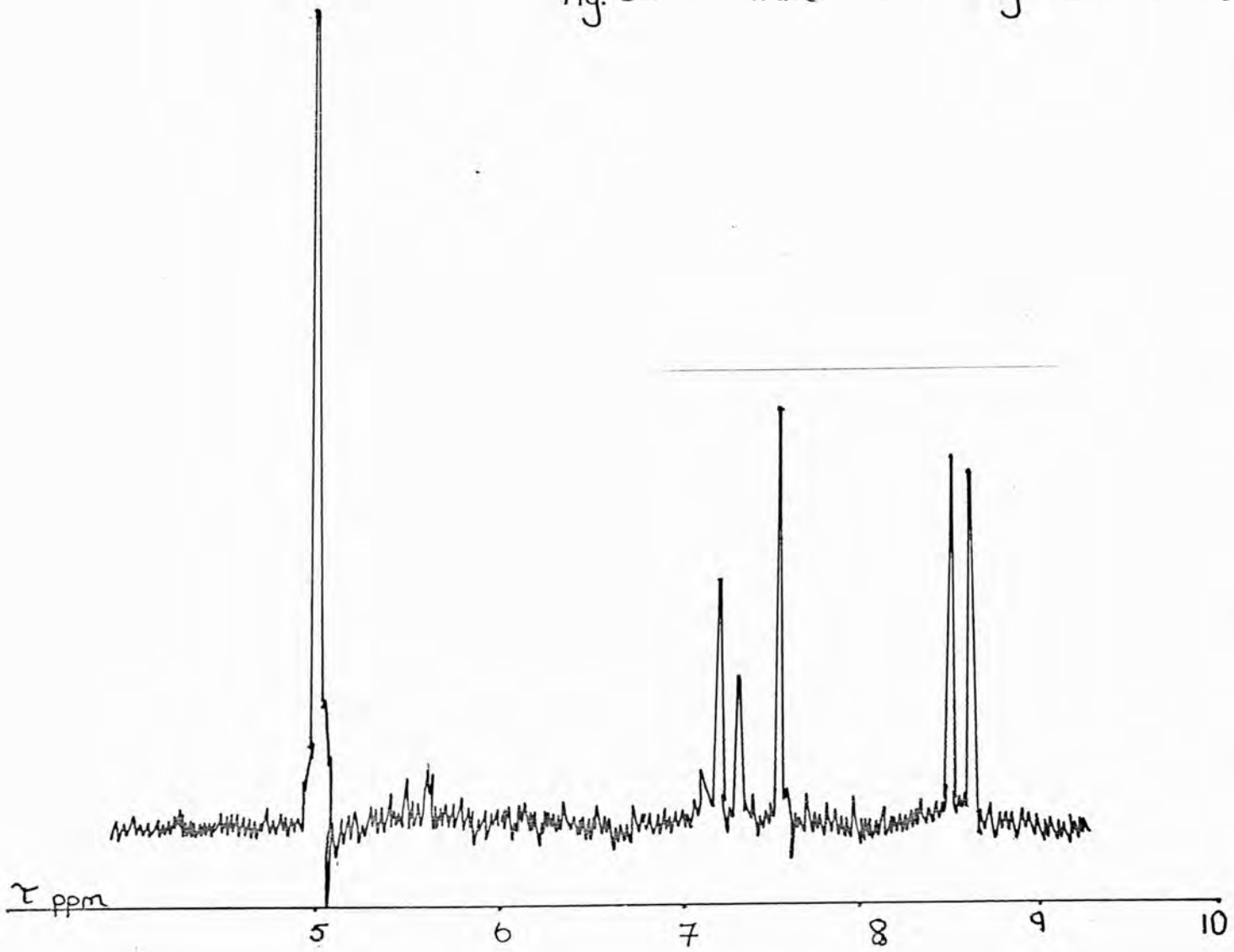


Fig 34 Pyruvic Acid in D_2O

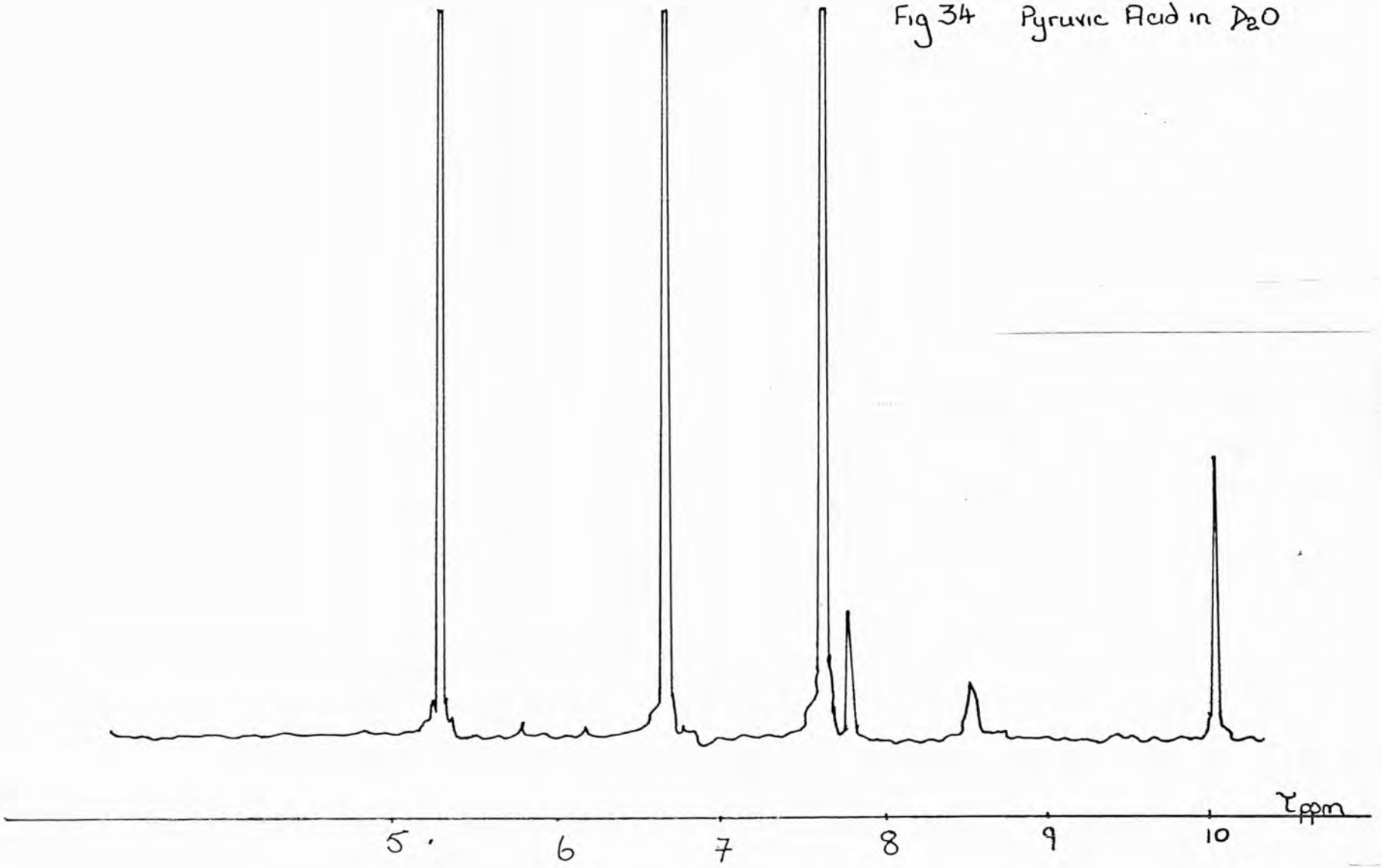
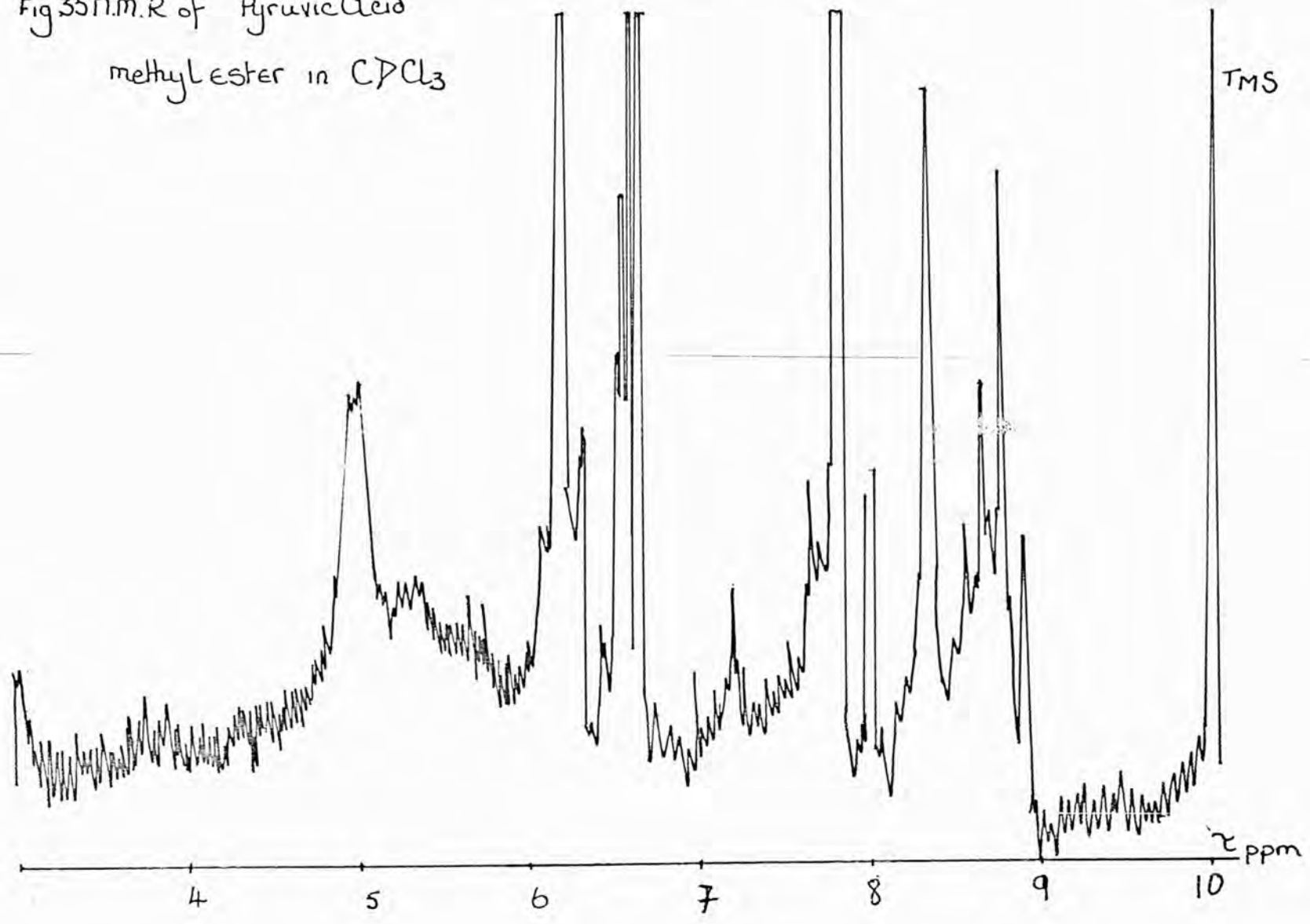


Fig 35 N.M.R of Pyruvic Acid methyl ester in $CDCl_3$



APPENDIX 1

Composition of Extracellular Polysaccharides of Soil Bacteria

TABLE A. Agrobacterium

Species	Constituents				Structure	Ref.
	glucose	galactose	uronic acid	others		
A. tumiefaciens	+	-	-	-	β-1,2-chains	66
	+	-	-	-	β-1,2-chains	67
	+	-	-	-	β-1,2-chains	68
	+	+	+	pyruvate, acetate		69
A. radiobacter	+	-	-	-	β-1,2-chains	66
	+	-	-	-	-	67
A. rhizogenes	+	-	-	-	β-1,2-chains	66
A. rubi	+	-	-	-	β-1,2-chains	66
A. gypsophilae	+	-	-	-	β-1,2-chains	66
A. pseudotsugae	+	+	-	-	β-1,2-and β-1,6-chains	66

TABLE B. Bacillus

Species	Constituents				Structure	Ref.
	glucose	fructose	uronic acid	others		
B. cereus	-	+	-	-	-	70
B. subtilis	-	+	-	-	β-2,6 chains β-2,1 branches	70-72
B. pumilis	-	+	-	-	-	77
B. mesentericus	-	+	-	-	β-2,6 chains	71, 73-75
B. megatherium	-	+	-	-	β-2,6 chains	76
B. megatherium 697	+	+	+			70
B. polymyxa 354[1]	+	-	+	mannose		70
354[1]	+	+	-	mannose		70
354	-	+	-	-		70
B. polymyxa	+	-	-	-	α-1,6 chains	77
B. polymyxa (S-33 var. Lactoviscosis)	+	-	-	fucose		78
B. polymyxa (C3 NRC 4901)	-	-	-	mannose	α-1,6 chains	79
B. brevis 799[1]	+	-	+	-		70
B. alvei 683[1]	+		+			70
B. circulans 294[1]	+		+	mannose		70
396[1]	+		+	mannose		70
295	+	-	+	mannose xylose		70
760	+		+	mannose		70
B. marcerans	+				Schardinger dextrin.	80

Note 1. The levans produced by the genus Bacillus are generally produced in a liquid sucrose medium. Those polysaccharides marked 1 have been grown in a solid medium.

TABLE C. *Corynebacterium*[81]

Species	Constituents					
	glucose	mannose	acid	galactose	fucose	rhamnose
C. flaccumfaciens	+	+	uronic		+	+
C. tritici	+	+	uronic			+
C. rathayi	+	+	uronic			
C. insidiosum	+		pyruvic	+	+	
C. michiganense	+	+	low molecular weight	+	+	
C. sepedonicum	+	+	low molecular weight	+	+	
C. faciens	+	+	not tested	trace		

TABLE D. Aerobacter or Klebsella

Species or Strain	Type No.	Constituents							
		glucose	galactose	mannose	fucose	rhamnose	uronic acid	others	Ref.
K. pneumoniae (A)	1	+					+		87,88 91,92 93,90
		+			+		glucuronic	pyruvic acid	94-97 92,93
K. pneumoniae (B)	2	+		+			glucuronic	pyruvic acid	97
		+					+		87,88 90
		+		+			glucuronic		99,98
K. pneumoniae (C)									
K. ozaenae			+	+			galacturonic	pyruvic acid	92,97
K. rhinoscleromatis	3								100,101
K. aerogenes									102-105
K. pneumoniae (C)	3	+					+	pyruvic acid	87,88 90,106
	4	+	tr.	+			glucuronic	pyruvic acid	92,97
	5	tr.	tr.	+			glucuronic	pyruvic acid	82,92,98
	5	+		+			glucuronic	pyruvic acid acetic acid	107,108

TABLE D. Aerobacter or Klebsiella (continued)

Species or Strain	Type No.	Constituents							
		glucose	galactose	mannose	fucose	rhamnose	uronic acid	others	Ref.
K. pneumoniae (C) (continued)	6	+		+	+		glucuronic	pyruvic acid	92,97
K. aerogenes	7	+	+	+			glucuronic		92
	8	+	+				glucuronic		92, 109 61
	9		+			+	glucuronic	pyruvic acid	92, 110 111
	10	+	+	+			glucuronic		51
	11	+	+	tr.			glucuronic		92, 112
	12	+	+			+	glucuronic		92
	13	+	+	tr.			glucuronic		92
	14	+	tr.	+		+	glucuronic		92
K. michigan	15	+	+				glucuronic		92
	16	+	+		+		glucuronic		92
	17	+				+	glucuronic		92
	18	+	+			+	glucuronic		92
	19	+	+			+	glucuronic		92

TABLE D. Aerobacter or Klebsiella (continued)

Species or Strain	Type No.	Constituents							
		glucose	galactose	mannose	fucose	rhamnose	uronic acid	others	Ref.
	20		+	+			glucuronic		92,112,113
	21		+	+			glucuronic	pyruvic acid	92,114
	22	+	+				+		92
	23	+	tr.			+	glucuronic		92
	24	+		+			glucuronic		92,115
	25	+	tr.				glucuronic		92
	26	+	+	+			glucuronic		92
	26		+	+			+		61
	27	+	+				glucuronic		92
	28	+	+	+			glucuronic		92
	29		+	+			galacturonic		61
	30	+	+	+			glucuronic		92
	31	+	+	+			glucuronic		92
	32		+			+		pyruvic acid	92,110

TABLE D. Aerobacter or Klebsiella (continued)

