A CHEMICAL AND IMMUNOLOGICAL INVESTIGATION OF THE LIPOPOLYSACCHARIDE OF *NEISSERIA GONORRHOEAE* COLONIAL TYPE 1

A Thesis

Presented to

the Faculty of Graduate Studies

University of Manitoba, Winnipeg

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

Department of Medical Microbiology

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by

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November 1978

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A dissertation submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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ACKNOWLEDGEMENTS

The author would like to thank Dr. G.M. Wiseman for his guidance and patience which was extended through the completion of this work.

The author would also like to express appreciation to the Medical Research Council for the financial support which they gave.

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LIST OF ABBREVIATIONS

BBL	Baltimore Biological Laboratories
cm	centimetre
CDC	Centre for Disease Control
CFU	colony-forming unit
°C	degrees centigrade
KDO	3-deoxy-d-manno-octulosonic acid
ESP	Enriched Single Phase
GLC	gas-liquid chromatography
GCBD	Gonococcal Base Defined
g.	gram
g	gravitational unit
HU	hemagglutination unit
Cetavlon	2% hexadecyltrimethylammonium bromide
IHA	indirect hemagglutination
IR	infra red
LPS	lipopolysaccharide
L	litre
µg	microgram
µl	microlitre
mg	milligram
ml	millilitre
nm	nanometre
N.	Neisseria
N	normal
PW	phenol-water
PBS	phosphate buffered saline
RNA	ribonucleic acid
SE	saline extract
TMS	trimethylsilyl
UV	ultraviolet
v/v	volume per volume
w/v	weight per volume

ABSTRACT

A study of some of the chemical and serological differences between type 1 and type 4 Neisseria gonorrhoeae strains and the serological differences between Neisseria gonorrhoeae, Neisseria sicca and Neisseria meningitidis was performed.

Infra-red spectrophotometry, ultraviolet spectrophotometry and gas-liquid chromatography (GLC) demonstrated that gonococcal saline extracted (SE) preparations contained lipopolysaccharide (LPS) typical of other Gram-negative bacteria. Differences were found between SE and phenol-water (PW) extracted LPS in chemical content and in the extent to which the extracts could be purified. Sugar analysis of type 1 gonococcal LPS indicated the presence of sugars which were known to be side chain sugars of LPS of other Gram-negative species.

Antigenic differences were observed between LPS of type 1 gonococci, type 4 gonococci and other Neisseria species. A more detailed comparison of three strains of bacteria was made possible by reciprocal absorption. Phenol-water extracts had greater serological activity than the SE LPS. Antigenic relationships as observed by indirect hemagglutination (IHA) indicated that differences in polysaccharide determinants were probable.

CHAPTER I

INTRODUCTION

Immunological classifications of strains of Neisseria gonorrhoeae have been attempted by investigators in the past, but few have been related to colonial morphology. Each strain of Neisseria gonorrhoeae consists of bacterial organisms which are genetically derived from the same bacterial cell. Every strain in vivo gives rise to two colony cell types, type 1 and type 2. When these two cell types are cultured and subcultured on solid media, in vitro, they give rise to two other colony types, type 3 and type 4. All of these colony types have distinctive appearances on solid media when viewed through a stereomicroscope. More recently, additional colony types have been described by various investigators. It has been shown (Kellogg et al.,1963) that of the five well-established colonial varieties, types 1 and 2 differ from 3, 4 and 5 in that they are virulent when inoculated into human volunteers. Undoubtably, there are serological and chemical differences between these types and a study was undertaken to investigate this, particularly with reference to type 1 strains.

A feature of gonorrhea is that those afflicted with it acquire no protective immunity, and a possible explanation of this is that a number of antigenic types exist. Infection

with one strain might not therefore confer immunity to re-infection with another antigenic type. Consequently, the present study aimed to assess the degree of immunological variation in strains of gonococci.

CHAPTER II

REVIEW OF THE LITERATURE

Colonial morphology

Neisseria gonorrhoeae was first described by Neisser in 1869 as the cause of gonorrhea. The bacterium was cultivated on artificial medium by Leistikow and Loeffler, in 1882 (cited by Davis, *et al.*, 1973).

Kellogg *et al.* (1963) described four colonial types present in every strain of the gonococcus. Types 1 and 2 were virulent forms isolated from patients who had gonorrhea. Types 3 and 4 arose from types 1 and 2, and appeared after in vitro subculture. They were avirulent when injected into human volunteers. Jephcott and Reyn (1971) and Reyn *et al.* (1971) described a fifth colonial type which was avirulent. Chan and Wiseman (1975) described a sixth colonial type, type 1', which had similar properties to the other virulent types, 1 and 2. The morphological properties of the six types are described in Table 1.

TABLE I

CHARACTERISTICS OF COLONIAL TYPES OF *NEISSERIA GONORRHOEAE*[1]

Property	T1	T1'	T2	T3	T4	T5
Diameter (mm)	0.5	0.5	0.5	1.0	1.0	1.0
Colour	grey gold	very dark gold	dark gold	pale brown	colourless	brown
Granularity	-[2]	++	+	+	-	++
Edge	smooth	irregular	smooth, defined	smooth	smooth	irregular
Opacity	translucent	opaque	translucent	translucent	translucent	opaque
Surface	smooth	uneven	smooth	smooth	smooth	uneven
Reflection of light	+	++	++	-	-	+
Convexity	++	++	++	+	+	+

1 After Chan and Wiseman (1975)

2 - to++= degrees of expression of the character viewed under oblique transmitted light with a Bausch and Lomb stereomicroscope at 20 hours growth on Gonococcal Base Defined Medium (GCBD), under 5% CO_2, 37°C.

Antigenic analysis of the lipopolysaccharide of Neisseria gonorrhoeae

Classification systems have been proposed for the gonococcus based mainly on immunological studies. Teague and Torrey (1907) used complement fixation and agglutination techniques to classify the gonococci, and were able to demonstrate strain differences. Watabiki (1910) proposed two categories of gonococci. Hermanies (1921) classified 85 strains into six groups by absorption experiments. Torrey and Buckwell (1922) studied 76 strains by cross-reacting them with antisera prepared to nine strains and found that 39 strains reacted strongly with the majority of the nine sera; 18 strains reacted to an intermediate extent, and 19 strains did not react. They concluded that certain gonococcal strains have antigenic compositions in common with those of many other gonococcal strains.

Tulloch (1922) differentiated 20 strains of the gonococcus into three groups by means of cross reaction agglutination experiments with four antisera. He demonstrated a reduction in agglutination titres when the antisera prepared to gonococcal antigens were initially absorbed with Neisseria meningitidis. Atkin (1925) classified gonococci into two groups according to source of isolation (i.e., chronic or acute cases of gonorrhea). Caspar (1937) divided his strains into two groups based on their type specific carbohydrates. Agglutination tests demonstrated that antisera to polyvalent antigenic strains gave strong reactions with strains of the predominant serotype. Stokinger et al.(1944) found that the

gonococci could not be grouped. He found closer immunological relationship between certain strains of Neisseria intracellularis and Neisseria gonorrhoeae than between some Neisseria gonorrhoeae strains. Unlike Stokinger, Wilson and Miles (1946) divided Neisseria gonorrhoeae into two serological groups. Wilson (1954) described eight antigens in the protein fraction of his strains; four of these were group specific and four were type specific. That is, group specific antigens were common to all strains of the gonococcus, whereas type specific antigens varied from strain to strain.

Chanarin (1954) studied the serological properties of many gonococcal strains by means of hemagglutination, hemolysis, and complement fixation techniques. He used sheep erythrocytes coated with an alkali extract of gonococci in these tests, and suggested that a polysaccharide factor was probably responsible for the adsorption to erythrocytes.

Hutchison (1970) studied 181 strains of N. gonorrhoeae and found it possible to classify the organism into different serological groups. Five different antisera were prepared to whole cell suspensions of gonococci by intravenous injections of gonococcal cells into rabbits and cockerels. These antisera were absorbed and then were reacted with the 181 strains: one hundred and forty-three strains reacted with one or other of the five antisera, thirty-eight of the 181 strains gave no reaction to any of the five antisera, and forty-three strains exhibited positive precipitin tests with two or more of the antisera. She suggested that a larger number of classifications of the gonococci could be developed

if a greater number of antisera were used. She found that repeated subculture of the bacteria reduced serological specificity.

Maeland (1967, 1968, 1969 a, b, c) and Maeland et al. (1971) studied endotoxin extracts of Neisseria gonorrhoeae. Endotoxins are defined by Davis and Dulbecco (1973) as complex LPS of the bacterial cell wall which are released into the surrounding medium when the organisms become autolyzed or are artificially disrupted by mechanical or chemical means. Endotoxin is composed of LPS and protein. Kadis et al. (1971) found that the LPS is the moiety in the endotoxin which is responsible for the biological activity manifested by the endotoxin. The proportions of LPS and protein in the endotoxin varies in accordance with the extraction method used. The nature and number of antigenic determinants of the endotoxin also varies with the extraction methods used. A variety of endotoxin products were studied by Maeland and co-workers. They found that endotoxin had two types of antigenic determinants. One determinant was polysaccharide and the other was protein in nature. These determinants were designated as determinants "a" and "b" respectively. Determinant "a" was the only determinant present and LPS was found to be the major component, in the PW extracted endotoxin. Extraction of endotoxin with boiling water, alkali, trichloroacetic acid and aqueous ether yielded endotoxins which contained both determinants "a" and "b". The aqueous ether extracted endotoxin yielded 90% dry weight

protein and less than 3% carbohydrate and lipid. Six "a" determinants were proposed (a_1-a_6) and were observed to vary with the gonococcal strain studied. Determinants "a" belonged to the same molecular complex, were multi-specific and responsible for antigenic specificity; and were thought to be analogous to the O-antigens of Salmonella. Determinants "b" were identical to each other and cross-reacted serologically. Other Gram-negative cocci excluding meningococci did not cross-react with determinant "b" of the gonococci, so determinant "b" was described as a group reactive antigen in gonococci and meningococci. Determinants "a" and "b" probably form a tightly bound complex on the bacterial surface.

