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STRUCTURAL AND IMMUNOLOGICAL STUDIES OF THE LIPOPOLYSACCHARIDE ANTIGENS OF PSEUDOMONAS AERUGINOSA

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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1984

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DEDICATION

This dissertation is dedicated to the most important forces in my life, my family and my fiancée, Karen E. Leonard. I would also like to dedicate this to the memory of my father, whose inspiration and guidance led me to this career choice.
ACKNOWLEDGMENTS

I would like to gratefully acknowledge the assistance of three persons affiliated with the Campus Chemical Instrument Center: Dick Weisenberger, David Chang, and Chuck Cottrell. Their assistance was invaluable in the completion of this project. I would also like to thank Professor Horton for his direction and help during my graduate career. Finally, I would like to express my appreciation to the Amoco Corporation and the Graduate Committee of the Chemistry Department for my selection as the recipient of the 1983 Amoco fellowship.
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29. Elution profile for the purification of monoclonal IgM antibodies, using immunotype 5 O-specific polysaccharide derivatized Sepharose 4B ........................................... 171
I. INTRODUCTION

*Pseudomonas aeruginosa*, until recently was considered to be a harmless Gram-negative bacterium. However, with the advent of modern antibiotics and antibiotic therapy regimens, new resistant bacterial strains emerged and *Pseudomonas aeruginosa* became a significant pathogen. Under normal circumstances in healthy individuals, this particular bacterium poses no threat; 7-25% of the population has *Pseudomonas* as a constituent of their intestinal flora (1). In these persons, there is no evidence of clinical symptoms nor are their immune systems stimulated to a measurable level. When the immune system of an individual is compromised in some way, *P. aeruginosa* can become a severe pathogen with colonization leading to septicemia and ultimately death.

Persons who are very susceptible to pathogenic infection can be divided into several groups. The major groups are: burn patients, cancer chemotherapy and radiation therapy patients, organ transplant patients, general surgical patients, newborns, and persons with cystic fibrosis. Infections of this type are most important for burn patients, where there is a high degree of mortality once colonization occurs (2-5).

Prevention of infections with this bacterium are complicated by the fact that it is found (11) in nearly any environment, including water, plants, and animals (both vertebrates and invertebrates). *Pseudomonas aeruginosa* was
first studied indirectly by Fordos when he observed blue and blue-green pigments associated with burn victims (6). Schroeter continued the study of these pigments and found that they were produced by a bacterium, which he termed *Bacterium aeruginosum* (7). It was not until 1882 when the bacterium responsible for the production of these pigments was isolated in pure culture. Upon its isolation, it received a new name, *Bacillus pycyaneus* (8). Several different methods of isolation were developed in the next decade, along with several new names for a bacterium that was "rediscovered" each time. While studies were being completed on this mammalian colonizer, parallel studies were being conducted on a bacterium found associated with plants. In 1942, Elrod and Braun reported that the two bacteria were identical (9). After this time the collective strains were classified as *Pseudomonas aeruginosa*. A complete study of the physical characteristics of this bacterium was completed in 1966 (10). This study reported that the bacteria belonging to this class were unicellular rods that possessed lateral or polar flagella. Molecular oxygen is the terminal oxidant of cellular respiration. The DNA found in this organism has a guanine/cytosine ratio of 58-69%.

Although *Pseudomonas aeruginosa* has always been found in association with infections since its discovery in 1882, it was not until recently that it has become recognized as a
serious pathogen, once it is established in a susceptible host, it is extremely difficult to control the progress of the infection. β-lactam and aminoglycoside antibiotics have little or no effect on established Pseudomonas infections (11). There are several reasons for this resistance. The most important facet of the resistance is the presence of a thick outer membrane on the exterior of the cell surface. This outer membrane is a glycocalyx composed of complex protein, phospholipid, and lipopolysaccharide. The glycocalyx represents both a physical and a hydrophilic barrier to the external environment. Thus the antibiotics are prevented from reaching their target sites within the cell. A secondary factor contributing to the drug resistance is the production of enzymes capable of degrading the antibiotics once they pass through the outer membrane. There are two major classes of the degradative enzymes, one class targetted for the β-lactams and the other targetted for the aminocyclitol antibiotics. The final component of the cellular resistance is the bacterium's ability to modify the target site of the antibiotic so that it is no longer able to bind to its receptor and is thus rendered inactive.

Because of the ineffectiveness of traditional antibiotic therapy, there was a major effort during the 1960s to develop and investigate passive immune therapy. Any
attempt to produce a vaccine that could act as a protective agent against *Pseudomonas* infections is greatly hampered by the numerous strains of the organism. Several laboratories have attempted to classify the strains of *Pseudomonas aeruginosa* into sub-groups according to *in vitro* serological reactions (12-14). Each of these methods grouped a majority of the 755 strains into one of 12-14 serotypes. However, the *in vitro* reactions have little or no correlation with the *in vivo* immunological reactions that they are attempting to monitor (15). M. W. Fisher developed a method, based on *in vivo* reactions that successfully addressed this problem (15). In this method, rats are inoculated with heat-killed bacterial cells and are permitted to develop antibodies to that strain for several days. At the end of this induction period, the rats are injected with a live culture of a second strain of bacterium. If the rats survive, the strains are identical in immunotype, if not then the strains are from different immunotypes. Using this procedure, all but eight strains could be placed into one of the Fisher immunotypes (15).

Many studies were conducted prior to this time to determine the site of the antigenic determinants of *P. aeruginosa* (16-18). It was found that, as can be seen with other Gram-negative bacteria, the antigenic determinants are
found in the outer membrane of the bacterium. Specifically these antigens reside within the lipopolysaccharide found in the glycocalyx (19). Any study of the immunotherapy of P. aeruginosa infections must also include an investigation of the relationship between the lipopolysaccharide and antigenicity.

The first attempt of passive immune therapy, in which high risk patients were immunized with an anti-Pseudomonas vaccine, was undertaken by Fisher (20). Using the lipopolysaccharides from each of the seven immunotypes, he prepared a vaccine that was marketed under the name Pseudogen. While the use of this vaccine produced a decrease in the mortality of burn victims and those undergoing anti-neoplastic therapy, it also produced several undesirable side-effects (2-5,21,22). The major problem with this first vaccine was its pyrogenic nature (fevers of 106 F and higher were not uncommon). In addition to vaccine therapy, a combined program of vaccination and gamma-globulin therapy was also introduced (23). This combined program and the use of gamma-globulin by itself produced marked decreases in the mortality of these high-risk individuals (24). However, the toxicity of the vaccine could not be diminished.

Shortly after the introduction of Pseudogen, a second vaccine was tested in burn wards of India (25). The results
of these preliminary tests were promising but also suffered many of the drawbacks of Pseudogen. A fair degree of protection was observed but with little or no ameliorative effect in well established infections.

At the present time these vaccines are the most promising anti-Pseudomonas agents available. From these preliminary results, it is evident that immune therapy of infections of this type will be most effective when a combined vaccination and gamma-globulin therapy is utilized (1,26). Several problems need to be addressed before an effective program can be developed. These include the production of a non-toxic vaccine with high protection capabilities and the production and purification of concentrated antibody preparations.

It was the attempt of this project to aid in the development of a new vaccine by providing an understanding of the chemical nature of the antigenic determinants of Pseudomonas aeruginosa. A second portion of this project deals with the development of a new method for the purification of antibodies raised to Pseudomonas aeruginosa.
II. The Biological Properties of *Pseudomonas aeruginosa*

II.1 Background

It is not possible to trace the virulence of *P. aeruginosa* to one single factor, thus the pathogenesis of this opportunistic organism requires further investigation. The overall virulence can be traced to the metabolic products of the cell. These cellular products or virulence factors will be discussed in this section.

Because of the large number of strains of this bacterium, several classification schemes have been developed to provide a means of placing the organisms into sub-groups with similar properties. The major classification schemes will be presented and contrasted to a new typing scheme based on the chemical structure of the somatic antigens of *Pseudomonas aeruginosa*.

I.2 Epidemiology

In early studies, it was assumed that pathogenic infections were the direct result of autoinfection stimulated by the event that compromised the host's immune system (1). This assumption was based on the observation that 25% of the population has *P. aeruginosa* in their gastrointestinal tracts. More recent studies have indicated that *Pseudomonas*
infections often result from exposure to an external source of the bacterium (27).

The exact nature of the spread of *Pseudomonas* sepsis is dependent on the nature of the infection, e.g. urinary vs. burn wound infections. However, the major trend observed is that whenever high-risk individuals are treated in a segregated unit, the probability of the establishment of a *Pseudomonas* infection increases (1). The magnitude of this problem can be best illustrated with the following statistics: 23-46% of cancer patients, 60-100% of burn patients, and 71-85% of persons with cystic fibrosis will develop pathogenic infections (1).

**II.3 Virulence Factors**

In *Pseudomonas aeruginosa* as in other pathogenic bacteria, virulence can not be related to a single factor. Rather it is seen as an effect brought about by many components that act independently. In *Pseudomonas* the major compounds responsible for virulence include: lipopolysaccharide, pili, outer membrane proteins, alkaline protease, elastase, hemolysin, leukocidin, phospholipase C, exoenzyme S, and exotoxin A (28). In all probability, exotoxin A is the most toxic factor produced by this organism (28). Exotoxin A is a potent protein synthesis inhibitor,
promoting the ADP-ribosylation of elongation factor 2 and the associated destruction of NAD (29). More and more evidence is pointing to this factor as being the single factor, or at least the most important factor, responsible for the lethality of *Pseudomonas aeruginosa* infections. The remaining factors, with the exception of the lipopolysaccharide, are generally responsible for the localized tissue damage caused by their proteolytic, hemolytic and degradative activities (1). While these add to the pathogenicity of the organism, they are not responsible for the lethality (30).

The lipopolysaccharide is included in this section as a toxin, however, its toxicity is a point of controversy. In *in vivo* studies of the lipopolysaccharide toxicity, Dyke and Berk found that the level of toxicity is largely dependent upon the method of isolation used to obtain the purified lipopolysaccharide (31). Thus much of the implied toxicity of this macromolecule is in all probability due to other cellular components that co-purify with the lipopolysaccharide. The functions of this molecule will be discussed in a later section.

Another aspect of the virulence of *P. aeruginosa* can be found in its resistance to antibiotics. The resistance to β-lactam antibiotics can be traced to two different "defense mechanisms". The major reason for the resistance is that the
antibiotic can not cross the outer membrane, as was indicated in a previous section. Additionally the bacterium produces a battery of β-lactamases that are capable of degrading the antibiotic if it does cross the outer membrane.

Aminocyclitol antibiotics are also largely ineffectual in treating \textit{Pseudomonas} sepsis. This lack of success can be traced to the presence of three classes of enzymes capable of derivatizing the molecule in such a manner as to inactivate the antibiotic. The three classes represented are: phosphorylases, acetylases, and nucleotidylases. Each of the modifications, separately or in combination, completely inactivates the aminoglycoside.

A final aspect of the virulence is the ability of the organism to survive in the host tissue. Iron is an essential nutrient for the growth of \textit{P. aeruginosa}. When found in tissue, iron is most often found bound to protein and thus in a form unavailable to the organism. Many bacteria are unable to survive in an environment such as this because they have no mechanism available to sequester iron (32). However, \textit{P. aeruginosa} produces a series of pyochelins, iron siderophores, in high concentration which enables this species to extract iron from its environment and survive (33).

As a result of these unique and unusual properties and metabolic processes, \textit{Pseudomonas aeruginosa} is an effective colonizer of a suitable host.
II.4 Classification Methods

At the present time, over 700 strains of *P. aeruginosa* have been identified. Any attempt to study this organism strain by strain would be tedious at best. Because of this, several groups have proposed classification methods that separate the strains into smaller sub-classes. There exist today, six major classification methods: biotyping, serotyping, antiobiograms, bacteriocin (pyocin) typing, bacteriophage typing, and combined methods.

Biotyping relies on a study of the metabolic processes of the organism, that are easily measured and quantitated in a clinical microbiology laboratory. Reactions that have been used are: production of indole, growth on specialized agar media, and catabolism of D-xylose (34).

Antiobiograms, bacteriocin, and bacteriophage typing are based on the interaction of the test bacterium and different classes of antimicrobial agents. In antiobiograms, antibiotics produced by the organism or synthetically are introduced into living cultures, which are then monitored for sensitivity or resistance (35). Bacteriocin typing is dependent upon the effect of pyocins, antibiotics produced by *P. aeruginosa*, on the various strains of the organism (36). Bacteriophage typing has its basis in the interaction between the bacterium and selected bacteriophages (37). In all of
these procedures, a fingerprint of susceptibility and resistance is obtained for each strain. Each strain is then compared to the others and similarities and differences are grouped accordingly.

The final typing method is serological typing. This type of classification scheme is based on the interaction between antigens and antibodies. Presently four independent methods are being used to classify P. aeruginosa. These include three in vitro and one in vivo serotyping procedure. The in vitro assays currently in use were developed by Habs (13), Lanyi (12), and Homma (14). The sole in vivo system was developed by Fisher (15) and is discussed in a previous section.

As more information pertaining to the primary structures of the somatic antigens of P. aeruginosa becomes available, the future may see a sixth typing system. Potentially this system could have a structural basis in the chemical repeating unit of the polysaccharide antigens of this gram-negative bacterium.

II.5 Cellular Organization

The final aspect of the biological properties of P. aeruginosa to be discussed is in effect the easiest to study, the cellular organization. The most salient feature of the
organism is its elaborate glycocalyx, which forms the outer membrane of the cell. This complex matrix contains the lipopolysaccharide, complex protein, and phospholipid of the outer membrane. In an electron micrograph, this layer is only one of three distinct electron dense layers seen. Moving toward the cell wall, a second membrane-like layer can be seen. By treating the bacterium with detergents, one can find that this layer is the peptidoglycan layer. The width of this section is much thinner than the equivalent structure in Gram-positive bacteria (Figure 1). In eukaryotic cells, a thin cytoplasmic membrane is sufficient to maintain structural integrity. However, in bacterial cells, elaborate or at least extensive structures are required for the same function. This is predominantly due to their habitation of a hypotonic environment. Lysis of the cell is prevented by the existence of the peptidoglycan matrix. The presence of three distinct membranes in the Gram-negative bacteria seems to indicate a higher degree of differentiation than is seen in the Gram-positive bacteria (38).