Species or Strain	Type No.	Constituents						
		glucose	galactose	mannose	fucose	rhamnose	uronic acid	others	Ref.
	33	+	+	+			glucuronic		92
	34	+				+	galacturonic		92
	35	+	+	+			glucuronic		92
	36	+	+			+	glucuronic		92
	37	+	+				+		92
	38	+	+				+		92
	39	+	+	+			glucuronic		92
	40		+	+		+	glucuronic		92
	41	+	+			tr.	glucuronic		92
	42		+			+	glucuronic		92
	43		+			+	glucuronic		92
	44	+				+	glucuronic		92
	45	+	tr.			+	glucuronic		92
	46	+	+	+			glucuronic		92
	47		+			+	glucuronic		110,116
	48	+				+	galacturonic		92

TABLE D. Aerobacter or Klebsiella(continued)

Species or Strain	Type No.	Constituents							
		glucose	galactose	mannose	fucose	rhamnose	uronic acid	others	Ref.
	49		+	+			galacturonic		92
	50	+	+	+			glucuronic		92
	51	+	+				glucuronic		92
	52		+			+	glucuronic	pyruvic acid	92, 110
	53		+	+		+	glucuronic		92
	54	+			+		glucuronic	acetic acid	61 117-120 121
	55	+	tr.			+	glucuronic		92
	56	+	+			+	+		92
	57		+	+			galacturonic		91,92
	58	+	+		+		glucuronic		92
	59	+	+	+			glucuronic		92
	60	+		+	+		glucuronic		92
	61	+	+	tr.			glucuronic		92
	62	+	+	+			glucuronic		92
	63		+		+	tr.	galacturonic		92

TABLE D. Aerobacter or Klebsiella (continued)

Species or Strain	Type No.	Constituents							
		glucose	galactose	mannose	fucose	rhamnose	uronic acid	others	Ref.
A. aerogenes	64	+		+		+	glucuronic		92,122
	65	+		+			glucuronic		92
	66		+	+			glucuronic		92
	67	+	+	+		+	glucuronic		92
	68	+	+	+	+		glucuronic	pyruvic acid	92,97
	69	+	+	+			glucuronic		92
	70	+	+			+	glucuronic		92
	71	+		tr.		+	glucuronic		92
	72	+	tr.			+			92
	73	+	+			+	glucuronic		123
	74		+	+			glucuronic	pyruvic acid	123
	75		+	+			galacturonic		123
	76		+	+			glucuronic		123
	77	+	+	+			glucuronic		123
	78	+	+				glucuronic		123
	79	+	+			+	glucuronic		123
	80		+	+		+	glucuronic	pyruvic acid	123

TABLE D. Aerobacter or Klebsiella (continued)

Species or Strain	Type No.	Constituents							
		glucose	galactose	mannose	fucose	rhamnose	uronic acid	others	Ref.
untyped a.			+	+			glucuronic		124
untyped b.			+	+			glucuronic	pyruvic acid	125
A. cloacae		+	+		+		+		61
A. aerogenes	(418)	+		+			glucuronic or mannuronic		126

TABLE E. Structural features of Capsular Polysaccharides of Klebsiella

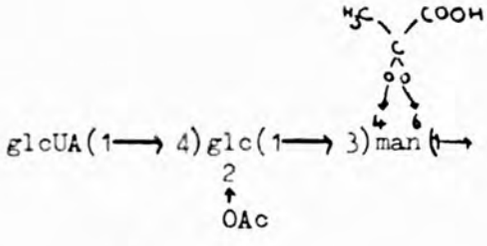
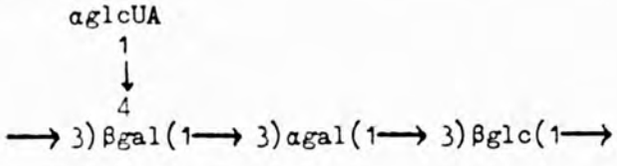
Type No.	Structure	Ref.
2	⟶ 3)glc(1⟶ 4)glcUA(1⟶ 3)man(1⟶ 4)glcUA(1⟶	98,99
3	mannose, non-reducing end group. galUA(1⟶ 4)man ⟶ 3(gal)(1⟶ 3)man(1⟶	100,101
5	 	108
8	 	109
9	βglcUA(1⟶ 4)rha ⟶ 3)αgal(1⟶ 3)αrha(1⟶ 3)αrha(1⟶ 2)rha 4 1 glcUA	111
20	— man - gal - man - gal glcUA gal	113

TABLE E. (continued)

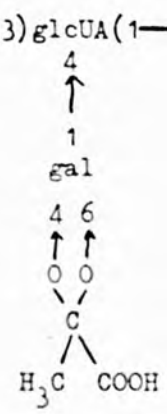
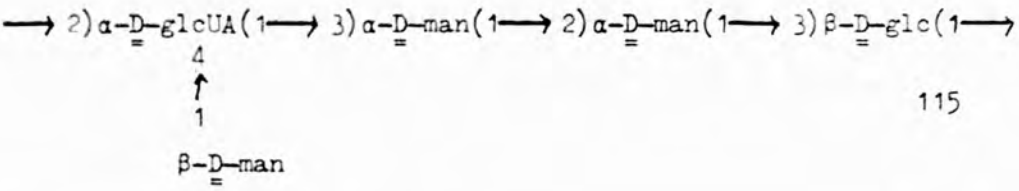
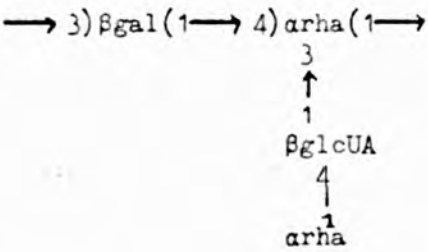
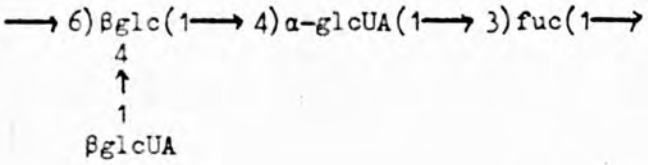
Type No.	Structure	Ref.
21		114
24		115
47		116
54		115, 118
64	rhanmose non-reducing end group, 1,2-linked and 1,4-linked glucose, mannose, glucuronic acid, 1,3-linked or at branch points.	122

TABLE E. (continued)

Type No.	Structure	Ref.
untyped a.	$\text{-}\longrightarrow 3)\text{gal}(1\longrightarrow 3)\text{man}(1\longrightarrow 3)\text{gal}(1\longrightarrow$ 2 ↑ 1 glcUA	124
untyped b.	glcUA(1⟶ 3)man, 1,3-linked gal. 1,2-linked man.	125

Key

gal galactose

glc glucose

man mannose

fuc fucose

rha rhamnose

glcUA glucuronic acid

galUA galacturonic acid

TABLE F. Pseudomonas

Species and Strain	Constituents							
	fructose or glucose	galactose	mannose	fucose	rhamnose	others	Structure	Ref.
Ps. pruni	+						β-2,6 chains	127
Ps. prunicola	+						β-2,6 chains	127,128
Ps. mors-prunorum	+						β-2,6 chains	127,128
Ps. syringae	+						β-2,6 chains	128
Ps. beta-gelatae	+						β-2,6 chains	129,130
Ps. aureofaciens	+						β-2,6 chains	131
Ps. chlororaphis	+						β-2,6 chains	131
Ps. fluorescens	+						β-2,6 chains	131
Ps. fluorescens 11		+		+		glucosamine		132
" 35B		+	+		+			132
" 25B		+	+	+		xylose		132
" 14		+			+			132
" 2A		+			+			132
Ps. fluorescens 21A		+						132
" 22A		+						132
" 23A		+			+			132
" 131		+		+	+			132
" 180								132
OSU 64								132

TABLE C. Serratia marcescens

Strain	Constituents					Ref.
	rhamnose	glucose	heptoses	mannose	glucuronic acid	
1st strain						
Capsule a		+		+	+	134
b	+	+				134
c		+	+			134
d	+	+	+			134
medium a	+	+	+			135,136
b	+	+	+	+		135,136
2nd strain						
Capsule a	+	+				137
b		+	+			137
c		+		+	+	137

TABLE H. Rhizobium

Species Strain or Host.	Constituents						
	glucose	galactose	mannose	rhamnose	glucuronic Ac.	4-O-MeGlcUA	pyruvic acid	acetic	Ref.
R. trifollii									
TA-1	+	+			+	9	4		110'
UNZ29	+	+			+	10	4		110
WA67	+	+			+	9	3		110
CC10	+	+			+	9	2		110
2480A	+	+			+	9	4		110
AR-3	48	10			13	15	14		69
Coryn KL	50	19			14	10	7		69
In2	50	10			19	11	10		69
K-8	51	11			16	11	11		69
Bart-A	35	9			15	11	5		139
0401	36	9			16	9	5		139
0402	30	8			15	8	5		139
0403	36	9			21	9	6		139
0412	47	1			21	6	3		139
0413	38	9			20	8	5		139
0416	25	6			15	6	5		139

TABLE H. (continued)

Species or Host	Strain	Constituents							Ref.
		glucose	galactose	mannose	rhamnose	glucuronic Ac.	4-OMeGlcUA	pyruvic acid	acetic	
	0417	38	10			17	9	5		139
	0418	48	9			18	10	6		139
	0419	43	11			18	10	6		139
	TA1	61	9			15	10	4		140
	205a	+	not examined			22				141
	205b	+				24				141
	205c	+				22				141
	201	+				25				141
	SU36	+				+	+			142
	SU46	+				+	+			142
	SU64	+				+	+			142
	SU91	+				+	+			142
	SU298/533	+				+	+			142
	NA30	+				+	+			142
	NZ19	+				+	+			142
R. Meliloti	B	+	+			tr.		5	4.5	110
	F	+	+			tr.		5	4	133
	BPe	77	10			tr.		5	8	69
	K24	75	8			tr.		6	9	69
	A145	83	8			tr.		3	6	69

TABLE H. (continued)

Species Strain or Host	Constituents							Ref.
	glucose	galactose	mannose	rhamnose	glucuronic Ac.	4-O-MeGlcUA	pyruvic acid	acetic	
A148	74	12			tr.		5	9	69
U27	70	10			4		5	4	143
100a	+				4				140
100b	+				7				140
100c	+				5				140
SU27	+					+			142
SU47	+					+			142
SU62	+					+			142
SU74	+					+			142
SU76	+					+			142
SU101	+					+			142
SU277	+					+			142
AH2	85	14			0.7				144
Be 1	82	13			0.6				144
Cl 1	82	16			0.8				144
Lb 1	84	15			1.2				144
Pl 2	85	13			0.6				144
$S_9$10	85	13			0.5				144
$S_9$10M	85	15			1.0				144
Sn 258	86	14			0.7				144
Sr 1	85	15			0.7				144
Ve 15	83	12			0.9				144
-	+	+	traces of unidentified components						145

TABLE H. (continued)

Species Strain or Host	Constituents							
	glucose	galactose	mannose	rhamnose	glucuronic Ac.	4OMeGlcUA	pyruvic acid	acetic	Ref.
R. leguminosarum								
H VIII	48	11			19		14	8	69
P8	48	11			19		13	9	69
PRE	48	11			19		13	9	69
310a	51	9			16		15	9	69
313	46	14			14		15	11	69
402	49	10			16		15	10	69
404	49	10			17		14	10	69
311a	+				19				141
311b	+				22				141
SU302	+				+	+			142
SU331	+				+	+			142
SU567	+				+	+			68
SU569	+				+	+			68
-	+	fructose			+				146
R. phaseoli									
SU312	+				+	+			68
SU330	+				+	+			68
Blink	48	11			16		13	12	69
460	50	11			17		11	12	69

TABLE H. (continued)

Species Strain or Host	Constituents								
	glucose	galactose	mannose	rhamnose	glucuronic Ac.	4QMeGlcUA	pyruvic acid	acetic	Ref.
Bokum	50	9			18		14	9	69
NA1	+				+	+			142
R. radicicolum									
Trifolii Bartel A	+				+		8	2	110
Clover Bartel A	+				+				147
R.japonicum									
SU442	+			+					68
SU511	+			+					68
SU564	+		+		+	+			68
R. lupini									
SU533	+		+			+			68
SU547	+		+						68
SU557	+		+	+		+			68

TABLE H. (continued)

Species Strain or Host	Constituents								Ref.
	glucose	galactose	mannose	rhamnose	glucuronic Ac.	4-O-Me-GlcUA	pyruvic acid	acetic	
Assorted SU421	+					+			68
SU506	+		+	+		+			68
SU509	+		+	+		+			68
SU522	+				+	+			68
SU531	+	+							68
SU536	+		+			+			68
SU553	+		+			+			68
SU560	+		+	+		+			68
Soya bean	+	+	+	+					148
clover	+	fructose			+				146
3Cla2-II crotolaria					7				149
3Clk3-I crotolaria	+	+	+		+				149
3C2d3a lupine	+	+	+		+				149
3D0a8-1 alfalfa	+	+							149
3D0a13-1 alfalfa	+	+	+		+				149
3D0a31-1 alfalfa									149

TABLE H. (continued)

Species Strain or Host.	Constituents								
	glucose	galactose	mannose	rhamnose	glucuronic Ac.	4O-MeGlcUA	pyruvic acid	acetic	Ref.
3Dlm 32-1 Clover	+	+							149
3Dly 8-1 Clover	+	+			+				149
SU289 Clover	+	+	+		+				149
3EOa3-I Lotus	+	+	+		+				149
3Fle 4-II Indigofer	+	+	+		+				149
3HOg 47a-I Pea	+	+	+		+				149
3HOg 13-I Pea	+	+	+		+				149
3I1b 66-II Soya bean	+		+						149
3I1b 118-II Soya bean	+	+	+		+				149
3I 613-II Bean	+	+			+				149

TABLE H. (continued)

Species Strain or Host.	Constituents						Ref.
	glucose	galactose	mannose	rhamnose	glucuronic Ac.	4-OMeGlcUA pyruvic acetic acid	
Lotus spp:-							150
NZP 2034, 2037, 2042, 2048, 2079, 2085, 2093, 2147, 2150, 2171 SU343 CC809a	50–60	21–24			20 - 30		150
NZP 2076, 2088, 2182, 2257, 5091, 5144	36–48	16–21	30–45		tr.		150
NZP 2075, 5026, 5274	0 - 9	0.3	88–100		-		150
NZP 2170, 2175, 2240	36–45	18–24	34–38		tr.		150
NZP 5059/2	40	15	35		10		150
NZP 2243	-	48	45		7		150
NZP 2227	53	18	11		18		150
WU 425	17	7	54		22		150
NZP 2021f, 2089 2193, 2200, 2254 CC 8145	41–49		18–25		27–39		150

TABLE H. (continued)

Species Strain or Host.	Constituents								Ref.
	glucose	galactose	mannose	rhamnose	glucuronic Ac.	4-OMeGlcUA	pyruvic	acetic acid	
NZP 5042	31		36		33				150
NZP 2186, 2192, 5052	36-39		11-4	25-32	21-23				150
NZP 2247, 5025, 5251/2	26-30	31-35	30-40		tr.		fucose	tr.	150
NZP 5113	42	23	22				fucose 12		150
NZP 2087, 2141, 2151 5032, 5033, 5035, 5044, 5074, 5087, 5088	26-29	6-15	18-20		22-40		also unknown	10-17	150
NZP 2128, 2183, 2189	19-28	20-40	12-28		16-25		also unknown	5-19	150
NZP 2073	27	36	33		tr.		also unknown	2-5	150
Clover spp:-									
NZP 5063	34	34	3		31				150
NZP 514, 5039, 5098, 5107, CB782 SU202, WU95, TA-1	34-45	15-28	2-5		30-40				150

TABLE H. (continued)

Species Strain or Host.	Constituents							Ref.
	glucose	galactose	mannose	rhamnose	glucuronic Ac.	4OMe-GlcUA	pyruvic acetic acid	
Carmichaelia spp:-								
NZP 5061,5083	49-65	16-33	4		15-30			150
NZP 5080,5105	34-45	15-28	2-5		30-40			150
Sophora spp:-								
NZP 5055	49-65	16-33	tr.		15-30			150
NZP 5057	34-45	15-28	2-5		30-40			150
CB 569			9		2-5			150
CB 562	50		32		18			150
Bean spp:-								
NZP 5097	34-45	15-28	2-5		30-40			150
CC511	27	18	7		48			150
Lucerne spp:-								
NZP 4008	75	21	4					150
SU47	54	13	32		tr.			150

TABLE H. (continued)

Species Strain or Host.	Constituents								
	glucose	galactose	mannose	rhamnose	glucuronic Ac.	4OMeGlcUA	pyruvic	acetic acid	Ref.
Peanut spp:-									
CB935	30.5	41	23	5	0.5				150
CB745	45	7	15		23		also unknown 10		150
Goats Rue spp:-									
NZP 5067, 5068	66-71	26-33	0-3.7		0.5				150
Chick Pea spp:-									
CB1189, 1199	51-57	13-14	7-9		23-25				150
Pea vetch spp:-									
NZP 5225 SU391	34-45	15-38	2-5		30-40				150
Crown vetch 3G161	34-45	15-38	2-5		30-40				150
Cow Pea CB756	37	9	20		20		also unknown 13		150
							fucose 1		150
Acacia NZP 5073	35		14	25	26				150

APPENDIX II

Culture conditions and properties of individual batches of polysaccharides.

