VIRULENCE FACTORS OF *HAEMOPHILUS DUCREYI*

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In Partial Fulfillment
of the Requirements for the
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by
Joseph Abiodun Odumeru
1985

VIRULENCE FACTORS OF *HAEMOPHILUS DUCREYI*

BY

JOSEPH ABIODUN ODUMERU

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

DOCTOR OF PHILOSOPHY

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A B S T R A C T

Factors mediating the virulence of Haemophilus ducreyi were investigated. Virulence in this organism was correlated with resistance of strains to the bactericidal activity of human serum and to phagocytosis by human polymorphonuclear leukocytes in vitro. In the present study, polymyxin resistance was not correlated with virulence. Polymyxin-sensitive mutants obtained from polymyxin-resistant parent strains by treatment with the mutagen N-methyl-N'-nitro-N-nitrosoguanidine, remained virulent. Similarly, polymyxin-resistant mutants obtained from polymyxin-sensitive parent strains by adaptive resistance to the antibiotic remained avirulent, indicating that polymyxin-resistance is not directly linked to virulence. SDS-polyacrylamide gel electrophoretic (SDS-PAGE) analysis of the outer membrane protein (OMP) of virulent and avirulent strains revealed differences in their electrophoretic mobility. A 47,000 molecular-weight protein which constitutes the major difference between OMP composition of the two groups was associated with polymyxin resistance, but not virulence.

Analysis of the lipopolysaccharide (LPS) of virulent and avirulent strains by gas liquid chromatography and SDS-PAGE also revealed differences in composition. The glycose-KDO ratio of the LPS of virulent strains exceeded that of avirulent strains indicating higher amounts of carbohydrates in the LPS of virulent strains in relation to the stable KDO component. The electrophoretic mobility of the LPS of these two groups of strains also differed. H. ducreyi has a "rough" type LPS lacking the monosaccharides and high-molecular weight subunits characteristic of LPS with O-side chains. The LPS contained fatty acid esters, hydroxyl and amide groups characteristic of the structure of LPS from other gram-negative bacteria, as indicated by infrared spectrophotometry.

Incubation of human serum with LPS of serum-sensitive avirulent strains for 30 min at 37°C resulted in inhibition of the serum bactericidal reaction in contrast to the LPS of serum-resistant virulent strains. This inhibitory effect was apparently due to depletion of serum complement. The classical pathway of complement activation was implicated in the serum killing of serum-sensitive avirulent strains.

These findings suggest that the LPS composition of H. ducreyi strains is important in serum susceptibility and virulence.

INTRODUCTION

Haemophilus ducreyi is the etiologic agent of chancroid, a sexually transmitted disease endemic throughout the tropics. There are occasional outbreaks in developed countries most of which are localized. Chancroid has been reported in Greenland (Lykke-Olesen et al., 1979), Canada (Hammond et al., 1980), United States (Nayyar et al., 1979; Hansfield et al., 1981; Carpenter et al., 1981; Hannah and Greenwood, 1982), Britain (Hafiz et al., 1981), Germany (Luders et al., 1975), Finland (Lassus et al., 1975), and France (Morel, 1974). There are reports of its persistence in Sheffield, England (Hafiz, et al., 1981; Kinghorn, et al., 1982).

Since the discovery of H. ducreyi in the ulcer exudate from patients with chancroid by Ducrey in 1889, and subsequent isolation of the organism from genital ulcers by Lenglet in 1898, relatively little effort has been directed toward the study of its virulence factors. A number of investigators (Reenstierna, 1921; Saelhof, 1924; Maximowa, 1936; and Feiner et al., 1945) were able to reproduce chancroid lesions in rabbits, monkeys, and in humans by intradermal inoculation of pure cultures of the organism. Stock strains kept in the laboratory by repeated transfer for 3-4 years failed to cause the disease. The ability to produce localized lesions in rabbits and humans has been associated with the virulence of the organism (Greenblatt et al., 1943 ; Dienst, 1948; and Kaplan et al., 1956a). Recently, Hammond et al. (1978) and Bertram (1980) used this criterion to determine the virulence status of stock strains. They found that virulent strains produced necrotic lesions in rabbits following intradermal inoculation of live organisms, in contrast to avirulent strains.

Another characteristic applied in the past in distinguishing virulent and avirulent strains is their in vitro antibiotic sensitivity. Thayer et al. (1955) found that virulent strains of H. ducreyi were more resistant to

polymyxin than avirulent strains. Similarly, Singer and Deacon (1956) noted that virulent strains were more resistant to penicillin G. The apparent relationship between the virulence of H. ducreyi and susceptibility of strains to various antibiotics was confirmed by the study of Hammond et al. (1978). They found that virulent strains were resistant to polymyxin, penicillin, ampicillin, cloxacillin and cephalothin, while avirulent stock cultures were susceptible. The most striking difference in the susceptibility of virulent and avirulent strains to these antimicrobials was their susceptibility to polymyxin.

To date, the only criteria of virulence applied to H. ducreyi have been the rabbit intradermal test and resistance to polymyxin. The present study was undertaken to explore relationships between these criteria and to identify other factors that mediate the virulence of this organism. Although H. ducreyi is gram-negative with typical ultrastructural details, no information is available on the relationship between its cell envelope components and virulence. Virulence factors investigated have included susceptibility of H. ducreyi strains to the bactericidal action of serum, resistance to phagocytosis and killing by human polymorphonuclear leukocytes. Also examined was the role of cell envelope components in resistance to host defenses.

LITERATURE REVIEW

A. Host defenses against bacterial infection

The host-pathogen relationship is a dynamic interaction of a number of host and microbial factors. The establishment of infection and disease in the host by microorganisms depends on which of these factors prevail. Host defenses against pathogens generally involve specific and non-specific immune responses. The skin, mucous membranes and the lining of the respiratory, gastrointestinal and genitourinary tract constitute a front-line defense against invading microorganisms (Bjorksten, 1980). Non-specific immune responses involved in the clearance of bacteria upon entry into the host, include the phagocytic system and the bactericidal effect of complement. These host defenses may be enhanced by specific antibody directed against the pathogen (Schultz, 1980; Quie, 1980). The mucosal membranes, especially in the gastrointestinal and respiratory tracts, are continuously exposed to microorganisms. In these sites, secretory IgA is the principal mediator of specific immunity (Hanson *et al*., 1980). It is believed that secretory IgA antibodies maintain the integrity of the mucous membranes by inhibiting its colonization by microorganisms, by preventing the penetration of microbial antigens through the surfaces and by neutralizing toxins and viruses (Heremans, 1974; Hanson *et al*., 1980).

Iron has been shown to be an essential requirement for bacterial multiplication (Weinberg, 1978). Iron in body fluids is very strongly bound to iron-binding proteins such as transferrin in blood and lymph (Aisen, 1980) and lactoferrin in secretions (Masson *et al*., 1966) and in neutrophils (Bullen and Armstrong, 1979). An increase in the concentration of free iron in body fluids has been associated with an increase in

infection (Barry and Reeve, 1977). Thus, deprivation of the invading pathogen of iron via these proteins is an important host defense mechanism. Certain bacteria however, can utilize lactoferrin as a source of iron while some have unique mechanisms by which they scavenge iron from their immediate environment. (Mickelsen et al., 1982; Finkelstein et al., 1983).

1. Complement in host defense.

The complement (C) system consists of a series of glycoproteins that circulate in extracellular fluids of vertebrates (Muller-Eberhard, 1980). It is an important component of the host defense against pathogens. Activation of the system may result in damage of biological membranes, inflammatory reactions, immediate and delayed hypersensitivity reactions, enhancement of immune adherence (opsonization) and phagocytosis by polymorphonuclear leukocytes and macrophages (Schultz, 1980; Gotze and Muller-Eberhard, 1971). Some of these manifestions may or may not be beneficial to the host.

There are two major C pathways, the classical and the alternative (properdin) pathway (Figure 1). The classical pathway can be activated by immune and non-immune factors. Immune factors such as antigen-antibody complexes or aggregated gamma globulins are considered the major activators of the classical C pathway (Gotze and Muller-Eberhard, 1971; Mayer, 1973). The lipid A moiety of the lipopolysaccharide (LPS) of gram-negative bacteria can activate the classical C pathway independent of antibody (Morrison and Kline, 1977). Similarly, DNA-lysozyme complexes (Willoughby et al., 1973), synthetic polynucleotides (Yachin et al., 1964) and non-specific lgG reacting with staphylococcal protein A (Sjoquist and Stalenheim, 1969) have been used to activate the classical pathway without

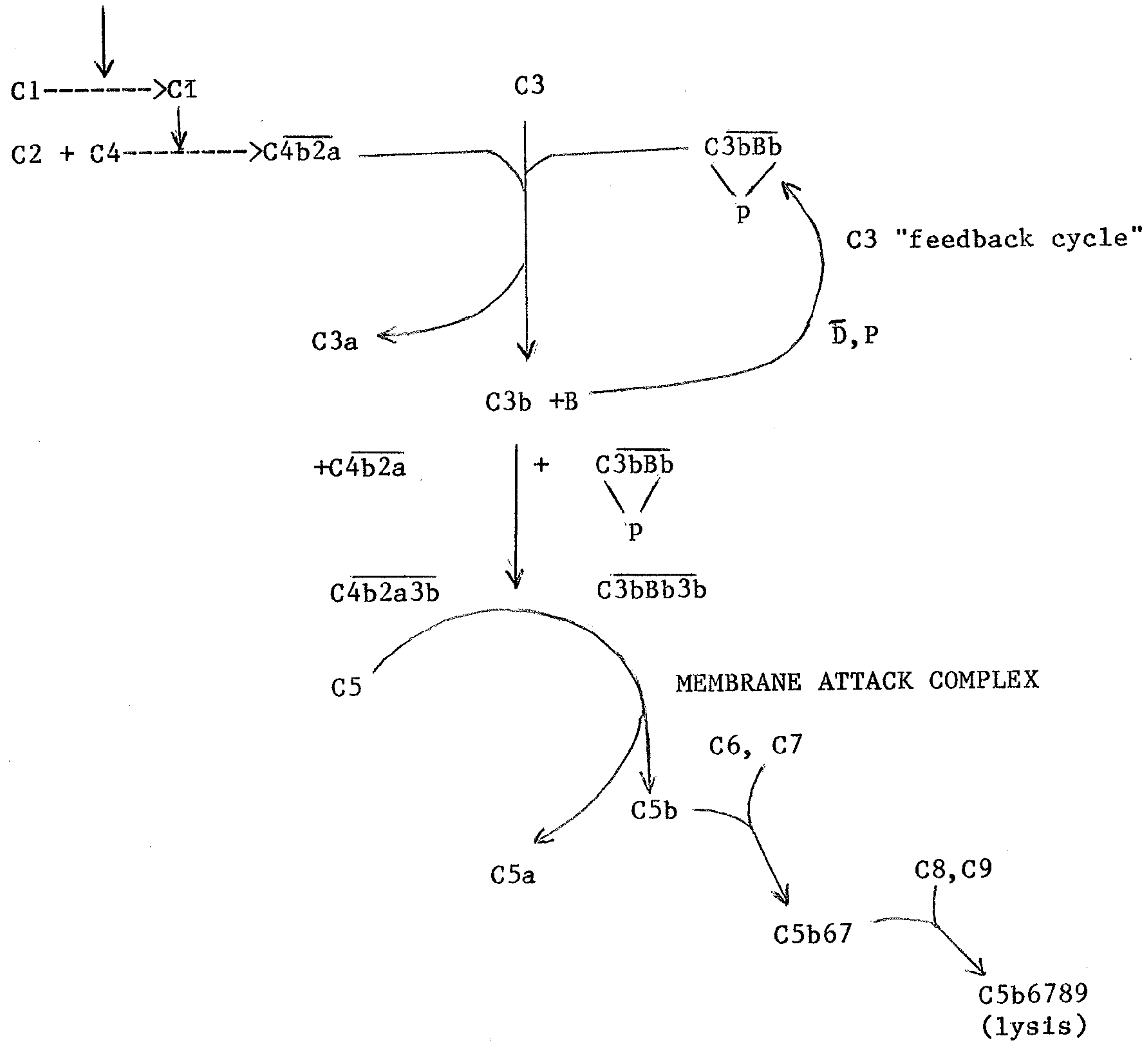


Figure 1: The Complement Cascade (Joiner et al., 1984a)

Fc: Fc region of Immunoglobulin (1g)
D : Factor D
P : Properdin

For explanation of fractions, see text.

the participation of specific antibody. The alternative pathway can be activated by bacterial LPS, naturally occuring polysaccharides as well as by aggregated immunoglobulins - IgA. (Osler and Sandberg, 1973).

Activation of the classical C pathway by immune complexes involves the binding of the Clq component of Cl molecules to the CH2 domain of IgG1, 2, and 3 or to the CH4 domain of IgM (Hurst et al., 1975). The Cl macromolecule consists of Clq, Clr and Cls molecules held together by calcium. The Clq is the receptor site for IgG or IgM Fc regions (Reid et al., 1977). Activation of the Cl components generally leads to generation of the enzyme Cl esterase which cleaves the next two C proteins C4 and C2. The C4b2a formed is a C3 convertase which is capable of initiating C3 - C9 consumption (Muller-Eberhard, 1969).

The alternative C pathway mechanism on the other hand bypasses Cl, C2, and C4 by binding C3 or C3b to a surface and forming C3bBb enzyme complex in the presence of factors B and D. This complex acts as a C3 convertase like the C4b2a complex of the classical pathway (Joiner et al., 1984a). Activation of either pathway generally leads to the initiation of C3 - C9 consumption with generation of biologically active C components such as the anaphylatoxins, chemotactic factors and factors promoting bacterial adherence to membrane receptors on phagocytic cells such as polymorphonuclear leukocytes and macrophages (Gotze and Muller-Eberhard, 1971). The C5b-9 complex formed is responsible for membrane damage and the ultimate lysis of the cell (Mayer, 1973; Muller-Eberhard, 1978).

A number of studies has demonstrated that C-mediated bactericidal activity of human serum is an important component of the host defense against many gram-negative bacteria (Taylor, 1983; Joiner et al., 1984a). The fact that the bactericidal and bacteriolytic activities of serum are

destroyed by heating at 56°C, strongly suggests that C plays a critical role in serum killing of bacteria. In a study designed to demonstrate the requirement of C in serum-mediated killing of E. coli, Inoue et al. (1968) treated the organism with guinea pig serum depleted of the third C component (C3), forming a complex of bacterial cells and antibody plus the first three C components (BAC142 complex). Purified C3, C5, C6, C7, C8 and C9 preparations were then added sequentially, and the bactericidal activity of the reconstituted serum was determined. Serum bactericidal activity was restored only when all of the C components were added to the reaction mixture. Other investigators (Goldman et al., 1969; Schreiber et al., 1979) also reported data similar to that of Inoue et al., 1968.

