STRUCTURAL STUDIES ON THE LIPOPOLYSACCHARIDES ISOLATED FROM BACTERIOPHAGES SELECTED STRAINS OF AEROMONAS HYDROPHILA

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STRUCTURAL STUDIES ON THE LIPOPOLYSACCHARIDES ISOLATED FROM BACTERIOPHAGE SELECTED STRAINS OF *AEROMONAS HYDROPHILA*

by

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A Thesis submitted in partial fulfillment
of the requirements for the degree of
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Department of Chemistry
Memorial University of Newfoundland

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St. John's Newfoundland

ABSTRACT

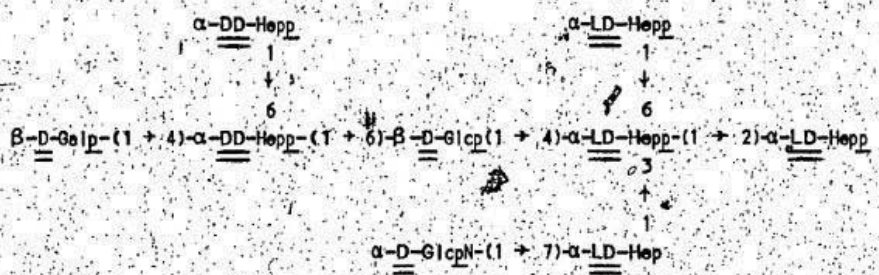
A. hydrophila represents a group of bacteria pathogenic to fish. Recently the same bacteria have been increasingly implicated in human infections, particularly as a secondary invader, and are now being recognized as a serious pathogen under these circumstances. As little is known of the biochemical basis of pathogenicity of this species, interest in the structure and immunological properties of the cell surface polysaccharides of the different chemotypes of this gram-negative bacterium has increased.

The three bacterial colonies used for this investigation were selected from a biochemically pure culture of A. hydrophila. One represented the overall strain, whereas the other two, mutants, were isolated as morphologically different clones. The original strain and one of the clones were sensitive to phage while the other clone was resistant to the same phage.

Lipopolysaccharides were extracted from all three variants, and the basic structures of the polysaccharide portion from each of the three strains exhibited distinct structural differences.

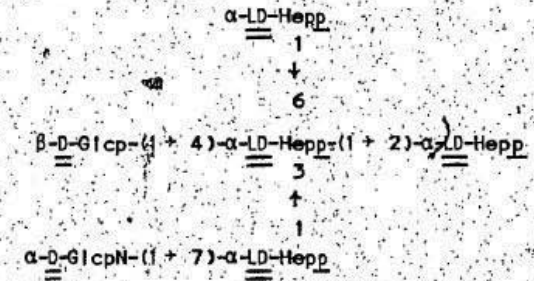
Chemical analysis (methylation, periodate oxidation and selective degradations) as well as spectroscopic techniques including mass spectrometry and high resolution n.m.r. were used for the structural investigations.

The core oligosaccharide isolated from the lipopolysaccharide of the overall strain (SJ-55) was shown to have the structure indicated below:



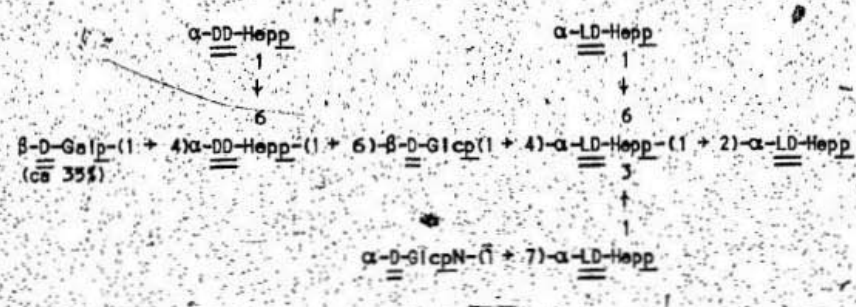
SJ-55

The core oligosaccharide isolated from the lipopolysaccharide of the phage resistant variant (SJ-48) was shown to be as follows:



SJ-48

Finally, the following structure was demonstrated for the core oligosaccharide, isolated from the lipopolysaccharide of the minor phage sensitive variant (SJ-47).



SJ-47

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NOTATIONS AND ABBREVIATIONS

SJ-48, SJ-47, SJ-55 represent A. hydrophila strain A6b, A6a and A6 whose origin is discussed in the experimental part (Section 1. p. 57)

- R- and SR- stand for "rough" and "semi-rough" oligosaccharides, lps for lipopolysaccharide.
- α-LD-Hepp stands for L-glycero-α-D-manno-heptopyranose.
- α-DD-Hepp stands for D-glycero-α-D-manno-heptopyranose
- KDO or dOclA stands for 3-deoxy-D-manno-2-octulosonic acid
- GlcpN stands for 2-amino-2-deoxy-D-glucopyranose
- ManpN stands for 2-amino-2-deoxy-D-mannopyranose
- Glcp stands for glucopyranose
- Galp stands for galactopyranose
- Rhap stands for rhamnopyranose
- Manp stands for mannopyranose
- Ara stands for arabinose

The following abbreviations will also be used: g.l.c. for gas liquid chromatography, g.l.c.-m.s. for gas liquid chromatography coupled with mass spectrometry, n.m.r. for nuclear magnetic resonance and H.V.E. for high voltage electrophoresis.

The nomenclature used inthe following text will be that of the IUPAC Joint Commission on Biochemical Nomenclature (JCBN) reported in the Journal of Biological Chemistry, Vol. 257, No. 7, pp 3352-3354 (1982).

PART I

LITERATURE SURVEY

ON

BACTERIAL LIPOPOLYSACCHARIDES AND

STRUCTURAL ANALYSIS OF POLYSACCHARIDES

INTRODUCTION

1. BACTERIAL LIPOPOLYSACCHARIDES

Several years ago Bennett (1) predicted that "an investigator in almost any biological field is likely to obtain a positive result if he tries lipopolysaccharides in the experimental system he is using."

The general term lipopolysaccharide (lps) denotes a unique class of macromolecules, and represents a characteristic attribute of the gram negative bacteria. The term has acquired a special connotation in chemical bacteriology, where lipopolysaccharide is often used as a synonym for endotoxin.

The development of lipopolysaccharide chemistry during the last thirty years has been remarkable both for its achievements and for the variety of disciplines from which contributions have stemmed (2-5). The literature of lipopolysaccharide research is immense and cannot be adequately reviewed in this presentation, therefore only the most recent and relevant studies have been cited with an attempt to refer to critical reviews whenever possible.

Lipopolysaccharides can be extracted from bacteria and exhibit antigenic properties in biological systems. They will react with certain antibodies in anti whole cell antisera, and when used as an immunoadsorbant, will remove these antibodies by precipitation or by binding.

On the other hand, injection of larger doses of lipopolysaccharide containing bacteria or of purified lipopolysaccharide into experimental

animals causes a wide spectrum of so-called endotoxic reactions. In contrast to the specific delayed immune response, these effects are, in general, non-specific and acute and would include such phenomena as fever, changes in leukocyte counts, shock, and in many cases death (6-12). Cleavage of lipopolysaccharide with mild acid yields free polysaccharide or oligosaccharide which, when coupled to protein, may be used for the induction of antibodies (13-14). Obviously, such studies are very important in the context of bacterial infections and the production of protective vaccines (15).

The basis of the antigen-antibody immunological phenomena is the recognition of partial structures of the polysaccharide antigens by antibodies, molecules or immune cell receptors. These partial structures are known as antigenic determinants (determinant groups) and may be oligosaccharides or even individual monosaccharide residues (16).

For consideration of the antigen-antibody reaction, the knowledge of the structure of the polysaccharide antigen is necessary (10, 11, 12, 16).

1.1. The Gram-Negative Bacterial Cell Wall

The bacterial cell wall consists of three layers: (A) the inner cytoplasmic membrane in which complex enzymatic systems for redox processes, the synthesis of complex macromolecules and the active transport mechanisms are located; (B) the peptidoglycan or murein layer which maintains the rigidity and shape of the bacterial cell wall;

(C) the outer membrane, containing a lipoprotein, phospholipid and the lipopolysaccharide layer (Fig. 1).

The carbohydrate chains of the lps extend outward from the outer membrane layer (17) and are responsible for the antigenic specificity of the organism (18). In addition, many bacteria such as Pneumococcus, Klebsiella and E. coli are surrounded by a capsule.

The cell envelope, which comprises the outer membrane and capsule, contains carbohydrate antigens which induce the formation of antibodies, and which react serologically with these antibodies in both man and animal (16). The cell envelope also contains receptors for bacteriophages, hence the bacterial surface contains components which play an important role in the immune recognition process. The best studied of these components are the polysaccharides which form the capsules and are known as capsular antigens or K-antigens and the lipopolysaccharides known as O-, or R-antigens. It is mainly through K-, O- and R-antigens that bacteria express their immunological individuality (12, 16).

1.2. General Architecture of Bacterial Lipopolysaccharide

The classical model for a bacterial lipopolysaccharide is that originally proposed by Lüderitz and Westphal for a product isolated from smooth "wild type" strains of Salmonella. As shown (Fig. 2), the model for the structural unit of a complete lipopolysaccharide (S-form) is composed of three covalently linked segments (O-side chain, the core, and lipid A) each with its distinctive composition, biosynthesis and biological function.

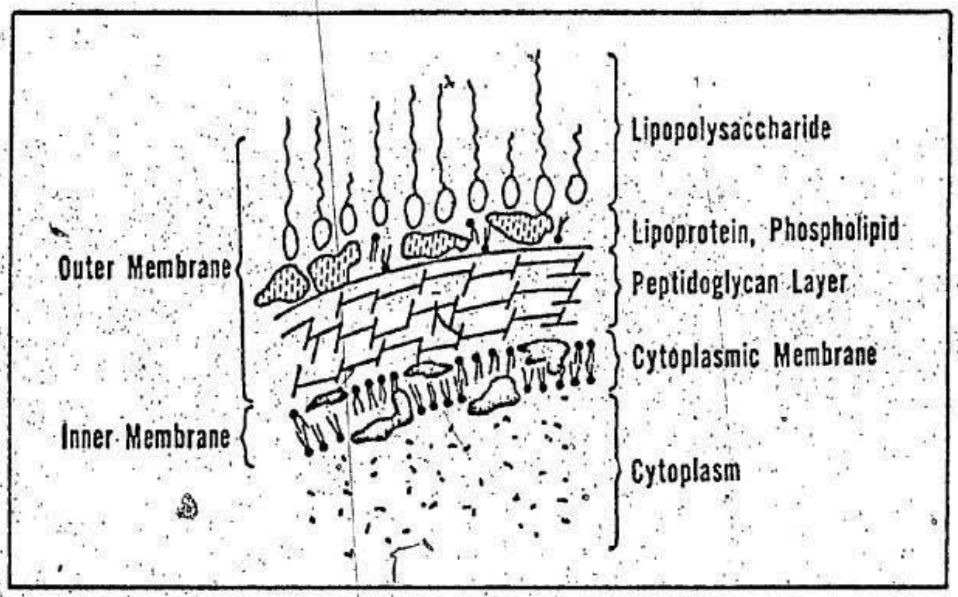


Fig.1. Structure of the bacterial cell membrane

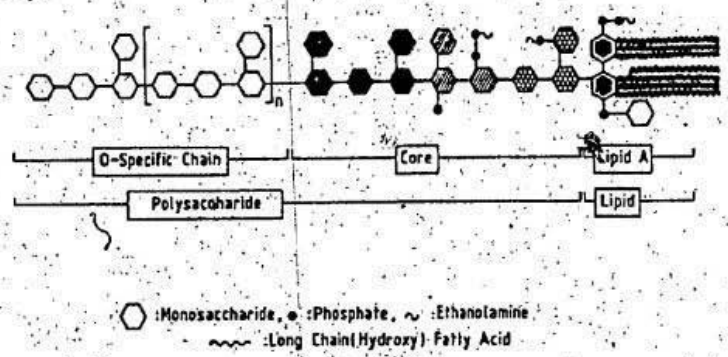


Fig. 2. Schematic structure of Salmonella lipopolysaccharides from Lüderitz et al. (12)

The O-side chain is the serologically dominant part of the molecule responsible for antigenic specificity. It is composed of repeating oligosaccharide units (often containing rare sugars) in which the serological determinant resides. It is absent from the R-form lipopolysaccharides isolated from rough strains, but is usually produced as a hapten by mutants in which genetic defects lead to the biosynthesis of an incomplete core (12).

The O-antigen portion of the lps molecule is potentially unique for each bacterial species and as such imparts serological specificity and identity to a particular organism.

The O-antigen is linked to the core oligosaccharide which is often species specific but can vary both within and between bacterial species, particularly when non-Enterobacteriacea are considered. The complete core oligosaccharide, composed of different sugar units, may be subdivided into an "outer region" to which the O-antigen is attached, and an "inner region" linked to lipid A. This oligosaccharide is generally linked to the lipid component of lps (lipid A) through a trisaccharide of 2-keto-3-deoxy-D-manno-octulosonic acid (KDO, dOclA) though this linkage may not be universal. Lipid A is apparently responsible for the endotoxic activities of the lipopolysaccharide and appears to be very similar if not identical in many bacterial species (19).

The lipopolysaccharide of Salmonella has been widely used as a basic model in the interpretation of compositional and structural data for the lipopolysaccharides of other gram-negative bacteria. With the

elucidation of the structures of lps from an increasing number of species however, gross differences from this "basic model" have been reported.

1.3. Isolation Structure and Biosynthesis of Lipopolysaccharides

1.3.1 Isolation and Purification of Lipopolysaccharides

A number of extraction procedures for the isolation of lipolysaccharide or lipopolysaccharide-protein complexes have been described by Wilkinson (20) and by Galanos et al (21) and are illustrated in the Table I.

The efficiency of the extraction and the composition of the extract depend on the organism, the starting material (whole cells or isolated envelopes) and the method used.

The method of choice is usually the phenol/water procedure (22) which yields a water soluble extract that is subsequently purified by high speed centrifugation. For R-form lipopolysaccharides which have reduced water solubility, low yields may be obtained by the phenol/water method. In this case superior yields may be obtained by the exceptionally mild and selective "PCP" method (23).

Because of the presence of carboxyl, phosphoryl and ethanolamine residues in the molecules, lipopolysaccharides are amphoteric; the overall net charge is negative. In the original lipopolysaccharide preparation the negatively charged groups are neutralized by Na^+, K^+, Mg^{++} and Ca^{++}. It has been found that the nature of the cations present

Table I. Methods for the Extraction of Lipopolysaccharides (20)

Treatment	Leading reference
- 45% Aqueous phenol, 65-68°C	Westphal and Jann (22)
- Aqueous phenol - chloroform - light petroleum ether, 5-20°C (PCP method)	Galanos et al. (23)
- 0.25M trichloroacetic acid, 4°C	Staub (24)
- Aqueous EDTA (pH 8.0-8.5), 37°C	Leive and Morrison (25)
- Aqueous butanol, (0-4°C)	Leive and Morrison (25)
- Dimethyl sulphoxide, 60°C	Adams (26)
- 1M sodium chloride - 0.1M sodium citrate (pH 7.0), 0-4°C	Raymond et al. (27)
- Aqueous diethyl ether, ambient temperature	Ribi et al. (28).

in lipopolysaccharides greatly influence their state of aggregation, and as a consequence their biological activities. Therefore, it is useful to convert lipopolysaccharides into a uniform salt form by electrodialysis (29).

1.3.2 Structure and Biosynthesis of the Lipopolysaccharide

1.3.2.1 Structure of the O-Chain

As previously mentioned, the O-specific chains of the lipopolysaccharides are made up of repeating units of identical oligosaccharides (30). These units usually contain different sugar constituents, and the O-chain is therefore a heteropolysaccharide. In some cases however the O-chain may be a homopolysaccharide consisting of units of an oligomer composed of a single sugar species with a distinct linkage sequence.

Each bacterial serotype synthesizes a unique lipopolysaccharide, characterized by a specific composition and structure of the O-chain and having a distinct individual antigenicity.

Figure 3 illustrates the structure of the lipopolysaccharide of S. typhimurium.

As indicated, Lipid A, core and O-chain are interlinked by covalent linkages. The O-chain contains repeating units of a pentasaccharide with mannosyl, rhamnosyl and galactosyl residues in the main chain, and 2-O-acetylated abequosyl and glucosyl residues as branches.

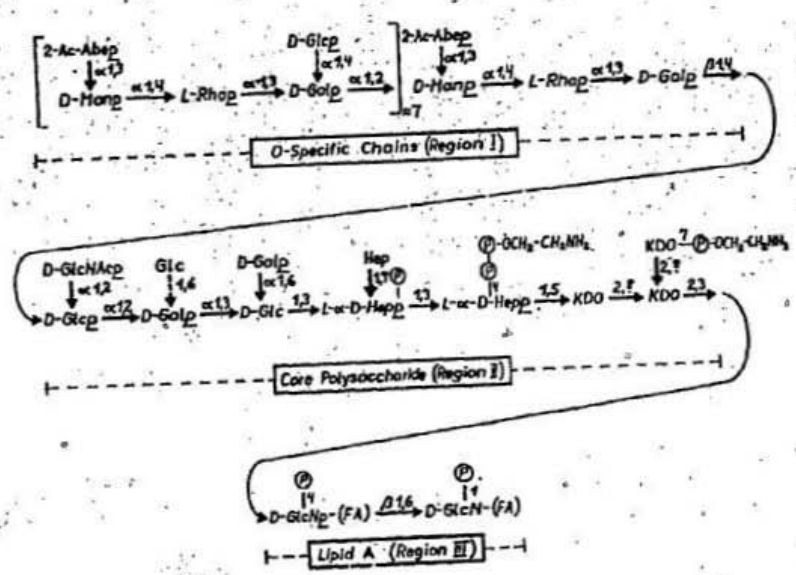


Fig. 3. Structure of the lipopolysaccharide from Salmonella typhimurium (12).

The oligosaccharide 1 which is formed and polymerized during the biosynthesis represents "the biological repeating unit". Due to the acid lability of the rhamnosyl and abequosyl linkages, mild acid hydrolysis of the lipopolysaccharide yields the "chemical" repeating unit 2 with a different sequence (11, 12). The concept of two types of repeating unit is quite important as chemical analysis will only reveal the chemical repeating units and only by luck will they be identical to the biological ones (12).

1 2

1.3.2.2. The Heterogeneity of O-Chains

The constituents of the true biological repeating unit Abe, Man, Rha and Gal of Figure 3 are constant. In contrast both the acetyl group of the abequose residue and the side chain glucose unit, which are added in the latter stages of the biosynthesis, vary and are often not present in equimolar ratio (31). Furthermore, their presence or absence may be subject to variation for other reasons (e.g. antigenic conversion by phages). This incomplete substitution of the basic O-chain causes the well known microheterogeneity found in lipopolysaccharides (12).

1.3.2.3. Structure of the Core

The core structure of S. typhimurium (Fig. 3) is common to all Salmonella species and also occurs in other enterobacterial lipopolysaccharides (11, 12, 20). The Salmonella core contains a lipid; a hexose oligosaccharide (outer core) consisting of D-glucosamine, D-glucose and D-galactose; and an inner core region consisting of an oligosaccharide of the core specific sugar L-glycero-D-manno-heptose (LD-Hep) and 3-deoxy-D-manno-2-octulosonic acid (KDO or dOclA), each forming a branched trisaccharide (10,32), this region is linked directly to the lipid which, by common agreement, is always referred to as Lipid A. The exact linkages in the KDO trisaccharide have not yet been definitively established (33).

The reducing end of the KDO trisaccharide links the polysaccharide to the lipid A in a relatively acid labile (ketosidic) linkage. The O-chains are attached to the terminal glucose unit of the core oligosaccharide.

A characteristic feature of the core is its substitution by polar groups such as phosphate and ethanolamine. The carboxyl group of KDO contributes to the acidic character of the lipopolysaccharide and this negative charge appears to be physiologically important (12). The lipopolysaccharides may be interlinked by ionic linkages to other components of the cell wall through divalent cations and polyamines. If this is the case such an arrangement would provide integrity and stability to the cell wall.

The core region of lipopolysaccharides exhibits a higher degree of heterogeneity. Substituents of the core, including P, EtN, and GlcNAc, are not necessarily present in molar amounts. Furthermore, alkali labile substituents such as glycine have occasionally been detected on the core (32, 34).

In recent years numerous core types have been identified in Enterobacteriacea (11, 12, 20, 35). Figure 4 illustrates a selection of these core oligosaccharides (Ra-R4 and K-12).

They all show major differences in the hexose region (outer core) but are quite similar in the Hep-KDO region (35). Table II lists different strains having different core oligosaccharides structures, some of them are R. mutants with incomplete biosynthesis.

Compositional analyses of the core of lipopolysaccharides isolated from various bacterial species, have revealed the presence of many unusual constituents such as D-glycero-D-manno-heptose, (44), uronic acid (45), or amino acid (42), and in the case of Vibrio cholerae strains, fructose (16, 46) or sedoheptulose may be present. These possibly replace KDO in linking the core to the lipid A.

Lipopolysaccharides containing a core devoid of heptose or KDO have also been identified (20, 21, 47-49).

1.3.2.4 Lipopolysaccharides of R-Mutants

R-mutants are defective in lipopolysaccharide biosynthesis. Depending on the defect, these mutants synthesise complete or incomplete

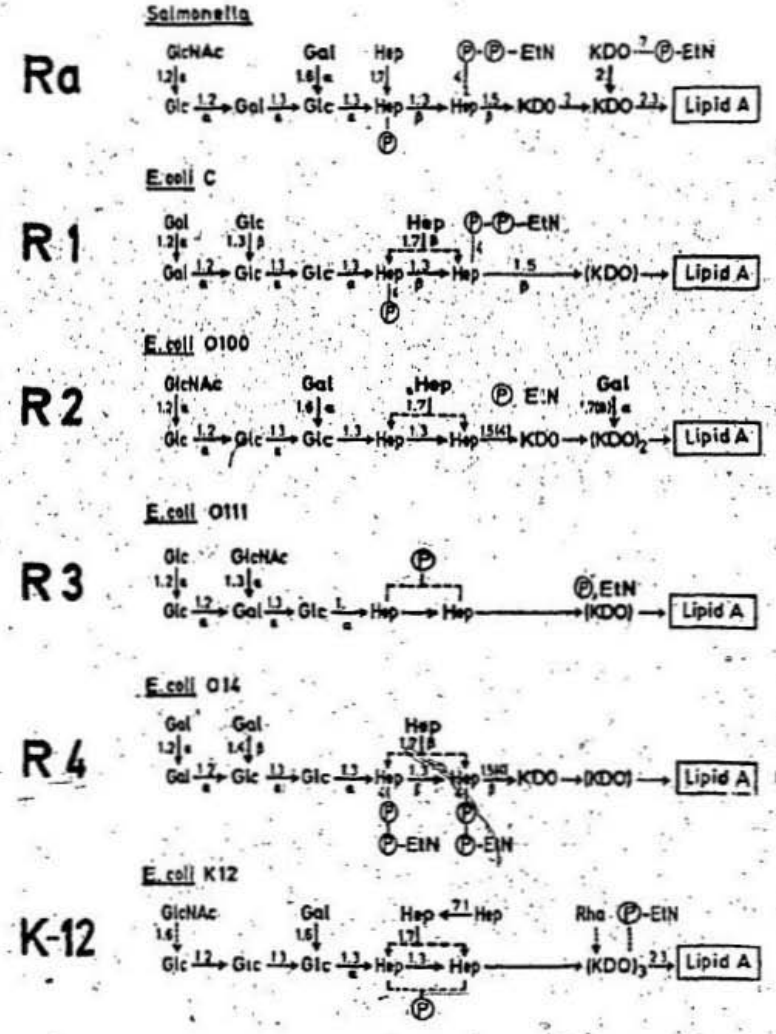


Fig. 4. Structure of enterobacterial core types Ra - K12 (12)

Table II. Core structures that have been partially evaluated (12).

Strains	Structure[a]	References
E. coli B	Glc → Glc → Hep → Hep → KDO → KDO (Hep: P, EtN; KDO: P)	Frehm *et al.* (36)
E. coli K-12		Ohkawa (37)
Ter-Mutants		Blache *et al.* (38)
CR 34		
E. coli 0111	Glc → Hep → Hep → KDO → X=H, Hep, or Glc → GlcN (X: P)	Fuller *et al.* (39)
E. coli ATCC 12408	Glc → Glc; (Hep → Hep)$_n$ → KDO → KDO (Hep: P)	Horton and Stewart (40)
Proteus mirabilis	Hep → P; Glc → Hep → KDO	Radziejewska-Lebrecht (41) *et al.*
Pseudomonas aeruginosa	Glc → Rha → Glc—Gal—Hep → Hep → KDO → KDO (Hep: P, EtN; Gal: Ala$_{1/2}$)	Drewry *et al.* (42)
Bordetella pertussis	GlcNAc; Hep; GlcNAc	Chaby *et al.* (43)
Xanthomonas sinensis	Man → Man → Glc → Man → KDO (Man: P; GalUA → Amide)	V. A. Volk (44) (unpublished data)

[a]Details of linkages are omitted.

core structures still linked to lipid A, but all devoid of the O-chain. Figure 5 shows a series of R-form lipopolysaccharides derived from Salmonella mutants defective in different steps of the biosynthetic pathway. Similar series of R mutants have also been isolated from E. coli (39), Shigella (50) and Proteus (51).

Ra mutants are defective in O-chain synthesis and produce lipopolysaccharides with complete core. All of the mutants from Rb to Re inclusive are core defective and synthesize glycolipids with incomplete core. Re lipopolysaccharides are the most defective and contain only KDO and Lipid A. The different R-mutants and their respective R lipopolysaccharides can be differentiated and identified by means of lectins (52) and antibodies (53) as the terminal sugar sequences (which are different) are recognized by such compounds. The mutants may also be identified by chemical analysis of their lipopolysaccharides and by their phage pattern (54). Occurrence of the R-forms is rare in nature although they can be cultivated under laboratory conditions and are generally non virulent, phagocytized by macrophages and are sensitive to toxic agents (12).

1.3.2.5. Structure of Lipid A

The lipid A moiety is usually liberated from lipopolysaccharides by mild acid treatment. The "free lipid A" thus obtained is a water-insoluble precipitate, soluble in organic solvents such as chloroform (12, 16, 20). Figure 6 shows the structure of Salmonella lipid A, which contains a central disaccharide of two D-glucosamine residues linked

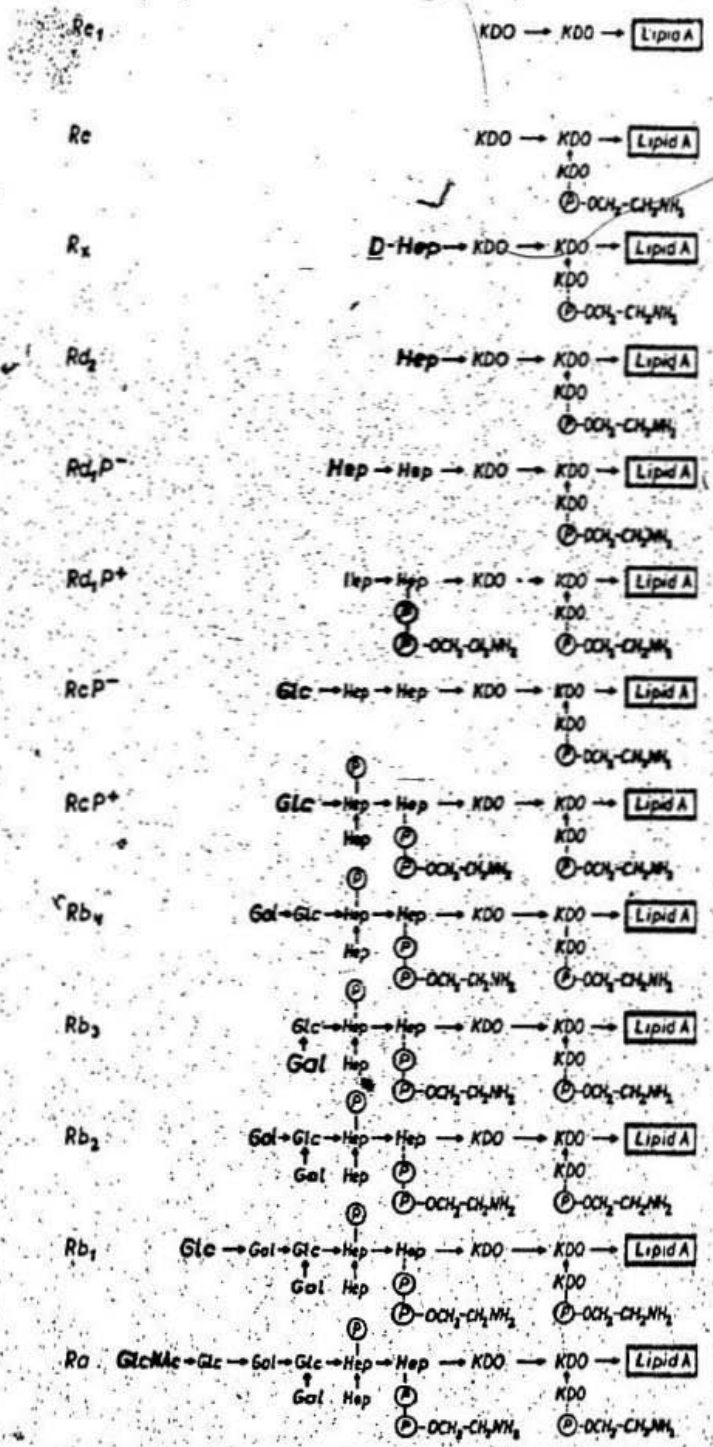


Fig.5. The structure of O-chain defective (chemotype Ra), and core defective (chembtype Rb-Re) lipopolysaccharides of Salmonella R-mutants. (12)

β1→6. Both glucosamine units are substituted by a phosphate group; one being bound in ester linkage to C-4 of the non reducing glucosamine (glucosamine II), and the other to C-1 of the reducing glucosamine (glucosamine I), thus occupying the glycosidic hydroxyl group and rendering the molecule non-reducing. In the lipopolysaccharide, the terminal KDO unit of the core is attached to C-3 of glucosamine II (12, 16, 19, 20). The P-GlcN-GlcN-P, moiety of the lipid A is termed "lipid A backbone" (Fig. 6).

The lipid A backbone contains both ester and amide linked fatty acids and is both hydrophobic and ionic due to the presence of the long chain fatty acids and negatively charged pyrophosphate groups.