The functions of the three membranes also differ. The outer membrane is predominantly a structural feature. Its secondary function is associated with the antigenicity and cellular recognition and will be discussed at a later time.
Figure 1: Generalized structures of the membranes of eukaryotic and prokaryotic cells. (CM, cytoplasmic membrane; CW, cell wall, OM, outer membrane; PG, peptidoglycan) (38).
ANIMAL CELL

+ CELL WALL (PEPTIDOGLYCAN)

+ OUTER MEMBRANE

GRAM-NEGATIVE

GRAM-POSITIVE

Figure 1
Since bacteria do not contain lysosomes, the degradative enzymes associated with these organelles in eukaryotes must be exported from the cell and maintained in the external environment. In the case of Gram-positive organisms, these enzymes diffuse into the external environment. In gram-negative organisms, these enzymes are trapped between the peptidoglycan and the cell wall, the space between serving as a diffuse lysosome. When the periplasmic space is compared to a lysosome, it can be seen that its function is even more complex in that it also contains proteins involved with facilitated amino acid and carbohydrate transport (37). The outer membrane must also provide for the diffusion of these nutrients through pores produced by the membrane proteins (porins).

A final function of the outer membrane is to provide a defensive barrier to the environment. This is provided primarily through the action of the lipopolysaccharide. The polysaccharide components of this complex make up a hydrophilic barrier, preventing the passage of hydrophobic molecules. In addition the lipopolysaccharides serve as receptors or at least an aid in the interaction of the organism with animal cells and other bacterial cells, especially during conjugation (38).
In conclusion, the functions of the outer membranes of Gram-negative bacteria are three-fold. They provide barriers to hypotonic lysis, aid in selective transport of nutrients, and facilitate cell to cell interactions. Perhaps its most important function is to provide the presence of an endotoxin, comprising a major portion of the membrane, which has been identified as the lipopolysaccharide (38).
III *Pseudomonas aeruginosa* Lipopolysaccharides

III.1 Background

As was mentioned earlier, the lipopolysaccharide (LPS) is a major component of the outer membrane and as such performs many of the functions that are associated with the membrane as a whole. In addition the LPS has several functions that can only be ascribed to it specifically. These include its endotoxicity (39), its function as a receptor for bacteriophages (39), and its function as a somatic antigen (40). When the LPS from *P. aeruginosa* is compared to the LPS from other Gram-negative bacteria, it appears to have the same basic structure as the majority of the other bacteria. Its general morphology is very similar, if not identical, to that of *Shigella* and *Salmonella* (41,42). The LPS is a complex phosphorylated heteropolysaccharide with three distinct structural domains: the O-antigenic polysaccharide (somatic antigen, O-antigen, or the O-chain), the core oligosaccharide (core), and lipid A (43). The O-chain contains the antigenic determinants that are the basis of the serological typing schemes described previously. The core region is an oligosaccharide that is specific for each species of bacteria and is thus conserved from strain to strain. The lipid A region is a glucosamine disaccharide to which fatty acids are
attached through amide and ester linkages. It is this region of the molecule that is believed to be conserved in all species of Enterobacteriaceae (44). In addition the majority of the endotoxicity is associated with this region.

A closer examination of the O-antigen shows that it is a heteropolysaccharide with a regular repeating unit. The repeat unit can be as small as a disaccharide or range in size up to a pentasaccharide repeating unit. In studies of various Gram-negative species, the O-specific polysaccharide appears to contain neutral hexoses, deoxy-hexoses, amino hexoses, and uronic acids. Amino sugars are especially evident in the O-antigens of *P. aeruginosa*. This bacterium apparently produces a polysaccharide greatly enriched in 2-amino-2-deoxyhexose, 2,3-diamino-2,3-dideoxyhexoses and the uronic acids derived from these classes of monosaccharides. In addition the monosaccharides within the repeating unit are often derivatized with acetate esters involving free hydroxyl groups within the sequence. In some Gram-negative strains this derivatization can include pyruvate acetals and methyl ethers. The different repeating units of the various strains of the organism are the actual antigenic determinants of the O-antigen.

The core oligosaccharide of the lipopolysaccharide appears to be a very stable unit within a given species of bacteria. The core is composed of neutral hexose, amino
hexose and heptose. The stoichiometry, linkage, and anomeric configuration are variable dependent upon the parent species. Alanine, phosphate esters, and acetate esters can also be found in direct association with the core. The structure of the core oligosaccharide of a *P. aeruginosa* mutant deficient in O-specific polysaccharide synthesis has recently been presented (Figure 2) (45). This structure is similar to the structure of the core proposed for the *Salmonella* core (46,47).

The final component, lipid A, is a 2-amino-2-deoxyglucose β-D-(1→6) linked disaccharide. The disaccharide is N- and O-acylated with saturated fatty acids and acylated hydroxy fatty acids (19). Additionally the lipid A contains phosphate esters. The core oligosaccharide is attached to lipid A in a ketosidic linkage with 3-deoxy-D-manno-octulosonic acid. In *Salmonella*, 4-amino-4-deoxyarabinose and 2-aminoethanol is also present in the lipid A region. However, neither of the substituents have been identified in the *P. aeruginosa* lipid A.

The structural characterization and the elucidation of the biosynthetic pathways involved in the synthesis of the LPS were greatly simplified through the use of a new approach combined with classical carbohydrate chemistry. The biosynthetic data was obtained by using *Salmonella* mutants.
Figure 2: Proposed structure of the core oligosaccharide of *Pseudomonas aeruginosa*.
Figure 2
These mutants synthesize a series of defective lipopolysaccharide molecules. Each mutant had a different stage in the biosynthetic pathway genetically blocked. Osborn and her co-workers used these mutants to develop the present understanding of the pathways involved in the total synthesis of the LPS (45).

III.2 Biosynthesis of the Lipopolysaccharide

The biosynthesis of lipid A is probably the least understood of the three regions of the LPS, but it is probably the first step in the biosynthesis. Many of the early events in the establishment of the acylated glucosamine disaccharide backbone remain unclear. Viable mutants with genetic blocks in these early steps have not been isolated. The first well characterized step in the biosynthesis of lipid A is the linkage of 3-deoxy-\(\beta\)-manno-octulosonic acid (KDO) to lipid A. In this step cytidine monophosphate-KDO acts as the KDO donor. This reaction precedes esterification of lipid A with fatty acids. In order to act as a KDO acceptor, the lipid A precursor must only be \(N\)-acylated (49). The transfer of KDO to the 3-hydroxyl group of the non-reducing glucosamine residue is especially important in translocation of the completed lipopolysaccharide to the outer membrane. Once the KDO residue(s) have been incorporated, the final processing of lipid A occurs. This
involves the esterification of all of the free hydroxyl groups with activated fatty acids.

Like that of the initial reactions in the biosynthesis of lipid A, the biogenesis and transfer of the heptose residues is poorly understood. The heptose region of the core is the next section added to the growing lipopolysaccharide. After the addition of heptose to the KDO-lipid A complex, the remainder of the reactions are readily characterized through the use of selective mutants.

The final components of the core are added sequentially using the appropriate nucleotide sugars. The only specificities in this series of reactions is that the proper activated sugars are available and that the required acceptor for the enzyme is available (50). The completed core acts as an acceptor for the O-specific polysaccharide, which is transferred as a polymer to the non-reducing terminus of the core-lipid conjugate.

The O-antigen is synthesized on an acceptor which differs from the core-lipid A conjugate. In studies with Salmonella typhimurium, it was found that the acceptor was a polyisoprenoid alcohol phosphate, termed the antigen carrier lipid (ACL) (51). It is on the ACL that the repeat unit of the polysaccharide is assembled. The nucleotide sugar diphosphates are required as the substrates of the
transferase enzymes. The intermediate formed in this reaction is the sugar-phosphate-ACL. Once the repeating unit is completed, it is transferred to a second molecule of the intermediate, thereby forming a dimer of the repeating unit bound to the ACL-phosphate.

Using pulse-chase labelling techniques, Robbins showed that the polymeric O-specific side chain was added to the non-reducing terminus of the newly formed repeat unit (52,53). Modifications of the repeating unit, acetylation or pyruvylation, occur after the repeat unit has been assembled, however, it is not known whether this occurs at the stage of the repeat unit monomer or polymer level or after the entire lipopolysaccharide has been assembled (54).

The LPS is synthesized on the inner cytoplasmic membrane (55). The completed molecule is then translocated, along with the other components of the outer membrane with concomitant assembly to form a functional membrane. The most recent model for the translocation of the lipopolysaccharide proposes transfer of the LPS to the outer membrane at zones of adhesion (56). These zones are said to be regions of contact between the cytoplasmic membrane and the outer membrane (56). The exact nature of the chemical and physical events responsible for initiating and promoting translocation and the factors responsible for its irreversibility are largely unknown.
III.3 Biological and Immunological Properties of the LPS

As was mentioned earlier, the LPS is thought to be one of the endotoxins produced by *P. aeruginosa*. However, the level of toxicity remains a matter of controversy. Of the toxicity that is ascribed to the LPS, the entire toxic response is due to lipid A. The antigenicity or immunogenicity of the complex is closely tied to the O-specific polysaccharide and is intimately associated with the repeating unit structure. Most of our understanding of the biological activity of the LPS has come from studies of *E. coli*, *S. typhimurium*, and *S. dysenteriae* (41,57). In addition to the deleterious effects of the endotoxic LPS, some beneficial effects have been observed. Administration of lipopolysaccharide preparations, especially using purified lipid A and lipid A analogues, necrosis of solid tumors has been observed (58) along with a mitogenic activity (59).

Earlier the vaccine developed by M. W. Fisher was discussed. While this vaccine did not fulfill all of the requirements of a good vaccine, it did show that the LPS could be used to induce hyperimmunity. Pseudogen induced formation of anti-*Pseudomonas* antibodies of the IgG class, while active *Pseudomonas* cultures induce IgM production (1). In terms of vaccine efficacy, this is an important observation. The purified LPS vaccine induced antibody formation of a class of antibodies with an intravascular half-life of 23 days, as compared to the half-life of IgM antibodies of 5.1 days. Alexander also showed that the IgG
antibodies formed were more effective than the same class produced by active immunization (60). The vaccine developed by Jones also stimulated antibody production, but produced antibodies of the IgM class similar to those produced by infection from external sources (61). Each year several additional or modifications of existing immunotherapy procedures are introduced, but none to date have provided the ideal immunotherapy procedure (62-64). All of these results show that one of the major biological activities of the lipopolysaccharide is the stimulation of the immune system of a host organism to produce antibodies. This property was the focus of much research into the production of new vaccines to prevent infections with *P. aeruginosa*. The problem of toxicity and short-terminated antibody production have yet to be adequately addressed. Combined approaches to immunotherapy have been proposed using a combination of vaccination and immunoglobulin therapy. Most promising in this field has been the development of monoclonal antibody technology to a point where it is now possible to isolate monoclonals to the seven Fisher immunotypes of *P. aeruginosa* (65). The solution to the prevention of *Pseudomonas* sepsis is the development of a suitable vaccine.
IV. Statement of the Problem

Until recently, the structures of the O-antigenic polysaccharides obtained from \textit{P. aeruginosa} have not been well characterized. In addition, without the structural information, attempts to produce new vaccines for the prophylaxis of infections caused by this organism met with little success. A knowledge of the chemical structure of the polysaccharides may lead the way to the production of a new generation of vaccines, that may have properties superior to those that have already been developed. In addition, with the knowledge of the repeating units of the O-antigens, it may be possible to prepare an immunoadsorbent to be used in the purification of anti-\textit{Pseudomonas} antibodies obtained from hyperimmune plasma or ascites fluid or continuous cell lines that produce immunotype specific monoclonal antibodies. The goals of this project were:

1. determination of the chemical structure of several of the O-antigens
2. identification of the linkage pattern and sequence of the O-antigens
3. develop a method to provide an immunoaffinity adsorbent suitable for the purification of antibodies raised to \textit{Pseudomonas aeruginosa}. 

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V. Purification of the Components of the Lipopolysaccharide of *P. aeruginosa*

V.1 Background

The first successful separation of lipopolysaccharides from *P. aeruginosa* was demonstrated by Boivin (66). This procedure involves the use of trichloroacetic acid to extract the carbohydrate containing components of the outer membrane and precipitate the cellular proteins. The aqueous extracts are treated with alcohol to precipitate the LPS. The LPS is redissolved in water, purified by dialysis and finally quantitatively purified through gel permeation chromatography.

A second method used to obtain bacterial lipopolysaccharides, developed by Westphal and his co-workers, utilizes hot monophasic aqueous phenol (67). The aqueous fraction obtained on cooling the mixture contains nucleic acids along with the LPS obtained from a cell extraction. A fairly complex work-up of this mixture results in a purified form of the LPS.

The lipopolysaccharides obtained from each of these procedures have apparent molecular weights of 100,000 daltons. The apparent molecular weight decreases in the
presence of deoxycholate, possibly indicating that the native LPS complex is aggregated in solution.

As mentioned previously, the O-antigen and core are covalently attached to lipid A through linkage to KDO. Because this linkage is extremely acid labile, it is possible to cleave lipid A from the polysaccharide by using dilute mineral acid (68). During the hydrolysis, the linkage between the core and O-antigen is also cleaved, providing a water soluble polysaccharide that can be further purified.

The O-antigen produced in this manner has been shown to be a homogenous, monodisperse polysaccharide of molecular weight range 14,000-27,000 daltons (69). These results simplified the study of these macromolecules by answering earlier questions of the polydispersity of the antigenic polysaccharides. The molecular weight information is vital to the design of new vaccines. Sadoff showed that a polysaccharide immunogen must have a molecular weight in excess of 20,000 daltons in order to elicit an immune response in humans (70). Thus it is evident that any potential immunogen developed in this laboratory must be coupled to high molecular weight carrier to provide the necessary size requirement.

The lipopolysaccharides used in this project were obtained from various strains of \textit{P. aeruginosa} that represent
the seven Fisher immunotypes. They were isolated by using a modified Boivin method developed at Parke-Davis Laboratories. The purified LPS preparations were donated by the Parke-Davis Company. The exact procedure for the LPS isolation and purification was reported by Haskell (71).

V.2 Separation and Purification of the components of the Lipopolysaccharides of *P. aeruginosa*

V.2.1 Hydrolysis of the Lipopolysaccharide

In this procedure, first described by Meadow (68), 500 mg of the LPS is dissolved in 1% aqueous acetic acid (600 mL). The solution is stirred and boiled under reflux for 90 minutes - 4 hours, depending on the LPS used. The completion of hydrolysis of the KDO linkages is determined by the cessation of precipitation of lipid A. The time of hydrolysis is variable, immunotype 1 LPS required four hours for complete hydrolysis, while that of immunotype six required only 90 minutes.