Maeland (1969b) studied the serological cross-reactions of the aqueous ether extracted endotoxin from Neisseria gonorrhoeae, and suggested a classification based on "a" determinants. Antigenic relationships between gonococci and meningococci, and between gonococci and non-pathogenic Neisseria were also noted. It was demonstrated that the serologically specific cross-reactions were due to the "a" factors shared by cross-reacting endotoxins, and not to the identical shared "b" determinants, which were found to be present in the gonococci and meningococci.

In another paper (Maeland and Kristoffersen, 1971), they observed that PW extracts had much greater serological reactivity with antiserum to "a" determinants than did the aqueous ether extracts. While both aqueous

ether and PW extracts inhibited agglutination by antibody to "a" determinants, that of PW inhibited agglutination most strongly. All of the aqueous ether preparations inhibited agglutination of antibody to "b", while only 2/3 of the PW extracts inhibited agglutination of antibody to "b".

Apicella and Allen (1973) isolated and characterized the β-antigen ("b" determinant) of Neisseria gonorrhoeae present in alkali-extracted endotoxin. The β-antigen comprised about 1% dry weight of the preparation. The absence of 3-deoxy-D-manno-octulosonic acid (KDO) in the β-antigen indicated that it was uncontaminated with LPS.

Apicella (1974) studied two antigenically-distinct populations of virulent colonial types 1 and 2 of the gonococcus. He isolated and characterized an acidic polysaccharide, GC1, from the first of these two groups, and the other, GC2, from the second. It seems very likely that these two polysaccharides are identical with two of the "a_1"-"a_6" determinants described earlier by Maeland (1969a,b,c). Apicella also found that a few strains contained determinants other than GC1 and GC2. The size, nature and charge of these antigens showed that they were unrelated to pili.

Methods other than antigenic analysis have been used in the attempt to classify the gonococci. Carifo and Catlin (1973) differentiated clinical isolates of gonococci on the basis of their patterns of growth responses on chemically defined media which either contained or did not contain

selected compounds. The compounds which elicited various growth responses were L-proline, L-arginine, L-ornithine, L-methionine, hypoxanthine, uracil, thiamine, and thiamine pyrophosphate. The inability of an organism to grow when certain nutritional sources are not available or to grow in the presence of certain nutrients, indicates that the nutritive requirements of bacteria are very specific and are closely related to the enzymes which are present in the different organisms. This auxotyping procedure allowed them to differentiate 251 clinical isolates on a set of 11 different chemically defined agar media. They could not reveal nutritional differences between colony types 1, 2, 3, and 4 of a given clinical isolate. The authors did not state whether their results indicated that different colonial types of a given clinical isolate always give similar growth patterns on all 11 defined growth media.

Catlin (1973) also compared Neisseria gonorrhoeae, Neisseria meningitidis, and Neisseria lactamica, on the basis of their growth on various chemically defined media. She found that all gonococcal strains had an absolute cysteine or cystine requirement but unlike meningococci, they showed great variation in their growth requirements. Seventy-four gonococcal strains were divided into 13 auxotypes on the basis of their requirement for hypoxanthine, thiamine pyrophosphate, isoleucine, and arginine. No attempt was made to correlate these auxotypes with other immunological classification systems. Auxotypes were stable

over a period of months.

Chemical composition of gonococcus LPS

While endotoxins from various species of Gram-negative bacteria have many properties in common, major differences in chemistry have been observed. Until recently it was not clear whether gonococcus endotoxin resembled that of other Gram-negative species. Tauber and Garson (1959) and Tauber and Russell (1961) extracted a protein and nucleic acid-free toxic LPS from the gonococcus. Chemically, this substance appeared to be a phosphoric acid ester and they concluded that it had properties in common with endotoxin as defined by Westphal and Jann (1965).

Stead et al. (1975) have studied the chemical composition of gonococcus LPS. All PW extracted preparations contained glucose, galactose, glucosamine, heptose, KDO and phosphate. They found beta-OH-10:0, beta-OH-12:0, beta-OH-14:0, 12:0, 14:0, 16:0, 16:1, 18:0, and 18:1 fatty acids in the lipid A moiety,with some variation from strain to strain in colonial types 1 and 4. They were unable to detect significant differences in sugar composition of the polysaccharide component of the two types and concluded that O-specific chains were absent from the virulent type 1. However, it does seem plausible that sugars characteristic of O-specific chains of other Gram-negative bacteria (for example, dideoxy sugars) are absent from the LPS of virulent gonococci. Perry et al. (1975) have also examined LPS of types 1 and 4 gonococci and found that type 1 LPS

possessed characteristic sugars absent or different in concentration from type 4 avirulent LPS, in contrast with the conclusions of Stead et al. (1975). Common to both types 1 and 4 LPS, however, were lipid A and a core fragment composed of D-glucose, D-galactose, glucosamine, heptose and KDO. In this connection, it should be pointed out that Maeland et al. (1971) found that galactose inhibited the reaction between determinants "a_2", "a_5", "a_6" and homologous antibody. This suggested that galactose is the terminal sugar in these polysaccharides. Since lactose inhibited the reaction to a greater degree than galactose,glucose may well be the second sugar in the terminal segment. The position of the linkage seemed important, since alpha and beta-galactosyl-(1,4)-glucose inhibited the reaction while alpha-galactosyl-(1,6)-glucose did not. They also noted that some of the glucose and galactose-inhibiting activity was not neutralized by antibody and it is possible that these sugars are present elsewhere than in the terminal segment of the polysaccharide.

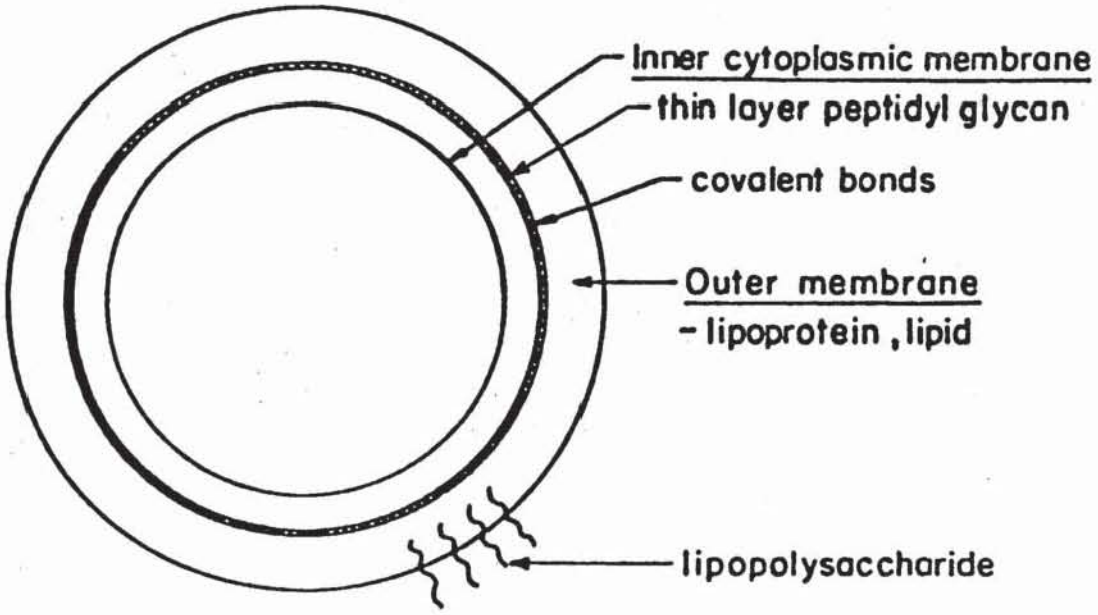
The findings of Stead et al. (1975) and Perry et al. (1975) are similar in many respects to those of Jennings et al. (1973) who studied the LPS of N. meningitidis. These authors found that LPS isolated from meningococcus serogroups A, B, X, and Y contained glucose, galactose, heptose, KDO and phosphate. In addition, the material contained lipid A, ethanolamine, protein and fatty acids similar to those characteristic of gonococcus LPS. There seems to be little information available

on the terminal sugar sequences in meningococcal serotypes.

All of the data available at present indicate that all the LPS of Neisseria spp. is in most respects similar to that of other Gram-negative bacteria. The main point of disagreement seems to centre on whether LPS from virulent strains of Neisseria contains the O-specific chains so characteristic of "smooth" enteric bacteria.

Structure of the cell envelope of the gonococcus and other Gram-negative bacteria

Figure 1 shows a diagram of the bacterial cell wall and its major constituents. The outer membrane which contains the LPS is the layer which has been of interest in this study. The LPS can be observed to contain lipid which retains toxicity and polysaccharide which is responsible for antigenic specificity. The core sugars are the common sugars which make up a constant polysaccharide to which O-specific residues attach. Side chains can be made of linear trisaccharide or branched tetra or pentasaccharide chains of up to 25 repeating units. The sugars in the core region are the sugars found to be present in all LPS preparations studied by Jennings et al. (1973).