Salmonella lipid A contains the following seven moles of long chain fatty acids: 4 moles of D-3 hydroxytetradecanoic (3-OH-14:0 or β-hydroxy myristic acid), and approximately 1 mole of each of dodecanoic acid (12:0 lauric acid), tetradecanoic acid (14:0, myristic acid) and hexadecanoic acid (16:0, palmitic acid) (12). Two moles of 3-OH-14:0 acylate the amino groups of the disaccharide. The remaining five moles of fatty acids are ester linked but not necessarily directly to the hydroxyl groups of the glucosamine (55).

In similar manner to O-chains and core, lipid A also exhibits structural heterogeneity. In approximately 50% of the lipid A molecules of Salmonella, the phosphate group linked to glucosamine I is substituted by a phosphoryl ethanolamine residue with a free amino group,

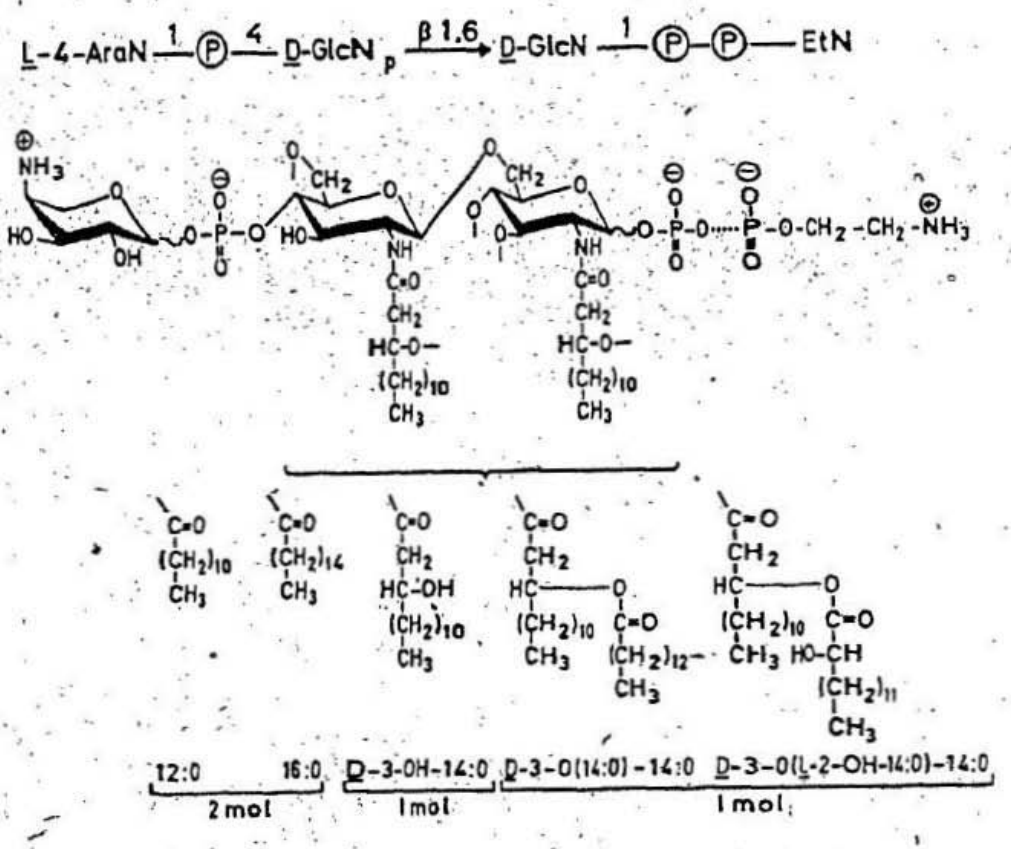


Fig. 6. Structure of lipid A from Salmonella. Dotted linkages indicate incomplete substitution(12).

In about 30-60% of the lipid A molecules the phosphate bound to glucosamine II is substituted by 4-amino-4-deoxy-L-arabinose (4-AraN); the linkage being through C-1. Both the amino group and the hydroxyl groups of 4-AraN appear to be unsubstituted (56).

1.3.3. Biosynthesis of Lipopolysaccharides

1.3.3.1 Principles of the Biosynthesis of the O-Chains

The biosynthesis of O-chains is catalysed by a multi-enzyme system that utilizes a membrane-bound polyisoprenoid compound as carrier for the glycosyl residues.

In Salmonella groups A, D, and E. (57), the synthesis begins with the reversible transfer of galactose 1-phosphate from UDP-galactose to the phosphorylated carrier lipid.

Sequential transfer of the additional sugars to the lipid linked intermediate leads to the formation of the O-chain repeating unit linked through a pyrophosphate bridge to the carrier molecule. The repeating units are then polymerised prior to the addition of other substituents (e.g. acetate) (12). Polymerisation occurs by constantly transferring the growing chain to a newly activated repeating unit (growth at the reducing end). This results in a long chain polymeric intermediate that is still linked at the reducing end of the carrier lipid. As a final step, the O-chains are transferred from the lipid-carrier to the independently synthesised core lipid A to form the complete molecule (56).

1.3.3.2. Biosynthesis of the Core

In a manner similar to O-chain biosynthesis a lipid-carrier is involved, in this case, lipid A, which remains linked to the core as part of the lipopolysaccharide molecule. The biosynthetic pathway of core-lipid A synthesis proceeds from the Re to Ra mutants, the sugar constituents being sequentially transferred by reactions catalysed by the respective sugar transferases (12, 16, 20). O-chain synthesis proceeds independently of the synthesis of the core and consequently bacteria with defects in core synthesis also synthesize O-chains, however, incomplete core is not capable of acting as a receptor for these O-chains.

1.3.3.3. Biosynthesis of the Lipid A

From pulse/chase experiments the biosynthesis of lipid A appears to occur via a precursor molecule which contains the diphosphorylated β-1,6-linked diglucosamine backbone, substituted by four 3-OH-14:0 residues, two in amide linkage and two in ester linkage (58). The precursor does not contain KDO and lacks the saturated fatty acids 12:0, 14:0 and 16:0 present in the complete lipid A. The precursor is also the acceptor for the polar groups of the lipid A region, including 4-aminoarabinose, phosphoryl ethanolamine and the KDO residues (59). The incorporation of the nonhydroxylated O-acyl residues of lipid A is not necessary for the extension of the core and O-chains, but is an essential function for the survival of the cells (12).

1.4. Biological and Endotoxic Properties of Lipopolysaccharides

Gram-negative bacteremia and septic shock in man are a direct result of the reaction to bacterial endotoxins (60). Early symptoms of gram-negative sepsis include fever, dyspnea, oliguria, and hypotension. Target organ involvement may result in pulmonary edema, haemorrhage, and hyaline membrane formation in the lung, tubular or cortical necrosis in the kidney, patchy necrosis in the myocardium, superficial ulceration in the gastrointestinal tract, and generalized thrombi in capillaries (60).

Laboratory findings include decreased levels of the third component of complement, deficiencies in several clotting factors, leukocytosis and decreased platelet counts.

The syndrome by which local tissue damage occurs as a result of increased vascular permeability together with the formation of fibrin thrombi in the capillaries is termed disseminated intravascular coagulation (DIC) (61).

Several aspects of this pathology can be mimicked in animal models by the injection of lps isolated from gram negative bacteria. The generalized Schwartzmann reaction in the rabbit, which is elicited by two properly spaced intravenous injections of lps, results in bilateral renal cortical necrosis due to fibrin deposition as a result of diffuse intravascular clotting (62). These effects are accompanied by a precipitous drop in both leukocytes and platelets (63). Immuno pathologic findings include glomerular deposition of IgG, IgM, C_3, fibrin and

albumin (64). The development of sophisticated in vitro techniques allowed a more direct demonstration of potential lps immunopathologic capabilities. Galanos et al. have surveyed the lps effects on plasma proteins, acute or inflammatory processes (65).

Many of these biological and endotoxic properties are now known to be associated with the lipid A which is common to lps from all bacterial species (12, 65, 66).

1.5 Relationship of lps Structure to Biological Activity

Although the structure of the lps molecule has been extensively studied, relatively little evidence is available concerning the mechanisms whereby specific portions of these molecules can effect the many activities described in the section I.4. The following approaches have been utilized to examine the relationships between chemical structure and biologic function of isolated lps preparations.

1.5.1. Naturally Occurring Variations in LPS Structure

The use of bacterial mutants deficient in polysaccharide synthesis has provided the most conclusive evidence that many of the biologic properties of lps are associated with the lipid A moiety of the endotoxic complex. These activities, which are elicited by preparations rich in lipid (such as S. minnesota R595 lps or isolated lipid A itself), include lethality in chick embryos and mice (67), the pyrogenic response in rabbits (67) activation of purified factor XII of the intrinsic coagulation sequence (66), mitogenic stimulation and

polyclonal activation of bone marrow derived lymphocytes (68), activation of the classical complement pathway (69), and induction of macrophage cytolytic capacity (70).

In addition to polysaccharide deficient preparations, natural variations in the O-chain component of smooth lipopolysaccharides provide a wide spectrum of molecules which can be used to investigate the contribution of the particular carbohydrate groups to (a) biological activity initiated by the polysaccharide itself and (b) the modulation of lipid A activity. The fractionation of E. coli serotypes O111:B4 and K235 (71, 72) into molecular species containing greater or lesser amounts of repeating O-polysaccharide may prove particularly valuable. Morrison and Leive (71) demonstrated that lps fraction I from E. coli serotypes O111:B4 (long O-chain) is considerably less toxic than fraction II (short O-chain). On the other hand, fraction I was found to be a more potent activator of serum complement due to activation of the alternative pathway by the polysaccharide component.

1.5.2. Chemical Modification of the LPS Molecule

1.5.2.1. Acid Hydrolysis

Mild acid hydrolysis (1% acetic acid, 100°C) cleaves the KDO-lipid A linkage liberating "free lipid A" (10, 12, 16, 20). Prior to the availability of polysaccharide deficient mutants, investigations using such isolated lipid A preparations provided the best evidence that many biologic and endotoxic properties were associated with the lipid

component. It should be noted however that "free lipid A" is not necessarily identical to the "bound lipid A" of the original molecule.

1.5.2.2. Alkaline Hydrolysis

Short periods of relatively mild alkaline hydrolysis (0.03N NaOH, 37°C) cause a substantial reduction in the ester bound fatty acid component of lps (73). However, the biological activities which are associated with lipid A and which might be expected to depend upon the fatty acid integrity of this molecule may actually be enhanced under these conditions. In general, however, longer periods of alkaline hydrolysis result in the loss of biologic properties associated with the lipid A component of lps, including mitogenesis (73).

1.5.2.3. Polymyxin B

Another approach which has proven extremely useful to the examination of structure-function relationships involves the use of the cyclic peptide antibiotic Polymyxin B. Polymyxin B has been reported to inhibit various in vivo effects of lps (74, 75). It has now been established that this antibiotic binds with lipid A interfering with several of the biological properties of the lps molecule (76).

2. STRUCTURAL ANALYSES OF POLYSACCHARIDES

The immunological, serological, and phage receptor properties of bacterial polysaccharides are usually expressed through regions of the polysaccharides known as the antigenic determinants (12, 16, 20). The basis of the antigen-antibody immunological phenomenon is the

recognition of partial structures of the polysaccharide antigens by antibody molecules or immune cell receptors. For a quantitative consideration of the different specificities of the antigenic determinants, the elucidation of the structure of the polysaccharide antigen is necessary (12, 16, 20).

In order to fully characterize a polysaccharide consisting of repeating units or an oligosaccharide, a structural study should determine:

a) the nature and number of sugars residues and their relative proportions;

b) the position of the linkages of the sugars residues;

c) the sequence of the component sugars;

d) the anomeric configurations of the sugars present;

e) the conformation of the polysaccharide.

The literature describing the techniques used to achieve these requirements cannot be reviewed adequately in a discussion of this nature. Therefore, only the techniques used in the course of this work will be discussed, with an attempt to refer to the latest critical reviews, and to update the applications of those techniques for which significant improvements have recently been made.

While some of the following discussion will deal with repeating units of polysaccharides, most of the methodology described can be

equally well applied to the investigation of oligosaccharide structures.

2.1. Total Hydrolysis

The hydrolysis of a polysaccharide and the subsequent analysis of the hydrolysis products is often performed qualitatively. However, the total hydrolysis of polysaccharides, particularly those with resistant glycosidic linkages, is a more difficult operation (77). Hydrochloric, sulfuric and trifluoroacetic acids are commonly used in hydrolysis and are sufficient to completely hydrolyse a neutral polysaccharide into its monomeric sugar units.

Most of the constituent monosaccharides released during the acidic hydrolysis have traditionally been identified by paper chromatography and t.l.c. (78), but the qualitative and quantitative analysis is now more usually performed by g.l.c. and g.l.c.-m.s. of suitable derivatives (79, 80). The sugars are usually transformed into acetylated alditols or aldonitriles. Stereoisomers give similar mass spectra and when this information is coupled with the relative retention times on g.l.c. identification of the individual sugar is possible, with the exception of the absolute configuration (79-83).

2.2. Methylation Analysis

Methylation analysis, developed by Haworth and co-workers, is still the most important single method in structural carbohydrate chemistry. It involves methylation of all free hydroxyl groups in the polysaccharide and hydrolysis of the methylated polysaccharide to a mixture of

partially methylated monosaccharides. The free hydroxyl-groups in these mark the positions at which the corresponding sugar residues were substituted in the polysaccharide. Qualitative and quantitative analysis of this mixture therefore gives information on how the different sugars are linked. It does not, however, give information on the sequence or on the anomeric configurations (81-83). Due to the insolubility of high molecular weight O-chain polymer in organic solvents, the method of Haworth (84), Purdie (85) and Kuhn (86) required several steps to achieve complete methylation, whereas now the Hakomori methylation (87) achieves complete methylation in just one step. The latter method consists of treating the polysaccharide in dimethylsulfoxide with the anion of sodium methylsulfinyl methanide and subsequently with methyl iodide (87). The Hakomori methylation, unlike the Kuhn and Purdie procedures, cannot be used on a polysaccharide containing alkali labile substituents which should be preserved. In the latter case, methylation with methyl trifluoromethanesulfonate in trimethyl phosphate, using 2,6-di-(t-butyl)-pyridine as a proton scavenger is an elegant alternative (88). The methylation analysis is based on the ability to fractionate and characterize the partially methylated monosaccharides generated by hydrolysis of the fully methylated polysaccharide. This is most conveniently accomplished by combined gas-liquid chromatography/mass spectrometry of the methyl ether alditol acetates derivatives (81-83, 89).

The interpretation of the mass spectra is usually a simple task. Primary fragments 2A and 2B are formed by fission of the carbon chain of

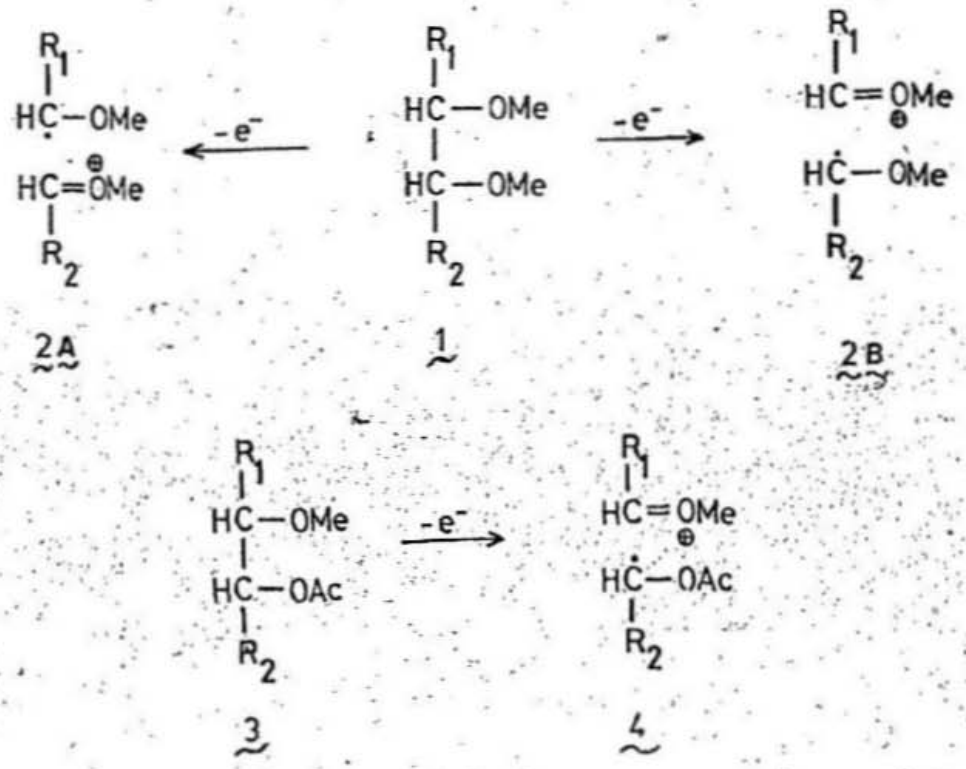
component 1 containing vicinal methoxylated carbons, the fission between these carbon atoms is preferred; either fragment may carry the positive charge. When a methoxylated carbon has an acetoxylated neighbour such as structure 3, tendency to fission between the methoxylated and acetoxylated carbon although not as great as between vicinical methoxylated carbon, gives significant primary ions and the fragment with the methoxylated carbon 4 always carries the positive charge (Scheme 1).

In general, if a residue contains a methoxy group, fragmentation between acetoxylated carbons becomes insignificant.

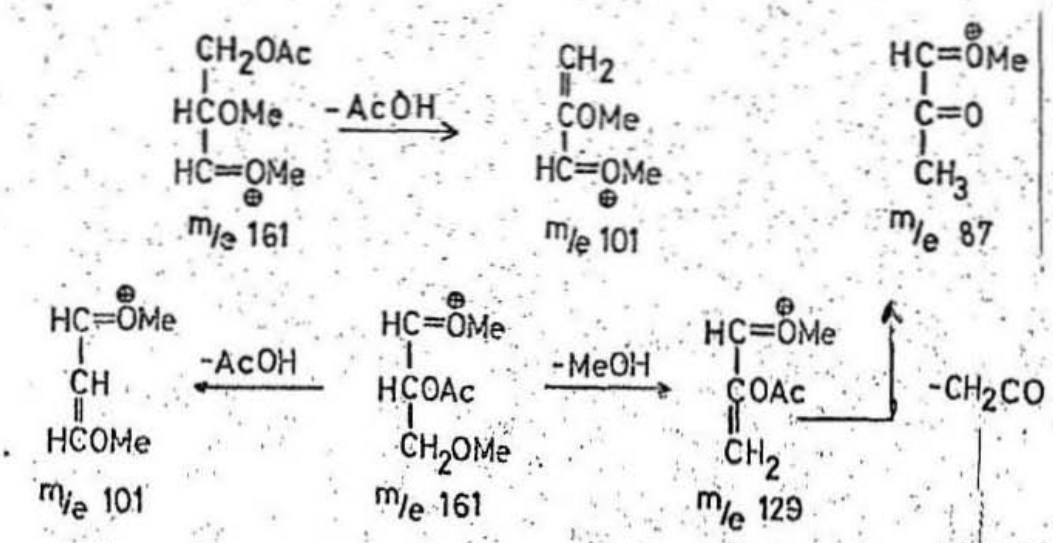
The primary fragments give secondary fragments by single or consecutive eliminations of methanol (m/z 32), acetic acid (m/z 60) and ketene (m/z 42). Eliminations of methanol, ketene and acetic acid from two primary fragments of m/z 161 (common in mass spectra of methylated sugars) are illustrated in Scheme 2.

Analysis and structural characterization of partially methylated alditol acetates of amino sugars has been the subject of several investigations (80, 90-93). For alditol acetates derived from 2-acetamido-2-deoxy-sugars 5 the primary fragment m/z 158 and the two secondary fragments m/z 116 and m/z 98 predominate. Other fragments are, however, strong enough to identify the methylation pattern (Scheme 3).

Other modifications of the methylation procedure such as the introduction of deuterium at C-1 in order to obtain unsymmetrical derivatives, dideuteration of C-6, trideuteriomethylation,



Scheme 1



Scheme 2

trideuterioacetylation, or ethylation give derivatives, the fragmentation patterns of which are quite analogous to those discussed earlier (82, 94, 102).

The reductive cleavage of the permethylated polysaccharide by boron trifluoride, triethylsilane and trifluoroacetic acid, to a mixture of anhydroalditols, can be used to distinguish between 4-linked aldohexopyranose and 5-linked aldohexofuranose residues in polysaccharides. Permethylated polysaccharides containing these residues will give only one partially methylated sugar, 2,3,6-tri-O-methylhexose after normal acid hydrolysis. This method can also be applied to 4- and 5-linked aldopentose residues and to 5- and 6-linked ketohexose residues (95).

As the methylation analysis has become so simple, it is also used in connection with different specific degradations of polysaccharides performed in order to determine the sequences of the sugar residues (96-98).

2.3. Sequence Analysis

Many different degradation techniques are used for sequence analysis of polysaccharides. There is no standard method, and each polysaccharide presents its own problems. Among the oldest methods are the partial acid hydrolysis and analysis of the resulting oligosaccharides, the Smith degradation and the deamination of the amino sugar residues. These different methods have been summarized in the literature (97).

2.3.1. Partial Hydrolysis with Acid

Capon (100) has comprehensively reviewed the first order rate constant for the acid catalysed hydrolysis of the glycosides of

monosaccharides, and several generalizations may be made:

a) - furanosides are more labile than pyranosides

b) - deoxy sugars are more easily hydrolysed than hexoses

c) - uronic acids are very resistant to hydrolysis

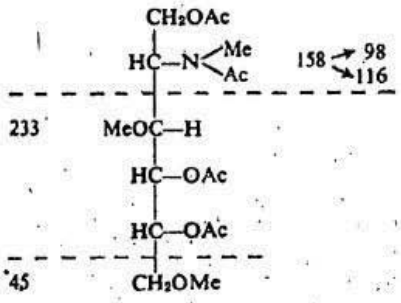
d) - amino sugars are more resistant to hydrolysis than common hexoses, due to the inductive effect of the protonated amino group

e) - pentopyranosides are more labile than hexopyranosides

f) - α-glycosidic bonds are usually more labile than β-glycosidic bonds

g) - residues present as "side chains" are often more easily hydrolysed than when present as "in-chain" residues.

It is therefore logical that given a heteropolysaccharide, there will be some glycosidic bonds that are relatively resistant to acid hydrolysis and others that are comparatively susceptible. Therefore, polysaccharides containing linkages of either type will tend to produce defined oligomeric subunits which can be isolated and identified. Considerably more structural information can be obtained if the methylated oligosaccharides derived from acidic hydrolysis of permethylated polysaccharide are reduced and realkylated using trideuteriomethyl or ethyl iodide. Thus the isolation of the alkylated disaccharide alditol 6 demonstrates: a) that the polysaccharide contains the disaccharide unit 7; b) that the non-reducing galactosyl group was linked to the 3-position in the polysaccharide; c) that the reducing residue was furanosidic; d) and that both sugars were chain residues and not branching



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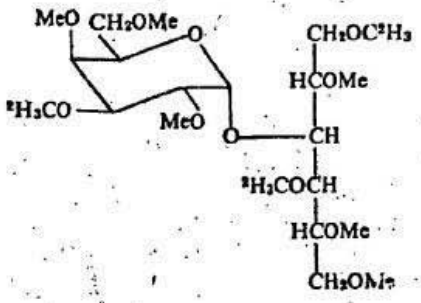
Scheme 3



6

α-D-Gal*p*-(1→3)-D-Gal

7

Scheme 4

residues (101) (Scheme 4). This latter technique has been further developed by Albersheim and his co-workers (102).

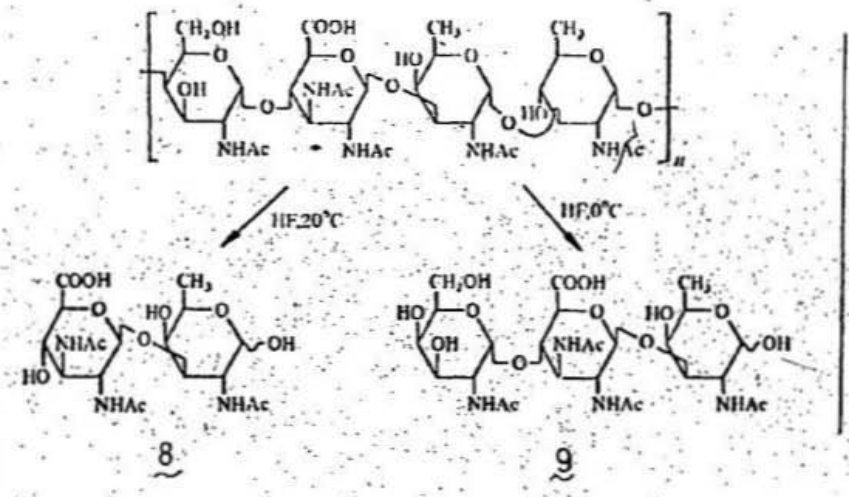
An alternative to partial acid hydrolysis is acetolysis (97, 103) and the relative rates by which the different glycosidic linkages are cleaved differ for this and the previous method. Pyranosidic (1→6)-linkages are cleaved more readily than other types of pyranosidic linkages on acetolysis. Acetolysis has been a valuable tool in structural analysis of fungal-α-D-mannans and for the isolation of oligosaccharides from glycoproteins derived from ovomucoid (104), and has also been suggested for use when N-acetylated amino sugars are components of the polysaccharide (90).

The relative rates of cleavage of different glycosidic linkages seem to differ according to the type of acidic hydrolysis and the reaction conditions used. The selective cleavage of hexosaminoglycans from Pseudomonas aeruginosa lipopolysaccharide (105) by hydrogen fluoride, yields either disaccharide 8 or a trisaccharide 9, depending on the reaction temperature used (Scheme 5).

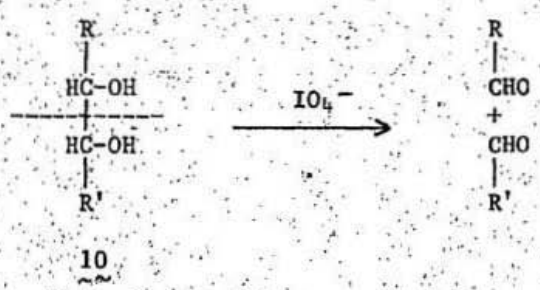
2.3.2 Periodate Oxidation and Smith Degradation

Since the discovery by Malaprade (106) in 1928 that sodium meta-periodate could cleave the carbon-carbon bond between vicinal hydroxy groups in aqueous solution, periodate oxidation has remained a powerful tool for structural investigations in carbohydrate chemistry.

Substances containing vicinal hydroxy-groups (e.g. 10) are oxidized



Scheme 5



Scheme 6

by periodate with the formation of carbonyl compounds as illustrated in Scheme 6; the simplest fragments being formaldehyde (R and R^1 = H). In the special case of three contiguous hydroxy-groups, formic acid is also formed.

The product of periodate oxidation of a polymer is termed a "polyaldehyde" which, when reduced with sodium borohydride, yields a "polyol". This derived polyol can provide considerable information on the original structure of the polymer.

Hydrolysis of the polyol will cleave all glycosidic linkages except those of unoxidized uronic acids and amino sugars. The hydrolysis products can be examined qualitatively by paper chromatography or by g.l.c. (107). The relative proportions of the surviving sugar residues and the fragments obtained from degraded sugars will give information regarding some of the glycosidic linkages present in the polysaccharide.

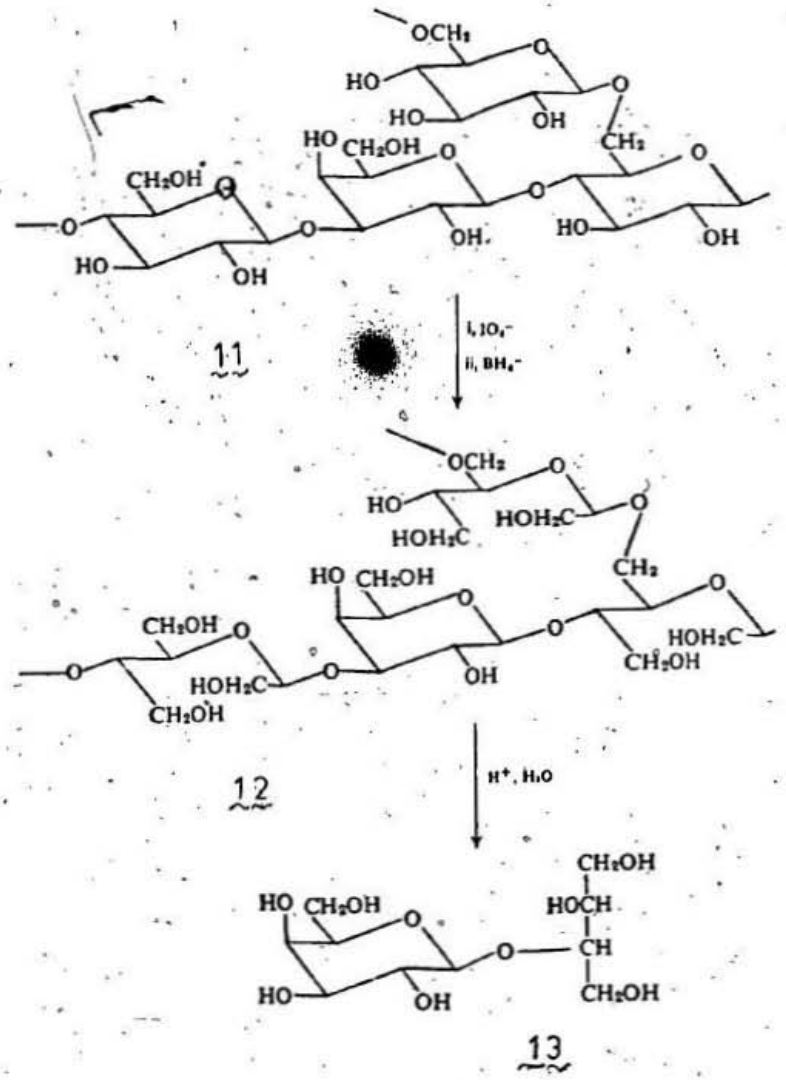
Much more information on the structure of the polysaccharide is obtained by the Smith degradation (108). In this degradation the periodate-oxidized polysaccharide (polyaldehyde) is reduced with borohydride to a polyalcohol, followed by mild hydrolysis with acid (usually at room temperature). The modified sugars contain acyclic acetal linkages which are hydrolysed (10^3 to 10^4 times) faster than the hemiacetal glycosidic linkages of the intact sugar residues. Characterization of the products, which may be either polymeric or low molecular weight glycosides, often gives considerable structural information. The Smith degradation of a galacto-glucan containing the

structural element 11, gives the 2-O-β-D-galactopyranosyl-D-erythritol 13 via the polyalcohol 12 (109) (Scheme 7).