V.2.2 Separation of Lipid A and the Polysaccharides

The solution obtained from the hydrolysis is centrifuged at 8000 rpm for 20 minutes at 4℃ in a Sorvall refrigerated centrifuge. The supernatant solution is decanted, concentrated, and lyophilized to yield the core and O-antigen. The lipid A precipitate is dissolved in benzene, dried, and lyophilized.
V.2.3 Purification of the Core and O-antigen

The products obtained from the aqueous layer above are separated using gel permeation chromatography. From previous studies, it was determined that Bio-Gel resins provided the best separation of the components (72). The buffer chosen was a pyridine-acetic acid-water (10:4:986) system that appears to minimize irreversible adsorption onto the column. The sample containing the O-antigen and core is dissolved in the buffer and loaded onto a column 1.5 x 75 cm of Bio-gel P-30 (200-400 mesh) The column was eluted with the buffer and fractions were collected. The effluent of the column was monitored by differential refractometry.

This procedure provides for the base-line separation of the O-antigen from the core and low molecular weight degradation products. On Bio-Gel P-30, the O-antigen eluted after the void \( (V_e/V_o = 1.6) \). The core eluted considerably later at \( V_e/V_o = 5.3 \). The core could be fractionated further by using Bio-gel P-6 (200-400 mesh). Each of the fractions were pooled, concentrated, and lyophilized to yield white amorphous powders. From 200 mg of LPS, 60 - 80 mg of pure O-antigen could be obtained, with the exception of immunotype 6 where the total amount of O-antigen that could be obtained was 10 mg.

The actual weight percentages of O-specific
polysaccharide that could be obtained from the lipopolysaccharide were: immunotype 1, 36%; immunotype 5, 32%; immunotype 6, 10%; and immunotype 7, 33%. These values are in good agreement with the initial work conducted in our laboratory, except for the isolation of immunotype 6 (73). However, the earlier preparations contained considerable amounts of 2-amino-2-deoxygalactose which not found in the O-specific polysaccharide of immunotype 6. Therefore, it is probable that these early preparations contained core contaminants.

VI. Structural Studies of the O-antigens of *P. aeruginosa*

VI.1 Background

The structural studies of the lipopolysaccharide antigens was started several years ago in this laboratory by presenting a complete characterization of the gross chemical constitution of the lipopolysaccharide (72). These results were based on classical chemical methods and quantitated the amount of neutral hexose, amino hexose, phosphate, amino acid, fatty acid, and KDO present in the macromolecule. Since that first report, the structures of several of the antigenic polysaccharides have been reported (74,75). Shortly after these results were available for the immunotype 2 and
immunotype 5 O-antigens, a second group reported the structures of two O-antigens from strains of P. aeruginosa that were classified according to the Lanyi serological typing procedure (76,77). These two different strains, Fisher immunotype 2 and Lanyi O:7 and Fisher immunotype 5 and Lanyi O:2, are thought to be identical strains of P. aeruginosa. The structures reported for the O-antigen derived from the immunotype 2 organisms were nearly identical. However, the repeating unit structures for the O-antigen derived from immunotype 5 differed significantly from the structure proposed for the Lanyi O:2 O-antigen. This difference between the two structures was one of the targets of this investigation.

The amount of information available on the O-specific polysaccharide structures has been rapidly increasing. Of the seven Fisher immunotypes, the structures of the repeating units of four of the O-antigens have been established (74-80). Recently several new research groups have added information relative to the O-antigens of P. aeruginosa. These groups have reported the structures of the O-antigens for two different strains of P. aeruginosa that can not be placed into any of the Fisher immunotypes or Lanyi serogroups (81,82). Even with this tremendous surge in the available information, there are still large gaps in our understanding.
of the O-antigens and their structure. One of the remaining questions is whether similar serotypes or immunotypes can be placed into one classification determined by the structure of the chemical repeating unit of the O-specific polysaccharide. If this is the case, the structural information could provide a new classification scheme based on chemotyping.

The structural studies of four of the O-antigens were conducted by using classical analytical methods (83-85) and several newer techniques (86,87). These methods included both destructive and non-destructive techniques. The analysis began with the quantitation and identification of the monosaccharides present within the repeating unit of the O-specific side chain. Once the composition of the polysaccharide was known, it was possible to determine the linkage sequence and anomeric configuration of each sugar in the sequence.

Since the O-specific side chains have a high proportion of amino hexose and amino hexuronic acid, quantitative acid hydrolysis was not always possible. When this problem was encountered, techniques were borrowed from other fields of chemistry in order to arrive at the actual structure of the repeating unit. The most important step in this respect was the use of anhydrous hydrogen fluoride (HF) to depolymerize the polysaccharide, thus facilitating the analysis.
The most important aid to the study of the antigenic polysaccharides was the introduction of high field, high resolution nuclear magnetic resonance (nmr) facilities. This permitted $^1$H nmr spectroscopy at fields of 200, 300, and 500 MHz and $^{13}$C nmr spectroscopy at fields of 50, 75, and 125 MHz.

Combining all of these techniques, it was possible to determine the structures of the repeating units for the O-specific polysaccharides of four of the Fisher immunotypes of *P. aeruginosa*. 
VI.2 Nuclear Magnetic Resonance Spectroscopy

VI.2.1 Introduction

Proton ($^1$H) and carbon ($^{13}$C) nmr spectroscopy were used primarily to determine the size of the chemical repeating unit, the anomeric configuration of the sugars within the repeating unit, and the type of monosaccharides that could be found in the repeating unit and their extent of derivatization. This information is readily obtained from these spectra. In the $^1$H nmr spectrum of a sugar having an equatorial C-2 substitent, a general rule is that the H-1 proton of an alpha linkage resonates at a relatively low field (4.8-5.1 ppm) and is characterized by a narrow coupling constant ($J_{1,2} = 1-2$ Hz). When a beta linkage is encountered, the chemical shift of the signal appears at higher fields (4.4-4.7 ppm) and has a relatively large coupling constant ($J_{1,2}=6-10$ Hz). Other obvious features of the $^1$H nmr spectrum are the methyl protons of 6-deoxy hexoses that can be found at 1.2-1.3 ppm, methyl protons of acetate esters at 2.2-2.3 ppm, and the protons of acetamido groups at 2.0-2.2 ppm. Several reviews of $^1$H nmr spectroscopy, as related to oligo- and polysaccharides are available (88,89).
The interpretation of the $^{13}_{\text{C}}$ nmr spectrum of polysaccharides is much easier than that of proton spectra. The majority of information about the chemical repeating unit is obtained from the $^{13}_{\text{C}}$ spectrum. The fully proton decoupled spectrum provides the most information. Several regions of the spectrum can be identified that are characteristic of various types of carbons that can be found in the polysaccharide. The most important region is the anomeric region. The signals of the C-1 atoms can be seen between 95 and 105 ppm. Carbons involved in an alpha linkage tend to resonate at a higher field, 95-100 ppm, and those involved in beta linkages resonate at lower field, 100-105 ppm. Another identifiable region contains the signals arising from the carbons of the methyl groups of 6-deoxy hexoses and acetyl groups, 16-18 and 20-22 ppm respectively. A third region shows the signals of carbonyl carbons, 172-176 ppm. A clearly separated region contains the signals of carbons bearing nitrogenous substituents, 48-54 ppm. The final region of interest shows the presence of neutral hexose through the signal for the hydroxy-methyl carbon of the hexose, 60-62 ppm. From this list of well defined regions of the spectrum, it is possible to obtain a great deal of information in a relatively short period of time.
Additional information can be obtained from the inverse gated proton coupled carbon spectrum. In this technique, the proton coupled spectrum is recorded with full nuclear Overhauser enhancement (n.O.e.). This is done by pulsing the decoupler frequency. The decoupler power is turned off during the frequency pulse and the acquisition of data. The decoupler is "gated" on during a relaxation delay programmed into the pulse sequence. If the relaxation delay is chosen so the delay is five times the relaxation time of the slowest relaxing species, a one bond, proton coupled spectrum with full n.O.e. is obtained.

The major portion of the spectrum that provides meaningful information is the anomeric region. The one bond carbon-hydrogen coupling constants, $J_{\text{C}1,\text{H}1}$, has been shown to be approximately 170 Hz in an alpha linkage and 160 Hz or less in a beta linkage (90). The field of $^{13}$C nmr spectroscopy has recently been extensively reviewed (91, 92).

As presented, these rules and trends represent a collection of empirical observations and are not meant to be deemed as absolutes. As with most rules there are nearly as many exceptions as there are instances that follow the observed trends. As a result, the interpretation of $^1$H and $^{13}$C nmr spectra must be carefully considered and weighed with all of the available information.
VI.2.2 $^1$H NMR Spectroscopy

Proton spectra were recorded at 200, 300, and 500 MHz using Bruker WH-200 and WH-300 nmr spectrometers. In all experiments dealing with oligosaccharides or polysaccharides dissolved in deuterium oxide, the spectra were recorded at a probe temperature of 343 K. The higher temperature was used to reduce line broadening and to shift the HOD resonance to a higher field (4.26 ppm) which removes the resonance from the anomeric region of the spectrum. All chemical shifts reported are relative to an external standard of 1,4-dioxane in deuterium oxide (D$_2$O). In a typical experiment, 10 mg of the O-antigen was dissolved in 0.5 mL of D$_2$O (100% isotopic purity). Each sample was exchanged three times with 99.8% D$_2$O This was done by dissolving the polysaccharide in 99.8% D$_2$O followed by lyophilization of the solution. This process was repeated two times and succeeded in minimizing the HOD resonance in the spectrum. After the final dissolution in 100% D$_2$O, the spectrum was recorded.
VI.2.3 $^{13}$C NMR Spectroscopy

Carbon spectra were recorded at 50, 75, and 125 MHz using Bruker WH-200, WH-300, and Nicolet NC-500 nmr spectrometers. For each experiment, 100-120 mg of the sample were dissolved in 2 mL of 99.8% D$_2$O. A broad-band proton decoupled spectrum of each polysaccharide was obtained at ambient probe temperature (309 K). The proton coupled spectra, using the the Nicolet instrument, were obtained using a gated decoupling procedure which produces the one-bond coupled spectrum with a concomitant nuclear Overhauser enhancement (n.O.e.) of each of the signals. The relaxation delay used in these experiments was 1.0 seconds. In order to obtain a spectrum with acceptable signal to noise ratio, 60000 scans were required at 50 MHz while 10000 scans were necessary at 75 MHz, and only 5000 scans were required at 125 MHz. The carbon chemical shifts are reported to an external reference sample of 1,4-dioxane in D$_2$O (singlet at 67.4 ppm).
VI.3 Determination of the Monosaccharide Composition

VI.3.1 Introduction

The initial step in the chemical analysis of the O-specific polysaccharide is the identification of the individual monosaccharides that are found in the polysaccharide. The primary way that this is done is to effect a total hydrolysis of the polysaccharide and an analysis of the resultant mixture of monosaccharides. The identification of the monosaccharides is carried out by using two independent methods. The methods chosen for this analysis were paper chromatography and gas-liquid chromatography (g.l.c.). In each separation method the mobility of each component is compared to that of known reference compounds.

In addition to the identification of each component, the quantitation of these is also important in order to determine the size of the repeating unit of the polysaccharide. Thus it is necessary to assure total hydrolysis of the polysaccharide and quantitative recovery of the hydrolysate components. While quantitation in this manner is not as crucial as it was perhaps a decade ago, because of the availability of high resolution nmr instruments, it is still important as a secondary confirmation of the size of the repeating unit of the polysaccharide.
VI.3.2 Acid Hydrolysis of the O-chain and Preparation of Alditol Acetates

In previous studies from this laboratory it was shown that a two-step hydrolytic procedure was the most effective method of depolymerizing the polysaccharide (72). One of the first proponents of this method was Lindberg (93).

In a typical experiment, 5 mg of the O-antigenic polysaccharide is dissolved in 5 ml of 87-90% formic acid and heated at 100 C for one hour. The solution is then evaporated in vacuo in a rotary evaporator (bath temperature <40 C). The residue remaining is dissolved in 5 ml of 2M trifluoroacetic acid and heated at 100 C for 4 hours. After the hydrolysis is complete the solution is evaporated to dryness in a rotary evaporator. The monosaccharide fraction is dissolved in M ammonium hydroxide, a small portion of which is saved for paper chromatography. To the ammoniacal solution, 2 mL of 2% sodium borohydride in dimethyl sulfoxide is added. The reduction is carried out for 90 minutes at 50 C. When the reduction is complete, the excess borohydride is decomposed by dropwise addition of glacial acetic acid. The final step in the reaction sequence is the acetylation of the alditols. This is accomplished by the addition of 0.2 mL of 1-methylimidazole to the reduction solution. This is followed by 2.0 mL of freshly distilled acetic anhydride. The reaction is complete after 60 minutes at room temperature (94).
The acetylation reaction is stopped by addition of the mixture to 5 mL of water. The fully acetylated alditols are extracted into dichloromethane (5 mL). The layers are separated and the organic layer is washed three times with deionized water (3 x 5 mL). The organic layer is then dried by storage over anhydrous sodium sulfate. When the water has been removed from the solution, the drying agent is removed by filtration through glass wool and the solution is concentrated in vacuo with the bath temperature below 40 C. At this time the sample is ready for analysis by g.l.c. and g.l.c.-m.s.

If the chromatogram shows the presence of a large number of contaminants, it is possible to purify the mixture of peracetylated alditols by using micro silica gel columns. The column is prepared by placing a glass wool plug into a Pasteur pipet and adding 0.5 g of silica gel to the column. The mixture, dissolved in dichloromethane, can be adsorbed on the columns and washed with dichloromethane. After the impurities have been removed, usually after 10 mL of solvent have been passed through the column, the alditol acetates can be recovered by changing the solvent to ethyl acetate. This method provides for a clean fraction for g.l.c. analysis.
VI.3.3 Gas-liquid Chromatography

The mixture of alditol acetates can be separated on a glass column (6' x 1/8" i.d.) which is packed with 3% OV-225 on GasChrom Q (Supelco, Inc., Bellefonte, Pennsylvania) using a Hewlett-Packard 5720A gas chromatograph equipped with a flame ionization detector. The carrier gas used was helium at a flow rate of 30 mL / minute. The separations were carried out isothermally at 220°C (inlet temperature: 265°C and detector temperature: 275°C). For quantitation purposes, the peak areas were determined planimetrically.

The peak areas were corrected for detector response differences by applying the following correction factors: 6-deoxy hexoses, 0.95; neutral hexose, 1.0; 2-amino-2,6-dideoxyhexose, 1.2; and 2-amino-2-deoxyhexoses, 1.65 (73). These factors permitted the accurate determination of the molar ratios of components found in the samples of the O-specific polysaccharides.