Studies on the mechanism of bacterial killing by C (Reed and Albright, 1974) suggest that serum-mediated killing of bacteria occurs as a result of the deposition of membrane attack complex (C5b-9) onto surfaces of susceptible bacteria. This complex appears cylindrical when viewed with an electron microscope. It has a hydrophilic central channel and a hydrophobic outer surface (Joiner et al., 1984a). The proposal has been advanced that the complex (C5b-9) penetrates through the membrane lipid-protein bilayer forming a channel (Tranum-Jensen et al., 1978), and damaging the membrane. The channels in the membrane result in increased membrane permeability to ions and cellular contents (Mayer, 1981). It is believed that simultaneous damage of the inner and outer membrane occurs when terminal complexes (C5b-9) are deposited on the bacterial surface at the points of contact between these membranes (Martinez and Carroll, 1980; Wright and Levine, 1981). The latter authors found that the kinetics of release of periplasmic enzymes and intracellular cations after exposure of E. coli cells to lysozyme-free serum are identical, an indication that the

disruption of both the inner and outer membrane by the terminal complexes of C are coupled events.

The role of lysozyme in the bacteriolytic activity of human serum has been demonstrated (Wardlaw, 1962). Removal of serum lysozyme results in reduction of serum bacteriolytic activity (Wright and Levine 1981). Some studies have shown that lysozyme gains access to its peptidoglycan substrate only after the C5b-9 complex has disrupted the outer membrane producing a permeability defect (Glynn, 1969; Inoue et al., 1968). Lysozyme bacteriolytic activity is therefore secondary to C activity in the cell.

2. The phagocytic system in host defense.

Phagocytosis is an important component of the host defense against invading microorganisms. The phagocytic system consists of polymorphonuclear leukocytes and monocytes which differentiate into macrophages in the tissues. A disturbance in tissue either as a consequence of microbial invasion or injury initiates an inflammatory response which attracts circulating leukocytes to the site of injury or infection (Quie, 1980). The undirectional movement of phagocytic cells to the site of infection is mediated by chemotactic factors produced either by the C cascade or the activation of the kinin system (Gallin and Quie, 1978).

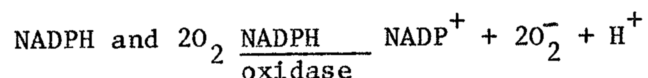
Phagocyte - pathogen interactions characteristic of phagocytosis involve three main events: attachment, ingestion and the subsequent killing of ingested microorganisms. Pathogens may avoid the microbicidal action of the phagocytic system by virtue of resistance to any of these stages of phagocytosis.

Attachment of microorganisms to phagocytes is facilitated by specific receptors on the phagocytes, and opsonins, which increase the affinity of microorganisms for these receptors by neutralizing antiphagocytic factors on the microbial surface (Quie, 1980). Opsonins such as specific antibacterial antibodies and C cleavage products (C3b), can mediate the attachment of the pathogen through Fc and the C3b receptors respectively (Roos et al., 1981). Attached microorganisms or particles are usually internalized by engulfment, and are contained in phagocytic vacuoles formed by the plasma membrane. Ingestion of microorganisms is accompanied by a triad of events: an increase in glucose uptake, an increase in O_2 consumption and the subsequent production of H_2O_2 by the phagocyte.

Two mechanisms by which phagocytes kill ingested microorganisms have been described. Microorganisms may be killed either by oxygen (O_2)-independent factors or by O_2-dependent factors (Gabig and Babior, 1981; Quie, 1980; Babior, 1978). Phagocytes have 'cytoplasmic granules' consisting of hydrolytic enzymes which mediate the O_2-independent microbicidal activity. Degranulation is initiated by contact between microorganisms or particles and the plasma membrane of the phagocyte (Quie, 1983). The granules fuse with the plasma membrane or vesicles containing ingested microbes and release their contents. In the granules are cationic proteins, proteases, lactoferrin, lysozyme, acid hydrolases and myeloperoxidase (Drutz and Mills, 1980). These proteins have been shown to participate in the killing of susceptible microorganisms (Gabig and Babior, 1981; Quie, 1980) especially under anaerobic conditions (Spitznagel and Okamura, 1983). A decline in intravacuolar pH resulting from accumulation of lactate also participates in O_2-independent microbicidal activity.

Oxygen-dependent killing of microorganisms by phagocytes is mediated by

oxidizing agents produced by the partial reduction of O_2 in a metabolic event commonly referred to as "respiratory burst". The O_2-consuming respiratory burst is believed to occur simultaneously with membrane response which leads to phagocytic vacuole formation (Quie, 1980; Babior, 1978). In addition to increased O_2 consumption by the phagocyte, reduced nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) is activated. The activation of this enzyme is associated with conversion of O_2 to the highly reactive superoxide anion (O_2^-). This enzyme utilizes NADPH as a source of electrons transferred to O_2 (Babior, 1978; Dechatelet *et al.*, 1983). The reaction catalyzed is as follows:



$$\text{NADPH and } 2O_2 \xrightarrow[\text{oxidase}]{\text{NADPH}} \text{NADP}^+ + 2O_2^- + H^+$$

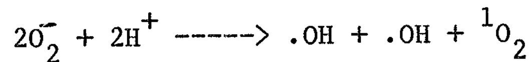
Root and Metcalf (1977) showed that all the O_2 consumed during the respiratory burst was converted to O_2^-, that 80 percent of this O_2^- was converted to H_2O_2 by dismutation, and that this dismutation reaction was the only important source of the H_2O_2 produced during the respiratory burst. The reaction leading to the production of H_2O_2, by the dismutation of O_2^- is as follows:

$$2O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$$

This reaction may occur spontaneously or via catalysis by superoxide dismutase (Root and Metcalf, 1977).

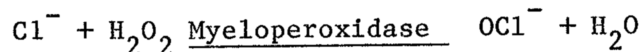
Other O_2 radicals produced in the phagocytic vacuoles during respiratory burst include hydroxyl radical (.OH) and singlet oxygen ($^1O_2$).

These O_2 intermediates are also formed by dismutation of O_2^-:



$$2O_2^- + 2H^+ \text{------>} .OH + .OH + {}^1O_2$$

H_2O_2, .OH and ${}^1O_2$ have all been shown to participate in O_2-dependent killing by neutrophils (Babior, 1978). Although O_2^- is the precursor of these O_2 intermediates, O_2^- has not been implicated in bacterial killing. H_2O_2 alone has some bactericidal activity. However, its ability to kill microorganisms is greatly enhanced through the action of myeloperoxidase present in the granules of most phagocytic cells (Bainton and Farguhar, 1968). Myeloperoxidase catalyzes the oxidation of halide ions to hypohalite ions by H_2O_2. Chloride ions, the most abundant halide in the cell, are oxidised by H_2O_2 to form hypochlorite ion:



$$Cl^- + H_2O_2 \xrightarrow{\text{Myeloperoxidase}} OCl^- + H_2O$$

The hypochlorous acid formed is believed to kill bacteria by mediating the oxidation of amino and sulfhydryl groups of proteins in the cell (Thomas, 1979).

In conclusion, bacterial killing by phagocytes is accomplished through O_2-independent mechanisms involving materials discharged from 'cytoplasmic granules' into vesicles containing the ingested microorganisms, and by O_2-dependent mechanisms involving highly reactive microbicidal agents produced by the partial reduction of O_2 during the respiratory burst.

The importance of O_2-dependent bacterial killing by neutrophil is exemplified by patients with chronic granulomatous disease (CGD). This is an inherited condition in which phagocytes fail to elicit a normal

respiratory burst and are unable to adequately kill many types of microorganisms (Holmes et al., 1967). Patients with this disease are highly susceptible to severe pyogenic bacterial infections, especially those caused by catalase-positive bacteria. The defect in CGD is believed to be due to lack of activation of NADPH oxidase involved in the production of O_2^-, H_2O_2 and other O_2 redicals that mediate O_2-dependent microbicidal activity of neutrophil (Quie et al., 1967; Babior, 1978; Roberts and Gallin, 1983). The failure of the oxidase activity has been linked to a deficiency of glucose-6-phosphate dehydrogenase which catalyzes electron transfer to NADPH in the hexose monophosphate shunt pathway (Babior, 1978).

Phagocytic dysfunction involving O_2-independent microbicidal activity of neutrophil can also affect host defenses against pathogens. A defect involving a delay in degranulation such as in the case of patients with the Chediak-Higashi syndrome, is known to result in a decreased rate of killing of bacteria by neutrophil, apparently caused by a delay in the fusion of lysosomal granules with phagocytic vacuoles containing ingested microorganisms (Roberts and Gallin, 1983).

3. Role of antibody in serum bactericidal reactions and phagocytosis.

The classical C pathway in the serum bactericidal reaction is initiated by antigen-antibody complexes on or near the bacterial cell surface (Taylor, 1983). Extremely small amounts of antibody are required for the activation of complement. Michael and Landy (1961) estimated that 10ng of antibody per ml can activate the classical C pathway. In another study, Bjornson and Michael (1970) found that 6-7 ng of purified IgM from rabbits immunized with P. aeruginosa was effective in bactericidal assays with homologous strains. Complement-activating antibodies belong mostly to the

IgM or IgG classes (Rowley, 1973), but IgA may also function in the C-mediated killing of bacteria by activating the alternative C pathway (Sirotak et al., 1976). The IgM antibody appears to be more effective in C activation than IgG. Schiller et al. (1984) found that both IgG and IgM antibodies were bactericidal for P. aeruginosa strains in the presence of C. However, IgM antibody was 10 times more effective. This has been attributed to the fact that one IgM molecule can combine efficiently with at least two CI binding sites, whereas two IgG molecules are required for such interactions (Taylor, 1983). Earlier studies by Gitlin et al. (1963) demonstrated a smaller bactericidal effect of cord serum from newborn infants compared to that of serum from the mothers. This study indicates that the bactericidal activity of human serum resides in the IgM fraction, since neonatal serum is normally deficient in IgM, while the IgG molecules which are capable of crossing the placental barriers predominate in the neonatal serum.

Complement-activating antibodies have been demonstrated in both normal and immune sera (Rice and Kasper 1980; Schoolnik et al., 1979; Muschel and Larsen, 1970). These antibodies may be directed against antigenic determinants carried by lipopolysaccharide (LPS) (Muschel, 1960; Roantree, 1971), outer membrane proteins (OMP) (Tratmont et al., 1974; Rice and Kasper, 1977) or capsular polysaccharide (Bjornson and Michael, 1970: Robbins and Robbins, 1984). Rough strains of Salmonella spp. and E. coli are generally susceptible to the bactericidal action of normal serum, while certain smooth variants are resistant (Taylor, 1983). The resistance of smooth variants has been attributed to a lack of adequate antibody in normal serum specific for the O-side chain of smooth LPS, (Muschel and Larsen, 1970). Antibody involved in the serum-bactericidal reaction

against rough strains of Salmonella spp. and E. coli was directed against the core moiety of the LPS (Rowley, 1968; Muschel and Larsen 1970).

It is believed that 'natural' antibodies found in normal serum are produced against commensal bacteria in the intestinal tract of humans. The presence of these bacteria ensures that small amounts of antibodies directed against the surface antigens of many types of bacteria are present in the blood and tissue fluids of humans (Kunin, 1962). IgM and IgG antibodies against surface antigens of E. coli (Cohen and Norins, 1966), other enterobacteria (Michael et al., 1962), Neisseria spp. (Cohen, and Norins, 1966; Rice and Kasper 1980) and H. influenzae (Norden et al., 1970) can be detected in the sera of adults in the absence of any history of immunization or specific infection although it is often difficult to rule out such past infection.

In addition to the role of antibodies in serum-bactericidal reactions, opsonizing antibodies against bacterial surface components such as LPS and capsular polysaccharide (Musher et al., 1983) can promote attachment and phagocytosis of microorganisms. Polymorphonuclear leukocytes and macrophages are known to have receptors for the Fc fragment of antigen-bound IgM, IgA and IgG (Roos et al., 1981).

B. Virulence factors of gram-negative bacteria.

The establishment of infection by a pathogen involves an interaction between the host defenses and microbial factors. For a pathogen to produce infection, it must be able to enter or adhere to the host cells, survive and proliferate in the presence of host defense, and in some cases produce tissue damage either by tissue invasion or by virtue of the ability to

produce toxins. The microbial products responsible for these processes are the determinants of pathogenicity or virulence of the organism (Lindberg, 1980; Sparling, 1983). Bacterial virulence can thus be defined as a factor or factors that enable the organism to successfully complete any of these stages of infection. Agents implicated in bacterial virulence include exotoxins, proteases, elastase, extracellular appendages such as pili and flagella, cell surface components, such as OMP, LPS and polysaccharide capsules. More than one of these components is usually involved in the pathogenesis of a given bacterial infection.

1. Bacterial extracellular products and virulence.

(i) Exotoxins.

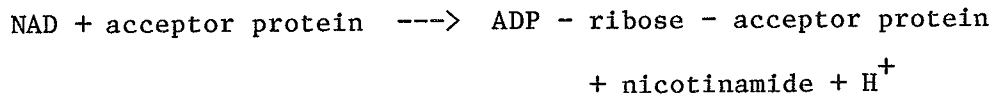
Invasive or noninvasive pathogenic microorganisms may cause disease at local or distant sites through the release of soluble products (toxins). There are two types of toxins: exotoxins and endotoxins. Exotoxins are extracellular heat-labile proteins which can be separated from the vegetative bacterial cell. Their production occurs in certain gram-negative and gram-positive bacteria. Endotoxins on the other hand, are heat-stable components of the outer membrane of gram-negative bacteria. They can be separated from the outer membrane only by cell lysis. Both types of toxins can either damage host cells or interfere with defense mechanisms. Gram-negative bacteria known to produce exotoxins include _S. dysenteriae_, _P. aeruginosa_, _V. cholerae_, _E. coli_, _Y. pestis_ and _B. pertussis_. Such toxins have been shown to contribute to the course of infection and disease produced by these organisms. The most potent toxin produced by gram-negative bacteria is that of _S. dysenteriae_ and is comparable in potency to tetanus toxin. It is a heat labile protein of

75,000 molecular weight (Keush and Grady, 1972). The precise mechanism of action of this toxin is unknown. It induces profound fluid loss from the intestine.