The lypophilised sample was suspended in 5 - 6 drops of sterile water and the suspension transferred to 10 ml of sterile water in a test tube. Samples of the solution were plated out on Burks agar slopes for 5 - 7 days at 25°C.

Individual colonies were then transferred to fresh agar slopes and grown for 3 - 4 days at 25°C.

Sterile water (5 ml) was added to each slope and the culture suspended in water by agitation. This organism produces a tenacious slime which is difficult to resuspend. This suspension (1.5 ml) was then transferred to conical flasks containing Burks citrate medium (200 ml). These seed stage flasks were incubated at 26°C for 2 - 3 days.

This seed stage culture (20 ml) was then inoculated into conical flasks containing Burks nitrate medium (200 ml) and the flasks were incubated for 1 - 7 days at 26°C and 220 rpm on the shaker.

Table I. Media

basic medium

dipotassium hydrogen phosphate	0.64 g/L
dihydrogen potassium phosphate	0.16 g/L
magnesium sulphate heptahydrate	0.2 g/L
sodium chloride	0.2 g/L
calcium sulphate dihydrate	0.05 g/L
sodium molybdate	0.0025 g/L
ferrous sulphate	0.015 g/L
sucrose	40 g/L

Table I (continued)

Additional ingredients

a) agar slopes

diammonium hydrogen citrate	0.8 g/L
agar	20 g/L

b) liquid seed stage

diammonium hydrogen citrate	0.8 g/L

c) final stage

potassium nitrate	0.72 g/L

Table II. Properties of Individual Batches

Sample	% yield based on sucrose provided in medium	Properties			
		milled powder		1% solution	
		protein %	moisture %	viscosity in cps	pH
S11A	26	20.6	14.6	570	4.6
S11B	26	7.0	12.9	470	4.6
S11C	26	7.0	21.6	47	6.7
S11D	19	9.36	19.6	1247	5.18
S11E	33.25	20.3	13.2	994	4.6
S11F	32	21.30	22.5	849	4.21
S11G	30	19.29	17.9	903	4.47
S11H	2.8 g/L	43	-	-	-
S11J	6.32 g/L	50.6	-	-	-

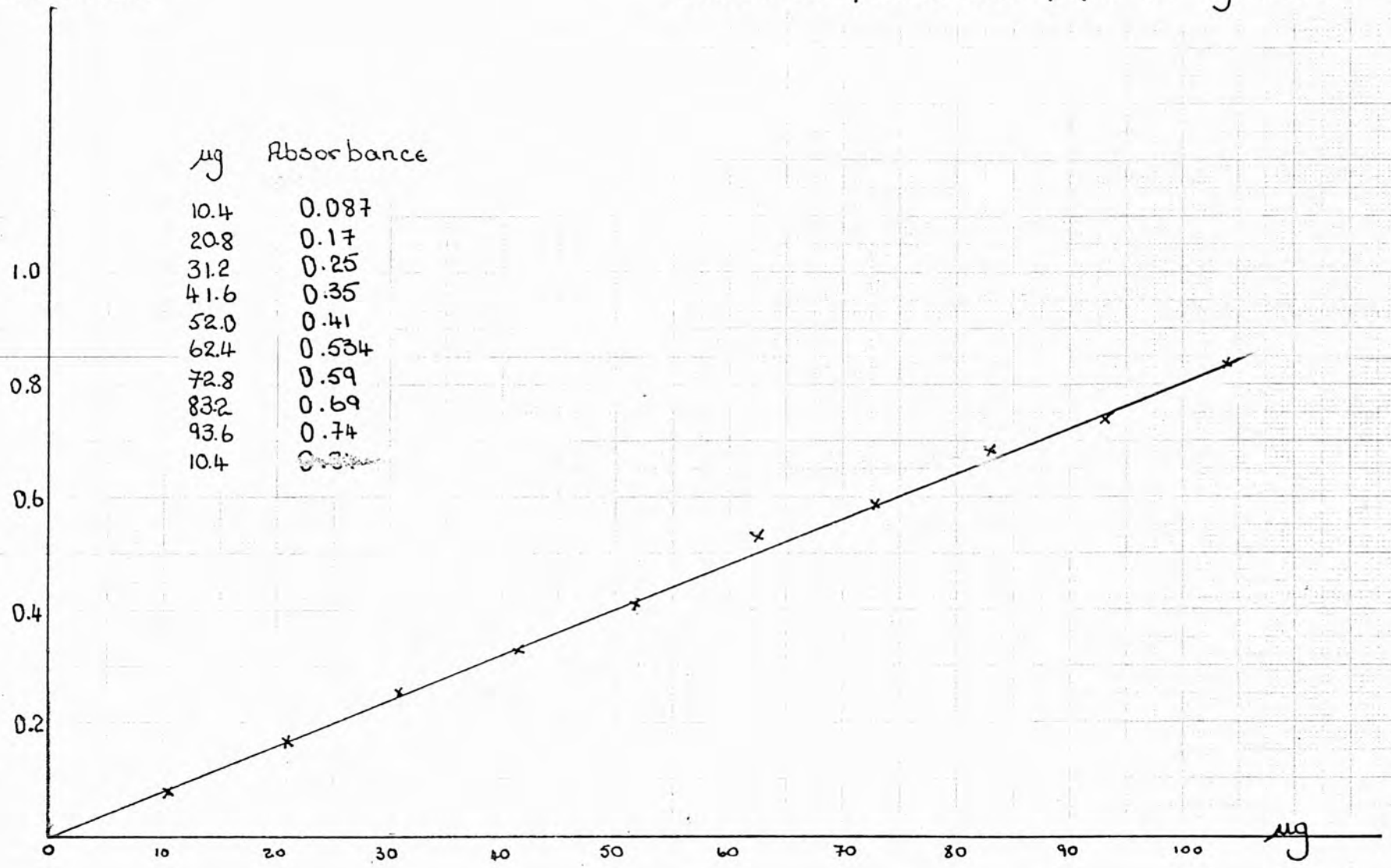
Appendix III

Standard graphs

1. Phenol–Sulphuric Acid graph Glucose
2. Phenol–Sulphuric Acid D-glycero-D-mannoheptose
3. Phenol–Sulphuric Acid L-guluronic Acid
4. Phenol–Sulphuric Acid Mannuronic
5. Phenol–Sulphuric Acid Total Polysaccharide
6. Phenol–Sulphuric Acid Acid Polysaccharide
7. Carbazole –α-guluronic acid
8. Carbazole D-glucuronic acid

Phenol-Sulphuric Acid Graph I Glucose

µg	Absorbance
10.4	0.087
20.8	0.17
31.2	0.25
41.6	0.35
52.0	0.41
62.4	0.534
72.8	0.59
83.2	0.69
93.6	0.74
10.4	[illegible]

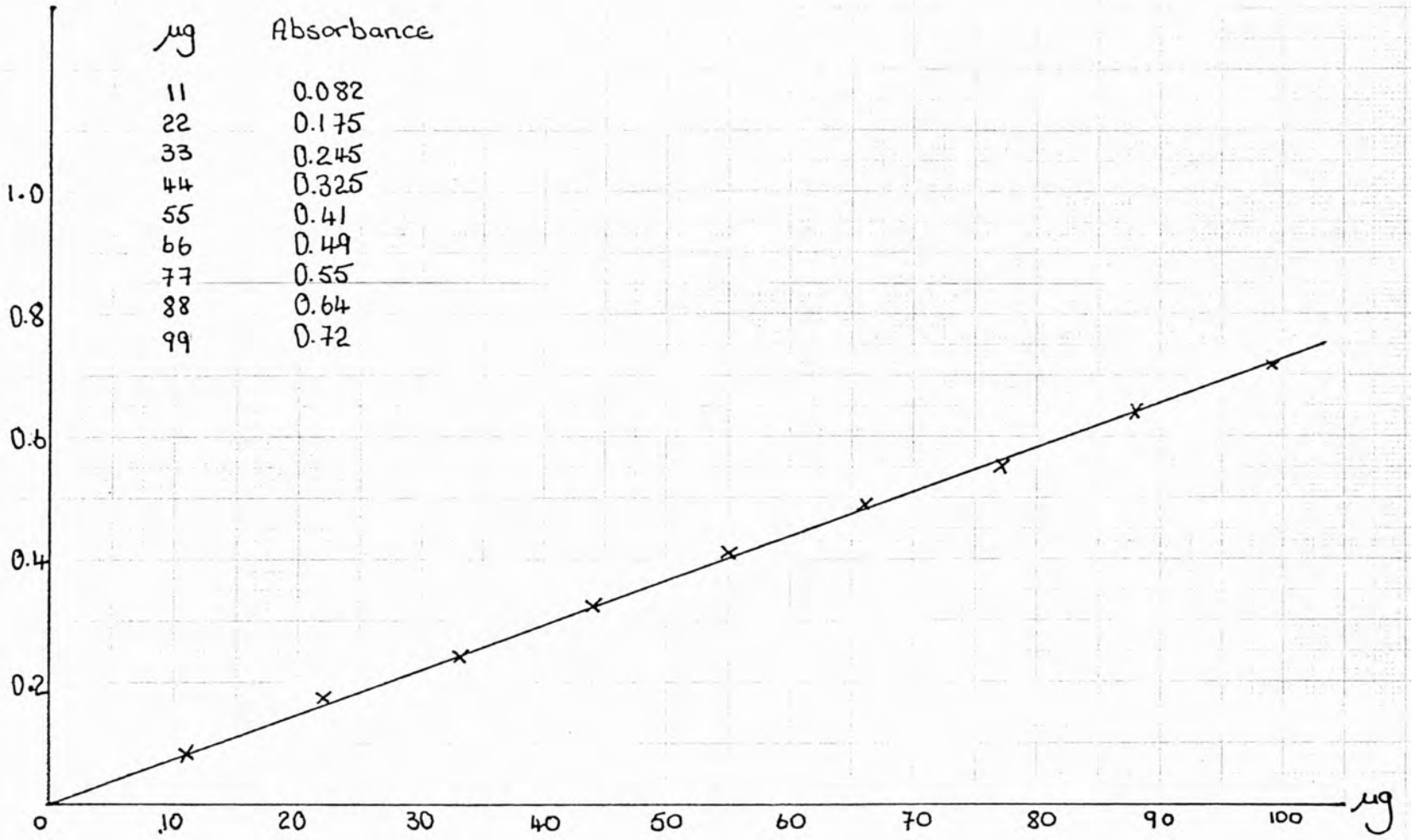


Phenol-Sulphuric Acid Graph II

D-Glycero-D-manno heptose

Absorbance

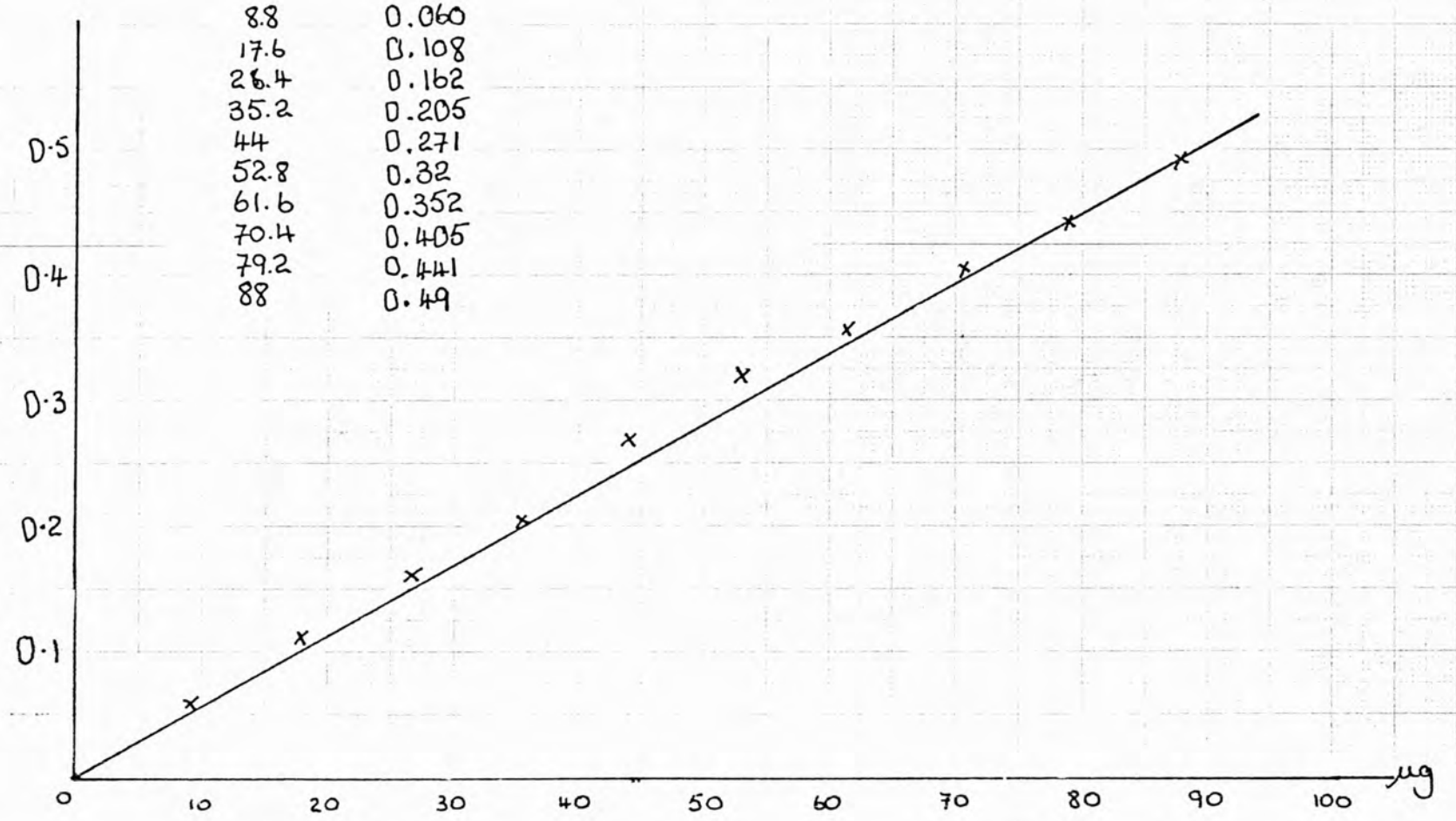
µg	Absorbance
11	0.082
22	0.175
33	0.245
44	0.325
55	0.41
66	0.49
77	0.55
88	0.64
99	0.72



Phenol- Sulphuric Acid Graph III

L-Guluronic Acid

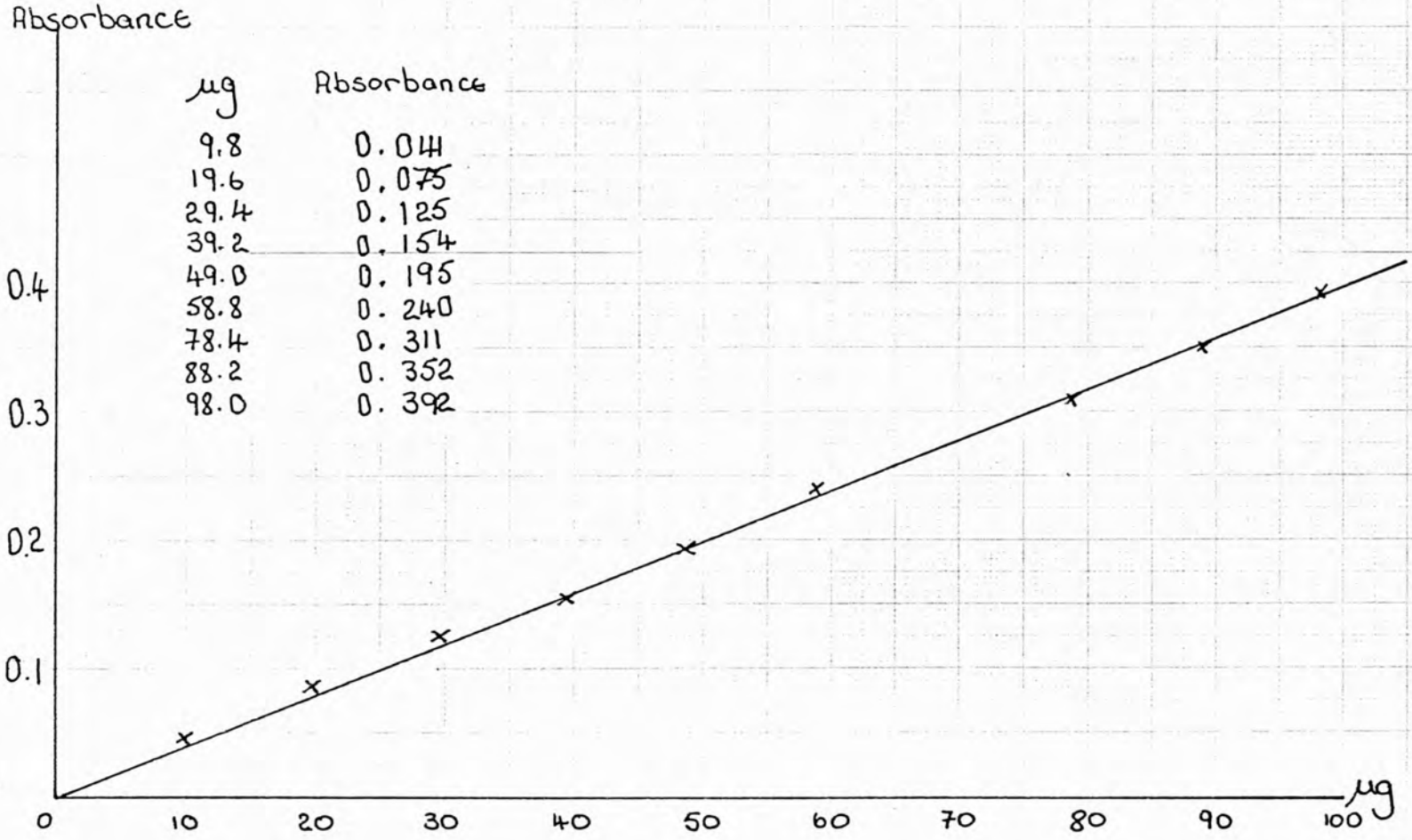
Absorbance	µg	Absorbance
	8.8	0.060
	17.6	0.108
	26.4	0.162
	35.2	0.205
	44	0.271
	52.8	0.32
	61.6	0.352
	70.4	0.405
	79.2	0.441
	88	0.49