The identification of substance 11 demonstrates that the polysaccharide contains β-D-galactopyranosyl residues resistant to periodate oxidation (because they are substituted in the C-3 position) and that these are linked to O-4- of the D-glucopyranosyl residues (109).

2,3.3. Deamination

Several polysaccharides contain amino sugars which are normally N-acetylated, these residues offer a starting point for specific degradations. After N-deacetylation by treatment with aqueous barium hydroxide, hydrazine or sodium hydroxide in aqueous dimethylsulfoxide containing thiophenol (110-111), the polysaccharides now containing free amino sugar residues offer a starting point for the nitrous acid deamination. The most common 2-amino-2-deoxyaldoses found in polysaccharides and glycoconjugates are 2-amino-2-deoxy-D-glucose and 2-amino-2-deoxy-D-galactose. The deamination of these 2-amino-2-deoxyhexoses or their hexopyranosides, in which the amino group is equatorially oriented in the most stable form, yields 2,5-anhydro-residues. This reaction has been adequately reviewed in the literature (97, 98, 112, 113). The rearrangement results from an attack on the intermediate diazonium ion by the ring-oxygen atom (which is in a trans and antiparallel disposition). Thus, deamination of a 2-amino-2-deoxy-D-glucopyranoside 14 via the diazonium ion 15 is known to give 2,5-anhydro-mannose 16,



11 12 13

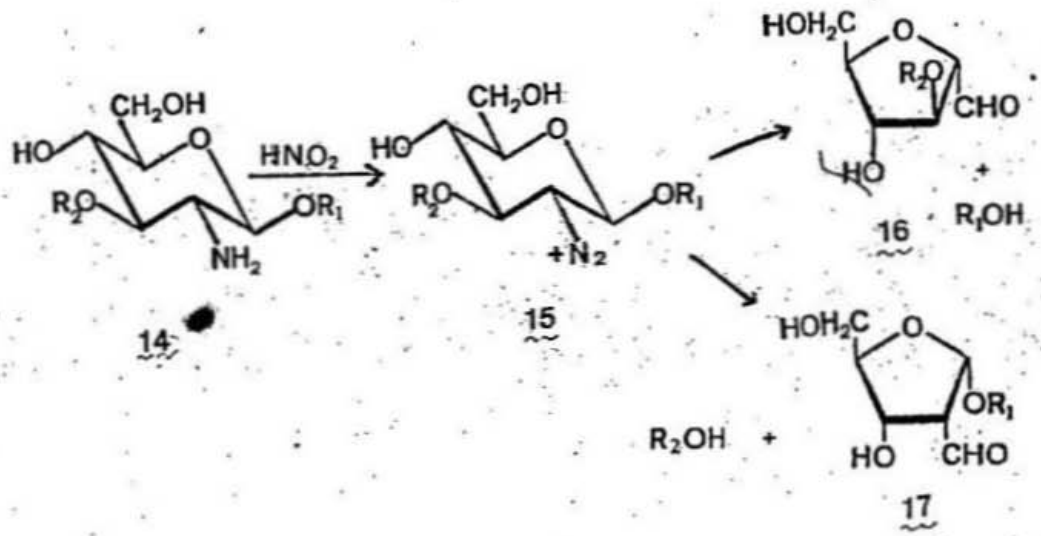
Scheme. 7

with release of the aglycon (Scheme 8).

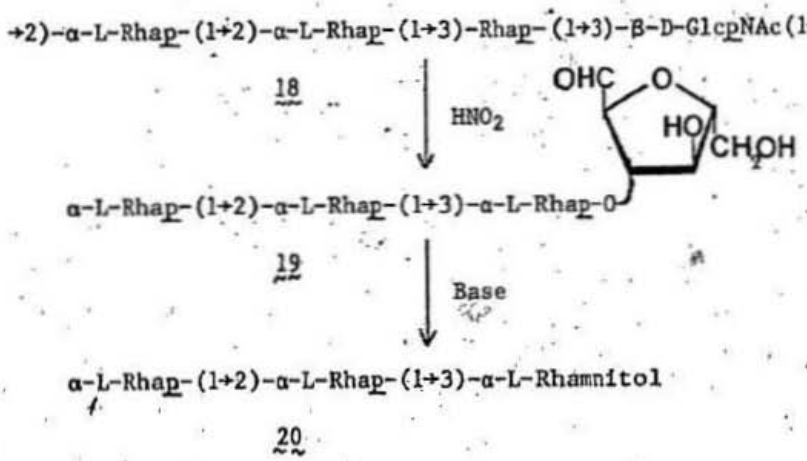
Similarly, 2-amino-2-deoxy-D-galactoside yields 2,5-anhydro-D-talose. Studies have revealed that part of the deamination reaction of 2-amino-2-deoxy-D-glucoside 14 may also take another course with formation of a 2-deoxy-2-C-formyl-D-ribo-hexoside 17. This unexpected rearrangement, results from the attack of carbon C-4 which is also trans and antiparallel to the diazonium ion (97, 98, 112, 113).

Aminosugar incorporated in a polysaccharide chain also reacts in the same manner. The O-specific polysaccharide from Shigella flexneri, polysaccharide variant Y, is composed of the tetrasaccharide repeating unit 18 (114). The N-deacetylated polysaccharide was deaminated by treatment with nitrous acid yielding tetrasaccharide 19 as the main product (Scheme 9). Methylation analysis of the reduced tetrasaccharide showed that one of the 2-substituted α-L-rhamnopyranosyl residues in the original polysaccharide had become the terminal non-reducing α-L-rhamnopyranosyl group in the tetrasaccharide, proving that the 2-acetamido-2-deoxy-D-glucose was linked to O-2 of this L-rhamnose. On treatment of tetrasaccharide 19 with base, the trisaccharide linked to O-3 of the 2,5-anhydro-mannose (produced from the deamination of the amino sugar residue) was released. Methylation analysis of trisaccharide alditol 20 provided the sequence of the three rhamnosyl residue in 18.

Deamination reactions have also been applied to polysaccharide containing other aminosugars, such as the polysaccharide from Vibrio cholerae containing a polymer composed of (1+2) linked 4-amino-4,6-



Scheme 8



Scheme 9

dideoxy-α-D-mannopynanosyl residues 21 (115); and polysaccharides containing 2-acetamido-4-amino-2,4,6,trideoxy-D-galactopyranosyl residues 22 (116, 117) (Scheme 10).

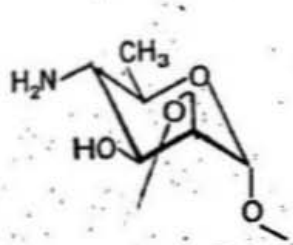
The deamination of 2-amino-2-deoxy aldopyranosides, having an axially attached amino group, affords complicated mixtures. Thus the deamination of 6-O-(2-amino-2-deoxy-β-D-mannopyranosyl)-D-glucose 23 gave the corresponding β-glycoside 24, together with rearrangement products, possibly 2-deoxy-β-D-erythro-hexo-pyranosid-3-uloses (118, 119) (Scheme 11).

2.4. Determination of Anomeric Configuration

In the past, determination of the anomeric configuration of the oligosaccharides obtained by graded hydrolysis was usually achieved by either enzymatic methods (120) or by measuring their optical rotations. More recently however, nuclear magnetic resonance spectroscopy spectra of the polysaccharide and its degradation products, as well as chromium trioxide oxidation of the fully acetylated polysaccharide have been added to these techniques and are being used as powerful tools to determine anomeric linkages.

2.4.1. Use of Proton and Carbon-13 Nuclear Magnetic Resonance Spectroscopy

^{1}H and ^{13}C-n.m.r. spectroscopy has been extensively used and has proven to be an extremely valuable non-destructive technique. Using the Fourier transform method, it allows spectra of the polysaccharides to be obtained from relatively small amounts of material. In ^{1}H n.m.r. it is



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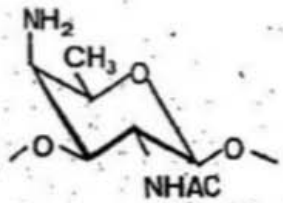

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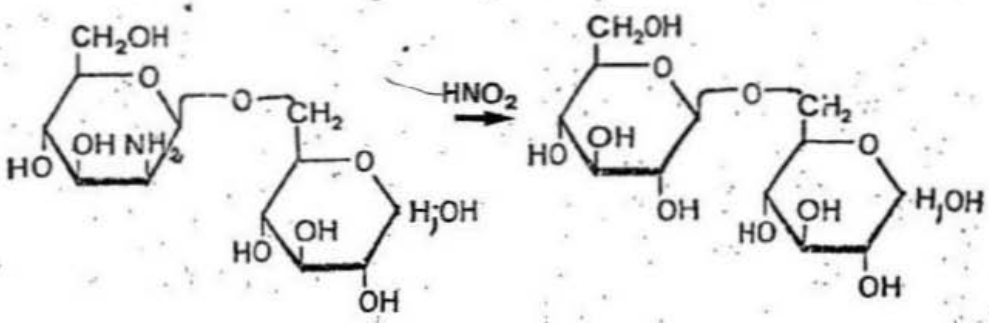
Scheme 10



23

24

Scheme 11

often sufficient to only determine the chemical shifts δ and the coupling constants $^{3}J_{H_1,H_2}$ of the anomeric protons. In general, the anomeric signals having chemical shifts greater than 5 ppm correspond to α-glycosidic linkages (H_1, equatorial), and the anomeric signals having chemical shifts below 5 ppm to β-glycosidic linkages (H_1, axial) (121).

Karplus (122) demonstrated that the values of the coupling constant $^{3}J_{H_1,H_2}$ depend on the dihedral angle Φ formed by the linkages involved as depicted in Scheme 12.

In the ^{13}C n.m.r. spectra similar but opposite effects are observed. In the anomeric region between 85 and 110 ppm, the anomeric carbons involved in an α-glycosidic linkage resonate at chemical shifts δ smaller (higher field) than those involved in β-glycosidic linkages (lower field). In effect, in proton coupled ^{13}C-n.m.r. spectra, coupling of the type ^{13}C-^{1}H can be observed, the major factor governing values of the coupling constant $^{1}J_{^{13}C_1-^{1}H_1}$ being the hybridization character of the anomeric carbon atom. In the D-glucose series for example ($^{4}\underline{C}_1$ conformation), the α-anomer C_1-O_5 bond exhibits a more pronounced double bond hybridization character (sp^2) than that of the β-anomer (anomeric effect). This effect results in the C_1-O_5 bond length being shorter for the α-anomer and hence the coupling constant, $^{1}J_{^{13}C_1-^{1}H_1}$ is greater than that of the β-anomer (123).

The coupling constant $^{1}J_{^{13}C_1-H_1}$ has considerable diagnostic value. In effect the $^{1}J_{^{13}C_1-^{1}H_1}$ values obtained for α- and β-anomers are

~ 170 Hz when H-1 is equatorial and ~ 160 Hz when H-1 is axial (124, 125). This general distinguishing feature is attributable to the fact that the equatorial C_1 - H_1 bond of the α-anomer has one trans and one gauche interaction (126).

The proton spin-lattice relaxation time (T_1) can have diagnostic value when determining the nature of the α- and β-glycosidic linkages. In effect, it has been shown that in the same magnetic environment the β H-1 protons relax faster than α protons (127).

Vliegenthart et al. have demonstrated that glycopeptide primary structure can be completely evaluated from the chemical shifts and the coupling constants of the anomeric and H-2 protons, by recording the ^{1}H-n.m.r. at 500 MHZ in deuterium oxide (128).

Two dimensional J-resolved ^{1}H-n.m.r. spectroscopy has been shown to be an efficient technique for resolving and assigning individual multiplets typically encountered in the one-dimension spectra of complex carbohydrates. In effect, the anomeric multiplets that are unresolved in the one dimensional 250 MHZ spectra of oligomannosidic glycopeptides can be separated and characterised by two-dimensional J-resolved ^{1}H-n.m.r. spectroscopy (129).

Recent reviews dealing with the well defined ^{13}C-n.m.r. spectroscopy of the carbohydrates and covering the current literature provide a wealth of information on the chemical and physicochemical properties of the polysaccharide (assignments of signals, sequencing and conformation) (130-136).

Finally, the ^{1}H and ^{13}C-n.m.r. spectra give valuable information on the presence or absence of pyruvate acetals, of N-acetyl and O-acetyl

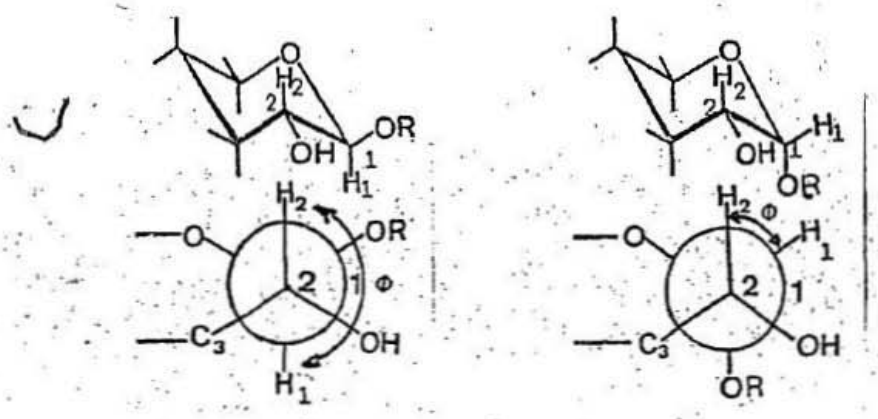
groups, 6-deoxy sugars and the deoxy functions carrying the amino group.

2.4.2. Chromium Trioxide Oxidation

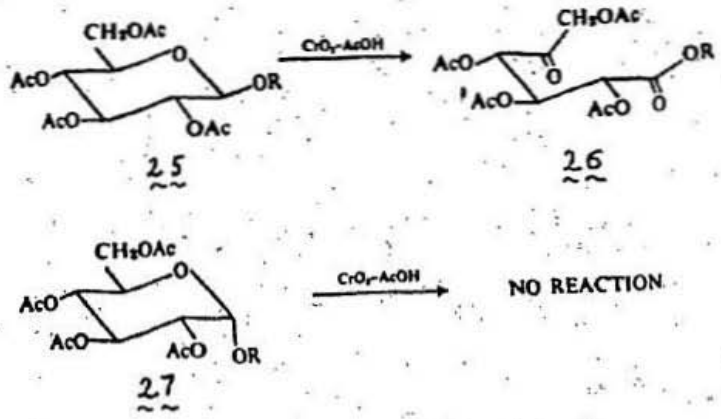
Angyal and James have demonstrated that the fully acetylated β-D-isomer of the aldopyranoside 25 with an equatorially oriented aglycon is oxidized by chromium trioxide in acetic acid to 5-aldulosonate 26 (137). The α-D-isomer 27 with axially oriented aglycon is only oxidized slowly under the same conditions (137) (Scheme 13).

Lindberg and co-workers have pointed out, however, that successful interpretation of the results of the chromium trioxide oxidation requires that the conformational stability of the chair form, having the axially oriented aglycon, be large enough to ensure that the proportion of the alternate form is negligible (138, 139). For α-D-linked pyranosides and some of the dideoxy-sugars (e.g. 3,6 dideoxy-D-arabino hexoside, tyvelose), the energy difference between the two chair forms is so small that the α-D-linked glycoside will also be oxidized (138-139). It has recently been confirmed that the α-linked glycosides of 3-amino-3,6-dideoxy-L-hexosides ($^1C_4$ conformation), having axially oriented aglycons, are resistant to chromium trioxide oxidation, whereas the β-L-linked glycosides having the gluco configuration ($^1C_4$ conformation and the α-L-linked glycoside having the talo configuration ($^4C_1$ conformation), where the aglycons are equatorially oriented, are readily oxidized to the corresponding 5-aldulosonates (140).

Sugar analysis of the oxidation product of the fully acetylated oligo- or polysaccharides, using myo-inositol hexa-acetate as an



Scheme 12



Scheme 13

internal standard, reveals which sugar residues have been destroyed and thus the anomeric configurations of the different sugar residues. Ester linkages formed during the oxidation can be cleaved during a subsequent methylation and by comparing the methylation analysis of the original polysaccharide with that of the oxidized sample, the sequence of the residues may be determined. For example, oxidation of the acetylated lipopolysaccharide from Salmonella kentucky revealed that the L-rhamnose and D-galactose residues were β-links in contrast to α-links for the D-mannose residues. On methylation analysis of the oxidized product, comparable amounts of 2,3,4,6-tetra-O-methyl-D-mannose and 3,4,6-tri-O-methyl-D-mannose were obtained. As the two D-mannose residues are the only α-linked sugars in the chain, it was concluded that they are adjacent, as shown in the structure of the oligosaccharide repeating unit 28 (141).

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2.5. Conformation Analysis

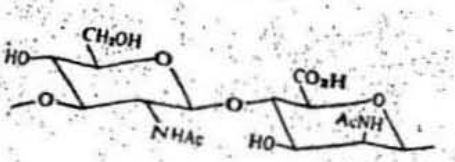
In studies aimed at a better understanding of the biological physical properties of a polysaccharide, determination of its structure

is only the first step; the next step should be the determination of its conformation in solution.

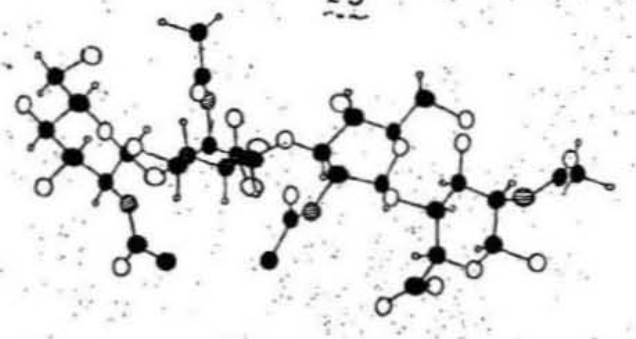
Recently, Lemieux et al. have used simple hard-sphere calculations, with correction for the exo-anomeric effect, for the determination of oligosaccharide conformations (142). They have also demonstrated, using high field ^{1}H-n.m.r. studies that there is good correlation between the calculated structures and those actually present in solution. The conformational preferences of the diastereoisomeric β-D-Gal-(1→4)-β-D-GalNAc-(1→6)-6-C-CH_3-D-Gal trisaccharide that binds specifically to the so-called anti I Ma monoclonal antibody, were established by ^{1}H-n.m.r. spectroscopy and rationalized by computer assisted molecular modeling (143). Similarly, the conformation of the bacterial polysaccharide from Haemophilus influenzae type e, composed of the disaccharide repeating unit 29 (144) has the conformation depicted in Scheme 14.

High resolution nuclear magnetic resonance data combined with hard-sphere molecular modeling (HSEA-calculations) has also permitted the study of the conformation of gentiobiose octaacetate in an organic solvent (145).

Finally, knowledge of the resonances associated with specific residues of a branched polysaccharide or oligosaccharide combined with measurements of carbon-13 spin lattice relaxation times (T_1) may also allow assignments of ^{13}C-n.m.r. resonances, as well as providing considerable information on the position of residues in branched polysaccharides or the order of saccharides in oligomers (146, 147), and



29



Scheme 14

hence permits measurement of the molecular motion of that polysaccharide.

2.6. Immunochemical Methods

When an antigen is injected into an animal (e.g. a rabbit), the immune system of that organism is stimulated into producing antibodies to counteract the "invading" antigen. The bacterial polysaccharides provide a rich source of antigenic material, and since they are often the principal antigenic determinants of the parent microorganisms, the corresponding antisera are also frequently available. Hence the cross reaction of a polysaccharide of unknown or uncertain chemical structure with antibodies to a polysaccharide of known structure may yield information on both position and linkage of one or more of the sugars residues. Conversely, cross reactivity of a polysaccharide of known structure with antibodies to a polysaccharide of unknown composition and linkage may be equally informative.

Heidelberger (148) has developed methods utilizing the precipitation reactions of antibody (precipitin) and antigen. Heidelberger et al. (149-151) have examined the cross reactions between approximately 60 of the serologically distinct capsular polysaccharides from Klebsiella types 1-80 and antisera to some selected Pneumococcus polysaccharides. The degree of cross reaction, as measured by the amount of precipitation, is an indication of the degree of similarity of the structure of the polysaccharide used to elicit the immune serum for

the test. A series of precipitin tests must be performed to deduce the fine partial structure of the unknown.

Cross reactions, when clear cut interpretation is possible, can yield structural information that could take months to obtain by purely chemical means. However, the complete structures of many of the Pneumococcus polysaccharides are not known and some strongly positive cross reactions between Pneumococcus antisera and Klebsiella polysaccharides cannot be fully interpreted (149-151). Furthermore, the O-polysaccharides of Salmonella and Escherichia species have been extensively studied with regard to their serological cross reactions. These reactions have been used to create an elegant classification scheme for these bacterial species (152).

2.7. Conclusion

The structural methods briefly discussed herein represent only some of the principal means used to elucidate the structure of a polysaccharide or oligosaccharide.

Although these methods are complementary, none are, by themselves, sufficient for the complete elucidation of the unknown molecule. Hence it is the combination of the results obtained by these different methods which permit tentative postulation of the structure.

PART II

STRUCTURAL STUDIES ON THE LIPOPOLYSACCHARIDES ISOLATED FROM BACTERIOPHAGE SELECTED STRAINS OF AEROMONAS HYDROPHILA

INTRODUCTION

Aeromonas hydrophila is a gram negative bacterium belonging to the family Vibrionaceae, and is a common inhabitant of freshwater lakes and streams (153-156)

The microorganisms of the genus Aeromonas are of particular interest to the fisheries scientist, as they are widely distributed, partly as saprophytes, and partly as agents causing disease in freshwater fishes.

According to Shubert (157) and Bergey's Manual of Determinative Bacteriology (158), three species are presently distinguished in the genus Aeromonas: A. hydrophila, A. punctata and A. salmonicida.

These microorganisms were discovered almost simultaneously toward the end of the last century under different circumstances. Sanarelli (159) identified A. hydrophila (ssp. hydrophila) as the agent causing an aquatic bacterial infection of frogs. In numerous experiments involving the artificial infection of fishes, amphibians, reptiles, birds and mammals, Sanarelli demonstrated its pathogenicity for both poikilothermic and homeothermic animals.

The genus Aeromonas hydrophila was proposed by Kluyver and Van Neil (160) to accommodate rod shaped motile (by means of polar flagella) bacteria possessing the general properties of the enteric group.

In the 7th edition of Bergey's Manual of Determinative Bacteriology Sniezko described three species of Motile Aeromonas, A. liquefaciens, punctata and A. hydrophila. The description of these

species was based on their pathogenicity and a few biochemical tests. It is obvious that since the initial proposal of the genus Aeromonas, the taxonomic classification of the motile Aeromonas species has presented a confusing picture, mainly due to the relatively minor biochemical differences which have been found between strains.

The present position accepted by the Judicial Commission (Bergey's Manual of Determinative Bacteriology 1974) is that the motile aeromonads consist of two species, viz. A. hydrophila and A. punctata, the former with three subspecies, and the latter with two. The major change represented by this latest disposition of the species is that A. liquefaciens has been amalgamated with A. hydrophila and no longer exists as either a separate species or subspecies.

On the basis of computer analysis of 203 biochemical characters for 68 motile Aeromonas, Popoff and Véron (161) have proposed the retention of the species A. hydrophila (biovars hydrophila and anaerogenes) and the introduction of a new species of A. sobria.

BIOLOGICAL ACTIVITIES OF AEROMONAS HYDROPHILA

Motile members of the genus Aeromonas are ubiquitous in fresh water and are known to cause haemorrhagic septicemia in both warm- and cold-water fishes (162). They are generally regarded as opportunistic pathogens causing disease in stressed fish or as secondary invaders in injured ones.

Little attention has been given to the involvement of Aeromonas hydrophila, as a cause of human infections. This may stem from the

frequency with which the organism has been incorrectly identified as another species and from the organism's reputation for low virulence; this has led clinicians to suspect that it is not contributing to disease even when it has been correctly identified.

Reports from around the world of this organism's involvement in infections in humans are becoming increasingly more frequent however, and it appears that this organism may have much greater clinical importance than was previously suspected.

It has been isolated in pure culture from both focal and systemic infections of varying severity, and can act in concert with other organisms. It has also been isolated from stool, urine and sputum samples without ascertainable pathogenicity, which suggests that it can be a constituent of normal human flora (163).

A. hydrophila has for some time been recognized as an opportunistic pathogen in hosts with impaired local or general defence mechanisms. However, reports of acute diarrheal disease caused by this organism in apparently healthy individuals are increasing in frequency. Compromised hosts are especially likely to incur progressive or even fatal infections (164-166)

Studies conducted on the serological identity of members of the Aeromonas hydrophila group of bacteria have indicated an extraordinary amount of heterogeneity (167). Recent investigations by traditional biochemical reactions (161), and by analysis of the composition of the core oligosaccharide of the cell-surface lipopolysaccharide (47) have indicated three major sub-groups.

Interest in the structures and immunological properties of the cell surface polysaccharides of the different chemotypes of this gram-negative bacterium has increased, and little is presently known of the biochemical basis of pathogenicity of these species.

The detailed core structure of Aeromonas hydrophila Chemotype III has recently been reported (48). This thesis presents the results of structural studies carried out on two distinct regions of the lipopolysaccharide (semi rough polysaccharide, and core oligosaccharide) isolated from Aeromonas hydrophila strain A6, as well as on the core oligosaccharide from two phage selected mutants (A. hydrophila strain A6a and A6b) of the original strain A6. These two mutants were isolated (from the same plate culture) as two morphologically different clones. The first one (Strain A6a) when cultivated in presence of phage, was unable to reproduce and was referred to as phage sensitive, conversely strain A6b grew when infected by the same phage and was referred to as phage resistant.

From these observations, it was of interest to look at the precise molecular structure of the outermost lipopolysaccharide layer, which is usually the receptor site for bacteriophages (12, 16, 20) and may contain the immunodeterminant sugar residue of these three genetically related species. Effort has been particularly concentrated on the core oligosaccharides from two of them (Strain A6 and Strain A6b), and the exact differences in these two molecules.

EXPERIMENTAL

1. BACTERIAL CULTURE

A. hydrophila strains were supplied by Dr. H. M. Atkinson, the South Australia School of Technology. *A. hydrophila* strain A6 (isolated from human feces) is the original strain from which two morphologically different clones were selected from plate culture; one of these (strain A6a) is sensitive to phage (not able to grow when infected by the phage), the other (strain A6b) is resistant (able to grow) when infected by the same phage. These strains were added to the collection of the Northwest Atlantic Fisheries Centre, St. John's as SJ-55 (original), SJ-47 (phage sensitive), and SJ-48 (phage resistant). The organisms were initially plated on Trypticase Soy Agar (Baltimore Biological Laboratory) to check purity and colonial morphology. Stock cultures were grown in Trypticase Soy Broth without dextrose, divided into 1 ml aliquots, made 15% with respect to glycerol, and frozen and stored at 80°C. The protocol for medium scale culture of the bacteria was generally identical for each strain. Trypticase Soy Broth (100 ml) was inoculated with the contents of a frozen vial, grown for 24 h at room temperature with reciprocating shaking, and used as the inoculum for 1 litre of the same medium. The 1 l culture grown for 18 h at 25°C (with aeration) was subsequently used as the inoculum for a 24 l batch grown in a commercial fermentor 25 l New Brunswick Scientific Co.) for 26 h at 25°C with an air flow of 4 l/min and no control of pH.

The culture was killed by adding formaldehyde to a final concentration of 0.3% and stirring for 18 h. The cells were collected by continuous centrifugation (Sorvall RC-2B high-speed centrifuge) and freeze-dried; cells were not washed.

2. GENERAL METHODS

2.1 Chromatographic Methods

Analytical paper-chromatography was performed on Whatman No. 1 paper, and Whatman No. 3 MM was used for preparative purposes, using the following solvent systems: (A) Freshly prepared 8:2:2 (v/v) ethyl acetate/pyridine/ water, (B) 4:1:5 (v/v) 1-butanol/ethanol/water (top layer), and (C) 10:4:3 (v/v) ethyl acetate/pyridine/water. Chromatograms were developed with alkaline silver nitrate (168) or aniline phthalate spray reagents (169).

High voltage paper-electrophoresis was conducted for 90 min in a Shandon flat bed, high voltage electrophoresis apparatus, using a buffer of 5:2:43 (v/v) pyridine/acetic acid/water, at pH 5.4, 2 kv and 170 mA. The aminosugars or aminosugar containing disaccharides were detected by spraying with ninhydrin, or by using the alkaline silver nitrate reagent. Mobilities (M_{GlcN}) are expressed relative to glucosamine. The aminosugars or disaccharides were eluted from the paper electropherogram with 0.05% HCl.

Thin layer chromatography was carried out on precoated cellulose (100 μm) plates (E. Merck, Darmstadt) with a solvent system (D) of

5:5:1:3 (v/v), ethyl acetate/pyridine/acetic acid/ water in an atmosphere saturated with 40:11:6 (v/v) ethyl acetate/pyridine/water (170)

Gas liquid chromatography of the acetylated alditols and partially methylated alditol acetates, was performed on packed columns (183 mm x 2mm i.d.) of 1.5% Silar 7CP on Gas Chrom Q (100-120 mesh) in a Perkin Elmer model 3920 gas chromatograph or a Hewlett Packard Model 402 gas chromatograph fitted with an hydrogen flame detector and a model 3380A electronic integrator. Temperature profiles varied as follows according to the type of derivative being analysed; profile A for partially methylated alditol acetates, isothermal at 180°C; profile B for alditols acetates, isothermal at 210°C and profile C for derivatized oligosaccharide, with a temperature program starting at 180°C for 32 min then increasing to 270°C at 8°/min (held for 45 min). Gas chromatography was also performed on a 25 m W.C.O.T. CP-Si15 (0.25μ film thickness) capillary column (Chrompack, The Netherlands) at 190° for methylated acetylated alditols (171).

