STRUCTURAL ELUCIDATION AND SYNTHESIS OF THE GLYCOCONJUGATE OF THE O-ANTIGEN ISOLATED FROM THE LIPOPOLYSACCHARIDE OF THE GRAM-NEGATIVE BACTERIA YERSINIA RUCKERI

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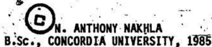
N. ANTHONY NAKHLA

STRUCTURAL ELUCIDATION AND SYNTHESIS OF THE GLYCOCONJUGATE OF THE O-ANTIGEN ISOLATED FROM THE LIPOPOLYSACCHARIDE OF THE GRAM-NEGATIVE BACTERIA <u>YERSINIA RUCKERI</u>

by



N. ANTHONY NAKHLA
B.Sc., CONCORDIA UNIVERSITY, 1985

A Thesis submitted in partial
fulfillment of the requirement for
the degree of Master of Science

Department of Biochemistry
Memorial University of Newfoundland
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Dedicated to my parents

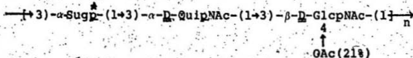
ABSTRACT

Y. Ruckeri represent a group of Gram-negative bacteria which are pathogenic to fish causing enteric red-mouth disease. Interest in the structure and immunological properties of the cell surface poly-saccharides of this bacterium has increased as little is presently known of the biochemical basis of pathogenicity of this species.

The O-specific antigen was isolated and purified and it was found to be composed of a trisaccharide repeating unit of 1→3 linked residues of 2-acetamido-2,6-dideoxy-α-D-glucose, 2-acetamido-2-deoxy-β-D-glucose and a novel 4-amino-4,6-dideoxy-4-(N-diacetyl)-hexose.

Sugar and methylation analyses, periodate oxidation and Smith degradation, chromium trioxide oxidation and partial acid hydrolysis allowed the determination of the sugar composition and the positions of linkage as well as the anomeric configurations.

The chemical structure was determined with the use of ^{1}H and ^{13}C-n.m.r. spectroscopy in addition to electron-impact (e.i.) and chemical ionization (c.i.) mass spectroscopy. The structure was determined to be the following.



$\rightarrow$[3)-α-Sugp*-(1→3)-α-D-QuipNAc-(1→3)-β-D-GlcpNAc-(1]$_n$$\rightarrow$

(4 ← OAc(21%) on GlcpNAc)

Within the course of this investigation, a novel mild method for the preparation of the artificial neoglycoprotein of *Y. ruckeri* O-antigen has been devised using 1,6-hexanediamine as the bridging arm, which linked the O-specific polysaccharide to the -aminolysyl groups of the bovine serum albumin protein carrier. This artificial neoglycoprotein elicited antibody production in rabbits.

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*where Sug*p* stands for 4-amino-4,6-dideoxy-4-(*N*-diacetyl)-hexose

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PART I

Literature Survey
on
Carbohydrate-containing macromolecules,
structural analysis of complex carbohydrates
and serological cross reactions of
carbohydrate antigens.

1. INTRODUCTION

The abundance of carbohydrate-containing macromolecules in nature is manifest in almost all the life forms. Thus, plants are known to consist of wood polysaccharides, gums, and mucilages, while algae, fungi and yeast contain their own polysaccharide matrices. Most bacteria produce carbohydrate polymers. Glycoconjugates consisting of proteins linked to carbohydrates e.g. glycoproteins, proteoglycans and peptidoglycans are widespread in animals and man, as are nucleic acids which are also carbohydrate containing biopolymers. Polysaccharide chains covalently attached to lipids (lipopolysaccharides, Lps) are a part of cell walls of many bacteria.

The walls of Gram-negative cells are much more complex than are those of Gram-positive cells (Figure 1.1.). Their accessory components consist of polypeptides, lipoproteins, and particularly, a very complex lipopolysaccharide whose structure has been the subject of remarkable achievements in a variety of disciplines within the last thirty-five years(1,2,3).

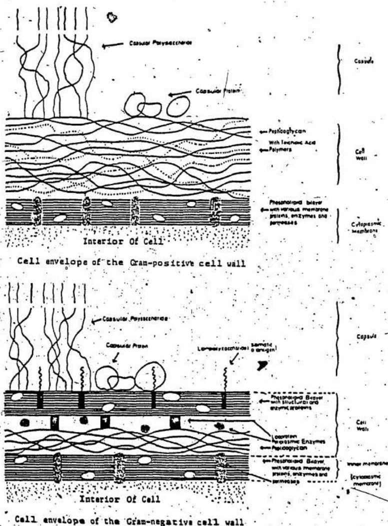


Figure 1.1. Schematic representation of Gram-positive and Gram-negative cell wall of bacteria(3).

The functions of microbial extracellular polysaccharides as storage and energy reserves and in virulence (protection against phagocytosis) have been known for a long time. However, their role in these areas was not clearly understood. It is now well established that polysaccharides are important in biological recognition functions where they (a) act as receptors for phage and bacteriocins (b) act as specific receptors in eukaryotes for viruses, bacteria, hormones, and toxins (c) have antigenic specificity (capable of combining with specific antibodies), and (d) are immunogenic (inducing the formation of antibodies)(4). In plants they function as regulators of growth, development, reproduction and disease.

The outermost cell component of bacteria, which is the antigen being recognized, is the crucial participant in an immune response. The location of microbial polysaccharide on the outer cell wall is therefore the reason for its antigenicity(5). These polysaccharides are either an integral part of the cell wall as in somatic lipopolysaccharides of Gram-negative Enterobacteriaceae (e.g. Salmonella) or form extracellular capsules as those of Pneumococcus, Klebsiella, and many Escherichia coli.

The polysaccharide antigens are of two classes (the K and O-antigens) with the third antigenic determinant (the H antigen) being composed of proteins. The K antigen is

in the form of a discrete capsule surrounding the cell. It may exist, also as a loose slime unattached to the cell surface (envelope). The somatic K antigen is capable of inhibiting agglutinations with O-antisera if present in sufficient quantity(6). The heat stable, somatic, smooth O-antigen is covalently linked to the phospholipid components in the outer membrane and is hence termed a lipopolysaccharide.

The general term lipopolysaccharide (Lps) which describes a unique category of macromolecules, is biologically part of the most significant components of the outer membrane of Gram-negative bacterial cells and is often used as a synonym for endotoxin. The Lps plays an important role in bacteriophage typing and serological classifications of the Gram-negative bacteria. Additionally, the Lps exerts endotoxic activities for which the lipid moiety is responsible(5).

Injections of significant amounts of bacterial Lps or purified Lps into experimental animals may result in a large range of endotoxic reactions. These effects, contrary to the specific delayed immune response, are in general non-specific and acute and can possibly cause such widespread phenomena as fever, changes in leukocyte counts, shock, and in some instances may be fatal(7-11).

Mild acid hydrolysis of Lps affords free poly-saccharide or oligosaccharide which, when coupled to

protein, may be utilized for the induction of antibodies, which in turn can be essential in the production of vaccines.

Three aspects of Lps have become of particular interest in biomedical investigation: the immunochemistry of O-antigenic chains as a basis for the diagnosis of, and vaccination against, bacterial infections; the search for inhibitors of the biosynthesis of core structures, especially those containing 3-deoxy-D-manno-2-octulosonic acid (KDO) as potential anti-infective drugs; and the study of lipid A or its derivatives as potential pharmacological agents in the therapy of immune disorders and cancer(12).

1.1. GENERAL ARCHITECTURE OF BACTERIAL LIPOPOLYSACCHARIDES

The bacterial cell wall consists of three layers (i) the inner cytoplasmic membrane in which complex enzymatic systems for redox processes, the synthesis of complex macromolecules and the active transport mechanisms are located; (ii) The peptidoglycan or murein layer which maintains shape and rigidity of the bacterial cell wall; (iii) the outer membrane containing phospholipid and the lipopolysaccharide(13,14) (see Figure 1.1.).

The classical model for a bacterial lipopolysaccharide is that originally proposed by Luderitz *et*

al. (15) for a product isolated from smooth "wild type" strains of Salmonella. As shown (Figures 1.2. and 1.3.) the model for the structural unit of a complete lipopolysaccharide (S-form) is composed of three covalently linked segments (O-specific chain, core oligosaccharide, and lipid A) each with its distinctive composition, biosynthesis and (presumably) biological function.

The O-specific chain is the serologically dominant part of the molecule responsible for its O-antigenic specifity. It consists of repeating oligosaccharide units in which the serological determinants reside. It is absent from the R-form lipopolysaccharides isolated from rough strains, but is usually produced as a hapten by mutants in which the genetic defect lead to the biosynthesis of an incomplete core(15,16). Generally the O-specific chains are made up of repeating units of di-, tri-, or higher oligosaccharides. The structure of the O-specific chain is unique to each bacterial serotype. O-specific chains (and core structures) contain many unique or unusual sugar constituents and in the past, many sugars of several categories have been identified in various lipopolysaccharides(12) (Table 1.1.).

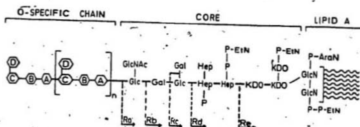


Figure 1.2. General structure of Salmonella lipopolysaccharide. Key: Glc, D-glucose; Gal, D-galactose; GlcN, D-glucosamine; GlcNAc, N-acetyl-D-glucosamine; Hep, L-glycero-D-manno-heptose; KDO, 2-keto-3-deoxy-D-manno-octulasonic acid; AraN, 4-amino-L-arabinose; P, phosphate, EtN, ethanolamine; ~, hydroxy and nonhydroxyfatty acids; R_a to R_e indicated are incomplete R form lipopolysaccharides; A–D, various sugar residues. Luderitz et al(15).

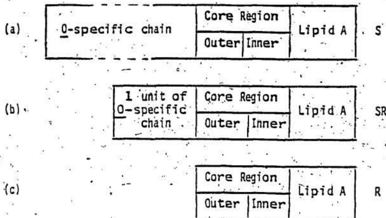


Figure 1.3. Architecture of (a) a complete structural unit (S, smooth) containing O-specific chain, core oligosaccharide and Lipid A, (b) a semi-rough (SR) structural unit containing only 1 unit of O-specific chain, core oligosaccharide and Lipid A and (c) a rough structural unit (R) consisting of only core oligosaccharide and Lipid A.

Table 1.1. Constituents Commonly Identified in Lipopolysaccharides From Various Gram-negative Bacteria.*

Neutral Sugars

- Pentose (5)
- 4-Deoxypentose (1)
- Pentulose (1)
- Hexose (3)
- 6-Deoxyhexose (7)
- 3,6-Dideoxyhexose (5)
- Hexulose (1)
- Heptose (4)
- 6-Deoxyheptose (1)
- Heptulose (1)

Amino Sugars

- 4-Amino-4-deoxypentose (1)
- 2-Amino-2-deoxyhexose (3)
- 2-Amino-2,6-dideoxyhexose (4)
- 3-Amino-3,6-dideoxyhexose (2)
- 4-Amino-4,6-dideoxyhexose (3)
- 2,3-Diamino-2,3-dideoxyhexose (1)
- 2,4-Diamino-2,4,6-trideoxyhexose (2)
- 2-Amino-2-deoxyheptose (1)

Acidic Sugars

- Hexuronic acid (2)
- 2-Amino-2-deoxyhexuronic acid (3)
- 3-Deoxyoctulosonic acid (1)
- 3-O-Lactyl-6-deoxyhexose (2)
- 4-O-Lactylhexose (1)
- 2,3-Diamino-2,3-dideoxyhexuronic acid (3)

Non-Sugar Constituents

- 2,4-Dihydroxybutanoic acid
- Glycine
- Alanine
- Lysine
- Pyruvic acid
- Ethanolamine

O-Methylated Sugars (30)

*( ): Number of isomers identified. For details see the reference of Luderitz et al(12).

The complete core may be subdivided into an "outer" region, to which the O-specific chain is attached, and an "inner" region linked to lipid A. Lipid A is apparently responsible for the endotoxic activities of the lipopolysaccharide, while both the lipid and proximal units of the inner core seem to be essential for viable bacteria. Core fractions are composed of an oligosaccharide which may frequently be species specific but may sometimes vary within bacterial species. The core oligosaccharide is attached to the lipid A portion via a trisaccharide of 2-keto-3-deoxy-D-manno-octulosonic acid (KDO) at the reducing end through a relatively mild acid labile linkage whereas the O-specific chains are attached to the terminal residue of the core oligosaccharide. However, the exact linkage in the KDO trisaccharide have not yet been definitely established(17). One of the main characteristics of the core oligosaccharide is its substitution by phosphoryl, pyrophosphoryl and ethanolamine residues, and this, as a whole expresses a net negative charge which appears to be physiologically important(18).

All rough (R-form) lipopolysaccharides as well as free lipid A (obtained via mild acid-hydrolysis of the KDO linkage) represent potent endotoxins, comparable in activity to complete lipopolysaccharides. This demonstrates that lipid A represents the component of

lipopolysaccharides which is responsible for its endotoxic properties.

The Lps of Salmonella has been widely utilized as a basic model in the interpretation of compositional and structural data for the lipopolysaccharides of other gram-negative bacteria. Structural analyses of Salmonella lipid A have shown the formula given in Figure 1.4. It contains a phosphorylated, β-(1→6)-linked D-glucosamine disaccharide, the so-called lipid-A-backbone, which is partly substituted by 4-amino-L-arabinose and phosphorylethanolamine.

In the lipopolysaccharide, the KDO of the core is linked to a hydroxyl group of the non-reducing glucosamine. The lipophilic character of lipid A is provided by seven long-chain fatty acid residues.

In summary the general features of the "classical" lipopolysaccharides can be seen in the following table. (Table 1.2.).

1.2. LOCATION, ISOLATION AND PURIFICATION OF LIPOPOLYSACCHARIDES

Although the biosynthesis of lipopolysaccharides takes place at the cytoplasmic membrane and complexes containing Lps can often be isolated from culture fluids, Lps is essentially a component of the characteristic outer

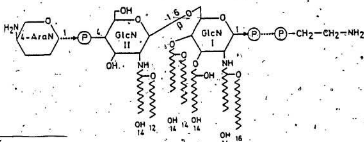


Figure 1.4. Proposed structure for Salmonella lipid A., Key: 4-AraN, 4-amino-L-arabinose; ,Ⓟ, phosphate GlcN, D-glucosamine; Ⓟ.....Ⓟ-CH_2-CH_2-NH_2, phosphorylethanolamine; ∿∿, long chain fatty acid residues(15).

Table 1.2. Properties characteristic of lipopolysaccharides from Gram-negative bacteria.

Cytological	Location in the outer membrane of the cell envelope as a complex with protein and phospholipid
Physico-chemical	Present in the aqueous phase after extraction with aqueous phenol. Polydisperse solutions with aggregates of particle weight over 10^6 daltons, dissociable by detergents and EDTA. Polymorphous in electron micrographs, but often filamentous. Hypsochromic shift with a carbocyanine dye.
Structural	Architectural principle: serotype-specific side-chain (polymerised oligosaccharide), attached to core oligosaccharide (inner region contains heptose, KDO and phosphate), attached to lipid A (contains glucosamine, hydroxy fatty acids and phosphate)
Biological	Polysaccharide side-chain contains thermostable O-specific antigen determinants. Extensive range of endotoxic activities (dependent on lipid A), including pyrogenicity, lethality, anticomplementary activity.

membrane of the gram-negative cell envelope (Figure 1.5.). However, the outer membrane also contains various common lipids (mostly phospholipids), a distinct range of proteins, a specific lipoprotein and possibly glycoproteins as well as lipopolysaccharide.

A number of extraction procedures for the isolation of Lps or Lps-protein complexes have been described and are illustrated in Table 1.3.

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 aqueous phenol procedure which yields a water soluble extract that is subsequently purified via high-speed centrifugation(19). For the R-form Lps which have reduced water solubility, low yields may be obtained via the phenol/water method, whereas superior yields may be obtained by the exceptionally mild and selective "PCP" method(20). There is evidence of carboxyl, phosphoryl, and ethanolamine residues in the molecules and it is due to these residues that the Lps character is amphoteric and the overall net charge is negative. In the original Lps preparation, the negatively charged groups are neutralized by Na^+, K^+, Ca^{++} and Mg^{++}. It has been found that the nature of the cations present in Lps greatly influence their state of

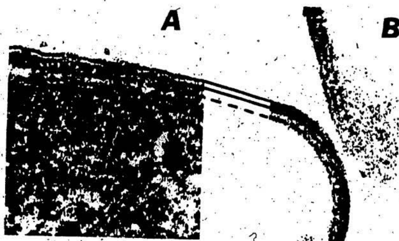


Figure 1.5. Electron micrographs of thin sections from Escherichia coli: A, whole cell; B, isolated cell wall. The broken tie-line indicates the "R-layer" containing peptidoglycan. The unbroken tie-lines indicate the trilaminar outer membrane. Wilkinson(14).

Table 1.3. Methods for the Extraction of Lipopolysaccharides

Treatment	Leading Reference
-45% Aqueous phenol, 65-68°C	Westphal and Jann(19)
-Aqueous phenol-chloroform-light petroleum ether, 5-20°C (PCP method)	Galanos *et al.*(20)
-0.25M trichloroacetic acid, 4°C	Staub(21)
-Aqueous EDTA (pH 8.0-8.5), 37°C	Lieve and Morrison(22)
-Aqueous butanol (0-4°C)	Lieve and Morrison(22)
-Dimethyl sulfoxide, 60°C	Adams(23)
-1M Sodium chloride - 0.1M sodium citrate (pH 7.0), 0-4°C	Raynaud *et al.*(24)
-Aqueous diethyl ether, ambient temperature	Ribi *et al.*(25)

aggregation, and as a consequence, their biological activities(26).