Phenol-Sulphuric Acid Graph IV Mannurone

178

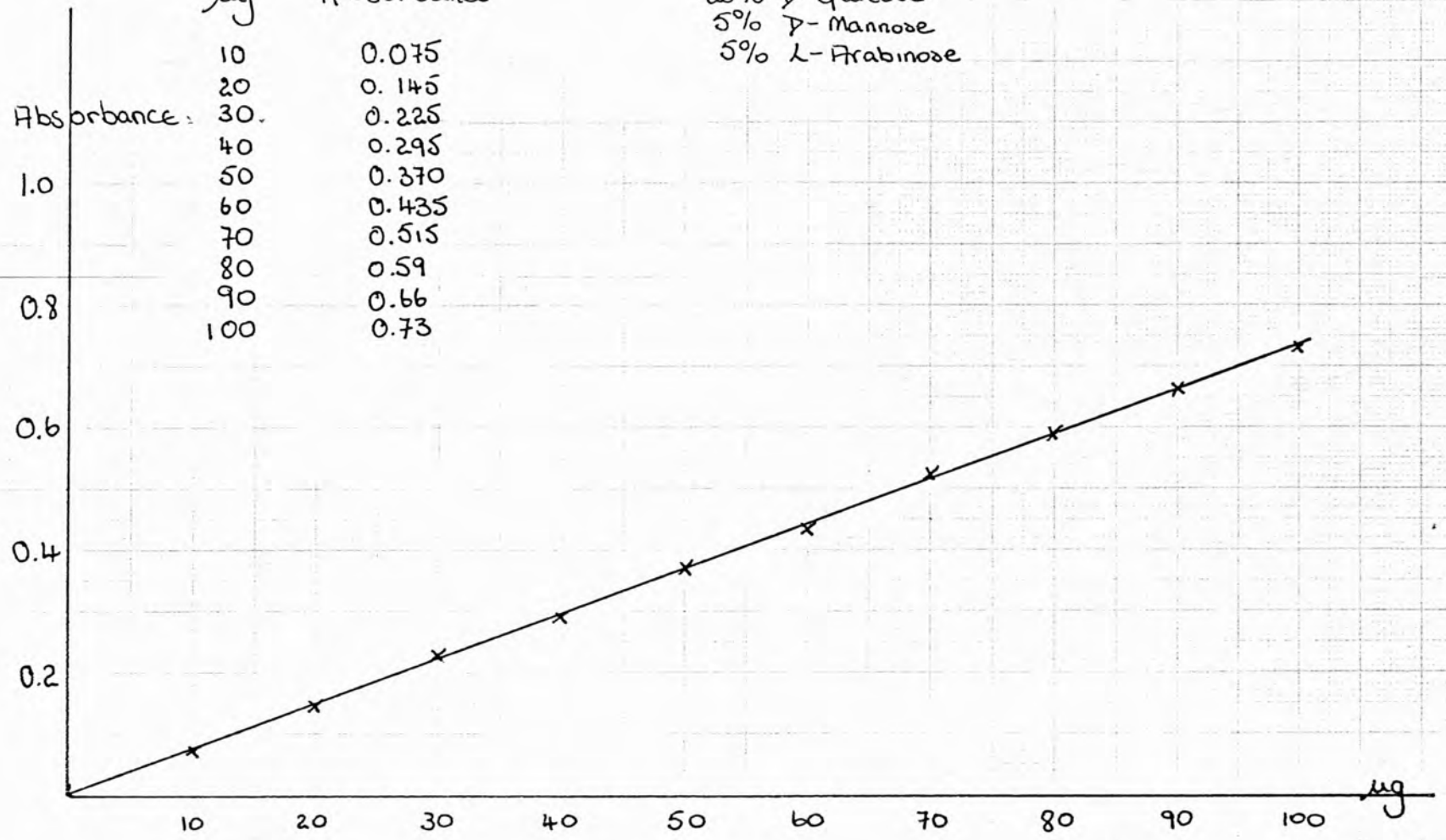
µg	Absorbance
9.8	0.044
19.6	0.075
29.4	0.125
39.2	0.154
49.0	0.195
58.8	0.240
78.4	0.311
88.2	0.352
98.0	0.392



Phenol-Sulphuric Acid Graph V

50% - Guluronic Acid
15% D-Glycero-D-mannoheptose
25% D-Glucose
5% D-Mannose
5% L-Arabinose

µg	Absorbance
10	0.075
20	0.145
30	0.225
40	0.295
50	0.370
60	0.435
70	0.515
80	0.59
90	0.66
100	0.73



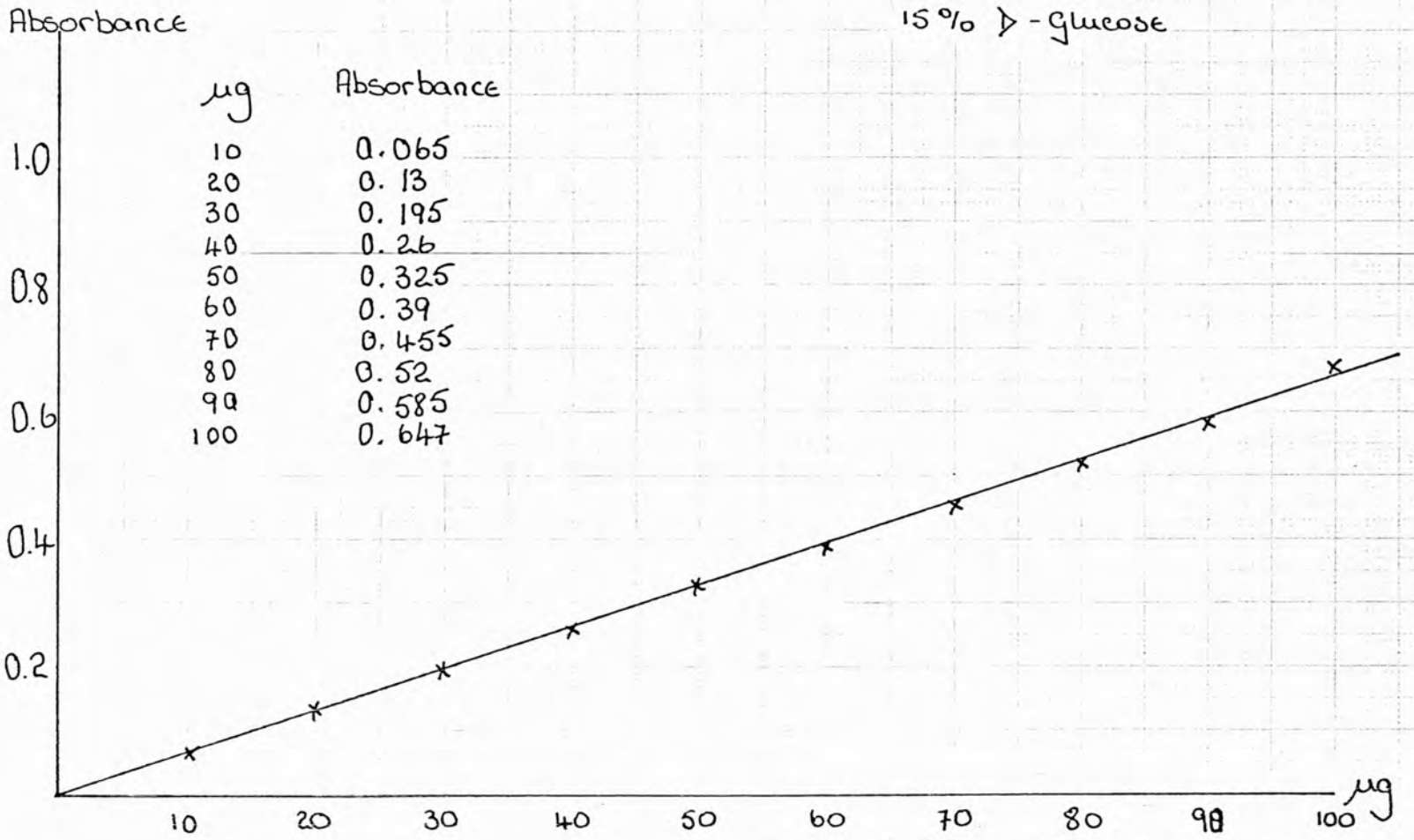
Phenol-Sulphuric Acid Graph VI

65% L-Guluronic Acid

20% D-Glycero-D-mannoheptose

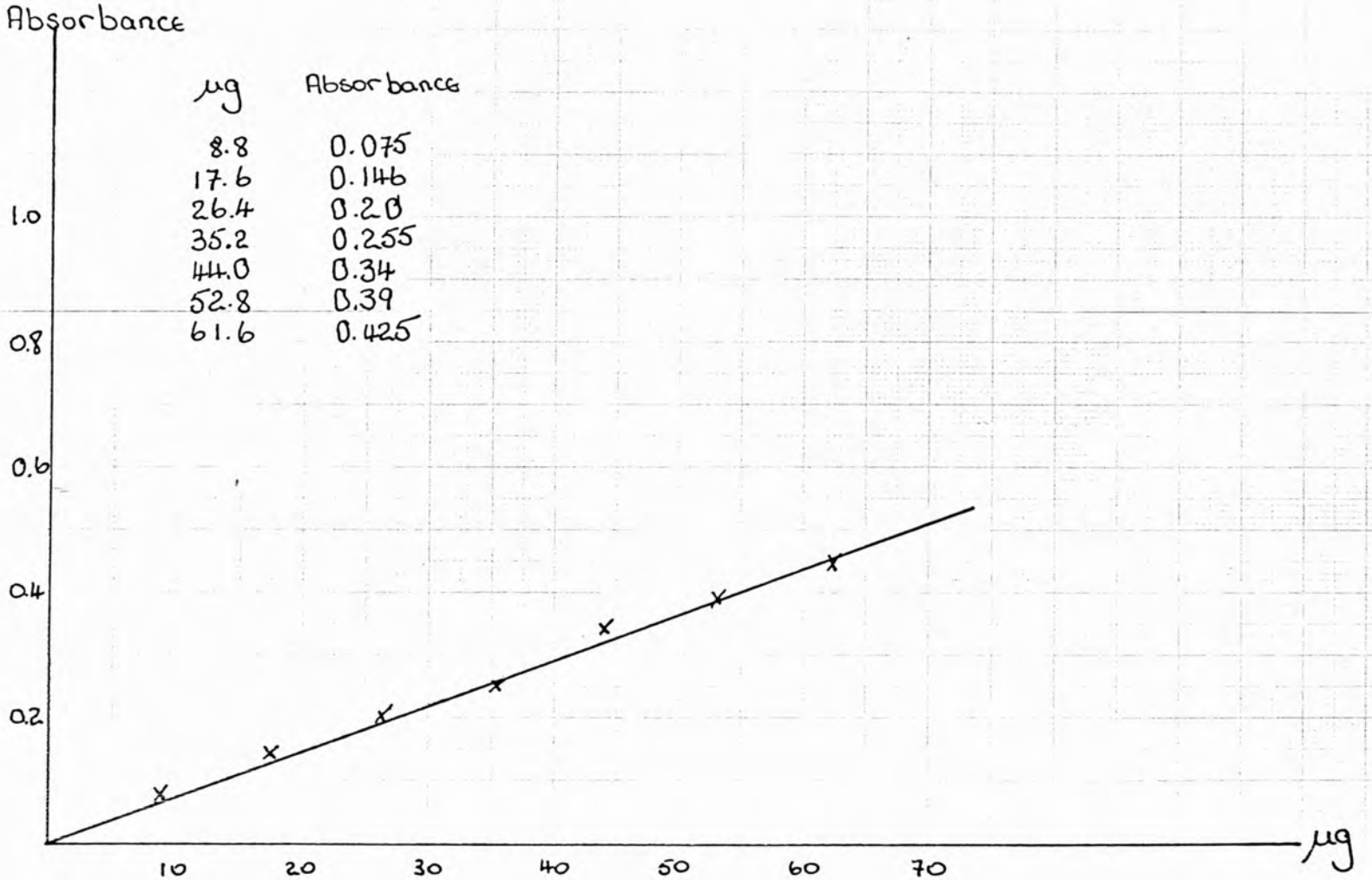
15% D-Glucose

µg	Absorbance
10	0.065
20	0.13
30	0.195
40	0.26
50	0.325
60	0.39
70	0.455
80	0.52
90	0.585
100	0.647



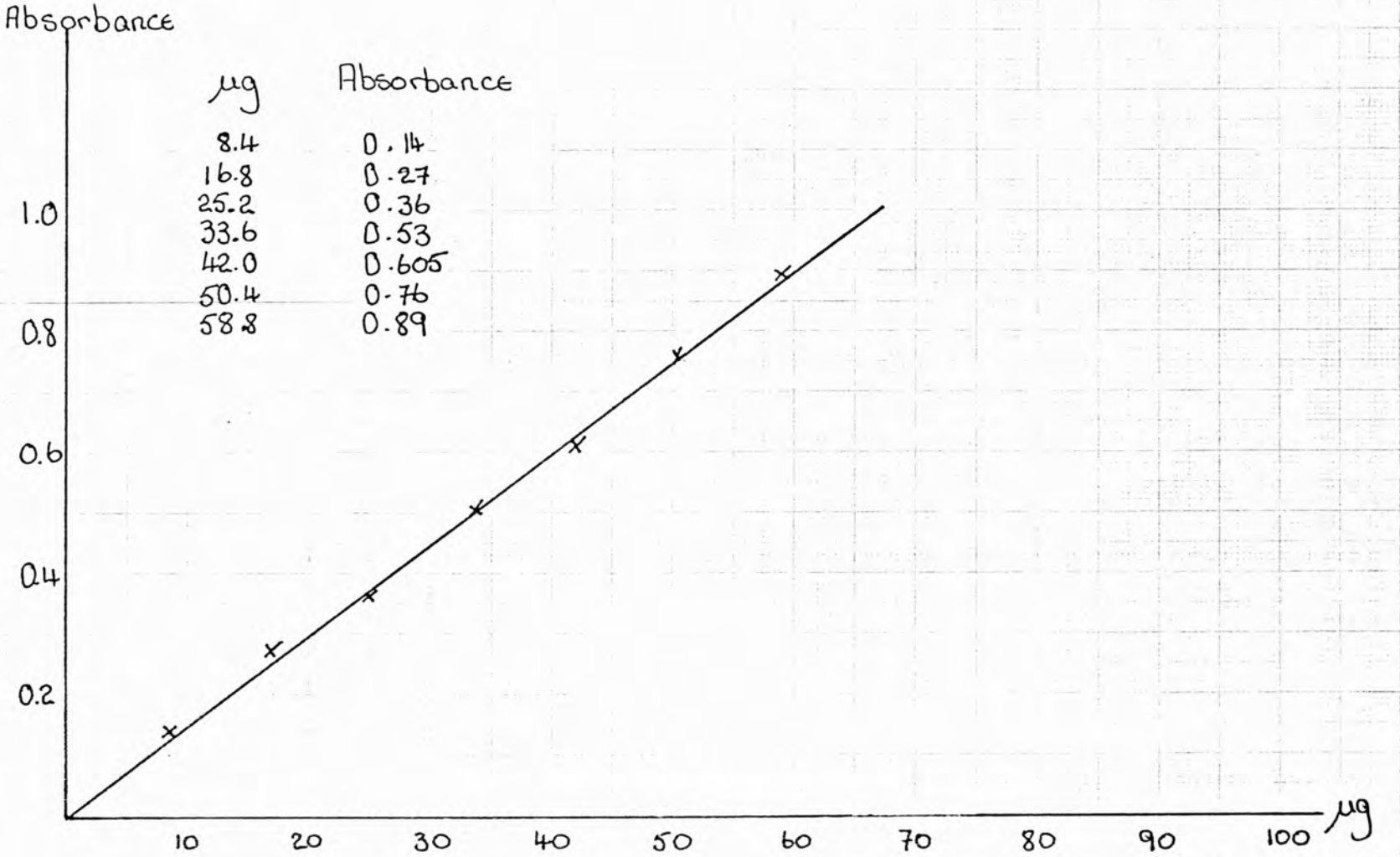
Carbazole Graph VII L-Guluronic Acid

µg	Absorbance
8.8	0.075
17.6	0.146
26.4	0.20
35.2	0.255
44.0	0.34
52.8	0.39
61.6	0.425



Carbazole Graph VIII D-Glucuronic Acid

µg	Absorbance
8.4	0.14
16.8	0.27
25.2	0.36
33.6	0.53
42.0	0.605
50.4	0.76
58.8	0.89



APPENDIX IV

Project at Product Development Department, Girvan

September - October 1971

Introduction

A characteristic property of alginates is their ability to form gels. It is well known that an essential of alginate gel formation is the conversion of a dissolved alginate to an insoluble form. Gel formation by conversion of sodium to calcium alginate is one of the important practical uses of alginates, but very little quantitative information is available on the effect of variables such as the nature of the alginate, its concentration, and the extent of conversion of sodium to calcium alginate, on the properties of the resulting gel. This work was therefore done in order to provide more information, which could be used to give a quantitative basis for the preparation of gels with required properties.