Combined gas chromatography-mass spectrometry was performed on a Hewlett Packard model 5981A GC/MS controlled by a 5934A data system, with a membrane separator, a source temperature of 160° and ionizing voltage of 70 e.v. The gas chromatographic conditions (profiles A, B, and C) described above were used.

Gel permeation chromatography was carried out using columns (1.5 x 90 cm) of Sephadex G-15 or G-50 using 47 mM pyridinium acetate buffer,

pH 4.26 as eluant. Elution of the components was monitored with a differential refractive index monitor (Waters Associates Model R403). The fractions were collected and freeze dried. Optical rotations were measured at 23°C in water in a 10-cm microtube using a Perkin-Elmer model 141 polarimeter.

2.2. Nuclear Magnetic Resonance

2.2.1. ^{1}H-n.m.r. Spectroscopy

Oligosaccharides were repeatedly treated with D_2O (99.8%), with lyophilization between treatments and finally dissolved in D_2O (99.99%). The 80, 100 and 250 MHz spectra were recorded at 85°C in the pulse Fourier Transform mode on a Bruker 80 MHz, Bruker 100 MHz and Cameca 250 MHz spectrometers respectively. In the 100 MHz and 250 MHz spectra the HOD signal was suppressed by selective saturation using an inversion recovery pulse sequence of T_1 measurement (T-180°-τ-90°-FID; in which a 180° pulse was applied followed by a 90° pulse after a τ time = 0.7 T_1 solvent) (172) which allowed zero magnetization for the solvent peak. Hence it became possible to eliminate the HOD solvent peak by taking advantage of the difference of relaxation speed between the protons of the studied sample and the protons of the solvent. Chemical shifts (δ, ppm) are measured with respect to internal acetone (δ = 2.225 ppm) and expressed relative to sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as external standard.

2.2.2. ^{13}C-n.m.r. Spectroscopy

The spectra were recorded at ambient temperature in 80% D_2O at 20.12 and 62.86 MHz in the pulse Fourier Transform mode on a BRUKER 80 MHz spectrometer and CAMECA 250 MHz respectively. The chemical shifts quoted (δ) in ppm are expressed relative to internal acetone (δ = 31.07 ppm from DSS) on spectra recorded with complete decoupling. Coupling constants $^1J_{C,H}$ are expressed in Hertz and were measured by the gated-decoupling technique. This technique consists of proton irradiation during a given time (1.6 sec was used) (taking advantage of the nuclear Overhauser effect) followed by switching off the irradiation during the acquisition time which permits observation of the $^1J_{C,H}$ coupling constants. The selective, heteronuclear, double irradiation spectra were obtained by application of a continuous wave of fixed frequency and with a weak field of ~ 0.1 Gauss (173).

3. PURIFICATION OF THE LIPOPOLYSACCHARIDE

The freeze dried cells were extracted by the phenol-water method of Westphal and Jann (22) with two wash cycles of the phenol layer. The combined aqueous layers were dialysed against tap water for 48 h and the dialysate was centrifuged at 5000 xg for 20 min to remove cellular and precipitated debris, prior to ultracentrifugation of the clear supernatant at 105,000 xg for 3 h. The resulting gel was redissolved in water, recentrifuged at 105,000 xg (twice), and freeze dried to give (usually) a purified lipopolysaccharide free

from ribonucleic acid. In the event that ribonucleic acid was still present, further centrifugation was carried out until a hydrolysate of the freeze-dried gel produced essentially no ribose.

4. ISOLATION OF THE CORE OLIGOSACCHARIDE

The pure lipopolysaccharide (100 mg) was hydrolysed with dilute acetic acid (1%) for 90 minutes at 100°. The released carbohydrate portion remained in solution, while the precipitated lipid A was removed by centrifugation (5000 rpm, 30 min). The obtained supernatant was freeze dried, redissolved in 1 ml of pyridinium acetate buffer (47 mM, pH 4.26) and recentrifuged to remove remaining traces of the lipid A. The resulting supernatant was fractionated on a column of Sephadex G-50 and afforded several components. The core oligosaccharide was defined as the peak immediately preceeding the salt peak eluting between Kav values at 0.6 and 0.7 with Kav = $(Ve-Vo)/V_t-V_o)$, where Ve is the elution volume of the specific material, Vo is the void volume of the system and Vt is the total volume of the system. In this study the fraction associated with such peaks were pooled and freeze dried.

5. METHODS OF QUANTITATIVE ANALYSIS

Protein was assayed according to the Lowry method (174), phosphorus by the method of Chen et al. (175), neutral glycoses by the phenol-sulfuric acid method (176), uronic acids by the carbazole method (177) and aminosugars by the Rondle-Morgan and Strominger assays (178, 179) (after hydrolysis with 3.9 M HCl for 12 h at 100°). They were also

quantified by amino acid analysis using a Durum D-500 model analyzer, stepwise elution was done with a citrate buffer ranging in molarity from 0.2 to 0.38 M and in pH from 3.8 to 4.25. 3-Deoxy-D-manno-2-octulosonic acid (KDO) was determined by the thiobarbiturate (180) and diphenylamine (181) assays.

6. HYDROLYSIS OF THE CORE OLIGOSACCHARIDE AND SUGAR ANALYSIS

For neutral glycose analysis, the core oligosaccharide (1 mg) was hydrolysed with either 2M trifluoroacetic acid or 0.5M sulfuric acid for 10 h at 100°, followed by neutralization and concentration. The liberated monosaccharides were converted into alditol acetates (182) by reduction and acetylation and were identified by g.l.c. and g.l.c.-m.s. using inositol as an internal standard for the quantitative analysis. For the analysis of 2-amino-2-deoxy-D-glucose, the sample (1 mg) was hydrolysed for 10 h at 100° with 4M hydrochloric acid followed by g.l.c. analysis (profile B) as alditol acetate derivative (183). The amino-glycose from the core oligosaccharide was isolated by preparative paper chromatography (solvent B), giving a single ninhydrin positive spot with R_{GlcN} 1.00 and $[\alpha]_D^{23}$ + 63° (c, 0.3, in water). After reduction ($NaBH_4$) and acetylation it gave on g.l.c. (profile B) a single peak with T_{Glc} 6.01, corresponding in retention time with authentic 1,3,4,5,6-penta-O-acetyl-2-acetamido-2-deoxy-D-glucitol. It was further characterized by ninhydrin degradation (184) carried out as follows: the isolated aminoglycose hydrochloride was oxidized by a 2% aqueous ninhydrin solution for 30 min at 100°C and gave after paper

chromatography (solvent C) a positive Trevelyan spot R_{Ara} 1.00. The oxidized product, after reduction ($NaBH_4$) and acetylation afforded Arabinitol pentaacetate which, on g.l.c. (profile B), corresponded in retention time (T_{Glc} 0.31) to an authentic derivative. The neutral sugars obtained after acidic hydrolysis (TFA 2M, 8h, 100°) were also isolated and characterized by paper chromatography (solvent A) and their optical rotations were recorded: D-glucose $[\alpha]_D^{23}$ + 43° (c, 0.01 in water); D-galactose $[\alpha]_D^{23}$ + 40° (c, 0.5, in water).

The identity of L-glycero-D-manno and D-glycero-D-manno-heptose were established by experimental comparison of their g.l.c. retention time and mass spectrum with that of authentic samples. L-glycero-D-manno-heptose was isolated (from SJ-48 core oligosaccharide) by paper chromatography (solvent B) It had $[\alpha]_D^{23}$ + 13.9° (c, 0.5, in water) (185).

7. METHYLATION ANALYSIS

All compounds were methylated by the method of Hakomori (87) as follows: the oligosaccharide (~ 1mg) was placed in a small serum vial containing a stirrer bar and fitted with rubber serum cap, and was dissolved in 0.5 ml of dry and distilled dimethyl sulfoxide. The flask and contents were flushed with dry nitrogen, and methyl sulfinyl anion (0.5 ml of a 2 M solution) was added; the reaction proceeded with stirring for approximately 3 hours. Methyl iodide (1 ml) was then added while the reaction was kept at 4°C in an ice bath for 4 h. The methylated oligosaccharide was then purified by passage of the reactants

through a small column of Sephadex LH-20 suspended in chloroform followed by elution with the same solvent. The purified permethylated oligosaccharide was then hydrolysed with 2M trifluoroacetic acid (10h, 100°).

The resulting partially methylated sugars were reduced, acetylated and analysed as alditol acetate derivatives by g.l.c.-m.s. (81-83, 171), using temperature profile A.

8. PARTIAL HYDROLYSIS OF THE CORE OLIGOSACCHARIDE

The core oligosaccharide (10 mg) was partially hydrolysed with 0.5 M sulfuric acid for either 30, 60 or 90 min, the solution was neutralized with barium carbonate and evaporated. The product was purified by chromatography on Sephadex G-15 and afforded a major fraction. This degraded oligosaccharide was subjected to sugar and methylation analysis. The permethylated product was hydrolysed with 2M trifluoroacetic acid (10h, 100°), borohydride reduced, acetylated and analysed by g.l.c.-m.s.

9. PERIODATE OXIDATION OF THE CORE OLIGOSACCHARIDE

The core oligosaccharide (50 mg) was dissolved in distilled water (10 ml) and 0.1 M sodium metaperiodate (10 ml) was added (186). the solution was kept in the dark at 5°C. After 72 h, $BaCO_3$ (25 mg) was added and the suspension was stirred overnight. After filtration, the solution was reduced with sodium borohydride and neutralized.

The polyol was purified by column chromatography on Sephadex G-15 and analysed for the component sugars.

10. SMITH DEGRADATION OF THE CORE OLIGOSACCHARIDE

The polyol (20 mg), obtained from the periodate oxidation of the core oligosaccharide, was hydrolysed with 1% acetic acid for 3 h at 100°C (108). After evaporation of the acid, the residue was borohydride reduced and purified by column chromatography on Sephadex G-15. The pure Smith degraded product was methylated followed by hydrolysis, reduction, acetylation and g.l.c.-m.s. analysis.

11. NITROUS ACID DEAMINATION OF THE CORE OLIGOSACCHARIDE

The core oligosaccharide (10 mg) in water (1 ml) was treated with 30% acetic acid (1 ml) and fresh 5% sodium nitrite solution (1 ml) (187), it was kept at room temperature for 60 min, prior to passage through a column of Rexyn 101 (H^+). The deaminated oligosaccharide was reduced with sodium borohydride, neutralized with acetic acid and then purified by chromatography on Sephadex G-15. The purified deaminated oligosaccharide was methylated, hydrolysed and analysed by g.l.c.-m.s. as partially methylated alditol acetates.

12. HYDROLYSIS OF THE CORE OLIGOSACCHARIDE WITH 2M HYDROCHLORIC ACID

The core oligosaccharide (30 mg) was hydrolysed with 2M hydrochloric acid for 1 h at 100°, followed by evaporation. The residue was purified by high voltage electrophoresis (H.V.E.) using a pyridinium

acetate buffer (pH 5.3). It afforded a fraction that had M_{glcN} = 0.6, which was recovered (by elution with 0.05% HCl) from the electropherogram. The disaccharide was subjected to acetylation, followed by methylation, hydrolysis, reduction, acetylation and g.l.c.-m.s. analysis. The isolated disaccharide was also subjected to a nitrous acid deamination, followed by borohydride reduction, acetylation and g.l.c.-m.s. analysis.

13. OXIDATION WITH CHROMIUM TRIOXIDE

The core oligosaccharide (20 mg) was dissolved in dimethylformamide (1 ml) and acetic anhydride (1 ml) and pyridine (1 ml) were added (139). The solution was stirred for 16 h at room temperature and extracted with chloroform. The residue was reacetylated in a mixture of (1:1) acetic anhydride/pyridine (1h, 100°)) to afford a peracetylated product. The residue was dissolved in chloroform, myo-inositol hexaacetate (2 mg) was added as internal standard, and the solution was divided into two parts and evaporated. A part was dissolved in glacial acetic acid (2 ml) and powdered chromium trioxide (40 mg) was added; the mixture was then agitated ultrasonically for 3 h at 53°C, poured into water, extracted with chloroform (10 ml), the extract washed twice with water and evaporated to dryness. The oxidized core and the remaining, original unoxidized material, were subjected to analysis for the individual sugars. The oxidized product was also methylated, hydrolysed with 2M trifluoroacetic acid for 10 h at 100°, reduced, acetylated and analysed by g.l.c. and g.l.c.-m.s.

RESULTS AND DISCUSSION

1. STRUCTURAL INVESTIGATIONS ON THE CORE OLIGOSACCHARIDE OF *AEROMONAS HYDROPHILA* STRAIN SJ-48

1.1. Isolation and Chemical Analysis of the Core Oligosaccharide

The lipopolysaccharide (lps) was isolated by the aqueous phenol method of Westphal and Jann (22). Analytical studies on the lps indicated that it contained phosphorus, 2-amino-2-deoxy-D-glucose and protein in the amounts indicated in Table I. The core oligosaccharide was obtained by mild hydrolysis of the lipopolysaccharide with acetic acid and purified by chromatography on a column of Sephadex G-50. As with the lipopolysaccharide of *Aeromonas hydrophila* Chemotype III (48), the core oligosaccharide of this strain (SJ-48) is the main constituent of the polysaccharide portion of the lps. The purified core oligosaccharide was a white, water-soluble powder that had $[\alpha]_D^{23}$ + 85° (*c* 0.85, water) and was essentially free from nucleic acid.

Conventional analysis of the core oligosaccharide indicated that it was apparently composed of residues of D-glucose, L-glycero-D-manno-heptose and 2-amino-2-deoxy-D-glucose in the molar ratio of 1:3:0.85 (Table II; A, p. 70). The optical rotations of the sugars isolated from the hydrolysate established that the glucose and 2-amino-2-deoxy-glucose residues had the D configuration. The 2-amino-2-deoxy-glucose was also confirmed by its degradation to ribose with ninhydrin (184). The identity of L-glycero-D-manno-heptose was established by experimental comparison

Table I: Analytical assays on SJ-48 lps, core and Lipid A

	dOclA[a] (KDO)	phosphorus[b] %	Hexosamine[c] A	B %	C	Protein[d] %
SJ48 LPS	(+)	3.5	nd	nd	10.1	2.1
SJ48 Core	(+)	0.35	7.2	12.8	6.2	1.7
SJ48 Lipid A	(nd)	6.34	10.6	11.2	nd	nd

[a]Thiobarbituric acid and diphenylamine assays (181). [b]Chen et al. assay (175). [c]Rondle Morgan (A) (178), Strominger assay (B) (179) and Amino Acid Analyser (C). [d]Lowry assay (174). nd: not determined.

Table II: Sugar Analysis of SJ-48 Core Oligosaccharide and Derived Product

Sugars (as alditol acetates)[a]	Given in molar proportions[c]								
	A	B	C	D	E	F	G	H	I
D-Glc	1.00	1.00	1.00	1.00	-	-	-	-	0.11
LD-Hep	2.91	3.85	2.15	3.05	1.00	1.00	1.00	1.00	2.70
D-GlcN[b]	0.85	-	0.73	-	-	-	0.82	-	0.58
2,5-anhydro-D-Man	-	0.65[d]	-	0.60[d]	-	-	-	0.69	-
Lyxitol	-	-	-	-	0.35	-	-	-	-
Threitol	-	-	-	-	0.75	-	-	-	-
Glycerol	-	-	-	-	1.40	0.60	-	-	-

[a] Alditol acetate derivatives of neutral sugars were analysed on a Silar 7CP column. [b] D-Glucosamine was quantified both by analytical assays, and g.l.c.-analysis (as alditol acetate after a 4M HCl hydrolysis for 16 hours at 100°C). [c] A - Original core; B - Deaminated core; C - Partially hydrolysed core (0.5 M H_2SO_4, 60 min, 100°); D - Deaminated product C; E - Periodate oxidized core; F - Smith degraded core; G - Disaccharide isolated by H.V.E (2M HCl, 1 h, 100°); H - Disaccharide G deaminated, reduced and acetylated; I - Chromium trioxide oxidized core. [d] 2,5 anhydro-D-mannose analysed on g.l.c. obtained after deamination, reduction and acetylation of the respective treated sample.

of its g.l.c. retention time and mass spectrum with that of an authentic sample, together with its optical rotation (185). Analytical studies on the core oligosaccharide indicated that it did not contain fatty acid, 2-aminoethanol, protein, or 3-deoxy-D-manno-2-octulosonic acid (dOclA) as indicated in Table I. Although small amounts of phosphorus were present, they were not present in quantities which would suggest even a close stoichiometric relationship.

1.2. Nuclear Magnetic Resonance

1.2.1. ^{1}H-n.m.r.

The ^{1}H-n.m.r. spectrum of the core oligosaccharide showed, inter alia, five signals in the anomeric region indicating an α-configuration ($J_{1,2}$ ~ 2-3 Hz) at 5.68 (1H), 5.53 (1H), 5.43 (1H) and 5.34 (2H) ppm, and one signal at 4.61 (1H) ppm ($J_{1,2}$ ~ 8Hz) indicating a β configuration (188). There were no signals for pyruvate, acetate or methyl protons of deoxy sugars (Fig. 1). These results suggested a core oligosaccharide made up of 6 glycose units; a result which would indicate that one residue was not accounted for by the chemical analysis.

1.2.2. ^{13}C-n.m.r.

The ^{13}C-n.m.r. spectrum of the core oligosaccharide showed, inter alia, five anomeric signals resonating at δ:103.10, 102.50, 100.47, 100.20 (double intensity signal) and 96.5 ppm respectively. These signals integrated for six anomeric carbon atoms.

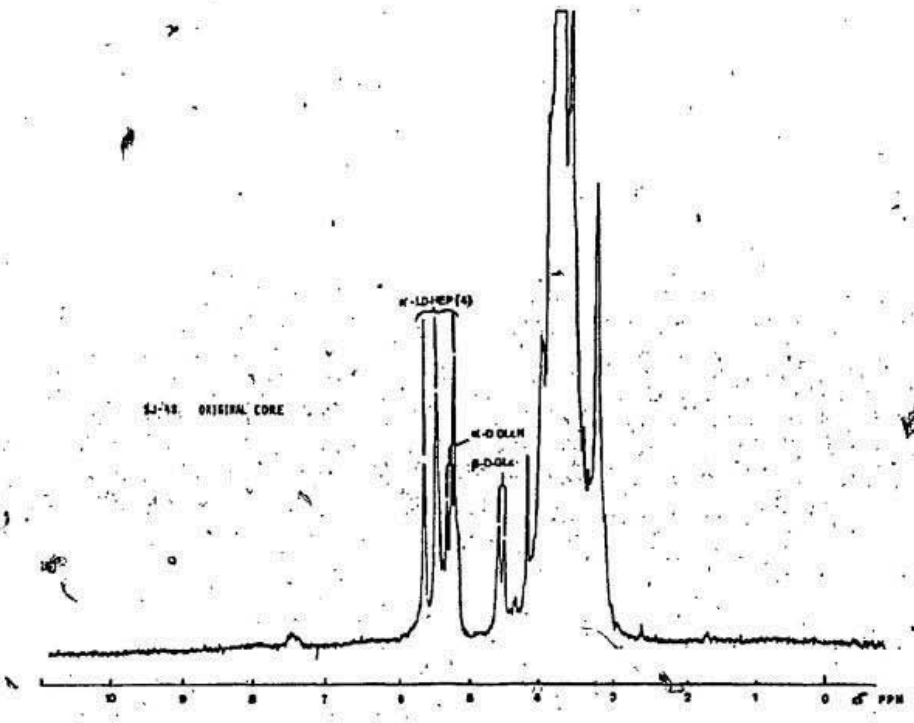


Fig.1. ^{1}H-n.m.r. spectrum of the original core from strain SJ-48.

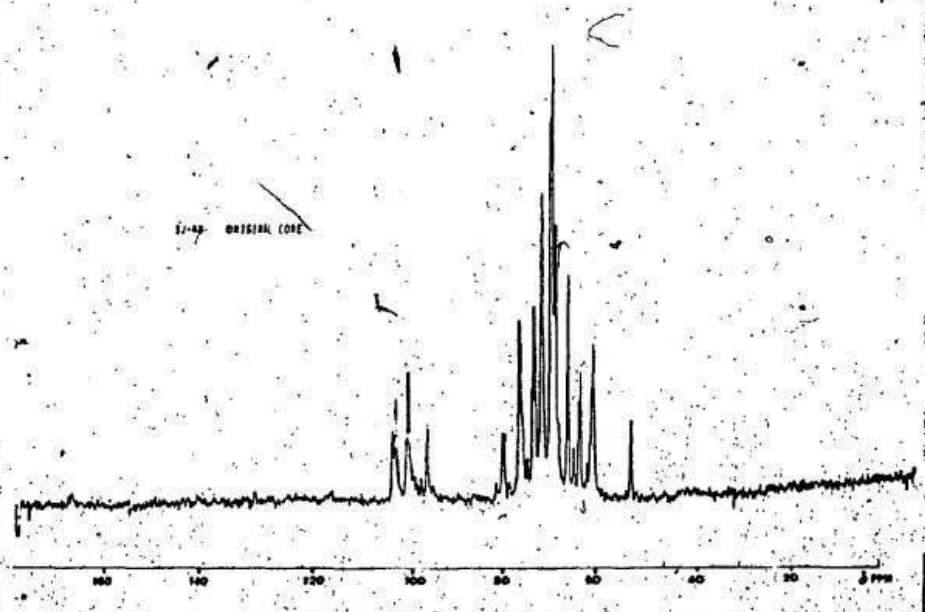


Fig.2. ^{13}C-n.m.r. spectrum of the original core from strain SJ-48.

By analogy with oligosaccharide models and by selective, heteronuclear double irradiation the signal at 103.10 ppm was assigned to the D-glucosyl unit; its $^{1}J_{C,H}$ value (~ 160 Hz) obtained by the gated decoupling technique suggested that it had a β configuration. Due to the lack of reported chemical shifts for L-glycero-D-manno-heptose, no attempt has been made to assign the anomeric signals between 102.5 and 106.5 ppm, but their $^{1}J_{C,H}$ coupling values lie between 170.0 and 170.4 Hz, suggesting α anomeric configurations (123).

The chemical shifts at 61,60 and 61.95 ppm correspond to the non-linked C-6 atom of the primary hydroxymethyl group of D-glucose and 2-amino-2-deoxy-D-glucose. The chemical shift at 54.95 corresponds to the C-2 carbon of the 2-amino-2-deoxy-D-glucose (Fig. 2).

The ^{13}C-n.m.r. spectrum further confirms the ^{1}H-n.m.r. postulate that the core oligosaccharide contains 6 glycose units.

1.3. Methylation Analysis

Methylation of the core oligosaccharide by the Hakomori method (87), followed by hydrolysis with 2M-trifluoroacetic acid, reduction, and derivatization as alditol acetates, afforded 2,3,4,6,-tetra-O-methylglucose, 2,3,4,6,7-penta-O-methylheptose, 3,4,6,7-tetra-O-methylheptose, and 2,7-di-O-methylheptose in approximately equimolar proportions (see Table III; A) identified by g.l.c.-m.s. (81-83, 171).

The presence of a 2,7-di-O-methylheptose is attributable to a double branch point, and that of 2,3,4,6-tetra-O-methylglucose and 2,3,4,6,7-penta-O-methylheptose, to two nonreducing, terminal groups.

Table III: Methylation analysis of SJ48 Core Oligosaccharide and derived products.

methylated sugar[a]	T[b] X	T[b] Y	In Mole % [c,d] A	B	C	D	E	F	Linkages
2,3,4,6-Glc.	1.00	1.00	25.7 (0.97)	19.8 (0.92)	34.4 (1.04)	25.1 (0.90)	5.0 (0.09)	4.4 (0.12)	D-Glc (1 →
2,3,4,6,7-LD-Hep	1.61	1.61	28.0 (1.05)	37.8 (1.75)	4.3 (0.13)	22.2 (0.80)	27.9 (0.87)	31.5 (0.86)	LD-Hep (1 →
2,4,6,7-LD-Hep	2.45	3.70	-	-	-	-	13.7 (0.43)	27.4 (0.75)	→5)LD-Hep(1 →
3,4,6,7-LD-Hep	2.38	3.72	26.5 (1.00)	21.6 (1.00)	32.9 (1.00)	27.8 (1.00)	32.0 (1.00)	36.6 (1.00)	→2)LD-Hep(1 →
2,3,4,6-LD-Hep	2.78	5.23	-	6.6 (0.30)	-	4.2 (0.15)	8.9 (0.28)	-	→7)LD-Hep(1 →
2,6,7-LD-Hep	2.71	4.80	-	-	28.4 (0.86)	20.7 (0.74)	-	-	→4)LD-Hep(1 → 3
2,4,7-LD-Hep	2.85	5.10	-	-	-	-	14.5 (0.45)	-	→6)LD-Hep(1 → 3
2,7-LD-Hep	3.69	8.47	19.8 (0.75)	13.8 (0.64)	-	-	-	-	→6)LD-Hep(→ 3

[a] 2,3,4,6-Glc stands for 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol, other methylated sugars are similarly designated. [b] Retention times relative to the alditol acetate derivative of 2,3,4,6-tetra-O-methyl-D-glucose on column X:WCOT CP-Sil.5 at 190° and Y:Silar-7CP at 180°C. [c] A-original core; B-deaminated core; C-partially hydrolysed core (0.5M H_2SO_4, 1h, 100°); D-deaminated product C; E-chromium trioxide oxidized original core; F-chromium trioxide oxidized product D. [d] Between brackets are given the molar ratios.

The 3,4,6,7-tetra-O-methylheptose is assigned to a residue linked through O-2. It should be noted that the relationship of branch points being equal to end groups minus one does not hold in the above methylation analysis, indicating that one end group is unaccounted for.

It is clear from the stoichiometric composition of the hydrolysis products of the methylated core oligosaccharide that, in fact, two glycose residues (one of which must be a 2-amino-2-deoxy-D-glucose unit) are unaccounted for, one of them presumably being the missing nonreducing terminal group. This observation appears to suggest that these two residues were glycosidically linked by a linkage resistant to the hydrolysis conditions used. When gas-liquid chromatography of the partially methylated alditol acetates was conducted under programmed temperature conditions (profile C), a slow moving component 1 was observed whose identity was established by mass spectrometry as 1,5-di-O-acetyl-6-O- or 1,5-di-O-acetyl-7-O-[2-deoxy-3,4,6-tri-O-methyl-2-(N-methylacetamido)-D-glucopyranosyl]-tetra-O-methyl-L-glycero-D-manno-heptitol 1. The structure of 1 was confirmed by interpretation of the fragmention pattern which gave primary ions at m/z 260 (for the nonreducing 2-amino-2-deoxy-glucosyl group) and m/z 335 (for the heptitol residue), together with major secondary ions at m/z 228 (260-32), 205, 196 (228-32), 161, 154 (196-32), 142, 129, 117, 101, 88, 87, 45 and 43 (see Fig. 3). The presence of the ions at m/z 205, 161 and 117 indicates that the linkage of the aminosugar to the heptose must be through position 6- or 7- of the heptose residue as indicated in the

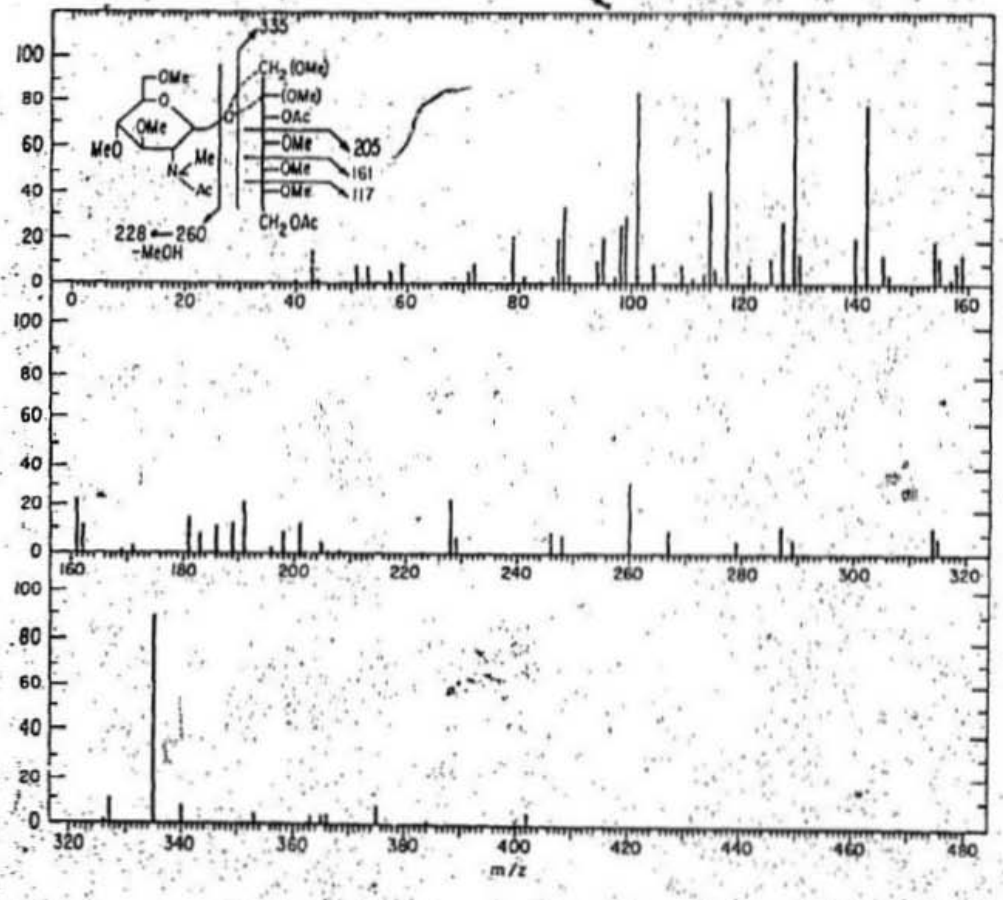


Fig. 3. Mass spectrum of 1,5-di-O-acetyl-6-O- or 7-O-[3,4,6-tri-O-methyl-2-(N-methylacetamido)-D-glucopyranosyl]-tetra-O-methyl-L-glycero-D-manno-heptitol.

following structure 1:

D-GlcpN(1 → 6 or 7)-LD-Hepp-ol

1

In order to establish the sequence and anomeric configuration of the glycosidic linkages of the sugar units of the core oligosaccharide, and to corroborate the existence of the 2-amino-2-deoxy-D-glucosyl moiety, subsequent work was directed towards the characterization of the oligosaccharides obtained after specific degradations such as nitrous acid deamination, periodate and Smith degradation, partial hydrolysis, and chromium trioxide oxidation.