2. STRUCTURAL ANALYSES OF POLYSACCHARIDES

In the course of studies conducted on bacterial polysaccharides, the existence of complex and immensely diverse patterns of polysaccharide chains has been revealed. The immunological serological and phage receptor properties of bacterial polysaccharide chains are usually expressed through regions known as antigenic determinants(27-29). For the elucidation and the definition of any one structure of such complexity, the use of a combination of different chemical analyses is necessary. These include the qualitative and quantitative estimation of the component sugars and analysis for the presence of non-sugar substituents (O or N-acetyl, phosphate, pyruvic acetal etc.). Thereafter, the determination of the position of linkage, the glycosidic configuration and finally the sequencing of the sugars in the polymer have to be embarked upon.

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 been recently made.

2.1. SEPARATION TECHNIQUES

Purity is an important factor in the analysis of any compound. The first step is to obtain the polysaccharide in pure form, separated from contaminants. Column chromatography (gel-permeation, ion-exchange, affinity) has been shown to be a useful tool for the purification of crude polysaccharides. To obtain information on the constituent sugars of the polysaccharide and its degradation products, separation of the mixtures into individual components is essential. Gas-liquid chromatography, gel-permeation and ion-exchange chromatography are all useful techniques in separating the mixtures of sugars and their derivatives. Some applications of these techniques are described briefly in the following section.

2.1.1. Gas-liquid chromatography (g.l.c.)

Structural studies are based initially on the identification of the component sugars. Gas-liquid chromatography is instrumental in the separation of a wide range of sugars and its use in carbohydrate chemistry is[1] extensive. This technique is based on the distribution of volatile components between a mobile gas phase and a stationary adsorbent phase. The nature of the stationary phase affords selectivity and a variation in polarity can

lead to better separations of the sugar derivatives(30). Since carbohydrates are non-volatile, early studies were carried out on the volatile methylated methyl glycosides(31). Conversion of sugars into their volatile trimethyl silyl (TMS) derivatives(32) afforded new applications of this technique for identification of sugars. Although the TMS derivations are easily formed, the existence of anomeric forms of sugar at equilibrium yields a complicated chromatograph of multiple peaks. In an attempt to overcome this problem, the acyclic sugar alditols were converted into volatile acetates, trifluoroacetates or trimethyl silyl ethers. Since the trifluoroacetates show partial de-esterification on the column and the TMS derivatives of the alditols showed poor separation(33), the readily formed alditol acetates(34) have proven to be the derivatives of choice having good resolution and short retention times. In recent times, g.l.c. has been coupled to mass spectrometry(35). For this application, alditol acetates are particularly useful since they yield simpler mass spectra than the other derivatives used for g.l.c.

Conventional packed columns made of non-polar stationary phase (e.g. silicone gums SE-J2, SE-30, XE-60 and carbowax) give good separations of TMS methyl glycosides, but not of alditol acetates(36). Base line separations of mixtures of many unalkylated and alkylated

alditol acetates(37) are possible on columns made up of ECNSS-M (ethylene succinate-cyanoethyl silicone copolymer) and SP-2330 (75% cyanopropyl silicone) which are polar fused silica capillary columns and are thermally stable at high temperatures (up to 350°), give good resolution, and, most importantly, can detect samples in the microgram range. Chemically bonded phases for fused silica columns have been obtained by covalent chemical bonding both in the form of cross-links within the polymer as well as bonds from the phase to the silica surface. Such phases have dramatically increased the capacity and dynamic range of capillary columns without a sacrifice in resolution. Studies on alditol acetates of amino sugars have revealed that shorter retention times are obtained with non-polar or medium-polar columns, and that chemically bonded capillary columns give better resolution than conventional columns. Oligosaccharide alditol acetates(34) and peralkylated oligosaccharide alditols(38) can also be separated by this technique. Therefore, the use of g.l.c., alone or in conjunction with mass-spectrometry (g.l.c.-m.s.), is a powerful tool in the analysis and sequencing of polysaccharides.

2.1.2. Gel permeation and ion-exchange chromatography

Gel-permeation chromatography is also known as gel

filtration, or molecular-sieve chromatography. As the names imply, the method is based on fractionation of molecules of different sizes on a column consisting of a gel of a three-dimensional network. The smaller molecules penetrate further into the pores of the gel than do the larger, and are retained longer on the column. The larger molecules are thereby eluted first. The applications of this technique include separations of mixtures of oligosaccharides, purification of polysaccharides and the determination of molecular weights(39). Amino sugars and amino acids can also be separated from neutral sugars or polysaccharides using ion-exchange resins.

2.2. SUGAR ANALYSIS

2.2.1. Total hydrolysis

The hydrolysis of a polysaccharide into individual monosaccharides with minimum degradation is the most important step in their analysis and is performed quantitatively. However, the total hydrolysis of polysaccharides, particulary those with resistant glycosidic linkages, is a more difficult operation. Hydrochloric, sulfuric and trifluoroacetic acids are commonly used in hydrolysis and are sufficient to completely hydrolyse a neutral polysaccharide into its monomeric sugar units. In

the past, identification of the constituent monosaccharides released during acidic hydrolysis were characterized with the use of paper chromatography and thin-layer chromatography (t.l.c.)(40). However, qualitative and quantitative analysis is now more frequently performed via g.l.c. and g.l.c.-m.s. of suitable derivatives(41). The sugars are usually transformed into acetylated alditols or aldonitriles. Stereoisomers give similar mass spectra and when this information is coupled with relative retention times on g.l.c., identification of the individual sugar is possible, with the exception of absolute configuration(40, 41).

2.2.2. Hydrolysis with acids

As mentioned earlier, depolymerizations of polysaccharides have been successfully carried out using sulfuric and hydrochloric acids. The latter may be removed by volatilization. However hydrochloric acid usually causes more degradation of sugars than sulfuric acid and, therefore, the more volatile and easily removable trifluoroacetic acid(42) is being used more frequently as compared to the mineral acids. Preliminary hydrolysis with acetic acid(43) has been helpful in avoiding N-deacetylation of amino sugars, and formic acid

is sometimes used in solubilizing methylated polysaccharides(44). Anhydrous hydrofluoric acid, being mild, does not remove the N-acetyl group from acetamido groups, and is thus able to hydrolyze amino sugar containing polysaccharides in quantitative yields(45).

The use of ion-exchange resins for hydrolysis is popular in the field of glycoproteins. The resin may be used alone or with mineral acid, the best results being obtained in conjunction with hydrochloric acid(37). Some sugars are more acid labile than others; for example 2-amino-2-deoxy-sugars and uronic acids are more resistant to hydrolysis than neutral sugars. Deoxy-sugars, ketoses, and sialic acids are extremely labile and are liberated by mild acid on short exposure. One of the problems in hydrolysis is in choosing ideal conditions. In many cases, no one method will cleave all the glycosidic linkages quantitatively. The use of strong acids (e.g. 4M HCl) (46) to cleave 2-amino-2-deoxy-sugars usually leads to the degradation of deoxy- and keto-sugars. Hence analysis usually differing acid strengths and exposure times are necessary in order to obtain information on all the sugars present.

2.3. ESTABLISHMENT OF LINKAGE POSITION

2.3.1. Methylation analysis

Methylation analysis, developed by Haworth(47) is still the most important single method in structural carbohydrate chemistry. The technique is based on the protection of free hydroxyl groups of a polysaccharide by etherification, which acts as a label in determining the originally unlinked positions of a sugar on its release by hydrolysis. Separation as their partially methylated alditol acetate derivatives by gas-liquid chromatography and identification by g.l.c.-m.s. gives an insight into the types of linkages existing in the polymer, but not of the sequencing, or the anomeric nature of the linkages. Due to the insolubility of high molecular weight O-specific chain polymer in organic solvents, the method of Haworth(47), Purdie and Irvine(48) and Kuhn(49) required several steps to achieve complete methylation, whereas now the Hakomori methylation(50) 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(50). 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 excellent alternative(51). The methylation analysis is based on the ability to fractionate and characterize the partially methylated monosaccharides generated by the hydrolysis of the fully methylated polysaccharide. Hydrolysis of the methylated polymer is usually performed with 2M trifluoroacetic acid in an oven at 100°C overnight. Uronic acid containing polysaccharides are carboxyl reduced before (carbodiimide reduction) or after (lithium aluminum hydride) the permethylation step. Amino sugar containing polysaccharides show better results with 2M hydrochloric acid at 95°C for 4-5 hours or on acetolysis(52,53) with 0.25 M sulfuric acid in 95% acetic acid at 80°C for 6 hours followed by dilution with water and further heating for 3 hours. Prolonged hydrolysis for liberation of glycosamines leads to degradation of neutral sugars and demethylation, particularly of 3-linked permethylated galactose.

2.3.2. Characterization of methylated sugars

The techniques for separating and identifying methylated sugars have evolved to the point where analysis by gas-liquid chromatography (see section 2.1.1.)

dominates all others.

The alditol acetate derivatives of the methylated sugars are used for more than any other derivative due to the simplicity of the chromatograms obtained and ease of quantitation. Reports on methylation analysis containing relative retention times on capillary g.l.c. columns are numerous(54) and many mass spectra of the methylated alditol acetates(55) have been published. The use of partially ethylated alditol acetates in identification of sugars is useful in separating sugars that are unresolved as the partially methylated derivatives on the g.l.c. as well as in distinguishing from already existing methyl ethers.

There has been considerable efforts in the analysis of the amino sugars and the retention times and mass spectral data for various methylated derivatives have been reported(53,41,56-60). The amino sugar derivatives have longer retention times than the corresponding neutral sugars.

The appearance of spurious peaks has been attributed to contamination by phthalates which can be differentiated by their characteristic peak at m/z 149 on mass spectral analysis.

Identification of derivatives is possible by comparison of retention times with known values on various columns, or by coelution with authentic samples, followed

by confirmation of the substitution pattern obtained on g.l.c.-m.s.

2.3.3. Applications of mass spectrometry

A mass spectrum consists of a plot of the relative intensities of gaseous ions formed by ionization and subsequent fragmentation of the volatilized molecules, against their mass-to-charge ratio (m/z). Different types of instrumentation can be used to record mass spectra. The inlet can be either a hot reservoir inlet, a direct probe inlet, or a g.l.c. inlet (g.l.c.-m.s.). Ionization of the molecules can be affected by electron impact techniques (e.i.), chemical ionization (c.i.)(61,62) field-desorption (f.d.)(63), field ionization (f.i.)(64), or fast atom bombardment (f.a.b.)(65,66).

The non-volatile carbohydrates are analyzed usually as their volatile derivatives. The fragmentation patterns depend on the stability of the fragments produced, and are characteristic of the derivatives. Stereoisomeric derivatives usually give near identical mass spectra with very small differences in intensity. Thus, this technique does not permit assignment of configuration.

The electron-impact technique is the standard method used in the analyses conducted in this laboratory. The high intensity (70 eV) beam of electrons used leads to

extensive fragmentation of the molecules and results in little or no molecular ion peak. Molecular ions and larger fragments can be obtained by c.i., f.i., and f.d. mass spectrometry which favour minimal fragmentation. F.a.b.-m.s. is becoming more interesting because it has the ability to give both molecular weight and fragment data and can be used in analysis of unmodified carbohydrates and glycolipids(67).

2.3.3.a. Characterization of monosaccharide derivatives

Direct derivatization of monosaccharides by permethylation, peracetylation, pertrimethylsilylation resulting in anomeric mixtures of glycosides has been studied extensively(68,69).

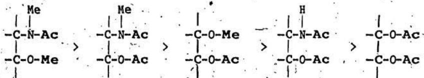
Alditols are of more importance than other derivatives due to the absence of anomers and ease of separation on g.l.c., and their mass spectra will be described in detail.

The alditol acetates of interest are the peracetylated and partially methylated. All these derivatives show no molecular ions, and those having the same substitution pattern give similar mass spectra that are typical of the substitution pattern. For instance, the mass spectrum of D-glucitol hexaacetate is representative of all peracetylated hexitols and that of

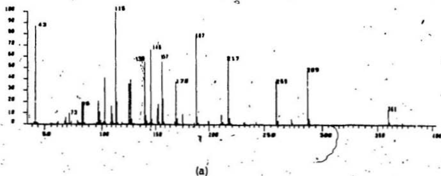
1,5-di-O-acetyl-2,3,4,6-tetra-O-methylglucitol is identical to any 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylhexitol (Figure 2.1.).

The fragmentation pattern simply consists of primary fragments resulting from α-cleavage of the carbon atoms in the alditol chain(70), the intensities of which decrease with increasing molecular weight. Secondary ions are obtained by loss of acetic acid (m/z 60), ketene (m/z 42), methanol (m/z 32), or acetamide (m/z 59) (see Figure 2.2.).

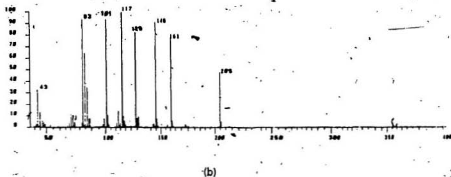
The fission between carbon atoms is governed by the stability of the resulting radical, the methoxylated radicals being more stable than acetoxylated radicals. Preference of bond cleavage decreases in the following order with no observable cleavage between two carbon atoms if either one is deoxygenated(71).



The primary fragmentation of amino alditol acetates is largely governed by the acetamido groups(43,56-60,72). The secondary fragments are formed as previously described (Figure 2.3.). Partially methylated amino alditol acetates show fission almost exclusively between the methylacetamido group and the adjacent methoxy or acetoxy



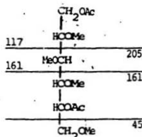
(a)



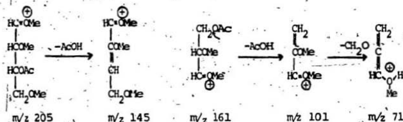
(b)

Figure 2.1. Mass spectra of (a) hexitol hexaacetate, and (b) 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylhexitol(54).

Primary fragmentation:



Secondary fragmentation:



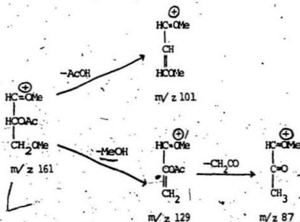
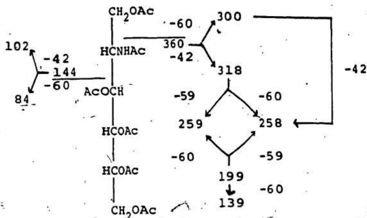
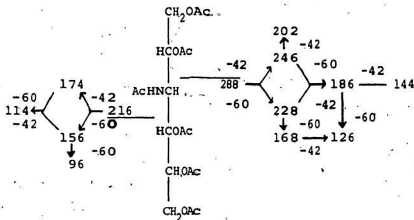


Figure 2.2. Fragmentation patterns of alditol derivatives.



(a)



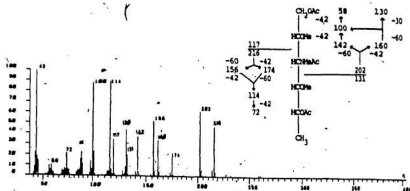
(b)

Figure 2.3. Fragmentation patterns of (a) 2-acetamido-2-deoxyhexitol pentaacetate and (b) 3-acetamido-3-deoxyhexitol pentaacetate.

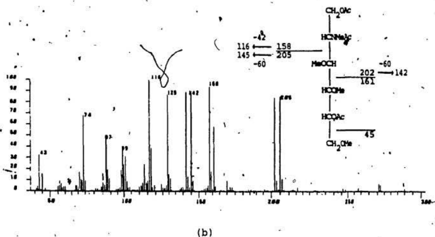
groups(55,58,60). For the amino sugars; 2-acetamido-2-deoxy-hexoses, 2-acetamido-2,4-dideoxy-pentoses and 2-acetamido-2,6-dideoxy-hexoses, the primary fragment (m/z 158) is formed by fission between C-2–C-3 which yields the secondary fragment (m/z 116) (formed by loss of Ketene). The latter fragment being stronger than the usual base peak(60,70) CH_3–C=O (m/z 43) (Figure 2.4.). Similarly, the same situation arises with 3-amino-3-deoxy sugars(73) but the fragmentation pattern is governed by the fission between C-2–C-3–C-4. Thus g.l.c.-m.s. is an invaluable tool in the analysis of amino sugars and methylated derivatives.

Labelling studies with deuterium to determine the reduction result in derivatives where the masses are shifted by one unit when compared with the undeuterated derivative as shown by g.l.c.-m.s. Reduction of uronic acids with deuterides followed by g.l.c.-m.s. analysis is useful in distinguishing the acidic sugar from the existing neutral sugars.

The identification of aldonitriles and methylated aldonitriles by g.l.c.-m.s.(74) is useful in the determinations of the degree of polymerization of oligosaccharides. The 2-amino-2-deoxyhexoses having the gluco-configuration at C-2 (e.g. glucosamine and galactosamine) form 2,5-anhydrohexitols upon deamination(75). G.l.c.-m.s. of the methylated anhydro



(a)



(b)

Figure 2.4. Mass spectra and fragmentation patterns of (a) 1,5-di-O-acetyl-3,6-dideoxy-3-N-methylacetamido-di-2,4-O-methylhexitol and (b) 1,5-di-O-acetyl-2-deoxy-2-N-methylacetamido-3,4,6-tri-O-methyl-glucitol(59).

alditols(76) is useful in further confirmation of the linkage position of the original amino sugar.

2.4. SEQUENCE OF SUGARS

2.4.1. Partial hydrolysis

Isolation of oligosaccharides generated by partial hydrolysis is a major key to the elucidation of the sequence of sugars in a polysaccharide. The method exploits the acid lability of some glycosidic linkages over others which are more resistant to hydrolysis. The rate of hydrolysis is affected by several factors which include the ring size, configuration, conformation, position of linkages, the polarity of the sugar as well as the size and polarity of the aglycone(77). Hence it is difficult to single out one factor in order to explain observed differences in hydrolysis rates. The following generalization can be made for broad classes of sugars:

(i) furanosides are more labile than pyranosides.

(ii) deoxy sugars and pentapyranosides are more easily hydrolyzed than hexopyranosides.

(iii) α-glycosides are generally more labile than β-glycosides.

(iv) 1→6 glycosidic linkages are more resistant to acid hydrolysis than are 1→4 and 1→2, with 1→3 linkages being

the most easily hydrolyzed. Terminal non-reducing sugars and those in side chains are more easily cleaved than in-chain glycosidic bonds.