Summary of Experimental work

The experiments carried out were:

(1) The alginate was converted into alginic acid and a pH titration curve was prepared.

(2) The standard gel was prepared using acid conditions for gelation, and then the acid content, alginate content and calcium content were varied. The acid used was gluconic acid. Attempts were made to study gelation with adipic acid, but without success.

(3) Gels were prepared with different additions of

(a) (i) Sugar. A percentage of the water was replaced by sugar. A range of calcium levels was studied.

(ii) Gels containing the same proportion of alginate to water as in

3a (i) but without sugar. In some cases the formulation was scaled up in order to fill the moulds.

(b) (i) Gels were made containing varying amounts of calgon (sodium hexametaphosphate).

(ii) Calgon (0.2 g) was dissolved in the 400 ml of water to make a standard gel, and amounts of hard water added.

(iii) Other sequestrants were added to the standard gels.

Materials used

Alginates: Two samples of sodium alginate made commercially by Alginate Industries Ltd., were used. Origin and analysis (using AIL standard methods) were as follows:

TABLE 1

Sample	Weed origin	% dry solids	% sodium alginate	viscosity cps	% Calciu
A	Ascophyllum nodosum	89.5	86.5	617[a]	0.13
B	Laminaria hyperborea	91.6	88.3	50[b]	0.23

(a) 0.895% solution as dry solids

(b) 0.80% solution as dry solids

Dicalcium Phosphate Dihydrate: This was Albright and Wilson grade O.

Other Chemicals Standard laboratory chemicals were used.

1. Conversion of Sodium Alginate to Alginic Acid

Method

45 g Sodium alginate were dissolved in water (c. 2L) and 50 mls 20% $CaCl_2$ solution and 12 ml glacial acetic acid were added with vigorous

stirring, the precipitated calcium alginate was left to harden, filtered through a nylon cloth, squeezed dry and transferred to a sintered glass funnel.

The Calcium Alginate was washed with 10% hydrochloric acid until the eluent gave no precipitate with ammonium oxalate on standing for 3 minutes; the Alginic acid was washed free of hydrochloric acid with distilled water until neutral to tropeolin 0.0.

The Alginic acid was then washed several times with acetone and air dried.

A suspension of Alginic acid was prepared in 0.1N-sodium chloride to approximately 0.025N and this was titrated against 0.1N-sodium hydroxide. The pH was read after each 5 ml aliquot had been added.

Results

pKa A = 3.35 (see graph)

B = 3.65 (see graph)

The pKa of the alginic acid was taken to be the pH at which it was half neutralised. This follows from the Henderson Hasselbalch equation.

$pH = pKa + n \log \frac{\alpha}{1-\alpha}$ where α = degree of neutralisation.

pKa mannuronic acid = 3.38 guluronic acid = 3.65. The pKa value of the polyacid is expected to be higher than that of the monomer in water because the ionisation of a second carboxyl is repressed by the negative charge on the first ionised group. However in salt solution the effect of this negative charge is suppressed, thus the pKa value approaches the value for the monomer. The pKa value is dependent on the proportion of mannuronic to guluronic acid.

Alginate from	M/G	pKa (H_2O)	pKa (0.1N-NaCl)
L. digitata	1.65	3.87	3.42
L. hyperborea	.45	4.41	3.74
A. nodosum	1.6	3.77	—

The values given in this paper were slightly higher than the values obtained in the described experiment.

2. Preparation and testing of gels with the variation in concentration of ingredients

Methods:

A standard gel was considered to consist of 3.2 g Alginate as dry solids, equivalent to 3.08 g sodium alginate by titration.

0.74 g dicalcium orthophosphate dihydrate (DCP $2H_2O$)

2.3 g glucono delta lactone (G.D.L.)

Distilled water(456.8 g)including moisture content of alginate.

A solution was made of 3.2 g dry sodium alginate in 350 mls H_2O. The weight was then made up to 400 g with water.

A slurry was made of 0.74 g DCP $2H_2O$ and 2.3 g G.D.L. in 60 mls water. This was added quickly to a briskly stirred solution of alginate and the stirring continued for 30 seconds, the solution was then poured into moulds. After 15 minutes the moulds were covered and then left for 18 hours.

After 18 hours the rupture strength, gel strength, amount of syneresis and pH were measured. (Table 2).

Rupture Strength

The method made use of equipment built in A.I.L. Laboratories. A piston is pressed on to the surface of the gel and the rupture strength is the force which is required to cause the piston to penetrate the gel.

Gel Strength

A standard F.I.R.A. jelly tester is used. A paddle is inserted into the gel and the paddle is made to turn by an increasing load. The load is measured for every 10° through which the paddle moves. The gel strength is the load required to rotate the paddle through 35°.

Syneresis

Drainage of free water from the gelled mass, this was measured by weighing the gel and container, the gel was then demoulded, dried with a tissue and reweighed. The container was dried and weighed. The syneresis was calculated as the proportion of water to gel by weight.

pH

This was measured on a pH meter which was calibrated with a standard buffer at pH 4.0.

The gel was first chopped up with a counter-rotating stirrer at high speed until most of the gel structure was destroyed.

Results

(a) Variation of DCP.$2H_2O$

Values taken were 1.0, 0.9, 0.8, 0.7, 0.74, 0.6, 0.5, 0.4, 0.3 g.

TABLE 2

Alginate A

$DCP.2H_2O$ g.	Equiv. Ca^{++} (%) Equiv. Alginate	Gel strength g.	Rupture strength g.	Syneresis %
1.0	81.5	55	847	7.4
0.9	73.35	48	772	2.1
0.8	65.2	39.5	654	1.7
0.74	60.3	34	524	1.35
0.7	57.05	31	448	0.89
0.6	48.9	24	410	0.85
0.5	40.75	21.5	365	0.73
0.4	32.6	18	265	0.57
0.3	24.4	7.5	201	0.14

Alginate B

1.0	81.5	115	929	6.9
0.9	73.35	91	769	5.3
0.8	65.2	71	486	2.3
0.74	60.3	58	440	1.4
0.7	57.05	50	318	0.9
0.6	48.9	39	283	0.5
0.5	40.75	27	224	0.1
0.4	32.6	21	164	
0.3	24.4	1	134	

Gels containing 0.4 and lower g. DCP. $2H_2O$ were not demouldable and so no syneresis effect could be measured.

The pH of the gels did not differ much from 4.1 for the standard gel.

(b) Variation of G.D.L. content

Values of 1.15 g., 2.3 g. and 4.6 g were taken. Alginate content was 3.08 g. D.C.P. content 0.74 g.

TABLE 3

Alginate A

G.D.L. (g)	pH	Equivalents as % of alginate As acid (unionised from pH)	Calcium % of total % of ionised gps.	Gel strength (FIRA)	Rupture strength g	Syneresis %
1.15	4.7	8.25	66.4	34	518	0
2.3	4.1	13.0	73.5	41	545	1.05
4.6	3.9	24.25	80.3	46.5	560	1.4
		Alginate B				
1.15	4.7	9.0	66.2	38	397	0
2.3	4.1	25.0	80.4	52.5	460	2.5
4.6	3.9	34.0	91.3	77	663	4.5

(c) Variation in Aglinate content

The amounts chosen were 3.2 and 2.24 g dry solids, corresponding to standard value and 70% of standard.

TABLE 4

Alginate A

Alginate g.	pH	Equiv. Ca^{++} (%) Equiv. Alginate	Gel Strength g.	Rupture Strength g.	Syneresis %
3.2	4.1	60.3	35	509	1.1
2.24	3.95	82.97	41	545	4
		Alginate B			
3.2	4.1	60.3	61	460	2.5
2.24	3.95	82.97	91	679	10

Gelation with Adipic Acid

Gels were attempted using adipic acid instead of glucono-lactone. But whereas the lactone gives a slow release of calcium as it is hydrolysed to gluconic acid, with adipic acid the calcium ions were released too fast from the dicalcium orthophosphate. Thus the gel started to form as soon as the acid was added and stirring to give a uniform solution broke up the gel structure. It was not possible to measure gel strength as the mix could not be poured into the moulds.

The effect was much more pronounced with alginate A than alginate B. Alginate A formed a gel structure round the stirrer immediately on the addition of the acid and calcium.

With low calcium content it was possible to prepare gels with alginate B without pre-gelation. However as a complete series could not be made gel strengths were not measured.

Discussion

It will be seen that alginate B. (L. hyperborea) gave gels of higher strength than those from alginate A. (Ascophyllum) for equal ratios of calcium to alginate when they are measured by the FIRA method. On the other hand the rupture strength of the gels from A were in a number of instances higher than the corresponding gels from B.

While the FIRA instrument gives results which to a fair approximation are proportional to the rigidity modules of the gel the rupture strength is a function of both the rigidity and the extent to which the gel can be deformed before it breaks (in non-scientific language the "elasticity" of the gel) and the higher rupture strength of the gels from A is due to their

much greater deformability without breaking. Although this is a general property of gels from Ascophyllum, in the present series of experiments the effect is probably accentuated by the very much higher degree of polymerisation of A compared with B (as shown by the viscosity in solution).

It is well-known that alginates from L. hyperborea stipes have a much higher proportion of guluronic acid units than do those from Ascophyllum and that in the base equilibrium of calcium and sodium with an alginate the proportion of calcium combined with alginate is higher for high guluronic acid than for high mannuronic acid alingates.

Although equilibria in presence of acid have not been studied quantitatively it can be expected that the alginate B will still be combined with more calcium than alginate A in equilibrium conditions. The figures given in tables 2, 3 and 4 are for the available calcium as percentage equivalents of the alginate and although the greater part can be expected to be combined with both of the alginates, this amount may be slightly higher for alginate B than for alginate A. It is probable, however, that other factors are also involved in the gel strength differences, as while alginate B has a rigidity of 58 g. in a standard jelly (60 g equivalents calcium), the maximum amount of calcium used with alginate A (approx. 81.5 equivalents calcium) gave a rigidity of 55 g.

3. Gels with different additions

a (i) Sugar

A proportion of the water in the weight of the gel was replaced by sugar. The proportions chosen were 10%, 20%, 45%, 65%. The amount of alginate was kept constant at 3.08 g and the G.D.L. level at 2.3 g. The amount of D.C.P.$2H_2O$ was taken at 3 levels, 0.5 g, 0.74 g, 0.85 g.

Results

TABLE 5

Alginate A

10% Sugar

gms. DCP $2H_2O$	pH	Equiv. Ca^{++}(%) Equiv. Alginate	Gel strength g	Rupture strength g	Syneresis %
0.5	4.2	40.75	28.25	353	0.69
0.74	4.05	60.3	41	609	0.93
0.85	3.95	69.3	51.25	705	1.35
20% Sugar					
0.5	4.19	40.75	25.2	385	0.675
0.74	4.0	60.3	45.2	653	0.78
0.85	3.97	69.3	50.7	1029	1.05
45% Sugar					
0.5	4.15	40.75	43	916	1.3
0.74	4.25	60.3	92.5	1125	2.1
0.85	4.31	69.3	110.5	1409	2.7
65% Sugar					
0.5	4.15	40.75	80	1700	undetectable
0.74	4.05	60.3	118	2100	
0.85	3.89	69.3	164	2400	

Alginate B

10% Sugar					
0.5	4.07	40.75	30	300	0.85
0.74	3.97	60.3	65.5	600	1.8
0.85	3.89	69.3	92.5	947	3.7
20% Sugar					
0.5	4.1	40.75	32	390	0.27
0.74	4.0	60.3	84	788	0.9
0.85	3.9	69.3	103	1150	1.3

TABLE 5 (continued)

Alginate B

gms. DCP $2H_2O$	pH	Equiv. Ca^{++}(%) Equiv. Alginate	Gel strength g	Rupture strength g	Syneresis %
45% Sugar					
0.5	4.05	40.75	44	700	0.11
0.74	4.02	60.3	128	1038	1.35
0.85	4.0	69.3	220	1459	2.3
65% Sugar					
0.5	4.01	40.75	113	1436	undetectable
0.74	4.0	60.3	170	1646	
0.85	3.95	69.3	200	2200	

The pH of the gels containing sugar were insufficiently different from those containing the same amount of D.C.P. $2H_2O$ to consider that the degree of conversion was different.

The gels containing 65% sugar were not true solutions, in fact were saturated solutions of sugars with the remaining sugar suspended in the viscous alginate solution. These gels were very sticky and crystals of sugar were visible in them. Possibly therefore the very high results, particularly the rupture strength, is due to suspended sugar. The gels were much more rubbery, and on demoulding the surface was sticky, possibly due to syneresis containing sugar. However this could not be measured.

(ii) Gels with same proportion of alginate to water but no sugar

These gels were prepared for comparison purposes to investigate whether the sugar strengthened the gel, or whether an increase in gel strength came from the increased proportion of alginate to water.

Most of these gels had to be scaled up in order to fill the measuring cubes to the correct level. All the ingredients were scaled up in proportion, and the DCP $2H_2O$ level was kept as in gels containing sugar.

TABLE 6

Alginate A

Equivalent to 10% sugar content

Proportion of Alginate to water increased by 11.5%

gms. DCP $2H_2O$	pH	Gel strength g	Rupture Strength g	Syneresis %
0.5	3.8	27.75	360	0.69
0.74	4.15	39	674	1.1
0.85	4.2	54.75	832	1.85

Equivalent to 20% sugar content

Proportion of Alginate to water increased by 26%

0.5	4.02	58.5	443	0.55
0.74	3.99	48.7	746	0.78
0.85	3.80	65.5	877	1.27

Equivalent to 45% sugar content

Proportion of Alginate to water increased by 82%

0.5	3.85	59.5	1,298	0.56
0.74	3.84	107.5	1,834	0.72
0.85	3.83	138	2044	1.0

Equivalent to 65% sugar content

Proportion of Alginate to water increased by 188%

0.5	3.87	147	2550	0.3
0.74	3.84	226	3042	0.3
0.85	3.82	Too strong to measure	3446	0.3

TABLE 6 (continued)

Alginate B

gms DCP $2H_2O$	pH	Gel strength g	Rupture Strength g	Syneresis %
Equivalent to 10% sugar content				
Proportion of Alginate to water increased by 11.5%				
0.5	3.71	52	285	1.3
0.74	3.75	81	477	2.0
0.85	3.82	111	775	4.8
Equivalent to 20% sugar content				
Proportion of Alginate to water increased by 26%				
0.5	3.78	37	316	1.1
0.74	3.8	92.5	645	2.5
0.85	3.81	127	1035	5.1
Equivalent to 45% sugar content				
Proportion of Alginate to water increased by 82%				
0.5	3.75	83	626	0.68
0.74	3.88	198	1332	1.0
0.85	3.9	219	1928	0.38
Equivalent to 65% sugar content				
Proportion of Alginate to water increased by 188%				
0.5	3.75	Too strong to	2163	
0.74	3.77	measure	3530	
0.85	3.78		4392	

Discussion

A standard gel is about a 0.8% solution, but with 65% of the water removed the gel is a 3.3% solution and so a large increase in the strength of the gel is expected. If gels with the same proportion of alginate to

water are compared those without sugar are stronger than the gels containing sugar. The available figures would enable comparisons to be made between gels with and without sugar, with an equal amount of alginate per unit volume. This would indicate whether other factors, such as availability of calcium, or the binding force between alginate chains, is affected by the sugar.

b (i) The addition of sequestering agents

Materials used

There are three main types:

(a) Phosphates e.g. Calgon (glassy sodium meta phosphate)
Sodium tripolyphosphate

(b) Organic amine derivatives e.g. EDTA

(c) Salts of organic acids e.g. Sodium Citrate

(ii) Calgon in distilled water

Gels in practice will often be made up with tap water which contains varying degrees of hardness. However for comparison purposes a calcium sequesterant was included in distilled water as well as water of different degrees of hardness. The amounts of Calgon given were dissolved in 400 g water. Gels were otherwise made to standard method.