Outer Membrane

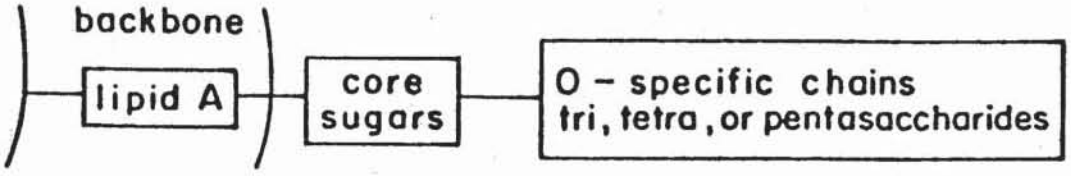


FIGURE 1 GRAM NEGATIVE BACTERIAL CELL WALL

CHAPTER III

MATERIALS AND METHODS

Growth of strains

Selection and identification: Neisseria gonorrhoeae strains 52875, 52778, 52698, 380657, 34590, 34559, X, 368311, and 3956, and Neisseria sicca strain 13248 were fresh isolates obtained from the Health Sciences Centre bacteriology laboratory in Winnipeg. Neisseria meningitidis strain N86 was obtained from Dr. A. Ronald of the University of Manitoba, Department of Medical Microbiology. Neisseria gonorrhoeae strain F62 was obtained from the Centre for Disease Control (CDC) in Atlanta, Georgia. Gonococcal strains GC2, GC46, GC50, and GC48 were obtained from Tulane University, New Orleans, Louisiana, and strain 9T1 was obtained from the CDC laboratory in Atlanta, through Dr. Ronald.

All Neisseria strains were Gram-stained and oxidase-tested to confirm that they belonged to the genus Neisseria. The bacteria oxidized tetramethylparaphenylenediamine while surface colonies turned first blue and then black.

Sugar fermentation tests were performed on Neisseria

meningitidis and Neisseria sicca to confirm species types. Muller-Hinton broth suspensions which contained sucrose, maltose, and glucose, were incubated with a loopful of organisms and incubated at 37°C under 5% CO_2 in a Hotpack incubator (Waterloo, Ontario). Tubes were observed for growth after 24 and 48 hours of incubation. N. meningitidis strain N86 was observed to ferment glucose and maltose, while N. sicca strain 13248 fermented glucose, maltose and sucrose. These fermentation patterns clearly identified the species of non-gonococcal Neisseria. Gonococcal strains could easily be differentiated from non-gonococcal strains visually with the aid of a stereomicroscope, and therefore did not need to be fermentation-tested.

Maintenance and preservation: Differentiation and identification of colonial types were performed according to the criteria of Kellogg et al. (1963), as given by Chan and Wiseman (1975) in Table I. Bacterial strains were maintained by daily selective subculture on Gonococcal base defined medium (GCBD) (BBL) agar with defined supplement (see Appendix A), and incubation at 37°C under 5% CO_2. The organisms were prepared for storage by use of the snap-freezing method of Ward and Watt (1971) in which cells were suspended in a solution of 8% v/v glycerol and 1% w/v proteose peptone in 1.0 ml ampoules, and stored in liquid nitrogen.

Growth in liquid media: Growth of N. gonorrhoeae type 1 colonies was tried in GCBD liquid medium and Enriched Single Phase (ESP) medium of Chan and Wiseman (1975). Neither of

the two liquid media provided good growth. However, growth in bulk was achieved with the use of GCBD solid medium.

Growth in bulk: Neisseria strains were grown in large quantities for LPS production. Twelve petri plates were streaked with the organism to be grown in bulk. These plates were incubated for 24 hours under the same conditions as those used to maintain the bacterial strains. Growth on these plates was examined under a stereomicroscope and those plates which had a high proportion of the desired colonial type, were harvested with Dulbecco's phosphate buffered saline (PBS) by means of a bent glass rod. Approximately two hundred plates of GCBD medium were then inoculated with the bacterial saline suspension at 10^4 colony-forming units (CFU)/ petri dish. The inoculated plates were incubated under maintenance conditions for 24 hours. Growth was harvested from the agar plates with PBS by means of a bent glass rod, and the proportion of colonial types was determined by the method of Miles and Misra (1938), which is described below under the heading 'Viable counts'. It was necessary to calculate the percentage of colonies present which were colonial types other than the desired type, as colony types 1 and 2 when grown in vitro give rise to colony types 3, 4, and 5. The harvested bacterial suspension was centrifuged at 3000 rpm for 15 minutes and the bacterial pellet was then stored at $-20^{\circ}C$. The amount of bacteria harvested per batch incubated ranged from 2 to 10 grams wet weight. Subsequent batches of the same strain of bacteria

were produced until approximately 20g. wet weight bacteria was grown. Lipopolysaccharide was then extracted from the pooled whole cell material.

Viable counts: Viable counts were performed according to the method of Miles and Misra (1938). Ten-fold dilutions of the bacteria were made in saline and 0.6 ml of each dilution was plated on GCBD agar by means of a calibrated pasteur pipette. Plates were incubated for 20 hours under maintenance conditions. Drops which contained between 30 and 300 CFU were counted, and the proportion of colonial types calculated. The proportion of colonies present which belonged to the colonial type that was cultivated ranged between 88% and 100% dependent on colony type and strain grown.

LPS extraction methods

Saline extraction: Lipopolysaccharide was extracted from the bacteria by the SE technique devised by Maeland et al. (1973). In this method, the cells were suspended for four days in PBS at a concentration of 1 g. wet weight bacteria per 4 ml PBS at 4°C. The suspension was then centrifuged at 30,000g for 20 minutes and the supernatant which contained the LPS was purified according to the procedure of Westphal and Jann (1965) described below, with the difference that supernatant fluid rather than whole cells was treated with the phenol. The SE extraction method as well as the PW extraction and purification

procedure is outlined in Figure 2.

Phenol-water extraction: Several gonococcal LPS samples were extracted by a second method, the hot PW method of Westphal and Jann (1965). Twenty g. wet weight of bacteria were suspended in 150 ml of water at 65°C. Phenol (150 ml of a 90% solution), preheated to 65°C was added and the mixture was stirred for 15 minutes. The solution was then cooled to 10°C in an ice bath and centrifuged for 45 minutes at 1500g to facilitate the separation of the aqueous phase and the phenol phase. The clear water phase was removed and the phenol phase was treated again with a second 150 ml volume of water. The water extracts were combined and dialyzed against running tap water for 48 hours, and then lyophilized. Ultracentrifugation of the LPS at a concentration of 0.3% in water at 105,000g for three hours was carried out two times to remove the nucleic acids and degraded products, which stayed in suspension.

The final LPS pellet was suspended in 2-3 ml of water and was freeze-dried. The LPS was treated with cetavlon (2% hexadecyl trimethylammonium bromide in water) which is a cationic detergent, in order to precipitate the nucleic acid present in the extract, and then the LPS was ethanol-precipitated, suspended again in water and dialyzed for 48 hours to remove the salts. After dialysis, the LPS was lyophilized and then stored at -20°C in dry powder form to keep it free from bacterial and fungal growth to which it is susceptible.

It should be made clear at this point that the essential

difference between SE and PW-LPS is that in the latter, whole cells are directly extracted while in the former, a saline extract is purified by the phenol-water method.

Immunological methods

Preparation of antisera: Antisera against Neisseria strains were prepared in New Zealand white rabbits. Type 1 and 4 cells of N. gonorrhoeae and cells of N. meningitidis and N. sicca grown on solid medium for 24 hours as previously described were harvested with PBS and treated with 1% v/v formalin at 4°C overnight. The formalin treatment killed the organisms.

The cells were washed 3 times with PBS and were re-suspended to concentrations of 10^8, 10^9, and 10^{10} per ml.

Two immunization schedules were followed, which were found to be equally satisfactory. Antisera to strains F62, X, GC50, GC2, 380657, 34590, and 368311 were obtained according to the method of Rudbach (1971) as modified by Chan (1974), in which intravenous injections of 10^8 CFU on days 1, 3, 5; 10^9 CFU on days 7, 9, 11; and 10^{10} CFU on days 13, 15, and 17, were given. Immunized rabbits were bled twice during the second week following the last injection. Apicella's (1974) schedule, which was the second method used, involved intravenous injections of 10^9 CFU injected on days 1, 7 and 14. Rabbits were bled 7 days after the last injection. The blood was allowed to clot overnight at 4°C. Sera were removed, centrifuged at 1600g for 15 minutes, and stored at