(v) uronic acids and 2-amino-2-deoxy glycosides need strong conditions to effect hydrolysis.

(vi) 2-acetamido-2-deoxy glycosides are easily hydrolyzed if the hydrolytic conditions prevent formation of the acid resistant 2-amino-2-deoxy derivatives by N-deacetylation.

In some instances, hydrolysis under non-aqueous conditions is necessary to preserve certain linkages or to avoid degradation of more labile sugars. Normal conditions of hydrolysis degrade 3,6-anhydrohexoses, sialic acid and KDO. Similarly neuraminidase can also be used for this purpose. In such cases methanolysis, acetolysis, trifluoroacetolysis, or mercaptolysis is favoured. N-acetyl hexosamine-containing oligosaccharides and glycoconjugates are degraded by trans-amidation during trifluoroacetolysis(78). The degradation occurs by a process in which the trifluoroacetyl groups stabilize the glycosidic linkages of the sugar residues, and subsequently cleave the peptide (amide) bonds(79). Anhydrous hydrogen fluoride is capable of cleaving glycosidic linkages of amino, neutral and acidic sugars without degradation of the sugars(80). This reagent is useful in quantitative analysis of polysaccharides and in cleavage of sugars from glycoproteins leaving the peptide moiety intact(80).

2.4.2. Periodate oxidation and Smith degradation

Oxidative cleavage of 1,2-diol groups(81) by sodium metaperiodate(82) or lead tetraacetate(83) is of analytical importance in structure determinations of polysaccharides. Oxidations are usually carried out in aqueous media with the water soluble metaperiodate ion, lead tetraacetate in acetic acid being used only in instances where the polysaccharide is insoluble in water.

Periodate oxidation is a quantitative reaction; each 1,2-diol consumes one mole of periodate and is oxidized to an aldehyde by cleavage of the carbon bonds. 1,2,3-triols liberate formic acid(84) by double cleavage of the carbon chain, and exocyclic diols produce formaldehyde(85), the products being analysed by titration and colorimetry, respectively. The reduction of the periodate ion to iodate can be monitored by titration or spectrophotometrically(84). Substituted rings are oxidized in various ways. The "polyaldehyde" produced may then be reduced with sodium borohydride into the polyol and hydrolyzed. The products of such a series of reactions can lead to information on the original substitution pattern (Figure 2,5.).

The rate of oxidation varies according to the configuration of the glycols. It is generally observed that open chain glycols are oxidized at a faster rate than cyclic cis-glycols. Cyclic trans-glycols are oxidized

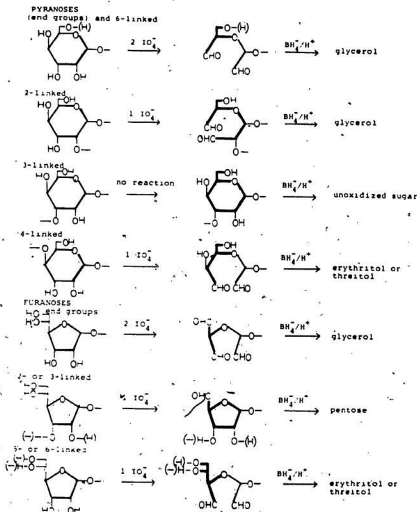


Figure 2.5. **Common products formed by terminal and mono substituted sugars on periodate oxidation followed by borohydride reduction and hydrolysis(84).**

more slowly or not at all if fixed in an unfavourable conformation as in some bicyclic anhydrohexoses(82).

Sugars containing free amino groups are oxidized if a 1,2-relationship between the free amine and a free hydroxyl group exists. An acetyl group on the amino or hydroxyl function prevents its oxidation. Hence a 2-acetamido-2-deoxy-glucopyranosyl unit is oxidized only if it is terminal in a side chain, or linked at position- 6; in both cases the 3,4-diol is the active participant. Under-oxidations involving amino sugars results from hydrogen bonding between the acetamido group and an oxidizable hydroxyl group on the neighbouring sugar residue(86,87) (Figure 2.6.).

The most important application of the periodate oxidation is in the generation of oligosaccharide fragments which can lead to conclusive structural evidence in a polysaccharide. The Smith degradation(88) involves mild acid hydrolysis of the polyol at room temperature wherein the acyclic acetals are cleaved in preference to the more resistant glycosidic linkages. A typical sequence of periodate oxidation and Smith hydrolysis is shown in Figure 2.7.

2.4.3. Deamination of amino sugars

Free amino groups in sugars can be deaminated by

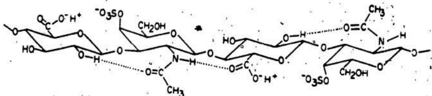


Figure 2.6. Proposed scheme for inter-residue hydrogen bonding between acetamido groups and oxidizable hydroxyl groups preventing the oxidation of the 4-linked β-glucuronic acid(86).

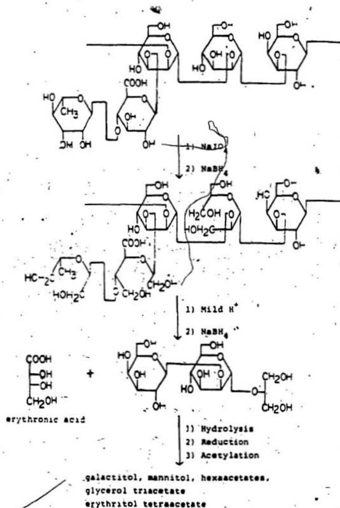
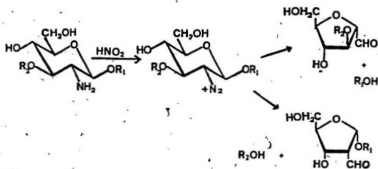


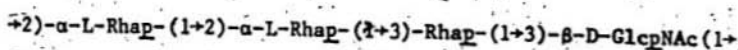
Figure 2.7. Typical sequence of periodate oxidation and Smith degradation(88).

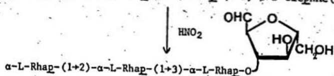
nitrous acid to form various derivatives. Since most polysaccharides contain N-acetyl groups, N-deacetylation is essential prior to deamination. Hydrazinolysis with anhydrous reagent in the presence of a catalytic amount of hydrazine sulfate(89), or treatment with sodium hydroxide in aqueous DMSO with thiophenol as an oxygen scavenger(90) results in removal of the acetamido function.

Depending on the position of the amino group and its configuration, the deamination process follows a basic pattern. Equatorial amino functions at position 2 of pyranoses (e.g. glucosamine) result in the formation of 2,5-anhydrohexoses. Thus glucosamine is converted into 2,5-anhydromannose(75) and galactosamine into 2,5-anhydrotalose(91). These occur as the major products resulting from the ring contraction which involves the O-C-1 bond migration, inversion of configuration at C-2, and subsequent cleavage of the glycosidic linkage. A small amount of 2-C-formyl pentafuranoside is formed by an alternate ring contraction involving the participation of the C-3 - C-4 bond as shown in Figure 2.8. Axial amino functions at position 2 (e.g. mannosamine) deaminate by a simple mechanism with replacement by OH. This results in inversion at C-2 without a change in ring size or cleavage of glycosidic linkages(91).

Hexoses containing equatorial amino functions at position 3 deaminate to form two C-formyl pentafuranosides







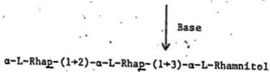


Figure 2.8. Deamination sequences of some amino sugars.(75)

without rupture of any glycosidic linkages (Figure 2.8.). The major product is formed by the migration of the C-4-C-5 bond. The furanoside from the involvement of the C-1 - C-2 bond is produced in small yield to lower nucleophilicity of C-1 which is bonded to two oxygen atoms(92). However, there have been several unsuccessful attempts to deaminate 3-amino-3,6-dideoxy-D-mannose(93).

2.5. DETERMINATION OF THE ANOMERIC CONFIGURATION OF LINKAGES

2.5.1. Nuclear magnetic resonance spectroscopy

The use of proton and carbon-13 n.m.r. spectroscopy is a valuable tool in the determination of the configuration of unknown carbohydrates and for ascertaining the conformations of known sugars in solutions. This technique can be used in polysaccharide analysis only if regular repeating units exist, making a uniform, homogenous polymer. Random structures result in broad lines with less information and poor resolution. Thus the sharp signals in the spectra obtained for bacterial polysaccharides serve as proof for the regular repeating-units present within them.

2.5.1.a. ^{1}H-n.m.r. spectroscopy

Proton magnetic resonance (p.m.r.) spectroscopy is a firmly established, widely used technique for the structural, configurational, and conformational analysis of carbohydrates and their derivatives. Since the first application of p.m.r. to carbohydrates(94) almost three decades ago, rapid progress has been made in instrumentation and techniques used. The introduction of magnets based on superconducting solenoids have increased the field strength available, thereby enhancing the resolution of the spectra (notably for polysaccharides). Fourier-transform techniques are important in enhancement of sensitivity(95,96) especially in averaging signals from dilute sample or from nucleii of low natural abundance (e.g. ^{13}C, ^{15}N). Other recently evolved methods include the nuclear Overhauser effect (n.O.e.), internuclear double- and triple-resonance techniques and many others. Many of these methods have limited applications in polysaccharide analysis due to the complexity of the spectra. High field ^{1}H-n.m.r. spectroscopy is advantageous in conformational analysis of both polysaccharides(97) and glycoproteins(98). The use of two dimensional homo- and hetero-nuclear n.m.r. gives enhancement of resolution. These methods are valuable in obtaining coupling information and assigning chemical

shift values(99).

Interpretation of the n.m.r. spectrum requires measurement of various parameters.

(i) The relative areas of integrals of individual signals.

The number of protons resonating at each particular frequency is proportional to the area of the signal produced. Thus the integrals are indicative of the relative number of anomeric linkages, 6-deoxy sugars, and *N*- and *O*-acetyl group substituents present in a sample; they also indicate the quantitative amounts of reducing sugars including pyranose and furanose forms in mono- and oligo-saccharides.

(ii) Coupling constants

Nuclear spin-spin coupling constants over two chemical bonds are designated as $^{2}J(N_1,N_2)$, and over three bonds as $^{3}J(N_1,N_2)$. Couplings for vicinal protons are hence expressed as $^{3}J_{H_1,H_2}$. These are particularly useful in determining the configuration of the protons (mainly anomeric) because of the existing relationship between the vicinal coupling constant (J) and the dihedral angle (Φ) between the protons.

In a first order spectrum, the magnitude of the coupling constant can be determined directly from the spectrum. Large vicinal couplings ($\sim$8-10 Hz) indicate antiparallel protons (diaxial, J_{aa}), whereas small values

(~2-4 Hz) are typical of protons in the gauche form (diequatorial, J_{ee} and axial-equatorial, J_{ae}) (Figure 2.9.). The coupling constants of anomeric protons are thus useful in establishing the configuration (α,β) as well as the overall conformation (pyranose, furanose, chair/boat forms) of the sugars.

Geminal coupling of the C-6 protons ($^2J_{H_{6A},H_{6B}}$) in pyranose forms are usually not measured because the signal is hidden in the ring proton region. However the presence of a functional group (acetate, for example) could enable measurement of this coupling constant by causing a downfield shift of the signals. The coupling constants for geminal protons are usually much larger (> 8 Hz) than for vicinal protons and sometimes are of negative sign(100). They are more useful in determining conformations of pentoses in the pyranose form.

iii) Chemical shift

The Chemical shifts of protons depend on many factors. Substitution, orientation of the molecule, electronegativity effects of neighbouring and distant groupings, and the nature of the solvent can induce protons to resonate at different field strengths. A polysaccharide spectrum contains three major regions: a) the anomeric region (δ= 4.5-5.5); b) the ring proton region (δ= 3.0-4.5) and c) the high field region (δ= 1.0-2.5) (Figure 2.10.).

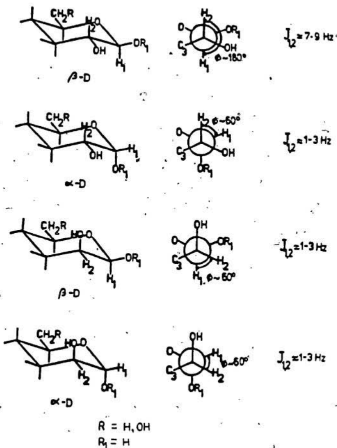


Figure 2.9. Relationship between dihedral angle (Φ) and coupling constants for α- and β-**D**-hexoses.

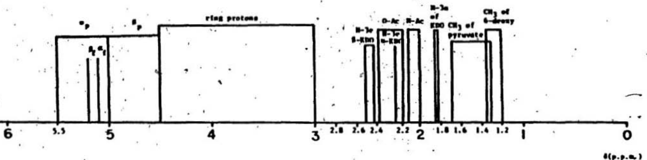


Figure 2.10. Schematic representation of different regions in the ^{1}H-n.m.r. spectrum of polysaccharides.(97)

The direct shielding effect caused by the ring-oxygen atom in carbohydrates accounts for the characteristic low-field shift of the anomeric protons. As a general rule, the axial ring protons resonate at a higher field than their equatorial counterparts. Hence for a pair of anomers in the pyranose form, the α-anomer has a lower chemical shift than the β-anomer (axial proton). The substituents at C-2 and C-3 have a large effect on the anomeric protons. Equatorial substituents at C-2 and C-3 yield an anomeric pair with a difference of 0.6 ppm in their chemical shifts (e.g. α-Glc δ= 5.23, β-Glc δ= 4.64). Axial substituent at C-2 yields an anomeric pair having a chemical shift difference of 0.2-0.3 ppm (e.g. α-Man δ= 5.10, β-Man δ= 4.90).

The chemical shift for the methyl group of the pyruvic acid acetal changes significantly with its orientation (axial or equatorial), and thus permits the determination of the configuration of the acetal carbon present on certain hexoses in some extracellular bacterial polysaccharides(101).

Line broadening of signals, and interference by exchangable protons (O-H and N-H) affect the quality of the high resolution ^{1}H-n.m.r. spectrum(97). The latter is minimized by the prior exchange of these protons with deuterium oxide (D_2O) and by the use of D_2O (preferably 99.95 atom %) as solvent. Nevertheless, a strong peak due

to residual water (HOD signal) is often obtained. The chemical shift of the HOD signal at room temperature ($\sim$4.8) interfere with the anomeric signals in this region. Elevating the temperature results in an upfield shift of the HOD signal ($\sim$4.3 at 90°) thereby exposing the valuable anomeric region. Fourier-transform (F.T.) techniques can also be used to minimize interference by the HOD signal.

2.5.1.b. ^{13}C-n.m.r. spectroscopy

The advent of Fourier-transform techniques has resulted in a steady increase in the use of ^{13}C-n.m.r. spectroscopy as a tool in the structure elucidation of polysaccharides(102). ^{13}C-n.m.r. is complementary to ^{1}H-n.m.r. spectroscopy, and has the ability to give better signal separation and thereby more information owing to the wider range of chemical shifts involved(103). This technique, which is rapid and non-destructive, has a great potential in the study of polysaccharides of biological origin where only small amounts of material are available for analysis.

The ^{13}C-n.m.r. spectrum also employs many of the same parameters used in the interpretation of p.m.r. spectra, a notable exception being the unsatisfactory integration caused by saturation phenomena and n.O.e. effects, which

result in loss of information of relative numbers of nuclei resonating at each chemical shift value(104). An important condition for obtaining correct integrals is a good signal to noise ratio (s/n). High field instruments, large sample tubes and increased concentrations will increase the s/n; but too high a concentration may lead to line broadening, which, in turn, has an adverse effect on the s/n(95).

Simple but well defined ^{13}C-n.m.r. spectra, which can be easily interpreted to yield a wealth of information, are obtained using proton decoupled conditions(104). However, single bond $^{13}C - ^{1}H$ coupling [$^{1}J_{C_1,H_1}$] is useful in differentiating anomeric pairs in the pyranose form, since the J values differ by approximately 10Hz(105). Identification of furanose forms by this procedure is unreliable due to the small difference ($\sim$2-3 Hz) between the coupling constants of the anomers(106).

The chemical shifts of individual, unsubstituted carbon atoms of poly- and oligo-saccharides show reasonable agreement with those obtained for previously assigned monosaccharides(107). In certain instances, the sensitivity of ^{13}C chemical shifts to effects of substitution renders this technique very useful in the determination of structures of unknown compounds and their linkages. Substitution by a glycosyl unit causes a large increase in the chemical shift of the carbon atom directly

involved in the linkage, with small changes (usually a decrease) in the chemical shifts of the neighbouring β-carbon atoms(103,107). A similar large shift occurs in the α-carbon atom on O-alkylation, with a much smaller shift occuring if the oxygen is acylated. Replacement of an oxygen by nitrogen (amino sugars) usually result in a large decrease (upfield shift) in the chemical shift of the carbon atom bonded to the nitrogen(102), thereby enabling its easy identification (Figure 2.11.). The enormous upfield shift in the α - carbon atom caused by substitution of an oxygen by a hydrogen atom reflects the influence of the changes in electronegativity brought about by substitution.

The potential of ^{13}C studies in conformational analysis depends on the sensitivity of the chemical shift towards conformational changes. Although many pairs of isomers give quite different signals for the anomeric carbon atoms, no general relationship has been discovered between the anomeric configuration and the chemical shift(102) of pyranoses. However, furanoses with trans-oriented substituents at C-1 and C-2 (e.g. β-galactofuranoside) show higher anomeric chemical shifts than the corresponding cis-isomer (α-galactofuranoside)(108). A change in the ring size from the six membered pyranose form to a five membered furanose ring results in a downfield shift of the anomeric signal. Glycosylation at

C-1 amounts to O-alkylation, and hence the anomeric carbon shows a corresponding substitutional shift to lower fields (usually ~ 3-8 ppm). Thus in a polysaccharide spectrum, the anomeric region (100 ± 8 ppm) is well separated from the ring carbon atom region (75 ± 5 ppm), with the primary hydroxy-methyl carbon atoms (at C-6) resonating away from the ring at a higher field (65 ± 5 ppm). Other characteristic chemical shift values are: carbonyl groups from uronic acid, pyruvic acid, N-and O-acetyl substituents at 175 ± 6 ppm; nitrogen containing (amino groups) ring carbon atoms at 48-55 ppm; methyl groups from O and N acetyl substituents at 20-28 ppm; methyl groups from pyruvate at 18-26 ppm; and methyl groups of 6-deoxy sugars at 15-17 ppm (Figure 2.11.).