TABLE 7

Alginate A

Calgon	pH	Gel strength g	Rupture strength g	Syneresis g
0.05	3.8	30.0	540	1.4
0.1	3.82	33	598	1.4
0.2	3.81	24.2	510	1.4
0.3	3.9	23.5	480	1.4
		Alginate B		
0.05	3.69	67	433	5.5
0.1	3.72	75	450	5.5
0.2	3.71	54	470	5.5
0.3	3.78	47.5	387	5.5

These figures appear to be slightly anomalous. 0.05 g gives very little difference in strength of gel. The strength then increases slightly and reaches a peak, after which the level falls.

A large increase in syneresis is noted.

(iii) Calgon in hard water

0.2 g of Calgon were dissolved in the 400 g of water together with varying amounts of water containing (100 - 600) ppm Ca CO_3. Other components of the gels were kept standard.

TABLE 8

Alginate A

Degree of hardness	pH	Gel strength g	Rupture strength g	Syneresis %
100 ppm	3.9	42	476	0
200 ppm	3.9	53	649	0
300 ppm	3.85	59	704	0.86
400 ppm	3.8	58.7	817	1.67
500 ppm	3.8	75	980	1.57
*600 ppm	3.8	76	741	0.77

TABLE 8 (continued)

Alginate B

Degree of hardness	pH	Gel strength g	Rupture strength g	Syneresis %
100 ppm	3.95	26.5	420	0
200 ppm	3.90	25.7	487	1.17
300 ppm	3.05	28.5	584	2.09
400 ppm	3.80	29.0	983	6.32
500 ppm	3.75	30.5	837	7.11
*600 ppm	3.70	46	1,131	7.2

* for 600 ppm 0.3 g calgon were used but the gels were very grainy and it is thought that some presetting had taken place especially with Alginate A.

These results do not seem to make much sense.

A large increase in syneresis was noted.

(iv) Other sequesterants

0.05 and 1 g of other sequestering agents were added to 400 g water. The gels were then made up by the standard method.

Agents used Tetron, Puron, EDTA, Sodiumtripolyphosphate, Sodium Citrate

TABLE 9

Alginate A

Agent	pH	Gel strength g	Rupture strength g	Syneresis %
0.05 g Puron	3.95	34.5	555	0.7
0.1 g Puron	3.90	45	575	0.9
0.05 g Tetron	3.97	44	463	0.65
0.1 g Tetron	3.98	41	535	0.85
0.05 g EDTA	3.9	41	430	0.82
1 g EDTA	3.89	45	475	0
0.05 g S.T.P.P.	3.82	45	524	0.85
0.1 g S.T.P.P.	3.9	40.5	530	0.79
0.05 g NaCitrate	4.05	36	422	.9

TABLE 9 (continued)

Alginate B

Agent	pH	Gel strength g	Rupture strength g	Syneresis %
0.05 g Puron	3.84	57	285	2.7
0.1 g Puron	3.83	60	400	3.3
0.05 g Tetron	3.90	72.5	320	3.0
0.1 g Tetron	3.89	83	275	3.5
0.05 g EDTA	3.85	86	424	2.5
0.1 g EDTA	3.82	61	412	2.7
0.05 g S.T.P.P.	3.8	88	270	3.0
0.1 g S.T.P.P.	3.9	73	264	1.8
0.05 g Na Citrate	3.9	61	432	1.45
0.1 g Na Citrate	3.95	56	426	1.85

Addition of these small quantities gave a very large increase in syneresis, a moderate increase in gel strength and little or no increase in rupture strength.

4. Conversion of Alginic Acid to salts

The alginic acid previously prepared was divided into 3 parts and weighed. The amount taken was to give a weight equivalent to 3.2 g of the dry salt.

The acid was wetted to about 25% moisture content and then ground up in a pestle and mortar with the carbonate salt of sodium, potassium or magnesium until the pH was about 6.0 – tested with Universal indicator.

The soft dough was then dissolved in water to make the total weight 400 g and gels were made in the standard way.

TABLE 10

Alginate A

	pH	Gel strength g	Rupture strength g
Sodium alginate	3.86	19	105
Potassium alginate	4.0	14.5	116
Magnesium alginate	4.4	8	29
Alginate B			
Sodium alginate	3.95	39	76
Potassium alginate	4.9	28	62
Magnesium alginate	4.6	15.4	33

None of the gels were demouldable so no syneresis effect could be measured. There appeared to have been a considerable amount of degradation of the alginic acid resulting in loss of gel strength.

However, it would seem from the figures that sodium alginate gives a higher gel strength than the potassium or magnesium salts.

APPENDIX V

Report on work carried out in Girvan, September - October 1972

Physical properties of Strain 11 (Extracellular polysaccharide of Beijerinckia mobilis).

Introduction

The extracellular polysaccharide of B. mobilis forms a highly viscous aqueous solution. The polysaccharide can be fractionated into acid and neutral constituents. The physical properties of the acid polysaccharide were compared with those of the unfractionated material.

Polysaccharide Samples

The crude material (S11E) had a nitrogen content of 3%. Centrifugation of the aqueous solution removed some protein and the nitrogen content was then found to be 0.5%. This material was used in the tests and called 'original polysaccharide'.

Fractionation

a) The material S11 E was fractionated as in Experiment XIX 3b (yield 8 g from 25 g). Only the fraction (A1) was used.

b) The material from batch S11E was also purified by Experiment XIX 3c (yield 16 g from 25 g) and used only in the viscosity/speed of spindle test. This polysaccharide appeared to have a higher viscosity than the acid polysaccharide obtained from the fractionation (a). This could be due either to higher grade material or to the different method of purification as few results are available of viscosities on the 'original' material of this batch.

This fractionated acid polysaccharide is referred to in the tests as 'Acid' material.

To eliminate differences in properties caused by varying calcium contents, the sodium salts of both the original and acid forms were prepared.

The mixed salt form was converted to the acid form by leaching acetone-precipitated material with a hydrochloric acid/acetone mix the excess acid was removed with an acetone/water mix. A portion of the acid form was retained, but the remainder was dissolved and the pH adjusted with either sodium hydroxide or calcium hydroxide to produce the pure sodium or calcium forms.

The pure form was then precipitated with acetone and left to dehydrate. Dry matter analysis was carried out on all forms of the original material, but only on the sodium form of the acid material due to lack of material, the calcium and acid forms of the acid material were assumed to be similar to those of the sodium form and were set at 80%. A rough check was carried out on the solids content of the calcium and acid forms by evaporating to dryness 10 mls, 1% solution weighed accurately and then drying in an oven for an hour in the usual way. These confirmed that the dry matter was about 80%.

The following tests were then performed on the samples:

1. Variation of viscosity with concentration.
2. Variation of viscosity with salt concentration. Both sodium and calcium chloride were added at constant polymer concentration.
3. Effect on viscosity of lowering the pH to 2.0 with hydrochloric acid. This was not carried out on the acid form.

4. Change in viscosity of selected samples from sections 1-3 after heating and cooling.

5. Attempted gelation using a method analogous to that used with alginates.

6. Variation of viscosity of a solution using different speeds of rotation on the Brookfield L.V.T.

Na^+ form (i) refers to acid Strain 11 in the sodium form

Na^+ form (ii) refers to original Strain 11 in the sodium form

	Original material	Acid material
N_2	2.61%	.63%
DM	82.2	79.5% (sodium form)
	85.25	~ 80% calcium form
	84.73	80% acid form

RESULTS

1. Viscosity concentration

Brookfield L.V.T measurements, T = 20°C, speed 60

No's in brackets are spindle numbers.

Table 1	1% DM	0.75% DM	0.5% DM	0.25% DM
Na^+ form (i)	123(2)	57.5(2)	32.5(2)	21.5(2)
Na^+ form (ii)	960(3)	510(3)	150(3)	69(2)
H^+ form (i)	-	1560(4)	274(3)	27(2)
H^+ form (ii)	-	9000(4)	1,250(4)	50(2)
Ca^{++} form (i)	2,190(4)	778(3)	130(3)	18.5(2)
Ca^{++} form(ii)	-	>10,000	1,200(4)	50(2)

2. Effect of Addition of salt on 0.5% DM Solution

The salt was added to 25 g of 1% DM solution in the form of aliquots of 10% w/v salt solution and the weight made up to 50 g.

Brookfield L.V.T. measurements Viscosity T = 20°C Speed 60

Table 2

	NaCl				$CaCl_2$			
	0.25%	0.5%	1%	2%	0.25%	0.5%	1%	2%
Na^+ form (i)	33(2)	191.5(2)	1,120 (3)	3,250 (4)	650(3)	1,350(4)	2,500(4)	3,500(4)
Na^+ form (ii)	130(2)	610(3)	3470 (4)	600* (4)	980(3)	1,430(3)	750*(3)	520*(3)
H^+ form (i)				1,500 (4)				2,800(4)
H^+ form (ii)				1,100* (4)				500*(4)
Ca^{++} form (i)				1,160* (4)				1,160 (4)
Ca^{++} form (ii)				1,280* (3)				300*(3)

* denotes broken or soft gel.

Table 3

Effect of varying the pH

Brookfield L.V.T. measurements T = 20°C Speed = 60 rpm

Na^+ form (i) 0.5% DM

pH	Viscosity
6.0	32.5 (2)
4.6	30 (2)
3.9	36.5 (2)
3.45	430 (3)
3.3	690 (3)
3.1	1,430 (4)
3.0	1,350 (4)
2.85	1,190 (4)
2.55	1,120 (4)

Na^+ form (ii) 0.25% DM solution

pH	Viscosity
5.7	57.5 (2)
3.5	294.0 (2)
3.2	720 (3)
3.0	1,800 (3)
2.6	1,350 (4)
2.4	450* (4)

Table 3 (contd)

Ca^{++} form (i) 0.5% DM		Ca^{++} form (ii) 0.25% DM	
pH	Viscosity	pH	Viscosity
6.7	118 (3)	6.6	44 (2)
4.5	122 (3)	4.0	60 (2)
3.8	164 (3)	3.8	87.5 (2)
3.3	280 (3)	3.4	162.5 (2)
3.0	740 (3)	3.1	440 (3)
2.8	532 (3)	2.9	610 (3)
2.5	508 (3)	2.65	500 (3)
2.0	494 (3)	2.4	190 (3)
		2.25	155 (2)

4. Change in Viscosity on heating

The samples were placed in a waterbath at 95°C for 10 minutes. The maximum temperature reached was 90°C. The solutions were cooled to 20°C in water.

The samples selected were:

A. 0.5% DM solution

B. 0.5% DM solution containing 2% NaCl.

C. 0.5% DM solution containing 2% $CaCl_2$.

D. 0.5% DM solution at pH 2.0. With the crude samples the solution was 0.25% at pH 2.5.

Table 4

Brookfield L.V.T. Measurements T = 20°C Speed = 60 rpm

	Viscosities							
	A		B		C		D	
	before	after	before	after	before	after	before	after
Na^+ form (i)	32.5(2)	30(2)	3250(4)	2150*(4)	560*(4)	3150(4)	1350(4)	950(4)
Na^+ form (ii)	150 (3)	45*(2)	600*(4)	10,000	520*(3)	10,000	450*(2)	165*(2)
H^+ form(i)	274 (3)	1,060(3)	1,500 (4)	3,560(4)	2800(4)	4630(4)	———	
H form (ii)	1,250 (4)	1,310(3)	1,100*(4)	350(4)	500*(4)	9,600(4)	———	
Ca^{++} form (i)	130 (3)	450(3)	1,160 (4)	3,250(4)	1,160 (4)	3,930(4)	508(3)	930(4)
Ca^{++} form (ii)	1200 (4)	1,090(3)	1,280(4)	10,000	300*(3)	10,000	155(2)	850(3)

5. Attempted Gelation

The method used was standard alginate method based on the formula:

0.665% S11 DM Purified form

0.160% DCP $2H_2O$

0.49% G.D.L.

The gel was made in a beaker as there was unsufficient material to use a cube. A gel was formed but it was very soft and not demouldable. No quantitative measurements were taken but the gel was of a similar consistency to those produced simply on the addition of calcium chloride to a solution of the polymer.

6. Variation of Viscosity with speed of Brookfield L.V.T. at 20°.

Table 5 -

Spindle speed	Viscosity
.6 (2)	3,650
1.5 (2)	3,500
3 (3)	3,600
6 (3)	3,200
12 (3)	3,191
30 (3)	2,436
60 (3)	1,844

Numbers in brackets refer to Spindle numbers. The reading obtained on the Viscometer at Speed 0.3 was too low to be accurate.

This experiment was carried out on an acid form of S11 which was purified in Girvan by a variation of the method used at R.H.C.

In order to minimise the degree of degradation to the available purified polymer no tests were carried out which involved autoclaving or sterilizing solutions of the material by making the pH alkaline, as both of these have previously been found to lead to degradation.

Comments

In general the viscosity of sodium forms were the lowest in all the tests and that of the acid form highest. However the viscosity of the acid form is the same order of magnitude as that of the calcium form and these are 10x viscosity of sodium form. This does not hold at very low concentrations when all viscosities are below 50 cps.

Viscosities of the original material were much higher than those of the acid form.

Effect of increasing salt on sodium form

With the original material viscosity reaches a peak and gel formation occurs. Gel formation does not occur up to a level of 2% salt, but it is possible that gelling might occur at higher salt concentration. The characteristics of the curve of viscosity v. salt content are however different. With the original material the viscosity rises sharply on addition of a small amount of salt and falls rapidly on formation of broken gel. The curve for the acid material is a more gradual increase up to 2% salt.

The maximum viscosity obtained before gel formation of the original material is similar whether sodium chloride or calcium chloride is used, but less calcium chloride is required to give the same effect.

Mechanical stirrers were used for dissolving the polymers in salt solution and formation of soft gel was prevented by stirring. However gel structure could be seen in the solution and this was generally indicated by a marked drop in viscosity.

Due to lack of material the only salt levels investigated for the calcium and acid forms were the 2% levels.

Addition of sodium chloride or calcium chloride to acid material caused a large increase in viscosity of the order of 10x the original viscosity.

With the original material a broken gel was formed, and it is thought that similar characteristics would be obtained if the complete range of salt contents was carried out for the calcium and acid forms to those obtained for sodium form.

It is possible that part of the salt effect on the original material could be due to the high percentage of protein present.

This could have to effects:

a) effect of salt on protein itself

b) relatively higher proportion of salt to polysaccharide because there is effectively less polysaccharide present.

Effect of reducing pH by adding acid

This was carried out on sodium and calcium forms only. It was found that viscosities became too high to measure with the original form of 0.5% DM so the test was carried out at 0.25% DM on the original material in order to get the same range of pH.

For both calcium and sodium forms a viscosity maximum is reached at pH 3 which is the normal pH of the acid form. The solution then forms a broken gel with resulting loss in viscosity.

The effect on the sodium for, both original and acid, was much more pronounced than that on the calcium form.

Increase in viscosity at maximum for sodium form was about 50x original viscosity but for calcium form increase was between 5 and 10x original viscosity.

These trends were followed by both original and acid materials.

Effects of heating

Heating the sodium form causes a drop in viscosity unless salt is present in which case a gel is formed which is quite strong. The solutions were not stirred after heating so the gel structure was not broken down at all and gave a reading on the viscometer of greater than 10,000.

The gel was not demouldable. Heating the acid and calcium forms with and without salt causes an increase in viscosity. However those containing salt form quite a stable gel, similar to that obtained with sodium form.

A difference between sodium and calcium forms was noted here. On heating the acidified form of both sodium and calcium, the viscosity of the sodium form decreased, but that of the calcium form increased. It is thought to be due to the formation of an unbroken gel of the calcium form.

These observations were the same for both crude and purified material.

Experiment

Attempted gelling of acid form on the same formulation as alginate gels. As S11(purified) has a high proportion of guluronic acid, it was thought that it may have gelling properties similar to those of the G blocks of alginate.

However the gel formed was not very strong. It was not demouldable and there was no evidence of syneresis.

The gel was no stronger than those obtained by adding salt to the solution of polymer and not as strong as those obtained after heating salt containing solutions.

Insufficient is known of the detailed structure of S11 but the presence of pyruvic acid, possibly acetyl groups and a neutral sugar unit could interfere with the gelling properties particularly if the guluronic acid units are not mutually linked, as in the G blocks of alginates.