The identity of the components was determined by comparison of the retention times of the unknown alditols to those of known alditols. A secondary confirmation of the assignments was possible by obtaining the fragmentation pattern of each component. The mass spectrum will provide information relative to the class of alditol, but it can not address the configuration of the parent sugar. The conditions used in the mass-spectroscopic analysis will be discussed in section VI.4.3.
VI.3.4 Paper Chromatography

Generally the g.l.c. analysis of the hydrolysate of the polysaccharide is sufficient to identify the sugars that can be found in the repeating unit. However, in polysaccharides that contain glycuronic acids or amino glycuronic acids, the analysis will be incomplete due to the low volatility of these derivatives. As a result an alternative procedure must be used to identify these components that are not visible to g.l.c. separations. Paper chromatography provides a sensitive method for doing this.

In this procedure, a small portion of the hydrolysate which has been dissolved in M ammonium hydroxide, is spotted onto Whatman 1 filter paper sheets (14 x 35 cm). On the same sheet, reference samples are spotted alongside the spot of the unknown mixture. The paper is developed by using the descending solvent technique. The solvent system that provided the best separation was the following mixed buffer system: ethyl acetate, pyridine, acetic acid, water; 5:5:1:3 (v/v). When the solvent front has moved to within 1-2 cm of the edge of the paper, the chromatogram is removed and dried. For each sample two chromatograms are developed. The chromatograms are sprayed with two different detecting agents. Any amino sugars present are detected by spraying the chromatogram with ninhydrin followed by heating at 105 C for
10 minutes (95). The remainder of the sugars are detected with alkaline silver nitrate (96). The relative mobilities of selected representative monosaccharides are shown in Table 1.

In addition to the analytical function of paper chromatography, it is possible to preparatively isolate the components of the polysaccharides. In this procedure 20-30 mg of the polysaccharide are hydrolyzed. After the acid has been removed, the residue is dissolved in a minimum amount of water. The solution is applied to Whatman 3MM filter paper to which a 10 cm strip of Whatman 1 has been sewn. This leader of Whatman 1 provides for better solvent flow. The chromatogram is developed in the same manner as was described for the analytical separation. To determine the location of the sugars, guide strips are cut from the edges of the dried chromatogram. The strips are detected with the two detection methods shown above. When the positions of the components have been detected, these areas are removed. The sugars can then be recovered by eluting the section with de-ionized water.

VI.3.5 Determination of Absolute Configuration

Once the identity of the monosaccharides have been determined, it becomes necessary to assign their absolute configuration. This is done by determining the optical rotation of a solution of each monosaccharide. The values
Table 1: Relative mobilities of selected derivatives on paper chromatograms.
<table>
<thead>
<tr>
<th>Sugar</th>
<th>Rg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>1.00</td>
</tr>
<tr>
<td>Mannose</td>
<td>1.10</td>
</tr>
<tr>
<td>Galactose</td>
<td>0.96</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>1.28</td>
</tr>
<tr>
<td>Xylose</td>
<td>1.16</td>
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<tr>
<td>N-Acetyl glucosamine</td>
<td>1.21</td>
</tr>
<tr>
<td>N-Acetyl galactosamine</td>
<td>1.08</td>
</tr>
<tr>
<td>N-Acetyl fucosamine</td>
<td>1.26</td>
</tr>
<tr>
<td>N-Acetyl quinovosamine</td>
<td>1.39</td>
</tr>
<tr>
<td>Glucosamine·HCl</td>
<td>0.82</td>
</tr>
<tr>
<td>Galactosamine·HCl</td>
<td>0.74</td>
</tr>
<tr>
<td>Fucosamine·HCl</td>
<td>0.86</td>
</tr>
<tr>
<td>Quinovosamine·HCl</td>
<td>1.07</td>
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<tr>
<td>Glucuronic acid</td>
<td>0.60</td>
</tr>
<tr>
<td>Galactosaminuronic acid</td>
<td>0.38</td>
</tr>
</tbody>
</table>

$R_f$ monosaccharide
$R_g = R_f$ Glucose

Solvent: ethyl acetate/acetic acid
water/pyridine, 5:1:3:5
obtained for the specific rotation of the sugar of unknown configuration can then compared to the specific rotation of pure compounds and the configurations can be determined.

In a typical experiment, a 1% solution of the monosaccharide in water is placed into a polarimeter cell with a 1mL volume and a 1 dm path length. The optical rotation of the solution is recorded at 25°C at a wavelength of 589 nm in a Perkin-Elmer 141 spectropolarimeter. By using the procedure as outlined here, the specific rotation can be calculated from the observed rotation by multiplying the reading by a factor of 100.
VI.4 Methylation Analysis

VI.4.1 Introduction

Once the composition of the polysaccharide is known, it is necessary to determine the linkage sequence of the monosaccharides within the polymer. The most accurate way of doing this is through methylation analysis. Several methods have been developed, but the most convenient and successful procedure is based on a modification of the Hakomori methylation (97). The Hakomori procedure was the first technique that was introduced that produced fully methylated polysaccharides in one step (98). The modified procedure provides for a reaction that produces fewer side products and in addition can be performed more rapidly. Once the methylated polysaccharide is obtained, it is possible to hydrolyze it and prepare partially methylated alditol acetates. These derivatives are ideally suited for g.l.c.-m.s. separation and characterization. The fragmentation patterns of these derivatives can be used to determine the positions of the methyl and acetyl groups and thus the positions through which the monosaccharides are linked to each other. The retention times of the derivatives also can confirm their identity. The fragmentation patterns and relative retention times of reference compounds were
VI.4 Modified Hakomori Methylation

VI.4.1 Introduction

This procedure, described by Phillips and Fraser, uses potassium dimethylsulfinyl carbanion in place of the sodium salt of the same anion as the deprotonation agent in the methylation (97). The solution of the carbanion can be prepared very easily in the following manner. The most important facet of this preparation is that all of the equipment and reagents must be scrupulously dry. Potassium hydride, 17.7 g of a 23.2% dispersion in oil, is weighed into a three-necked 500 mL round bottom flask, equipped with a large magnetic stir bar. A pressure equalizing addition funnel, gas inlet and outlet are placed into the flask. During the extraction of the oil and preparation of the carbanion, a stream of dry nitrogen gas is passed through the assembled apparatus. The oil is removed from the suspension by washing 8-10 times with 30 mL portions of dry hexane. After the final hexane wash is removed, the potassium hydride is allowed to dry in the nitrogen stream for 1-2 hours at room temperature. When the off-white potassium hydride is dry and powdery, dry dimethyl sulfoxide (Me₂SO, 40 mL) is slowly

compiled and meticulously catalogued by Bengt Lindberg and his co-workers (99).
and cautiously added dropwise to the powder with stirring. After the initial vigorous reaction subsides and hydrogen evolution slows, the rate of Me$_2$SO addition can be increased to 5 mL/minute. When all of the Me$_2$SO has been added to the solution, it should be a faint green color; a brown color indicates that either the materials were wet or the oil was not completely removed. The solution of dimethyl sulfinyl carbanion in Me$_2$SO at a concentration of 2 M, can be stored under nitrogen at 0°C for several months. When thawed, this solution can be used directly in the methylation analysis.

In a typical methylation reaction, 5 mg of the polysaccharide were dissolved in dry Me$_2$SO which was freshly distilled from calcium hydride. The solution was placed under a slow stream of nitrogen in a reaction flask. Dissolution was facilitated by placing this solution in an ultra-sound bath for 30 minutes. To this solution was added 5.0 mL of dimethyl sulfinyl carbanion in Me$_2$SO. The reaction was allowed to proceed for 16-18 hours at room temperature. After this reaction period, the solution was cooled to 4°C and was methylated with methyl iodide (5.0 mL, introduced by syringe). Upon addition of the methyl iodide, the solution was allowed to warm to room temperature and subsequently was placed into an ultra-sound bath for 30 minutes. To ensure complete reaction, the solution was stirred at room
temperature for 2-4 hours after sonication. The final dark red solution is carefully transferred to dialysis membranes, SpectraPor nominal molecular weight cut-off, 1500 daltons, and dialyzed against deionized water for 12-16 hours. The retentate was lyophilized and prepared for the final steps of the analysis.

The methylated polysaccharide is successively hydrolyzed, reduced, and acetylated using the procedures described in sections VI.3.2 and VI.3.3. The mixture of partially methylated alditol acetates can be purified by using micro silica gel columns. The purified residue from the ethyl acetate fractions can be subjected to g.l.c./m.s. analysis.

VI.4.3 Gas-liquid Chromatography/Mass Spectroscopy

All mixture analyses were obtained using a Finnigan 4021 g.l.c./m.s. system. In this system the stationary phase used for the separation was also 3% OV-225 on GasChrom Q. An ionization potential of 70 eV was used in the electron impact mode. The acceleration voltage applied to the sample was 8000 volts. When necessary, chemical ionization was conducted with ammonia as the reagent gas. The detection system for the Finnigan system is of the quadrupole design.
In the separation of the partially methylated alditol acetates, the oven temperature was not an isothermal program. Rather it was found that the following temperature program improved resolution. The initial oven temperature was 170°C. After an eight minute temperature hold after sample injection, the oven temperature was increased linearly at a rate of 8°C/minute. The retention times of the major components of the *P. aeruginosa* O-antigens are shown in Table 2. In fragmentation, each of these derivatives produce several distinct fragments which aid in the identification of the structure. These characteristic fragments are shown in Table 3. The results of the methylation analyses for each of the polysaccharides studies are given in the results section.

VI.4.4 Non-alkaline Methylation Reactions

VI.4.4.1 Introduction

In an earlier section, it was mentioned that many of the bacterial O-specific polysaccharides are derivatized with acyl groups involved in ester linkages. During the strongly alkaline conditions of the Hakomori methylation, these groups are quantitatively removed. This prohibits the identification of the sites of esterification during this methylation procedure. In order to be able to determine the position of these derivatives, several procedures have been
Table 2: Relative retention times of selected derivatives on 3% OV-225 on GasChrom Q.
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<td>3,4-di-$Q$-methyl rhamnose</td>
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<td>2,3-di-$Q$-methyl rhamnose</td>
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<td>2-(N-methyl)acetamido-2,6-dideoxy-4-$Q$-methyl glucose</td>
<td>3.57</td>
</tr>
<tr>
<td>2-acetamido-2-dideoxy-3,6-di-$Q$-methyl galactose</td>
<td>3.49</td>
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</table>

$^1$Based on separation on 3% OV-225
Table 3: Primary fragment ions for selected representative partially methylated alditol acetates and alditol acetates.
| Monosaccharide derivative                                                                 | 101 | 102 | 113 | 115 | 117 | 129 | 131 | 144 | 157 | 159 | 161 | 170 | 175 | 187 | 189 | 200 | 201 | 205 | 217 | 233 | 239 | 261 | 288 |
|----------------------------------------------------------------------------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 1,2-di-O-acetyl-6-deoxy-2,3,4,6-tetra-O-methyl hesitol                                  |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 1,2,5-tri-O-acetyl-6-deoxy-2,3,4,6-tetra-O-methyl hesitol                              |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 1,4,5-tri-O-acetyl-6-deoxy-2,3,4,6-tetra-O-methyl hesitol                              |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 1,2,3,5-tetra-O-acetyl-6-deoxy-4-O-methyl hesitol                                      |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 1,2-di-O-acetyl-2,3,4,6-tetra-O-methyl hesitol                                         |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 1,2,5-tri-O-acetyl-2,3,4,6-tetra-O-methyl hesitol                                      |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 1,4,5,6-tetra-O-acetyl-2,3-di-O-methyl hesitol                                         |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 2-acetamide-1,2,5-tri-O-acetyl-2,3,4,6-tetra-O-methyl hesitol                          |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 2-([R-methyl]acetamide-1,2,5-tri-O-acetyl-2,3,4,6-tetra-O-methyl hesitol               |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 2-acetamide-1,4,5-tri-O-acetyl-2,3,4,6-tetra-O-methyl hesitol                          |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 6-deoxy hesitol pentamucetate                                                         |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| hesitol hexamucetate                                                                |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 2-acetamide-2,6-dideoxy hesitol tetramer                                              |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
developed. The first of the two to be discussed involves the formation of mixed acetals between methyl vinyl ether and free hydroxyl groups on the polysaccharide (100). The acetalated polysaccharide is then subjected to the standard Hakomori methylation. The second procedure makes use of a more modern methylating agent, methyl trifluoromethylmethane-sulfonate, also known as methyl triflate (101).

In each of these methods, the results of the non-alkaline methylation are compared to the normal methylation results and any differences in substitution patterns indicate the presence of acyl or base-labile groups.

VI.4.4.2 Acetalation with Methyl Vinyl Ether

The dried O-antigen, 30 mg, is dissolved in 4 mL of dry DMSO and 30 mg of p-toluenesulfonic acid are added. The solution is cooled to 15 C and methyl vinyl ether (5 mL), condensed at -78 C, are added to the mixture. The solution is stirred at 15 C for five hours. After the reaction is complete, the red solution is applied to a Sephadex LH-20 column (2.6 x 30 cm) which is eluted with anhydrous acetone. The acetalated polysaccharide elutes in the void volume of this column and is recovered from the acetone on evaporation in vacuo (bath temperature<35 C) as a light oil. The oil is dissolved in dry DMSO and subjected to the modified Hakomori procedure described previously.
VI.4.4.3 Methylation with Methyl Triflate

The O-specific polysaccharide, 5 mg, is dispersed into 1.0 mL of trimethyl phosphate to which 0.2 mL of 2,6-di-t-butylpyridine is added as a proton scavenger. The dispersion is placed into a sonic bath and reacted for four hours at 50 C. After one hour 0.2 mL of methyl triflate is added to the solution. After four hours of sonication, the solution is placed into dialysis membranes (nominal molecular weight cutoff, 1500) and dialyzed against deionized water. The retentate is lyophilized and subjected to hydrolysis, reduction and acetylation as described previously.

VI.5 Additional Chemical Methods

VI.5.1 Introduction

During the structure analysis of the O-specific polysaccharides, several different chemical methods were required to obtain the final structure. Each different O-chain presents unique problems. The techniques described in this section were not required in each study. The results of these reactions will be discussed in later sections.