The chemical shift values are also useful in the determination of the stereochemistry of acetal carbons in pyruvates since axial methyl groups resonate at ~18 ppm and equatorial groups at ~26 ppm(109). The carboxylic acid at C-1 of KDO(110) is sensitive to the configuration at the anomeric centre (C-2) and resonates at characteristic values corresponding to the α- or β-anomer. Thus the use of ^{13}C-n.m.r. in the structure elucidation of polysaccharides leads to valuable information on the nature of linkages and anomeric centres, the presence of functional groups, and the stereochemistry of certain substituents.

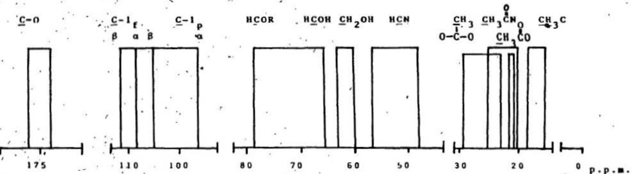


Figure 2.11. The characteristic regions for resonances of carbon atoms belonging to different monosaccharide residues in polysaccharides(97)

2.5.2. Chromium Trioxide Oxidation

Chromium trioxide in acetic acid can be used to oxidize ethers and acetals into esters without affecting acetates or other existing ester groups. Thus a fully acetylated glycopyranoside is oxidized into a 5-hexulosonate(111) (Figure 2.12.). The rate of oxidation of α-glycosides is considerably slower than that of the β -anomers, thus permitting the estimation of anomeric configuration by comparison with the original unoxidized sugars(112). The α-anomers would therefore survive, but the oxidized β-anomers are conspicuous by their absence on g.l.c.-analysis.

This supposition is valid for the α-pyranosides of the common hexoses, 6-deoxy-hexoses, 2-acetamido-2-deoxy-hexoses, 3-acetamido-3,6-dideoxy-hexoses and xylose. However substitution can change the conformational equilibrium of α-rhamnosyl and α-fucosyl residues, making them vulnerable to oxidation.

Methylation by the Hakomori procedure cleaves all ester linkages and methylates the exposed hydroxyl groups, thereby yielding structural information valuable for sequence analysis(113).

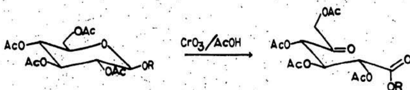


Figure 2.12. Chromium trioxide oxidation

3. SEROLOGICAL CROSS-REACTIONS OF ANTIGENS

It was in the early 1920's that Heidelberger, the pioneer in the immunological field, made use of the observation by Dochez and Avery(114), that a certain "soluble substance" capable of precipitating specific anti-pneumococcal sera of a homologous type was present whenever Pneumococci were grown in fluid media(115). This soluble substance was shown to be composed of carbohydrates and to be type specific(116) (having the ability to precipitate antibodies of its own type) (117).

The antibodies recognize certain immunodeterminant groups on the antigen. Therefore the shape of the antibody combining site must conform quite closely to the shape of the immunodeterminant group(118). The antigen specificity or ability to react with antibodies depends on the binding of the immunodeterminant group with the specific antibody. Serological cross-reactions occur when different antigens give precipitin reactions with the same antiserum on account of the similar nature of their immunodominant group. Thus immunochemical analyses of polysaccharide antigens, which combine serological and chemical studies, are important in defining the chemistry of the oligosaccharide structure of immunological significance within the polysaccharide(119).

The antigenic specificity of the polysaccharide or

the immunodeterminant group, is made up of several regions centering around a particular sugar unit. The immunodominant sugar is the major contributor to the serological specificity(5). These sugars may be terminal non-reducing or within the polysaccharide chain. Most branched polysaccharides possess immunodominant sugars in their side chains. The antigen specificity is not confined to the immunodominant sugar but extends along the polysaccharide chain. Hence, the nature of the sugar, its anomeric configuration, and even its position of linkage to the adjacent sugar are contributory factors. This is best illustrated in Figure 3.1. where the three sugars glucose, galactose, and mannose, despite having the same sequence, exhibit completely unrelated serological specificities owing to the differences in their linkages and anomeric configurations.

Studies on the O-antigens of Salmonella serotypes have shown that a polysaccharide may contain more than one antigenic determinant. Figure 3.2. shows the different antigenic determinants of Salmonella typhimurium, the specificities being denoted by their antigenic numbers.

Different and separable antibodies may combine with the same immunodominant sugar by reacting with it from different sites of the molecule. Hence sugar A in Figure 3.3. can combine with antibody I as well as antibody II, even though the antibodies have different specificities.

	Serological specificity (O factor)
Glc α 1→4; ^{3}Gal $\xrightarrow[\alpha]{1\ 2}$ Man $\xrightarrow[\beta]{1}$	12_2
Glc α 1→6; ^{3}Gal $\xrightarrow[\alpha]{1\ 2}$ Man $\xrightarrow[\alpha]{1}$	1_{12}
Glc α 1→4; ^{3}Gal $\xrightarrow[\beta]{1\ 6}$ Man $\xrightarrow[\alpha]{1}$	34

Figure 3.1. Chemical nature of the serological specificities expressed by O factors 12_2 and 1_{12} in *Salmonella typhi* and *S. typhimurium* and by O factor 34 in *S. illinois*(118).

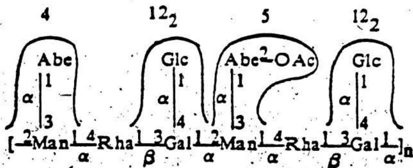


Figure 3.2. Illustration of the different antigenic determinants in the LPS of S. typhimurium(28). Key: Abequose; Abe, Mannose; Man, Rhamnose; Rha, Glucose; Glc, Galactose; Gal.

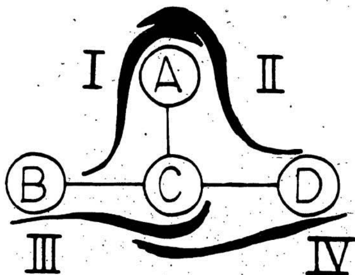


Figure 3.3. Various regions on a tetrasaccharide (determinants) possibly reacting with different antibodies(118). A,B,C, and D are different sugar residues possessing antigenic determinants. Hence, sugar A can combine with antibody I as well as antibody II even though the antibodies have different specificities. Therefore, different and separable antibodies may combine with the same immunodominant sugar by reacting with it from different sites of the molecule.

Thus cross-reactions are not always indicators of the presence of identical sugars or structural features in a polysaccharide as is illustrated by the above two examples. However, cross-reactions can be important indicators when used with antisera of known antigenic structural specificities.

3.1. IMMUNOLOGICAL SIGNIFICANCE OF ANTIGENS

The structural elucidation of the polysaccharides has been embarked upon in order to clarify two important issues: what makes a polysaccharide immunogenic and what is the chemical basis of its antigenicity (serological specificity)? Cross-reactions are useful in understanding the antigenic specificity. Knowing the chemistry of the structure alone is obviously not enough to solve such a highly complex biological process, but structural investigation must be carried out in the attempt to understand this phenomenon.

Immune responses produced by an immunogen fall into two classes, humoral and cell mediated. The former category is where the response can be transferred from one animal to another via the serum containing the antibody, while the latter class needs sensitized cells and not serum for transfer of the immune response. T lymphoctyes (thymus derived cells) are found to be responsible for the

cell mediated response while B lymphocytes (bone marrow derived), responsible for antibody formation, are involved in humoral responses. The antibodies secreted by B cells are protein molecules (immunoglobins, Ig). When T cells are involved in activating B cells into production of antibodies (IgM and IgG) the immune response is termed T cell dependent and results in a retention of immunological memory. The antigens involved in T cell dependent responses are capable of producing an even larger immune response on subsequent interaction due to the memory retained by the T cells following the first encounter with the antigens (T cell independent responses), where B cells secrete immunoglobins (IgM) without T lymphocyte cooperation, lack the retention of memory. Polysaccharide antigens usually stimulate T cell independent responses while protein antigens are T cell dependent response stimulators(120). It should be noted however that this is a simplified description of the complex functions occuring in an immune response; T cells have many other functions in addition to those mentioned above.

The virulence of bacteria depends on their ability to survive and propagate within the host by evading the host's immune system. The direct interaction of their surface antigens with the host immune system is important in bacterial pathogenesis and in the stimulation of an immune response. The capsular nature of the antigens is

useful in protecting the bacteria from phagocytosis.

Consideration of the various fish diseases caused by Gram-negative bacteria, together with the emergence of their widespread multiple antibiotic resistance, has given rise to a renewed interest in the prevention of these diseases by immunization (Table 3.1.)

Table 3.1. Diseases caused by bacteria

Bacterial Species	Diseases
Aeromonas salmonicida	Furunculosis
KD bacteruim	Bacterial Kidney Disease
RM bacterium (*Yersinia ruckeri*)	Enteric Redmouth Disease
Vibro angillaruim	Vibrosis
Pseudomonas sp	Pseudomonad Septicemia
Motile *Aeromonas* sp.	Motile Aeromonad Septicemia
Myxosoma cerebralis	Myxobacterial infections.

For reference see Rucker(121).

PART II

Elucidation of the Molecular Structure of the Lipopolysaccharide of *Yersinia ruckeri*

1. INTRODUCTION

Fish disease caused by the different bacterial species of the Vibrionaceae family has had a major influence on the success or failure of many fish culture operations, both public and private. In the past, fish disease problems have been handled primarily by chemical or antibiotic treatments. In some cases effective management of stocks has proven beneficial. The emergence of drug resistance in bacterial fish pathogens, as well as concern that indiscriminate use of antibiotics might transfer drug resistance factors to human pathogens, has necessitated the search for other disease prevention techniques. Vaccination is a logical alternative. The method is effective in higher animals and, in addition, is prophylactic rather than therapeutic. The problems faced in fish vaccine development have consisted primarily of developing efficacious preparations administered to large populations in an economic manner.

Red-mouth disease in rainbow trout has been known for over twenty years(121). The syndrome can be produced by certain Aeromonads and Pseudomonads, as well as by Yersinia ruckeri(121-123), which will be discussed in this thesis. The disease is systemic, and its major gross characteristic is inflammation in the areas of the mouth

and throat. It is also known as pink mouth and pink or red throat. Clinical and pathological aspects of the disease have been reviewed by others(122,123). The disease is enzootic in some private and federal hatcheries and has become epizootic on occasion. As an epizootic in hatcheries, the disease constitutes an important economic problem. An outbreak of redmouth disease, in which the RM bacterium was incriminated, has been reported in Saskatchewan, Canada in 1973.(123)

Although there appear to be at least two serological varieties of the RM bacterium, it is the serovar I or Hagerman strains which are associated with almost all occurrences of the disease(124,125). McCarthy and Johnson(125) suggested that the lower virulence of the serovar II or Oregon strains makes them of little concern with respect to inducing effective protection. However, the regulations of many fish health protection programmes recognize *Y. ruckeri* as a certifiable pathogen, with no discrimination being made on the basis of the relative virulence of different strains or serological varieties.

1.1. BACTERIAL CHARACTERISTICS OF *YERSINIA RUCKERI*

In 1966, Ross *et al*(122) gave a description of a Gram-negative, rod-shaped, oxidase-negative, peritrichous, fermentative bacterium that was isolated on numerous

occasions from kidney tissues of rainbow trout (Salmo gairdneri) afflicted with redmouth disease. It was concluded that the redmouth (RM) bacterium, Yersinia ruckeri, was a member of the family Enterobacteriaceae, but at that time it was not possible to determine its taxonomic position within the family.

The Gram-negative bacterium Yersinia ruckeri, formally called enteric redmouth bacterium causes disease in salmon and trout as mentioned previously. However, this organism has not been known to ever infect humans. Y. ruckeri grows best at 22-23°C; some strains will not grow at 37°. Y. ruckeri could have been included as a species in either the genera Yersinia or the genera Serratia of the Enterobacteriaceae family due to the data concerning their biochemistry and DNA hybridization. It is 25-30% related to species from both genera. Y. ruckeri DNA contains about 48% guanine and cytosine. DNA from Yersinia contains 47-49% guanine and cytosine whereas DNA from the Serratia species contains 56-59% guanine and cytosine. Genetically therefore, Y. ruckeri is closer to Yersinia than to Serratia.

1.2. CHEMICAL ELICIDATION OF THE STRUCTURE OF THE CELL-SURFACE POLYSACCHARIDE OF YERSINIA RUCKERI. AN OVERVIEW

The lipopolysaccharide antigens from the Vibrionaceae family have been studied extensively with the hope of understanding their pathogenicity in fish and the chemical structure elucidations of approximately 10-15 species of Vibronaceae have been completed. Unlike the simple sugars and structures found in Klebsiella, E. coli and Salmonella, the lipopolysaccharide antigens of the Vibrionaceae contain diverse sugars and are of varying degrees of complexity(126-130).

The precise molecular structure of the Gram-negative bacterium Yersinia ruckeri, which causes the redmouth fish disease, has not yet been investigated. Interest in the chemical structure and immunological properties of the cell-surface antigens of this species has increased, as little is presently known on the biochemical basis of pathogenicity of Yersinia ruckeri.

This thesis presents the results of the chemical and structural studies carried out on the two distinct regions of the lipopolysaccharide of Y. ruckeri, namely the O-specific antigen and the core oligosaccharide. The O-specific antigen, when coupled to either of the protein carriers bovine serum albumin (B.S.A.), or chicken serum

albumin (C.S.A.) may be used for the induction of antibodies, as well as the production of semi-synthetic vaccines.

The understanding of the chemical composition and molecular structure of the cell-surface lipopolysaccharide of *Y. ruckeri* may provide better understanding of the host-parasite interactions and may define the receptor sites and the immunodeterminant portion of the carbohydrate antigen.

2. METHODS AND MATERIALS

2.1. BACTERIAL CULTURES

Y. ruckeri strain RS3 (II) isolated from Chinook salmon was supplied by Dr. Roselyn Stevenson of Guelph University. This strain was added to the collection of the Northwest Atlantic Fisheries Centre, St. John's as SJ-103.

The organisms were initially plated on Trypticase Soy Agar, (Baltimore Biological Laboratory), to check purity and colony morphology. Stock cultures were grown in Trypticase Soy Broth (T.S.B.) without dextrose, divided into 1 ml aliquots, made 15% with respect to glycerol, and frozen and stored frozen at -80°C. The protocol for medium scale culture of the bacteria was generally identical for each strain. T.S.B. (100 ml) was inoculated with the contents of a frozen vial, grown for 24 hours at room temperature with reciprocating shaking, and used as the inoculum for 24 litre of the same medium. This culture was grown in a 25 litre commercial fermentor (New Brunswick Scientific Co.) for 26 hours 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 hours.

The cells were collected by continuous centrifugation (Sorvall RC-2B high-speed centrifuge) and freeze-dried; the cells were not washed.

2.2. CHROMATOGRAPHIC METHODS

2.2.1. Thin layer chromatography

Thin layer chromatography was carried out on precoated cellulose (100 gm) plates (E. Merck, Darmstadt) as well as on Baker-flex silica gel 1B2 7.5x2.5 cm or aluminum silica gel sheets 20x20 cm (Baker) with a solvent system 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(131).

2.2.2. Gas-liquid chromatography and g.l.c.-m.s. spectroscopy

Analytical gas-liquid chromatography separations of the acetylated alditols and partially methylated alditols were performed using a Perkin Elmer model 8310 gas chromatograph fitted with flame ionization detectors. Analyses were carried out on packed columns (183cm x 2mm i.d.) of 1.5% Silar 7CP on Gas Chrom Q (100-120 mesh) and on open tubular column of DB-225 and Megabore SP-2330 (30m

x 0.75mm i.d.). Temperature profiles varied as follows according to the type of derivative being analysed;

(a) 1.5% Silar 7CP on Gas Chrom Q (100-120 mesh) operated isothermally at 215°C for alditol acetates.

(b) 1.5% silar 7CP on Gas Chrom Q (100-120 mesh) programmed with a starting temperature of 170°C for 20 minutes and then increasing to 250°C at 7°C/min. where it is held for 20 minutes for partially methylated alditols.

(c) Megabore DB-225 isothermally at 210°C for alditol acetates and isothermally at 195°C for partially methylated alditols.

(d) Megabore SP-2330 programmed with an initial temperature of 180°C for 10 minutes and increasing at 2°C/min until 220°C where it is held for 7 minutes for alditol acetates.

(e) Megabore SP-2330 programmed at 165°C initially for 10 minutes followed by a ramp rate of 2°C/min until 240°C where it was held for 15 minutes for partially methlated alditols.

For the packed column, helium was used as a carrier gas at a flow rate of 40 ml/min. In the case of the open tubular columns, helium was under a pressure of approximately 200 kpa.

Combined gas-liquid chromatography-mass spectroscopy analyses were performed on a Hewlett Packard model 5980A GC/MS controlled by a 5934A data system, with a membrane

separator, a source temperature of 160°C and an ionizing voltage of 70 e.v. The gas chromatographic conditions (profiles a-e) as described above were used.

2.3. GEL-PERMEATION AND ION-EXCHANGE CHROMATOGRAPHY

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 (Water Associates Model R403). The k_{av} values are defined as (elution vol. of peak)-(void vol. of column)/(packed bed vol. - void vol. of column). The fractions were collected and freeze-dried.

Ion-exchange chromatography for deionizations of neutral and amino-sugars were carried out with Amberlite IR-120 (H^+) resin. The neutral sugars were eluted with water whereas the amino-sugars were eluted with 1-5% hydrochloric acid.