1.4. Nitrous Acid Deamination of the Core Oligosaccharide

The core oligosaccharide was treated with nitrous acid followed by reduction with sodium borohydride (187). The 2,5-anhydro-D-mannitol residue released was acetylated and the ester identified by g.l.c. and g.l.c.-m.s. by comparison with an authentic sample. The resulting deaminated core oligosaccharide was purified by chromatography on Sephadex G-15. Sugar analysis of this product gave D-glucose and L-glycero-D-manno-heptose in the molar ratios ~ 1:4. Sugar analysis performed on the deamination mixture prior to purification on Sephadex G-15 gave 2,5-anhydro-D-mannitol, D-glucose and L-glycero-D-manno-heptose in the molar ratios 0.65:1.00:3.85 (~ 1:1:4) - (see Table II; B, p. 70). These are the expected molar proportions for the neutral glycose

residues of the core oligosaccharide and also confirm the previous n.m.r. data (i.e. a total of 6 sugars residues).

The ^{13}C-n.m.r. (Fig. 4) of the deaminated core oligosaccharide showed inter alia, four anomeric signals at δ 103.4, 102.8, 100.6 and 100.5 (double signal) ppm integrating for five anomeric carbon atoms. By comparing this ^{13}C-n.m.r. spectrum with that of the original core, it is evident that one residue has been lost. In fact, we notice the disappearance of the anomeric signal at 96.5 ppm (which may be attributable to C-1 of the missing residue), together with the disappearance of the signals at 61.35 ppm (C-6 bearing the primary hydroxyl group) and 54.95 ppm (C-2 bearing an amino group) which must have originated from the 2-amino-2-deoxy-D-glucose residue. These results showed that the deamination reaction was quantitative and indicated that the deaminated oligosaccharide was made up of 5 glycose units, a result compatible with the chemical analysis.

The ^{1}H-n.m.r. spectrum of the deaminated oligosaccharide (Fig. 5) showed four signals in the anomeric region, integrating for 5 protons. These consisted of 3 signals at δ:5.68, 5.53 and 5.34 (2H) ppm with $J_{1,2}$ (~ 2Hz) corresponding to an α-configuration, and a signal at 4.61 ppm ($J_{1,2}$ ~ 8Hz) corresponding to a β-configuration. The comparison of the ^{1}H-n.m.r. spectra of the original core with that of the deaminated one (Fig. 5) indicated the disappearance of the signal at 5.43 ppm (α-anomeric proton) which may therefore be assigned to H-1 of the α-linked glucosamine residue.

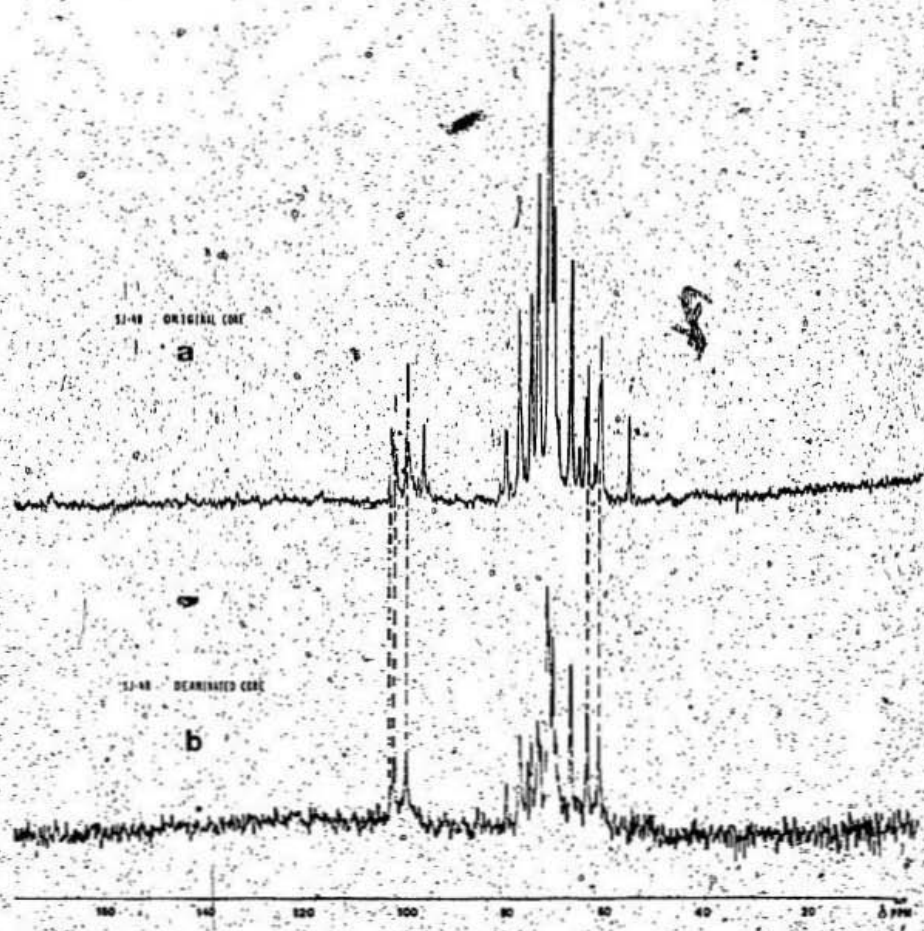


Fig.4. ^{13}C-n.m.r. spectra of the original (a) and the deaminated (b) core oligosaccharide of strain SJ-48.

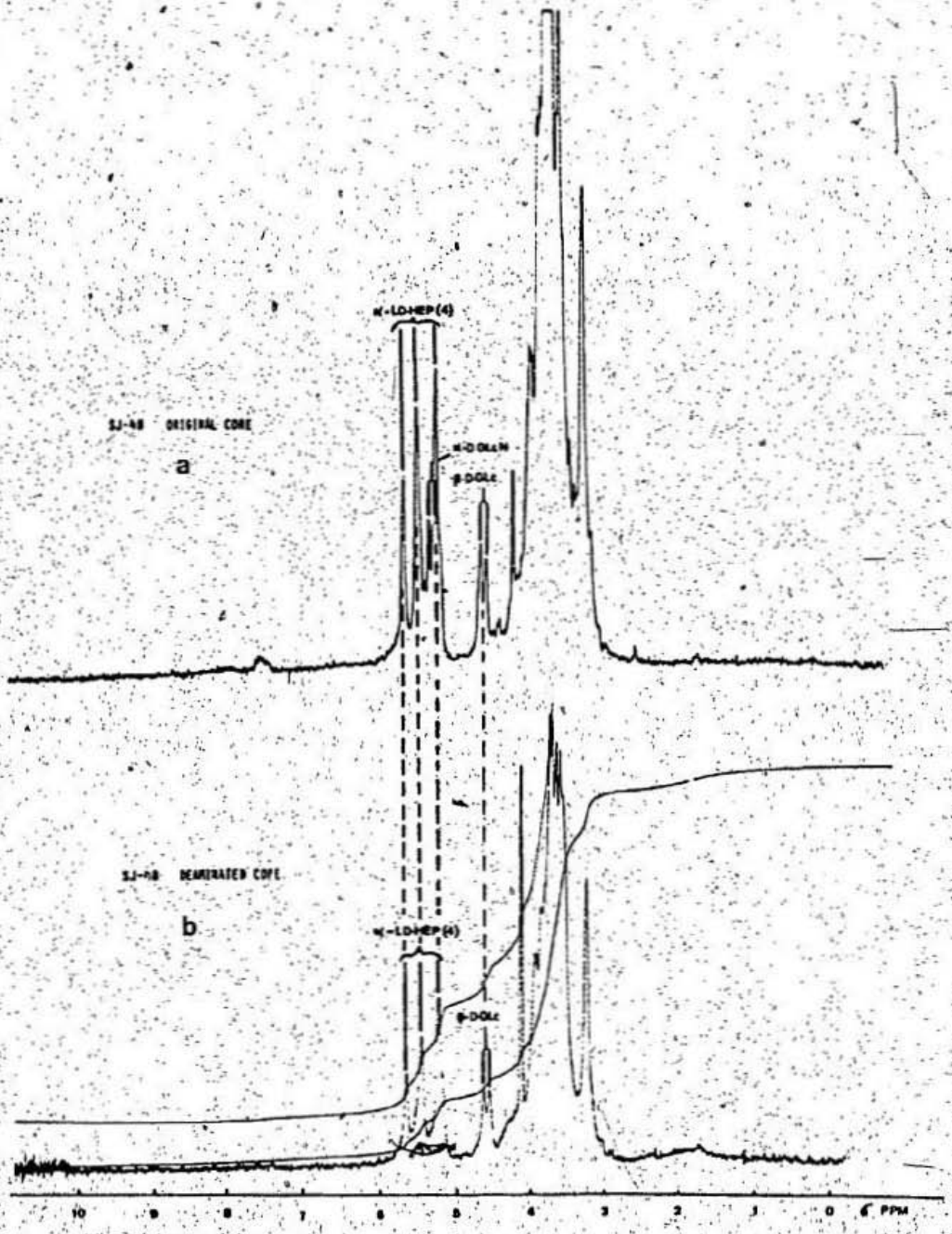


Fig.5. ^{1}H-n.m.r. spectra of the original (a) and the deaminated (b) core oligosaccharide of strain SJ-48.

Methylation analysis of the deaminated oligosaccharide gave 2,3,4,6-tetra-O-methylglucose, 2,3,4,6,7-penta-O-methylheptose, 3,4,6,7-tetra-O-methylheptose, 2,3,4,6-tri-O-methylheptose and 2,7-di-O-methylheptose in the following molar ratios: 0.92:1.75:1.00:0.30:0.64 (see Table III; B, p. 74). The low molar proportion of 2,3,4,6-tetra-O-methylheptose arising from the 7-O-substituted LD-heptosyl unit, considered together with the net increase of 2,3,4,6,7-penta-O-methylheptose confirmed that the 2-amino-2-deoxy-D-glucosyl group was linked to O-7 of the L-glycero-D-manno-heptose residue. These results are in good agreement with those obtained by Erbing et al. (189) who have shown that during deamination of a 2-amino-2-deoxy-D-glucopyranoside, part of the reaction (~ 20%) instead of releasing the aglycon, takes another course with formation of a 2-deoxy-2-C-formyl-D-ribo-pentoside (189).

In conclusion, direct formation of the 2,5-anhydro-D-mannose residue during the deamination reaction together with formation of 2,3,4,6-tetra-O-methylheptose, confirmed the gluco configuration of the amino sugar residue, that it was terminal nonreducing, and that it was glycosidically linked to O-7 of a heptosyl residue by a linkage extremely resistant to normal hydrolysis conditions.

1.5. Periodate Oxidation of the Core Oligosaccharide

The core oligosaccharide was oxidized with sodium metaperiodate, borohydride reduced and purified by chromatography on Sephadex G-15.

Sugar analysis of the purified polyol indicated the presence of L-glycero-D-manno-heptose, lyxitol, threitol and glycerol in the molar ratios of ~ 1:0.3:0.7:4.4 (Table II; E, p. 70). Formation of threitol can only be from the 7-O-substituted L-glycero-D-manno-heptose residue. The lyxitol residue may arise from the incomplete oxidation of this 7-O-linked heptose; in fact oxidation of the vicinal 3- and 4-hydroxyl groups was very slow, probably due to steric hindrance and to possible hydrogen bonding between the amino group of the glucosamine moiety and the 4-hydroxyl group of the heptose residue. In repeat experiments there were quantitative differences in the amounts of lyxitol and threitol with respect to each other, however, the sum of these amounts was invariably very close to 1 mole. These results also showed that 1 mole of D-glucose, 1 mole of 2-amino-2-deoxy-D-glucose and 3 moles of L-glycero-D-manno-heptose had been oxidized. This is in agreement with the results of the methylation analysis.

1.6. Smith Degradation of the Core Oligosaccharide

The core oligosaccharide was subjected to Smith degradation (108), that is, periodate oxidation, borohydride reduction and mild hydrolysis with acid.

The Smith degraded product was purified by chromatography on Sephadex G-15 and sugar analysis of the purified product 2 indicated the presence of L-glycero-D-manno-heptose and glycerol in the molar ratio 1:0.6 (Table II; F, p. 70). Methylation analysis of the Smith degraded product 2 yielded 2,3,4,6,7-penta-O-methylheptose and 1,3-di-O-

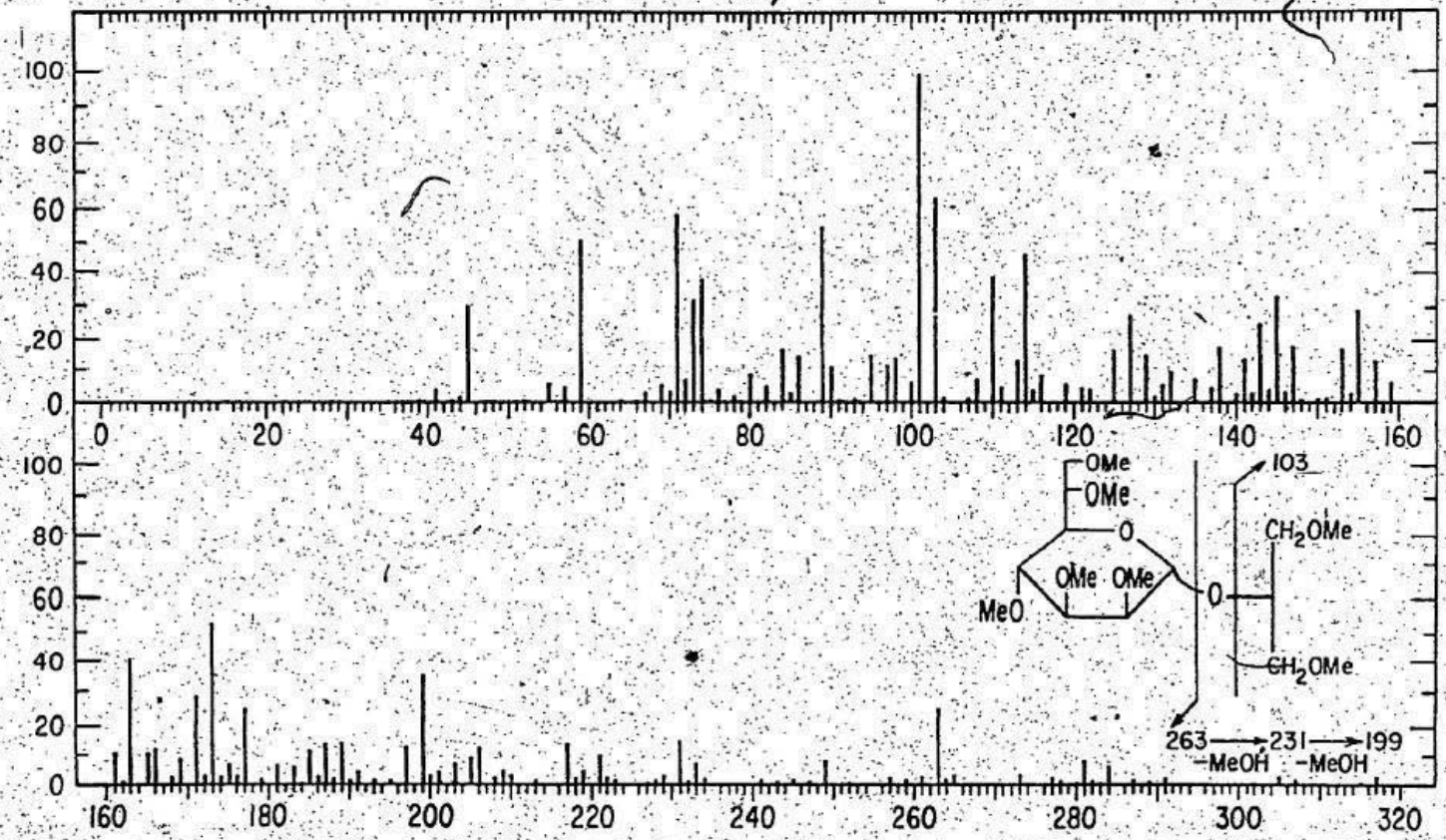


Fig.6. Mass spectrum of the permethylated Smith oligosaccharide of strain SJ-48.

methylglycerol in the molar ratio of 1:0.4 (the non stoichiometric amount of methylated glycerol detected by g.l.c. is due to its great volatility during the successive concentrations necessary for its derivatization).

Hence the structure of the Smith oligosaccharide 2 is as shown.

LD-Hepp-(1 → 2)-glycerol

2

The permethylated Smith oligosaccharide 2 was analysed by g.l.c.-m.s. (profile C) and its structure was confirmed from the fragmentation pattern (79, 190). Primary ions (of the A series) characteristic of a terminal nonreducing heptosyl residue at m/z 263 (a A_1), 231 (a A_2), 199 (a A_3), those of a glycerol moiety m/z 103 (b A_1) and 163 (b J_1), together with the expected secondary ions at m/z, 173, 155, 145, 101, 89, 71, 59 and 45 (see Fig. 6) were observed.

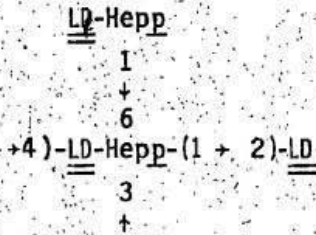
1.7. Partial Hydrolysis of the Core Oligosaccharide

1.7.1. Partial Hydrolysis with 0.5M Sulfuric Acid

Partial hydrolysis of the core oligosaccharide with 0.5M sulfuric acid for 1 h at 100°, followed by purification on Sephadex G-15 yielded a degraded oligosaccharide. Sugar analysis indicated that it was composed of D-glucose, L-glycero-D-manno-heptose and 2-amino-2-deoxy-D-glucose in the molar ratios of 1:2.15:0.73 (Table II; C, p. 70).

This result indicates that 1 mole of L-glycero-D-manno-heptose has been lost during the acid degradation.

Methylation of the degraded oligosaccharide followed by hydrolysis, reduction and g.l.c.-m.s. of the alditol acetates, yielded 2,3,4,6-tetra-O-methylglucose, 2,3,4,6,7-penta-O-methylheptose, 3,4,6,7-tetra-O-methylheptose and 2,6,7-tri-O-methylheptose in the following molar ratios 1.04:0.13:1.00:0.86 (see Table III; C, p. 74)[1]. This result indicated that the 2,7-di-O-methylheptose found on methylation and hydrolysis of the original material had been replaced by 2,6,7-tri-O-methylheptose and that the terminal LD-heptosyl moiety was almost quantitatively removed and must have been linked to the branched L-glycero-D-manno-heptose residue through O-6 as in the following partial structure.



The ^{13}C-n.m.r. spectrum of the degraded oligosaccharide showed five anomeric signals resonating at δ:103.2, 102.5, 100.45, 100.2 and 96.5 ppm. By comparison of the ^{13}C-n.m.r. spectrum of this degraded

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1. When g.l.c.-m.s. was conducted under program (profile C) disaccharide alditol 1 was present.

oligosaccharide with that of the original core, (see Fig. 7) it is evident that the double signal at 100.45 has been reduced to a single signal, thus confirming the loss of a sugar residue. This makes it possible to assign this signal to the anomeric carbon of the L-glycero-D-manno-heptose end group. It is also interesting to note the presence of the signals at 54.95 ppm and 62.0 ppm corresponding to C-2 and C-6 carbons of the D-glucosamine residue, which is obviously still present.

1.7.2. Deamination of the Partially Hydrolysed Core

Nitrous acid deamination of the partially hydrolysed oligosaccharide, followed by borohydride reduction and sugar analysis yielded D-glucose, L-glycero-D-manno-heptose and 2,5-anhydro-D-mannose (1:3:0.6) (Table II; D, p. 70). This result indicates that destruction of the 2-amino-2-deoxy-D-glucose has occurred.

Purification of the above deamination reaction mixture by chromatography on Sephadex G-15 afforded a deaminated product. Methylation analysis of this deaminated product gave: 2,3,4,6-tetra-O-methyl-glucose, 2,3,4,6,7-penta-O-methylheptose, 3,4,6,7-tetra-O-methylheptose and 2,6,7-tri-O-methylheptose in the molar ratio of ~ 1:1:1:1 (Table III; D, p. 74). By comparison of this methylation analysis with that of the partially hydrolysed oligosaccharide (Table III; C, p. 74) it is clear that the 7-O-substituted L-glycero-D-manno-heptosyl residue arising from the already mentioned D-GlcN-1→7 LD-Hep oligosaccharide has become a terminal nonreducing residue. Hence the partial structure is as follows:

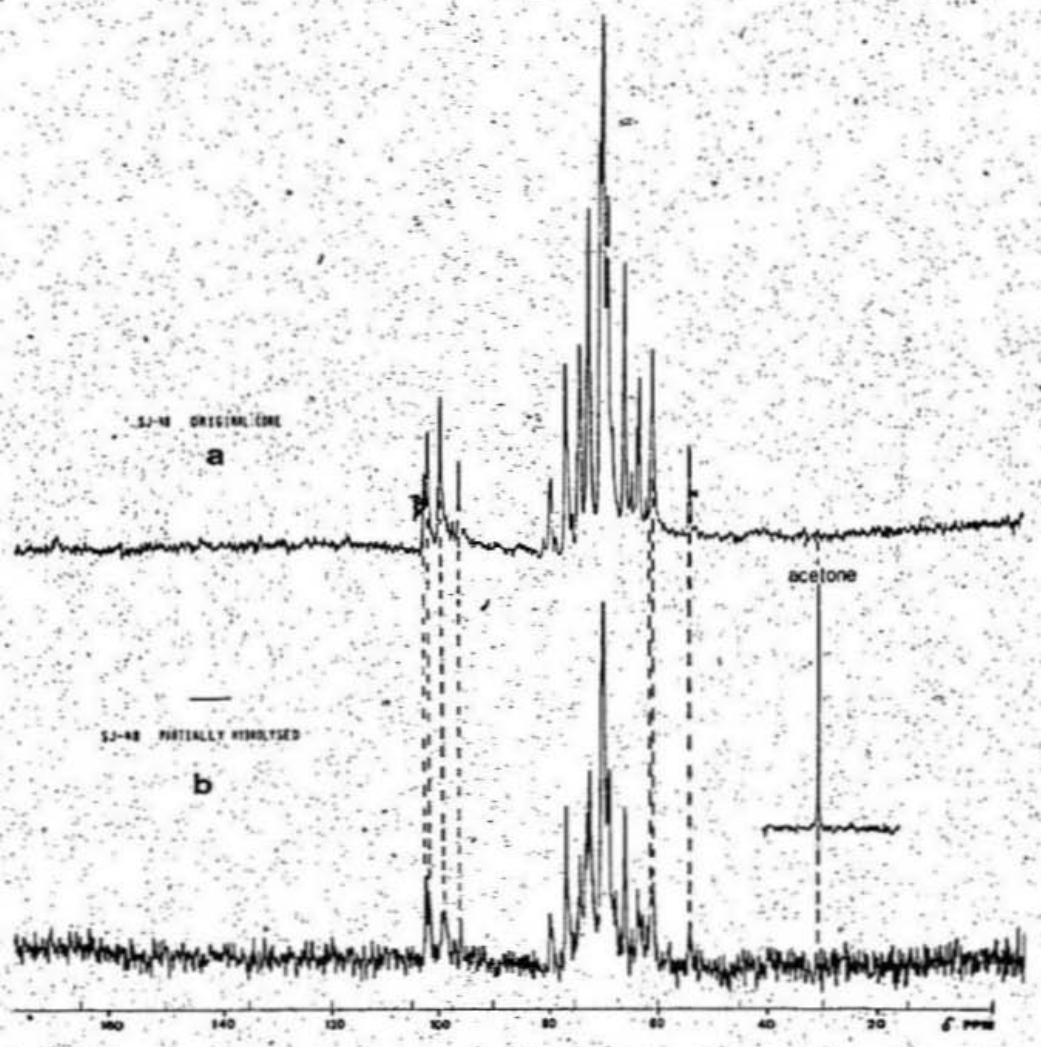


Fig.7. ^{13}C-n.m.r. spectra of the original (a) and the partially hydrolyzed (b) core oligosaccharide of strain SJ-48.

D-Glcp-(1→4/3)-LD-Hepp-(1→2)-LD-Hepp
4/3
↑
1
LD-Hepp

1.7.3. Partial Acid Hydrolysis of the Core Oligosaccharide with 2M Hydrochloric Acid

Attempts were made to isolate an oligosaccharide containing the 2-amino-2-deoxy-D-glucose unit. Thus, partial hydrolysis of the core oligosaccharide with 2M hydrochloric acid for 1h at 100°, followed with purification by high voltage paper electophoresis, yielded a disaccharide 3, that had M_{GlcN} 0.6. When this disaccharide was submitted to nitrous acid deamination followed by reduction and acetylation of the products, it afforded 1,3,4,6-tetra-O-acetyl-2,5-anhydro-D-mannitol and the alditol acetate of LD-heptose in stoichiometric amount (Table II; H, p. 70).

Acetylation of the disaccharide 3, followed by methylation, hydrolysis, reduction, acetylation and g.l.c.-m.s. of the alditol acetates confirmed the position of the linkage through O-7 of the LD-heptosyl residue by the formation of 2,3,4,6-tetra-O-methylheptose. Hence structure of the isolated disaccharide 3 is as follows:

D-GlcpN-(1→7)-LD-Hepp

3

1.8. Chromium Trioxide Oxidation

1.8.1. Chromium Trioxide Oxidation of the Original Core

The anomeric configurations of the various glycosyl groups of the core oligosaccharide were also ascertained. The core oligosaccharide was peracetylated, and the product subjected to oxidation with chromium trioxide (138,139). Sugar analysis of the resulting oxidized core (Table II; I, p. 70) demonstrated that during the oxidation, the D-glucose moiety was the only sugar destroyed and was β-linked.

Methylation analysis of the oxidized core oligosaccharide (Table III; E, p. 74) gave 2,3,4,6,7-penta-O-methylheptose, 2,4,6,7-tetra-O-methylheptose, 3,4,6,7-tetra-O-methylheptose, 2,3,4,6-tetra-O-methylheptose and 2,4,7-tri-O-methylheptose in the molar ratio of ~ 1:0.4:1:0.3:0.5, together with the methylated acetylated disaccharide alditol 1. Even though the interpretation of the chromium trioxide oxidation seems quite complicated it can nevertheless be rationally explained. The 2,7-di-O-methylheptose found in the methylated original material has been replaced by either 2,4,7-tri-O-methylheptose (45%), indicating that the β-glucosyl residue was linked through O-4 of this branched heptose unit or by 2,4,6,7-tetra-O-methylheptose (43%) arising from the parallel (but unexpected) loss of the terminal nonreducing heptosyl residue linked through O-6 of the branched heptose unit. Partial acid hydrolysis has already confirmed the lability of this terminal residue, and it would be reasonable to postulate similar

behaviour under the relatively strong acetic acid conditions unavoidably used during the chromium trioxide oxidation.

Peracetylation of the core oligosaccharide prior to oxidation and methylation would have afforded an N-acetyl amino sugar with a glycosidic linkage much less resistant to hydrolysis than that of the free amino sugar (83). This would explain the presence of 2,3,4,6-tetra-O-methylheptose arising from the previously acid resistant amino sugar containing oligosaccharide 3.

The chromium trioxide oxidation has therefore shown that the D-glucose residue had a β-configuration and that it was linked through O-4 of the double-branched heptose unit, as in the following partial structure:

$$\beta\text{-D-Glcp-(1}\rightarrow\text{4)-}\alpha\text{-LD-Hepp (1}\rightarrow \quad \begin{matrix} \uparrow 6 \\ \uparrow 3 \end{matrix}$$

1.8.2. Chromium Trioxide Oxidation of the Partially Hydrolysed and Deaminated Core

The partially hydrolysed and deaminated core oligosaccharide (see Section 1.7.2) was peracetylated and oxidized with chromium trioxide. During the oxidation only the D-glucose was oxidized, substantiating the β-link. By comparing the methylation analysis of this chromium trioxide oxidized oligosaccharide (Table III; F,

p. 74) with that of its parent precursor (Table III; D, p. 74) it was evident that the 2,6,7-tri-O-methylheptose has been replaced by a 2,4,6,7-tetra-O-methylheptose, thus indicating that the β-D-glucosyl group was linked through O-4 of the branched LD-heptose residue. Though the glucose residue has not been totally oxidized (0.12 mole has survived the oxidation), we can assign a structure to the major product, as follows:

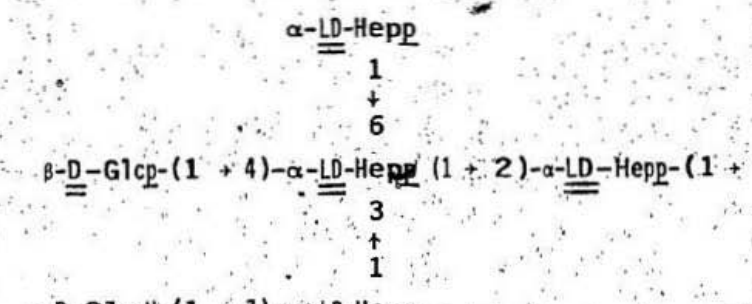
α-LD-Hepp-(1 → 2)-α-LD-Hepp
3
↑
1
α-LD-Hepp

1.9. Determination of the Reducing End of the Core Oligosaccharide

In most of the lipopolysaccharide structures studied to date, three molecules of 3-deoxy-D-manno-2-octulosonic acid (dOclA) were found to join the core oligosaccharide to the lipid A. In our study, determination of dOclA by the published assay methods seemed to indicate that it was absent. However, recent investigations conducted in our laboratory (191) suggest that at least one mole of dOclA may be present in the lps of A. hydrophila, though certainly not linked in a conventional manner.

1.10. Conclusion

From the combined chemical and spectroscopic evidence, the core oligosaccharide of Aeromonas hydrophila strain SJ-48 is made up of six glycose units and has the following structure:



2. STRUCTURAL INVESTIGATIONS ON THE CORE OLIGOSACCHARIDE OF *AEROMONAS HYDROPHILA* STRAIN SJ-55

2.1 Introduction

A. hydrophila Strain SJ-55 would be classified as Chemotype I on the basis of the neutral glycose analysis of the core oligosaccharide (47), which consists of D-galactose, D-glucose, L-glycero-D-manno- heptose, and D-glycero-D-manno-heptose. The results of the structural investigations on this core oligosaccharide will be discussed.