VI.5.2 Periodate oxidation/Smith Degradation

The polysaccharide, 50 mg, or reduced oligosaccharide, 25 mg, is dissolved in 10 mL of 0.2% aqueous sodium periodate solution. The solution is stirred in the dark at 4 C for 72
hours. After the oxidation is complete the reaction is quenched with an equimolar amount of ethylene glycol, approximately 13 µl, and the solution is stirred for an additional hour. The oxidized polysaccharide is immediately treated with an excess of sodium borohydride, 50 mg, for 2 hours at room temperature. The excess of borohydride is decomposed by the dropwise addition of glacial acetic acid (if no effervescence is observed an additional 25 mg of sodium borohydride is added and the reduction is continued for another hour). The reduced, degraded polysaccharide is dialyzed against de-ionized water. After dialysis, the retentate is lyophilized. The purified polysaccharide is hydrolyzed under mild conditions to effect cleavage of the non-cyclic acetal linkages. This is done by dissolving the degraded polysaccharide in 10 mL of 0.2 M hydrochloric acid. The hydrolyzate is evaporated to dryness in the presence of 1-propanol. The residue is next dissolved in pyridinium acetate buffer and chromatographed on a Bio-gel P-2 column (1.6 x 40 cm). The carbohydrate fractions are recovered and lyophilized. The oligosaccharides obtained in this manner are then characterized by the same methods as are used for the intact polysaccharide.
VI.5.3 Carboxyl Reduction (87)

In a typical experiment, 20 mg of the polysaccharide is dissolved in 5 mL of de-ionized water with the pH being adjusted to 4.75 by the addition of 10 mM HCl. The solution is stirred at room temperature. In small increments, a total of 30 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) are added while maintaining the pH at 4.75 by the addition of 10 mM hydrochloric acid. After the EDC has been added and no further hydrogen ion uptake is observed, 75 mg of sodium borohydride are slowly added maintaining the pH between 6.5-7.0. After 2 hours at room temperature, the excess of borohydride is decomposed by dropwise addition of glacial acetic acid. Caution must be exercised at this point to prevent excess foaming. The dialyzed against deionized water and lyophilized.

VI.5.4 Depolymerization by Anhydrous Hydrogen Fluoride

The use of anhydrous hydrogen fluoride was required in the analysis of several of the O-antigenic polysaccharides. These O-chains contained either acid sensitive components readily destroyed by mineral acid or residues that were intractable toward acid hydrolysis.

After the polysaccharide had been extensively dried in vacuo in the presence of phosphorus pentoxide, the sample of 50-100 mg of the O-antigen is transferred to a 30 mL
polypropylene screw cap bottle. Anhydrous hydrogen fluoride, 25 mL condensed at -78 C, is added to the sample and the bottle is sealed. The solution kept at room temperature for three hours. After this time, the cap is removed and the hydrogen fluoride is permitted to evaporate under mild aspiration. The residue remaining is dissolved in pyridinium acetate buffer and chromatographed on Bio-gel P-2 and is eluted with pyridinium acetate buffer. The carbohydrate containing fractions are recovered and lyophilized.

While the handling of anhydrous hydrogen fluoride can be hazardous, this procedure provides for the ready depolymerization of polysaccharides with little degradation.
VI.6 Results

VI.6.1 Immunotype one

The first step in the analysis of this polysaccharide was nuclear magnetic resonance spectroscopy. The 300 MHz $^1$H nmr spectrum, Figure 3, shows the following structural details. It is evident from the signal at 1.25 ppm that the repeating unit contains 6-deoxyhexoses. In addition, the high field region shows the presence of both N- and O-acetyl groups by the presence of methyl proton signals at 2.00, 2.02, and 2.05 ppm. The signal at 2.05 ppm is most probably attributable to the O-acetyl derivatives. One additional signal is seen in this region. The signal at 2.21 ppm was previously thought to be a signal due to the presence of a deshielded methyl group from an acetate ester or amide. Its position, however contradicted this assignment. In addition this signal disappears upon treatment of the O-antigen with aqueous triethylamine. As will be shown later in this discussion, this signal can be attributed to the 2-methyl group of an imidazoline-type ring system found in association with a 2,3-diamino-2,3-dideoxymannopyranuronic acid residue in the repeating unit.

The next region of interest is that containing the sharp singlet at 8.17 ppm. The only protons reported to
Figure 3: 300 MHz $^1$H nmr spectrum of the O-specific polysaccharide of immunotype 1.
resonate at this low field are the protons associated with formyl groups.

The final region of interest is the anomeric region, 4.8-5.2 ppm. In this region, there appear to be four signals: 4.82 ppm ($J_{1,2'}$ 9 Hz) and 4.94, 5.05, and 5.14 ppm ($J_{1,2'}$ low). One of these signals could also be due to an incompletely exchanged carboxyl group of a glycuronic acid, 5.2 ppm. From the signals in the anomeric region, it appears as if the polysaccharide has a tetrasaccharide repeating-unit. From this spectrum, it is possible to tentatively assign one beta linkage and three alpha linkages.

The size of the repeating unit is confirmed by $^{13}$C nmr spectroscopy. The 75 MHz $^{13}$C nmr spectrum, Figure 4, shows the presence of four distinct anomeric carbon atoms, 97.6, 99.0, 99.6, and 99.8 ppm. The nature of the anomeric configuration of each of these residues was confirmed by obtaining the gated proton coupled spectrum of the polysaccharide. The anomeric region of the 125 MHz spectrum is shown in Figure 5. The spectrum provides information relative to the one bond carbon-hydrogen coupling constants associated with these carbon atoms. The constants obtained in this manner show that three of the carbons have $^{1}J_{C1-H1}$ values of 170 Hz or greater. The remaining coupling constant has a value of 154 Hz, indicative of beta linkages.
Figure 4: 75 MHz $^{13}$C nmr spectrum of the O-specific polysaccharide of immunotype 1.
Figure 5: 125 MHz $^{13}$C gated, proton coupled nmr spectrum of the O-specific polysaccharide of immunotype 1, anomeric region expansion.
The broad band decoupled spectrum provides additional information regarding the structural features found in the polysaccharide. In the region between 50 and 58 ppm, there appear three signals showing the presence of three carbons bearing nitrogenous substituents. The presence of two different 6-deoxyhexoses is confirmed by the signals at 17.5 and 18.0 ppm. In addition, it can be seen that the repeating unit contains both N-acetyl and O-acetyl groups. This is due to the presence of the signals at 23.1 and 23.4 ppm. These derivatives can also be seen from the carbonyl signals at 173.9, 174.5, 175.2, and 175.4 ppm.

Another interesting feature of the spectrum are the two signals at 31.1 and 168.8 ppm. From recent studies (76-80), it seems apparent that these resonances arise from the 2,3-imidazoline ring system described previously. These same resonances have been found in two other strains of P. aeruginosa corresponding to immunotype 3 and 7. These signals can be removed on treatment of the polysaccharide with 5% aqueous triethylamine and new signals can be seen in the carbonyl region and the region of N-acetyl methyl carbons. These observations are consistent with the ring system proposed, since it is expected that a 1-acetyl-2-methyl-imidazoline ring would be subject to solvolysis.

One final signal in the carbon spectrum requires
further clarification. The signal at 165.5 ppm has been assigned to the carbonyl carbon of a formyl derivative. The assignment was confirmed when this signal appeared as a sharp doublet, $^{1}J_{C1-H1} = 194$ Hz, in the gated decoupled spectrum. This is consistent with the assignment of this signal to a formamide group on the repeating unit, since a formate ester would be expected to have a coupling constant of 210-220 Hz while formamides have coupling constants of 190-196 Hz (102).

Thus from the results of the nmr experiments, it is evident that the repeating unit is composed of a tetrasaccharide which contains 6-deoxyhexose, hexose and aminohexose. It is also found derivatized with both N- and O-acetyl groups and at least one N-formyl group.

The nmr spectra provided general information about the repeating unit of the O-antigen. The next logical step in the analysis of the polysaccharide is the determination of the sugars present and their relative amounts. Classical sugar analysis revealed three sugars: glucose, rhamnose, and quinovosamine (2-amino-2,6-dideoxyglucose). Isolation of these sugars enabled the determination of their specific rotations. The following rotations were obtained: rhamnose, $+8.1$; glucose, $+52.9$; quinovosamine, $+46.5$. These values are in good agreement with the accepted values for the following pure sugars: $L$-rhamnose, $+8.2$; $D$-glucose, $+52.7$;
\[ \text{\(\alpha\)-quinovosamine, } + 47.8. \text{ All rotations were measured by using one percent aqueous solutions of the sugars and a } 1 \text{ dm cell.} \]

In order to determine the presence of non-volatile components in the repeating unit, the hydrolysate was separated by paper chromatography. The analysis of the components revealed the same three sugars described above and a fourth component that was apparently degraded during the hydrolysis step. The mobility of this component was 0.2 relative to glucose. The unknown component was reactive to ninhydrin and alkaline silver nitrate. Attempts to isolate this component after acid hydrolysis were unsuccessful.

Methylation analysis of the polysaccharide revealed four major components. The components were identified from their fragmentation patterns obtained from their mass spectra, Table 4. The identity of the components were the following: \(1,5\text{-d}\alpha\text{-acetyl-6-deoxy-2,3,4-tri-}\beta\text{-methylmannitol, 1,2,5-tri-}\beta\text{-acetyl-6-deoxy-3,4-di-}\beta\text{-methylmannitol, 1,4,5-tri-}\beta\text{-acetyl-2,3,6-tri-}\beta\text{-methylglucitol, and 2-acetamido-1,3,5-tri-}\beta\text{-acetyl-4-}\beta\text{-methyl glucitol.} \)

This indicates that the glucose residues are linked in a \(1\rightarrow4\) fashion, the rhamnose residues in a \(1\rightarrow2\) fashion and the quinovosamine residues in a \(1\rightarrow3\) fashion. No other methylated derivatives were found in the mixture. The unknown component remained unidentified at this point.
Table 4: Mass spectral data for the methylation analysis of the O-specific polysaccharide of immunotype 1.
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I : 1,5-di-0-acetyl-6-deoxy-2,3,4-tri-Q-methyl hexitol  
II : 1,2,5-tri-Q-acetyl-6-deoxy-3,4-di-Q-methyl hexitol  
III : 1,4,5-tri-Q-acetyl-2,3,6-tri-Q-methyl hexitol  
IV : 2-(N-methyl)acetamido-2,6-dideoxy-4-Q-methyl hexitol  
V : 1,4,5,6-tetra-Q-acetyl-2,3-di-Q-methyl hexitol
If the results of the structural work at this time are combined, it seems apparent that the missing component of the repeating unit must be a diaminohexose and possibly a diaminohexuronic acid. If it was the latter compound, its acid instability would be explained. Because this fourth component was acid sensitive, it was necessary to develop a new method to depolymerize the polysaccharide. The method chosen was depolymerization by anhydrous hydrogen fluoride solvolysis.

This procedure appeared to work very well. Upon purification of the mixture on Bio-gel P-2, one major component was obtained prior to the salt and monosaccharide fractions. The elution occurred at a $V_e/V_0$ value of 2.2, corresponding to a molecular weight of approximately 350 daltons. The $^{13}$C nmr spectrum of this fraction suggests the presence of a disaccharide. From the spectrum it can also be seen that the spectrum arises from an equilibrium mixture of the two anomeric forms of a reducing disaccharide (Figure 6). In the spectrum there are signals for 6-deoxyhexose, 17.3 ppm, two N-acetyl methyl carbons, 22.7 and 23.0 ppm, two carbons bearing nitrogen, 50.1 and 53.4 ppm, and three anomeric signals, 91.8, 95.4, and 99.1 ppm. In addition there are three distinct carbonyl signals and a total of twelve signals in the region bounded by 66 and 76 ppm. The spectrum shows
Figure 6: 50 MHz $^{13}$C nmr spectrum of the disaccharide obtained from the anhydrous hydrogen fluoride depolymerization of the O-specific polysaccharide of immunotype 1.
that the non-reducing terminus is probably the rhamnose residue. This leaves the identity of the remaining sugar to be a diaminohexuronic acid.

In order to simplify the analysis of the disaccharide, it was reduced and subjected to periodate oxidation and subsequent borohydride reduction. The glycoside obtained was analyzed in two parts. The first portion of the glycoside was methylated and subjected to chemical ionization mass spectrometry (c.i./m.s.). The c.i. mass spectrum is shown in Figure 7. The molecular ion is seen at m/z 452. The structure of the fragment and the major fragmentation pathways are shown in Figure 8. The major fragments are seen at m/z 77, 350 and 375. The addition product between dimethylsulfinyl carbanion has been observed previously in the methylation of uronic acids in the presence of excess anion (85,103).

The c.i. mass spectrum provides a general description of the unidentified component. The configuration of the diaminosugar was confirmed by obtaining the 200 MHz $^1$H nmr spectrum of the peracetylated glycoside. A solution of the acetylated glycoside in chloroform-$d$ was prepared. The signals for each of the ring protons were well separated and could be assigned by homonuclear decoupling. The assignments as well as the calculated coupling constants are shown in Table 5. The observed coupling constants in combination with
Figure 7: Chemical ionization mass spectrum of the permethylated glycoside obtained from the Smith degradation of the HF disaccharide.
Figure 7
Figure 6: Fragmentation pathways available to the perethylated glycoside.
Figure 8
Table 5: $^1$H nmr data for the acetylated glycoside obtained on oxidation of the hydrogen fluoride depolymerization product of immunotype 1.
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the Karplus rule permitted the assignment of the relative configuration, assuming a pyranose derivative in the $\text{C}_4$ or the $\text{C}_1$ conformation. The configuration of the diaminohexuronic acid is the $\beta$-manno configuration.

This data indicates that the hexuronic acid is $\beta$-linked to the rhamnose derivative. The above reaction sequence therefore serves to establish one half of the chemical repeating unit of the O-specific polysaccharide. Since the linkage pattern and the anomeric configuration of the remaining sugars is known from the methylation analysis and gated carbon nmr data, it is possible to construct two possible repeating units for this polysaccharide. The two structures are shown in Figure 9.

The structures also summarize two other pieces of data. First, the non-alkaline methylation results indicate that glucose is the only sugar that is O-acetylated. The position was confirmed to be the six position. This is also consistent with the carbon spectrum. If the glucose residue was not derivatized in this position a signal would be seen at 61.2 ppm. The signal at this position is of low intensity possibly indicating incomplete esterification. The second summarization addresses the formylation of quinovosamine. From the results of the non-alkaline methylation and nmr studies, it can be concluded that the formyl group can only be found as an $N$-formyl derivative on quinovosamine.
Figure 9: Possible repeating units for the O-specific polysaccharide of immunotype 1.
The final step in the structural analysis of this O-antigenic polysaccharide was to determine which of the two structures was the actual repeating unit. The method of choice to facilitate this analysis was a periodate oxidation of the native polysaccharide.