2.4. NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

Polysaccharides were repeatedly treated with D_2O (99.8%), with lyophilization between treatments and finally dissolved in D_2O (99.99%). ^{1}H-NMR Spectra were recorded using a Bruker AM-500 MHz in the pulsed Fourier

transform mode at 343K, the HOD signal being suppressed by selective saturation using an inversion recovery pulse sequence. Chemical shifts are quoted in ppm downfield from external 2,2,3,3-tetradeutero-4,4-dimethyl-silapentanoate, but were actually measured by reference to internal acetone (δ = 2.225 ppm).

^{13}C-NMR spectra were measured in D_2O with a Varian CFT-20 spectrometer operating at 20 MHz in the pulse Fourier transform mode with complete proton decoupling. Chemical shifts are reported in ppm downfield from external tetramethylsilane (TMS). Proton-coupled and-decoupled ^{13}C-NMR spectra were also recorded at 125 MHz on a Bruker AM-500 MHz in D_2O at 310°K.

2.5. PURIFICATION OF THE LIPOPOLYSACCHARIDE

The freeze-dried cells were extracted by the phenol-water method of Westphal and Jann(19) with two wash cycles of the phenol layer. The combined aqueous layers were dialysed against tap water for 72 hours and the dialysate was centrifuged at 5000 rpm for 20 minutes to remove cellular and precipitated debris, prior to ultra centrifugation of the clear supernatant at 39,000 rpm for 16 hours. The resulting gel was redissolved in water, recentrifuged at 39,000 rpm (twice), and freeze-dried to give (usually) a purified lipopolysaccharide free of

of 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, indicating the lack of ribonucleic acid.

2.6. ISOLATION OF THE O-SPECIFIC ANTIGEN AND CORE OLIGOSACCHARIDE UNITS

The pure lipopolysaccharide (100 mg) was hydrolysed with dilute acetic acid (1%) for 2 hours at 100°C. The released carbohydrate portion remained in solution, while the precipitated lipid A was removed by centrifugation (5000 rpm, 30 min x 3). The combined 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 O-specific antigen was defined as the first peak to be eluted, whereas the core oligosaccharide was defined as the peak immediately preceeding the salt peak.

2.7. HYDROLYSIS AND SUGAR ANALYSIS

The O-specific antigen or core oligosaccharide (0.5-1

mg) resulting from fractionation on Sephadex G-50, was hydrolyzed with 1 M trifluoroacetic acid for 18 hours at 100°C, or with anhydrous hydrogen fluoride (8 hours, 0°C), followed by removal of the hydrogen fluoride in vacuo, to afford a residue which was dissolved in water and neutralized with 0.01 M ammonium hydroxide. Sugar analyses were also performed with 48% hydrogen fluoride (12 hrs, 0°C) followed by neutralization with 0.01 M lithium hydroxide. The hydrolyzate containing the monosaccharide was dissolved in water (0.5-1 ml): $NaBH_4$ (1-2 mg) was added to the solution which was left at room temperature for 1 hour. The excess borohydride was destroyed with a few drops of 50% acetic acid, evaporated to dryness, and coevaporated (3 times) with a solution of methanol/acetic acid (4:1) in order to destroy the carbohydrate-borate complex completely. The reduced products were acetylated with pyridine-acetic anhydride 1:1 (2 ml) at 100°C for 1 hour. The excess pyridine and acetic anhydride were removed with successive evaporations with methanol and chloroform. The resulting alditol acetate derivative was subjected to analysis by g.l.c. and g.l.c.-m.s.

2.8. METHYLATION ANALYSIS

All compounds were methylated by the method of

Hakomori(50) as follows: the O-specific polysaccharide (~2 mg) was dissolved in 0.5 ml of dry and distilled dimethyl sulfoxide and placed in a small serum vial fitted with a teflon silicone septum and a stirrer bar. The flask and contents were flushed with dry argon, and methyl sulfinyl anion (0.5 ml of a 2M solution) was added; the reaction proceeded with stirring for approximately 1 hour. Methyl iodide (0.5 ml) was then added while the reaction was kept at 4°C in an ice bath for 4 hours. The methylated polysaccharide was then purified by passage through a small column of Sephadex LH-20 suspended in chloroform followed by elution with the same solvent. The methylated polysaccharide in the fractions was detected by charring with 10% H_2SO_4 in ethanol (95%). The purified methylated polysaccharide was then hydrolysed either with 1 M trifluoroacetic acid (8 hours, 100°C) or anhydrous hydrogen fluoride (4 hours, 0°C).

The resulting partially methylated sugars were reduced, acetylated and analysed as alditol acetate derivatives by g.l.c. and g.l.c.-m.s.

2.8.1. Preparation of methyl sulfinyl anion

Sodium hydride (300 mg) dispersed in oil was washed three times with benzene (6 ml) in order to remove the excess oil. The washed sodium hydride is placed in a

small serum vial containing a stirrer bar and is dissolved in dimethyl sulfoxide (6 ml). The vial was fitted with a teflon-faced silicone septum and the contents were flushed with argon. The solution was heated at 50-60°C for 1 hour.

2.9. SMITH DEGRADATION

The O-specific antigen (50 mg) was dissolved in distilled water (10 ml) and 0.1 M sodium metaperiodate (10 ml) was added(132). The solution was kept in the dark at 5°C. After 72 hours, $BaCO_3$ (25 mg) was added and the suspension was stirred overnight. After filtration, the solution was borohydride reduced and neutralized. The resulting polyol was purified by column chromatography on Sephadex G-15 and analysed for the component sugars.

The polyol (20 mg) obtained from the periodate oxidation was hydrolysed with 1% acetic acid for 3 hours at 100°C(133). After evaporation of the acid, the residue was reduced with sodium borohydride and purified by column chromatography on Sephadex G-15. The purified Smith degraded product was subjected to sugar and methylation analyses and the resultant alditol acetates and partially methylated alditol acetates were identified by g.l.c. and g.l.c.-m.s.

2.10. N-DEACETYLATION OF O-SPECIFIC POLYSACCHARIDE

The O-specific polysaccharide (15-20 mg) was dissolved in water (0.5 ml) and dimethyl sulfoxide (2.5 ml), sodium hydroxide (200 mg) and a drop of thiophenol were added. The mixture was stirred in a stoppered vial for 15 hours at 80°C. The excess base was neutralized with 2M hydrochloric acid and the inorganic salts and the reagents were removed by dialysis against water (for 72 hours in a bag with molecular cutoff of 1000 daltons). The resulting solution was centrifuged and the supernatant was freeze-dried.

2.11. NITROUS ACID DEAMINATION OF O-SPECIFIC POLYSACCHARIDE

The O-specific polysaccharide (10 mg) in water (1 ml) was treated with 30% acetic acid (1 ml) and fresh 5% sodium nitrate solution (1 ml)(134), and it was kept at room temperature for 60 minutes, prior to passage through a column of Rexyn 101 (H^+). The deaminated polysaccharide was reduced with sodium borohydride, neutralized with acetic acid, and then purified by chromatography on Sephadex G-15. The purified, deaminated polysaccharide was methylated, hydrolysed and analysed by g.l.c.-m.s. as partially methylated alditol acetates.

2.12. PARTIAL HYDROLYSIS WITH 0.5 M SULFURIC ACID

The core oligosaccharide and O-specific antigen (10 mg) were partially hydrolysed with 0.5 M sulfuric acid for either 30, 60 or 90 minutes or 0.5 M trifluoroacetic acid (30, 60 and 90 minutes) and the solutions were respectively either neutralized with barium carbonate or evaporated. The degraded products were purified by chromatography on Sephadex G-15 and afforded a major fraction. This presumed degraded oligo- or polysaccharide was subjected to sugar and methylation analyses, and the resultant alditol acetates and methylated alditol acetates were identified by g.l.c. and g.l.c.-m.s.

2.13. LOCATION OF O-ACETYL GROUPS

2.13.1. Treatment with methyl vinyl ether

A dried sample of polysaccharide was dissolved in dimethyl sulfoxide, together with p-toluenesulfonic acid, sealed and flushed with N_2 and left stirring overnight. Methyl vinyl ether was introduced in two aliquots (3 ml each) at -60°, with stirring for 4 hours each time. The product was purified on a column of Sephadex LH-20 by elution with acetone.

2.13.2. Methylation of the methyl vinyl ether-protected derivatives.

The protected sample was dissolved in dimethyl sulfoxide and treated with dimsyl anion ($CH_3SOCH_2^-Na^+$) for 2 hours. Iodomethane was added to the cooled solution which was stirred for 1 hour and extracted with chloroform. The product was purified on Sephadex LH-20 with methanol as the eluent.

2.13.3. Removal of the vinyl ether protection

The methylated, protected product was dissolved in a mixture of acetone/1% sulfuric acid (2:3) and stirred at room temperature for 24 hours. The acid was neutralized with 2N sodium hydroxide, and the solution was evaporated to dryness, dissolved in dimethyl sulfoxide, filtered, and evaporated to yield the product.

2.14. OXIDATION WITH CHROMIUM TRIOXIDE

The polysaccharide (20 mg) was dissolved in dimethylformamide (1 ml) and acetic anhydride (1 ml) and pyridine (1 ml) were added(135). The solution was stirred for 16 hours at room temperature and extracted with chloroform. The residue was reacetylated with a mixture

of (1:1) acetic anhydride/pyridine (1 hour, 100°C) to afford the peracetylated product. The residue was dissolved in chloroform and _myo_-inositol hexaacetate (2 mg) was added as internal standard. The solution was divided into two parts and evaporated. A part was dissolved in glacial acetic acid (2 ml) and powdered chromium trioxide (40 ml) was added; the mixture was then agitated ultrasonically for 3 hours at 53°C, poured into water, extracted with chloroform (10 ml), the extract washed twice with water and evaporated to dryness. The oxidized polysaccharide and the remaining original unoxidized material, were subjected to sugar analysis. The oxidized product was also subjected to methylation analysis and the partially methylated alditol acetates g.l.c. and g.l.c.-m.s.

2.15. IMMUNOCHEMICAL STUDIES

2.15.1. Preparation of 1-(6-isothiocyanate)-hexane alkylamine derivative

The _O_-specific polysaccharide (82 moles) dissolved in 2.0 ml of water was added to a rapidly stirred mixture adjusted to pH 8.0 of 1,6-hexanediamine (8 mmoles) and 0.82 mmoles of sodium cyanoborohydride in 2.0 ml of water. The

reaction was allowed to proceed for 48 hours while maintaining constant pH by titration with 0.1M sodium hydroxide. The reaction mixture was then applied to a Bio-Gel P2 column (90 cm x 2.5 cm) and eluted with 0.1 M acetate buffer, pH 5.0. The Bio-Gel P2 column facilitated the separation of the alkylamine derivative from the starting material. Fractions were collected and assayed for carbohydrate and alkylamine by the phenol-sulfuric acid method (Dubois et al,) (136) and by the glucose-phosphoric acid reagent, respectively (Merck) (137).

Synthesis of the polysaccharide-1-(6-isothiocyanate)-hexane alkylamine derivative was performed essentially as described by McBroom et al(138). To 74 moles of the polysaccharide amine derivative, dissolved in 80% aqueous ethanol (2 ml), thiophosgene, (3.9 mmoles), was added dropwise while a constant pH of 7.0 was maintained by titration with 1M sodium hydroxide in 80% aqueous ethanol. When consumption of sodium hydroxide had ceased, the reaction mixture was freed of excess thiophosgene by repeated evaporation with methanol.

2.15.2. Coupling to bovine serum albumin

Saccharides were covalently linked to BSA by reacting their 1-(6-isothiocyanate)-hexane alkylamine derivatives with the free ε-aminolysyl groups of the protein. For

production of BSA conjugate (D.S.40), 177 moles of the polysaccharide-isothiocyanate derivative, dissolved in phosphate-buffered saline (4 ml, pH 9.0), was added to a rapidly stirred solution of 1 moles of BSA dissolved in the same buffer. Coupling was then allowed to proceed for 48 hours at room temperature, constant pH being maintained by titration with 10 mM sodium hydroxide. When coupling was complete, the resulting conjugates were extensively dialysed and purified by column chromatography.

2.15.3. Preparation of the partially deacylated Lps used for the hemagglutination

Lipopolysaccharide (6 mgs) were dissolved in 1 ml of 0.25 M NaOH and placed in a 56°C water bath for 60 minutes. The solution was cooled to room temperature and centrifuged at 2000 rpm for 15 minutes at 4°C. The pellet was discarded whereas the supernatant was neutralized with 1 N acetic acid and dialyzed against distilled water overnight followed by lyophilization.

2.15.4. Coating of the erythrocytes with either the lipopolysaccharide or the artificial glycoconjugate

A 5% solution of formalinized human type O red blood

cells was washed twice with phosphate-buffered saline, pH 7.2, followed by centrifugation in order to obtain a packed pellet of red blood cells and the supernatant was discarded. A sample of the packed red blood cells was added to a 250 g sample of the prepared lipopolysaccharide which has been dissolved in 10 mls of saline. For the coating of the artificial glycoconjugate (250 mg) the packed red blood cells were added directly to the saline solution with shaking (without incubation). The solution was incubated at 37°C for 30 minutes and then washed three times with phosphate-buffered saline. The coated red blood cells were suspended in 10 ml of phosphate-buffered saline (1:200).

2.15.5. Production of antisera

Antisera were prepared from albino rabbits weighing 2-3 Kg which were immunized intravenously without adjuvant by injection of the lipopolysaccharide (1.68 mg/ml) or glycoconjugate (1.68 mg/ml) solution by the Neter method(139) in the following manner;

Day 1	2 ml of 1/10 dilution	0.34 mg/ml
Day 2	2 ml of 1/10 dilution	0.34 mg/ml
Day 3	2 ml of 1/5 dilution	0.67 mg/ml
Day 4	2 ml of 1/5 dilution	0.67 mg/ml
Day 5	2 ml of 1/2 dilution	1.68 mg/ml

15 days following the final injection, the rabbits were boosted with 2 mls of a neat solution (i.e. 3.36 mg/2mls). On the 24th day, the rabbits were bled and the antiserum was isolated.

Antisera against whole *Yersinia ruckeri* bacteria was obtained by intravenous immunization with heat-killed bacteria ($1x10^{10}$ cells/dose) twice a week for 8 weeks followed by a booster injection 2 weeks later. Rabbits were bled approximately 1 week after the last injection.

2.15.6. Hemagglutination test

The antisera was heat inactivated at 56°C for 30 minutes. Sixteen tubes were prepared to which 0.2 ml of phosphate-buffered saline was added to each. This was followed by addition of 0.2 ml of antiserum to the first tube and the antiserum was serially diluted sequentially. 200 ml of the lipopolysaccharide-coated human type O red blood cells or the artificial glycoconjugate-coated human type O red blood cells were added to each tube and the solutions were incubated at 37°C for 30 minutes. The test was completed by centrifugation at 1000 rpm for 1 minute. The agglutination is read tentatively. The titer is the reciprocal of the highest dilution which gave visible agglutination.

2.15.7. Demonstration of antibody production

A 1% standard agar was plated out in standard diffusion plates. Small circular wells were cut around the circumference of a central well. Antiserum (0.1 ml) was placed in the central well, while serial dilutions of Lps were placed in each of the peripheral wells. The plates were incubated at room temperature for 24 to 48 hours in a humidity chamber.

2.15.8. Slide agglutination

An innoculating loopful of a 24 hour culture was suspended in a drop of phosphate-buffered saline (PBS) pH7. To the suspension one drop of antiserum was added, the liquid was rotated so that if antibodies were present, the cells would agglutinate.

3. STRUCTURAL INVESTIGATION ON THE O-SPECIFIC POLYSACCHARIDE OF YERSINA RUCKERI SJ-103

RESULTS

3.1. ISOLATION AND CHEMICAL ANALYSIS OF THE O-SPECIFIC POLYSACCHARIDE AND THE CORE-OLIGOLIGOSACCARIDE

24 l of cultured medium afforded 56 g of dried cells which gave 326 mg of lipopolysaccharide after extraction by the phenol/water method of Westphal and Jann(19) i.e. a yield of 0.58% based on dry weight of the bacteria. The purified lipopolysaccharide was free of ribonucleic and deoxyribonucleic acids, had a protein content of 2% and was not further purified.

Analytical studies on the Lps indicated that it contained phosphorous and 3-deoxy-D-manno-2-octulosonic acid KDO in the amounts shown in Table 3.1.

The lipopolysaccharide had an apparent monosaccharide composition after hydrolysis with 1 M trifluoroacetic acid (18 hours at 100°) of D-glucose,2-acetamido-2,6-dideoxy-D-glucose (D-quinovosamine), D-glycero-D-manno-heptose, L-glycero-D-manno-heptose and 2-acetamido-2-deoxy-D-glucose (D-glucosamine) in the approximate molar ratio of 1:1.5:0.5:2:2. (Table 3.2.).

TABLE 3.1. ANALYTICAL STUDIES ON SJ-103 LIPOPOLYSACCHARIDE, CORE-OLIGOSACCHARIDE AND O-SPECIFIC ANTIGEN

	KDO[a] %	Phosphorous[b] %	Hexosamine[c] %	Protein[d] %
SJ-103 Lps	6.61	5.70	14.3	3.5
SJ-103 O-specific antigen	0.02	-	8.4	ND
SJ-103 Core-oligosaccharide	3.12	0.41	6.7	1.2

[a] Thiobarbituric acid assay(140)

[b] Chen *et al* assay(141)

[c] Rondle Morgan assay(142)

[d] Lowry assay(143)

nd: not determined

TABLE 3.2. SUGAR ANALYSIS OF SJ-103 LIPOPOLYSACCHARIDE, CORE-OLIGOSACCHARIDE AND O-SPECIFIC ANTIGEN

	In mole percent			
Sugars as alditol acetates	Lps	Core oligosaccharide	O-specific antigen	
	a	a	a	b
D-Glc	12.24(1.0)[c]	8.80(1.0)	-	-
D-QuiNAc	19.33(1.5)	8.27(1.0)	42.87(2.0)	26.06(1.0)
D-D-Hep	5.63(0.5)	9.41(1.0)	-	-
L-D-Hep	24.11(2.0)	8.89(1.0)	-	-
D-GlcNAc	23.63(2.0)	18.48(2.0)	20.32(1.0)	28.14(1.0)
4,6-dideoxy-4-(N-diacetyl)-hexose	-	-	-	16.15(0.6)

a. Hydrolysis with 0.5M trifluoroacetic acid.

b. Hydrolysis with anhydrous hydrogen fluoride.

c. Value in parentheses are approximate relative molar ratios.