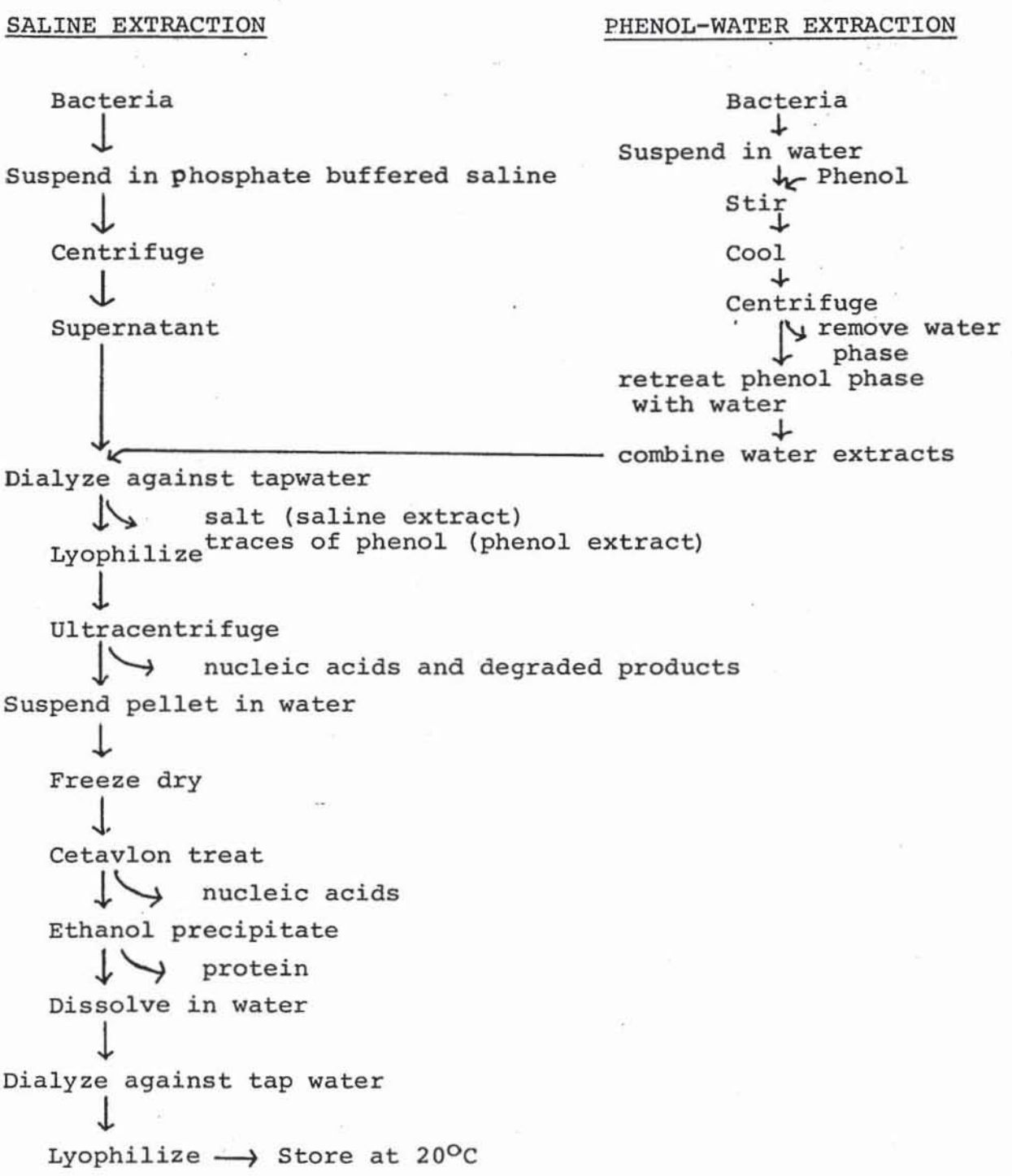


FIGURE 2. EXTRACTION OF BACTERIAL LIPOPOLYSACCHARIDE

-20°C until required.

Antisera were heated for 30 minutes at 56°C. The sera were also absorbed with unsensitized sheep red cells prior to use in order that any antibodies to unsensitized sheep red cells would be absorbed and would not interfere in IHA assays. Absorption was performed with 0.2 ml packed sheep red blood cells per 1.0 ml sera at room temperature for 1 hour.

Sensitization of sheep erythrocytes to LPS: Sheep erythrocytes were centrifuged (600 - 1000g) and washed three times with PBS. Sensitization of the cells to LPS was performed by the method of Ciznar and Shands (1970) in which 5 ml of a 1% solution of washed sheep erythrocytes was incubated with 0.1 ml of a 1% (w/v) solution of LPS in water at 37°C for 1 hour. The erythrocytes were then resuspended to a 1% solution for use in the IHA test. Sensitized erythrocytes were kept as 4% suspensions v/v at 4°C. Unfixed sensitized erythrocytes could be kept for about two weeks without deterioration.

Lipopolysaccharide was alkali-treated when it was to be used to sensitize sheep erythrocytes for use in IHA. According to Chan (1974), untreated gonococcal LPS did not sensitize erythrocytes. This treatment affected the LPS surface such that the LPS would adhere to the erythrocyte surface more readily. Alkali treatment of the LPS was performed according to the method of Davies et al. (1958). An equal volume of a 1% (w/v) solution of LPS in water was incubated with 0.04 N NaOH at 37°C for 18 hours. The

solution was then neutralized with 0.2 N HCl as described by Davies et al. (1958).

Lipopolysaccharide for sensitization of sheep erythrocytes which were to absorb antisera, was not alkali-treated. The LPS in this case was untreated because alkali-treated LPS preparations when compared with non-alkali-treated preparations showed a decreased antibody-neutralizing activity in hemagglutination tests, according to Chan (1974). Absorption of antisera with sensitized erythrocytes was performed by incubation of 0.16 ml sensitized packed sheep erythrocytes with 0.15 ml antisera, at 37°C for 1 hour. Absorption was performed once. The supernatant of the newly sensitized erythrocytes was able to sensitize a different batch of sheep cells. This indicated that there was an excess of antigen present during the first absorption procedure and that all the possible sensitization sites were filled.

Indirect hemagglutination test (IHA): This was performed according to the method of Tomiyama et al. (1973), with the use of microtiter equipment. Doubling dilutions of antisera were prepared in PBS, and sensitized erythrocytes were added to each dilution well. Controls used in the IHA test were: 1) Antisera diluted 1:2 with PBS and unsensitized sheep red blood cells, 2) PBS and sensitized sheep erythrocytes.

The end point in the IHA test was considered to be the highest dilution of antiserum at which agglutination of erythrocytes was observed. This was recorded as the recipro-

cal of the dilution (concentration) in units per ml (HU/ml).

Analytical techniques

Gas-liquid chromatography: Sugar components of the LPS were analyzed by GLC. Several mg of LPS from each strain tested, were hydrolyzed with 1 ml of 1N H_2SO_4 in sealed ampoules for 10 hours at 100°C. The hydrolyzed sugars were neutralized with barium carbonate, the mixture centrifuged at 3000g for 15 minutes and the supernatant lyophilized. The sugars were treated with 1 ml "Sil-prep" reagent (pyridine 9 parts, hexamethyl-disilazane 3 parts, trimethylchlorosilane 1 part) obtained from Applied Science Laboratories, State College, Pa., for 1 hour at room temperature. This procedure converted the sugars to their trimethylsilyl derivatives (TMS). The TMS sugar preparations were then injected into the GLC for analysis.

Samples were applied to the column by means of a 10µl Hamilton syringe. Authentic carbohydrate standards were obtained from Applied Science Laboratories, and used as references. For a summary of GLC operating conditions, see Appendix C.

Infra-red spectophotometry (IR): Infra-red spectrophotometry was performed with a Unicam SP200 spectrophotometer. Lipopolysaccharide samples were powdered with KCl in a concentration of 50 mg LPS to 500 mg KCl and formed into a disc by the use of a press. The procedure was performed in

the Biochemistry Department at the Medical School. Lipopolysaccharide samples were scanned at various stages of purification, and comparisons made.

Ultraviolet scan (UV): Ultraviolet scans were performed with a Pye-Unicam SP800 spectrophotometer in the wavelength range of 200 to 400 nm. Lipopolysaccharide samples were dissolved in water in a concentration of 0.5 mg LPS/ml.

CHAPTER IV

RESULTS

A. Production of gonococci in liquid and solid media.

Liquid media: The ESP medium (see Appendix B) devised by Chan and Wiseman (1975) was initially reinvestigated. In this experiment, 25 and 250 ml volumes of ESP medium were inoculated with about 4×10^4 CFU of type 1 cells. The cells were grown at 37°C in a New Brunswick shaker in an atmosphere of 16% CO_2. The rate of shaking was 160 rpm and incubation was terminated at 27 hours in accordance with the procedure of Chan.

Results are shown in Table II in which it is observed that in 25 ml volumes, an increment of about 10^4 cells was obtained which confirms Chan and Wiseman's findings. Stability of the type 1 cells was maintained throughout the incubation period. In contrast, the use of larger volumes of ESP medium (250 ml in 1L flasks) under the same conditions resulted in such poor yields of cells that measurements were not performed. The disadvantage of the ESP medium was that although yields in 25 ml volumes were satisfactory, those in larger volumes were not. Consequently, solid media were considered.

Solid media: ESP medium to which agar had been added was investigated, but growth of gonococci was poor.

TABLE II

GROWTH OF TYPE 1 GONOCOCCI

IN ESP LIQUID MEDIUM[1]

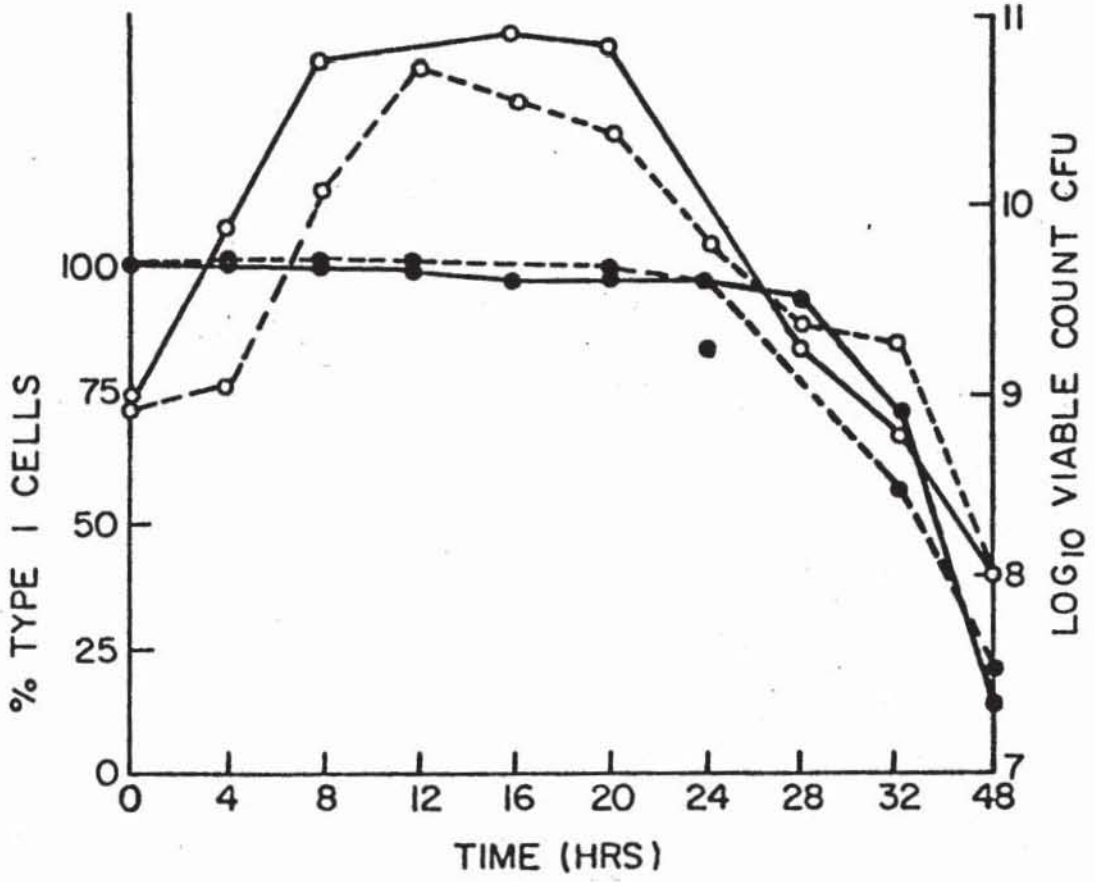
Strain	Viable Count (CFU/ml) initial	final
380657	$4x10^4$	$4.02x10^8$
369003	"	$4.66x10^8$
368311	"	$2.84x10^8$
F62	"	$5.92x10^8$