Comparing the two structures, it can be seen that two sets of products would be expected from the oxidation of the polysaccharide. Structure A would produce an erythritol glycoside of 2,3-diamino-2,3-dideoxymannuronic acid and a glycerol glycoside of quinovosamine. Structure B would produce the glycerol glycoside of 2,3-diamino-2,3-dideoxymannuronic acid and the erythritol glycoside of quinovosamine.

After a 72 hour oxidation, the polysaccharide was reduced, dialyzed and hydrolyzed to obtain the glycosides described above. The two glycosides were separated by using ion exchange chromatography. Using Dowex 1-X8, the erythritol glycoside of quinovosamine was obtained in the water wash and the glycerol glycoside of 2,3-diamino-2,3-dideoxymannuronic acid was obtained by eluting with M sodium chloride. The $^1\text{H}$ nmr spectrum of the component eluting with the salt fraction was identical to the peracetylated glycoside obtained from the hydrogen fluoride depolymerization product. The fraction that was obtained from the aqueous washings was lyophilized,
and subjected to classical sugar analysis. The results are shown in Table 6. It can be concluded that the two glycosides obtained were identical to those expected from the periodate oxidation of structure B in Figure 9.

Combining all of these results it is possible to assign the structure of the chemical repeating unit of the O-specific polysaccharide of immunotype 1 is:

\[ \rightarrow \]

\[ 2\text{)-}\alpha-L\text{-Rhamnopyranose-(1→3)-}\alpha-D\text{-N-formylquinovosamine-(1→4)-}\alpha-D\text{-glucopyranose-(1→4)-}\beta-D\text{-mannopyranuronic acid(imidazoline[3,2d]-1)} \].
Table 6: Mass spectral data of the product of the Smith degradation of the immunotype 1 O-specific polysaccharide.
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I: erythritol tetraacetate  
II: N-acetyl quinovosamine tetraacetate
Figure 10: Actual repeating unit of the O-specific polysaccharide of
immunotype 1.
VI.2 Immunotype 6

The analysis of this polysaccharide presented several problems. The first major problem is that the O-specific polysaccharide appears to comprise only 5-10% of the total weight of the lipopolysaccharide. In order to determine if this low yield was due to degradation of the polysaccharide, the hydrolysis was effected in several different ways. The factors varied were reaction time, temperature of hydrolysis, acid strength, and concentration as well as several different acids. The pH studies showed that no hydrolysis occurred above pH 4.5. Below pH 4.5, the polysaccharide could be obtained free of the core and lipid A portions. However, the recovery was no more than 5 mg of O-chain/100 mg of lipopolysaccharide. Subsequent reactions were carried out at pH 4.5. The reaction did not appear to be affected by the actual acid used, as evidenced by reaction with formic, acetic, and trifluoroacetic acids. For the sake of simplicity, all hydrolyses were carried out in 1% aqueous acetic acid. The optimum time of hydrolysis was found to be 60 minutes at 100 C. By varying any of these parameters, it was not possible to increase the yield of the O-specific polysaccharide above 10%. The final hydrolysis conditions were: a 0.1% solution of the lipopolysaccharide in 1%
aqueous acetic acid, pH 4.5, heated at 100 C for 60 minutes. Purification of the lyophilized aqueous fraction yielded 10 mg of pure O-antigen from 100 mg of lipopolysaccharide. The structural studies on this antigen were conducted on pooled O-chain isolates obtained from the same production batch of the lipopolysaccharide.

As was the case with the O-antigen from immunotype 1, the structural characterization of the polysaccharide began with nmr spectroscopy. The 200 MHz \(^1\)H nmr spectrum of this polysaccharide is rather simple, Figure 11. The anomeric region showed the presence of three signals at 4.90, 4.98, and 5.13 ppm. The only other feature of the spectrum is the broad singlet, which through resolution enhancement appears as two overlapping doublets, centered at 1.19 ppm, indicative of two different 6-deoxyhexoses.

The 75 MHz \(^13\)C nmr spectrum confirms a trisaccharide repeating unit by the presence of three signals in the anomeric region, 101.7, 102.9, 103.1 ppm, Figure 12. The region of methyl carbons of 6-deoxyhexoses shows a singlet of high intensity, corresponding to two carbon signals. The remainder of the spectrum contains twelve signals, at least two of which appear to be due to two or more overlapping signals (as determined by their intensity relative to the anomeric carbons). The spectrum also confirms the absence of
Figure 11: 200 MHz $^1$H nmr spectrum of the O-specific polysaccharide of immunotype 6.
Figure 12: 75 MHz $^{13}$C nmr spectrum of the O-specific polysaccharide of immunotype 6.
Figure 12
O-acetyl derivatization as well as the absence of amino sugars and the absence of uronic acids. If neutral hexose is present in the polysaccharide, it is probably linked in a 1→6 fashion as is predicted by the absence of signals in the 60–62 ppm region, indicative of the hydroxymethyl group of a neutral hexose. In each of the other O-specific polysaccharides from the other immunotypes of *P. aeruginosa* there is as high content of amino sugars. However, as predicted by the carbon spectrum, these sugars are totally absent in the O-chain of immunotype 6.

The next step in the analysis of this polysaccharide was a sugar analysis and methylation analysis. Sugar analysis revealed the presence of two sugars: glucose and rhamnose. The isolation of these sugars showed them to be identical to D-glucose and L-rhamnose.

The modified Hakomori methylation showed four components, listed in Table 7. Analysis of the fragmentation patterns of the components showed them to be the following: 1,5-di-6-acetyl-2,3,4,6-tetra-6-methylglucitol; 1,5,6-tri-6-acetyl-2,3,4-tri-6-methylglucitol; 1,2,5-tri-6-acetyl-6-deoxy-3,4-di-6-methylmannitol; and 1,3,5-tri-6-acetyl-6-deoxy-2,4-di-6-methylmannitol. These results show that glucose is the non-reducing terminus and that the internal glucose residues are linked in a 1→4 fashion. Two different
Table 7: Mass spectral data for the methylation analysis of the immunotype 6 O-specific polysaccharide.
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I : 1,5-di-\( \text{Q} \)-acetyl-2,3,4,6-tetra-\( \text{Q} \)-methyl glucitol
II : 1,5,6-tri-\( \text{Q} \)-acetyl-2,3,4-tri-\( \text{Q} \)-methyl glucitol
III : 1,2,5-tri-\( \text{Q} \)-acetyl-6-deoxy-3,4-di-\( \text{Q} \)-methyl mannitol
IV : 1,3,5-tri-\( \text{Q} \)-acetyl-6-deoxy-2,4-di-\( \text{Q} \)-methyl mannitol
rhamnose derivatives were found, indicating that both 1→3 and 1→2 linked rhamnose residues are found in the repeating unit of the polysaccharide. The results are consistent with those expected from the nmr studies. Glycosidation through position six in a neutral hexose causes a downfield shift of the signal for C-6 corresponding to 5-7 ppm shift (105). Thus the signal expected at 61-62 ppm would be shifted to 68-69 ppm.

This information can be combined to produce two possible structures for the O-antigen repeating unit of immunotype 6, Figure 13. In order to distinguish between these two, the native O-antigen was subjected to a Smith degradation. As was the case with the immunotype 1 polysaccharide, two possible glycosides can be obtained. The glycoside was isolated by gel permeation chromatography on Bio-gel P-2. This derivative also eluted prior to the salt fraction, with an apparent molecular weight of 200 daltons. After lyophilization of the sample, it was subjected to methylation analysis. Upon g.l.c./m.s. analysis, two methylated products were obtained. The first was identified as 2-O-acetyl-1,3-di-O-methylglycerol. The second component was identical to 1,5-di-O-acetyl-6-deoxy-2,3,4-tri-O-methylmannitol. The fragmentation patterns of these derivatives are shown in Table 8. These two compounds show
Figure 13: Possible repeating units of the O-specific polysaccharide of immunotype 6.
Table 8: Mass spectral data for the Smith degradation product obtained from the immunotype 6 O-specific polysaccharide.
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I : 1,5-di-\(\beta\)-acetyl-6-deoxy-2,3,4-tri-\(\beta\)-methyl mannitol
II : 2-\(\beta\)-acetyl-1,3-di-\(\beta\)-methyl \(\alpha\)glycerol
that the correct structure is the repeating sequence shown in Figure 14. The anomeric configurations of the linkages were assigned as the $\beta$-configuration based on the position of the carbon signals in the $^{13}$C nmr spectrum. However, this repeating sequence does not account for the apparent lability of the polysaccharide. A possible explanation of the low yield of O-chain from the lipopolysaccharide is that these strains of *P. aeruginosa* produce a lipopolysaccharide that is deficient in O-antigen. While the repeating unit of this O-specific polysaccharide is consistent with the available data, further investigation of these strains of the bacterium is warranted.
Figure 14: Actual repeating unit of the O-specific polysaccharide of immunotype 6.
Figure 14
VI.6.3 Immunotype 5

The O-antigenic polysaccharide of immunotype 5 was isolated from the lipopolysaccharide by hydrolysis with 1% acetic acid followed by Bio-gel chromatography. The polysaccharide was isolated in 27% yield from the lipopolysaccharide.

Physical studies were the first objective of the structure analysis. This particular polysaccharide was first studied in our laboratory by D.A. Riley (72). In this work, the repeating unit was reported to be $\rightarrow$3)$L$-Rhap$\alpha$-(1$\rightarrow$3)$L$-Rhap$\alpha$-(1$\rightarrow$3)$D$-QuiNAcp-(1\text{)}. This structure is shown in Figure 15. Shortly after this report, N.K. Kochetkov reported the structure of the repeating sequence for an O-antigen from the Lanyi 02 serogroup (77). This serotype is thought to be identical to the Fisher immunotype 5. The structure described by this group differed considerably from the structure reported from our laboratory. The structure is shown in Figure 16. Because of this possible discrepancy between supposedly homologous O-antigens, a re-examination of this polysaccharide was necessary.

Nuclear magnetic resonance provided the first insight into this problem. The 300 MHz $^1$H nmr spectrum, Figure 17, reveals two signals in the anomeric region: 5.05 ($J_{1,2}$=6 Hz, 114
Figure 15: Structure proposed for the repeating unit of the immunotype 5 O-specific polysaccharide. (72)
Figure 15
Figure 16: Repeating unit of the O-specific polysaccharide obtained from *Pseudomonas aeruginosa* Lanyi serogroup 02. (??)
Figure 17: 300 MHz $^1$H nmr spectrum of the immunotype 5 O-specific polysaccharide.
1H) and 4.88 ppm ($J_{1,2}$, low, 2H). Two other regions showed the presence of 6-deoxyhexose, 1.27 ppm, and the presence of O- and N-acetyl groups, 1.96, 2.02, and 2.16 ppm.

The 20 MHz $^{13}$C nmr spectrum, Figure 18, provided more information. The anomeric region shows three signals at 98.6, 99.2, and 99.8 ppm, indicating a trisaccharide repeating unit. The presence of 6-deoxyhexose is confirmed by the presence a double intensity signal at 17.7 ppm. In a similar fashion, N-acetyl groups are present evidenced by the double intensity signals at 23.3 ppm and 175.5 ppm. An O-acetyl substituent is seen by the signals at 21.6 and 174.5 ppm. Signals of two carbons bearing nitrogen can be seen at 50.4 and 52.6 ppm. A final carbonyl signal is seen at 173.6 ppm which may indicate the presence of a glycuronic acid.

The next stage in the characterization of the polysaccharide was a classical sugar analysis. Two sugars were revealed as their alditol acetates, rhamnose and quinovosamine. However analysis of the hydrolysate by paper chromatography revealed these two sugars in addition to a third component that was reactive to both ninhydrin and alkaline silver nitrate. Since the nmr spectra indicate a possible glycuronic acid, the native polysaccharide was subjected to carboxyl reduction. Sugar analysis of the reduced polysaccharide revealed the presence of galactosamine.
Figure 18: 20 MHz $^{13}$C nmr spectrum of the immunotype 5 0-specific polysaccharide.
as well as rhamnose and quinovosamine. The absolute configurations of these sugars were confirmed by comparison of the specific rotations of the sugars, isolated by paper chromatography, with known sugars. The sugars were found to be: D-quinovosamine, L-rhamnose, and L-galactosamine. Thus the native polysaccharide contains 2-amino-2-deoxy-L-galactopyranuronic acid.

Methylation analysis of the carboxyl reduced polysaccharide revealed four components, Table 9. These components were: 1,5-di-D-acetyl-6-deoxy-2,3,4-tri-D-methylmannitol, 1,3,5-tri-D-acetyl-6-deoxy-2,4-di-D-methylmannitol, 2-acetamido-1,3,5-tri-D-acetyl-2,6-dideoxy-4-D-methylglucitol, and 2-acetamido-1,4,5-tri-D-acetyl-2-deoxy-3,6-di-D-methylgalactitol. These results show that rhamnose is the non-reducing terminus and linked in a 1→3 fashion within the polysaccharide. The quinovosamine residues are linked in a 1→4 fashion as are the galactosaminuronic acid residues. Again this data provides for two different repeating units, Figure 19. In order to determine the correct repeating sequence, partial acid hydrolysis was performed on the polysaccharide. Since the repeating unit contains an aminoglycuronic acid, the anomeric linkage of this residue should be relatively stable to acid hydrolysis. The hydrolysate was separated using gel permeation
Table 9: Mass spectral data for the methylation analysis of the carboxyl reduced immunotype 5 0-specific polysaccharide.
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I: 1,5-di-0-acetyl-6-deoxy-2,3,4-tri-0-methyl mannitol
II: 1,3,5-tri-0-acetyl-6-deoxy-2,3-di-0-methyl mannitol
III: 2-acetamido-1,3,5-tri-0-acetyl-2,6-dideoxy-4-0-methyl glucitol
IV: 2-acetamido-1,4,5-tri-0-acetyl-2-deoxy-3,6-di-0-methyl galactitol
Figure 19: Possible repeating units of the O-specific polysaccharide of immunotype 5.
Figure 19
and indeed a disaccharide was obtained. Carboxyl reduction and sugar analysis of this disaccharide revealed it to be composed of quinovosamine and galactosamine. This indicates that the actual repeating unit is represented in Figure 19A, since solvolysis of structure 1B would result in the formation of a galactosamine-rhamnose disaccharide.