D-Glc = D-Glucose

D-QuiNAc = D-Quinovosamine

D-D-Hep = D-glycero-D-manno-Heptose

L-D-Hep = L-glycero-D-manno-Heptose

D-GlcNAc = D-Glucosamine

Mild acid hydrolysis of the lipopolysaccharide with 1% acetic acid followed by Sephadex Chromatography of the supernatant solution gave two carbohydrate-containing components corresponding to an O-specific polysaccharide with K_{av} value of 0.4 - 0.5 and a core-oligosaccharide with K_{av} value of 0.6 - 0.7. The amounts of these components averaged 72% and 11%, respectively, of the recovered carbohydrate.

Unlike the lipopolysaccharides of Aeromonas hydrophila chemotypes I-III(126,127,144) and Vibrio Ordali(129), the O-specific antigen of Yersinia ruckeri is the main constituent of the polysaccharide portion of the cleaved lipopolysaccharide.

The purified O-specific antigen was a white water-soluble powder that had a $[\alpha]_D^{23}$ + 42.5° (c. 0.24, water) and was free of uronic acid and fatty acid, when estimated by their respective assay methods (145,146).

During treatment of the O-specific polysaccharide with anhydrous liquid hydrogen fluoride (8 hours, 0°)(147) followed by purification on high-voltage paper electrophoresis, a positively charged unknown amino sugar migrating faster than 2-acetamido-2,6-dideoxy-D-glucose and 2-acetamido-2-deoxy-D-glucose was observed. This amino sugar did not survive acid hydrolysis with relatively mild acid such as 0.5 M trifluoroacetic acid (4 hours at 100°) and was totally degraded. This observation

may explain the absence of this unknown amino sugar in the sugar analysis of the lipopolysaccharide.

The unknown amino sugar gave on thin-layer chromatography (t.l.c.) a spot staining pink with ninhydrin with R_{GlcN}=1.4. This unknown amino sugar was also visualized by the Trevelyan reagent(148). Analytical assay(149) of this unknown amino sugar indicated that it may contain an N-acetyl group which was confirmed by mass spectral studies.

In effect, the identity of this unknown amino sugar has been established by mass spectra of its alditol acetate derivative using electron-impact and chemical ionization (methane) as 1,2,3,5-tetra-O-acetyl-4,6-dideoxy-4-(N-diacetyl)-Hexose (Figure 3.1.).

In the electron-impact mass spectrum of the alditol acetate of the 4-amino-4,6-dideoxy-4-(N-diacetyl)-hexose, the fragmentation pattern was governed by fission between C-2-C-3 and C-3-C-4 carbon atoms to afford respectively the primary fragment-ions at m/z 145 and 200 (Figure 3.1.a.). These ions were degraded to secondary fragment-ions by elimination of ketene (42 a.m.u.) and acetic acid (60 a.m.u.). Another important mode of fragmentation occurred by elimination of one molecule of acetic acid from the ion at m/z 344 (which occurs by fission between C-1 and C-2) to yield the secondary fragment-ion at m/z 284. The latter eliminates consecutively, one molecule of

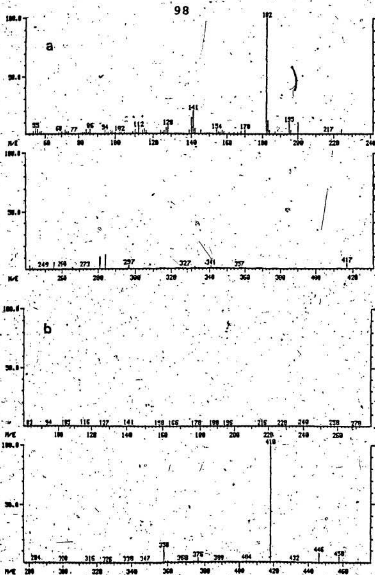


Figure 3.1. Mass spectrum of 1,2,3,5-tetra-O-acetyl-4,6-dideoxy-4-(N-diacetyl)-hexose, (a) Electron-impact and (b) chemical-ionization.

acetic acid and one molecule of ketene to yield the fragment-ion at m/z 182 (Figure 3.2.).

The chemical-ionization may spectrum of the alditol acetate of 4-amino-4,6-dideoxy-4-(N-diacetyl)-hexose showed the protonated molecular-ion $[M+H]^+$ at m/z 418 (base peak) and the secondary fragment-ion at m/z 358 generated by the loss of one molecule of acetic acid from the $[M+H]^+$ ion (Figure 3.1.b.).

The electron-impact and the chemical-ionization mass spectra and fragmentation pattern of the alditol acetate of 4-amino-4,6-dideoxy-4-(N-diacetyl)-hexose confirms the presence of the 4-N-diacetyl group on this unique 4,6-dideoxy-amino sugar.

Treatment of O-specific polysaccharide with 48% hydrogen fluoride (12 hours, 0°) followed by reduction with sodium borohydride and acetylation with pyridine/acetic anhydride (30 min, 100°) afforded (in addition to D-quinovosamine and D-glucosamine) an alditol acetate of a 4-acetamido-4,6-dideoxy-hexose which resulted from the N-deacetylation of the 4-amino-4,6-dideoxy-4-(N-diacetyl)-hexose.

The identity of this 4-acetamido-4,6-dideoxy-hexose residue was confirmed by electron-impact and chemical-ionization mass spectra which are shown in Figure 3.3.

The fragmentation pattern of this alditol acetate derivative was governed by fission between C-3–C-4 to

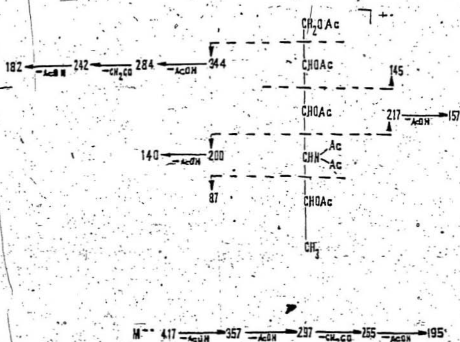


Figure 3.2. Fragmentation pattern of 1,2,3,5-tetra-*O*-acetyl-4,6-dideoxy-4-(*N*-diacetyl)-hexose.

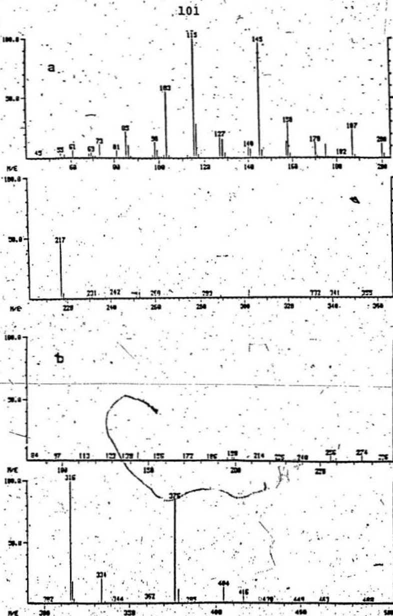


Figure 3.3. Mass spectrum of 1,2,3,5-tetra-O-acetyl-4-acetamido-4,6-dideoxy-hexose (a) Electron-impact and (b) Chemical ionization.

afford the primary fragmentation ions at m/z 217 and m/z 158. These ions were degraded to secondary fragment ions by elimination of acetic acid (60 a.m.u.) and ketene (42 a.m.u.). (Figure 3.4.).

The c.i.-m.s. of the peracetylated 4,6-dideoxy-4-acetamido-hexitol derivatives showed the protonated molecular ion $[M + H]^+$ at m/z 376 (base peak) and the secondary fragment ion at m/z 316 generated by the loss of 1 molecule of acetic acid from the protonated molecular ion. [Figure 3.3.(b)].

It is evident that 48% hydrogen fluoride has caused partial N-deacetylation in removal of one acetyl residue from the N-diacetyl group at C-4 as confirmed by E.I. and C.I. mass spectrometry.

Sugar analysis of the O-specific polysaccharide with the anhydrous hydrogen fluoride method indicated that it was apparently composed of D-glucosamine and 4-amino-4,6-dideoxy-4-(N-diacetyl)-hexose in the approximate molar proportions of 1:1:0.6 (Table 3.2.).

Due to the lack of known standards of this dideoxy-amino sugar series, the g.l.c. response factors of the alditol acetates and the partially methylated alditol acetates have not been calculated and therefore the molar ratios proposed for all sugar and methylation analyses are tentative.

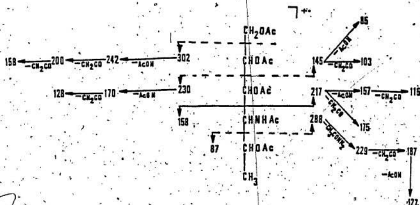


Figure 3.4. Fragmentation pattern of 1,2,3,5-tetra-O-acetyl-4-acetamido-4,6-dideoxy-hexose.

The identity of 2-acetamido-2,6-dideoxy-hexose and 2-acetamido-2-deoxy-hexose were established by experimental comparison of the g.l.c. retention time and mass spectrum with that of authentic samples together with their optical rotations.

The D-configuration of the 2-acetamido-2,6-dideoxy-glucose and 2-acetamido-2-deoxy-glucose was established by the optical rotation of the sugars isolated from the hydrolysate [D-GlcNAc; $[\alpha]_D^{23}$+60 → 40° (c. 0.02, water), D-QuiNAc; $[\alpha]_D^{23}$+56 → 11° (c. 0.01, water)] and by the g.l.c. of the trimethylsilylated (−)-2-butyl glycoside derivatives of the isolated mono-saccharide by the method of Gerwig et al(150). The configurations of the 4-amino-4,6-dideoxy-4-(N-diacetyl)-hexose and its 4-acetamido derivatives are still under study and have not yet been determined due to their extreme acid liability.

3.2. NUCLEAR MAGNETIC RESONANCE

3.2.1. ^{1}H-n.m.r.

The ^{1}H-n.m.r. (500 MHz) of the O-specific poly-saccharide showed inter alia, two anomeric signals at 5.02 (S, 1H, $J_{1,2}$ 0.2 Hz, H-1) and at 4.90 (S, 1H, $J_{1,2}$ 0.2 Hz, H-1') ppm indicating an - configuration and one anomeric signal at 4.50 (d, 1H, $J_{1,2}$=8.5 Hz, H-1") ppm indicating a - configuration. The spectrum showed two

signals for methyl protons at 1.23 (d, 3H, $J_{5,6}$=7 Hz, H_3C-6) and at 1.32 (d, 3H, $J_{5,6}$=7 Hz, H_3C-6') ppm of 6-deoxy sugars and three signals for N-acetyl groups at 1.95 [S, 6H, N-$(COCH_3)_2$], 2.04 (S, 3H, N-$COCH_3$') and 2.18 (S, 3H, N-$COCH_3$") ppm. There was also a signal for an O-($COCH_3$) group at 2.14 ppm integrating for 0.21 of the 6-deoxy methyl protons, indicating partial substitution of the O-specific polysaccharide with 21% of O-acetyl groups. There were also various signals resonating at 3.20-4.20 ppm which corresponded to the ring protons of the various glycosyl portions of the O-specific polysaccharide. The results suggested that the O-specific polysaccharide was composed of a trisaccharide repeating unit (Figure 3.5.).

3.2.2. ^{13}C-n.m.r.

The ^{13}C-n.m.r. spectrum of the O-specific polysaccharide showed, <u>inter alia</u>, five carbonyl signals at 178.62, 174.90, 174.61, 174.39 and 174.16 ppm which are consistent with the presence of four N-acetyl groups and one O-acetyl group (Figure 3.6.).

In the anomeric region, three anomeric carbon atoms were observed resonating at 101.07, 98.01 and 97.60 ppm which were attributed to the 2-acetamido-2-deoxy-β-D-glucose, 4-amino-4,6-dideoxy-4-(N-diacetyl)-α-hexose and 2-acetamido-2,6-dideoxy-α-D-glucose respectively.

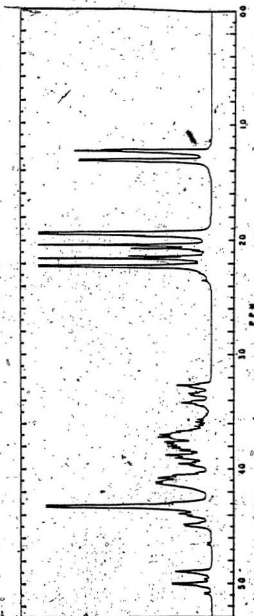


Figure 3.5. ^{1}H-n.m.r. spectra of the O-specific polysaccharide.

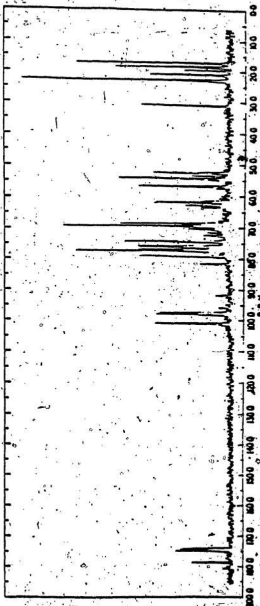


Figure 3.6. ^{13}C-n.m.r. spectra of the O-specific polysaccharide.

Similarly, in the region upfield of 90 ppm, there were three signals at 81.60, 79.10 and 77.40 ppm which were attributed respectively to the C-3 glycosylated carbon atoms of the aforementioned amino sugar residues, in the same order. In the region for carbon linked to nitrogen, there are three signals resonating at 54.64, 54.42 and 52.53 ppm assigned to the two C-2 deoxy carbon atoms and one C-4 deoxy carbon atom of the 1 β- and 2 α-dideoxy-amino sugar residues, respectively. The presence of N-acetyl groups and O-acetyl groups were observed at 23.89 and 21.08 ppm, respectively.

Therefore, in the region upfield from 110 ppm, we observed in total, twenty-two carbon atom resonances which tend to indicate that the O-specific polysaccharide consists of a trisaccharide repeating unit.

The absence in the ^{13}C-n.m.r. spectra of signals in the region of 82 to 88 ppm, characteristic of furanosides, indicates that all the monosaccharide residues in the O-specific polysaccharide were in the pyranose form.

3.3. METHYLATION ANALYSIS

Methylation analysis of the O-specific polysaccharide by the Hakomori method(50), followed by hydrolysis with 1 M trifluoroacetic acid, reduction with sodium borohydride and derivatization as alditol acetates afforded: 1,3,5-

tri-O-acetyl-2,6-dideoxy-4-O-methyl-2-(N-methyl-acetamido)-D-glucose; 1,5-di-O-acetyl-4,6-dideoxy-2,3-di-O-methyl-4-(N-diacetyl) hexose; 1,3,5-tri-O-acetyl-2-deoxy-4,6-di-O-methyl-2-(N-methylacetamido)-D-glucose; and 1,3,5-tri-O-acetyl-4,6-dideoxy-2-O-methyl-4-(N-diacetyl)-hexose in the approximate molar ratios shown in Table 3.3.

The identity of this novel series of methylated alditol acetate derivatives was effected by mass spectrometry.

The e.i.-m.s. of 1,5-di-O-acetyl-4,6-dideoxy-2,3-di-O-methyl-4-(N-diacetyl)-hexitol is shown in Figure 3.7.(a). The fragmentation pattern is governed by fission between C-3-C-4 carbon atoms to yield the primary fragment-ions at m/z 161 and 200 respectively. These ions are degraded into secondary fragment-ions by elimination of acetic acid (60 a.m.u.), Ketene (42 a.m.u.) and methanol (32 a.m.u.). An important route of fragmentation arises also by fission between C-4-C-5 carbon atoms which produce the minor primary fragment-ion at m/z 274. This latter ion is the precursor of the fragment-ion at m/z 214 (Figure 3.8.) which yields the fragment-ion at m/z 182.

The c.i.-m.s. of 1,5-di-O-acetyl-4,6-dideoxy-2,3-di-O-methyl-4-(N-diacetyl)-hexitol [Figure 3.7.(b)] showed the protonated molecular-ion $(M+H)^+$ at m/z 362 and the secondary fragment-ions at m/z 330, and 302 which results from elimination of methanol and acetic acid respectively

TABLE 3.3. METHYLATION ANALYSIS OF SJ-103 O-SPECIFIC POLYSACCHARIDE

methlated sugar[a]	T[b]	in mole %
4-QuiNMeAc[c]	1.09	26.34 (1.3)
2,3-SugN(Ac)$_2$[d]	1.05	1.6 (0.13)
4,6-GlcNMeAc[e]	1.59	24.12 (1.1)
2-Sug (NAc)$_2$[f]	1.66	20.19 (1.0)

a As alditol acetate derivative.

b Retention times relative to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol, on column C.

c 4-QuiNMeAc=1,3,5-tri-O-acetyl-2,6-dideoxy-4-O-methyl-2-(N-methylacetamido)-D-glucitol.

d 2,3-Sug(NAc)$_2$=1,5-di-O-acetyl-4,6-dideoxy-2,3-di-O-methyl-4-(N-diacetyl)-hexitol.

e 4,6-GlcNMeAc=1,3,5-tri-O-acetyl-2-deoxy-4,6-di-O-methyl-2-(N-methylacetamido)-D-glucitol.

f 2-Sug(NAc)$_2$=1,3,5-tri-O-acetyl-4,6-dideoxy-2-O-methyl-4-(N-diacetyl)-hexitol.