Measurement of Viscosity with different speeds

The viscosity as measured does vary with speed of viscometer spindle. Wherever possible speed 60 was used so that the results would be fairly consistent.

Conclusions

The original and acid materials show a good many similarities, although in general the viscosities of the original materials were much higher.

The effect on lowering the pH was the same as was the effect of heating the solution.

A difference was noted in the effect of salt on the solutions.

Addition of salt causes gelling with the original material, but did not occur at the concentrations of salt used with the acid form.

References

1. H. Halvorson in 'The Bacteria' eds A.C. Gunsalus and R.Y. Stanier, Vol. IV p.223
2. J. Pochon, Y.T. Tchan and T.L. Wang, Ann. Inst. Pasteur, 74 (1948) 182.
3. H.C.J. Gram, Fortsch. Med., 2 (1884) 185.
4. H. Henry and M. Stacey, Nature (London), 151 (1943) 671.
5. H. Henry and M. Stacey, Proc. Roy. Soc. London, B, 133 (1946) 391.
6. P. Mitchell and J. Moyle in 'Bacterial Anatomy', (1956) Cambridge University Press p.150.
7. C.S. Cummings, Intern. Rev. Cytol. 5 (1956) 25.
8. J-M. Ghuysen, J.L. Strominger and D.J.Tipper, Comprehensive Biochemistry, 26A (1968) 53.
9. J. Baddiley J. Roy. Inst. Chem., (1962) 366
   Federation Proc., 21 (1962) 1804.
10. M.D. Lilly, P.H. Clarke and P. Meadow, J. Gen. Microbiology, 32 (1963) 103.
11. B.A. Newton, Bact. Rev., 20 (1956) 14.
12. C.R. Amies, Brit. J. Exptl. Pathol., 32 (1951) 259.
13. E. Englesburg and J.B. Levy, J. Bacteriol., 67 (1954) 438.
14. E. Englesburg, T.H. Chen, J.B. Levy, F.E. Foster and K.T. Meyes, Science, 119 (1954) 413.
15. J. Tomesik in 'Bacterial Anatomy', Cambridge University Press, (1956) p.41.
16. S. Levene, H.J.R. Stevenson,E.C. Tabor, R.H. Bordner and L.A. Chambers, J. Bacteriol., 66 (1953) 664.
17. E.J. Bourne and H. Weigel, in 'Methods in Carbohydrate Chemistry V eds. R.L.Whistler (1965) 78.
18. E. Ito, and J.L. Strominger, J. Biol. Chem. 239 (1964) 210.
19. J.F. Petit, E. Munoz and J-M Ghuysen, Biochemistry, 5 (1966) 2765.
20. M. Leyh-Bouille, J-M Ghuysen, D.J. Tipper and J.L. Strominger, Biochemistry, 5 (1966) 3079.
21. C.S. Cummings and H. Harris, J. Gen. Microbiol. 14 (1956) 583.
22. P. Brookes, A.R. Crathorn and G.D. Hunter, Biochem J., 73 (1959) 396.
23. P.A. Albertsson, Biochim. Biophys. Acta., 27 (1958) 378.

24. J.J. Armstrong, J. Baddiley and J.G. Buchanan, Biochem J., 76 (1960) 610. Biochem J., 80 (1961) 254.

25. S.A. Barker and P.J. Somers in 'The Carbohydrates' ed. Pigman and Horton IIB (1970) 575.

26. O. Luderitz, K. Jann and R. Wheat, Comprehensive Biochemistry, 26A (1968) 105.

27. O. Luderitz, A.M. Staub and O. Westphal, Bacteriol. Rev., 30 (1966) 192.

28. N. Sharon, Ann. Rev. Biochem., 35 (1966) 485.

29. F. Kauffman, O. Luderitz, H. Steirlin and O. Westphal, Zentr. Bakteriol. Parasitenk Abt. I. 178 (1960) 442.

30. F. Kauffman 'Die Bakteriologie der Salmonella Species', Munksgaard Copenhagen (1961).

31. F. Kauffman in 'The world Problem of Salmonellosis' ed E. van Oye Junk Den Haag 1964.

32. O. Westphal and K. Jann in 'Methods in Carbohydrate Chemistry' V eds. R.L. Whistler (1965) p.83.

33. C.G. Hellerquist, B. Lindberg, K. Samuelsson and R.R. Brubaker Acta Chem. Scand., 26 (1972) 1389.

34. J.F. Wilkinson, Bact. Rev., 22 (1958) 46.

35. M. Stacey and S.A. Barker in 'Polysaccharides of Microorganisms', Oxford University Press, 1960 p.24.

36. R.E. McKinney, J. Bactariol., 66 (1953) 453.

37. J.P. Duguid, J. Pathol. Bacteriol., 63 (1951) 673.

38. F. Kauffman 'The Bacteriology of the Enterobacteriaceae' , Munksgaard Copenhagen 1966.

39. S.S. Bhatnagar, Brit. J. Exptl. Pathol., 16 (1936) 375.

40. S.S. Bhatnagar, C.G.J. Speechly and M. Singh, J. Hyg., 38 (1938) 663.

41. K. Jann and Munder see Comprehensive Biochemistry 26 (1968) p.196.

42. T.W. Burrows and G.A. Bacon, Brit. J. Exptl. Pathol. 37 (1956) 481.

43. W.O. Lawton, R.L. Erdman and M.J. Surgalla, J. Immunol., 91 (1963) 179.

44. S. Ben-Efraim, M. Aronson and L. Bichowsky-Slomnicki, J. Bacteriol., 81 (1961) 704.

45. E.S. Andersen and A.H. Rogers, Nature (London) 198,(1963) 714.

46. M.H. Proctor, Nature (London), 184 (1959) 1934.

47. O. Wyss, M.G. Neumann, and M.D. Socolofsky, J. Biophys. Biochem. Cytol., 10 (1961) 555.

48. L.P.T.M. Zevenhuizen, Carbohyd. Res., 6 (1968) 310.

49. J.P. Martin, J. Bacteriol., 50 (1945) 349.

50. J.P. Duguid and J.F. Wilkinson, J. Gen. Microbiol., 9 (1953) 174.

51. J.F. Wilkinson, J.P. Duguid and P.N. Edmunds, J. Gen. Microbiol., 11 (1954) 59.

52. J. Farrell and A. Rose, Ann. Rev. Microbiol., 21 (1967) 101.

53. A. Markovitz and S. Sylvan, J. Bacteriol., 83 (1962) 483.

54. A. Markovitz, J. Bacteriol., 83 (1962) 483.

55. J.F. Wilkinson, W.F. Dudman and G.O. Aspinall, Biochem J., 59 (1955) 446.

56. G.H. Cohen and D.B. Johnstone, J. Bacteriol., 88 (1964) 329.

57. W.H. Taylor and E. Juni, J. Bacteriol., 81 (1961) 688.

58. R.S. Jones, I. Radcliffe and A. Linker, Proc. Soc. Exptl. Biol. Med., 110 (1962) 323.

59. E. Juni and G.A. Heym, J. Bacteriol., 87, (1964) 427.

60. R.G. Doggett, G.M. Harrison and E.S. Wallis, J. Bacteriol., 87 (1964) 427.

61. W.F. Dudman and J.F. Wilkinson, Biochem J., 62 (1956) 289.

62. A.M. Staub in 'Methods in Carbohydrate Chemistry V' eds. 1965 p.5.

63a B. Lindberg and S. Svensson in 'Carbohydrates' ed. G.O. Aspinall MTP Int. Rev. Sci. Organic Chem. Ser. 1 Vol. 7 Butterworth 1973 p.319.

b Specialist Periodical reports; Carbohydrate Chemistry, the Chemical Society, London.

64. S. Winogradsky, Ann. Inst. Past. 39 (1925) 299.

65. F.E. Clark in 'Soil Biology' ed. Burgess and Raw Academic Press N.Y. (1967) p.15.

66. E.W. Putman, A.L. Potter, R. Hodgson and W.Z. Hasid, J. Am. Chem. Soc., 72, (1950) 5024.

67. P.A.J. Gorin, J.F.T. Spencer and D.W.S. Westlake, Can. J. Chem., 39 (1961) 1067.

68. P.H. Graham, Antonie van Leeuwenhock, 31 (1965) 109.

69. L.P.T.M. Zevenhuizen, J. Gen. Microbiol., 68 (1971) 239.

70. W.G.C. Forsyth and D.M. Webley, Biochem J., 44 (1949) 455.

71. S. Hestrin and S. Avineri-Shapiro Biochem J. 38, (1944) 2.

72. D.J. Bell and R. Dedonder, J. Chem. Soc., (1954) 2866.

73. F.C. Harrison, H.L. Tarr and H. Hibbert, Can. J. Res., 3 (1930) 449.

74. H. Hibbert, R.S. Tipson and F. Brauns, Can. J. Res., 4 (1931)221.

75. S.W. Challinor, W.N. Haworth and E.L. Hirst, J. Chem. Soc., (1934) 676.

76. R.R. Lyne, S. Peat and M. Stacey, J. Chem. Soc., (1940) 237.

77. K. Misaki and
J. Ferment, Technol. 32 (1954) 147, 191, 311, 315, 341.

78. F-C.Chao, Industr. Agric. et Aliment. (Paris) 68, (1951) 13.

79. D. Murphy, C.T. Bishop and G.A. Adams, Can. J. Biochem. Physiol., 34 (1956) 1271.

80. F. Schardinger, Zbl. Bakt. II, 14 (1905 772, 22 (1909) 98.

81. P.A.J. Gorin and F.J.T. Spencer, Can. J. Chem., 39, (1961) 2274.

82. P.A.J. Gorin and F.J.T. Spencer, Can. J. Chem., 42 (1964) 1230.

83. O. Emmerling, Chem. Ber., 33 (1900) 2477.

84. F. Schardinger, Zentr. Bakteriol., 8 (1902) 175.

85. E. Toenniesen, Zentr. Bakteriol., 85 (1921) 225.

86. E. Kramar, Zentr. Bakteriol., 87 (1922) 40.

87. M. Heidelberger, W.F. Goebel and O.T. Avery, J. Exptl. Med., 42 (1925) 701.

88. W.F. Goeber and O.T. Avery, J. Exptl. Med., 46 (1927) 601.

89. P.R. Edwards and F. Fife, J. Infect. Disc., 91 (1952) 92.

90. W.F. Goebel, J. Biol. Chem., 74 (1927) 619.

91. S.D. Henriksen, J. Eriksen and V. Maini, Acta Pathol. Microbiol. Scand., 51 (1961) 259.

92. W. Nimmich, Z. Med. Mikrobiol Immunol., 154 (1968) 117.

93. S. Levene, H.J.R. Stevenson, R.H. Bordner and P.R. Edwards, J. Infect. Disc., 96 (1955) 193.

94. S.A. Barker, J.S. Brimacombe, J.L. Eriksen and M. Stacey, Nature (London) 197 (1963) 899.

95. J.L. Eriksen, and S.D. Henriksen, Acta, Pathol. Microbiol. Scand., 55 (1962) 65.

96. J.L. Eriksen and S.D. Henriksen, Acta, Pathol. Microbiol. Scand., 45 (1959) 387.

97. B.J. Gormus, R.W. Wheat and J.F. Porter, J. Bacteriol. 107 (1971) 150.

98. J.L. Eriksen, Acta Pathol. Microbiol. Scand., 64 (1965) 511.

99. L.C. Graham, P.A. Sandford and H.E. Conrad, Biochem., 6 (1967) 2755.

100. J.L. Eriksen and S.D. Henriksen, Acta Pathol. Microbiol. Scand., 54 (1962) 387.

101. S. Surjam and M. Surjam, Pathol. Microbiol., 24 (1961) 370.

102. S.D. Henriksen and J.L. Eriksen, Acta Pathol. Microbiol. Scand., 54, (1962) 391.

103. J.L. Eriksen, Acta Pathol. Microbiol. Scand., 64 (1965) 347.

104. J.L. Eriksen, Acta Pathol. Microbiol. Scand., 64 (1965) 522.

105. J.L. Eriksen, Acta Pathol. Microbiol.Scand. 64 (1965) 527.

106. R.W. Wheat, C. Dorsch and G. Godoy J. Bacteriol. 89 (1965) 539.

107. G.G.S. Dutton and M.T. Yang, Can. J. Chem., 50 (1972) 2382.

108. G.G.S. Dutton and M.T. Yang, Can. J. Chem., 51 (1973) 1828.

109. I.W. Sutherland, Biochem., 9 (1970) 2180.

110. M. Heidelburger, W.F. Dudman and W. Nimmich, J. Immunol., 104 (1970)1321.

111. B. Lindberg, J. Loungren, W. Nimmich and J.L. Thompson, Carbohyd. Res., 25, (1972) 49.

112. J. Eriksen and S.D. Henriksen, Acta Pathol. Microbiol. Scand., 58 (1963) 245.

113. Y.M. Choy and G.G.S. Dutton, J. Bacteriol., 112 (1972) 635.

114. Y.M. Choy and G.G.S. Dutton, Can. J. Chem., 51 (1973) 198.

115. Y.M. Choy and G.G.S. Dutton, Can. J. Chem., 51 (1973) 1819.

116. H. Bjorndal, B. Lindberg, J. Lonngren and K.G. Rosell, Carbohyd. Res., 27, (1973) 373.

117. I.W. Sutherland and J.,F. Wilkinson, Biochem. J., 110 (1968) 749.

118. P.A. Sandford and H.E. Conrad, Biochem., 5 (1966) 1508.

119. H.E. Conrad, J.R. Bamburg, J.D. Epley and T.J. Lindt, Biochem., 5 (1966) 2808.

120. P.E. Jones, Acta. Pathiol. Microbiol. Scand., 54 (1962) 121.

121. G.O. Aspinall, R.S.P. Jamieson and J.F. Wilkinson, J. Chem. Soc., (1946) 3483.

122. S.A. Barker, A.B. Foster, S.J, Pirt, I.R. Siddiqui and M. Stacey, Nature, (London) 181 (1958) 999.

123. W. Nimmich, Acta. Biol. Med. Ger., 26 (1971) 397.

124. E.C. Yurewicz, M.A. Chalambor and H.C. Heath, J. Biol. Chem., 246, (1971) 5596.

125. M.P. Veniamin, J. Chromatog. Sci., 8 (1970) 173.

126. S.A. Barker, A.B. Foster, I.R. Siddiqui and M. Stacey, J. Chem. Soc., (1958) 2358.

127. J. Eriksen, Acta Pathiol. Microbiol Scand., 66 (1966) 71.

128. E.A. Cooper and J.F. Preston, Biochem. J., 29 (1935) 2267.

129. J.L. Eriksen, Ann. Appl. Biol., 32 (1954) 44.

130. B. Delaporte and H. Belval, Compt. Rend., 22 (1946) 1011.

131. B. Delaporte and H. Belval, Ann. Inst. Past., 73 (1947) 862.

132. A. Fuchs, Nature (London) 178, (1956) 921.

133. R.G. Eagon and R. Dedonder, Compt. Rend., 241 (1955) 527.

134. R.G. Eagon and C.I. Randles, Archives of Biochem. (1955).

135. G.A. Adams and R. Young, Can. J. Biochem., 43 (1965) 1499.

136. G.A. Adams and S.M. Martin, Can. J. Biochem., 42 (1964) 1403.

137. R. Young and G.A. Adams, Can. J. Chem., 43 (1965) 2929.

138. G.A. Adams and R. Young, Ann. N.Y. Acad. Sci., 133 (1966) 527.

139. C.M. Hepper, Antonie van Leeuwenhoek, 38 (1972) 437.

140. A.S. Chandhari, C.T. Bishop and W.F. Dudman, Carbohyd. Res., 28(1973)221.

141. E.W. Hopkins, W.H. Peterson and E.B. Fred, J. Am. Chem. Soc., 52 (1930)3659.

142. B.A. Humphrey and J.M. Vincent, J. Gen. Microbiol., 21 (1959) 477.

143. H. Bjorndal, C. Erbing, B. Lindberg, G. Fahreus and H. Loungren, Acta. Chem. Scand., 25 (1971) 1281.

144. N. Amarger, M. Obaton and H. Blachere, Can. J. Chem., 13 (1967) 99.

145. W.F. Dadman, J. Bacteriol., 88 (1964) 782.

146. J. and A. Kleczkowski, J. Gen. Microbiol., 7 (1952) 340.

147. E. Schluctererand M. Stacey, J. Chem. Soc., (1945) 776.

148. M. DeLaeizaola and R. Dedonder, C.R. Acad. Sci. (Paris), 240 (1955)1825.

149. C.E. Clapp and R.J. Davis, Soil Biol. Biochem., 2 (1970) 109.

150. R.W. Bailey, R.M. Greenwood and A. Craig, J. Gen. Microbiol., 65 (1971) 315.

151. L.F.T.M. Sevenhuizen, Carbohyd. Res.,26(1972) 409.

152. S. Winogradsky, Ann. Inst. Pasteur, 60 (1938) 351.

153. H.L. Jensen, Bacteriol. Rev., 18 (1954) 195.

154. A.W. Hofer, J. Bacteriol., 48 (1944) 697.

155. H.G. Derx, Proc. Komnkl., 53 (1950) 140.

156. H.G. Derx, J.F. de Vries and E.L. Wittenberns-Klerkx, Ann. Bogonenses, 1 (1950) 11.

157. A.J. Kluyver and J.H. Becking, Ann. Acad. Sci. Fennicae Ser. A2 Chem., 60 (1955) 367.

158. D.B. Johnstone, Soil. Microbiol. Methods Newsletter, 1 (1962) 6.

159. B. Kauffmann and R, Repaske, Bacteriol Proc., (1958) 126.

160. B. Kauffman 'The Physiology of Carbohydrate formation by Azotobacter vindlandii', Ph. D. Thesis Inidana University, Bloomington (1960).