[1]Cells grown at 37°C in 25 ml volumes in New Brunswick incubator in an atmosphere of 16% CO_2, and shaken at 160 rpm for 27 hours.

Reasonable yields were obtained on GCBD agar when incubated under 5% CO_2. It was decided to examine the importance of CO_2 tension on growth. Strain 3956 type 1 was incubated on GCBD under CO_2 concentrations of 0, 5 and 10%. Reference to Figure 3 indicates that there was little difference between 5% and 10% CO_2 tensions in terms of their effect on yield. A concentration of 0% CO_2 tension (not shown), did not permit growth. With respect to colonial type stability, at both 5% and 10% CO_2 tensions, the percentage of type 1 remained near 100% at the end of 24 hours, but rapidly decreased beyond that time. Continued incubation up to 48 hours favored the appearance of type 4 colonies, and viable counts decreased.

One point worth mentioning is that a fairly high initial inoculum always seemed to be required in the order of 4×10^4 CFU/ml for liquid medium and 10^9 CFU gonococci on solid medium. Increments in viable counts over the 24 hour growth period, were approximately 10^4 CFU/ml in 25 ml of liquid ESP medium and 10^2 on solid GCBD medium. At the larger liquid medium volume, growth was negligible. Since large production of bacteria in small volumes of liquid medium was not practical because of the few flasks which could be shaken in the incubator at one time, growth on solid medium was used.

As a result of this experiment, all inocula on GCBD plates for production of cells were incubated under 5% CO_2 tension and harvested at 24 hours. Although the viable counts at 24 hours growth were lower than at 12-20 hours



⊙- - - -⊙ % type 1 cells at 10% CO_2 tension
⊙———⊙ % type 1 cells at 5% CO_2 tension
○- - - -○ viable counts at 10% CO_2 tension
○———○ viable counts at 5% CO_2 tension

FIGURE 3 EFFECT OF CO_2 ON GROWTH AND COLONIAL TYPE STABILITY ON GCBD AGAR IN GONOCOCCUS STRAIN 3956

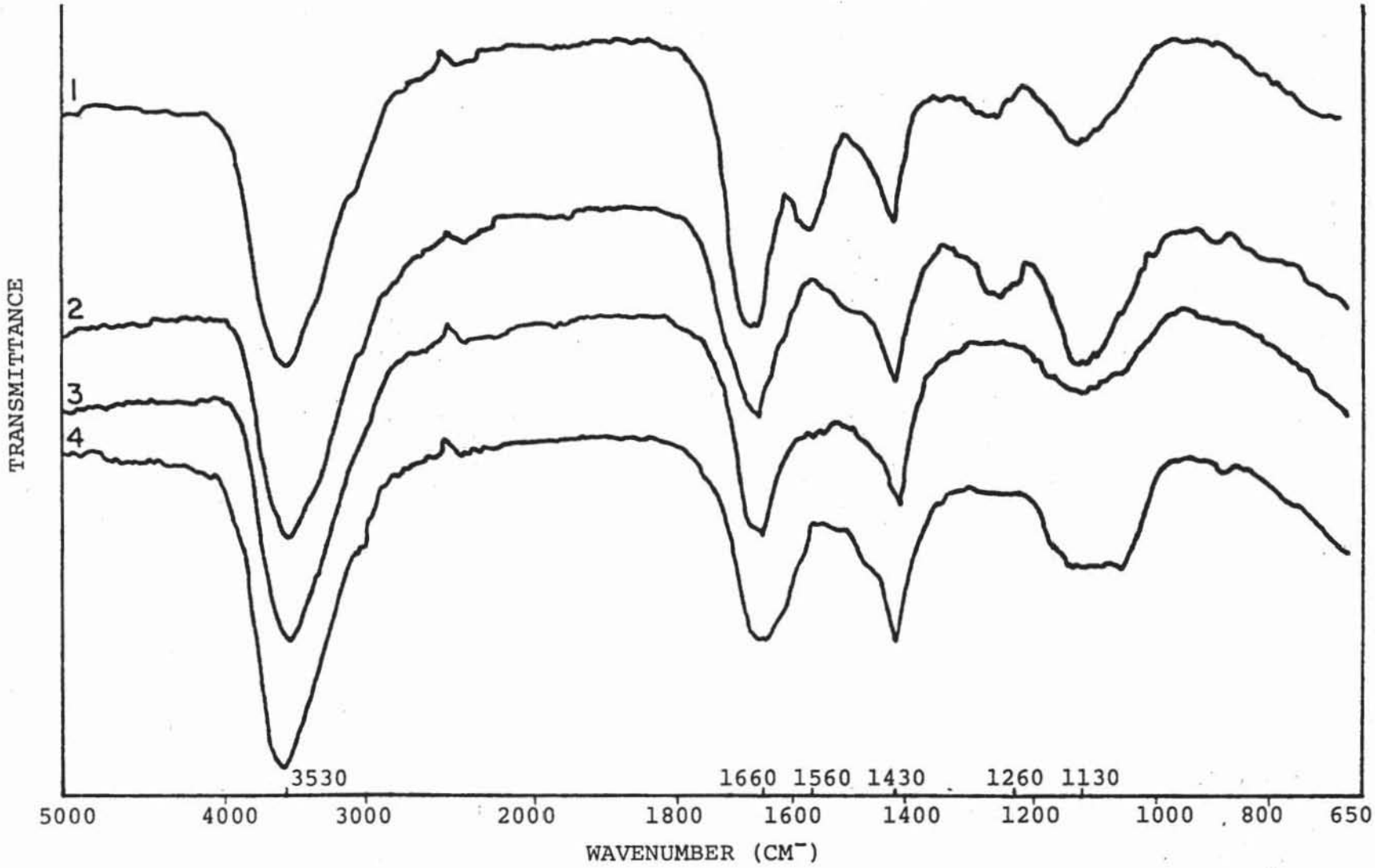
growth, the percentage of type 1 cells at 24 hours was approximately 100 percent of the viable cells, so harvesting was done at 24 hours growth.

B. Properties of gonococcus LPS: comparison of saline extracted and phenol-water extracted LPS

Spectrophotometry: Lipopolysaccharide fractions were prepared from several gonococcus strains according to the SE method of Maeland et al. (1973) and the hot PW method of Westphal and Jann (1965) as described elsewhere in Materials and Methods.