The production of exotoxin A by P. aeruginosa has been associated with virulence (Woods et al., 1982). Exotoxin A is a potent inhibitor of protein synthesis. Its mechanism of action is identical to that of diphtheria toxin in that it inhibits eucaryotic protein synthesis by catalyzing the transfer of adenosine - S^1 - diphosphate ribosyl (ADPR) moiety of nicotinamide adenine dinucleotide (NAD) to enlongation factor - 2 (EF_2) resulting in an inactivated EF_2-ADPR complex (Pollack et al., 1980). Although the intracellular mechanism of action of exotoxin A is similar to that of diphtheria toxin, the enzymatic activity of the toxin can be neutralized with antibody produced against the toxin, but not with antibody to diphtheria toxin (Iglewski and Kabat, 1975). The toxin produced demonecrosis following injection of culture filtrates of P. aeruginosa isolates into shaved backs of guinea pigs (Pollack et al., 1977). Woods et al. (1982) found that Tox^- mutants and mutants that produce inactive toxin were less virulent in the rat lung model than the toxigenic parental strain. The infections caused by these mutants were restricted to bronchi and immediate peribronchial areas whereas the parental strain produced parenchymal invasions and dense mononuclear cell infiltrations in the alveolar spaces. This study suggests that exotoxin A plays a major role in the pulmonary infections caused by P. aeruginosa.

V. cholerae secretes an exotoxin, known as cholera toxin, after colonization of the small intestine. The toxin binds to receptors on the mucosal cells and stimulates intestinal adenylate cyclase activity. Increase in cyclic AMP causes diarrhoea and fluid loss by inhibiting uptake

of NaCl by the villi and also by stimulating active chloride secretion by crypt cells (Holmgren, 1981). The mechanism of action of this toxin is very similar to that of diphtheria toxin, although the metabolic and clinical effects are different. Moss <u>et al</u>. (1976) demonstrated that cholera toxin, like diphtheria toxin has ADP-ribosyltransferase activity which catalyses the reaction:



$$\text{NAD} + \text{acceptor protein} \longrightarrow \text{ADP - ribose - acceptor protein} + \text{nicotinamide} + H^{+}$$

The guanyl nucleotide-binding component of the membrane bound adenylate cylase is the protein that is ADP-ribosylated (Gill and Mehren, 1978). GTPase hydrolyses GTP to GDP. GTP is required for adenylate cylase activity. Hydrolysis of GTP results in inactivation of adenylate cyclase. Cholera toxin stabilizes this enzyme in an active conformation by blocking GTPase action (Cassel and Selinger, 1977).

Acute diarrheal disease caused by enterotoxigenic strains of <u>E. coli</u> (ETEC) is mediated by release of a heat-stable and a heat-labile enterotoxin either singly or together. These toxins stimulate adenylate cyclase activity by a mechanism similar to that of cholera toxin (Sack, 1975).

(ii) Extracellular enzymes.

Extracellular enzymes known to be produced by certain gram-negative bacteria include proteases and elastase. Most clinical isolates of <u>P. aeruginosa</u> produce elastase and alkaline proteases. These enzymes have been associated with virulence. The proteases are capable of inducing alveolar necrosis in the lungs of experimental animals and hemorrhage similar to that seen in patients with pneumonia caused by <u>P. aeruginosa</u>

(Gray and Kreger, 1975). Elastase has been implicated in hemorrhagic skin lesions (Kawaharajo et al., 1975) and the destructive vascular lesions associated with P. aeruginosa septicemic infections (Mull and Callahan, 1965). Schultz and Miller, (1974) showed that elastase is capable of inactivating human C components in vitro including C3. They found that the phagocytic activity of polymorphonuclear leukocytes on Pseudomonas cells was impaired as a result of the destruction of cell-bound C3b by the enzyme.

A number of gram-negative bacteria produce IgA protease which cleaves human IgA_1 molecules at a specific site in the hinge region of the chain, releasing Fab and Fc fragments. Such cleavage results in complete loss of antibody activity (Plaut et al., 1977). The enzyme specifically cleaves human serum IgA_1 and secretory IgA, but not IgA_2, IgG or IgM classes (Plaut, 1978; Kilian et al., 1979). Most IgA protease-producing bacteria are pathogens that colonize mucosal surfaces where secretory IgA is the principal mediator of specific immunity. Kilian (1982) screened 625 bacterial and Mycoplasma strains representing 62 species, for IgA protease production. He found that strains of S. sanguis, N. meningitidis, N. gonorrhoeae, H. influenzae and S. pneumoniae produce this proteolytic enzyme. Five H. ducreyi strains tested were not producers of IgA proteases. Slaney et al. (1983) tested 8 strains of H. ducreyi for IgA protease production and found that none of them was positive. Male (1979) suggested that the selective mucosal penetration of H. influenzae type b at an inflamed site containing transudated serum components may be accomplished because of the ability of its protease to destroy protective IgA_1 antibody. The fact that all colony types of N. gonorrhoeae and all biotypes of H. influenzae produce IgA proteases (Male, 1979) indicates that

the ability of microorganisms to produce IgA_1 protease may not necessarily be a virulence factor.

(iii) Hemolysin production.

Hemolysin production by E. coli strains has been related to their pathogenicity. Three types of hemolysins produced by E. coli have been described. A filterable hemolysin, a cell bound non-filterable hemolysin termed beta-hemolysin and gamma-hemolysin (Smith, 1963). Hemolysin production is more common in E. coli from human extraintestinal infections than in those isolated from stools (DeBoy et al., 1980). Hemolytic E. coli have been shown to be more virulent for mice than hemolysin-negative mutants (Emody et al., 1980). Also the ability of E. coli strains to produce experimental pyelonephritis in rats has been associated with hemolysin production (Fried et al., 1971).

P. aeruginosa produces a heat stable and heat-labile hemolysin. It is believed that these hemolysins promote invasion of tissue on the basis of their necrotizing activities (Liu, 1966).

2. Surface components of gram-negative bacteria in relation to virulence.

(i) Cell envelope of gram-negative bacteria.

As shown in Figure 2, the cell envelope of a gram-negative bacterium is composed of three macromolecular components; the outer membrane, the cytoplasmic membrane and the rigid peptidoglycan layer. The outer membrane consists of phospholipids, OMP and LPS. External to the outer membrane are the polysaccharide capsular layer and bacterial appendages such as the pili and flagella. These cell-surface components play a critical role in primary interactions with the host cell during

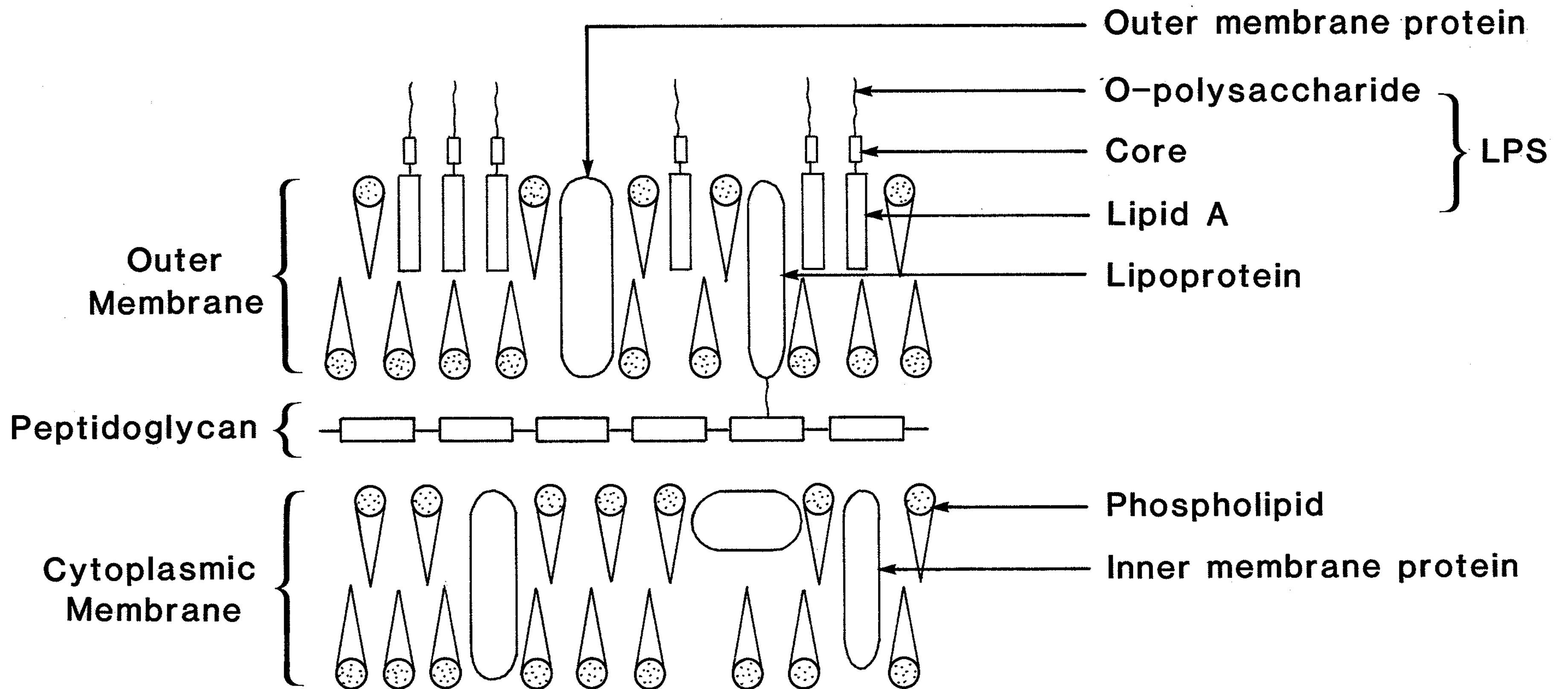


Figure 2: Schematic representation of the cell envelope of gram-negative bacteria.

infection. They facilitate bacterial attachment, invasion of the host cells, resistance to phagocytosis, resistance to or interaction with C-mediated bactericidal activity of serum and host tissue damage.

(ii) Role of extracellular appendages in bacterial virulence.

Adherence of bacteria to host cells is usually the first stage of infection. Attachment is mediated by cell surface ligands (termed adhesins) and specific receptors for these structures in the host cell (Beachey, 1981; Korhonen *et al.*, 1981). Pili are implicated in the adherence of most gram-negative bacteria to the host cell (Sparling, 1983). Virulence of *N. gonorrhoeae* for example has been associated with piliation (Punsalang and Sawyer, 1973; Buchanan and Pearce, 1976). The virulence of piliated gonococci correlated with attachment to human cells (Buchanan and Pearce, 1976; Wiseman *et al.*, 1981), and with resistance to phagocytosis (Thongthai and Sawyer, 1973; Thomas *et al.*, 1973). Punsalang and Sawyer (1973) reported enhanced attachment of piliated gonococci to sheep, chicken, guinea pig, rabbit, human blood group O, Rh+ erythrocytes and to human buccal cells as compared to nonpiliated organisms. Antibody to partially purified pili inhibited both hemagglutination and adherence of gonococci to epithelial cells *in vitro*. This inhibition was removed only by adsorption of the antiserum with piliated gonococci (Punsalang and Sawyer, 1973), an indication that pili are the cell component involved in the attachment of gonococci to epithelial cells.

Adherence of *H. influenzae* to human epithelial cells has also been shown to play a role in pathogenesis. Adherence is mediated by pili (Pichichero *et al.*, 1982) and can be blocked with glucose but not mannose (Kaplan *et al.*, 1983). Lewis and Dajani (1980) in their study of the adherence of typable and nontypable *H. influenzae* strains to human buccal

and conjuctival cells, found that most of their nontypable strains adhered to these epithelial cells while type b strains did not. Similar observations were reported by Lampe *et al.* (1982) using pharyngeal, nasal and buccal epithelial cells. The increased adherence of nontypable strains of *H. influenzae* to human epithelial cells may explain the differences in colonization between type b and nontypable *H. influenzae*. Pichichero *et al.* (1982) found that two of their *H. influenzae* type b isolates from the nasopharynx were exceptionally adherent, and their ability to adhere to human buccal cells correlated with agglutination of human erythrocytes. The adherent cells were piliated. They postulated that *H. influenzae* type b has the potential for piliation and that the piliated variants may be necessary for nasopharyngeal colonization and the subsequent hematogenous spread of the organism. Kaplan *et al.* (1983) found that cerebrospinal fluid and blood isolates cultured from infant rats inoculated with an adherent piliated *H. influenzae* type b were non-piliated. The loss or suppression of pili may enhance the survival of the organism in the circulation by reducing its adherence to polymorphonuclear leukocytes (Turk, 1984). It has been suggested that piliated strains interact with host defense mechanisms such that they are preferentially eliminated thus allowing subpopulations of nonpiliated organisms to proliferate (Tosi *et al.*, 1985).

Woods *et al.* (1980a) demonstrated that pili mediate the adherence of *P. aeruginosa* organisms to human buccal cells. In another study (Woods *et al.*, 1980b), they found a correlation between the *in vitro* adherence of the organism to upper respiratory tract epithelium of humans and colonization of the respiratory tract of patients with cystic fibrosis.

The attachment of other pathogenic bacteria such as E. coli (Salit and Gotschlich 1977), S. typhimurium (Lindberg, 1980; Korhonen et al., 1981), P. mirabilis (Silverblatt and Ofek, 1978) and N. meningitidis (Craven et al., 1980) to epithelial cells has been shown to be pilus mediated.

Flagella are also known to contribute to pathogenesis. For example, motility of P. aeruginosa is known to be mediated by flagella (Pitt, 1981; Montie et al., 1982). Flagella specific antibodies inhibit motility (Montie et al., 1982) and were protective against pseudomonal infections in burned mice (Holder et al., 1982). Montie et al. (1982) found one isogenic mutant of P. aeruginosa lacking flagella to be less virulent in the burned-mouse model than the parent strain with flagella.

(iii) Bacterial capsules and virulence.

Resistance of many gram-negative bacteria to host defenses such as the bactericidal effects of serum and phagocytosis has been related to the presence of a capsular layer. For example the polyribosyl phosphate (PRP) capsule is an important virulence determinant of H. influenzae type b (Moxon and Vaughan, 1981; Zwahlen et al., 1983). H. influenzae strains having a type b capsule have been shown to be more resistant in vitro to lysis by C than other capsular types while the non typable strains were very sensitive (Sutton et al., 1982). The relative susceptibility of strains to the bactericidal effect of C correlated with their virulence in humans.

Moxon and Vaughn (1981), in their study of the role of capsular polysaccharides in the virulence of H. influenzae strains, found that all capsulated strains caused bacteremia and meningitis in some animals following intraperitoneal inoculation, whereas the non-capsulated strains were non-invasive. Strains with a type b capsule produced bacteremia in

all experimental animals, and only the type b strains were invasive following intranasal inoculation. Zwahlen et al. (1983) also found type b H. influenzae capsular transformants to be more virulent in rats than other capsular types. Type b produced the greatest density of bacteremia in their experimental animals. Thus the possession of a capsule enhances the virulence of H. influenzae, but the PRP type b capsule appears to provide the best protection against the bactericidal effect of human serum and phagocytosis by polymorphonuclear leukocytes.