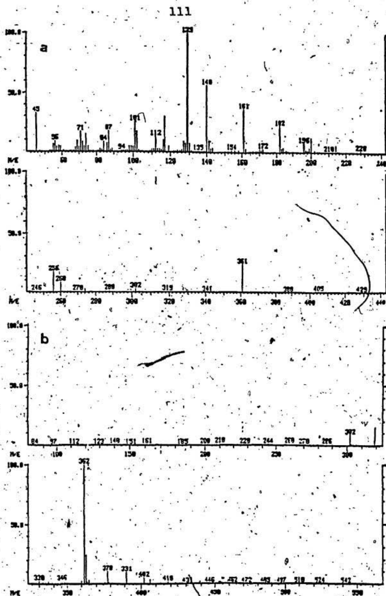


Figure 3.7. Mass spectrum of 3,5-di-O-acetyl-4,6-dideoxy-2,3-di-O-methyl-4-(N-diacetyl)-hexitol a) electron-impact and b) chemical-ionization

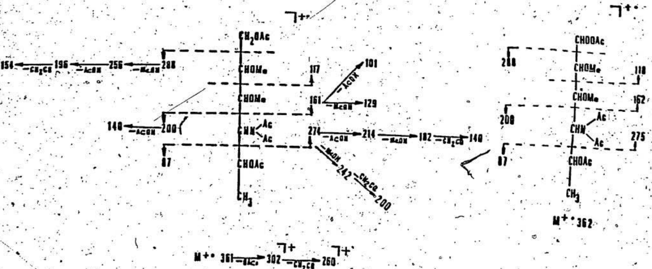


Figure 3.8. Fragmentation pattern of 1,5-di-O-acetyl-4,6-dideoxy-2,3-di-O-methyl-4-(N-diacetyl)-hexitol and its 1-deuterio derivative.

from the $(M+H)^+$ ion.

The e.i.-m.s. of 1,3,5-tri-O-acetyl-4,6-dideoxy-2-O-methyl-4-(N-diacetyl)-hexitol is shown in Figure 3.9.(a). The most prominent cleavages of the alditol carbon chain occurs between C-1-C-2 and C-3-C-4 carbon atoms to afford respectively the primary fragment-ions at m/z 316 and 200. These ions are degraded into secondary-ions by elimination of acetic acid (60 a.m.u.), ketene (42 a.m.u.) and methanol (32 a.m.u.) (Figure 3.10.).

The e.i.-m.s. of 1,3,5-tri-O-acetyl-4,6-dideoxy-2-O-methyl-4-(N-diacetyl)-hexitol (Figure 3.9.(b)) showed the protonated molecular ion $[M+H]^+$ at m/z 390. The secondary fragment-ion at m/z 330 occurs by elimination of a molecule of acetic acid from the $[M+H]^+$ ion.

Interpretation of the results of the g.l.c.-m.s. analysis of the partially methylated products of the O-specific polysaccharide showed the presence of the fully methylated monosaccharide unit 1,5-di-O-acetyl-4,6-dideoxy-2,3-di-O-methyl-4-(N-diacetyl)-hexose which was attributed to the nonreducing terminal group. The 1,3,5-tri-O-acetyl-2-deoxy-4,6-di-O-methyl-2-(N-methlacetamido)-D-glucose is assigned to a residue linked through O-3 and the presence of 1,3,5-tri-O-acetyl-2,6-dideoxy-4-O-methyl-2-(N-methylacetamido)-D-glucose and 1,3,5-tri-O-acetyl-4,6-dideoxy-2-O-methyl-4-(N-diacetyl)-hexose is also

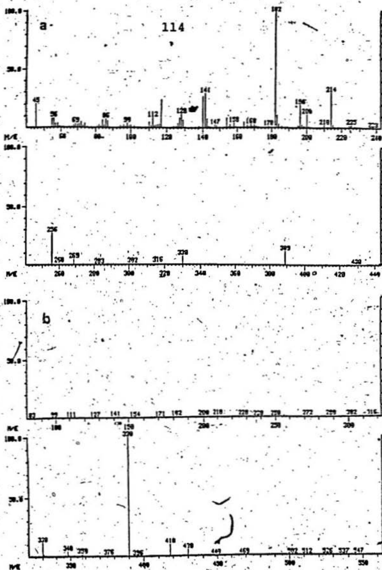


Figure 3.9. Mass spectrum of 1,3,5-tri-*O*-acetyl-4,6-dideoxy-2-*O*-methyl-4-(*N*-diacetyl)-hexitol a) electron-impact and b) chemical-ionization.

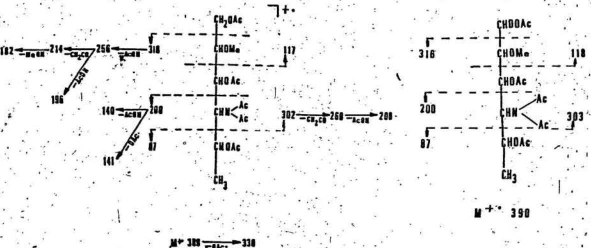


Figure 3.10. Fragmentation pattern of 1,3,5-tri-*O*-acetyl-4,6-dideoxy-2-*O*-methyl-4-(*N*-diacetyl)-hexitol and its 1-deuterio-derivative.

consistent with residues linked through O-3. The e.i.- and c.i.-mass spectrum of 1,3,5-tri-O-acetyl-2-deoxy-4,6-di-O-methyl-2-(N-methylacetamido)-D-glucose and 1,3,5-tri-O-acetyl-2,6-dideoxy-4-O-methyl-2-(N-methylacetamido)-D-glucose are illustrated in Figures 3.11 and 3.12 respectively.

It is clear from the above observations that the O-specific antigen is composed of trisaccharide repeating unit of (1→3)-linked D-glucosamine, (1→3)-linked D-quinovosamine and (1→3)-linked 4-amino-4,6-dideoxy-4-(N-diacetyl)-hexose and that the 4-amino-4,6-dideoxy-4-(N-diacetyl)-hexosyl residue is situated at the non-reducing end.

From the molar ratio of the non-reducing terminal residue dideoxy-amino sugar to any one of the constituents of the trisaccharide repeating unit, we can extrapolate that the O-specific polysaccharide is composed approximately of 12-13 trisaccharide repeating units.

3.4. LOCATION OF THE O-ACETYL GROUPS IN THE O-SPECIFIC POLYSACCHARIDE

Treatment of the O-specific polysaccharide with methyl vinyl ether by the method of De Belder and Norrman(151), was followed by Hakomori methylation, hydrolysis, reduction and acetylation gave 1,3,5,6-tetra-

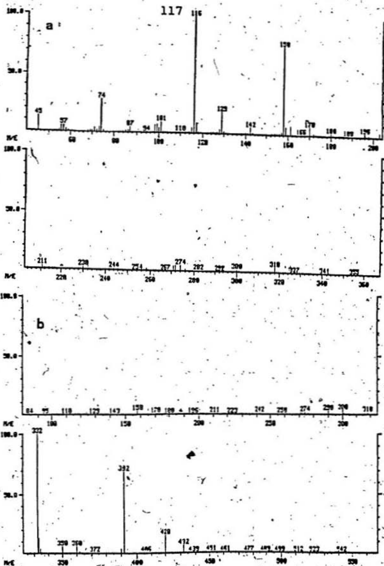


Figure 3.11. Mass spectrum of 1,3,5-tri-O-acetyl-2-deoxy-4,6-di-O-methyl-2-(N-methylacetamido)-D-glucose (a) electron-impact and (b) chemical-ionization.

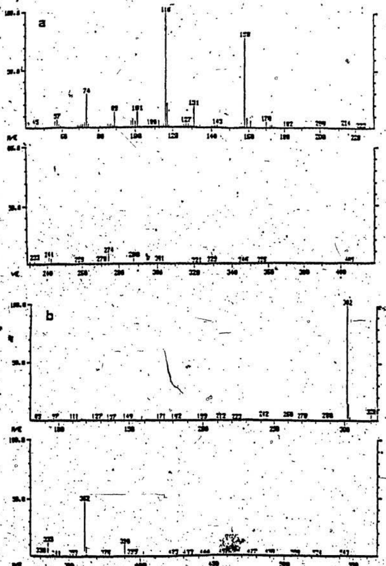


Figure 3.12. Mass spectrum of 1,3,5-tri-O-acetyl-2,6-dideoxy-4-O-methyl-2-(N-methylacetamido-D-glucose (a) electron-impact and (b) chemical-ionization.

O-acetyl-2-deoxy-4-O-methyl-2-(N-methylacetamido)-D-glucose and the alditol acetates of 2-(N-methylacetamido)-2,6-dideoxy-D-glucose, 2-acetamido-2-deoxy-D-glucose and traces of 4-acetamido-4,6-dideoxy-hexose in the approximate molar ratio of 0.2:1:0.7:0.1 (Table 3). This result indicates that approximately 20% of the 2-acetamido-2-deoxy-D-glucose has been methylated at position O-4.

3.5. SMITH DEGRADATION

The O-specific polysaccharide was oxidized with sodium metaperiodate, reduced with sodium borohydride, purified by chromatography on Sephadex G-15 and acetylated. Sugar analysis of the purified polyol using the 48% hydrogen fluoride method indicated the presence of 2-acetamido-2,6-dideoxy-D-glucose, 2-acetamido-2-deoxy-D-glucose and 4-acetamido-4,6-dideoxy-hexose in the molar ratios of 1:1:0.4.

The periodate-oxidized polysaccharide was subjected to a Smith degradation with 1% acetic acid (3 hours, 100°) and purified by chromatography on Sephadex G-15 to yield mainly a polysaccharide which was almost identical to the native O-specific polysaccharide. Sugar analysis of the purified Smith degraded polysaccharide was identical to that of the periodate-oxidized O-specific polysaccharide.

Methylation analysis of the Smith degraded polysaccharide gave; 1,5-di-O-acetyl-2,6-dideoxy-3,4-di-O-methyl-2-(N-methylacetamido)-D-glucose; 1,3,5-tri-O-acetyl-2,6-dideoxy-4-O-methyl-2-(N-methylacetamido)-D-glucose; 1,3,5-tri-O-acetyl-4,6-didoexy-2-O-methyl-4-(N-diacetyl)-hexose in the appropriate molar ratio of 0.1:1:1.2:0.8; which were identified by g.l.c.-m.s. (E.I. and C.I.) (Table 3.4.).

The ^{1}H-n.m.r. (500 MHz) and the ^{13}C-n.m.r. (125 MHz and 20 MHz) spectra of the Smith-degraded O-specific polysaccharide were almost identical to that of the native O-polysaccharides.

3.6. NITROUS ACID DEAMINATION

The O-specific polysaccharide was N-deacetylated by the method of Kenne and Lindberg(90). Deamination of this N-deacetylated O-specific polysaccharide, followed by sugar analysis (1 M trifuoracetic acid) afforded a mixture of decomposition products that could not be identified by g.l.c.-m.s. The N-deacetylated polysaccharide was deaminated once more, and without acid hydrolysis was reduced with sodium borohydride and acetylated. G.l.c.-m.s. analysis of the deaminated products indicated that it was composed of 1,3,4-tri-O-acetyl-2,5-anhydro-6-deoxy-D-hexose and 1,3,4,6-tetra-O-acetyl-2,5-anhydro-D-mannose in the appropriate molar ratios of 1:1.2.

TABLE 3.4. METHYLATION ANALYSIS OF SMITH-DEGRADED O-SPECIFIC POLYSACCHARIDE

methylated sugar[a]	T[b]	in mole %
3,4-QuiNMeAc[c]	0.84	1.8 (0.12)
4-QuiNMeAc[d]	1.09	21.6 (1.0)
4,6-GlcNMeAc[e]	1.59	24.84 (1.15)
2-Sug $(NAc)_2$[f]	1.66	19.45 (0.9)

[a] As alditol acetate derivative.

[b] Retention times relative to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol, on column C.

[c] 3,4-QuiNMeAc=1,5-di-O-acetyl-2,6-dideoxy-3,4-di-O--methyl-2-(N-methylacetamido)-D-glucitol.

[d] 4-QuiNMeAc=1,3,5-tri-O-acetyl-2,6-dideoxy-4-O-methyl-2-(N-methylacetamido)-D-glucitol.

[e] 4,6-GLcNMeAc=1,3,5-tri-O-acetyl-2-deoxy-3,4-di-O-methyl-2-(N-methylacetamido)-D-glucitol.

[f] 2-Sug$(NAc)_2$=1,3,5-tri-O-acetyl-4,6-dideoxy-2-O-methyl-4-(N-diacetyl)-hexitol.

Hakomori methylation of the reduced deaminated polysaccharide afforded 2,5-anhydro-6-deoxy-1,3,4-tri-*O*-methyl-*D*-hexose and 2,5-anhydro-1,3,4,6-tetra-*O*-methyl-*D*-mannose in the appropriate molar ratios of 1:1.3. (Table 3.5.).

3.7. PARTIAL ACID HYDROLYSIS

Hydrolysis of the *O*-specific polysaccharide with 0.5 M sulfuric acid for 30, 60 and 90 minutes at 100° resulted in the total decomposition of the 2-acetamido-2,6-dideoxy-*D*-glucose and 4-acetamido-4,6-dideoxy-4-(*N*-diacetyl)-hexose.

Milder acid hydrolysis with 0.5 M trifluoroacetic acid for 30, 60 and 90 minutes at 100° did not cleave the *O*-polymer into oligosaccharides or disaccharides but yielded some of its constituent monosaccharides. Attempted hydrolyses of the permethylated *O*-specific polysaccharide with 0.5 M trifluoracetic acid at 100° for 30, 60 and 90 minutes resulted in the partial cleavage of the permethylated polymer into its constituent monosaccharides.

3.8. CHROMIUM TRIOXIDE OXIDATION

The *O*-specific polysaccharide was dissolved in

TABLE 3.5. METHYLATION ANALYSIS OF THE DEAMINATED

O-SPECIFIC POLYSACCHARIDE

methlated sugar	T[2]	in mole %
2,5-anhydro-6-deoxy-1,3,4-tri-O-methyl-D-hexose	0.41	29.25 (1)
2,5-anhydro-1,3,4,6-tetra-O-methyl-D-mannose	0.63	38.80 (1.3)

[2] Retention time relative to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol on column e.

dimethylformamide and was acetylated with a mixture of acetic anhydride/pyridine (4 hours, 100°). The peracetylated O-specific polysaccharide was oxidized with chromium trioxide which produced a dark product. Purification by chromatography on Sephadex G-15 followed by sugar analysis indicated the oxidation of the 2-acetamido-2-deoxy-D-glucose, and the complete decomposition of the remaining dideoxy-amino sugars.

3.9. DISCUSSION

Although various O-specific polysaccharides isolated from the lipopolysaccharides of different species of *Yersinia enterocolitica* have been studied, this thesis presents the first investigation on the structure of the O-specific polysaccharide isolated from the virulent strain *Yersinia ruckeri*.

The lipopolysaccharide from this strain of *Y. ruckeri* was obtained from the dry cells in a relatively low yield. This lipopolysaccharide was composed of large amounts of an O-specific polysaccharide and lipid A in addition to a small quantity of core-oligosaccharide. The structure of this core-oligosaccharide will not be discussed in this thesis. The presence of 2-acetamido-2,6-dideoxy-D-glucose (D-quinovosamine), 2-acetamido-2-deoxy-D-glucose (D-glucosamine) and 4-amino-4,6-dideoxy-4-(N-diacetyl)-hexose

contributed enormously to the difficulty in formulating the chemical structure of the O-specific polysaccharide. Definite analyses of the monosaccharide present in the O-specific polysaccharide and derived products was only possible when anhydrous hydrogen fluoride or 48% hydrogen fluoride were used. Treatment of the O-specific polysaccharide with anhydrous hydrogen fluoride reagent, followed by acid hydrolysis under mild conditions gave a good yield of 4-amino-4,6-dideoxy-4-(N-diacetyl)-hexose which was confirmed by e.i.- and c.i.- mass spectrometry. It is to be noted that hydrolysis of the O-specific polysaccharide with mineral acid such as 0.5 M sulfuric acid resulted in the total decomposition of some sugar residues constituting the O-specific polysaccharide whereas treatment of the O-polymer with 48% hydrogen fluoride afforded the partial N-deacetylation of the 4-amino-4,6-dideoxy-4-(N-diacetyl)-hexose (along with the release of D-glucosamine and D-quinovosamine) to form the 4-acetamido-4,6-dideoxy-hexose. The identity of this dideoxy-amino sugar was established by e.i.- and c.i.-mass spectrometry. Mild acid hydrolysis of the lipopolysaccharide and the O-specific polysaccharide with 0.5 M trifluoroacetic acid (4 hours 100°) afforded all the respective constituent residues with the exception of 4-amino-4,6-dideoxy-4-(N-diacetyl)-hexose. The confirmation of the presence of the 4-amino-4,6-dideoxy-4-(N-diacetyl)-

hexose is corroborated from the results of the methylation analysis.

Methylation analysis of the O-specific polysaccharide indicated that it was composed of three methylated alditol residues namely: 1,3,5-tri-O-acetyl-2,6-dideoxy-4-O-methyl-2-(N-methylacetamido)-D-glucose; 1,3,5-tri-O-acetyl-2-deoxy-4,6-di-O-methyl-2-(N-methylacetamido)-D-glucose and 1,3,5-tri-O-acetyl-4,6-dideoxy-2-O-methyl-4-(N-diacetyl)-hexose whose identities were established by e.i. and c.i.-mass spectrometry. Structurally these results indicate the presence on each molecule of the O-specific polysaccharide of three unbranched, internal (different) amino sugars residues which are linked through O-3.

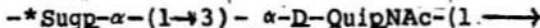
The presence of small amounts of 1,5-di-O-acetyl-4,6-dideoxy-2,3-di-O-methyl-4-(N-diacetyl)-hexose indicates a terminal, non-reducing end group. It logically follows that the O-specific polysaccharide is a linear heteropolymer of different (1→3)-linked amino sugar residues terminated at the non-reducing end by the 4-amino-4,6-dideoxy-4(N-diacetyl)-hexose.

The foregoing data suggests that the linear heteropolymer is constructed of a trisaccharide repeating unit.

The complex methylation analysis of the O-specific polysaccharide basically indicated that the only residue susceptible to periodate oxidation would be the non-

reducing, terminal 4-amino-4,6-dideoxy-4-(N-diacetyl)-hexose residue. If this was indeed correct, an ideal situation would then exist for the use of periodate oxidation followed by Smith degradation. This, in turn, would reveal the identity of the first sugar residue in the trisaccharide repeating unit and hence, in this particular case, describe the sequence of the sugar residues.