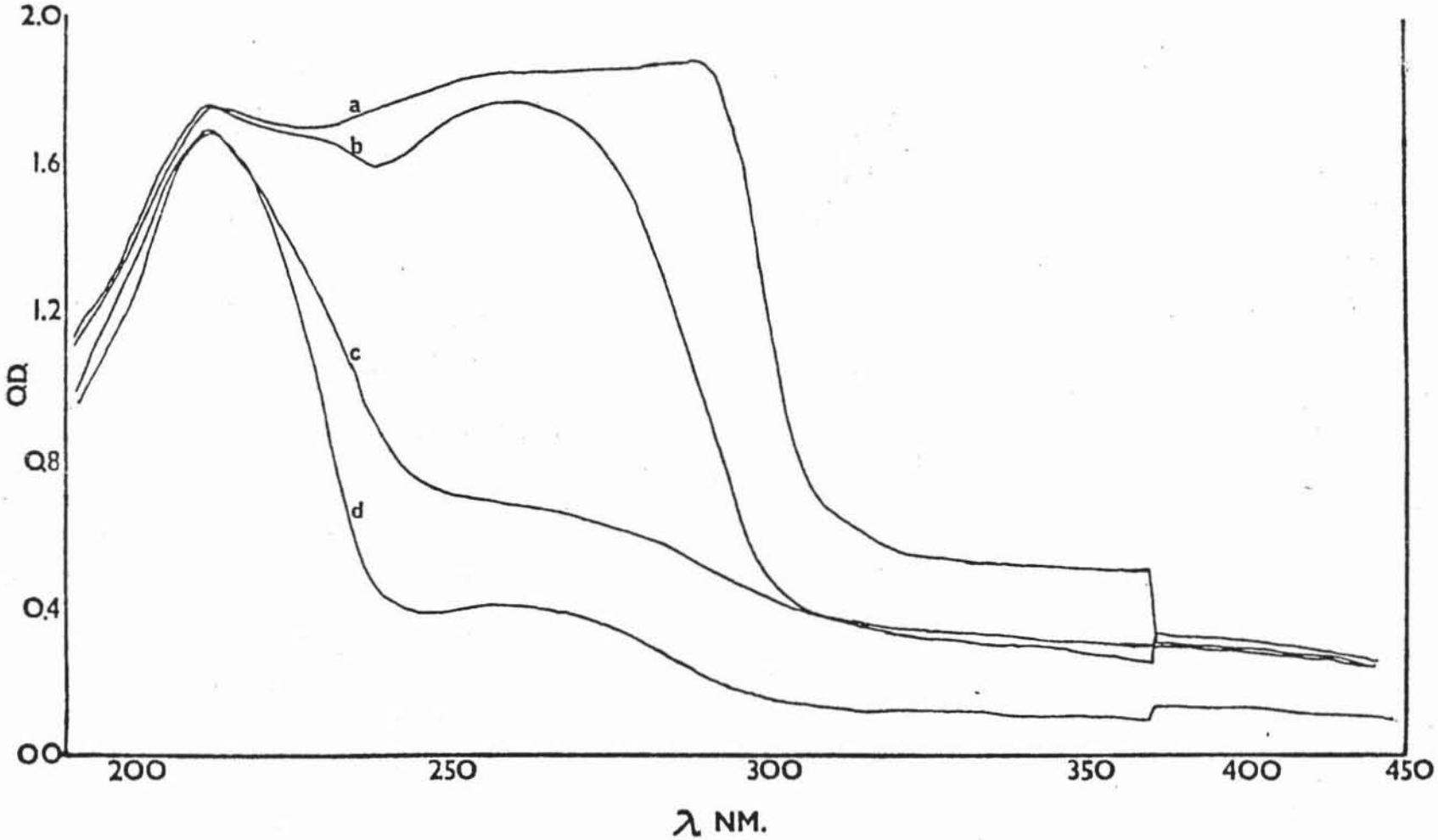
Infra-red scans obtained with these preparations in both crude and purified stages of preparation are shown in Figure 4. While spectra of both crude and purified preparations are characteristic of LPS of Gram-negative bacteria (Davies et al. 1958), certain differences were observed between these preparations. Crude SE material showed a peak at 1560 cm^{-1} which is absent from the PW preparation. Crude SE and PW preparations contain peaks at 1260 cm^{-1} which are not present in the purified preparations. Peaks at 3530 cm^{-1}, 1660 cm^{-1}, 1430 cm^{-1} and 1130 cm^{-1} are present in all four preparations.

Spectrophotometry in the UV range shown in Figure 5 indicates that there are differences in the 9T1 and F62 LPS preparations related to the treatment they have received. Comparison of PW extracted purified LPS



(1) saline extracted crude LPS; (2) phenol water extracted crude LPS;
(3) saline extracted purified LPS; (4) phenol water extracted purified LPS.
The ordinate scale has been omitted so that several curves may be simultaneously compared.

FIGURE 4 INFRA RED SPECTROPHOTOGRAMS OF LIPOPOLYSACCHARIDE OF N. GONORRHOEAE STRAIN 3956 TYPE 1



(a) phenol extracted purified LPS strain 9T1
(b) saline extracted crude LPS strain F62
(c) saline extracted purified LPS strain F62
(d) saline extracted purified LPS strain 9T1

FIGURE 5. SPECTROPHOTOMETRIC SCAN IN UV REGION OF SPECTRUM OF N. GONORRHOEAE TYPE 1 LPS

(curve a) and SE-extracted purified LPS (curve d) of the 9T1 strain shows that the latter preparation contains less material which absorbs in the 260-280 nm region. There is a similar difference between SE-extracted crude and purified LPS from the F62 strain (curves b and c).

C. Analysis of selected LPS preparations for sugar content.

The LPS of several strains of gonococci, extracted by the SE method, were analysed for neutral sugars as described in Materials and Methods. Preparations of LPS obtained by the PW method were not analyzed.

Table III presents results of an analysis of neutral sugars from four colonial type 1 and one type 4 N. gonorrhoeae LPS preparations.

The type 1 samples all contained KDO, D-mannose, D-glucose, and D-xylose. Two of the type 1 preparations, of strains 3956 and F62, contained D-galactose, while strains GC50 and GC48 did not. Type 1 colonies of strains F62 and GC48 contained D-ribose, while strains 3956 and GC50 type 1 colonies did not. One strain, GC50 contained rhamnose.

The colonial type 4 LPS of strain 3956 contained KDO, D-galactose, D-glucose, D -ribose, and did not contain rhamnose, D-xylose and D-mannose.

The concentrations of each sugar in the LPS ranged from 0 to 0.12 µg/µg LPS for rhamnose, from 0 to 0.87 µg/µg LPS for D-ribose, from 0 to 0.173 µg/µg LPS for D-xylose, from 0.02 to 0.232 for D-glucose, from 0 to 0.065

TABLE III

ANALYSIS OF NEUTRAL SUGARS IN GONOCOCCUS LPS PREPARATIONS BY GAS-LIQUID CHROMATOGRAPHY

Strain	Colony type	Sugar Constituents (µg/µg LPS)							Total sugar concentration (µg)	Total sugar/ KDO ratio
		Rhamnose	D-Ribose	D-Xylose	D-Glucose	D-Mannose	D-Galactose	KDO		
3956	1	0	0	0.002	0.02	0.018	0.157	0.087	0.482	5.5
GC50	1	0.012	0	0.070	0.232	0.065	0	0.141	0.520	3.7
F62	1	0	0.087	0.173	0.086	0.049	0.006	0.005	0.406	81.2
GC48	1	0.09	0.0162	0.018	0.113	0.043	0	0.034	0.314	9.2
3956	4	0	0.059	0	0.056	0	0.050	0.135	0.300	2.2

for D-mannose, from 0 ÷ 0.157 for D-galactose, and from 0.005 to 0.141 µg/µg LPS for KDO.

The total concentrations of these seven neutral sugars in each preparation ranged from 0.300 µg/µg LPS to 0.520 µg/µg LPS. The type 4 LPS preparation had the lowest concentration of neutral sugars in the analysis at 0.300 µg/µg LPS. Also, examination of total sugar/KDO ratios revealed that the type 4 preparation was exemplified by the lowest value.

D. Comparison of IHA titres of SE and PW-extracted LPS

The LPS of two type 1 gonococcus strains, GC2 and 9T1, was obtained by the SE and PW methods of extraction. Erythrocytes were sensitized to these LPS preparations and were then titrated with a number of antisera to type 1 strains in the IHA test as shown in Table IV. As indicated in this table, titres were invariably higher when PW-extracted LPS was used as the sensitizing agent.

However, it was decided to use the SE-extracted material in subsequent antigenic cross-reaction experiments because it was felt that the SE-extracted LPS more nearly represented the natural state of the bacterial LPS, as it was less likely to contain whole cell material.

E. Antigenic cross-reactions of gonococcus strains in the IHA test

Antisera to a number of strains of type 1 gonococci were prepared in rabbits as described elsewhere, and

TABLE IV

COMPARISON OF SALINE EXTRACTED AND PHENOL-WATER EXTRACTED LPS IN THE INDIRECT HEMAGGLUTINATION TEST

	IHA titre (units/ml) with erythrocytes sensitized to type 1			
Antisera[1]	GC2 SE[2]	GC2 PW[3]	9T1 SE	9T1 PW
52875	4	96	4	768
3956	4	64	6	32
F62	8	128	8	512
52778	8	128	8	64
34559	2	64	2	96
GC50	8	256	8	1024
GC46	2	512	2	32
59568	2	512	2	64
59576	2	32	6	32
N86 (*N. meningitidis*)	4	32	6	64
13248 (*N. sicca*)	4	16	2	32

[1] To type 1 strains

[2] Saline extract

[3] Phenol-water extract

titrated against erythrocytes sensitized to the respective SE-extracted LPS. Results are shown in Table V. As observed, cross-reactions were noted in varying degree amongst the 10 LPS preparations studied. The N86 (meningococcus) and 13248 (N.sicca) strains also cross-reacted with the gonococcus strains. On the basis of this experiment, strains were classified according to the degree of cross-reaction as shown in Table VI. Most strains showed little difference in titre when tested against the various antisera but in several (3956, 380657, N86, and 13248), the differences were frequently greater than two tubes dilution factor when homologous were compared to heterologous titres. That is, there was a more frequent number of weak cross-reactions with these strains. It was observed that in several cases, cross-reactions of antisera with the homologous LPS-sensitized erythrocytes (eg. 380657 antisera vs 380657 LPS-sensitized cells) did not yeild the highest titres when compared with cross-reactions of antisera with heterologous LPS-sensitized cells (eg. 380657 antisera vs GC2 or GC50 LPS-sensitized cells). These results cannot be explained.