Abscess-potentiating ability of capsulated Bacteroides fragilis has been related to their capsular polysaccharide (Onderdonk et al., 1977). These authors found that capsulated B. fragilis alone can produce intraabdominal abscesses in rats, whereas a non-capsulated strain of B. fragilis and capsular polysaccharide from other organisms such as E. coli and S. pneumoniae type III did not produce this effect. Thus the capsular polysaccharide of B. fragilis may represent a virulence factor of this species.

Glynn and Howard (1970) demonstrated a relationship between capsular (K1) polysaccharide and the resistance of E. coli strains to C-mediated bactericidal activity of serum. The level of C resistance correlated with the amount of K1 antigen produced by the organism. In another study (Howard and Glynn, 1971), they found that E. coli rich in K1 antigen was resistant to phagocytosis and C killing and was virulent for mice following intracerebral injection. Strains of E. coli possessing the K1 antigen have been implicated in more than 80% of cases of neonatal E. coli meningitis (Robbins et al., 1974). Guerina et al. (1983) found that a high percentage of neonatal rats fed with E. coli K1 strains developed bacteremia, whereas non-K1 strains or a non-capsulated mutant of K1 strain

did not produce bacteremia. Mutation resulting in loss of the ability of E. coli strains to produce K1 antigens results in loss of serum resistance (Opal et al., 1982). Cross et al. (1984) also found a correlation between resistance of E. coli strains to phagocytic killing and the presence of K1 antigen. Pluschke and Achtman (1984), however, demonstrated that the presence of K1 capsule is not the only surface component involved in serum-resistance of K1 E. coli strains. K1 E. coli strains with certain "O" serotypes were serum-sensitive. Their study indicates that both the capsular polysaccharide and the O antigen determine serum resistance and virulence of K1 E. coli strains.

The capsular polysaccharide (Vi antigen) of S. typhi has also been implicated in resistance of the organism to the bactericidal action of serum complement and phagocytosis (Robbins and Robbins, 1984).

P. aeruginosa produces an extracellular polysaccharide referred to as the glycocalyx or mucoid substance. The glycocalyx which functions as a capsule, has been shown to mediate bacterial attachment to host cells (Costerton et al., 1979). Glycocalyx production has been considered a virulence factor primarily in the lungs of patients with cystic fibrosis. Most isolates of P. aeruginosa from the lung of patients with cystic fibrosis produce glycocalyx (Doggett et al., 1971). The antiphagocytic property of the glycocalyx has also been described (Schwarzmann and Boring 1971; Baltimore and Mitchell, 1980).

(iv) LPS composition of gram-negative bacteria.

(a) Structure.

Structurally, LPS consist of three regions of contrasting chemical and biological properties (Westphal, 1975). Figure 3 shows the LPS structure of S. typhimurium. The first region is the O-specific

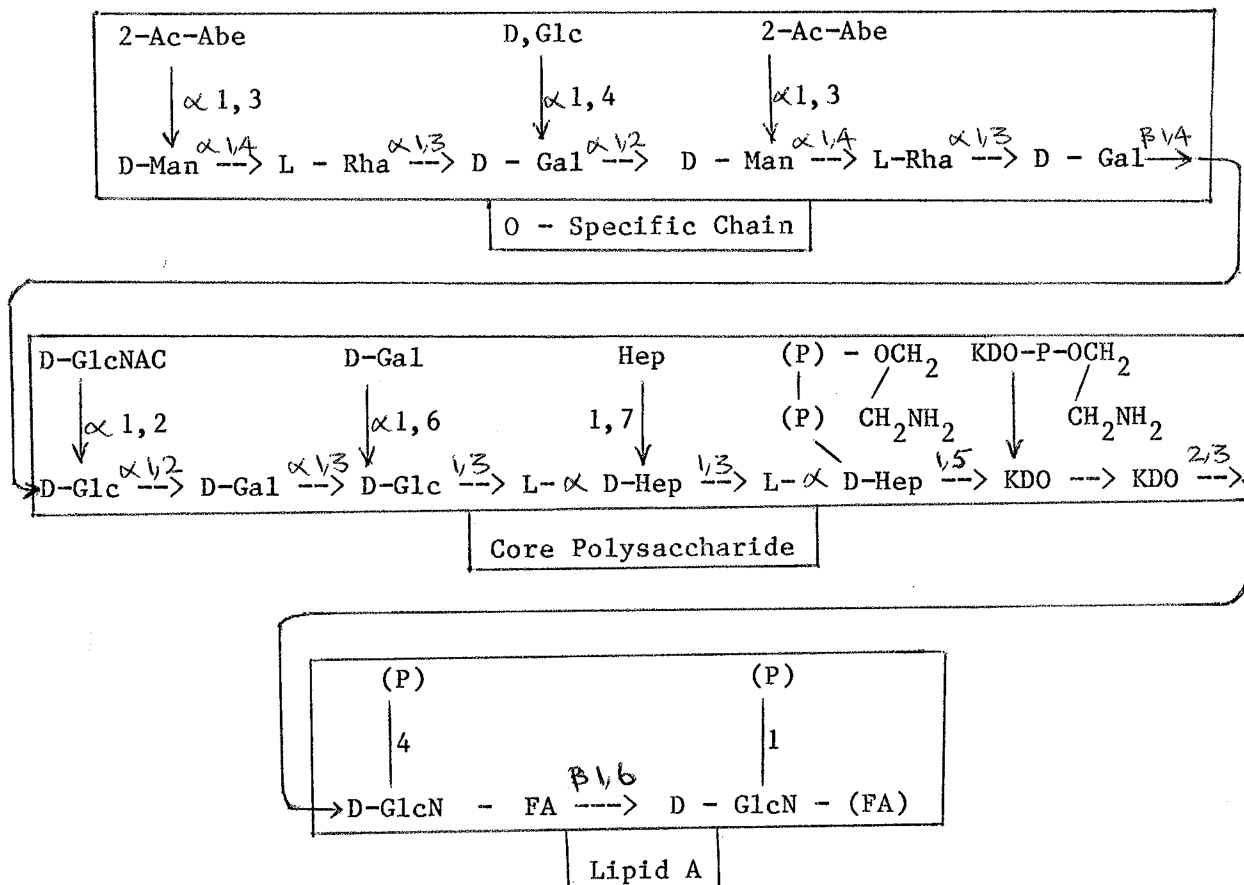


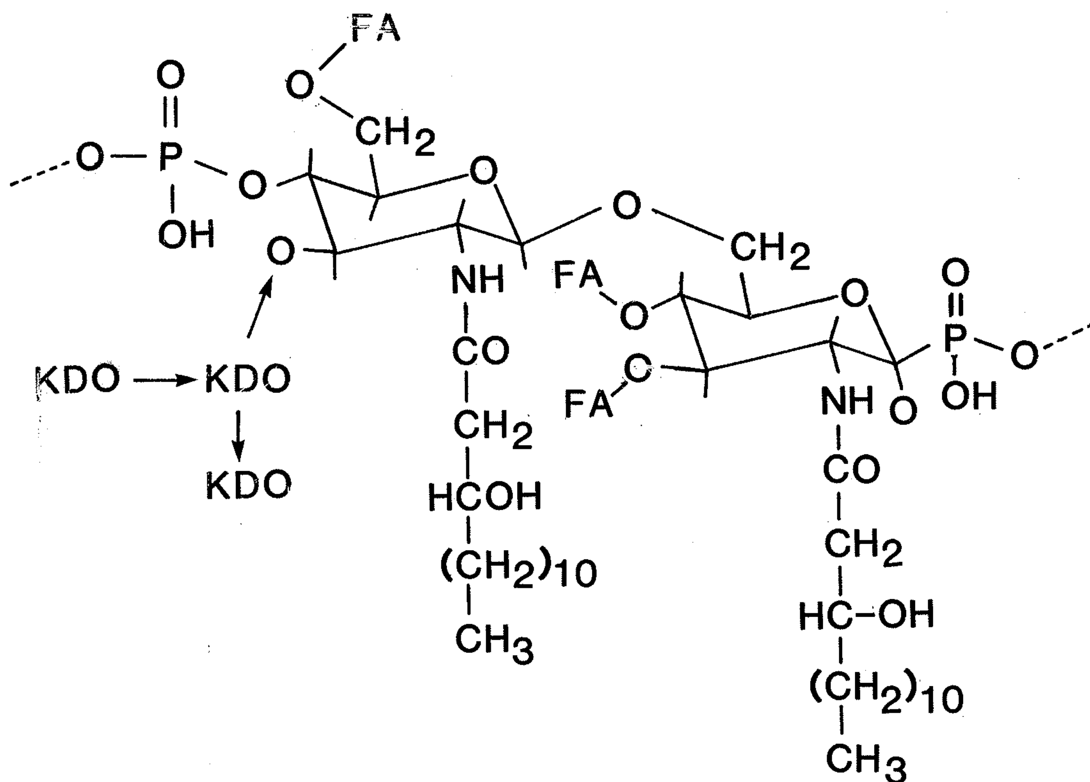
Figure 3: Structure of the lipopolysaccharide chain of S. typhimurium[a]

[a] Modified from Lindberg (1980).

Abe, Abequose; Rha, L-rhamnose; Man, D-mannose; Gal, D-galactose; Glc, D-glucose; OAc, O-acetyl; GlcNAc, N-acetyl-D-glucosamine; Hep, L-glycero-D-mannoheptose; P, phosphorus; KDO, 2-keto-3-deoxyoctulosonic acid; GlcN, D-glucosamine; FA, fatty acids

polysaccharide which confers serological specificity. The O-polysaccharide chain is exposed on the surface of the bacterium. It is composed of polymerized oligosaccharide repeating units (usually tri-to-pentasaccharide units). This structure varies between strains belonging to different serogroups (Lindberg, 1980). The O-specific polysaccharide is covalently linked to the second region - the core polysaccharide, which is present in the LPS of most gram-negative bacteria. The rough core oligosaccharide consists of 10 to 12 sugars and contains a variety of hexoses and most of the cellular octose and heptose (Luderitz et al., 1971). The core is covalently linked through a 2-keto-3-deoxyoctonate (KDO) trisaccharide to the third region, the lipid component, termed lipid A. Lipid A is composed of a phosphorylated β (1 --> 6)-linked D-glucosamine disaccharide with ester-linked fatty acids (Figure 4). The structure usually contains five to six attached fatty acyl chains most of which are 2- or 3-hydroxyl fatty acid (Nikaido and Nakae, 1979). The amino groups are substituted with β-hydroxy-myristic acid and the 3 hydroxyl groups are esterified with fatty acids such as lauric (C_{12}), myristic (C_{14}), palmitic (C_{16}) and an additional β-hydroxyl-myristic acid (Westphal, 1975). Toxic properties of bacterial LPS reside in the lipid A moiety (Luderitz et al., 1984).

Glycoses which have been identified in the LPS of gram-negative bacteria are listed in Table 1. D-mannose, L-rhamnose and dideoxyhexoses are found only in the O-side chain of the LPS. In addition to these glycoses, 6-deoxy-L-talose has been detected in the O antigens of Enterobacteriaceae. Other glycoses may be found in the core region of the LPS. The presence of ribose in hydrolyzates of LPS indicates contamination with ribonucleic acid (Ashwell and Hickman, 1971).



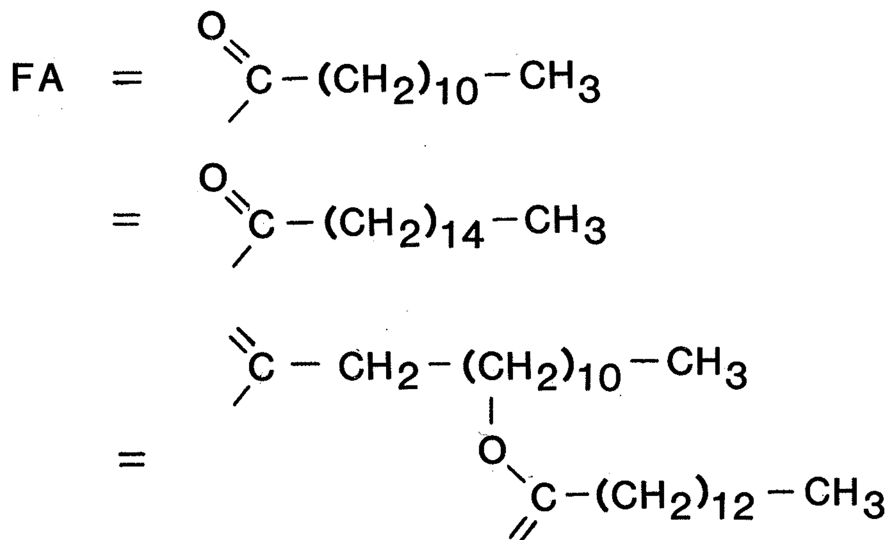


Figure 4: Structure of lipid A (Westphal, 1975)

Table I. Monosaccharide constituents of the LPS of gram-negative bacteria[a].

Hexosamines	Deoxyhexo-samine	Hexoses	6-Deoxyhexoses	3,6-Dideoxy-hexoses
				Abequose[b]
D-Galactosamine	L-Fucosamine	D-Galactose	L-Fucose	Colitose[c]
	D-Fucosamine	D-Glucose		Paratose[d]
D-Glucosamine	D-Viosamine	D-Mannose	L-Rhamnose	Tyvelose[e]
			6-Deoxy-L-talose	Ascarylose[f]

2 - keto-3-deoxyoctonate	L-Glycero-D-mannoheptose
Neuraminic acid	D-Glycero-D-galactoheptose
Ribose	D-Glycero-D-mannoheptose
Xylose	

[a]Luderitz *et al.*, 1966
[b]3,6 - Dideoxy-D-galactose
[c]3,6 - Dideoxy-L-galactose
[d]3,6 - Dideoxy-D-mannose
[e]3,6 - Dideoxy-L-mannose
[f]3,6 - Dideoxy-D-glucose

(b) LPS and bacterial virulence.

Virulence of smooth strains of E. coli and S. typhimurium has been related to the composition of the O-antigenic side chain of their LPS. Medearis et al. (1968) in their study of the virulence of E. coli O111B_4 in mice found a relationship between the LPS composition of the organism and its resistance to phagocytosis by macrophages in vivo and polymorphonuclear leukocytes in vitro. Mutants which contained LPS lacking galactose, glucose, N-acetylglucosamine or colitose in the LPS were avirulent for mice, whereas the parent strain E. coli O111B_4 was virulent. The capacity of the organism to kill mice was correlated with their ability to resist phagocytosis and persist in the site of inoculation. Edebo and Norman (1970) also found a relationship between the virulence of S. typhimurium mutants in mice and the presence of O-specific side chains in their LPS. The most virulent had O-specific antigen at their surface whereas none could be demonstrated in the least virulent mutants. A similar study was reported by Makela et al. (1973). They found that qualitative alterations of the structure of the O-specific chain of S. typhimurium LPS resulted in changes affecting the virulence of the organism for mice.