161. R. Lopez and A. B cking, Microbiol. Espanol., 21 (1968) 53.

162. A. Portoles, R. Lopez and A. Hildago, Can. J. Microbiol., 14 (1968) 763.

163. D. Claus, Biochem. Biophys. Re Comm.,20(1965) 745.

164. J.W. Newton, P.W. Wilson and R.H. Burris, J. Biol. Chem., 204 (1953) 445.

165. P.A.J. Gorin and J.F.T. Spencer, Can. J. Chem., 44 (1966) 993.

166. A. Linker and R.S. Jones, Nature (London), 204 (1964) 187, J. Biol. Chem., 241 (1966) 3845.

167. D.M. Carlson and L.R. Mathews, Biochem., 5 (1966) 2817.

168. A. Haug, B. Larsen and O. Smidsrod, Acta. Chem. Scand., 20 (1966) 183.

169. A. Haug, B. Larsen and O. Smidsrod, Acta. Chem. Scand., 21 (1967) 691.

170. A. Haug, Report No.30, Norwegian Institute of Seaweed Research, Trondheim, Norway 1964.

171. A. Haug, B. Larsen, and E. Baardseth, 'Proc. 6th Intern. Seaweed Symp. 1968', Madrid 1969, p.443.

172. A. Haug and B. Larsen, Biochem. Biophys. Acta., 192 (1969) 557.

173. B. Larsen and A. Haug, Carbohyd. Res., 17 (1971) 287.

174. A. Haug and B. Larsen, Carbohyd. Res.,17 (1971) 297.

175. G.H. Cohen and D.B. Johnstone, J. Bacteriol. 88 (1964) 1695.

176. L. Warren, J. Biol. Chem., 234 (1959) 1971.

177. E.A. Cooper, W.D. Daker and M. Stacey, Biochem. J. 32 (1938) 1752.

178. G.J. Lawson and M. Stacey, J. Chem. Soc., (1954) 1925.

179. M.W. Beijerinck, Folia Microbiol., 1 (1912) 1.

180. A. Fuchs 'On the Synthesis and Breakdown of Levan by Bacteria' Uitegervery Waltman 1959.

181. C.M. Quinnell, S.G. Knight and P.W. Wilson, Can. J. Microbiol., 3, (1957) 277.

182. J.K.N. Jones, M.B. Perry and W. Sowa Can. J. Chem., 41 (1963) 2712.

183. Z. Dische, J. Biol. Chem., 183 (1950) 489.

184. Q.M. Parikh and J.K.N. Jones, Can. J. Chem., 41 (1963) 2826.

185. W.N. Howarth, J. Chem. Soc. (London), 107, (1915) 8.

186. R. Kuhn, H. Frischmann and I. Low, Angew. Chem., 67 (1955) 32.

187. A. Haug and B. Larsen, Acta. Chem. Scand., 24 (1970) 1855.

188. C.A. Knutson and A. Jeanes, Anal. Biochem., 24 (1968) 476.

189. A. Haug, and B. Larsen, Acta Chem. Scand., 15 (1961) 1395.

190. H.G. Derx, Ann. Bogoriensis, 1 (1950) 1.

191. R.L. Starkot and P.K. De, Soil Ser., 47 (1939) 329.

192. H.G. Derx, Koninkl. Ned. Akad. Proc. Ser. C., 53 (1950) 140.

193. J. Kauffman and P. Toussaint, Rev. Gen. Botan., 58 (1951) 553.

194. Y.T. Tchan, Proc. Linnean Soc., N.S. Wales, 78, (1953) 171.

195. H. Stockdale, D.W. Ribbons and E.A. Dawes, J. Bacteriol., 95 (1968) 1798.

196. P.W. Wilson and S.G. Knight in Experiments in Bacterial Physiology, Burgess Publishing Co. Minneapolis 1952.

197. J.F. Saeman, W.G. Moore, M.A. Millett, in 'Methods in Carbohydrate Chemistry III' Academic Press London 1963 ed. R.L. Whistler p.55.

198. F.G. Fischer and H. Dor l, Z. Physiol. Chem. Hoppe Seyler's, 301 (1955)224.

199. H. Weigel, Adv. Carb. Chem., 18 (1962) 61.

200. D. Gross, Chem. Ind., (1959) 1219.

201. J.A. Nunn, H. Parolis and I. Russell, Carbohyd. Res., 29 (1973) 281.

202. W.G. Trevelyan, D.P. Proctor and J.S.Harrison, Nature, 166 (1950) 444.

203. L. Hough, J.K.N. Jones and W.H. Wadman, J. Chem. Soc., (1950) 1702.

204. Modification of spray used in ref. 189.

205. M.R. Slaton, Nature,186 (1960) 966.

206. M. Duckworth and W. Yaphe, Chem. Ind., (1970) 747.

207. R. Dedonder, Bull. Soc. Chim. Biol. (Paris) 34, (1952) 44.

208. M. Dubois, K.A. Gillies, J.K. Hamilton, D.A. Rebers, and F. Smith, Analytical Chem., 28 (1956) 350.

209. T. Bitter and H.H. Muir, Analytical Biochem. 4 (1962) 330.

210. R.L. Sidebotham, Ph.D. Thesis, London (1969).

211. T.E. Timell, Svensk Papperstidning, 63 (1960) 668.

212. S. Peat, W.J. Whelan and J.G. Roberts, J. Chem. Soc., (1956) 2258.

213. A. White, P. Handler and E.L. Smith in 'Principles of Biochemistry', McGraw Hill 1956, p.11.

214. D. Sandford and H.E. Conrad, Biochem, 5, (1966) 1508 .

215. T.J. de Boer and H.J. Backer, Organic Syntheses Coll. vol. 3 (1955) 250.

216. W.J. Whelan in 'Methods in Carbohydrate Chemistry IV' ed. R.L. Whistler, Academic Press London 1964 p.76.

217. C.C. Sweeley, R. Beatty, M. Makita and W.W. Wells, J. Am. Chem. Soc., 85, (1963) 2497.

218. H. Bjorndal, B. Lindberg and S. Svensson, Acta Chem. Scand., 21 (1967)1801.

219. S.I. Hakomori, J. Biochem. (Tokyo), 55 (1964) 205.

220. H. Bjorndal and B. Lindberg, Carbohyd. Res., 10 (1969) 79.

221. C.T. Bishop and O. Perila, Can. J. Chem., 39 (1961) 823.

222. I.O. Maskonardi, S.M. Flematti, J.O. Deferrari and E.G. Gros, Carbohyd. Res., 3 (1966) 177.

223. G.O. Aspinall and R.J. Ferrier, Chem. Ind., (1957) 1216.

224. J.K. Wold and B. Smestad, Acta. Chem. Scand., 24 (1970) 2472.

225. F.A.Kuehl in Methods in Carbohydrate Chemistry Vol. I eds. R.L. and M.L.Wolfrom (1962) p.271.

226. J.A. Dominque, J. Am. Chem. Soc., 73 (1951) 849.

227. E.L. Hirst, J.K.N. Jones and E.A. Woods, J. Chem. Soc., (1946) 1048.

228. H.S. Isbell and H.L. Frush, in 'Methods in Carbohydrate Chemistry I' ed. by R.L. Whistler and M.L. Wolfrom Academic Press 1962, p.145.

229. R.K. Hulyalkar, J.K.N. Jones and M.B. Perry, Can. J. Chem., 41 (1963)1490.

230. J.E. Scott, in 'Methods of Biochemical Analysis VIII' ed. D. Glick, Interscience 1960, p.145.

231. S. Hestrin, J. Biol. Chem., 180 (1949) 249.

232. O.L. Brady, J. Chem. Soc., (1931) 756.

233. S. Hirase, Bull. Chem. Soc. (Japan) 30, (1957) 70.

234. J.H. Sloneker and D.G. Orentas, Can. J. Chem., 40 (1962) 2188.

235. A.G. Hadjivassilou and S.V. Rieder, Clin Chim Acta., 19 (1968) 357.

236. H. Bjorndal, B. Lindberg and S. Svensson, Carbohyd. Res., 5 (1967) 433.

237. H. Meier in 'Methods in Carbohydrate Chemistry V' editor R.L. Whistler Academic Press,London,1965, p.45.

238. R.L. Whistler and J.L. Sannella in 'Methods in Carbohydrate Chemistry V' ed. R.L. Whistler, Academic Press, London 1965, p.34.

239. J.K.N. Jones and R.J. Stoodley in 'Methods in Carbohydrate Chemistry V' ed. R.L. Whistler, Academic Press, London 1965, p.36.

240. J.D. Blake and G.N. Richards, Carbohyd. Res., 8 (1968) 275.

241. P.A.J. Gorin and J.F.T. Spencer, Can. J. Chem. 44 (1966) 1230.

242. J.H. Sloneker and D.G. Orentas, Nature, 194 (1962) 478.

243. D.G. Orentas, J.H. Sloneker and A. Jeanes, Can. J. Microbiol. 9 (1963) 427.

244. R.J. Fielder, S.F. Grappel, C.T. Bishop and F. Blank, Can. J. Microbiol., 18, (1972) 701.

245. S. Hirase, Bull. Chem. Soc. Japan, 30, (1957) 75.

246. S. Hirase and K. Watanabe, Bull. Inst. Chem. Res. Kyoto Univ. 50, (1972) 332.

247. Y-MChoy and G.G.S. Dutton, Can. J. Chem., 51 (1973) 198.

248. P.A.J. Gorin and T. Ishikawo, Can. J. Chem., 45 (1967) 52.

249. G.M. Beboult, Y.M. Choy, G.G.S. Dutton, N. Funnell, A.M. Stephen and M.T. Yang, J. Bacteriol., 113 (1973) 1345.

250. A.N. de Belder and B. Norrman, Carbohyd. Res., 8 (1968) 1.

251. P.J. Garegg, B. Lindberg, T. Onn and I.W.Sutherland, Acta. Chem. Scand. 25 (1971) 2103.

252. I.W. Sutherland, Biochem. J., 115 (1969) 935.

253. P.J. Garegg, B. Lindberg, T. Onn and T. Holme, Acta. Chem. Scand., 25 (1971) 1185.

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Note

Methyl ethers of L-gulose

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Complete methylation of uronic acid-containing polysaccharides is difficult, and it is frequent practice to reduce the uronic acid to the corresponding sugar and to methylate the derived, neutral polysaccharide. The occurrence of L-guluronic acid in alginic acid[1] and in a number of bacterial extracellular polysaccharides[2] has made it important to prepare and characterise the methyl ethers of gulose. In the present note, L-gulose was partly methylated and the derived mixture of methylated methyl L-gulosides was analysed by g.l.c. (Table I). The peaks corresponding to the respective methyl ethers were identified by comparison with those of the methyl ethers of D-glucose. An aliquot was also converted into a mixture of the corresponding alditol acetates which was analysed and characterised by g.l.c.–mass spectrometry. The relative size of the individual peaks of the alditol acetates was used to confirm the identity of the methyl L-gulosides. The retention times are given in Table I. No evidence for the formation of methyl gulofuranosides could be found. Although 2,4–

TABLE I

METHYL ETHERS OF L-GULOSE (RETENTION TIMES)

Methyl L-gulosides		*Gulitol acetates*	*Corresponding L-gulose ether*
		Column 3	
Column 1	*Column 2*		
1.53	1.37	0.96	2,3,4,6-tetra-*O*-methyl
2.1, 3.5	1.5, 2.1	1.48	2,4,6-+3,4,6-tri-*O*-methyl
2.9, 4.0	1.7, 1.93	1.65	2,3,6-tri-*O*-methyl
3.2, 4.5	1.37, 1.93	1.74	2,3,4-tri-*O*-methyl
	3.1	2.26	2,6-+4,6-di-*O*-methyl
	3.63, 3.2	2.66	3,6-di-*O*-methyl
			2,3-di-*O*-methyl
	3.2, 2.46	2.82	2,4-di-*O*-methyl
		3.1	6-*O*-monomethyl
		4.0	2-*O*-monomethyl
		4.24	4-*O*-monomethyl
		5.5	Hexa-acetate

and 3,5-di-*O*-methylgulitol acetates, 3- and 4-, and 2- and 5-mono-*O*-methyl-gulitol acetates would be expected to give the same fragmentation pattern[3] in mass spectrometry and therefore the alditols derived from furanosides would not be detected, the 5,6-di-*O*-methylgulitol acetate should give a strong primary fragment with *m/e* 89. This fragment was not given by any of the products. Furthermore, it is unlikely that the sugars in each of the above pairs would have identical retention times on g.l.c., or that methyl furanosides, if present as di- and mono-*O*-methyl sugars, would have been completely missing in the tri-*O*-methyl series. It is concluded that, under the conditions of glycosidation, the amount of furanosides formed was extremely small.

Whereas partial methylation of methyl β-D-xylopyranosides[4] by the Purdie method gave mainly the 2,4-dimethyl ether and the reactivity sequence was HO-2>HO-4>HO-3, with methyl L-guloside the 6-*O*-monomethyl sugar was present in much the largest proportion, with roughly equal amounts of the 2-, 3-, and 4-monomethyl ethers. Due to partial overlap of peaks, it was difficult to assay the di-*O*-methyl derivatives accurately, but the 2,3- and 3,6-isomers were present in least amount and the 2,4-di-*O*-methyl derivative was equal in quantity to the combined 2,6- and 4,6-di-*O*-methyl derivatives. The main tri-*O*-methyl derivative was the 2,3,6-isomer, corresponding to the combined amount of 2,4,6- and 3,4,6-compounds which, as the alditol acetates, gave an overlapping peak. The 2,3,4-tri-methyl ether was present in least amount.

EXPERIMENTAL

G.l.c.[5] of the methyl glycosides was performed on columns of acid-washed Celite coated with 15% by weight of poly(butane-1,4-diol succinate) (column 1), and 10% polyphenol ether [*m*-bis(*m*-phenoxyphenoxy)benzene] (column 2) at an operating temperature of 175°. The methylated alditol acetates were injected into a glass column (12 ft) packed with 3% OV225 on Gas Chrom Q (column 3) and fitted in a Perkin–Elmer F11 combined gas-chromatograph RMS4 mass spectrometer at 195°. The mass spectra were recorded at an inlet temperature of 200°, ionising potential of 80 eV, ionising current of 50 μamp, and a temperature of the ion source of 240°.

Chromatographically and ionophoretically pure L-gulose (50 mg from Cambrian Chemicals) was treated with 0.5% methanolic hydrogen chloride (20 ml) for 18 h under reflux; these are conditions expected to give the highest yield of methyl pyranosides. The recovered syrup was non-reducing to Fehling's solution and the Nelson reagent. Partial methylation of the derived methyl gulopyranosides was effected by the Purdie[6] procedure, using refluxing methyl iodide and silver oxide for 1 h. The derived mixture of methylated L-gulosides was analysed by g.l.c., and an aliquot was converted into the corresponding alditol acetates[7], which were analysed by g.l.c.–m.s.

REFERENCES

1 See E. PERCIVAL AND R. H. MCDOWELL, *Chemistry and Enzymology of Marine Algal Polysaccharides*, Academic Press, London, 1967.
2 P. A. J. GORIN AND J. F. T. SPENCER, *Can. J. Chem.*, 44 (1966) 993; A. LINKER AND R. S. JONES, *J. Biol. Chem.*, 241 (1966) 3845; D. M. CARLSON AND L. R. MATTHEWS, *Biochemistry*, 5 (1966) 2817; A. HAUG AND B. LARSEN, *Acta Chem. Scand.*, 24 (1970) 1855.
3 H. BJORNDAL, C. G. HELLERQVIST, B. LINDBERG, AND S. SVENSSON, *Angew. Chem. Int. Ed. Engl.*, 9 (1970) 610.
4 YU. S. OVODOV AND E. V. EVTUSHENKO, *Carbohyd. Res.*, 27 (1973) 169.
5 G. O. ASPINALL, *J. Chem. Soc.*, (1963) 1676.
6 T. PURDIE AND J. C. IRVINE, *J. Chem. Soc.*, 83 (1903) 1021.
7 H. BJORNDAL, B. LINDBERG, AND S. SVENSSON, *Acta Chem. Scand.*, 21 (1967) 1801.