The final results indicate that Figure 19A is the repeating unit of the native immunotype 5 O-specific polysaccharide. The fine points of the structure were determined in several ways. The anomeric configurations were determined from the nmr spectra and the nmr spectrum of the disaccharide obtained from partial acid hydrolysis. The 1H nmr spectrum of the acetylated disaccharide showed that the galactosamine residue was linked to the quinovosamine by an alpha linkage (J1,2,low). The anomeric assignment for the quinovosamine residue was seen to be a beta linkage as seen from the anomeric mixture obtained in the disaccharide. This leaves only the anomeric configuration of rhamnose to be assigned. The proton spectrum showed two signals, one with a high coupling constant, the quinovosamine residue, and two protons with low coupling constants. A beta linked rhamnose would have an H1 coupling constant on the order of 6-10 Hz. Since this is not observed, it is assumed that the rhamnose residues are alpha linked.
The non-alkaline methylation revealed only one component not found in the Hakomori methylation. This was identified as 1,2,3,5-tetra-$\text{O}$-acetyl-6-deoxy-4-$\text{O}$-methyl mannitol. This indicated that the internal rhamnose residues are acetylated at position 2.

When the final structure of the repeating unit of the O-specific polysaccharide is compared with the structure reported by Kochetkov and his co-workers, it can be seen that they are identical. This lends support to the idea that the ultimate classification of bacterial O-antigens may lie in a chemotyping procedure. This result also explains the similarity/identity between the Fisher immunotype 5 strains and those that are classified into the Lanyi O2 serogroup.
VI.6.4 Immunotype 7

Initial studies of the chemical repeating unit of the immunotype 7 O-specific polysaccharide showed that it was extremely resistant to acid hydrolysis. After prolonged hydrolysis, only small amounts of fucosamine, 2-amino-2,6-dideoxygalactose, were obtained. However physical analysis revealed a more complex structure was probable.

The 200 MHz $^1$H nmr spectrum, Figure 20, revealed the presence of 6-deoxyhexose, 1.08 ppm, and acetyl groups, 1.85, 1.92, and 1.97. In addition, a signal at 2.31 ppm was also present. This signal has been previously assigned to the 2-methyl group of a 2,3-imidazoline ring system found associated with several diamino sugars, isolated from P. aeruginosa. The anomic region shows three distinct resonances, two with low coupling constants and a third with an associated coupling constant of 6.9 Hz. Tentatively the information indicates two alpha and one beta linkage, along with a trisaccharide repeating unit. Since the polysaccharide contained a 2,3-imidazoline ring system, which is labile to alkaline hydrolysis, the native polysaccharide was treated with aqueous triethylamine. The 200 MHz $^1$H nmr spectrum showed no changes except in the acetate methyl proton region. The signal at 2.31 ppm was removed and a new acetyl methyl proton signal appeared at 1.98 ppm.
Figure 20: 200 MHz $^1$H nmr of the O-specific polysaccharide of immunotype 7.
The 75 MHz $^{13}$C nmr spectrum, Figure 21, was relatively complex. It is apparent that the spectrum contains signals for both the sugar derivatized as the 2,3-imidazoline derivative and as the free diacetyl derivative. To simplify the spectrum, it was treated with aqueous triethylamine. The readily interpreted spectrum obtained in this manner is shown in Figure 22.

The spectrum indicates a trisaccharide repeating unit by the presence of three anomeric signals at 96.0, 99.8, and 100.9 ppm. The 6-deoxyhexose is seen by the resonance at 16.3 ppm. In the region of carbons bearing nitrogen substituents, five distinct signals can be seen, 47.0, 48.3, 48.4, 52.4, and 53.2. It is also shown that the repeating unit contains only $\text{N}$-acetyl derivatives, 22.8 and 23.3, and no $\text{O}$-acetyl groups. The carbonyl region is somewhat crowded, however, it is possible to observe seven signals.

The results of the nmr experiments provide for an initial description of the polysaccharide as being one highly enriched in amino sugars, possibly comprised solely of amino sugars. Since this polysaccharide could not be hydrolyzed using standard procedures, the anhydrous hydrogen fluoride depolymerization was applied. When this was done with the O-antigen from immunotype 7, a trisaccharide was obtained. The $^1\text{H}$ nmr spectrum of the trisaccharide was nearly identical to
Figure 21: 75 MHz $^{13}$C nmr spectrum of the O-specific polysaccharide of immunotype 7.
Figure 22: 75 MHz $^{13}$C nmr spectrum of the O-specific polysaccharide of immunotype 7 after treatment with 5% aqueous triethylamine.
the spectrum of the native polysaccharide, Figure 23. The major difference lies in the signals attributable to the 6-deoxyhexose. These differences would be expected if the 6-deoxyhexose was at the reducing terminus of the trisaccharide. The added complexity in the anomeric region can be explained in realizing that the spectrum is a combined spectrum of the two anomeric forms of the trisaccharide.

The $^{13}$C nmr spectrum confirms that the compound obtained is a mixture of the anomers of a reducing trisaccharide. The similarity between this spectrum and the spectrum of the native polysaccharide supports the idea that the chemical repeating unit was isolated in the solvolysis.

The next step in the analysis was the reduction of the trisaccharide and subsequent periodate oxidation and final reduction. The $^{13}$C spectrum, Figure 24, shows that the 6-deoxyhexose has been degraded, but no loss of carbons bearing nitrogen have been lost. Since fucosamine could be isolated from the polysaccharide, it is evident that this sugar can be found at the reducing terminus of the trisaccharide. (Isolation of the fucosamine revealed it to have the D-configuration). The spectrum also shows that the imidazoline ring was hydrolyzed during the alkaline reduction step. This hydrolysis simplified this spectrum as it did that of the native polysaccharide. Two anomeric signals at 100.8
Figure 23: 200 MHz $^1$H nmr spectrum of the trisaccharide obtained after anhydrous hydrogen fluoride depolymerization of the O-specific polysaccharide of immunotype 7.
Figure 24: 50 MHz $^{13}$C nmr spectrum of the derivative obtained after Smith degradation of the trisaccharide obtained from the depolymerization of the O-specific polysaccharide of immunotype 7.
and 100.6 were observed. Several of the signals in the region of 70-80 ppm have merged into a double intensity singlet. This appears to indicate that the two monosaccharides would have very similar if not identical structures. When the fragment was subjected to carboxyl reduction, two carbonyl signals were removed and two signals due to hydroxymethyl carbons became evident. By combining the nmr results, it is possible to propose a structure for the degraded trisaccharide. The trisaccharide fragment is consistent with the 2-amino-2-deoxy-erythritol glycoside of a diaminoglycuronic acid disaccharide.

The anomeric configurations of the two diaminoglycuronic acids were determined from the $^1$H nmr spectrum of the peracetylated carboxyl reduced disaccharide glycoside. Using selective homonuclear decoupling it was possible to assign each proton resonance and thereby determine the relative configuration of the two sugars as the beta manno-configuration.

In order to determine the absolute configuration of the components of the disaccharide, it was necessary to hydrolyze the glycoside and recover the monosaccharides by paper chromatography. The only sugar obtained was 2,3-diamino-2,3-dideoxy-$D$-mannose. The specific rotation of this sugar was $-35$, which is in good agreement with the accepted value of $-38$. 
From these results, it is possible to deduce the structure of the repeating unit of the immunotype 7 polysaccharide. There remains some ambiguity relative to the location of the imidazoline ring system. The $^{13}$C nmr spectrum indicates that there is only one of these derivatives per repeating unit. Due to the partial hydrolysis during isolation of the lipopolysaccharide, it was not possible to successfully assign the position of the derivative within the repeating unit. Thus there are two possible structures for the repeating unit, Figure 25.

These results only address the nature of the chemical repeating unit. Further investigation is required before the biological repeating unit will be known. This would most easily be probed with a series of phase shift variant trisaccharides based on the two sugars found in the repeating unit. By reacting these derivatives with specific antibodies, it would be possible to discern the structural requirements for binding.

Prior to the completion of this work, several reports were published that addressed the structures of the repeating units of the O-specific polysaccharides from Lanyi serogroup O3 (78). There are several members of this serogroup that are thought to be identical to the strains classified into Fisher immunotype 7. The structure assigned to the serogroup O3a,b
Figure 25: Possible repeating units for the O-specific polysaccharide of immunotype 7.
is shown in Figure 26. This structure is very similar to the result obtained for the Fisher immunotype 7 O-antigen. This is especially true if the $^{13}$C spectra of the trisaccharide obtained after HF solvolysis are compared. Signal for signal these two spectra match to within 0.2 ppm. As a result, it can be seen that the similarities predicted by the two classification schemes have a basis in chemical structure. Thus far this result has been confirmed with both the Lanyi O2-Fisher 5 and Lanyi O3-Fisher 7, implying that strains of \textit{P. aeruginosa} that have similar immunological properties also have similar chemical compositions.
Figure 26: Repeating unit of the O-specific polysaccharide obtained from *Pseudomonas aeruginosa* Lanyi serogroup O3a,b. (78)
VII. Affinity Chromatography

VII.1 Introduction

Affinity chromatography first developed as a new method for the purification of proteins. This method exploits the highly specific reaction between a protein and its associated ligand. In general, this method attaches the ligand to an insoluble support, which in turn is placed into a chromatographic column. Theoretically, only the desired protein will be bound to the ligand and will be effectively removed from the solution. This occurs, provided the ligand is chosen so that it contains the appropriate receptors for the desired protein. Any other components of the mixture can be washed from the column. Once the contaminants have been removed, the adsorbed protein can be removed by selective modification of the elution buffer. Three generalized methods have been developed to displace the protein. The most commonly used process involves an addition of free ligand to the buffer in concentrations high enough to elute the protein. The second method relies on a modification of the pH which changes the ionization of the protein causing a conformational change which disrupts the protein-ligand binding. The final method used is the addition of chaotropic ions to the buffer solution. This produces a change in the hydration sphere of the protein, facilitating its displacement.
Modern affinity chromatography has its basis in enzyme purification (106,107). Porath and his co-workers developed a novel purification method using hydrophilic porous support materials, principally agarose. The term affinity chromatography implies the application of the ability of biomolecules to form stable reversible complexes with other substances. When one of the components of this complex is fixed to a solid support, it is possible to isolate the second component. However, this assumes that binding of the ligand does not interfere with its ability to complex with the other portion of the complex. As was previously mentioned, this technique was first applied to enzyme purification. By binding an enzyme substrate to a solid, insoluble support it was possible to isolate the enzyme specific for that substrate from a mixture of proteins. More recently there are numerous classes of compounds that have been purified by an affinity chromatography approach.

One of the major problems associated with this technique is the covalent linkage of the ligand to the solid support. This problem was recognized early in the development of this method and has been successfully addressed. By incorporating a spacer arm into the ligand-solid support complex, it was possible to reduce or eliminate most of the negative effects of the covalent attachment. Cuatrecasas
found that the size requirements for the spacer arm were relatively specific (108). In this and subsequent studies, it was found that binding to the ligand was greatly diminished when the ligand was attached directly to the support. The size of the spacer arm is dependent on the system being studied. However, Hipwell showed that binding of dehydrogenases to their specific substrates increased as the number of methylene groups separating the ligand and the support increased (109). Maximum binding occurred when 5-6 methylene units were incorporated into the spacer arm. Combining the results of several laboratories, it can be seen that the size of the spacer arm is variable, but binding is facilitated when the distance between the ligand and the support is 15-50 Å (110-112). The use of a spacer arm is therefore imperative. However, the spacer arm should not impart adverse properties to the ligand. Negative interactions with the chosen spacer are most important when the spacer acts as an adsorption center itself. Additionally, the ligand should not be bound in such a way that it loses all or part of its specificity (113). Once an affinity adsorbent is prepared, it is possible to prepare relatively large amounts of purified ligate in a short period of time.

The most important developments in this separation method, relative to this project, were first introduced by
Campbell in 1951 (114). This was the first report of affinity chromatography as applied to antibody-antigen interactions. In these first experiments, antigenic determinants were covalently attached to agarose and the antibodies specific for the antigen could be purified. The field of immunological reactions was an ideal area to apply affinity based purification methods. The specificity of the antibody for its antigen and the relatively strong interaction between the two components of the system are readily exploited. The association between these two classes of biomolecules is relatively tight; dissociation constants of $10^{-5-10^{-8}} \text{M}$ are common (115). Since this first purification procedure was developed, it has become recognized as the single most effective purification method in the field of immunochemistry (116). As a result, immunoaffinity chromatography was chosen as the most satisfactory purification method available for obtaining pure fractions of anti-	extit{Pseudomonas} antibodies.

In the immunoaffinity chromatographic separation of antibodies obtained from serum samples or the ascites fluid of continuous cell lines, it is necessary to provide a solid support to which the appropriate antigen can be attached. The production of this type of matrix consists of three steps: activation of the solid support, attachment of the spacer, and attachment of the antigen. Most immunoaffinity methods
use Sepharose gels as the solid support. These agarose gels provide excellent stability and flow properties which are superior to the other support materials. The activation of Sepharose can be achieved in many ways. The most common methods include reaction with: cyanogen bromide (106), bis-oxiranes (117), reductive amination (periodate oxidation-Schiff base formation-cyanoborohydride reduction) (118), or divinyl sulfone (119). The second step, spacer arm incorporation, is most often directly associated with the activation step. This permits one reaction to provide both activation of the matrix and the presentation of an activated spacer to the ligand. If this is not done within the activation step, the antigen is most often reacted with the spacer arm and the spacer-antigen composite is then reacted with the activated support to form an immunoaffinity adsorbent. Generally, once the adsorbent has been prepared, it is stable enough to most elution conditions so that it can be used for multiple separations, without loss of the ability to bind or a loss of its specificity.
VII.2 Preparation of Immunoadsorbents based on the Immunotype 5 O-antigen

VII.2.1 Introduction

The ability of antibodies to react to the O-antigenic polysaccharides of Gram-negatives is readily observed. The use of the native antigen as a specific adsorbent for affinity chromatography was ignored due to the extremely tight binding of the antibody to the carbohydrate antigen. The conditions that were required to dissociate the antigen-antibody complex were sufficient to cause denaturation of the protein and thus a loss of antibody activity. The method chosen to effect the displacement was modification of the pH of the buffer. The pH required for this was 2.0, which is too drastic of a pH environment to expect protein stability. As a result of this complication, immunoaffinity chromatography involving carbohydrate ligands generally used synthetic haptens and antigen analogues to bind the antibody. The initial intent of this project was to couple artificial antigens based on the chemical repeating unit of the O-chain to a solid support. This type of matrix would have permitted the purification of anti-Pseudomonas antibodies. Unfortunately, the structural studies on the antigenic determinants of P. aeruginosa are
showing that the repeating unit structures are relatively complex, making the effort to synthesize the repeating unit a very difficult and time consuming process. Unless the synthetic approaches make significant advances, the amounts of synthetic haptens that can be prepared would have little clinical significance. Therefore, the target immunoabsorbent had to be modified in order to address the high clinical demand for purified antibodies. The system to be described in this section is based on the activation of Sepharose resins with divinyl sulfone. The activated Sepharose support is then reacted with intact O-specific polysaccharide. In this initial exploratory work, the O-antigen of immunotype 5 was coupled to Sepharose. One reason for selecting this particular immunotype is that in clinical infections, these strains of P. aeruginosa are the most virulent. An affinity adsorbent prepared in this manner should have a high capacity and high affinity for the desired antibodies.