2.2. Isolation of the Core Oligosaccharide

The lipopolysaccharide (lps) was isolated by the aqueous phenol method of Westphal and Jann (22). Analytical studies showed that the lps preparation contained very little protein as contaminant (Table IV). The carbohydrate portion obtained after mild hydrolysis of the lipopolysaccharide with acetic acid was fractionated on a Sephadex G-50 column as shown in Figure 8. The material which eluted with a K_{av} value between 0.6 and 0.7 (Fraction PII) represents the core oligosaccharide. The peak with a K_{av} value between 0.45 and 0.55 (Fraction PI) corresponds to a fraction eluting before the core oligosaccharide and represents the "Semi Rough" (SR) oligosaccharide (i.e. the core oligosaccharide plus one repeating unit of the O-polysaccharide). This fraction usually represented no more than 15% (w/w) of the core

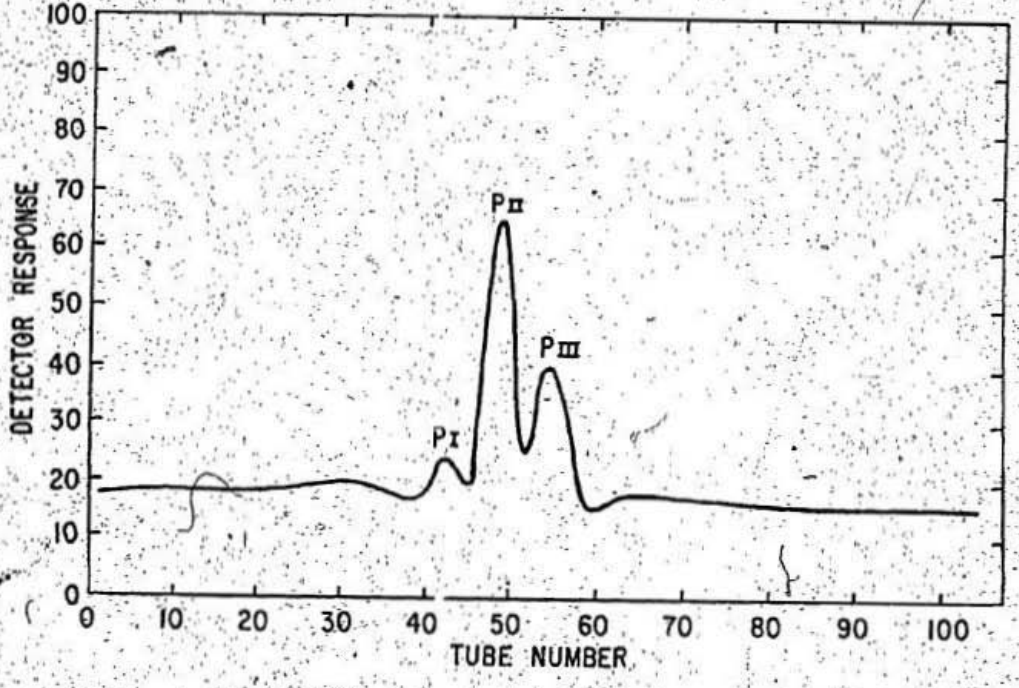


Fig.8. Sephadex G-50 chromatography of 1% acetic acid hydrolysis fragments of the lipopolysaccharide from *A. hydrophila* strain SJ-55.

Table IV. Analytical data on the fractions of A. hydrophila SJ-55 lps

	dOclA[a]	Phosphorus[b] %	Hexosamine[c] % A	B	C	Protein[d] %	Uronic[e] acid
LPS	(-)	2.75	nd	nd	nd	2.0	(-)
CORE	(-)	0.25	4.90	7.63	3.8	0.7	(-)
SR form	(-)	0.19	9.85	11.20	nd	1.2	nd
Lipid A	(-)	6.3	11.7	nd	nd	nd	nd

[a]Thiobarbiturate (180) and diphenylamine assays (181). [b]Chen et al. assay (175). [c]Rondle-Morgan (A) (178), Strominger (B) (179) and Amino acid analysis (C). [d]Lowry assay (174). [e]Carbazole assay (177).
nd: not determined.

oligosaccharide fraction. The core oligosaccharide had $[\alpha]_D$ + 77.5° (c 2.58, in water). The fraction P III was essentially free of carbohydrate and consisted of various minor components associated with the original lps. This fraction was not studied any further.

The analytical data of the lps, R (core) and SR form are summarized in Table IV.

2.3 Sugar Analysis of the Core Oligosaccharide

Acid hydrolysis of the original and deaminated core oligosaccharide indicated that the original core consisted of D-galactose, D-glucose, D-glycero-D-manno-heptose, L-glycero-D-manno-heptose and 2-amino-2-deoxy-D-glucose residues in the molar proportions of ~ 1:1:2:4:1 (see Table V; A and B, p. 97).

The optical rotations of the sugars isolated from the hydrolysate established that the galactose, glucose and 2-amino-2-deoxy-glucose had the D-configuration. The 2-amino-2-deoxy-D-glucose had a free amino group, as indicated by its complete destruction and formation of 2,5-anhydromannose after deamination of the core oligosaccharide.

The heptoses were identified as D-glycero-D-manno-heptose and L-glycero-D-manno heptose by the relative retention times of their alditol acetates in g.l.c. (profile B) (185) coupled with their mass spectra.

Analytical studies on the core oligosaccharide (Table IV, p. 95) indicated that it did not contain fatty acid, 2-aminoethanol, protein, uronic acid or 3-deoxy-D-manno-2-octulosonic acid (dOclA).

Table V: Sugar Analysis of S155 Core Oligosaccharide and Derived Products

Sugars (as alditol acetates)[a]	Given in molar proportions[c]									
	A	B	C	D	E	F	G	H	I	J
D-Gal	0.93	1.07	0.32		0.30	-	-	-	-	-
D-Glc	1.00	1.00	1.00	1.00	1.00	-	-	-	-	0.18
DD-Hep	2.01	1.94	1.67	0.96	1.70	-	-	-	-	1.95
LD-Hep	2.92	3.80	1.93	2.10	2.91	1.00	1.00	1.00	1.00	2.82
D-GlcN[b]	0.78	-	0.83	0.65	-	-	-	0.73	-	0.60
2,5-Anhydro-D-Man	-	0.61	-	-	-	-	-	-	0.78	-
Ribitol	-	-	-	-	-	0.85	-	-	-	-
Lyxitol	-	-	-	-	-	0.30	-	-	-	-
Threitol	-	-	-	-	-	0.70	-	-	-	-
Glycerol	-	-	-	-	-	6.20	0.65	-	-	-

[a] Alditol acetate derivatives of neutral sugars were analysed on a Silar 7CP column at 200°. [b] D-glucosamine was quantified both by analytical assays, and g.l.c. analysis (as alditol acetate after a 4M HCl Hydrolysis for 10 h at 100°C). [c] A - Original core; B - Deaminated core; C - Partially hydrolysed core (0.5M H_2SO_4, 30 min, 100°); D - Partially Hydrolysed core (0.5M H_2SO_4, 1 h 30, 100°); E - Oligosaccharide C, deaminated; F - Periodate oxidized core; G - Smith oligosaccharide; H - Disaccharide isolated by HVE (2M HCl, 1 h, 100°C); I-Disaccharide H deaminated, reduced and acetylated; J-Chromium trioxide oxidized original core.

2.4. Nuclear Magnetic Resonance

2.4.1 ^{1}H-n.m.r. Spectrum of the Core Oligosaccharide

The 250 MHz ^{1}H-n.m.r. spectrum of the deuterium exchanged SJ-55 core oligosaccharide, recorded in D_2O at 90°, showed characteristic resonances of the anomeric protons in the region between 4.5 and 6.0 ppm (Fig. 9). Four signals integrating for 7 protons ($J_{1,2}$ ~ 2-3Hz) at δ:5.70 (1H), 5.45 (1H), 5.20 (4H) and 5.08 (1H) p.p.m. were assigned to α-linked sugar residues (188), whereas the two signals at δ:4.65 and 4.60 ppm, each integrating for one proton, and showing a coupling constant of 8Hz, correspond to β-linked sugar residues. There were no signals of O-acetyl, N-acetyl, or methyl protons of deoxy-sugars.

2.4.2. ^{13}C-n.m.r. Spectrum of the Core Oligosaccharide

The 62.84-MHz ^{13}C-n.m.r. spectrum of the original SJ-55 oligosaccharide (Fig. 10) was recorded at 70° in D_2O. In the anomeric region (between 90 and 110 ppm) 8 signals could be distinguished, at 103.8 (double signal), 103.7, 102.3, 99.85, 99.7, 99.6, 98.7 and 96.5 ppm. The $^1J_{C,H}$ couplings were measured by the gated decoupling technique and showed coupling values of ~ 160 Hz for the signals at 103.8 and 103.7 ppm, which therefore correspond to the β-anomeric configuration (123). The anomeric signals resonating at higher field had a coupling value of ~ 170 Hz and might therefore have an α-configuration. At higher field beyond 70 ppm, the two signals at 61.15 and 59.7

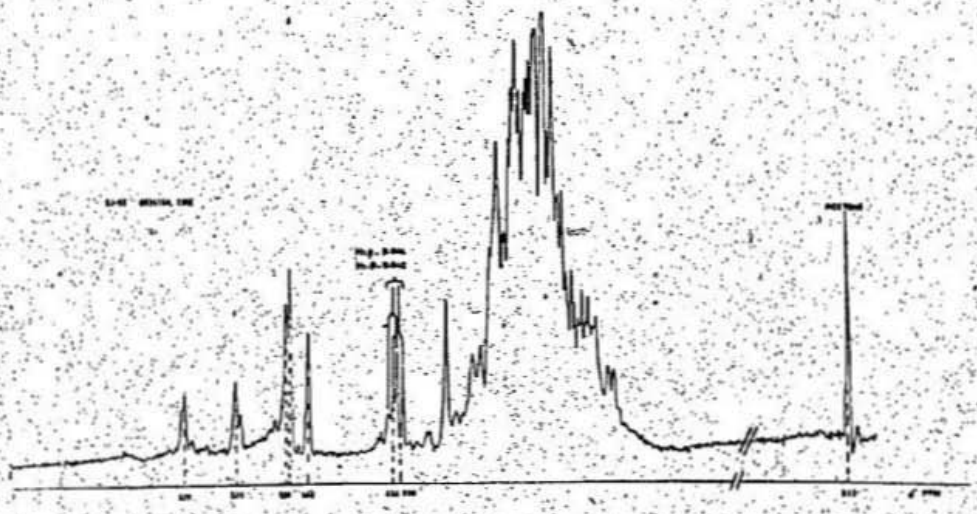


Fig.9. ^{1}H-n.m.r. spectrum of the original core oligosaccharide from strain SJ-55.

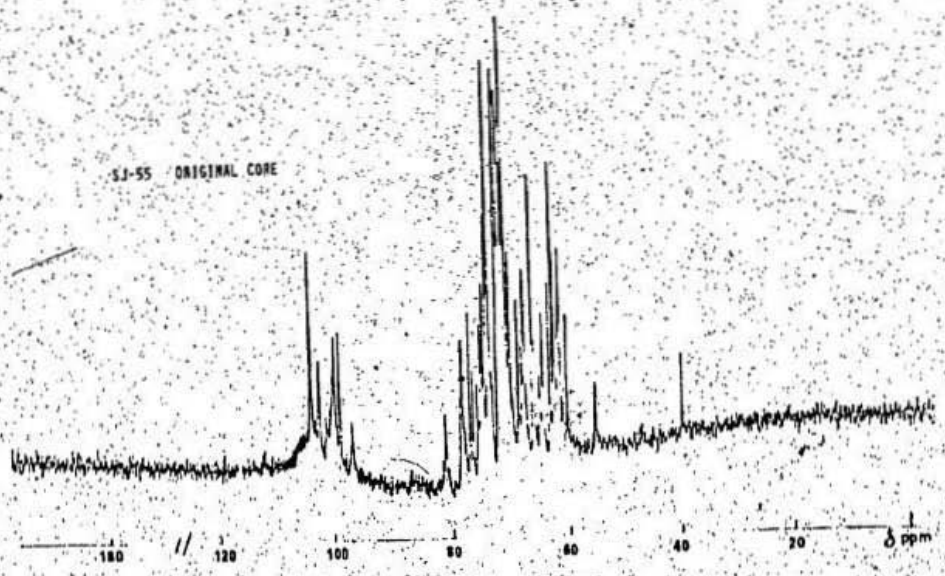


Fig.10. ^{13}C-n.m.r. spectrum of the original core oligosaccharide from strain SJ-55.

correspond to two non-linked C-6 carbons (bearing a primary free hydroxyl group) of two hexose residues (D-galactose and D-glucosamine). Finally, the signal at 54.95 is attributable to the C-2 deoxy carbon bearing an amino group of the 2-amino-2-deoxy-D-glucose residue and the weaker signal at 38.25 ppm may correspond to the C-3 deoxy function of a suspected but not established dOclA reducing molecule (191).

2.4.3. Conclusion

The proton and carbon-13 n.m.r. spectra seem to corroborate the results of the chemical analysis and indicate that the core oligosaccharide consists of 9 glycose residues. Furthermore, the results unambiguously confirm the presence of 2-amino-2-deoxy-D-glucose.

2.5. Methylation Analysis of the SJ-55 Core Oligosaccharide

Methylation of the core oligosaccharide by the method of Hakomori, followed by hydrolysis with 2M trifluoroacetic acid, reduction, and derivatization as the alditol acetates, afforded 2,3,4,6-tetra-O-methyl-galactose, 2,3,4,6,7-penta-O-methyl-DD-heptose, 2,3,4,6,7-penta-O-methyl-LD-heptose, 2,3,4-tri-O-methylglucose, 3,4,6,7 tetra-O-methyl-LD-heptose, together with 2,3,7-tri-O-methyl-DD-heptose[1], and 2,7-di-O-

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[1] Throughout the argument and for purposes of clarity this residue will be assumed to be D-glycero-D-manno-heptose; no g.l.c. standard of this compound was available during this study in order to conclusively differentiate it from 2,3,7-LD-heptose. The configuration is, however, proved later in the Discussion (Section 2.8).

Table VI: Methylation analysis of SJ55 Core Oligosaccharide and derived products.

methylated sugar[a]	T[b] X	T[b] Y	In Mole %[c,d] A	B	C	D	E	F	G	Linkages
2,3,4,6-Gal	1.07	1.21	13.3 (0.97)	11.7 (0.96)	6.3 (0.32)	-	5.1 (0.28)	-	-	Gal 1 →
2,3,4-Glc	1.48	2.25	14.1 (1.03)	14.4 (1.20)	18.7 (0.91)	21.3 (0.82)	15.4 (0.85)	1.9 (0.10)	2.4 (0.10)	→6 Glc 1→
2,3,4,6,7-DD-Hep	1.61	1.61	16.2 (1.18)	13.6 (1.13)	17.1 (0.85)	27.1 (1.04)	19.9 (1.10)	22.5 (1.19)	23.0 (0.95)	DD-Hep 1→
2,3,4,6,7-LD-Hep	1.76	2.00	16.1 (1.17)	21.3 (1.77)	1.0 (0.05)	-	13.7 (0.76)	21.5 (1.16)	17.1 (0.71)	LD-Hep 1→
2,3,4,7-DD-Hep	2.23	3.37	-	-	13.5 (0.66)	-	11.8 (0.65)	12.8 (0.67)	14.0 (0.58)	→6 DD Hep1→
2,4,6,7-LD-Hep	2.45	3.70	-	-	-	-	-	15.9 (0.84)	19.2 (0.79)	→3 LD-Hep1→
3,4,6,7-LD-Hep	2.38	3.72	13.7 (1.00)	12.0 (1.00)	20.5 (1.00)	26.0 (1.00)	18.1 (1.00)	18.9 (1.00)	24.2 (1.00)	→2 LD-Hep1→
2,6,7-LD-Hep	2.71	4.80	-	-	19.2 (0.94)	25.0 (0.96)	15.4 (0.85)	-	-	→4 LD-Hep1→ 3 ↑
2,3,4,6-LD-Hep	2.78	5.23	-	4.0 (0.33)	-	-	4.0 (0.22)	4.9 (0.26)	-	→7 LD-Hep1→ ↓ 3
2,3,7-DD-Hep	2.90	5.41	14.9 (1.09)	11.9 (0.99)	3.1 (0.15)	-	1.8 (0.10)	-	-	→4 DD-Hep1→ ↓ 6
2,4,7-LD-Hep	2.85	5.10	-	-	-	-	-	2.0 (0.11)	-	→5 LD-Hep1→ ↓ 6
2,7-LD-Hep	3.69	8.47	11.3 (0.82)	8.2 (0.68)	-	-	-	-	-	→4 LD-Hep1→ 3 ↑

[a] 2,3,4,6-Gal stands for 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-galactitol, other methylated sugars are similarly designated.

[b] Retention times are relative to the alditol acetate derivative of 2,3,4,6-tetra-O-methyl-D-glucose on column 7:WCOT OV-SII-5 at 190° and Silar 7CP at 180°. [c] A - Original core; B - deaminated Core; C - partially hydrolysed (0.5N H_2SO_4, 30 min, 100°) core; D - Partially hydrolysed (0.5N H_2SO_4, 1 h 30, 100°) core; E - Deaminated product C; F - Chromium trioxide oxidized original core; G - Chromium trioxide oxidized product E. [d] Between brackets are given the molar proportion.

methyl-LD-heptose in approximately equimolar amounts (see Table VI; A, p. 101).

The presence of a 2,7-di-O-methyl-LD-heptose is attributable to a double branched heptose residue linked through positions O-6, O-4 and O-3 and that of 2,3,7-tri-O-methyl-DD-heptose to a single branched heptose residue linked through O-6 and O-4. The existence of 2,3,4,6-tetra-O-methylgalactose, 2,3,4,6,7-penta-O-methyl-LD-heptose and 2,3,4,6,7-penta-O-methyl-DD-heptose indicates that at least three residues are non reducing end groups. The presence of 2,3,4-tri-O-methylglucose is attributable to a glucose residue linked through O-6, and that of 3,4,6,7-tetra-O-methyl-LD-heptose to a heptose residue linked through O-2. By comparison of the stoichiometric composition of the sugar analysis with that of the hydrolysis products of the methylated core oligosaccharide, it is clear that the alditol acetates derived from 2-amino-2-deoxy-D-glucose and 1 mole of L-glycero or D-glycero-D-manno-heptose are missing. This result suggested that these last two residues were glycosidically linked by a linkage resistant to the hydrolysis conditions used. In fact, when gas-liquid chromatography of the partially methylated alditol acetates was conducted using the program temperature (profile C), a slow moving component 4 was observed whose identity was established by mass spectrometry. The fragmentation pattern of this compound was identical to that of compound 1 already described in paragraph 1.3. (Fig. 3, p. 76).

Therefore, the tentative structure of component 4 is as shown:

D-GlcpN 1→7-LD or DD-Hepp-ol

4

Although it is theoretically possible for D-glycero-D-manno-heptose to be the reducing sugar, the identical g.l.c. retention times of 1 and 4 makes this extremely unlikely.

When the permethylated SJ-55 core oligosaccharide was hydrolysed with 90% formic acid at 100° for 90 min, followed by reduction of the hydrolysate and derivatization as alditol acetates, another slow moving component 5 was observed on g.l.c. when temperature profile C was used. The identity of this component was established by mass spectrometry as a 1,5-di-O-acetyl-6-O-[di-O-acetyl-tri-O-methylheptopyranosyl]-2,3,4-tri-O-methylhexitol. The structure of this disaccharide alditol 5 was confirmed by its fragmentation pattern (Fig. 11) which gave primary ions at m/z 319 (for the nonreducing heptosyl group), and m/z 291, 205, 117, 161 (for the hexitol moiety), together with secondary ions, inter alia, at m/z 259, 227, 199, 167, 157 (formed through elimination of CH_3OH, CH_3COOH and $CH_2=C=O$ from the primary ions of m/z 319 and 291), 129, 101, 88, 71, 45 and 43.

The fragmentation pattern therefore indicated that disaccharide alditol 5 consisted of a nonreducing heptosyl group, glycosidically linked to O-6 of a hexitol residue. Since the methylation analysis of the original core has shown the presence of a branched D-glycero-D-manno-heptose residue (linked through its O-6 and O-4 positions), and a

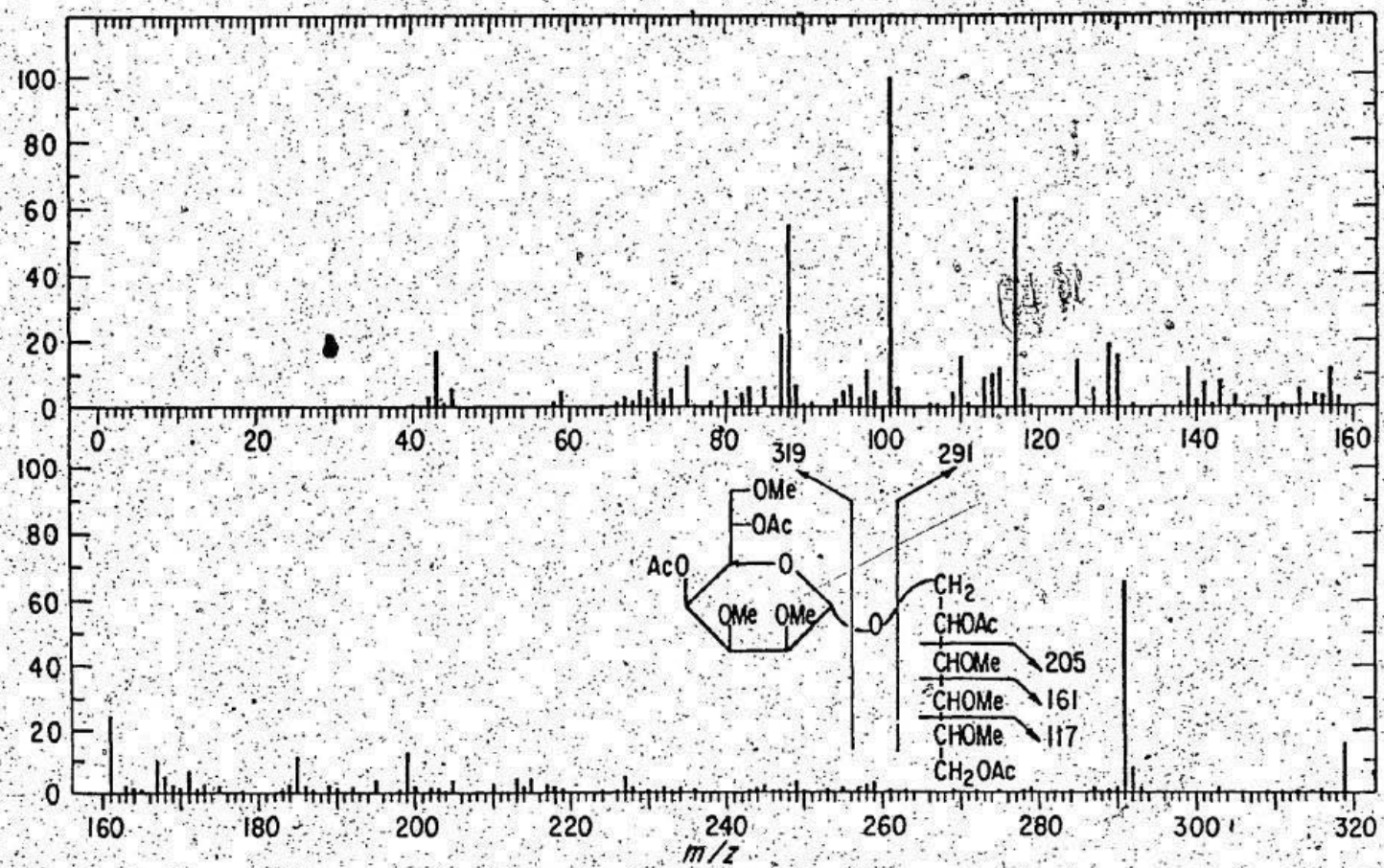


Fig. 11. Mass spectrum of the 1,5-di-O-acetyl-6-O-[4,6-di-O-acetyl-2,3,7-tri-O-methylheptopyranosyl]-2,3,4-tri-O-methylglucitol.

2,3,4-tri-O-methyl glucose, we can assign the same O-6 (6-OAc) and O-4 (4-OAc) substitutions for the heptosyl group containing disaccharide alditol (see Fig. 11), and furthermore, we may also assign the D-glycero-D-manno- and D-gluco configurations respectively to these two residues. Hence the structure of disaccharide alditol 5 is as shown below:

DD-Hepp-(1 → 6)-D-Glcp-ol

5

2.6. Deamination of SJ-55 Core Oligosaccharide

The core oligosaccharide was deaminated with nitrous acid, followed by reduction of the product with borohydride (187). The 2,5-anhydro-D-mannitol released during the treatment was acetylated and identified by g.lm.s. Sugar analysis of the deaminated oligosaccharide purified on Sephadex G-15, gave D-galactose, D-glucose, D-glycero-D-manno-heptose and L-glycero-D-manno-heptose in the molar ratios of ~ 1:1:2:4 (Table V; B, p. 97). It is clear by comparison of this sugar analysis with that of the original core that one extra mole of L-glycero-D-mannoheptose has now been detected.

The release of 2,5-anhydro-D-mannose together with the assay of one extra heptose residue indicated that the 2-amino-2-deoxy-D-glucose was present as a nonreducing end group, and that it was glycosidically linked to an L-glycero-D-manno-heptose residue.

Thus, disaccharide alditol 4 discussed in Section 2.5 is identical to compound 1 obtained from the core oligosaccharide SJ-48 (Section 1.3.) and is as shown below.

D-GlcpN(1 → 7)-LD-Hepp-ol

4

The methylation analysis of the purified deaminated core oligosaccharide afforded the partially methylated alditol acetates as indicated in Table VI; B, p. 101. The presence of 2,3,4,6-tetra-O-methyl-LD-heptose again confirms that the 2-amino-2-deoxy-D-glucosyl moiety is glycosidically linked to O-7 of this L-glycero-D-manno-heptose unit.

2.7. Partial Hydrolyses of the Core Oligosaccharide

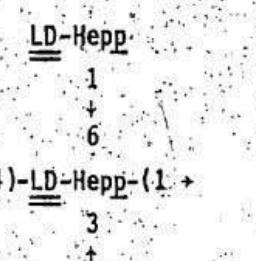
2.7.1. Partial Hydrolysis with 0.5 M Sulfuric Acid for 30 Min at 100°

Partial hydrolysis of the core oligosaccharide with 0.5M sulfuric acid for 30 min at 100°, followed by purification on Sephadex G-15, yielded a degraded oligosaccharide. Sugar analysis (Table V; C, p. 97) revealed that 0.7 mole of D-galactose, 0.3 mole of D-glycero-D-manno-heptose and approximately one mole of L-glycero-D-manno-heptose had been removed by the acid hydrolysis.

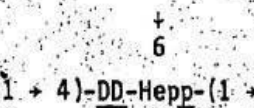
Methylation of the degraded oligosaccharide, followed by hydrolysis, reduction, and g.l.c.-m.s. of the alditol acetates, yielded methylated sugars indicated in Table VI; C, p. 101.

When gas liquid chromatography was carried out with temperature profile C the presence of the methylated disaccharide alditol acetate 4 was observed, indicating that it had survived the partial hydrolysis.

The above methylation analysis indicates that the 2,7-di-O-methyl-LD-heptose (double branch point) found in methylation and hydrolysis of the original material had been replaced by 2,6,7-tri-O-methyl-LD-heptose, and that the terminal nonreducing LD-heptosyl unit was originally linked to this heptose residue through O-6, as in the following partial structure.



The incomplete removal of 2,3,4,6-tetra-O-methylgalactose and the presence of 2,3,7-tri-O-methyl-DD-heptose, together with the appearance of 2,3,4,7-tetra-O-methyl-DD-heptose seem to indicate that the terminal galactosyl unit was linked to the branched D-glycero-D-manno-heptose residue through O-4, as in the partial structure shown below:

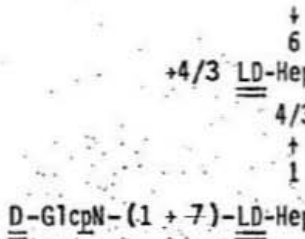


2.7.2. Nitrous Deamination of the Partially Hydrolysed Oligosaccharide

The degraded oligosaccharide was treated with nitrous acid, followed by reduction of the product by borohydride. The 2,5 anhydro-D-mannitol released was acetylated and the ester identified by g.l.c.-m.s.

Sugar analysis gave D-glucose, D-glycero-D-manno-heptose and L-glycero-D-manno-heptose in the molar ratios of 1.0:1.7:2.9 (see Table V; E, p. 97).

The deaminated degraded oligosaccharide was purified by gel filtration on Sephadex G-15, and the resulting product was methylated. Subsequent hydrolysis, reduction and g.l.c.-m.s. of the alditol acetates yielded the methylated sugars shown in Table VI; E, p. 101. Again (as in Section 2.6.) the limited proportion of 2,3,4,6-tetra-O-methyl-LD-heptose arising from the 7-O-substituted LD-heptosyl unit, considered together with the new appearance of 2,3,4,6,7-penta-O-methyl-LD-heptose, reconfirms that the 2-amino-2-deoxy-D-glucose was a nonreducing end group linked to O-7 of the L-glycero-D-manno-heptose unit, which in turn, was linked to the branched L-glycero-D-manno-heptose unit of the degraded product either through O-4 or O-3, as in the following partial structure:



2.7.3. Partial Hydrolysis for 90 Min at 100° with 0.5M Sulfuric Acid

Partial hydrolysis of the core oligosaccharide with 0.5M sulfuric acid for 90 min at 100°, followed by purification on Sephadex G-15, afforded a further oligosaccharide composed of D-glucose, D-glycero-D-manno-heptose, L-glycero-D-manno-heptose and 2-amino-2-deoxy-D-glucose residues in the molar ratios of ~ 1:1:2:1 (see Table V; D, p. 97).