Mintz and Deibel (1983), in their study of Salmonella-mediated gastroenteritis in rabbits, found that mutation affecting the LPS structure of S. typhimurium caused reductions in invasiveness and ability to produce Salmonella enterotoxin. Rough mutants without the O-side chain in their LPS elicited reduced ileal loop response in rabbits and produced less enterotoxin compared to the parent strain. Young (1972) reported that the opsonizing activity of sera from human volunteers given purified P. aeruginosa LPS was removed by absorption with whole live bacteria, heat-killed intact bacteria or LPS. The heat stable opsonizing antibody

was type specific, and purified LPS could block phagocytosis of the strain from which the LPS was obtained. The author concluded that the LPS of *P. aeruginosa* mediates resistance to phagocytosis which can be neutralized by specific antibody. Takasaki *et al*. (1983a) discovered that substitution of tyvelose, N-acetylglucosamine, mannose and glucose for abequose, rhamnose, mannose and galactose in the O-side chains of *S. typhimurium* LPS, lowered the resistance of the organism to phagocytosis by macrophages *in vitro*. The LD50 of the organism for mice was significantly increased, suggesting that the composition of the O-side chains is important in virulence of *S. typhimurium*. In another study (Takasaki *et al*., 1983b), they observed that the extent of activation of C3 and the subsequent rate of phagocytosis were inversely proportional to virulence for mice. They postulated that C activation by the polysaccharide component of the LPS will result in the ingestion of bacteria via the C receptors on the phagocytic cells, but if polysaccharide activates C poorly, bacteria will escape ingestion and may cause disease.

(c) LPS and bacterial serum resistance.

Bacterial LPS appears to be the cell surface component most frequently associated with resistance of gram-negative bacteria to the C-mediated lethal action of serum. Wardlaw (1963) first associated cell wall components of bacteria with serum resistance. He compared the cell envelope composition of a serum-resistant smooth strain of *E. coli* with that of a serum-sensitive rough strain. The strains contained comparable amounts of envelope protein and lipid, but there was a ninefold difference in the amount of LPS that could be extracted from the cell envelopes of the strains. He suggested that the outer membrane rich in LPS protected the cell against the C reaction. However, the phenol-water procedure he used

in the extraction of LPS has been shown to be more efficient in extracting smooth LPS than rough LPS (Galanos et al., 1969). Thus, serum-resistant smooth strains may not necessarily have contained more LPS than serum-sensitive strains. In fact, mutations to serum resistance occurred independently of the amount of LPS in E. coli (Taylor, 1975). The acquisition of serum resistance was associated with the ability of E. coli strains to synthesize O-side chain. Also, phenotypic-induced transition from serum-resistance to serum-susceptibility was not accompanied by a decrease in the amount of LPS recovered (Taylor et al., 1981a). Thus the quality and not quantity of cell envelope LPS may be the determinant of serum-resistance of E. coli strains.

Many other studies have associated serum resistance of smooth enterobacteria to the presence of O-side chains in their LPS. Taylor et al. (1981b) obtained serum-sensitive strains in the presence of subinhibitory concentrations of 6-β-amidinopenicillanic acid (mecillinam). The LPS of these serum-sensitive strains contained reduced amounts of the O-side chain components (Taylor et al., 1982). Nelson and Roantree (1967) also derived serum-sensitive mutants from serum-resistant smooth virulent S. typhimurium and S. enteritidis strains by selection for resistance to cephalosporin or penicillin. The majority of the mutants were rough or part rough as a result of partial or total loss of the O-side chains. However, a few of the serum-sensitive mutants possessed LPS with complete O-side chains indistinguishable from the smooth serum-resistant parent strains, an indication that other factors could be involved in the serum-sensitivity of these mutants.

Feingold (1969) obtained serum-sensitive strains of E. coli and P. aeruginosa from serum-resistant parent strains by growth in media

supplemented with sublethal concentrations of diphenylamine. Cells grown on diphenylamine media had LPS with lower O-side chain to core polysaccharide ratio compared with that of the parent strains. Therefore phenotypic conversion to serum-sensitivity by diphenylamine was associated with alteration in LPS composition. The incorporation of diphenylamine into the cytoplasmic membrane of these organisms probably resulted in defective synthesis of O-antigen or interfered with some specific step in the synthesis, transfer or polymerization of O-antigen.

Muschel and Larsen (1970) also found that a smooth strain of E. coli $0111B_4$ was resistant to the bactericidal action of normal serum, but rough strain E. coli J-5 was sensitive. However, both strains were sensitive to serum containing $0111B_4$ antibody. They suggested that the difference in resistance of the two organisms to normal serum may be attributed to a lack of adequate antibody against $0111B_4$ (O-antigen) in normal serum, whereas antibody to core polysaccharide is usually present in normal serum because of widespread distribution of core polysaccharide in relatively large numbers of organisms compared to the limited distribution of the specific determinants in strains similar to $0111B_4$. Rowley (1968), however, found a smooth strain of S. minnesota 218S to be resistant to the bactericidal action of antiserum raised against the strain while the three rough strains were sensitive. The bactericidal activity of this serum can be removed by absorption with a rough strain but not with the smooth strain. The rough strain however became resistant to the bactericidal action of 218S antiserum when coated with 1 mg of 218S LPS per ml prior to the bactericidal assay. Rowley suggested the possibility that the antigenic components which are important in serum sensitivity were shielded by the smooth LPS thereby blocking access of antibody to the antigens involved in

bacterial sensitivity. Although several studies have demonstrated a relationship between serum resistance and the presence of LPS with O-side chains, many serum-sensitive clinical isolates have smooth LPS (Taylor, 1983). Phenotypic and perhaps genotypic variation in the O-side chain length and degree of substitution of the core polysaccharide may account for the differences in the serum sensitivity of smooth strains (Goldman and Leive, 1980; Taylor et al., 1981a).

The chemical composition of the core polysaccharide has also been shown to contribute to serum resistance, especially in gram-negative bacteria in which O-side chain LPS has not been unequivocally demonstrated. For example, Guymon et al. (1982) obtained three LPS mutants of N. gonorrhoeae lacking certain sugars found in the core polysaccharide of the LPS. They found that mutants lacking heptose but containing reduced amounts of glucose, galactose and N-acetylglucosamine and mutants lacking all these sugars in the core polysaccharide were rapidly killed by normal human serum. The parent strain and mutants lacking only galactose were resistant.

A number of studies have shown that LPS is the target antigen involved in the killing of N. gonorrhoeae by antibody and complement (Glynn and Ward, 1970; Ward et al., 1978; Tramont et al., 1977). Glynn and Ward (1970) showed that the bactericidal antibodies in normal and immune sera could be absorbed by heat-killed or trypsinized gonococci and by erythrocytes coated with gonococcal LPS. Tramont et al. (1977) also demonstrated that the bactericidal activity of convalescent sera from patients with disseminated gonococcal infection was inhibited by absorption with LPS purified from the homologous strain. Ward et al. (1978) raised monovalent rabbit antisera against purified gonococcal LPS, pili and two

major outer membrane proteins. They found that antibody to the major outer membrane proteins and LPS was bactericidal for the homologous and some heterologous gonococci, whereas antibody to pili was not. LPS antibody, however, was most effective at inducing serum killing. More recently, Shafer et al. (1984) showed that the LPS of N. gonorrhoeae strains determines their serum sensitivity. Purified LPS of serum-sensitive strains was capable of inhibiting bactericidal activity of normal human serum against homologous strains. The inhibitory effect was associated with the anticomplementary effect of LPS, but LPS of one of the strains inhibited serum bactericidal activity at concentrations which were not anticomplementary.

Joiner et al. (1982), in their study of the mechanism of resistance of a smooth strain of S. minnesota, found that serum-resistant organisms did not block C activation. Rather, the terminal C components involved in the bactericidal activity of serum were deposited on the bacterial surface but failed to remain surface bound. The C5b-9 complex referred to as the membrane attack complex was released from the bacterial surface without bacterial killing. The release of C5b-9 was associated with its failure to bind hydrophobically in the outer membrane. The C5b-9 remained stable and bound to the outer membrane of the serum-sensitive strains resulting in membrane damage and cell death. In another study (Joiner et al., 1984a) they found that increased LPS content and O-side chains of serum-resistant E. coli $0111B_4$ were associated with release of bound C5b-9 from the bacterial surface and outer membrane. The major receptor site for C3 deposition on E. coli 0111B4 was the LPS and C3 attached preferentially to the longest LPS molecule within the bacterial membrane. This was especially true of molecules with the largest number of O polysaccharide

subunits (Joiner et al., 1984b). Based on these observations, Joiner and coworkers postulated that the LPS molecules containing long O-side chains sterically hinder access of the C3b to the shorter molecules, preventing the insertion of the C5b-9 into the hydrophobic domains. The attachment of C3b to short chain length LPS, however, provides a focus of attachment for C5b-9 near the outer membrane, leading to C-mediated killing.

Harriman et al. (1982) also demonstrated that both serum-resistant and serum-sensitive strains of N. gonorrhoeae activate C1s and C5 C components in human serum. The ratio of C9/C5 bound to the resistant strains was comparable to that of the sensitive strains, suggesting that the C5b-9 is formed on the outer membrane of both types of strains. The C5b-9 deposited on the outer membrane of both serum-resistant and serum-sensitive strains could be seen in the electron microscope. However, the molecular configuration of the C5b-9 bound to serum-resistant gonococci was different from that bound to serum-sensitive strains or to resistant strains rendered sensitive by presensitization with bactericidal antibody (Joiner et al., 1983). Sucrose density gradient ultracentrifugation of detergent-extracted C5b-9 complexes indicated that the major bactericidal form of C5b-9 on the surface of serum-sensitive or presensitized serum-resistant gonococci sediments as a 33S complex whereas the nonbactericidal C5b-9 on serum-resistant gonococci exists in larger 35S and 43S aggregates of C5b-9, or as C5b-9 membrane constituents (Joiner et al., (1983). Joiner et al. (1985) showed that the bactericidal C5b-9 was associated with four distinctive outer membrane proteins. The non-bactericidal C5b-9 was not associated with these proteins. The role of such proteins in the bactericidal activity of C5b-9 complex remains to be determined.

(v) Role of outer membrane proteins in bacterial virulence.

In contrast to the large number of studies concerned with the role of LPS and capsular polysaccharide in serum-resistance and virulence of gram-negative bacteria, relatively few studies have implicated outer membrane proteins (OMP) in resistance to host defenses.

The ability of gonococci to cause disseminated infection (DGI) has been associated with a particular antigenic type of principal OMP-1. Hildebrandt et al. (1978) found protein-1 with subunit molecular weights of approximately 36,500 in 88% of strains from DGI, but only 30% of serum-sensitive strains from localized infections had this protein antigen. The subunit molecular weight of protein-1 found in strains from localized infections ranged from 36,000 to 39,000. Transformation of serum-sensitive strains with DNA from DGI strains resulted in acquisition of serum-resistance. All the serum-resistant transformants acquired the 36,500 molecular weight protein-1. Guymon et al. (1978) performed similar transformation experiments and found that most of the transformants which acquired protein-1 from a serum-resistant donor, also acquired the donor serum resistance phenotype. However, some of the recipients remained fully serum-sensitive. LPS from serum-resistant transformants was 10-fold less efficient than that from the serum-sensitive parent in blocking serum killing of the parent strain. They suggested that the loci for major outer membrane phenotype and serum-resistance are closely linked but not identical and that the locus for serum-resistance probably affects LPS structure.

A relationship between colony opacity, protein-1 and serum-sensitivity of gonococci has been established. James et al. (1982) found that *N. gonorrhoeae* of transparent colonial type were more serum-resistant than

isogenic opaque colonial type. Gonococci with protein-1 (36,500) were more serum-sensitive than those with low-molecular weight protein-1 (35,000). Stephens and McGee (1983) also detected an association between the serum resistance of *N. meningitidis* colony opacity and the molecular weight of heat-modifiable OMP. Like gonococci, meningococci of transparent colonial type were more resistant to killing by normal human serum than were opaque colonial types. Meningococci isolated from patients with meningitis and septicemia usually produced transparent colonies, whereas organisms isolated from asymptomatic carriers generally formed opaque colonies. Heat-modifiable OMP of molecular weight (26,000 - 32,000) were found in disease-associated isolates, but not in isogenic clones that formed opaque colonies (Stephens and McGee, 1983).

Serum resistance in *E. coli* has been associated with a protein factor. Taylor and Parton (1977) found a correlation between the serum resistance of *E. coli* strains and the presence of a 46,000 molecular weight protein in their cell envelope. The serum resistance of *E. coli* strains has also been associated with a major OMP, the tra T gene product, encoded by a plasmid (Moll *et al.*, 1980).

Goldman *et al.* (1984) found no correlation whatsoever between OMP composition and the degree of serum resistance of serum-resistant mutants of *E. coli* 0111B4. However, the serum resistance of the mutants correlated with the amount of LPS present in their cell envelope.

The virulence of *Aeromonas salmonicida* (the causative agent of systemic furunculosis in salmonid fishes) has been correlated with the presence of a major surface protein referred to as A-protein, and the LPS composition of the organism (Munn *et al.*, 1982). *A. salmonicida* with both A-protein (molecular weight 49,000) and LPS displayed high resistance to the

bactericidal activity of C both in the presence and absence of specific antibody.

Chang and Doyle (1984) used an antiserum that specifically agglutinates mouse virulent but not avirulent strains of Yersinia enterolitica, to identify virulence-associated proteins by Western blot techniques. They identified four major OMP present only in the virulent strains. The expression of these proteins correlated with antiserum agglutination reactions. Their importance in the virulence of Y. enterolitica remains to be determined.

Taylor et al. (1981a) found that phenotypic induced transition from serum resistance to serum susceptibility was accomplished by the loss of ability of E. coli strains to produce extractable K1 antigen and a reduction in the amount of 46,000 molecular weight envelope protein. Whether these changes were directly responsible for the observed increase in serum sensitivity is not clear.