As was mentioned earlier, the bound ligate can be desorbed from the column by using one of three different procedures. The variation of the pH of the buffer is not acceptable for this system because of the denaturation of the antibody that has been encountered in previous work. The second method would be to use a high concentration of the ligand. In this particular system this is not practical due
to the relatively low availability of the O-antigen and its relatively low water solubility. This leaves only the last elution method as a viable technique. The use of chaotropic ions in high concentration in the buffer is the only method that will provide for elution of antibodies from this adsorbent. Initial studies with this elution method used bound proteins and antibodies raised to these protein ligands (120-123). Several different chaotropic ions have been studied with this protein-protein system. The order of effectiveness is the following: thiocyanate = iodide > chloride > acetate > citrate (123). In addition to the relatively mild nature of the elution conditions, Avrameas demonstrated that antibodies isolated in this manner were more stable and retained a higher percentage of their original titer than antibodies eluted with acids (122). As a result this procedure was chosen for use as the elution method in the immunotype 5 system.

The final component of the adsorbent is the activator of the solid support. Divinyl sulfone (DVS) was chosen as the activator for several reasons. Perhaps the most important reason is its high selectivity in reactions with carbohydrate ligands. Studies conducted by Porath indicated that the majority of the ligands are attached through the reducing end of the monosaccharide or oligosaccharide (124). This is
particularly of importance in this system because a linkage of this type would imitate the manner in which the O-antigen is presented to the antibody in the native lipopolysaccharide. Secondarily, the conditions used to achieve activation and binding of the ligand are very mild, preventing degradation of the O-specific polysaccharide. These two properties are sufficient to warrant the use of divinyl sulfone as the activator. However, it provides for a third beneficial property. During the activation of Sepharose, it is impossible to avoid a certain amount of cross-linking of the polymer. This is not an undesirable side reaction. Porath and his co-workers showed that agarose gels cross-linked in this manner have increased mechanical strength, chemical stability and superior flow rates (125). In addition, they have increased thermal stability which permits sterilization in an autoclave. Thus by using the O-specific polysaccharide coupled to DVS activated Sepharose, a stable immunoadsorbent capable of purifying anti-Pseudomonas antibodies can be obtained.
VII.2.2 Experimental procedure

VII.2.2.1 Preparation of the Immunoadsorbent

A sample of Sepharose 4B was extensively washed with distilled water (10 x 100 mL) under mild suction to remove any contaminants and the merthiolate added as a bacteriostat. The washed resin was suspended in 20 mL of carbonate buffer, pH 8.5. 2 mL of DVS is added and the suspension is stirred at room temperature for two hours. The suspension is filtered and washed with carbonate buffer (3x20 mL, pH 10.0). The activated resin is suspended in 30 mL of carbonate buffer, pH 10.0 to which 50 mg of Immunotype 5 O-chain has been added. The suspension is stirred at 4 C for 72 hours.

After the coupling is complete, the immunoadsorbent is washed with carbonate buffer (3 x 30 mL, pH 11.0) and resuspended in carbonate buffer, pH 11.0, to which 2 mL of 2-mercaptoethanol has been added. This provides for the blocking of unreacted vinyl sulfone groups. After two hours at room temperature, the adsorbent is washed with carbonate buffer, phosphate buffered saline (PBS, 0.1 M Na$_2$HPO$_4$ in 0.11 M NaCl, pH 7.1), and stored at 4 C in PBS to which 0.01% sodium azide is added as a bacteriostat.
VII.2.2.2 Determination of the Extent of Coupling

The amount of O-antigen bound can be determined in two different ways. The first and simplest method is to recover the unbound material from the coupling process. After gel permeation chromatography to remove the salts, the amount of material can be determined. This method is also the least accurate and most subject to overestimation of the amount of polysaccharide bound.

A more accurate method is to chemically analyze the adsorbent to determine the amount of bound polysaccharide. Since the immunotype 5 O-antigen contains galactosaminuronic acid, the polysaccharide should produce the characteristic color in the carbazole colorimetric determination of uronic acids. If a standard curve is produced from different concentrations of the O-antigen, it would be possible to accurately quantitate the amount of bound polysaccharide.

In this method samples of the polysaccharide (5 mg), the affinity adsorbent (20 mg), and Sepharose 4B (5 mg) are hydrolyzed for two hours in 2 M trifluoroacetic acid. This provides for solubilization of the adsorbent. The residues obtained after evaporation of the trifluoracetic acid are dissolved in 2.0 mL of water. These solutions are then used in the carbazole assay. The standard curve is prepared by taking samples of the polysaccharide solution and diluting
with varying amounts of water to obtain a range of concentrations. This is done while maintaining the volume of the sample to be used in the carbazole assay at 1.0 mL. The sample to be analyzed, 1 mL, is placed into a culture tube and cooled in ice. Sulfuric acid containing 0.25 M, 1 mL, borate is added to the sample solutions. The solution is heated in a boiling water bath for 10 minutes. The tubes are cooled and carbazole, 0.5 mL, 1% in ethanol, is added and the tubes are heated in the boiling water bath an additional 10 minutes. The tubes are removed and read at 527 nm.

VII.2.2.3 Antibody Purification

The affinity adsorbent is placed into a chromatography column, 1.5x10 cm, and equilibrated with PBS. When the column has reached thermal and flow equilibrium, the antibody preparation is carefully layered onto the column. The antibody is washed onto the column with three portions of buffer, 3 x 2 mL, and the column is connected to a solvent reservoir. The flow rate of the column in this initial work was 15 mL/hour. The column eluate is monitored at 280 nm. Once the non-bound protein has eluted from the column, the buffer reservoir is replaced with a gradient generator, capable of producing a linear gradient of 2-4 M potassium thiocyanate (KSCN). The elution of the bound fraction is also
monitored at 280 nm. The fractions containing the desorbed protein are pooled and dialyzed against deionized water to remove the salt. The retentate is recovered and lyophilized. The dried antibody is stored at 4°C.
VII.3 Results

The affinity adsorbent prepared in this manner appears to be a very effective way of purifying anti-*Pseudomonas* antibodies. The antibody preparations that were purified were the kind donation from J. Sadoff affiliated with The Walter Reed Army Institute of Research. These antibodies were derived from continuous cell lines producing monoclonal antibodies to the seven different Fisher immunotypes of *P. aeruginosa*. The purification procedure was first applied to the ascites fluid containing an immunotype 5 IgG antibody. The second trial was conducted on ascites fluid containing antibodies of the class IgM.

Prior to the purification, the extent of derivatization of the Sepharose 4B was determined. The carbazole quantitation showed that 1.96 mg of O-chain / gram of adsorbent was bound to the column, see Figure 27. This agrees quite well with the amount predicted by recovery of the unbound polysaccharide, 2.1 mg O-chain / gram of adsorbent. At the present time, the optimal conditions have not been identified in this sequence. It may be possible to increase or decrease the amount of bound polysaccharide by varying the coupling conditions. It can be seen that this somewhat low value permits ready elution of the antibody from the column. Higher
Figure 27: Calibration curve used to determine the extent of coupling of the immunotype 5 O-specific polysaccharide to Sepharose 4B.
Figure 27

A<sub>527</sub> vs ug Immunotype 0-chain
concentrations of the ligand may cause broadening of the elution pattern.

The first sample, IgG, was separated on this column using a linear KSCN gradient to displace the antibody. The elution profile can be seen in Figure 28. As can be seen the ascites fluid contains a large amount of material that is not bound to the column. Once this material was washed from the column, the gradient was started. However, no protein was seen to elute before the introduction of 4 M KSCN. This antibody was recovered in a manner differing from the final recovery scheme presented above. The protein was recovered from the fractions by addition of an equal volume of saturated ammonium sulfate solution. However, once the protein was recovered it could not reconstituted. As a result, no information is known relative to the recovery of the antibody activity after the purification step.

The second sample was recovered using the procedure described in the experimental section. This sample was an IgM antibody that appeared to have superior protective capabilities in animal studies. The affinity purification was conducted in the same manner, with the same column as the first separation. Before application of the ascites fluid, the column was extensively washed and re-equilibrated with PBS. The sample was applied and the eluate was monitored at
Figure 23: Elution profile for the purification of monoclonal IgG antibodies using immunotype 5 O-specific polysaccharide derivatized Sepharose 4B.
280 nm. Since the gradient appeared to be unnecessary, the antibody was displaced by direct elution with 4 M KSCN. The elution profile of this experiment is shown in Figure 29. As was the case with the first antibody separation, the antibody elutes in a narrow band after introduction of the 4 M KSCN. The fractions containing this displaced antibody were pooled, dialyzed and lyophilized. The antibody appears to be more stable in this storage form. It is possible to reconstitute the activity by dissolving the antibody in PBS.

This purified antibody was sent to WRAIR for evaluation of its ability to bind antigen and its efficacy in animal protection studies. The initial results from this laboratory indicate that the antibody retained 90% of its activity as measured by a simple titer determination. The results of the animal studies were not available at the time of the preparation of this report.

The results indicate that this method can be successfully applied to the purification of antibodies raised to the native lipopolysaccharide. The technique can also be applied to a broad range of antibody classes: IgG, IgM, and potentially IgA antibodies. Once the coupling conditions are completely optimized, this technique represents a rapid method for the purification of anti-glycosyl antibodies. In addition, by using the well characterized and homogenous
Figure 29: Elution profile for the purification of monoclonal IgG antibodies using immunotype 5 O-specific polysaccharide derivatized Sepharose 4B.
native antigenic determinant it is possible to produce the adsorbent in quantities sufficient to meet the clinical demand for pure antibody preparations. Thus this technique alleviates the most pressing problem associated with the production of immunoaffinity adsorbents, a ready source of the appropriate ligand.
VIII. Discussion and Future Directions

The goal of this project was to increase the understanding of the chemical structures of the O-specific polysaccharides of \textit{P. aeruginosa}. With this report, the structures of the seven Fisher immunotypes are now available. This data comes from the contents of this report combined with the work completed in this laboratory and several other laboratories. The structures that are now available show the diverse nature of this organism. Due to the varied immunological reactivity seen in the strains of \textit{P. aeruginosa}, these differences are to be expected. However, this organism differs from other Gram-negative organisms in the extent of these differences. For example within the \textit{Citrobacter} genus, the structures of the lipopolysaccharides differ only in the outer core region (126). The O-antigens are identical in all of the species. The \textit{S. flexneri} O-antigens share a very similar set of allowed repeating sequences with minor variations in substitution and derivatization (127,128). In the \textit{P. aeruginosa} O-specific polysaccharides only three immunotypes share any type of sequence homology. Immunotypes 3,4 and 7 all have very similar repeating sequences. The remaining immunotypes have dramatically varied structures. It is obvious that a detailed
investigation into the biosynthetic pathways of the O-specific polysaccharides in this organism are indicated.

The analysis of the O-antigens was complicated by the presence of acid and alkali labile groups found associated with the polysaccharides. Of especial interest are the diaminoglycuronic acids found as the 2,3-imidazoline derivative. The degradation of these derivatives is evident from the analysis of the nmr spectra of the purified O-antigens. This suggests that the milder conditions of the Westphal lipopolysaccharide isolation procedure should be used in the isolation of the lipopolysaccharides in future studies. It is assumed that this hydrolysis occurs in the isolation step. It is possible that it is not an artifact of isolation but rather an example of incomplete processing during the biosynthesis. This question could initially be addressed through an examination of the lipopolysaccharides isolated from the cells by using the Westphal procedure.

With the knowledge of the repeating units of the O-specific polysaccharides available, it is now possible to begin the synthesis of the repeating units of the O-antigens. These artificial haptens could have applications in both artificial vaccines and a new class of immunoadsorbents. The synthesis of many of the repeating units will require a great deal of creativity and technological advancement. While the
synthetic haptens would be beneficial in the determination of immunodominant sugars within the repeating unit, they may not be practical for their most important role, artificial vaccines and immunoadsorbents.

A possible solution to the problem of obtaining an effective artificial vaccine and immunoadsorbent may lie in the exploitation of the initial results of the antibody purification studies. While the O-chain is not a stimulus to the immune system by itself, the immunoadsorbent work showed that it was recognized when bound to a high molecular weight carrier. This knowledge can lead to the production of a protein-polysaccharide conjugate that may act as an immunogen. The practical aspects of this type of vaccine are acceptable. The advances in fermentation science are permitting the isolation of vast quantities of bacterial products. If this technology could be applied to the growth of \textit{P. aeruginosa}, the amounts of O-specific polysaccharide that are potentially available are sufficient to study this type of approach to both artificial antigens and immunoadsorbents. Thus now that the structures of the O-antigens are available these synthetic routes can be approached with confidence.

The entire approach to develop an anti-\textit{Pseudomonas} vaccine may be targeted to the wrong section of the lipopolysaccharide. Early investigators have supported the
hypothesis that the core region of the LPS is conserved
within a given species of gram-negative bacteria. If this is
the case with *P. aeruginosa*, it could be possible to design a
vaccine around this oligosaccharide. The major obstacle to
this is that little is known about the core region. The early
studies from our laboratory indicate that it is an
oligosaccharide with a molecular weight of approximately 2000
daltons. A molecule of this size is ideally suited to
structural analysis by fast atom bombarment mass
spectroscopy. The molecule could also be studied by two-
dimensional nmr. It seems a likely target for future studies
of the lipopolysaccharide antigens of *P. aeruginosa*.

While there is a large amount of information available
for the structures of the antigenic determinants of *P.
aeruginosa*, there remains a great deal of unknown material.
The structures of the O-antigens begin to address these
problems, but also create new questions. The chemical
repeating unit structures do not address the actual
biological immunological determinant. This may be represented
as the chemical repeating unit, a phase shift variant of the
repeating sequence, multiple copies of the repeating unit or
only one or two of the sugars found in the sequence. What
still needs to be addressed is the biological specificity
represented by these complex polysaccharides.


65. Sadoff, J.C., personal communication.


72. Riley, D.A., Ph.D. Dissertation, The Ohio State University, Columbus, Ohio, 1980.