Methylation analysis of this oligosaccharide gave 2,3,4,6,7-penta-O-methyl-DD-heptose, 2,3,4-tri-O-methylglucose, 3,4,6,7-tetra-O-methyl-LD-heptose and 2,6,7-tri-O-methyl-LD-heptose in the approximate molar ratios of 1:1:1:1 (see Table VI; D, p. 101. When gas liquid chromatography was performed using the program temperature profile C, the presence of the disaccharide alditol 4 indicated that it was still a part of the degraded oligosaccharide. By comparing the methylation analysis of this degraded product (Table VI; D, p. 101) with that of the degraded product after 30 min hydrolysis (Table VI; C, p. 101), we notice the virtual disappearance of 2,3,4,7-tetra-O-methyl-DD-heptose (which had originated from the 2,3,7-tri-O-methyl-DD-heptose of the original core). This same residue is the one from which the 2,3,4,6,7-

penta-O-methyl-DD-heptose unit, now present in the degraded oligosaccharide, is formed. It is also clear that all of the 2,3,4,6-tetra-O-methylgalactose and 2,3,4,6,7-penta-O-methyl-DD-heptose, from the original end groups, are no longer present in the methylation pattern.

The partial (0.5 M sulfuric acid) hydrolysis has thus in a first step (30 min; 100°) removed some of the end groups from the core oligosaccharide (~ 0.2 mole of D-glycero-D-manno heptose, ~ 1 mole of L-glycero-D-manno-heptose and ~ 0.7 mole of D-galactose) showing unequivocally the linkage positions of these last two residues (through O-6 of the double branched L-glycero-D-manno-heptose and O-4 of the branched D-glycero-D-manno-heptose respectively); in a further step (90 min; 100°) all of the neutral end groups have been removed leaving an oligosaccharide in which the original branched D-glycero-D-manno-heptose has become the new nonreducing end group.

Hence the terminal nonreducing DD-heptose residue present in the original core was linked to O-6 of the original branched DD-heptose as in the following partial structure.

$$\begin{array}{c} \text{DD-Hepp} \\ 1 \\ \uparrow \\ 6 \end{array}$$

$$\text{D-Galp-(1} \rightarrow \text{4)-DD-Hepp-(1} \rightarrow \text{6)-D-Glcp (1} \rightarrow$$

2.7.4 Partial Hydrolysis of the Core Oligosaccharide with 2M Hydrochloric Acid

Attempts were made to isolate an oligosaccharide containing the 2-amino-2-deoxy-D-glucose unit. Thus, partial hydrolysis of the core oligosaccharide with 2M hydrochloric acid for 1 h at 100°, followed by purification on high voltage electrophoresis, yielded a disaccharide 6 that had M_{GlcN} 0.6.

When disaccharide 6 was subjected to nitrous acid deamination and the resulting product reduced and acetylated, it afforded 1,3,4,6-tetra-O-acetyl-2,5-anhydro-D-mannitol and the alditol acetate of L-glycero-D-manno-heptose in stoichiometric amount (see Table V; I, p. 97).

Acetylation of disaccharide 6, followed by methylation, hydrolysis, reduction, acetylation and g.l.c-m.s., confirmed the position of the linkage through O-7 of the LD-heptosyl residue by the formation of 2,3,4,6-tetra-O-methyl-heptose, results which are in agreement with the previous deamination analysis data. Hence the structure of disaccharide 6 is as follows:

D-GlcNp-(1 → 7)-LD-Hepp

6

It should be noted that disaccharide 6 is identical to the disaccharide 3 afforded on hydrolysis of the SJ-48 core oligosaccharide SJ-48 with 2M HCl, described in Section 1.7.3.

2.8. Periodate Oxidation of SJ-55 Core Oligosaccharide

The core oligosaccharide was oxidized with periodate, and the product was reduced and purified by gel filtration on Sephadex G-15. Sugar analysis of the purified polyol indicated the presence of L-glycero-D-manno-heptose, ribitol, threitol, glycerol and lyxitol. (NB. lyxitol would be the reduction product from either arabinose, or lyxose) in the molar ratios of 1:0.85:0.70:6.2:0.3 (see Table V; F, p. 97). It is evident that the 7-O-substituted L-glycero-D-manno-heptose is the parent residue of the threitol; thus confirming the L-glycero configuration for this residue. The only way in which the oxidation product ribitol can be produced from an oligosaccharide which gives the demonstrated methylation pattern is if 2,3,7-tri-O-methylheptose has the D-glycero-D-manno configuration (this has, to this point, been assumed: see footnote p. 100).

The lyxitol residue must arise from incomplete oxidation of the 7-O-substituted L-glycero-D-manno-heptose residue; as already discussed in the case of SJ-48 in Section 1.5.

Collectively these results demonstrate that 1 mole of D-galactose, 1 mole of D-glucose, 1 mole of 2-amino-2-deoxy-D-glucose, 2 moles of D-glycero-D-manno-heptose and 3 moles of L-glycero-D-manno-heptose have been oxidized. From the original core residues only one mole of L-glycero-D-manno-heptose survives the oxidation, thus confirming the results of the methylation analysis.

2.9. Smith Degradation of the Core Oligosaccharide

The core oligosaccharide was subjected to a Smith degradation, that is, periodate oxidation, followed by borohydride reduction, and mild hydrolysis with acid.

The Smith degraded product was purified by gel filtration on Sephadex G-15. Sugar analysis of the resulting product 7 indicated the presence of L-glycero-D-manno-heptose and glycerol in the molar proportions of ~1:1 (see Table V; G, p. 97).

Methylation analysis of the Smith-degraded product 7 yielded 2,3,4,6,7-penta-O-methyl-LD-heptose and 1,3-di-O-methylglycerol in the molar ratio of 1:0.6. This result indicated that the original double branched heptosyl residue was linked through O-2 of the L-glycero-D-manno-heptose unit that yielded the glycerol residue.

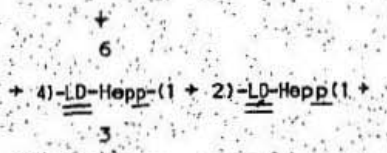
Hence the structure of oligosaccharide 7 is as shown.

LD-Hepp-(1 → 2)-glycerol

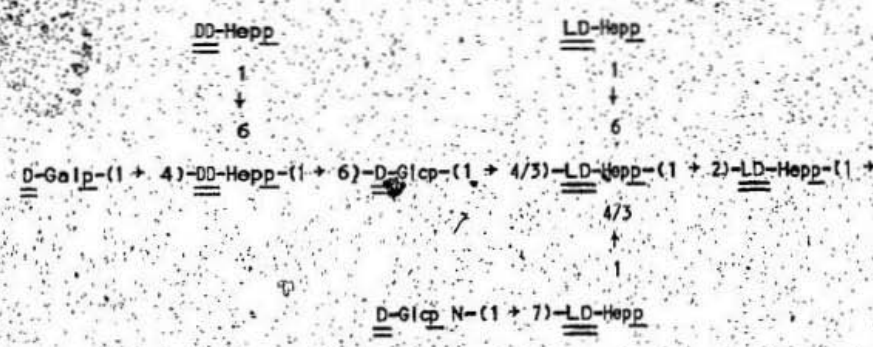
7

Methylation of the Smith degraded product 7 followed by g.l.c.-m.s. analysis gave a mass spectrum with the expected fragmentation for the above postulated structure, this fragmentation pattern(Fig. 6), and structure are identical to that already reported for compound 3 from SJ-48 core oligosaccharide (discussed in Section 1.7).

In conclusion, the Smith oligosaccharide fragment provides evidence for the following partial structure:



At this point of the investigation, a partial structure of the core oligosaccharide may be proposed as shown:



Missing from the above postulated structure, are the anomeric configurations and the linkage position of the two substituents D-glucose and 7-O-linked L-glycero-D-manno-heptose on the double branched L-glycero-D-manno-heptosyl residue.

2.10.- Chromium Trioxide Oxidation of SJ-55 Core Oligosaccharide

The core oligosaccharide was acetylated and the product subjected to oxidation with chromium trioxide. Sugar analysis of the resulting

oxidized product revealed that the D-glucose and D-galactose residues had been oxidized, whereas all of the heptose residues as well as the 2-amino-2-deoxy-D-glucosamine survived the oxidation (see Table V; J, p. 97). This indicates that the former two residues were β-linked and the latter α-linked.

These results are in good agreement with the ^{1}H and ^{13}C-n.m.r. data which indicated two β-anomeric configurations, and with the high positive value of the optical rotation found for the core oligosaccharide.

Methylation analysis of the oxidized oligosaccharide gave the methylated sugars shown in Table VI; F, p. 101, together with the methylated disaccharide alditol acetate 4 (observed when g.l.c. was performed using profile C.).

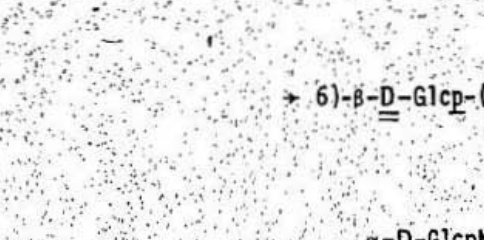
The result of the methylation analysis of the oxidized core oligosaccharide appears to be confusing, but may be rationalized, if we consider that the nonreducing D-glycero-D-manno and particularly L-glycero-D-manno-heptosyl end groups, are potentially labile under the relatively strong acidic conditions of the chromium trioxide treatment.

This result showed that the 2,7-di-O-methyl-LD-heptose found in the original methylated core had been replaced by 2,4,6,7-tetra-O-methyl-LD-heptose (0.84 mole), together with 2,4,7-tri-O-methyl-LD-heptose (0.1 mole).

These observations therefore indicate that the β-D-glucose residue is linked to the double branched L-glycero-D-manno-heptose unit through O-4, and consequently that the amino sugar containing disaccharide must

be linked through O-3 of the same double branched L-glycero-D-manno-heptose.

Hence the additional structural features provided by the chromium trioxide oxidation are shown below:



Since the previous results were not absolutely conclusive, due to the low molar proportion of 2,4,7-tri-O-methyl-LD-heptose recovered after the chromium trioxide treatment, the partially hydrolysed deaminated oligosaccharide (See Section 2.7.2.) was peracetylated and subjected to oxidation with chromium trioxide. Sugar analysis of the resulting oxidized product revealed that the D-glucose unit had been oxidized indicating that it was β-linked.

Methylation analysis of this oxidized product afforded the methylated products shown in Table VI; G, p. 101. By comparison of the methylation analysis of the degraded oligosaccharide (Table VI; E, P. 101 with that of the above oxidized product it is obvious that the 2,6,7-tri-O-methyl-LD-heptose has been replaced by 2,4,6,7-tetra-O-methyl-LD-heptose, indicating, once again, that the β-D-glucosyl residue was

linked through O-4 of the branched L-glycero-D-manno-heptose. Hence the partial structure provided by the oxidized compound is as shown.

$$\rightarrow 6)\text{-}\beta\text{-D-Glc}p\text{-}(1 \rightarrow 4)\text{-}\alpha\text{-LD-Hep}p\text{-}(1 \rightarrow 2)\text{-}\alpha\text{-LD-Hep}p$$

$$\begin{array}{c} 3 \\ \uparrow \\ 1 \end{array}$$

$$\alpha\text{-LD-Hep}p$$

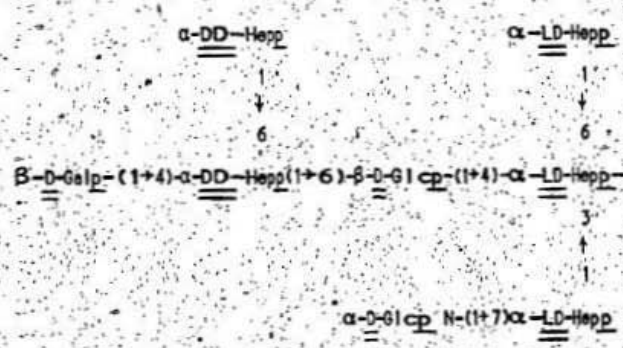
2.11. Determination of the Reducing End

As with the core oligosaccharide of SJ-48, determination of dOclA by the usual methods seemed to indicate that it was absent. However, recent investigations conducted in our laboratory would suggest that dOclA is also present in the lps of this A. hydrophila strain.

2.12. Conclusion

From all the foregoing chemical and spectroscopic evidence the core oligosaccharide of A. hydrophila strain SJ-55 appears to have nine glycose units. This core oligosaccharide possesses three branch points: the L-glycero-D-manno-heptose, which is a double-branched residue carrying the disaccharide (containing the amino sugar), and the nonreducing terminal L-glycero-D-mannoheptosyl unit; and the branched D-glycero-D-manno-heptose which carries the terminal nonreducing D-glycero-D-manno-heptosyl residue.

The core oligosaccharide of *A. hydrophila* strain SJ-55 has the following structure:



3. STRUCTURAL STUDIES ON THE "SEMI ROUGH" OLIGOSACCHARIDE PORTION AND ON THE LINKAGE BETWEEN THE O-SPECIFIC POLYSACCHARIDE AND THE CORE REGION OF A. HYDROPHILA STRAIN SJ-55

3.1 Introduction

The lipopolysaccharide (lps) isolated from A. hydrophila Strain SJ-55, when submitted to mild acid hydrolysis and fractionation on Sephadex G-50, afforded two carbohydrate fractions [SR-oligosaccharide (PI) and core-oligosaccharide (PII)]. (See Fig. 8, p. 94).

The structural studies of the core oligosaccharide of the lps were discussed in the previous chapter. In this section the partial structure of the "Semi Rough" oligosaccharide (PI fraction) from lps SJ-55 will be briefly discussed.

3.2. Sugar Composition of the SR-Oligosaccharide

The "Semi-Rough" oligosaccharide portion was isolated as indicated in Section 2.2. It had $[\alpha]_D^{23}$ + 103.5 (c 0.57, in water). Preliminary analytical results are reported in Table IV, p. 95). In common with the core fraction, the "SR" oligosaccharide does not contain phosphorus (at least not in a significant amount), dOclA or protein. Acid hydrolysis of the original SR-oligosaccharide with 2M trifluoroacetic acid indicated that it consisted of L-rhamnose, D-mannose, D-galactose, D-glucose, D-glycero-D-manno-heptose, L-glycero-D-manno-heptose, 2-amino-2-deoxy-D-glucose and 2-amino-2-deoxy-D-mannose in the molar ratios of ~ 2:1:1:1:2:3:1:1 (see Table VII).

Table VII. Sugar analysis of SJ55 "Semi Rough" (SR) oligosaccharide.

Sugars (as Alditol acetates)	T[a]	Molar Ratios
Rha	0.25	1.60
Man	0.77	0.90
Gal	0.87	1.00
Glc	1.00	1.00
DD-Hep	1.84	1.90
LD-Hep	2.18	2.85
GlcN[b]	5.96	0.70
ManN[c]	6.25	0.80

[a] Retention times are given relative to glucitol hexaacetate on a SIlar-7CP column at 200°

[b,c] Glucosamine as well as Mannosamine were also quantified by analytical assays

Table VIII. Methylation analysis of SJ-55 (SR) oligosaccharide

Methylated sugars	T[a] Y	Mole %[b]	
2,3,4-Rha	0.53	6.6	(0.60)
2,4-Rha	0.81	8.2	(0.75)
2,3,4,6,7-DD-Hep	1.61	11.4	(1.05)
2,4,6-Man	1.92	9.8	(0.90)
2,4,6-Gal	1.98	10.3	(0.95)
2,3,4,6,7-LD-Hep	2.00	10.9	(1.00)
2,3,4-Glc	2.25	8.7	(0.87)
3,4,6,7-LD-Hep	3.70	10.9	(1.00)
2,3,7-DD-Hep	5.41	9.7	(0.89)
2,7-LD-Hep	8.47	7.1	(0.65)
4,6 (N $^{Me}_{Ac}$) ManN[c]	21.60	6.6	(0.60)

[a] Retention times are given relative to 2,3,4,6-tetra O-methyl galactitol on column Y: SIlar 7CP at 180°.

[b] Between brackets are given the molar ratios.

[c] 4,6 (N $^{Me}_{Ac}$) ManN stands for 4,6-di-O-methyl-2-(N-methyl acetamido)-2-deoxy-D-mannose.

By comparison of the sugar analysis of the SR-oligosaccharide with that of the core oligosaccharide (see Table V; A, p. 97), we notice the addition of L-rhamnose (2 moles), D-mannose (1 mole) and 2-amino-2-deoxy-D-mannose (1 mole) residues, together with the correct stoichiometric amounts of the original glycoses of the core oligosaccharide.

3.3. Methylation Analysis of the SR-Oligosaccharide

Hakomori methylation of the SR-oligosaccharide followed by hydrolysis, reduction, and g.l.c-m.s. analysis of the alditol acetates yielded 2,3,4-tri-O-methyl rhamnose, 2,4-di-O-methylrhamnose, 2,3,4,6,7-penta-O-methyl-DD-heptose, 2,4,6-tri-O-methyl mannose, 2,4,6-tri-O-methylgalactose, 2,3,4,6,7-penta-O-methyl-LD-heptose, 2,3,4-tri-O-methylglucose, 3,4,6,7-tetra-O-methyl-LD-heptose, 2,3,7-tri-O-methyl-methyl-DD-heptose, 2,7-di-O-methyl-LD-heptose, and 4,6-di-O-methyl-2-(N-methyl acetamido)-2-deoxy-D-mannose in approximately equimolar amounts (see Table VIII, p. 120).

If we compare the methylation pattern of the SR-oligosaccharide with that of the core oligosaccharide, we notice that 2,3,4,6-tetra-O-methylgalactose has been replaced by 2,4,6-tri-O-methyl galactose, with no change in the other methylated residues, indicating that the O-specific chain of the lps was linked through O-3 of the terminal galactosyl nonreducing residue of the core oligosaccharide. The presence of 2,3,4,-tri-O methylrhamnose is attributable to a nonreducing end group, probably the outermost residue of the "SR" oligosaccharide.

The presence of 2,4 di-O-methylrhamnose is attributable to a rhamnose residue substituted at C-3 and that of 2,4,6-tri-O-methyl-mannose to a glucose residue substituted at C-3. Finally, the 4,6-di-O-methyl-2-(N-methylacetamido)-2-deoxy-D-mannose is attributable to a mannosamine residue substituted at C-3.

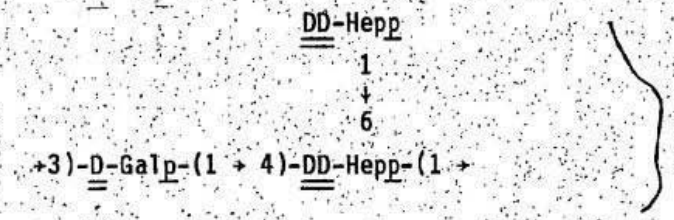
The methylation analysis of the SR-oligosaccharide seems to indicate that the added sugar units are linearly attached to the core and to each other. These results also demonstrate that the reducing sugar component of the O-specific chain is bound to the D-galactosyl residue of the core oligosaccharide of the lps A. hydrophila strain SJ-55, i.e. the galactose residue is the one responsible for the linkage of the O-chain to the core oligosaccharide.

3.4 Conclusion

The following linkage positions have been determined for the non-core sugars of the SR-oligosaccharide, but the sequence, (other than the terminal nonreducing rhamnose residue) is not known:

rhap 1 → ; → 3 manp 1 → ; → 3 rhap 1 → ; → 3 manpN 1 →

The oligosaccharide which they constitute is linked to the core through its terminal galactosyl residue as shown in the following partial structure:



4. STRUCTURAL INVESTIGATIONS ON THE CORE OLIGOSACCHARIDE OF A. HYDROPHILA STRAIN SJ-47

4.1 Introduction

A. hydrophila strain SJ-47 is one of the two selected clones isolated from the plate culture of the original strain SJ-55. This strain was shown to be sensitive to phage as was most of the original culture. In the following section, the structure of the core oligosaccharide isolated from the lps of this mutant will be briefly discussed.

4.2. Isolation of the Core Oligosaccharide

The lipopolysaccharide was isolated by the aqueous phenol method of Westphal and Jann (22). Analytical studies on the lps indicated that it contained phosphorus and very little protein contaminant (see Table IX). The core oligosaccharide was obtained by mild hydrolysis of the lipopolysaccharide with acetic acid and purified by gel filtration on Sephadex G-50. The purified core oligosaccharide had $[\alpha]_D^{23}$ + 86° (c 2.5, in water) and was free of nucleic acid.

4.3. Chemical Analysis of the Core Oligosaccharide

Acid hydrolysis of the core oligosaccharide before and after deamination indicated that it was composed of D-galactose, D-glucose, D-glycero-D-manno-heptose, L-glycero-D-manno-heptose and 2-amino-2-deoxy-D-glucose in the molar ratios of ~ 0.3:1:2:4:1 (see Table X, p. 126).

Table IX: Analytical assays on SJ-47 lps, core and lipid A.

	dOclA[a]	Phosphorus[b]	GlcN[c]		Protein[d]
		%	A %	B %	%
SJ-47 lps	(-)	2.53	7.2	7.5	1.6
SJ-47 core	(-)	0.12	5.3	7.75	1.0
SJ-47 lipid A	(-)	5.5	9.65	10.5	nd

[a]Thiobarbituric acid and diphenylamine assays. [b]Chen et al. assay.
[c]Rondle Morgan (A) and Strominger (B) assays. [d]Lowry assay

Table X. Sugar Analysis of SJ-47 core oligosaccharide

Sugars (as alditol acetates)[a]	given in Molar ratios[c] A	B
D-Gal	0.35	0.33
D-Glc	1.00	1.00
DD-Hep	2.00	1.97
LD-Hep	2.85	3.90
D-GlcN[b]	0.75	-
2,5-Anhydro-D-Man	-	0.60

[a]Alditol acetates derivatives of neutral sugars were analysed on a Silar 7CP column at 210°. [b]D-glucosamine was also quantified by analytical assays. [c]A-Original core; B-Deaminated core;

Analytical studies on the core oligosaccharide indicated that it did not contain phosphorus, fatty acids, 2-amino-ethanol, protein or 3-deoxy-D-manno-2-octulosonic acid (dOclA) as indicated in Table IX.

Similar deamination, partial hydrolyses and other experiments carried out on SJ-55 were also performed on SJ-47 strain. The results however indicated an identical inner core structure as well as an identical positioning of the DD-Hep + DD-Hep + D-Glc trisaccharide, and only where the results differ from those found for SJ-55 core oligosaccharide will they be discussed.

4.4. Methylation Analysis of the Original Core Oligosaccharide

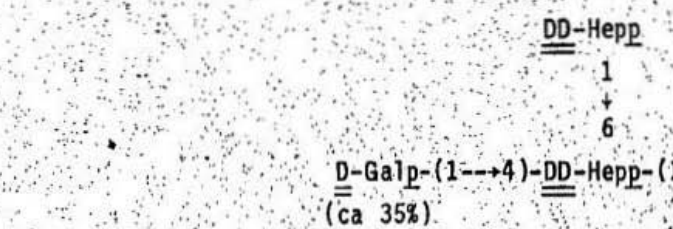
Hakomori methylation of the core oligosaccharide followed by hydrolysis, reduction and g.l.c.-m.s. of the alditol acetates in the normal way afforded the methylated sugar residues shown in Table XI; A, p. 128. By comparison of the methylation analysis of the core oligosaccharide "Strain SJ-47" with that of the original core oligo saccharide "Strain SJ-55" (Table VI; A, p. 101), we notice that the sole difference in the methylation pattern is a change in the molar proportions of 2,3,4,6-tetra-O-methylgalactose (0.3 mole), 2,3,7-tetra-O-methyl-DD-heptose (0.3 mole), and the formation of 2,3,4,7-tetra-O-methyl-DD-heptose (0.7 mole). This suggests that the non equimolar nonreducing galactosyl end group (0.3 mole) is linked to O-4 of the DD-heptosyl residue that formed the 2,3,7-tri-O-methyl-DD-heptose (0.3 mole).

Table XI: Methylation analysis of SJ-47 Core Oligosaccharide

methylated sugar[a]	T^{b} X	Y	in Mole %[c,d] A	Linkages
2,3,4,6-Gal	1.07	1.21	5.8 (0.35)	Gal 1→
2,3,4-Glc	1.48	2.25	15.6 (0.95)	→6 Glc 1→
2,3,4,6,7-DD Hep	1.61	1.61	17.2 (1.07)	DD-Hep 1→
2,3,4,6,7-LD-Hep	1.76	2.00	15.9 (0.97)	LD-Hep 1→
2,3,4,7-DD-Hep	2.23	3.37	11.5 (0.70)	→6-DD-Hep 1→
3,4,6,7-LD-Hep	2.38	3.70	16.4 (1.00)	→2-LD-Hep 1→
2,3,4,6-LD-Hep	2.78	5.23	- -	→7-LD-Hep 1→
2,3,7-DD-Hep	2.90	5.41	5.2 (0.32)	→4-DD-Hep(6↓) 1→
2,7-LD-Hep	3.69	8.47	12.3 (0.75)	→4-LD-Hep(6↓, 3↑) 1→

[a] 2,3,4,6-Gal stands for 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-galactitol etc. [b] Retention times are relative to 2,3,4,6-tetra-O-methyl glucitol on column X: WCOT CP-Sil5 at 190° and Y: Silar 7CP at 180°. [c] A-Original core. [d] Molar ratios are given in parentheses.

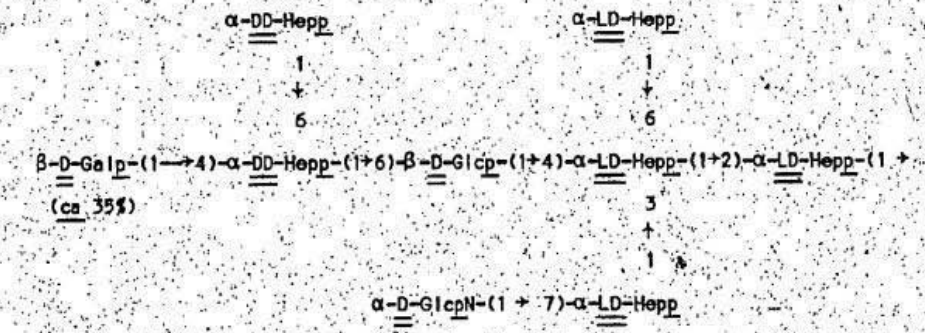
The foregoing results confirm that the terminal nonreducing galactosyl unit is present in the core oligosaccharide in non stoichiometric amount, and would be linked (when present) to the branched DD-heptose through O-4 as in the following partial structure:



4.5. Conclusion

The above chemical evidence strongly suggested that 35% of this core oligosaccharide had a structure identical to that of the core oligosaccharide isolated from the lps of Strain SJ-55, while the remaining 65% of the SJ-47 core molecules would represent a galactose defective mutant of the original strain SJ-55 core. It is also of interest to note the net positive increase in the optical rotation value when one goes from SJ-55 core to SJ-47 core, an increase which indicates partial loss of a β-linked residue (i.e. D-galactose). This last observation seems to be in agreement with the previously reported n.m.r. and chromium trioxide data.

Therefore, from the foregoing evidence a tentative structure may be proposed for the core oligosaccharide of A. hydrophila Strain SJ-47, which is as shown:



SUMMARY AND CONCLUSIONS

The lipopolysaccharide of A. hydrophila strain A6 (SJ-55) is mainly of the R-form (since it does not contain an O-specific chain) and its polysaccharide component is made up of D-glucose, D-galactose, L-glycero and D-glycero-D-manno-heptose as neutral sugars. This has led to its classification as Chemotype I (47); additional to the neutral sugars 2-amino-2-deoxy-D-glucose is also an integral part of the core oligosaccharide.

Hydrolysis, methylation, periodate oxidation and nitrous acid deamination permitted determination of the sugar composition and the nature of their linkages. Selective degradations including hydrolysis, Smith degradation and chromium trioxide oxidation gave information on the sequence of the different sugar residues in the molecule. Finally, combined with spectroscopic techniques (mass spectrometry and ^{1}H, ^{13}C-nuclear magnetic resonance), the structure of SJ-55 core oligosaccharide could be determined (see Fig. 12a).

Some unusual compositional and structural features are of interest, particularly the presence of D-glycero-D-manno-heptose in the outer core (hexose region) of the lipopolysaccharide. The first tentative identification of this uncommon sugar was reported by Davies (192) in a polysaccharide isolated from Chromobacterium violaceum and later unequivocal identification was made by Jones et al. (193) and Young et al. (194) in the extracellular polysaccharides of Azotobacter indicum and Serratia marcescens respectively. In lps, D-glycero-D-

manno-heptose was first identified in the core structure of several Proteus mirabilis strains (195, 196) with the concomitant presence of its 6-epimer, namely L-glycero-D-manno-heptose. More recently DD-heptose has also been found together with LD-heptose in the R-form lps of Yersinia pestis (197). However, Weckesser et al. (198) interestingly reported DD-heptose as the sole neutral sugar in Rhodopseudomonas gelatinosa lps and Adams et al. (185) reported it in the L-glycero-D-manno-heptose free lps of Xanthomonas campestris.

Lehmann et al. (199) have shown that the LD-heptose of Salmonella typhimurium R-form was replaced to a large extent by DD-heptose in some rfa mutants and transductants. These isolated R-form lipopolysaccharides contain DD-heptose linked to dOclA-lipid A, supporting the biosynthetic pathway proposed by Eidels et al. (200) and Ginsburg et al. (201) in which sedoheptulose-7-phosphate (acting as an intermediate of heptose synthesis) is isomerized in the bacterial cell to DD-heptose-7-phosphate. The latter is subsequently converted to its corresponding activated dinucleotide NDP-DD-heptose which is, after enzymatic epimerization in position 6 by the NDP-DD-heptose-6-epimerase, transformed to the activated NDP-LD-heptose and finally transferred to dOclA.

The incorporation of DD-heptose in the core oligosaccharide of A. hydrophila strain SJ-55 does not seem to follow the same biosynthetic pathway as it occurs solely in the outer core. It is presumably an intrinsic part of the outer core rather than a manifestation of imbalance in the synthesis of the inner core.

Another pertinent structural feature is the presence of a free amino sugar (2-amino-2-deoxy-D-glucose) in the inner core (heptose region) of the core oligosaccharide. The presence of this free amino sugar residue in the inner part of a core oligosaccharide is rare but has been observed in the E. coli (0111) core (39), and (linked in a configuration the same as that found in A. hydrophila) in the Shigella flexeneri Serotype 6 core (202).