C. Bacterial virulence and plasmid carriage.

Plasmid-mediated factors associated with virulence of gram-negative bacteria include production of toxins, colonization antigens, serum resistance and bacterial invasiveness (Elwell and Shipley, 1980). Translucent colonial variants (form I cells) of Shigella sonnei which are virulent, readily become opaque colonial variants (form II cells) which are avirulent upon repeated passages on agar media. Form I cells have smooth LPS and are invasive while form II cells are rough and non-invasive (Kopecko et al., 1980). These authors found that the form I cells harbor a 120 Mdal plasmid absent from form II cells. Conjugal transfer of this

plasmid to other Shigella spp. or Salmonella typhi, was accompanied by the ability of these organisms to synthesize the form I antigen (smooth LPS). Sansonetti et al. (1981) obtained form II S. sonnei transconjugants harboring the form I plasmid. The form II transconjugants acquired both virulence and the ability to produce form I antigen.

A 60 Mdal plasmid has been associated with adhesive, invasive, and virulent phenotypes of six S. typhimurium strains (Jones et al., 1982). Strains which were cured of the plasmid were significantly less adhesive and invasive in Hela cells, and were less virulent for orally infected mice as compared with the parent strain.

Invasiveness and local tissue damage by enteropathogenic Y. enterolitica have been shown to be under control of a plasmid. Gemski et al. (1980) found that invasive strains harbored a 42.2 Mdal plasmid not present in non-invasive strains. Portnoy et al. (1981) also reported that virulent Y. enterolitica harbor a particular species of plasmid of molecular weight range 40-48 Mdal. Strains cured of the plasmid had altered colonial appearance and were less virulent for laboratory animals and less able to produce damage in HEp-2 tissue cell monolayers. Recently, Skurnik et al. (1984) using an isogenic pair of Y. enterolitica strains (one with and one without a plasmid coding for 240,000 molecular weight protein P1), demonstrated that the plasmid-associated protein mediates autoagglutination in Yersinia spp. The strain with the plasmid autoagglutinated when grown at 37°C, the one without plasmid did not.

Serum resistance of E. coli strains has been associated with plasmid carriage. Reynard and Beck (1976) reported that the E. coli K12 strain bearing the F-like plasmids R1 or R100 was more resistant to the bactericidal action of rabbit serum than the isogenic strain without a

plasmid. Taylor and Hughes (1978), however, found no association between plasmid carriage and the susceptibility of six E. coli K-12 strains to fresh human or rabbit serum. The discrepancy in results was attributed to a different source of serum.

Binns et al. (1979) cloned various fragments of ColV and I-K94 plasmids specifying production of colicins V and I. The BamHI-generated fragment increased the virulence of E. coli, causing a 100-fold reduction in LD50 in chicks and a significant increase in resistance to fresh rabbit serum. The gene specifying serum resistance, the iss gene, was mapped to a 5,300 base-pair sequence within the BamHI-generated fragment. The determinants of serum resistance and colicin V synthesis were found closely linked in the Col V, K94 plasmid (Binns et al., 1979). Another plasmid gene, tra T gene, specifying serum resistance in E. coli, was identified in the R6-5 plasmid by Moll et al. (1980). The tra T gene codes for a major OMP (tra T protein) which mediates serum-resistance in E. coli. The tra T protein is present at about 21,000 copies per cell and is located on the outer surface of the outer membrane.

The colonization factor antigens and both the heat stable and heat labile enterotoxins produced by enterotoxigenic strains of E. coli have been shown to be plasmid-mediated (Elwell and Shipley, 1980) and contribute to virulence. The loss of colonization antigens and enterotoxin production was correlated with the loss of a single 60 Mdal plasmid (Evans et al., 1975; Elwell and Shipley, 1980).

D. Characteristics of *Haemophilus ducreyi*.

1. Taxonomy and nutritional characteristics

The inclusion of *H. ducreyi* in the genus *Haemophilus* has been based on (1) its nutritional requirement (2) its gram staining reaction and metabolic activity and (3) its DNA base composition. All organisms in the genus *Haemophilus* demonstrate a requirement for either or both of the two nutritional factors; hemin (X-factor) or nicotinamide adenine dinucleotide (V-factor) (Kilian, 1976). *H. ducreyi* is a facultative gram-negative bacillus that requires hemin (X-factor) for growth, reduces nitrate to nitrite and has a DNA guanosine and cytosine content of 0.38 mole fractions. The range in the genus *Haemophilus* is 0.36-0.40 mole fractions. *H. ducreyi* can be differentiated from other hemin-requiring strains of *Haemophilus* by its lack of requirement for V-factor, production of alkaline phosphatase and its inability to produce H_2S, catalase or indole. A recent study of the genetic relatedness of *H. ducreyi* and other members of the genus *Haemophilus* by Albritton *et al*. (1984) suggests a distant relationship between *H. ducreyi* and *H. influenzae*. Their relative DNA homology is only 0.18%.

The requirement for hemin for growth has been demonstrated by a number of investigators (Kilian, 1976; Hammond *et al*., 1978; Albritton *et al*., 1980). *H. ducreyi* lacks the enzymes involved in the biosynthesis of hemin including ferrochelatase or heme synthetase which catalyzes the insertion of Fe^{++}/Fe^{+++} into the protoporphyrin nucleus. Most strains have been found to be non-reactive with a number of carbohydrate substrates. They are positive in the Voges-Proskauer reaction and produce weak acid from glucose and arabinose, and are biochemically inert when tested for a

chemical reaction with various substrates present in the API-20E system (Albritton et al., 1980) and Minitek system (Oberhofer and Back, 1982).

2. Ultrastructure.

Electron microscopic examination of a thin section of H. ducreyi has revealed the trilaminar appearance of the cell wall, characteristic of gram-negative organisms (Ovchinnikov et al., 1976; Marsch et al., 1978; Bertram, 1980). In addition to having a gram-negative cell wall, the organisms also possess a microcapsule. Bertram (1980), using ruthenium red and alcian blue stains demonstrated microcapsules on the surface of H. ducreyi cells. These could not be seen under the light microscope with the wet india ink method of capsular visualization probably because of their small size.

3. Pathogenesis of infection.

The pathogenesis of chancroid, an acute autoinoculable venereal disease caused by H. ducreyi, is poorly understood. It is believed that the organism penetrates the epidermis through areas of scarification, abrasion or trauma of the skin. Experimental chancroid can be induced in humans or rabbits by introducing pure cultures of H. ducreyi through the broken skin (Greenblatt et al., 1943; Kaplan et al., 1956a; Hammond et al., 1978). The mechanism by which the organism resists the host defenses to establish an infection has not been investigated. The incubation period for development of chancroid in some patients was reported to be as short as 1-2 days (Gaisin, 1975; Hammond et al., 1980), but may be as long as two weeks (Tavs, 1978). A longer incubation period has been attributed to infection with strains of lower virulence. There is an association between sexual

promiscuity, poor hygiene, and the incidence of the disease (Gaisin and Heaton, 1975). Uncircumcized males appear to be more vulnerable (Alergant, 1972). H. ducreyi has a predilection for cutaneous surfaces, mucous membranes are seldom affected (Tavs, 1978). The initial lesion usually develops within one to five days of infection on the genitalia or anal areas and rarely on other anatomic sites except for experimentally induced chancroid. Occurrence of extragenital lesions of the finger, tongue, lips and breast has been reported, but is rare (Hart, 1964). Lesions in the male are frequently found on the prepuce, especially on the internal surface, coronal sulcus, frenulum and shaft of the penis. The glans is rarely affected. In females, the initial lesions occur mostly on the fourchette, and the ulcers may spread to the labia, vestibule and perianal skin (Tavs, 1978). Ulcerations rarely occur on the introitus or cervix. The organisms may be present on mucosal surfaces without producing ulcers, establishing an asymptomatic carrier state in the female (Tavs, 1978).

The soft chancre which develops at the site of inoculation begins as a tender papule with a narrow zone of surrounding erythema and rapidly becomes a pustule within 24-48 hours (Alergant, 1972; Gaisin and Heaton, 1975). The pustule ruptures and develops into a painful, shallow ulceration with ragged undermined edges. The base of the ulcer is frequently covered by a grey necrotic sticky purulent exudate. Lesions occur in single or multiple forms. Multiple ulcers are characteristic of this disease owing to the autoinoculability of the ulcers (Greenwald, 1943; Hammond et al., 1980). Autoinoculation is one of the oldest methods of diagnosis of chancroid. In the test, a drop of pus from one of the ulcers is placed on the skin and a sterile needle is used to puncture the skin through the drop (Alergant, 1972). Several pustules usually develop within 24 hours in positive cases.

In lesions, H. ducreyi can be seen within macrophages, neutrophils and as free clumps in the interstitium (Marsch et al., 1978). The acute regional lymphadenitis and suppuration of the inguinal lymphatic glands, a common complication of the disease, is due to a pyogenic inflammatory response and the build-up of neutrophils respectively. The inguinal swelling is red, tender, and may rupture to discharge pus. Thus, considerable tissue damage may occur as a result of the inflammatory reactions in the lesion. There is no evidence of systemic spread of the disease.

It is unknown whether H. ducreyi produces toxins or extracellular enzymes which may be involved in the ulceration of the infected sites. The role of host defences in the pathogenesis of H. ducreyi infection has not been determined. Delayed type hypersensitivity to H. ducreyi antigens develops within 7-14 days of infection, especially in patients with inguinal buboes (Greenblatt et al., 1944; Dienst, 1948). This response is readily demonstrated by a skin reaction to an intradermal injection of heat-killed cells, and may persist for months or years. Hypersensitivity response to H. ducreyi antigens has also been demonstrated in rabbits (Feiner et al., 1945; Bertram, 1980).

An important characteristic of chancroid is its recurrence without any evidence of host resistance. In the animal model, rabbits previously infected with H. ducreyi develop no immunity to subsequent infections. Repeated infections generally produce more severe ulcerations of the infected site (Feiner et al., 1945; Kaplan et al., 1956b; Bertram, 1980). The role of immune response in the disease process is largely unknown.

4. Virulence studies.

There are few reports on the factors which determine virulence of H. ducreyi, and these were largely reported more than 30 years ago. Ducrey, in 1889, first described H. ducreyi as the causative agent of chancroid. Although he was unable to grow the organism in vitro, he produced an experimental chancroid by inoculating volunteer patients with pus from the ulcers. The organisms were found in the pus as bacilli 1.5u in length to 0.5 u wide with rounded ends, but less frequently in the pus of buboes. Experimental chancroid in animals was first demonstrated by Davis (1903), who inoculated a rhesus monkey intradermally with the pus from chancroid lesions. Saelhof (1924) also produced lesions in the skin of monkeys by intradermal inoculation of pure cultures of H. ducreyi, but failed to infect rabbits with the organism. Other investigators (Maximowa, 1936; Greenblatt et al., 1943; Feiner et al., 1945; Dienst, 1948) successfully reproduced chancroid lesions in humans and rabbits by intradermal inoculation of viable organisms. They were unable to produce the disease in rats, hamsters, mice, and dogs.

Greenblatt et al. (1943), in their studies of virulence of H. ducreyi, found that recent isolates produced cutaneous ulcers in humans in contrast with laboratory strains. Strains lost their virulence after several months of repeated passages in artificial media (Dienst, 1948; Thayer et al., 1955; Kaplan et al., 1956a). Deacon et al. (1956) also found that fresh isolates from patients with clinical evidence of chancroid produced cutaneous lesions in rabbits and humans, but isolates transferred frequently on artificial media were avirulent. Virulent strains manifested smooth type colonies and were less fastidious in their cultural characteristics than the non-virulent strains which produce rough type

colonies (Deacon *et al.*, 1956; Gaisin and Heaton, 1975). Strains remained virulent in the lyophilized state after 18 months of storage (Dienst, 1948). Kaplan *et al.* (1956a) observed that the size and intensity of the lesions produced in rabbits varied with the amount of the inoculum, virulence of the strain and tissue susceptibility of the individual rabbits. The back and the abdominal regions of the rabbits were equally susceptible to infection. The dose-dependent effect on the results of virulence testing of *H. ducreyi* was recently demonstrated by Bertram (1980). At least 10^7 CFU/ml of organisms were required for production of chancroid lesions in rabbits.

There are some indications that fresh isolates of *H. ducreyi* may be resistant to the bactericidal action of fresh human serum. Sanderson and Greenblatt (1937), in their study of nutritional requirements, found that the addition of 20% defibrinated rabbit blood into agar slants or soft infusion agar favoured growth of clinical isolates. Resistance of virulent *H. ducreyi* strains to the bactericidal action of rabbit serum was first noted by Greenblatt *et al.* (1944). In their study of experimental chancroid in humans, they found that rabbit serum mixed with fresh cultures for 1-2 hours had no inhibitory effect on the experimental production of chancroid. Deacon *et al.* (1956) reported that old avirulent strains differed from freshly isolated virulent cultures by their inability to grow in fresh human blood. The lack of growth of avirulent strains was thought to be due to the bactericidal properties of fresh blood. Kaplan *et al.* (1956b) were able to select more virulent organisms from those with reduced virulence, by repeated daily passages in fresh rabbit blood clots. The organisms gave chancroid lesions of greater size and intensity than the parent strain.

Greenblatt *et al*. (1944) also observed that the leukocytes in blood from patients with chancroid were highly phagocytic for *H. ducreyi*, but no details of the experimental procedures were given.

Virulence of *H. ducreyi* has been correlated with their *in vitro* antibiotic resistance. Thayer *et al*. (1955) suggested that there might be a relationship between *in vitro* antibiotic sensitivity of *H. ducreyi* and their ability to produce lesions in rabbits. They found virulent strains to be more resistant to polymyxin than the avirulent strains. Virulent strains also differed from avirulent strains in their resistance to penicillin G and other antibiotics (Singer and Deacon, 1956). The apparent relationship between the *in vitro* sensitivity of *H. ducreyi* to various antibiotics and its virulence was confirmed recently by Hammond *et al*. (1978) and Bertram (1980). All 19 isolates from patients obtained during an outbreak of the disease in Winnipeg in 1977, were virulent (as determined by the rabbit intradermal test), and were resistant to polymyxin and penicillin G. By contrast, the avirulent strains were highly susceptible.

E. A review of LPS isolation methods.

The three methods most commonly used for the extraction of bacterial LPS are (1) the trichloroacetic acid (TCA) extraction procedure originally described by Boivin and Meserobeanu (1935) and later modified by Staub (1965), (2) the hot phenol-water extraction procedure described by Westphal and Jann (1965) and (3) the petroleum ether, chloroform and phenol method developed by Galanos *et al*. (1969).

The TCA method (Figure 5) involves the extraction of wet or acetone-dried bacteria with 0.25N trichloroacetic acid at $4^{o}C$. The extracts are precipitated in the cold with 2 volumes of alcohol, resuspended in small amounts of water, dialyzed and lyophilized. Such extracts, however, are not homogeneous. The composition of the extracts varies from species to species and even (slightly) between preparations obtained from the same species (Luderitz et al., 1966). The extracts are composed of polysaccharide, lipid and protein.