In another experiment, five antisera were adsorbed with homologous LPS and titrated against erythrocytes sensitized with SE-extracted LPS of various strains, as indicated in Table VII. As expected, some differences in strains are revealed, and these are set out in Table VIII. For example, adsorption of antiserum 52875 with the homologous LPS shows a strong similarity with LPS 13248 and 380657, but a weak

TABLE V

ANTIGENIC CROSS-REACTIONS BETWEEN ANTISERA TO LPS OF

N. GONORRHOEAE, *N. SICCA* (13248) AND *N. MENINGITIDIS* (N86)

Unabsorbed antisera to strain	Titres (HU/ml) with saline extracted LPS of Neisseria strains									
	52875	3956	X	F62	52778	380657	GC50	GC2	N86	13248
52875	16	16	16	8	16	32	8	4	8	32
3956	32	64	16	16	16	16	8	4	8	8
X	32	32	64	32	32	64	128	24	32	64
F62	32	--[1]	64	32	64	128	64	8	4	8
52778	16	16	32	8	16	16	64	8	8	16
380657	16	32	32	16	16	64	128	512	8	8
GC50	32	32	32	16	32	32	64	8	16	8
GC2	16	16	32	16	16	64	64	2	16	8
N86	16	16	32	16	32	32	64	4	256	8
13248	32	16	64	16	16	64	64	4	4	128

[1] no data

TABLE VI

RELATIONSHIPS BETWEEN STRAINS OF N. GONORRHOEAE, N. SICCA (13248) AND N. MENINGITIDIS (N86)

	Difference between homologous and heterologous titres (HU/ml)		
Antisera to LPS	None - 1 tube	>1 - 2 tubes	>2 tubes
52875	3956, X, F62, 52778, 380657, GC50 N86, 13248	GC2	---
3956	52875	F62, 52778, 380657	GC50, GC2, N86, 13248
X	52875, 3956, F62, 52778, 380657, GC50 N86, 13248	GC2	---
F62	52875, X, 52778 GC50	380657, GC2, 13248	---
52778	52875, 3956, X, F62 380657, GC2, N86 13248	GC50	---
380657	3956, X, GC50	52875, F62, 52778	GC2, N86, 13248
GC50	52875, 3956, X, 52778, 380657	F62, N86	GC2, 13248
GC2	52875, 3956, X, F62, 52778, 380657, GC50 N86	13248	---
N86	---	GC50	52875, 3956, X, F62, 52778, 380657, GC2, 13248
13248	X, 380657, GC50	52875	3956, F62, 52778, GC2, N86

[1]none - 1 tube difference is considered a strong cross-reaction. >1-2 tubes difference is considered partial cross-reaction and >2 tubes, a weak cross-reaction.

TABLE VII

ADSORPTION OF ANTISERA TO LPS WITH THE HOMOLOGOUS LPS ANTIGEN IN SELECTED NEISSERIA STRAINS

Antisera[1]	Adsorbed with LPS	Titres (HU/ml) with saline-extracted LPS of strains											
		52875	3956	X	GC2	13248	F62	52778	368311	380657	34590	GC50	N86
52875	--	16	16	16	4	32	8	16	32	32	32	8	8
	52875	<2	4	8	-	4	4	6	8	4	8	6	4
3956	--	32	64	16	4	8	16	16	16	16	8	8	8
	3956	4	8	16	-	8	<2	16	4	16	4	8	4
X	--	32	32	64	24	64	32	32	64	64	256	128	32
	X	<4	<4	<4	-	<4	<4	<4	4	<4	4	6	<4
GC2	--	16	16	32	2	8	16	16	32	64	32	64	16
	GC2	4	-	4	-	8	6	4	-	-	8	16	-
13248	--	32	16	64	4	128	16	16	2	64	128	64	4
	13248	4	6	-	-	6	<4	4	6	4	4	12	4

[1] 13248 is N. sicca. Others are N. gonorrhoeae

TABLE VIII

RELATIONSHIPS BETWEEN LPS OF *NEISSERIA SPP.* ADSORBED WITH HOMOLOGOUS LPS

Antisera[2]	Adsorbed with LPS	Difference in titre after absorption with homologous LPS[1]		
		None-2-fold	2-fold-4-fold	>4-fold
52875	52875	X, F62, GC50, N86	3956, 52778, 368311, 34590	13248, 380657
3956	3956	X, 13248, 52778, 380657, 34590, GC50 N86	52875, 368311	F62
X	X	---	---	52875, 3956, 13248, F62, 52778, 368311, 380657, 34590, GC50, N86
GC2	GC2	13248	52875, X, F62 52778, 34590, GC50	---
13248	13248	N86	3956, 52778, 368311	380657, 34590, 52875 F62, GC50

[1]none-2-fold difference is considered a weak relationship; 2-fold-4-fold a partial relationship; and >4-fold a strong relationship.

[2]13248 is *N. sicca*. Others are *N. gonorrhoeae*.

relationship with LPS of strains X, F62, GC50, and N86. It is noteworthy that adsorption of antiserum X with the homologous LPS distinguished no weak or partial relationships.

The last experiment in the series was an attempt to analyze the antigens present in three strains by means of the classical adsorption experiment. The complexity of analysis and lack of appropriate data precluded the investigation of more than three strains. The results shown in Table IX illustrate the method. It was noted in this experiment that there were several cases of incomplete absorption. This is evident in the example of the absorption of antiserum 3956 with 52875 LPS. If complete absorption had taken place, cross-reaction of the 3956 antiserum (after absorption by 52875 LPS) and 52875 LPS-sensitized cells would have yeilded a titre of 0 (<2). The incomplete absorption in this experiment was a critism of the results. Cross-agglutination (lines 1, 4 and 7) indicates that LPS 52875 is related to that of 3956 and 13248 (N.sicca) and in turn, 3956 and 13248 are related to each other. These relationships are explored by reciprocal adsorption. Antiserum 52875 adsorbed with LPS 3956 and 13248 (lines 2, 3) only weakly reacted with LPS 52875, 3956 and 13248. Thus the antigen adsorbed out is common to all three. Adsorption of the antiserum 3956 with LPS 52875 (line 5) reduced the titre and also reduced it to some extent for LPS 3956 and 13248, although slightly less for the latter. Adsorption of the antiserum 13248 with LPS 52875 (line 8) removed virtually all antibody to LPS 52875, 3956 and 13248, and adsorption

with LPS 3956 (line 9) removed none. Thus LPS 3956 contains a determinant not found in 52875 or 13248.

TABLE IX

ANTIGENIC ANALYSIS OF THREE STRAINS OF *NEISSERIA* BASED ON RECIPROCAL ADSORPTION OF THE ANTISERA

Line	Antiserum	Adsorbed with LPS	Titre (HU/ml) when tested with LPS of: 52875	3956	13248[1]
1	52875	unadsorbed	16	16	32
2	"	3956	4	<2	4
3	"	13248	4	4	4
4	3956	unadsorbed	32	64	8
5	"	52875	8	8	4
6	"	13248	6	6	6
7	13248	unadsorbed	32	16	128
8	"	52875	<2	<2	2
9	"	3956	32	16	256

[1] *N. sicca*

CHAPTER V

DISCUSSION

The gonococcus has been known to be a very fastidious organism ever since it was first cultivated in vitro. The experiment performed on type 1 organisms in ESP medium at 16% CO_2 demonstrated this fact. When liquid medium was used to support the growth of the gonococci, 25 ml volumes in 250 ml flasks gave adequate yields, while larger volumes of 250 ml in 1 litre flasks did not grow. This could have been due to the decreased surface/volume ratio in the larger flasks. Thus, the amount of CO_2 absorbed by the medium at a constant rate of shaking would be decreased because of the reduced area. This observation tends to confirm the findings of many who agree that adequate O_2 and CO_2 tensions are critical for gonococcal growth. At the same time, an investigation of solid GCBD medium indicated that no significant differences could be observed in either viable counts or type 1 proportional counts at CO_2 tensions of 5% and 10%.

Jennings et al. (1973) studied serogroup LPS in Neisseria meningitidis. According to them, the broad absorption at 3375 cm^{-1} is attributable to hydroxyl groups which is characteristic of carbohydrates. The IR spectra we obtained from gonococcal LPS preparations indicates a peak at 3530

cm^{-1} which could be attributable to hydroxyl-groups in carbohydrates or which might also be attributed to H-OH groups, which suggests that the KCl-LPS pellet used for the IR spectrum could have had some moisture in it. The absorption peak at 1560 cm^{-1} might be due to C=O bond stretching and N-H deformation, which absorbs in the region of 1560 to 1508 cm^{-1} or C-NO_2 groups which are absorbed at approximately 1560 cm^{-1}. Bonds in this region are characteristic of monosubstituted amide groups (NH·CO·CH_3). On the gonococcal scans, there was no significant absorption at 1735 cm^{-1}, which is the location of absorption of ester carbonyl bonds. Absorption in this region combined with absorption in the ranges 1270 to 1150 cm^{-1} and 1120 to 1030 cm^{-1}, where C-O-C bondages absorb, are characteristic of fatty acid esters. While no absorption is evident at 1735 cm^{-1} in SE crude LPS and PW extracted crude LPS, there are absorption peaks at 1260 cm^{-1}. The purified preparation by contrast did not produce an absorption peak in this region. This variation between the crude extracts and purified extracts at this adsorption band(characteristic of fatty acid esters), may indicate differences in fatty acid content of the polysaccharides. The absorption at 1660 cm^{-1} could indicate one of several possible molecular groups: aldehydes $\alpha\beta,\delta\delta$-unsaturated absorb in the region 1680 to 1660; ketones, $\alpha\beta,\alpha'\beta'$-unsaturated and diaryl absorb in the region of 1670 to 1660; secondary -CONH- in solid state absorb between 1680 and 1630 cm^{-1}; tertiary -CONH- absorb

between 1670 and 1630 cm^{-1}; and ureas, N-CO-N absorb at approximately 1660 cm^{-1}.

Molecular vibrations which are associated with individual bonds or functional groups are useful for the identification of these groups because when a molecular bond vibrates, it causes absorption at specific wavelengths. The absorption in the gonococcal LPS spectra at 1430 cm^{-1} could represent groups of $CH_2<$, CH_3 or $>C-H$ deformations. These bond types show up as absorption bonds in the region of 1470 to 1430 cm^{-1} and are difficult to distinguish.

The bond at 1260 cm^{-1} could represent one of several molecular structures. The region 1270 - 1250 cm^{-1} is the bond where nitrates ($O-NO_2$) show up. Also, amide I $\begin{matrix} & C \\ & \| \\ & S \end{matrix}-N<$ are indicated in the region from 1300 to 1100 cm^{-1}. The last possibility is that of O-H bond bending, which presents itself in the region between 1410 and 1260 cm^{-1}. The last definite peak in the gonococcal spectra is that at 1130 cm^{-1}, between the region 1150 and 1040 cm^{-1}, which represents C-O bond stretching as in $>C-OH$ groups.