During this study, it was observed that the glycosidic linkage of the glucosamine residue was extremely resistant to normal acidic conditions. This, obviously has considerably hampered the sugar analysis, but the difficulty was overcome by application of the nitrous acid deamination reaction. Interpretation of the methylation analysis of the core oligosaccharide has also been made difficult by the presence of the free amino sugar glucosamine, since even after N-acetylation of the amino group prior to methylation and hydrolysis, as suggested for the methylation analysis of glycosaminoglycans (83), no nonreducing end group glucosamine could be detected. However, its neutral sugar aglycon (i.e. 2,3,4,6-tetra-O-methylheptose) was observed. The literature (202, 45) indicates that when a free amino sugar (hexosamine), in a gluco or galacto-configuration, is glycosidically linked 1 → 7 or 1 → 6 to a heptose or hexose residue respectively, it is very difficult to detect the nonreducing end group during the methylation analysis.

The structure of the core oligosaccharide isolated from the minor phage sensitive variant (SJ-47) has been shown to be similar to that of

SJ-55 strain (see Fig. 12b) but exhibits a microheterogeneity such that only 35% of the core oligosaccharides have had all of the galactosyl end groups transferred to the molecule. This reconfirmed the linkage position of the galactose end groups on the branched DD-heptose (i.e. D-Gal 1 → 4 DD-Hep). It is difficult to rationalize the microheterogeneity in the core structure of this mutant, however, a decrease in the amount of the diphosphonucleotide galactosyl-transferase (or in that of NDP-galactose) would lead to incomplete substitution of the core by the galactose end groups. This series of experiments involved bacteria grown well beyond the log phase (to obtain maximum yield of lps) and the relative nutrient deficiency of the medium during the late growth phase may be a contributing factor to the microheterogeneity.

The core oligosaccharide isolated from the phage resistant variant SJ-48) contains no galactose or D-glycero-D-manno-heptose and has the structure shown on Fig. 12c. As it corresponds also to the inner core of SJ-55 and SJ-47, it is reasonable to assume that such a mutant represents an earlier stage in the biosynthesis of the core. The fact that it is phage resistant may indicate a loss of the phage receptors, in this case probably the DD-heptose end group; less likely is the galactose end group since the SJ-47 mutant, which has lost most of the galactose, is phage sensitive.

Isolation of the SR-form from lps of strain SJ-55 allowed the determination of the linkage postion of the O-specific polysaccharide on the core. It was shown that the galactose end group is linked through its O-3 position to the terminal reducing sugar residue of the O-chain

(whose identity has not been determined). Preliminary study indicates that the 4 moles of sugar residues added to SJ-55 core oligosaccharide (Rha:2 mole; Man:1 mole; ManN:1 mole) are linearly linked to each other, a rhamnose residue being the terminal nonreducing end group of the SR-oligosaccharide.

It would be of interest to follow this study with one designed to investigate serological cross reactions between these lipopolysaccharides and the specific antisera against each of these three R-form antigens.

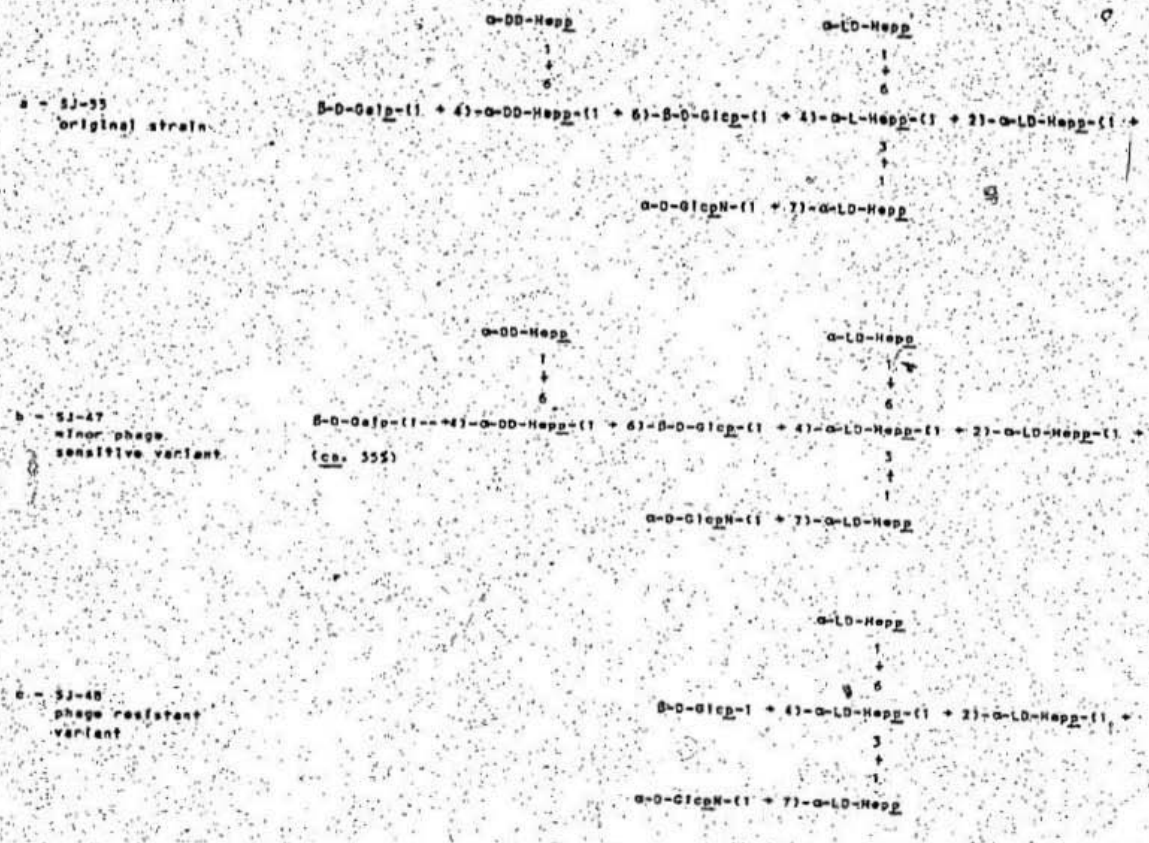


Fig. 12. Core oligosaccharide structures of A. hydrophila strain SJ-55, SJ-47, and SJ-48.

BIBLIOGRAPHY

1 - I.L. Bennet, Bacterial Endotoxins, (Landy and Brown, eds.), Rutgers University Press, New Brunswick, New Jersey, xiii (1964).

2 - A. Novotny (ed.), Annals of the New York Academy of Sciences. 133 (1966).

3 - M. Florkin and E.H. Stotz (eds.), Comprehensive Biochemistry, Elsevier, New York, 26A, 105 (1968).

4 - G. Weinbaum, S. Kadis and S.J. Ajil (eds.), Microbial Toxins, Academic Press, New York and London, 4, 473pp (1971).

5 - L. Leive (ed.), Bacterial membranes and walls, Marcel Dekker, New York (1973).

6 - E.H. Kass and S.M. Wolff (eds.), Bacterial Lipopolysaccharides, The Chemistry, Biology and Clinical Significance of Endotoxins, University of Chicago Press, Chicago, 304pp (1973).

7 - S. Kadis, G. Weinbaum and S.J. Ajil (eds.), Microbial Toxins, Academic Press, New York, 5, 507pp (1971).

8 - D. Schlessinger, Microbiology, 219 (1977).

9 - K. Jann and O. Westphal, The Antigens, (M. Sela, ed.), Academic Press, New York, 3, 1 (1973).

10 - O. Lüderitz, C. Galanos, H.J. Risse, E. Ruschmann, S. Schlecht, G. Schmidt, H. Schulte-Holthausen, R. Wheat and O. Westphal, Ann. N.Y. Acad. Sci., 133, 349 (1966a).

11 - O. Lüderitz, A.M. Staub and O. Westphal, Bacteriol. Rev., 30, 192 (1966b).

12 - O. Lüderitz, M.A. Freudenberg, C. Galanos, V. Lehmann, E. Th. Rfetschel and D.H. Shaw, Current Topics in Membranes and Transport, (F. Bronner and A. Kleinzeller, eds.), Academic Press, New York, 17, 79 (1982).

13 - R.U. Lemieux, D.A. Baker and D.R. Bundle, J. Am. Chem. Soc., 97, 4076 (1975).

14 - C.P. Stowell and Y.C. Lee, Adv. Carbohydr. Chem. Biochem., 37, 225 (1980).

15 - H.J. Jennings and C. Lugowski, J. Immunol., 127, 1011 (1981).

16 - K. Jann and B. Jann, Surface Carbohydrates of the Prokaryotic Cell, (I. Sutherland, ed.), Academic Press, New York, N.Y., 247 (1977).

17 - J.W. Shands, J. Bacteriol., 90, 266 (1965).

18 - O. Lüderitz, K. Jann and R. Wheat, Comprehensive Biochemistry, (Florkin and E. H. Stotz, eds.), American Elsevier Publishing Co., Inc., New York, 26A, 105 (1968).

19 - S. Hase and E. Th. Rietschel, Eur. J. Biochem., 63, 101 (1976).

20 - S.G. Wilkinson, Surface Carbohydrates of the Prokaryotic Cell, (I. Sutherland, ed.), Academic Press, New York, 97 (1976).

21 - C. Galanos, O. Lüderitz, E. Th. Rietschel and O. Westphal, Int. Rev. Biochem., 14, 239 (1976).

22 - O. Westphal and K. Jann; Methods Carbohydr. Chem., 5, 83 (1965).

23 - C. Galanos, O. Lüderitz and O. Westphal, Eur. J. Biochem. 9, 245 (1969).

24 - A.M. Staub, Methods. Carbohydr. Chem. 5, 92 (1965).

25 - L. Leive and D.C. Morrison, Methods Enzymology, 28B, 254 (1972).

26 - G.A. Adams, Can. J. Biochem., 45, 422 (1967).

27 - M. Raynaud, B. Kovznetzova, M.J. Navarro, J.C. Chermann, M. Digeon and A. Petitprez, J. Infect. Dis., 128, 535 (1973).

28 - E. Ribi, K.C. Milner and T.D. Perrine, J. Immunol., 82, 75 (1959).

29 - C. Galanos and O. Lüderitz, Eur. J. Biochem., 54, 603 (1975).

30 - P.W. Robbins and T. Uchida, Biochemistry, 1, 323 (1962).

31 - A. Wright and S. Kanegasaki, Physiol. Rev., 51, 748 (1971).

32 - C.G. Hellerqvist and B. Lindberg, Carbohydr. Res., 16, 39 (1971).

33 - W. Dröge, V. Lehman, O. Lüderitz and O. Westphal, Eur. J. Biochem., 175 (1970).

34 - N. Gentner and P. Berg, Abstr. Am. Soc. Biol. Meet., 100 (1971).

35 - P-E. Jansson, A.A. Lindberg, B. Lindberg and R. Wollin, Eur. J. Biochem., 115, 571 (1981).

36 - P. Prehm, S. Stirm, B. Jann and K. Jann, Eur. J. Biochem., 56, 41 (1975).

37 - T. Ohkawa, Biochem. Biophys. Res. Commun., 95, 938 (1980).

38 - D. Blache, M. Bruneteau and G. Michel, Eur. J. Biochem., 113, 563 (1981).

39 - N.A. Fuller, M.C. Wu, S.G. Wilkinson and E.C. Heath, J. Biol. Chem., 248, 7938 (1973).

40 - J.J. Morton and J.C. Stewart, Eur. J. Biochem., 29, 308 (1972).

41 - J. Radziejewska-Lebrecht, U. Feige, M. Jensen, K. Kotelko, H. Friebolin and H. Mayer, Eur. J. Biochem., 107, 31 (1980).

42 - D.T. Drewry, K.C. Symes, G.W. Gray and S.G. Wilkinson, Biochem. J., 149, 93 (1975).

43 - R. Chaby, M. Moreau and L. Szabo, Eur. J. Biochem., 76, 453 (1977).

44 - K. Kotelko, W. Gromska, M. Papierz, K. Szer, D. Krajewska and Z. Sidorczyk, J. Hyg. Epidemiol. Microbiol. Immunol., 18, 405 (1974).

45 - D.J. Neal and S.G. Wilkinson, Eur. J. Biochem., 128, 143 (1982).

46 - J.W. Redmond, Biochem. Biophys. Acta., 584, 346, (1979).

47 - D.H. Shaw and H.J. Hodder, Can. J. Microbiol. 24, 864 (1978).

48 - J.H. Banoub and D.H. Shaw, Carbohydr. Res., 98, 93 (1981).

49 - K. Hisatsune, S. Kondo, T. Iguchi, M. Machida, S. Asou, M. Imaguma, and F. Yamamoto, Microbiol. Immunol. 26, 649 (1982).

50 - H. Mayer and G. Schmidt, Z. Bakteriol. Parasiten. Infekt. Hyg., Abt 1 Orig. Reihe A, 224, 345 (1973).

51 - J. Gmeiner, Eur. J. Biochem., 58, 621 (1975b).

52 - N.M. Ahamed, J. Radziejewska-Lebrecht, C. Widemann and H. Mayer, Z. Bakteriol. Parasiten. Infekt. Hyg., Abt 1. Orig. Reihe A, 247, 468 (1980).

53 - K.K. Nixdorff and S. Schlecht, J. Gen. Microbiol., 71, 425 (1972).

54 - S.G. Wilkinson, P. Gemski and B.A.D. Stocker, J. Gen. Microbiol., 70, 525 (1972).

55. H-W. Wollenweber, K.V. Broady, O. Lüderitz, and E.Th. Rietschel, Eur. J. Biochem., 124, 191 (1982).

56 - W.A. Volk and O. Lüderitz, Methods Carbohydr. Chem., 9, in press.

57 - P.W. Robbins and A. Wright, Microbial Toxins, (G. Weimbaum, S. Kadis and S.J. Ajil, eds.), Academic Press, New York, 4, 351 (1971).

58 - V. Lehmann, E. Rupprecht and M.J. Osborn, Eur. J. Biochem., 76, 41 (1977).

59 - M.J. Osborn, Bacterial Outer Membranes, Biogenesis and Functions, (M. Inouye, ed.), John Wiley and Son, New York, 15 (1979).

60 - S.M. Wolff and J.V. Berinet, N. Engl. J. Med., 291, 733 (1974).

61 - R.G. Petersdorf, Harrisson's Principles of Internal Medicine, G.W. Thorn, R.D. Adams, E. Braunwald, K.J. Isseltacher and R.J. Petersdorf, eds.), 8th ed., McGraw-hill, New York, 770 (1977).

62 - R.J. Elin and S.M. Wolff, Ann. Rev. Med., 27, 127 (1976).

63 - R.G. Horn and R.D. Collins, Lab. Invest., 18, 101 (1968).

64 - J.M. Bergstein and A.F. Michael, Arch. Pathol., 97, 230 (1974).

65 - C. Galanos, O. Lüderitz, E. Th. Rietschel and O. Westphal, International Review of Biochemistry, Biochemistry of Lipids II, (T. W. Goodwin, ed.), University Park Press, Baltimore, 14, 241 (1977).

66 - D.C. Morrison and C.G. Cochrane, J. Exp. Med., 140, 797 (1974).

67 - O. Westphal, J. Gmeiner, O. Lüderitz, A. Tanaka and E. Eichenberger, Colloq. Int. Cent. Natl. Rech. Sci., 174, 69 (1969).

68 - J.M. Chiller, B.J. Skidmore, D.C. Morrison and W.O. Weigle, Proc. Natl. Acad. Sci., USA, 70, 2129 (1973).

69 - C. Galanos, E. Th. Rietschel, O. Lüderitz and O. Westphal, Eur. J. Biochem., 19, 143 (1971).

70 - W. Doe, D. Yang and P. Henson, Fed. Proc., 36, 1263 (1977).

71 - D.C. Morrison and L. Leive, J. Biol. Chem., 250, 2911 (1975).

72 - G.W. Goodman and B.M. Sultzer, Infect. Immun., 17, 205 (1977).

73 - M. Niwa, K.C. Milner, E. Ribi, and J.A. Rudback, J. Bacteriol., 97, 1069 1969).

74 - D. Rifkind and R.B. Hill Jr., J. Immunol., 99, 564 (1967).

75 - J.J. Corrigan and B.M. Bell, J. Lab. Clin. Med., 77, 802 (1971).

76 - J. Bader and M. Teuber, Z. Naturforsch., 28C, 422 (1973).

77 - G.G.S. Dutton, Adv. Carbohydr. Chem. Biochem., 28, 11 (1960).

78 - G. Hämmerling, O. Lüderitz, O. Westphal and P.H. Mäkelä, Eur. J. Biochem., 22, 331 (1971).

79 - J. Lönngren and S. Svensson, Adv. Carbohydr. Chem. Biochem., 29, 41 (1974).

80 - C.G. Wong, S.S.J. Sung and C.C. Sweeley, Methods. Carbohydr. Chem., 8, 55 (1980).

81 - B. Lindberg, Methods Enzymol., 28B, 178 (1972).

82 - P-E. Jansson, L. Kenne, H. Liedgren, B. Lindberg and J. Lönngren, Chem. Commun. Univ. Stockholm, 8, 1 (1976).

83 - B. Lindberg and J. Lönngren, Methods Enzymol., 50, 3 (1978).

84 - W.W. Haworth, J. Chem. Soc., 107, 8 (1915).

85 - T. Purdie and J.C. Irvine, J. Chem. Soc., 83, 1021 (1903).

86 - R. Kuhn, H. Trishman and I. Low, Angew. Chem. Internat. Ed. 67, 32 (1955).

87 - S. Hakomori, J. Biochem. (Tokyo), 55, 205 (1964).

88 - P. Prehm, Carbohydr. Res., 78, 372 (1980).

89 - H. Björndal, B. Lindberg and S. Svensson, Carbohydr. Res., 5, 433 (1967).

90 - K. Stellner, H. Saito and S-I. Hakomori, Arch. Biochem. Biophys., 155, 464 (1973).

91 - G.O.H. Schwartzmann, and R.W. Jeanloz, Carbohydr. Res., 34, 161 (1974).

92 - J. Finne and H. Rauvala, Carbohydr. Res., 58, 51 (1977).

93 - J.H. Banoub, Carbohydr. Res., 100, C17, (1982).

94 - J.H. Banoub and F. Michon, Carbohydr. Res., 100, C24 (1982).

95 - D. Rolf and G.R. Gray, J. Am. Chem. Soc., 104, 3539 (1982).

96 - G.O. Aspinall and A.M. Stephen, Int. Rev. Sci. Org. Chem. Ser. 1, 7, 285C (1973).

97 - G.O. Aspinall, Int. Rev. Sci. Org. Chem. Ser. 2, 7, 201 (1976).

98 - B. Lindberg and S. Svensson, Int. Rev. Sci. Org. Chem. Ser. 1, 7, 319 (1973).

99 - B. Lindberg, J. Lönngren and S. Svensson, Adv. Carbohydr. Chem. Biochem., 31, 185 (1975).

100- B. Capon, Chem. Rev., 69, 407 (1969).

101- B. Lindberg, J. Lönngren and W. Nimmich, Carbohydr. Res., 23, 47 (1972).

102 - S. Valent, A.G. Darvill, M. McNeil, B.K. Robertsen, and P. Albersheim, Carbohydr. Res., 79, 165 (1980).

103 - L. Rosenfeld and C.E. Ballou, Carbohydr. Res., 34, 15 (1974).

104 - B. Bayard and J. Montreuil, Carbohydr. Res., 24, 427 (1972).

105 - B.A. Dmitriev, N.A. Kocharova, Y.A. Knirel, A.S. Shaskov, N.K. Kochetkov, E.S. Stanislavsky, and G.M. Mashilova, Eur. J. Biochem., 125, 229 (1982).

106 - L. Malaprade, Bull. Soc. Chim., 43, 683 (1928).

107 - G.G.S. Dutton, K.B. Gibney, G.D. Jensen and P.E. Reid, J. Chromatog., 36, 152 (1968).

108 - I, J. Goldstein, G.W. Hay, B.A. Lewis and F. Smith, Methods. Carbohydr. Chem., 5, 361 (1965).

109 - P-E. Jansson, L. Kenne, B. Lindberg, H. Ljunggren, J. Lönngren, U. Ruden and S. Svensson, J. Am. Chem. Soc., 99, 3812 (1977).

110 - C. Erbing, K. Granath, L. Kenne and B. Lindberg, Carbohydr. Res., 47, C5 (1976).

111 - L. Kenne and B. Lindberg, Methods Carbohydr Chem., 8, 295 (1980).

112 - J. Defaye, Adv. Carbohydr. Chem. Biochem., 25, 181 (1970).

113 - J.M. Williams, Adv. Carbohydr. Chem. Biochem., 31, 9 (1975).

114 - L. Kenne, B. Lindberg, K. Petersson, and E. Romanowska, Carbohydr. Res., 56, 363 (1977).

115 - L. Kenne, B. Lindberg, P. Unger, T. Holme, and J. Holmgren, Carbohydr. Res., 68, C14 (1979).

116 - B. Lindberg, B. Lindquist, J. Lönngren and D.A. Powell, Carbohydr. Res., 78, 111 (1980).

117 - L. Kenne, B. Lindberg, K. Petersson, E. Katzenellenbogen, and E. Romanowska, Carbohydr. Res., 78, 119 (1980).

118 - S. Hase and Y. Matsushima, J. Biochem. (Tokyo), 72, 1117 (1972).

119 - S. Hase, Y. Tsuji and Y. Matsushima, J. Biochem. (Tokyo), 72, 154 (1972).

120 - J.J. Marshall, Adv. Carbohydr. Chem. Biochem., 30, 257 (1974).

121 - A. De Bruyn, M. Anteunis, R. De Gussem and G.G.S. Dutton, Carbohydr. Res., 4, 158 (1976).

122 - M. Karplus, J. Chem. Phys., 30, 11 (1959).

123 - K. Bock and C. Pedersen, J. Chem. Soc. (Perkin trans.), 2, 293 (1974).

124 - A.S. Perlin and B. Casu, Tetrahedron lett., 2921 (1969).

125 - K. Bock and C. Pedersen, Acta. Chem. Sand., B29, 258 (1975).

126 - A.S. Perlin, Isot. Org. Chem., 3, 171 (1977).

127 - J.P. Joseleau, F. Michon and V. Vignon, Carbohydr. Res., 101, 175 (1982).

128 - F.G. Vliegenthart, H. Jan Halbeeck and L. Dorland, Pure. Appl. Chem., 53, 45 (1981).

129 - R.C. Bruch and M.D. Bruch, J. Biol. Chem., 257, 3409 (1982).

130 - A.S. Shashkov and O.S. Chizov, Biorg. Khim. (English version)., 2, 437 (1976).

131 - H.J. Jennings and I.C.P. Smith, Methods Enzymol., 50 C, 39 (1978).

132 - A.S. Perlin, MTP Int. Rev. Sci. Org. Chem., Ser. 2, 7, 1 (1976).

133 - A.S. Perlin, Fed. Proc., 36, 106 (1977).

134 - P.A.J. Gorin, Adv. Carbohydr. Chem. Biochem., 38, 13 (1981).

135 - D.R. Bundle and R.U. Lemieux, Methods. Carbohydr. Chem., 6, 79 (1976).

136 - H.J. Jennings and I.C.P. Smith, Methods Carbohydr. Chem., 8, 97 (1980).

137 - S.J. Angyal and K. James, Austr. J. Chem., 23, 1209 (1970).

138 - J. Hoffman, B. Lindberg, S. Svensson, Acta. Chem. Scand., 26, 661 (1972).

139 - J. Hoffman and B. Lindberg, Methods. Carbohydr. Chem., 8, 117 (1980).

140 - J.H. Banoub and F. Michon, Can. J. Biochem., 60, 517 (1982).

141 - C.G. Hellerqvist, J. Hoffman, A.A. Lindberg, B. Lindberg and S. Svensson, Acta. Chem. Scand., 26, 3282 (1972).

142 - R.U. Lemieux, K. Bock, L.T.J. Delbaere, S. Koto and V.S. Rao, Can. J. Chem., 58, 631 (19809).

143 - R.U. Lemieux, J.C. Wong and H. Thøersen, Can. J. Chem., 60, 81 (1982).

144 - P. Branefors-Helander, L. Kenne, B. Lindberg, K. Petersson and P. Unger, Carbohydr. Res., 88, 77 (1981).

145 - K. Bock and M. Vignon, Nouv. J. Chem., 6, 301 (1982).

146 - F.R. Seymour, R.D. Knapp and S. Bishop, Carbohydr. Res., 72, 229 (1979).

147 - M. Vignon, F. Michon, J.P. Joseleau and K. Bock, Macromolecules, in press.

148 - M. Heidelberger, Bacteriol. Rev., 3, 49, (1939).

149 - G.G.S. Dutton and M. Heidelberger, J. Immunol. III, 875 (1973).

150 - G.G.S Dutton, J. Erikson, C.T. Fang, M. Heidelberger, W. Nimmich and S. Stirm, Acta. Path. Microb. Scand. Sect. C, 83, 397 (1975).

151 - M. Heidelberger and W. Nimmich, Immunochem., 13, 67 (1976).

152 - F. Kauffman, Classification of Bacteria, Munksgaard, Copenhagen, 169 pp, (1975).

153 - A. Von Graevenitz and L. Zinterhofer, N. Engl. J. Med., 278, 245 (1968)

154 - C. R. Blaise and J. R. Amstrong, Appl. Environ. Microbiol., 26, 733 (1973).

155 - V.J. Cabelli, Abstract, American Society of Microbiology, Washington, D.C., 32 (1973).

156 - W.H. Ewing, Manual of Clinical Microbiology, (E.H. Spaulding, E.H. Lennette, and J.P. Traut, eds.), Washington, D.C., American Society of Microbiology, 230 (1974).

157 - R.H.W. Schubert, Arch. Hyg., 150, 681 (1967).

158 - Bergey's Manual of Determinative Bacteriology, 8th Edition, The Williams and Wilkins Company, Baltimore (1974).

159 - G. Sanarelli, Z. Bakt. Orig., 9193 (1891).

160 - A.J. Kluyver and C.B. Van Neil, Z. Bakteriol. Parasiten. Infekt. Hyg. Abt 1. Orig. Reihe A, 369 (1936).

161 - M. Popoff and M. Véron, J. Gen. Microbiol., 84, 11 (1972).

162 - Acuigrup, Bull. Centr. Rech. Sci. Biarritz, 12, 493 (1979).

163 - T.J. Trust and D.C. Chepman, CMA Journal, 120, 942 (1979).

164 - W.A. Davis, J.G. Kane and V.F. Garagusi, Medicine, 57, 267, (1978).

165 - J.F. Lopez, J. Quesada and A. Saied, Am. J. Clin. Pathol., 50, 587 (1968).

166 - I.J. Slontnik, Ann. N.Y. Acad. Sci., 174, 503, (1970).

167 - S. Krug, Z. Binnenfisch. DDR, 26, 178 (1979).

168 - W.E. Trevelyan, D.P. Proctor and J.S. Harisson, Nature, 166, 444 (1950).

169 - S.M. Partridge, Nature, 164, 443, (1949).

170 - F.G. Fisher and H. Dörfel, Hoppe-Seyler's Z. Physiol. Chem., 301, 224 (1955).

171 - J. Radziejewska-Lebrecht, D.H. Shaw, D. Borowiak, I. Fromme and H. Mayer, J. Chromatogr., 179, 113 (1979)

172 - R. Freeman, H. Hill and R. Kaptein, J. Magn. Reson., 7, 327 (1972).

173 - D.Y. Gagnaire, F.R. Taravel and M. Vignon, Carbohydr. Res., 51, 157 (1976).

174 - H.O. Lowry, N.T. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem., 193, 265 (1951).

175 - P.S. Chen, T.Y. Toribara and H. Warner, Anal. Chem., 28, 1756 (1956).

176 - M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers and F. Smith, Anal. Chem., 28, 350 (1956).

177 - Z. Dische, J. Biol. Chem., 167, 189 (1947).

178 - C.J.M. Rondle and W.T.J. Morgan, Biochem. J., 61, 586 (1955).

179 - J.L. Strominger, J.T. Park and R.E. Thompson, J. Biol. Chem., 234, 3263 (1959).

180 - R. Chaby and L. Szabo, Eur. J. Biochem., 59, 277 (1975).

181 - R. Chaby, S.R. Sarfati and L. Szabo, Anal. Biochem., 58, 123 (1974).

182 - J.S. Sawardeker, J.H. Sloneker and A.R. Jeanes, Anal. Chem., 12, 1602 (1965).

183 - M.B. Perry and A.C. Webb, Can. J. Biochem., 46, 1163 (1968).

184 - P.J. Stoffyn and R.W. Jeanloz, Arch. Biochem. Biophys., 52, 373 (1954).

185 - G.A. Adams, C. Quadling and M.B. Perry, Can. J. Microbiol., 13, 1605 (1967).

186 - G.W. Hay, B.A. Lewis and F. Smith, Methods Carbohydr. Chem., 5, 357 (1965).

187 - B.A. Dmitriev, Y.A. Knirel and N.K. Kochetkov, Carbohydr. Res., 30, 45 (1973).

188 - A. De Bruyn, M. Anteunis, R. De Gussem and G.G.S. Dutton, Carbohydr. Res., 47, 158 (1976).

189 - C. Erbing, B. Lindberg, and S. Svensonn, Acta Chem. Scand., 27, 3699 (1973).

190 - N.K. Kochetkov and O.S. Chizov, Adv. in Carbohydr. Chem., 21, 39 (1966).

191 - J.H. Banoub, F. Michon and D.H. Shaw, unpublished results.

192 - P.A.L. Davies, Nature, 180, 1129 (1957).

193 - J.K. Jones, M.P. Perry and W. Sowa, Can. J. Chem, 41, 2712 (1963)

194 - R. Young and G.A. Adams, Can. J. Chem., 43, 2929 (1965).

195 - G. Badgian, W. Dröge, K. Kotelko, O. Lüderitz and O. Westphal, Biochem. Z., 344, 197 (1966)

196 - K. Kotelko, O. Lüderitz and O. Westphal, Biochem. Z., 343, 227, (1965).

197 - J.L. Hartley, G.A. Adams, and T.G. Tornabene, J. Bacteriol., 118, (1974).

198 - J. Weckesser, H. Mayer, G. Drews and I. Fromme, J. Bacteriol., 123, 449 (1975).

199 - V. Lehmann, G. Hämmerling, M. Nurminen, I. Minner, E. Ruschmann, O. Lüderitz, T.T. Kuo and B.A.D. Stocker, Eur. J. Biochem., 32, 268, (1973).

200 - L. Eidels and M.J. Osborn, Proc. Nat. Acad. Sci., U.S.A., 68, 1673, (1971).

201 - V. Ginsburg, P.J. O'Brien and C.W. Hall, J. Biol. Chem., 237, 497, (1962).

202 - E. Katzenellenbogen and E. Romanowska, Eur. J. Biochem., 113, 205, (1980).