The hot phenol-water extraction procedure described by Westphal and Jann (1965) can be used for the extraction of smooth and rough type LPS, to give pure preparations accounting for 1-4% of the bacterial cell dry weight (Luderitz et al., 1971). However, the Galanos method is more effective in extracting rough type bacterial LPS. The yields are generally higher than those obtained by phenol-water extraction and smooth type LPS is partially excluded from the extract (Galanos et al., 1969). An important factor in the phenol-water procedure for LPS extraction, is the distribution of LPS between (water-saturated) phenol and (phenol-saturated) water. Smooth LPS which is hydrophilic is generally excluded from the phenol phase and is found mostly in the water phase. Rough type LPS lacking O-side-chains and in some cases part of the core-polysaccharide, may be found in any of the phases depending on whether they are hydrophobic or hydrophilic. Therefore the yield of rough type LPS obtained by the Westphal method depends on the distribution of the LPS between the phases.

Recently, Darveau and Hancock (1983) described a method of LPS isolation which would extract both smooth and rough forms of LPS. The method involved treatment of bacterial cells with DNase and RNase followed by breakage of cells with a french pressure cell. Then cells were treated

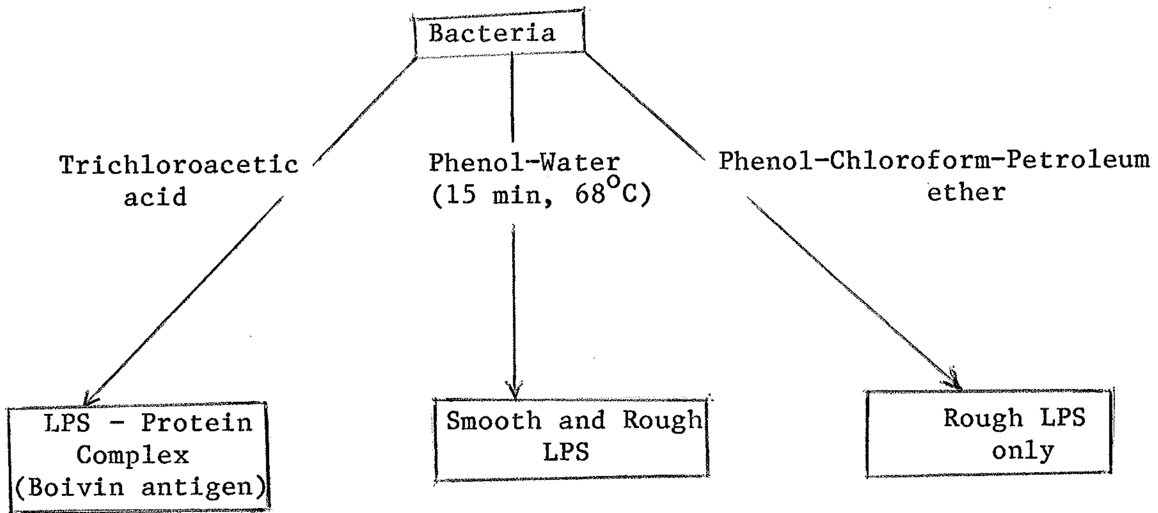


Figure 5: Methods of extraction of bacterial LPS (Luderitz et al., 1966).

with a mixture of EDTA and SDS to solubilize the cell components. The peptidoglycan was removed by centrifugation and the supernatant was treated with pronase. LPS was precipitated with 95% ethanol and centrifuged to obtain a pellet. These authors obtained higher yields of both smooth and rough type LPS compared to other methods of LPS extraction. The degree of purity of the LPS obtained was comparable to that of the Westphal and Galanos methods.

MATERIALS & METHODS

A. Culture media and growth conditions.

1. Solid media

H. ducreyi were cultured on hemoglobin agar composed of GC Agar Base, 1% CVA enrichment (Gibco Diagnostics, Madison, Wisconsin) and 1% bovine hemoglobin (Gibco). Cultures were incubated at 35°C under 5% CO_2 in air and high humidity (approx. 90%).

Strains were also cultured on a special medium composed of GC Agar Base supplemented with 50 ug hemin per ml, 1% CVA and 20% Sheep serum.

2. Liquid media.

H. ducreyi strains were grown in a special medium composed of 40% Eagle's Minimal Essential Medium (MEM) 1% Isovitalex, 20% foetal calf serum or lamb serum heated at 56°C for 30 min. Tubes were incubated at 35°C and rotated on a rotator (Canlab, Toronto) at 10 rpm.

B. Identification of bacterial strains.

H. ducreyi strains used in this study, and their sources, are listed in Table 2. Growth on hemoglobin agar was identified as H. ducreyi by the nonmucoid, yellow-gray appearance and the cohesive nature of the colonies, which enabled them to be pushed intact across the agar surface. All strains required hemin (X factor) for growth. Biochemically, they were oxidase-positive and catalase, urease and ornithine decarboxylase negative, did not produce indole or H_2S, and did not ferment glucose, sucrose, lactose, mannitol or xylose. Microscopically, all organisms were small, pleomorphic, gram-negative rods.

Table 2. *H. ducreyi* strains and sources.

Strain No.	Source
409, BG411, V1159, C148, 54211, 54213 078, 557, C147, 3019, 5439	Nairobi, Kenya
35000, 78118, 78226	Winnipeg, Manitoba
A77, A75, A76	Institute Pasteur, Paris, France
CH39	Thailand, S.E. Asia
36-F-2	Received from M. Kilian

C. Virulence testing.

Virulence of stock strains was tested by the intradermal injection of 0.2 ml of a sheep brain heart infusion broth suspension containing 10^9 CFU of H. ducreyi per ml into 2-3 kg 1-year-old New Zealand female rabbits. Induration and necrosis were determined from day 1 to day 11 postinoculation. The criteria for virulence were as follows: by day 4, induration exceeded 0.5 cm and the lesion progressed to an eschar by day 11. Each test was performed in duplicate.

D. Polymyxin sensitivity testing of H. ducreyi.

The minimum inhibitory concentration (MIC) for polymyxin B (Sigma Chemical Co., St. Louis, Mo.) was determined by the agar dilution method as described by Hammond et al. (1978). S. aureus ATCC 24923 and E. coli ATCC 25992 were used as the resistant and sensitive controls respectively. The agar dilution technique was performed in duplicate using hemoglobin agar. Polymyxin B was incorporated into the agar at $50^{o}C$ to yield final concentrations increasing in a log_2 dilution series from 0.005 to 128 ug/ml. Inoculated plates were incubated at 35° under 5% CO_2 and high humidity. The plates were read after 24 and 48h incubation. The MIC of polymyxin B was considered to be the lowest concentration that allowed growth of three colonies or less.

E. Isolation of polymyxin-resistant and sensitive strains.

1. Polymyxin-resistant isogenic strains.

Strains inhibited by $<$ 32 ug of polymyxin B/ml were considered susceptible. Polymyxin-susceptible strains were made resistant by cultivation of the strains on hemoglobin agar with increasing concentration of polymyxin, until they grew on media containing 250 ug of polymyxin/ml.

2. Polymyxin-sensitive mutants.

Polymyxin-sensitive mutants were obtained from resistant parent strains by treatment with the mutagen N-methyl-N^1-nitro-N-nitrosoguanidine. An overnight culture (18h) on hemoglobin agar was harvested into sheep brain heart infusion broth supplemented with hemin (50 ug/ml), glucose (50 mg/ml), glutamine (5 mg/ml), and cysteine (12.5mg/ml). A 0.5 ml portion of N-methyl-N^1-nitro-N-nitrosoguanidine (0.5 ug/ml) was added to 2 ml of bacterial suspension (approximately 10^9 CFU/ml) along with 2 ml of fetal calf serum heated at 56°C for 30 min. After 2h of incubation at 35°C, the cell suspension was centrifuged at 10,000 x g for 5 min., the pellet was washed twice in fresh broth, and appropriate dilutions were plated on hemoglobin agar supplemented with CVA enrichment. The surviving organisms were screened for loss of resistance to polymyxin by the replica plating method described by Lederberg and Lederberg, (1952).

F. Determination of the viability of *H. ducreyi*.

A 16h culture of *H. ducreyi* was harvested into buffers of varying composition (see Appendix B) to a concentration of 5 x 10^8 CFU/ml. At time

zero, 0.1 ml of the bacterial suspension (5 x 10^7 CFU) was added to each tube containing 0.9 ml of the buffer. Tubes were incubated at 35°C and rotated on a rotator at 10 rpm. Samples were taken at time intervals of 0, 30, 60, and 120 min., and serially diluted in the buffer and 0.3 ml of the appropriate 10-fold dilutions was plated on hemoglobin agar in 0.05 ml volumes (6 drops per plate) for the determination of viable counts as described by Miles and Misra (1938).

G. Serum bactericidal assay.

Blood from five healthy young adults with no history of chancroid was allowed to clot at room temperature for 30 min and then centrifuged at 2,000 x g for 10 min at 4°C. The serum specimens were pooled and stored in small samples at -70°C until required. Normal rabbits were bled from the ear and serum was separated and stored similarly. A 16h culture of _H. ducreyi_ was harvested into proteose peptone-saline (PPS) buffer (1% Difco Proteose Peptone in physiological saline), pH 7.2. The cell suspension was diluted to a concentration of 5 x 10^8 CFU/ml with PPS. At time zero, 0.1 ml of the bacterial suspension (5 x 10^7 CFU) was added to a tube containing 0.4 ml of PPS and 0.5 ml of undiluted normal serum. Control tubes contained serum heated at 56°C for 30 min to destroy complement. Tubes were incubated at 35°C and rotated on a rotator at 10 rpm. At time intervals of 0, 30, 60 and 120 min, samples were taken and serially diluted in PPS and the appropriate 10-fold dilution was plated as described in the previous section, for the determination of viable counts. Serum resistance was defined as a <50% reduction of the original bacterial inoculum during 120 min of incubation in 50% serum. Intermediate resistance was defined as

10-49% survival, strains with <10% survival were considered susceptible.

H. Determination of the mechanism of complement activation by *H. ducreyi*.

Classical C pathway activity in serum was selectively inhibited by chelation with 20 mM ethylene glycol-bis (β aminoethyl ether)-N,N,^{1}-tetra-acetic acid (EGTA) plus 2mM $MgCl_2$ as described by Fine *et al*. (1972) and Schiller *et al*. (1984). EGTA chelates Ca^{2+} while it binds Mg^{2+} much less efficiently (Fine, 1977). The classical pathway requires both Ca^{2+} and Mg^{2+} ion, in contrast with the alternative pathway which requires only Mg^{2+} (Sandberg and Osler, 1971). Both pathways were inhibited by treatment of serum with 20 mM ethylenediamine tetra acetic acid (EDTA) which chelates both Ca^{2+} and Mg^{2+}.EGTA or EDTA (20 mM final concentration) was added to serum prior to its use in serum bactericidal assays.

Serum was also treated with inulin (2 mg/ml) according to the method of Gotze and Muller-Eberhard (1971) or heated at 50°C for 20 min. Inulin was added directly to serum in this concentration, immediately before the serum bactericidal assay. Treatment of serum with inulin or heating at 50°C for 20 min. has been shown to deplete factor B required for alternative pathway activity (Gotze and Muller-Eberhard, 1971; Eidinger *et al*., 1977).

I. Absorption of serum with whole cells of *H. ducreyi*.

Absorption of serum with heat-treated (100°C for 10 min) whole cells of *H. ducreyi* or *H. influenzae* NCTC 8143 was performed as follows: serum (1 ml) was mixed with a heavy suspension of heat-killed whole cells in PPS (1 ml), tumbled slowly on a rotator at 4°C for 5h, and then centrifuged to

remove bacteria. The supernatant was either used immediately in serum bactericidal assays or stored at $-70^{o}C$ in small portions.

J. Isolation of human polymorphonuclear leukocytes.

Human polymorphonuclear leukocytes (PMNL) were prepared as described by Quie et al. (1967) with slight modifications. Heparinized venous blood from a single donor with no history of chancroid was sedimented for 60 min by adding 3 ml of 6% dextran in saline to 10 ml of blood. The supernatant fluid containing PMNL and a few erythrocytes was collected, the cells recovered by centrifugation, and washed twice in saline. The pellet was suspended in 10 ml of 0.84% NH_4Cl (in distilled water) and incubated in a water bath at $37^{o}C$ for 10 min to lyse the residual erythrocytes. The PMNL were then washed free of NH_4Cl with Hanks balanced salt solution (HBSS) containing 0.1% gelatin (pH 7.2). Viability was determined by trypan blue exclusion, the cells were counted in a hemocytometer, and resuspended in HBSS to give a concentration of 10^7/ml.

K. Phagocytosis assays.

1. Viable Count Method

The susceptibility of H. ducreyi strains to the killing activity of human PMNL was determined by the method of Quie et al., (1967), with some modifications. The reaction mixture in each siliconized tube (12 by 75 cm) consisted of 5×10^7 CFU (0.1 ml), 5×10^6 human PMNL (0.5 ml), 2% normal human serum (NHS) and HBSS (containing 0.1% gelatin). The control tubes contained no PMNL. Heat-inactivated serum was used in some of the

experiments. The tubes were rotated on a rotator at 10 rpm. Samples were taken at zero time and after 30, 60 and 120 min of incubation. These were serially diluted in PPS and "vortexed" to lyse PMNL. Dilutions were plated on hemoglobin agar, and incubated at 35°C for 48h, under 5% CO_2 in air, and high humidity. Colonies were counted to determine the total number of surviving bacteria.

$$\text{Percent survival} = \frac{\text{viable count (t)}}{\text{viable count (to)}} \times 100$$

to = time zero

t = incubation time

2. Microscopic method.

The kinetics of ingestion of *H. ducreyi* by human PMNL was also determined with a sensitive acridine orange fluorescence method described by Smith and Rommel (1977), combined with the use of crystal violet to quench fluorescence of non-ingested organisms (Hed, 1977). Acridine orange-labelled bacteria fluoresce green when viable and red if nonviable (Smith and Rommel, 1977). Acridine orange undergoes metachromatic changes in its absorption spectra depending on concentration. The green fluorescence of viable cells is associated with acridine orange binding to nucleoprotein complexes. In dead cells, additional dye-binding sites on nucleic acids are released as the DNA becomes denatured. High concentration of dye results in a shift to visible absorption bands producing red fluorescence (Wolf and Aronson, 1961).