The gonococcal LPS showed the same absorption bonds as those observed by Jennings et al. (1973) in N. meningitidis.

The SE crude LPS preparation is the only one of the four preparations which contains the absorption peak at 1560 cm^{-1}. Since this peak indicates the presence of nitrogen in N-H or $C-NO_2$ bonding, the appearance of this peak in the SE crude preparation may show that the SE method of extraction may extract more protein than the PW

method.

The presence of the absorption peak at 1260 cm^{-1} in both the PW crude extract and the SE crude LPS preparation and not in the purified preparations suggests that this peak which could represent a structural group that contains nitrogens (as in amino acids) has been eliminated in both purification procedures, and that this peak most likely represents protein material.

The UV spectrophotometric scans demonstrate that there is nucleic acid and protein in the PW-purified LPS strain 9T1 (scan a of Figure 4) and in the SE crude LPS strain F62 (scan b). The similarity in peak height in these two samples indicates that approximately the same amounts of nucleic acid and protein are present in both preparations. The PW scan indicates that either purification of the PW LPS extract does not remove much of the protein and nucleic acids or that the PW extraction method extracts more protein and nucleic acid than the SE method and that with purification, only part of this material is removed. Scans (c) and (d) show UV absorptions of two SE purified LPS. There are small peaks at 260 and 280 nm, which indicate that the nucleic acids and protein have not been removed completely. Since a UV scan was not performed on crude PW extracted LPS, it is not possible to tell whether the difference in scans of the PW and SE LPS indicates that the SE LPS may be more amenable to the purification process

used than is the PW extracted LPS. If PW and SE preparations are equally amenable to the purification process, then the PW extraction method must extract far more protein and nucleic acids than does the SE procedure. However, IR scans have shown that SE LPS contains additional peaks associated with the presence of protein so it is most probable that SE LPS is more amenable to the purification process used than is PW extracted LPS.

Jennings and co-workers (1973) found that all the meningococcal LPS preparations which they studied contained glucose, galactose, heptose, N-acetyl glucosamine, ethanolamine, lipid A, KDO, phosphate and fatty acids. Others have confirmed that these chemical substances are common not only to N. meningitidis but to most if not all Gram-negative bacteria, including N. gonorrhoeae.

In the gonococcal strains examined in this study, all of the preparations contained KDO although not all of the LPS contained glucose and galactose which are usually found in Gram-negative bacterial LPS. Two type 1 LPS preparations did not contain D-galactose. The type 4 LPS studied contained KDO, glucose and galactose. The absence of these sugars in some preparations could indicate that there was incomplete hydrolysis of the sugars before silylation was performed or incomplete silylation before gas chromatography was done.

The presence of ribose in several strains may indicate incomplete removal of nucleic acid from the LPS. Two of

the five preparations did not contain ribose, so it is possible that nucleic acids in these two strains were less firmly bound.

Rhamnose is a sugar which is known to be a side chain sugar in the LPS of Salmonella typhimurium, and its presence in some type 1 gonococcus strains could indicate that at least some type 1 LPS contain side chain sugars in addition to core sugars. The absence of rhamnose from strains 3956 and F62 type 1 LPS preparations indicates that these two strains do not have side chain sugars or that their side chains do not contain rhamnose. The type 4 LPS preparation did not contain rhamnose, xylose or mannose. When the type 4 LPS is compared to the type 1 preparation from the same strain, 3956, they do not resemble one another in concentrations of shared sugar constituents. It is possible that the type 1 LPS changes when the type 1 colony is subcultured in vitro, and in the process the side chains on the LPS structure are removed. Jennings et al. (1973) found that the LPS of N. meningitidis lacked the O-antigenic side chains, but the LPS still exhibited serotype specificity.

In regard to the limited antigenic cross-reaction studies performed in our investigation, we confirmed the observations of O'Reilly et al. (1973) that there are antigenic variations amongst the strains of gonococci as well as between gonococci and other species of Neisseria. Our method of comparison was the IHA test in contrast with the fluorescent antibody technique of O'Reilly et al. (1973).

The reciprocal adsorption test employed in this study (Table VI) indicates that it is possible to demonstrate antigenic differences between strains and species in a logical way as was shown for N. gonorrhoeae 52875, 3956 and N. sicca 13248. Unfortunately, the data required for the application of this test to a larger number of strains were not available, but the method illustrates the possibilities of this kind of analysis. Reciprocal adsorption permits the comparison of any two or more organisms but as one would expect, analysis of more than a few strains at a time becomes increasingly complex.

Maeland and Kristoffersen (1971) compared PW and aqueous ether-extracted endotoxin (LPS) preparations of the gonococci and found that the two gave essentially the same reactions in the hemagglutination-inhibition test except that the PW endotoxin had greater serological reactivity. We observed the same phenomenon when PW and SE-extracted LPS were compared, although it is unlikely that SE and aqueous ether-extracted materials are equivalent. It may be that the PW-LPS had greater activity because of a larger amount of polysaccharide in the extract, as suggested by Maeland. As indicated previously, we felt that the SE-extracted LPS was more likely to contain smaller amounts of whole cell contamination than the PW preparations extracted directly from whole cells, even though IHA titres were lower. Otherwise, we cannot explain the differences.

In conclusion, it is recognized that the SE-extracted LPS probably contains some protein on the basis of the scans obtained. Consequently there is the question whether the

relationships and differences shown to exist between gonococci are due to polysaccharide determinants on the LPS or to protein associated with it. One possibility for resolution of this question would be the treatment of the extracted LPS with protease. Also, it might be possible to rid the LPS of ribonucleic acid (RNA) by digestion with ribonuclease since it would appear that the use of cetavlon in the purification scheme is not entirely successful. Future studies of antigenic relationships between the gonococci would have to deal with this question.

CHAPTER VI

SUMMARY

The main features of the thesis are summarized as follows:

1. Infra-red spectroscopy indicated that LPS extracted by the SE method as compared to the PW method contained more protein. These two extraction methods yielded LPS extracts which contained characteristic LPS structural groups as shown by IR peaks at 3530, 1660, 1430 and 1130 cm^{-1}.
2. Ultra-violet spectroscopy showed that SE preparations were more amenable to the purification process used, as a greater amount of protein and nucleic acid was removed from SE as compared to PW extracts during purification.
3. Sugar analysis by gas-liquid chromatography of N. gonorrhoeae indicated that all preparations contained KDO, which is known to be present in Gram-negative bacterial LPS. However, all preparations did not contain all the sugars which are thought to be present in most if not all Gram-negative bacteria LPS. The presence of sugars known to occur as side chain sugars in Salmonella typhimurium were found in some type 1 LPS and not in the type 4 LPS studied. Type 1 and 4 vary considerably in concentrations of shared sugar

constituents.

4. Antigenic variation was found to be present amongst type 1 colonies of gonococcal strains as well as between gonococcal strains and other Neisseria species.
5. Reciprocal adsorption experiments permitted comparison of three strains of bacteria. It was evident that analysis would become very complicated with the involvement of more than a few strains. It was found that LPS 3956 contains a determinant not found in 52875 or 13248.
6. Phenol-water extracts had greater serological activity than SE preparations as could be seen by the higher IHA titres produced when PW-LPS-sensitized erythrocytes reacted with antisera as compared with SE-LPS-sensitized erythrocytes.
7. Since the SE preparations of LPS probably contained some protein, antigenic relationships could have been due to the protein associated with the LPS as well as the polysaccharide determinants present.

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APPENDIX

APPENDIX A

GONOCOCCAL BASE WITH DEFINED SUPPLEMENT (GCBD)[1]

Formula per liter distilled water:

Peptone	15.0g.
Corn Starch	1.0
Dipotassium phosphate	4.0
Monopotassium phosphate	1.0
Sodium chloride	5.0
Agar	10.0

Final pH 7.2 $\pm$ 0.2

Defined supplement per liter of above solution:

Dextrose	11.0g.
L-glutamine	0.35
Cocarboxylase	0.0015

[1] Baltimore Biological Laboratories, Baltimore, MD.

APPENDIX B

ENRICHED SINGLE PHASE MEDIUM (ESP)

Formula per liter distilled water:

Proteose Peptone #3 (Difco)	15.0 g
Potassium monohydrogen phosphate	4.0
Potassium dihydrogen phosphate	1.0
Sodium chloride	5.0
Dextrose	11.0
L-glutamine	0.35
Cocarboxylase	0.0015
Ferric nitrate	0.0002
Vitamin B12	0.0001
Adenine	0.001
Guanine hydrochloride	0.0003
p-aminobenzoic acid	0.00013
L-cystine	0.011
Diphosphopyridine nucleotide oxidized (Coenzyme 1)	0.0025
Thiamine hydrochloride	0.00003
Cysteine hydrochloride	0.259

APPENDIX C

CONDITIONS FOR USE OF GAS-LIQUID CHROMATOGRAPHY

Gas-liquid chromatography was performed on a Pye series 104 gas chromatograph, equipped with a temperature programme control. The columns used for the separation of the sugars measured 1.52 x 4 mm and contained 10% E30 on 200/120 gas chrom Q. The detection system was dual flame ionization. The optimal operating conditions for the separation of the TMS derivatives of the LPS of the gonococcus, found by Mr. J. D. Caird of this laboratory, were as follows:

Gas flow rates for carrier gas (nitrogen) ml/min.	45
" " " " hydrogen	45
" " " " air	500
Attenuation (sensitivity)	5×10^2
Temperature programme	100-250°C by increments of 2°C/min.
Recorder speed (cm/hour)	76.5