Methylation analysis of the Smith-degraded polysaccharide yielded the results shown in Table 3.4. These results indicate that the 1,5-di-O-acetyl-4,6-dideoxy-2,3-di-O-methyl-4-(N-diacetyl)-hexose found in the analysis of the original material has been replaced by 1,5-di-O-acetyl-2,6-dideoxy-3,4-di-O-methyl-2-(N-methylacetamido)-D-glucose and that the non-reducing, terminal 4-amino-4,6-dideoxy-4-(N-diacetyl)-hexose unit is linked to the 2-acetamido-2,6-dideoxy-D-glucose through O-3 as is in partial structure I.



-*Sugp-α-(1→3)- α-D-QuipNAc-(1 ⟶

1

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* Sugp stands for 4-amino-4,6-dideoxy-4-(N-diacetyl)-hexose)

Deamination of the N-deacetylated O-specific polysaccharide, followed by borohydride reduction and acetylation afforded 2,5-anhydro-D-mannose and 2,5-

anhydro-6-deoxy-D-hexose (Table 3.5.). Similarly, the methylation of the reduced deamination product afforded the fully methylated derivatives of 2,5-anhydro-D-mannose and 2,5-anhydro-6-deoxy-D-hexose respectively.

The appearance of 2,5-anhydro-D-mannose and 2,5-anhydro-6-deoxy-D-hexose without any acidic hydrolysis of the native O-polysaccharide indeed confirms that the latter is solely composed of different amino sugar residues.

The failure to observe the deamination products of the 4-amino-4,6-dideoxy-4-(N-diacetyl)-hexose indicates that the latter probably has decomposed during N-deacetylation.

The ^{13}C-n.m.r. spectra of the native O-specific polysaccharide and the Smith-degraded polysaccharide were assigned tentatively according to known values of chemical shifts within the literature(102).

The ^{13}C-n.m.r. spectra of the native O-specific polysaccharide showed in the anomeric regions three equally intense resonances at 101.07, 98.01 and 97.60 ppm, indicating that the O-specific polysaccharide was composed of a trisaccharide repeating unit. The configurational assignment on the hexapyranosyl constituents of the repeating unit were made using the anomeric one-bond ^{13}C-^{1}H coupling constants ($^{1}J_{^{13}C-^{1}H}$).

The magnitude of the $^{1}J_{^{13}C-^{1}H}$ coupling constant has been demonstrated to be sensitive to anomeric configurations.

In the proton-coupled ^{13}C-n.m.r. spectrum of the native O-specific polysaccharide, the anomeric carbon atom resonating at 101.07 ppm had a $^{1}J_{^{13}C-^{1}H}$ value of 160 Hz, suggesting the β-configuration and was attributed to the 2-acetamido-2-deoxy-D-glucopyranose. The two anomeric carbon atoms resonating at 98.01 and 97.60 ppm had a $^{1}J_{^{13}C-^{1}H}$ value of approximately 170 Hz, which indicated the α-configuration, were attributed to the 4-amino-4,6-dideoxy-4-(N-diacetyl)-hexose and 2-acetamido-2,6-dideoxy-D-glucose, respectively.

In the region for carbon linked to nitrogen, the carbon atoms resonating at 56.65, 54.42 and 52.53 ppm were assigned to the C-2 deoxy carbon atoms of 2-acetamido-2-deoxy-D-glucose, and 2-acetamido-2,6-dideoxy-D-glucose and the C-4 deoxy carbon atom of 4-amino-4,6-dideoxy-4-(N-diacetyl)-hexose, respectively.

The data presented so far for the proton-coupled ^{13}C-n.m.r. (125 MHz) and the ^{1}H-n.m.r. (500 MHz) spectra of the O-specific polysaccharide, indicates the presence of a repeating trisaccharide unit composed of three different amino-sugar residues.

The ^{13}C-n.m.r. linkage assignments of the trisaccharide repeating unit of the native O-specific

polysaccharide is consistent with its methylation analysis.

^{1}H-n.m.r. spectroscopy of the O-specific polysaccharide has suggested the presence of approximately 21% of O-acetyl group (relative to the 6-deoxy-CH_3 protons) partially substituting the natural polymer. The presence of the 4-O-methyl ether of 2-acetamido-2-deoxy-D-glucose, after methyl vinyl ether treatment and methylation analysis of the O-specific polysaccharide, confirmed that the O-acetyl group was on the O-4 position of the 2-acetamido-2-deoxy-D-glucose. Using the ratio of the 4-O-methyl-derivative to that of the unchanged 2-acetamido-2-deoxy-D-glucose it is clear that 21% substitution is in excellent agreement with that shown by ^{1}H-n.m.r.

It is to be noted that no structural information was obtained on the O-specific polysaccharide when this latter was subjected to partial hydrolysis with acid as total decomposition of the residues occurred.

It has been established that the 4-amino-4,6-didoexy-4-(N-diacetyl)-hexose is the nonreducing, terminal residue of the main backbone of the O-specific polysaccharide and that this nonreducing, terminal dideoxy-amino sugar is glycosidically linked through O-3 of the 2-acetamido-2,6-dideoxy-D-glucose. It is also known that the biosynthetic polymerization of the O-specific polysaccharide occurs via growth at the nonreducing end which is accomplished by

transferring the newly activated repeating unit to the growing O-chain (growth at the nonreducing end). Since the native O-polysaccharide is a linear heteropolymer composed of a trisaccharide repeating unit formed of three different (1→3) linked amino sugars, it therefore follows that the O-3 substituted 4-amino-4,6-dideoxy-4-(N-diacetyl)-hexose is the last residue of the repeating unit.

By process of elimination, it seems logical, with the evidence presented thus far, to postulate that the O-3 linked 2-acetamido-2-deoxy-D-glucose, presently unassigned, must be located as the first residue of the trisaccharide repeating unit.

In an attempt to confirm the anomeric configurations of the various glycosyl residues, the O-specific polysaccharide was acetylated and the product subjected to oxidation with chromium trioxide. During the oxidation reaction, the 2-acetamido-2-deoxy-D-glucose was oxidized which was consistent with the fact that the D-glucosamine was indeed β-linked. It is obvious that the α-linked 2-acetamido-2,6-dideoxy-D-glucose and 4-amino-4,6-dideoxy-4-(N-diacetyl)-hexose would not survive such harsh treatment as it has been proved(152,153,154) that they are extremely labile.

There are two points which are not fully resolved in this investigation. The assignment of the relative and

absolute configurations of the 4-amino-4,6-dideoxy-4-(N-diacetyl)-hexose have not yet been established.

Notwithstanding these two relatively minor points, all of the evidence presented is consistent with the following structure formula for the O-specific polysaccharide of Yersinia ruckeri SJ-103.

$$\left[\rightarrow 3)\text{-}\alpha\text{-Sug}p^{*}\text{-}(1\rightarrow 3)\text{-}\alpha\text{-D-Qui}p\text{NAc-}(1\rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{NAc-}(1\rightarrow\right]_n$$

$$\underset{\text{OAc(21\%)}}{\uparrow 4}\ \text{(on GlcpNAc)}$$

---

*Where Sugp stands for 4-amino-4,6-dideoxy-4-(N-diacetyl)-hexose.

4. NOVEL PREPARATION OF THE O-SPECIFIC POLYSACCHARIDE -BOVINE SERUM ALBIUMIN CONJUGATE FOR USE AS ARTIFICIAL ANTIGEN

4.1. INTRODUCTION

During the last decade, artificial polysaccharide protein conjugates have been extensively used for lectin-polysaccharide and antibody-polysaccharide interactions. The use of soluble and unsoluble polysaccharide-conjugates has found great utility in biological studies and numerous methods have been developed for their syntheses each for their own purpose and having their own particular advantages(155,156). Most studies of antibody-hetero-saccharide reactions have resorted to the use of naturally occuring oligo- and polysaccharides.

One method that has shown great versatility is the direct, covalent attachment of oligosaccharides to the amino groups of proteins(157,158), lipids(159) or derivatized gels(160) by reductive amination using sodium cyano-borohydride. Advantages of this method are that it is direct, requires minimal chemical manipulation and is performed under extremely mild conditions. One of the major disadvantages of this method is that it opens up the ring structure of the terminal reducing sugar to generate

an acyclic amine. This could prove, in certain cases, to be detrimental to the biological properties of the conjugate. Another drawback to this method is that it is not possible to conjugate the polysaccharide having a reducing ketose residue. This is consistent with the fact that a successful reductive amination would require the introduction of a small functionalized spacer molecule at the ketonic carbonyl group.

4.2. RESULTS

4.2.1. Reductive amination of the O-specific polysaccharide of *Y. ruckeri* with the novel 1,6-hexanediamine linker arm

The O-specific polysaccharide of *Yersinia ruckeri* which is under study in this thesis, as already mentioned, is composed of a regular heteropolymeric structure (O-polysaccharide) linked to a core-oliogosaccharide. The structure of this core-oligosaccharide has been partially investigated and it was found that it contained a terminal, reducing 3-deoxy-*D*-*manno*-2-octulosonic-acid (KDO).

The O-specific polysaccharide, dissolved in water, was reacted with an excess of the novel linker, 1,6-

hexanediamine in the presence of sodium cyanoborohydride. At pH 8.0, the N-imine derivative I (Figure 4.1.) formed between the terminal reducing KDO residue and the aklylamino group was selectively reduced to the secondary amine II. The O-specific polysaccharide-1-(6-amino)-hexane alkylamine derivative was purified by gel chromatography. Fractions positive for sugar, as well as alkylamine, were pooled and concentrated.

4.2.2. Preparation of O-specific polysaccharide-1-(6-isothiocyanate)-hexane alkylamine derivative

To a solution of the polysaccharide-1-(6-isothiocyanate)-hexane alkylamine derivative II in 80% ethanol, thiophosgene was added dropwise under vigorous stirring. The pH of this reaction mixture was maintained at 7.0. When the formation of the isothiocyanate derivative was complete (4-6 hours), the reaction mixture was co-distilled with methanol to remove the excess thiophosgene.

The formation of the isothiocyanate derivative III from the corresponding amine II was essentially quantitative (Figure 4.1).

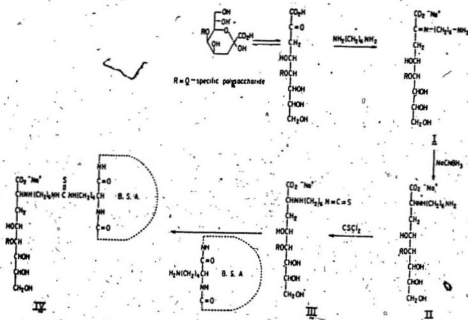

Figure 4.1. Reaction sequence of formation of polysaccharide - protein conjugate.

4.2.3. Coupling of the Q-specific polysaccharide-1-(6-isothiocyanate)-hexane alkylamine derivative to the bovine serum albumin protein

Coupling of the Q-specific polysaccharide was achieved by addition of the isothiocyanate derivative III to the appropriate amount of bovine serum albumin protein in phosphate buffer saline. After dialysis the product IV (Figure 4.1) was purified by chromatography on Bio-Gel P-150.

To evaluate the efficiency of the coupling reaction, different molar amounts of the polysaccharide-isothiocyanate derivate III were reacted with BSA. The resulting polysaccharide-protein conjugates were purified and the degree of substitution were determined by analytical assays. It was established that the highest degree of substitution (D.S. 10) was obtained using a 3.6 molar input ratio of the isothiocyanate derivate over ϵ-aminolysyl groups of the BSA.

4.2.4. Immunological properties of the glycoconjugate IV

The immunological specificity of the anti-Q-specific polysaccharide-BSA conjugate IV sera was determined by passive hemagglutination. Antisera elicited by immunization with this artificial glycoconjugate IV were

used. Lipopolysaccharide extracted from *Y. ruckeri* SJ-103 was used for coating of the erythrocytes. The antibody response, seen after immunization of rabbits with the glycoconjugate IV was compared to that obtained with heat-killed whole-cell bacteria of *Yersina ruckeri*. In rabbits immunized with glycoconjugate IV, a reasonable titer (640) was obtained when the Lps-coated human red blood cells (HRBC) were used as antigens. As expected in the rabbits immunized with heat-killed whole cell bacteria, the titer obtained when the Lps-coated HRBC were used, was considerably higher (2560) higher. Thus, it is evident that immunization with the glycoconjugate IV had an antigenic specificity similar to the whole cell bacteria.

4.3. DISCUSSION

Immunization against bacterial enteric infections has met with little success. Existing vaccines of killed whole bacteria are intrinsically toxic because of the endotoxin content of the cell envelope. The protective value of these vaccines has long since been attributed to the anti-O-antigen moiety, although antibodies to other cell wall constituents are probably also of importance. Attempts to immunize with the purified O-antigen have not been successful, because when lipid A is cleaved from the

Lps the resulting O-polysaccharide fraction is not immunogenic. In order for the polysaccharide to be immunogenic, it has to be covalently linked to a suitable carrier molecule.

Several elegant coupling methods for the preparation of polysaccharide-protein conjugates have been described in the literature(161,162). These methods are based on the coupling of polysaccharide derivatives of 2-(4-aminophenyl)-ethylamine, p-nitrophenyl and p-nitrobenzyl with the protein carrier by the methods of the diazonium reaction and of McBroom et al(138), respectively.

Although these methods are meritorious in many respects, they share the common disadvantage of eliciting antibodies which are directed toward the aromatic bridging-arm portion of the polysaccharide-protein conjugate.

It has been shown by Lemieux et al(163,164) that the introduction of a bridging-arm composed of a polymethylene chain between the haptenic polysaccharide and the protein carrier produced an antisera which mainly recognized the epitopes of the polysaccharide portion of the glycoconjugate.

The novel coupling method presented in this thesis is based on the introduction of the 1,6-hexanediamine bridging-arm to the terminal reducing end of the core-oligosaccharide portion of the native O-specific

polysaccharide.

The anticipated success of this novel method was based on the experimental results of conjugation by reductive amination of simple monosaccharides such as D-fructose and KDO and known synthesized oligosaccharides having free ketose residues with the bridging-arm 1,6-hexanediamine.

As mentioned earlier the O-specific polysaccharide is formed of a trisaccharide repeating unit composed of 1→3 linked 2-acetamido-2,6-dideoxy-α-D-glucose, 2-acetamido-2-deoxy-β-D-glucose, and 4-amino-4,6-dideoxy-4-(N-diacetyl)-hexose. These deoxy-amino sugars are extremely acid sensitive and contain the 1→3 interchain linkage which contributes enormously to their lability. The mild conditions used in the present coupling procedure allowed the coupling of the unstable (but intact) O-specific polysaccharide to the macromolecular protein carrier.

The primary amino group was introduced in the KDO terminal, reducing end of the polysaccharide by reacting it with the 1,6-hexanediamine under very mild conditions at pH 8.0.

After subsequent conversion to the corresponding polysaccharide-isothiocyanate derivative III, it was covalently linked to the free ε-aminolysyl group of the carrier protein bovine serum albumin. The resulting glycoconjugate was highly immunogenic and elicited in

rabbits, both anti-haptenic and anti-carrier BSA specific antibodies. Some of the advantages of this novel coupling procedure are that they can be used with polysaccharide containing highly acid or alkaline labile structures and/or glycosidic linkages. Also, this method does not grossly affect the immunogenic specificities of the carrier protein. In effect the resulting conjugate eluted as a single peak in gel chromatography, indicating that no cross linking or major alterations of the carrier-protein has occurred.

The antisera raised in rabbits against the polysaccharide-BSA conjugate had high titers against the heat killed whole-cell bacteria and the Lps of Yersinia ruckeri.

It is anticipated that the O-specific polysaccharide - BSA conjugate may be used as a potential vaccine against the enteric red-mouth disease and that the produced antisera will possess good bacteriostatic activity against the homologous organism.

5. SUMMARY AND CONCLUSION

The lipopolysaccharide of *Yersinia ruckeri* strain SJ-103 is mainly composed of the O-specific polysaccharide chain and lipid A and a relatively small amount of core-oligosaccharide.

The O-specific polysaccharide was made up of a trisaccharide repeating unit of 1→3 linked residues of 2-acetamido-2,6-dideoxy-α-D-glucose, 2-acetamido-2-deoxy-β-D-glucose and 4-amino-4,6-dideoxy-4-(N-diacetyl)-hexose, respectively.

Sugar and methylation analyses, periodate oxidation and Smith degradation permitted the determination of the sugar composition and the nature of the linkages. Smith degradation also revealed information on the sequence of the different deoxy-amino sugar residues in the molecule.

The β-anomeric configuration of the 2-acetamido-2-deoxy-D-glucose was confirmed by chromium trioxide oxidiation.

The use of ^{1}H- and ^{13}C-n.m.r. spectroscopy combined with e.i. and c.i. mass-spectrometry, in addition to the foregoing chemical evidence, permitted the establishment of the chemical structure of SJ-103 O-specific polysaccharide.

Some unusual compositional and structural features

are of interest particularly the presence of the novel 4-amino-4,6-dideoxy-4-(N-diacetyl)-hexose in the repeating unit of this O-specific polysaccharide. This is the first time that this uncommon N-diacetylated-dideoxy-amino sugar has ever been found in nature. The identification of this rare sugar residue was made by use of e.i.- and c.i.-mass-spectrometry.

Finally, a pertinent structural feature of this O-specific polysaccharide is its extreme instability towards acid hydrolyses which has contributed considerably to the difficulty in formulating the chemical structure.

During this study, a novel mild method for the preparation of the artificial neoglycoprotein of *Yersinia ruckeri* O-antigen has been devised (using 1,6-hexanediamine as the bridging-arm) which linked the O-specific polysaccharide to the ε-aminolysyl groups of the bovine serum albumin carrier protein.

The artificial neoglycoprotein of *Y. ruckeri* O-specific polysaccharide has elicited antibody production in rabbits. It is hoped that this artificial antigen and its rabbit-raised antisera may be used for passive immunization experiments for the prevention of enteric red-mouth fish disease caused by the aquatic gram-negative *Yersinia ruckeri*.

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