Leukocyte suspensions (0.2 ml) obtained by the dextran sedimentation method were deposited on a series of cover slips (22 by 22 mm) and

incubated at 35°C in a humidified 5% CO_2 incubator for 60 min. The cover slips with monolayers of leukocytes were rinsed with HBSS (pH 7.2) at 35°C. Bacterial cells (10^8 CFU/ml of HBSS plus 0.1% gelatin) were pre-opsonized in 2% NHS for 15 min at 35°C, and 0.2 ml was added to each cover slip. The cover slips were reincubated at 35°C. They were removed at various times and washed in HBSS at 35°C. The adherent cells were stained with acridine orange (14.4 mg/litre) in Gey's balanced salt solution (Appendix B) at 35°C for 45S washed in HBSS at 35°C to remove excess stain. Identification of intracellular organisms was achieved by flooding the adherent cells with a crystal violet solution (1 mg/ml in 0.15M NaCl) at 35°C for 45 sec. The cover slips were washed in HBSS to remove excess dye, and each cover slip was placed on a glass slide with the adherent leukocytes in contact with the slide. This wet-mount preparation was examined under a Leitz UV epiluminescent microscope, with a Leitz 100x oil immersion objective lens. Two hundred cells were counted, and the percentage of leukocytes containing ingested *H. ducreyi* cells was referred to as percent phagocytosis.

L. Isolation and analysis of outer membrane proteins of *H. ducreyi*.

1. Isolation procedure.

Sarcosinate - insoluble outer membrane proteins of *H. ducreyi* were prepared according to the procedure described by Barenkamp *et al*. (1981), with some modifications. A 24h culture of *H. ducreyi* was harvested from hemoglobin agar into 0.01 M Tris HCl buffer (pH 7.0), and the organisms pelleted by centrifugation at 10,000 xg for 20 min. They were resuspended in 10 ml of 10 mM HEPES buffer (pH 7.0) and "sonicated" for 20 sec four times while cooling in an ice bath. This material was centrifuged at

10,000 xg for 20 min to remove intact cells, and the supernatant centrifuged at 100,000 xg for a further 60 min at $4^{o}C$. The pellet obtained was the total membrane preparation. The outer membrane fraction was extracted by suspending the pellet in 1 ml of 10 mM HEPES buffer (pH 7.4) and then adding 1 ml of 2% sodium lauryl sarcosinate in 10 mM HEPES buffer (pH 7.4). Extraction continued for at least 30 min at room temperature (about $23^{o}C$). The sarcosinate-insoluble fraction was harvested by centrifugation at 100,000 xg for 60 min at $4^{o}C$ and then analyzed by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

2. SDS-PAGE.

The protein preparations were solubilized by boiling for 5 min in a solution containing 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.0625 M Tris-HCl, all at pH 6.8, as described previously by Odumeru et al. (1983). Samples containing 30 ug of proteins were analyzed in a 1.5mm-thick slab gel. Electrophoresis was performed at a constant current of 16 mA for about 6h. Gels were fixed and stained overnight in a solution of 0.05% Coomasie blue, 50% methanol, and 10% (w/v) trichloroacetic acid and destained in a solution of 7.5% acetic acid. The protein content of the samples was measured by the method of Bradford (1976), as described elsewhere in this section.

M. Isolation of the LPS of H. ducreyi.

1. Phenol-water extraction method

Since the present study constitutes the first study of the LPS composition of H. ducreyi, the LPS extraction method of Westphal and Jann

(1965), which will extract both smooth and rough type LPS, was chosen.

Eighteen to twenty g wet-weight stationary phase cultures of H. ducreyi were suspended in 200 ml of water at 65-68°C in a beaker placed in water bath. Then 200 ml of 90% phenol, preheated to 65-68°C, was added with vigorous stirring, and the mixture was held at this temperature for 15 min. The solution was cooled to 10°C in an ice bath, and centrifuged at 10,000 xg for 30-45 min to enhance separation of the water and phenol phases. The water phase was removed and the phenol layer and the insoluble residue were treated again at 65-68°C with a further 200 ml of water as described above. The water extracts were combined and dialyzed against running tap water (10-15°C) for 72h to remove phenol and traces of low-molecular-weight substances. The dialyzed solution was then lyophilized.

In order to remove nucleic acids from the extract, the crude LPS preparation was dissolved in water to give a 3% solution, and centrifuged at 105,000 xg for 3h. The pellet was resuspended in distilled water and the centrifugation repeated. Nucleic acids and degraded products remained in solution. The final LPS pellet was resuspended in 2-3 ml of water and was lyophilized. The LPS was dissolved in 150 ml of water and 15 ml of aqueous 'cetavlon' (2% hexadecyl trimethylammonium bromide in water), to remove nucleic acids. The mixture was stirred for about 15 min at room temperature, and centrifuged for 20 min at 10,000 xg to remove the precipitated nucleic acid. The opalescent supernatant was lyophilized and the fluffy residue dissolved in 50-60 ml of 0.5M aqueous NaCl. This solution was poured into 500-600 ml ethanol to precipitate the LPS. The ethanol-LPS mixture was held at 0-4°C for 4h, then the precipitate was centrifuged and redissolved in water. After dialysis for 2 days against distilled water to remove NaCl, the solution was lyophilized. About 60-100

mg of purified LPS per 18g wet weight of cells was obtained. The LPS was stored at -20°C.

The LPS of S. typhimurium and E. coli $0111B_4$ were purchased from Difco laboratories, Detroit, Michigan, U.S.A. The LPS of N. gonorrhoeae P^-6472 was supplied by Dr. G.M. Wiseman.

2. Rapid isolation method.

LPS was also isolated by the rapid isolation micro method (RIM) for LPS described by Inzana (1983). This is a microadaptation of the phenol-water extraction procedure of Westphal and Jann (1965). The advantage of this method is that it allows for rapid isolation of LPS from several strains. About 2-4 ml of bacterial suspension (10^9 CFU/ml) was centrifuged in a 15 ml Corex tube (Fisher Scientific Ltd.) at 10,000 xg for 5 min and washed once with phosphate-buffered-saline (PBS) pH 7.2, containing 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$. The washed cells were resuspended in 500 ul of water and transferred to a 3 ml vial containing a magnetic stirring bar. An equal volume of hot (65-70°C) 90% phenol was added to the bacterial suspension, and stirred vigorously at this temperature for 15 min. The suspension was chilled on ice and transferred to a 1.5 ml polypropylene tube, and centrifuged at 8,500 xg for 15 min. The aqueous supernatant was transferred to another 1.5 ml polypropylene tube. The phenol phase was reextracted with another 500 ul of water. The aqueous phases were pooled and adjusted to 0.5 M NaCl, and 1 ml of 95% ethanol was added to precipitate the LPS. The ethanol-LPS mixture was left at -20°C overnight, then centrifuged at 2,000 xg for 10 min. The opaque pellet obtained was resuspended with 100 ul of distilled water and transferred to a 1.5 ml prolypropylene tube. The precipitation with ethanol was repeated, and the

precipitate obtained was suspended in 50 ul of distilled water. The LPS preparation was stored at $-20^{o}C$ until required.

N. Measurement of Limulus amebocyte lysate gelation by LPS.

The gelation of Limulus amebocyte lysate by H. ducreyi LPS was measured as described in the Sigma Technical Bulletin No. 210, using E-Toxate concentrate obtained from Sigma Chemical Co., St. Louis, Mo. LPS suspended in 0.2 ml of 'endotoxin-free' water obtained from Sigma, was added directly into the ready-to-use E-Toxate vial, 'vortexed' to ensure homogeneity and incubated for 1h at $37^{o}C$. S. flexneri LPS was used as a positive control and endotoxin-free water as a negative control. The LPS of H. ducreyi strains produced a gelation at concentrations $\geq$40 ug. S. flexneri LPS was positive at a lower concentration (20 ug).

O. Preparation of lipid A from LPS.

Lipid A was prepared as described by Stead et al. (1975). About 30 mg of LPS was hydrolysed with 5 ml of 1% (v/v) acetic acid at $100^{o}C$ for 2h in a sealed ampoule. The precipitated lipid A was removed by centrifugation, washed with 1% acetic acid, lyophilized and weighed.

P. Alkaline hydrolysis of lipid A moiety of LPS.

H. ducreyi LPS was hydrolysed by alkaline treatment as described by Shafer et al. (1984). LPS (2 mg/ml) in PPS, was extensively dialyzed against sterile distilled water at $4^{o}C$. The dialyzed LPS was incubated in

0.5 N NaOH at 56°C for 30 min. HCl was added to the LPS suspension to neutralize the reaction. Untreated LPS was included as control. Both treated and untreated samples were dialyzed against sterile distilled water at 4°C, for 48h. The samples were then used in the inhibition of serum bactericidal reactions.

Q. Analysis of the LPS composition of *H. ducreyi*.

1. Physical methods.

(a) SDS-PAGE.

The LPS preparations from *H. ducreyi* strains were analyzed by SDS-PAGE with the discontinuous gel system described by Laemmli (1970). The gel was 15% polyacrylamide and contained 4M urea (see Appendix C). In the electrophoretic analysis of LPS preparations, 2 ug of purified LPS was loaded onto the gel, and electrophoresed at 20 mA (constant current) per slab. Equivalent amounts of LPS obtained by the rapid isolation method, were also analyzed.

(b) Silver staining procedure.

After electrophoresis, the LPS bands were visualized by the silver staining method of Tsai and Frasch (1982). The LPS in polyacrylamide gel was fixed by placing the gel into a 40% ethanol - 5% acetic acid solution (200 ml) in a clean glass dish overnight. After fixation, the gel was placed in 0.7% periodic acid in 40% ethanol - 5% acetic acid and oxidized for 5 min. The gel was washed in 500-1000 ml distilled water in another dish for 15 min, three times. The water was drained off, and 150 ml of freshly prepared silver staining reagent (Appendix C), was poured on the gel and agitated vigorously (about 70 rpm on the rotor) for 10 min. The

gel was then washed for 10 min in distilled water, three times. The water was replaced with 200 ml of formaldehyde developer containing 50 mg of citric acid and 0.5 ml of 37% formaldehyde/litre. The LPS usually stained dark brown within 5-10 min. Development was terminated when the stain was of desired intensity or whenever the clear gel background showed the first sign of discoloration. The gel was washed and stored in distilled water.

(c) Infra red spectrophotometry.

Infrared (IR) spectra of the LPS preparation were determined in order to identify the funtional groups present in the LPS of H. ducreyi strains, E. coli 0111B_4 and N. gonorrhoeae P^-6472. The characteristic absorption frequencies of various functional groups present in bacterial LPS are shown in Table 3. The magnitude of the absorption of a given molecule is directly proportional to the concentration of the molecule (Smith, 1979).

The IR spectra of the LPS preparations were obtained with the assistance of the Chemistry Department, Fort Garry Campus. Lyophilized dried LPS was ground with Nujol (mineral oil), placed in cells and scanned.

(d) Gas liquid chromatography (GLC).

(i) Glycoses.

Glycose analyses were performed as described by Wiseman and Caird (1977), with slight modifications. About 30 mg of LPS was hydrolyzed with 1N H_2SO_4 for 4h at 100^oC in a sealed ampoule. The hydrolysate was neutralized with solid $BaCO_3$ and centrifuged and the clear solution was freeze-dried. The freeze-dried material was treated with 0.1 ml Sil-Prep reagent (Pyridine 0.5 ml; hexamethyldisilazane 0.15 ml;trimethyl-chlorosilane 0.1 ml) for each mg of material and shaken vigorously. Sil-prep reagent was purchased from Sigma Chemical Co., St. Louis, Mo.

Table 3. Characteristic absorption frequencies of various functional groups[a].

Functional group	Absorption Frequency
Carboxylic acids	
OH	3500 - 3580W
C=O	1740 - 1800S
C-O	1075 - 1190S
Carbohydrate	
OH	3300S
C-O	1000 - 1125S
Amides	
O (double-bonded to C) / R-C-N-R / N (bonded to N)	1510 - 1530S
Esters	
R'OR'	1210 - 1310S

S - Strong (intense band)

W - Weak

[a] Modified from Smith, 1979.

The reaction was allowed to proceed for 60 min at room temperature and the trimethysilyated monosaccharides were analyzed by a Pye 104 gas-liquid chromatograph equipped with dual hydrogen flame ionization detectors. Separation was achieved in a stainless steel column (0.65 by 180 cm) prepacked with 10% SE30 on 100 - 120 mesh Gas-Chrom Q in a temperature program over the range 100 - 250°C and rising 2°C/min. The detector temperature was kept at 250°C.

(ii) Aminoglycoses.

Aminoglycoses were also determined by GLC, as described by Laine et al. 1972. Lyophilized LPS hydrolysate obtained as described above, was heated with Sil-Prep reagent for 30 min at 80°C. The mixture was concentrated under a stream of nitrogen and 0.1 ml of n-hexane was added. The sample was analyzed in the GLC operated under the same conditions described above.

(iii) Fatty acids.

Methyl esters of fatty acids in lipid A were prepared as described by Jennings et al. 1973. Lyophilized lipid A was dissolved in chloroform, reprecipitated in acetone (1 part lipid to 3 parts acetone), and refluxed in methanolic HCl (5 parts methanol to 1 part concentrated HCl) for 8h. The fatty acid methyl esters were extracted with ethyl ether-petroleum ether (1:1), concentrated and then analyzed by GLC on a stainless-steel column (0.65 by 180 cm) prepacked with 10% DEGA and 2% phosphoric acid on 100-120 mesh Gas-Chrom Q. The oven temperature was programmed as follows: 120°C for 4 min, temperature rising at 6°C/min, and 250°C for 5 min. The detector temperature was held at 250°C. Known standards were used as a comparison in all GLC methods.

2. Chemical methods.

(a) Protein determination.

The protein content of the LPS preparations was determined by the method described by Bradford (1976), using bovine serum albumin (Sigma, St. Louis, Mo.) as the standard. Protein solution containing 1-10 ug of bovine serum albumin (BSA) in volumes of 0.1 ml of distilled water was pipetted into test tubes. LPS (0.2 mg) was weighed and added into a test tube with 0.1 ml of distilled water and "vortexed". Protein reagent (Appendix C), 1 ml portion, was added to the test tube and mixed. The mixture was left at room temperature for (2-45) min. The absorbance was measured at 595 nm (Unicam Spectrophometer) against a reagent blank (1 ml protein reagent + 0.1 ml distilled water). The weight of BSA was plotted against the corresponding absorbance (see Appendix D). The weight of protein in 0.2 mg LPS was determined from the standard curve.

$$\% \text{ Protein} = \frac{\text{Protein (ug) in LPS}}{\text{200 ug LPS}} \times 100$$

(b) Nucleic acid determination.

The DNA content of the LPS preparations was determined by the colorimetric method of Ceriotti (1952). DNA from salmon testes (Sigma, St. Louis, Mo.) was used as a standard. Distilled water (2 ml) containing (2-20)ug of DNA or 1-2 mg of LPS was added into test tubes. Then 1 ml of indole reagent (Appendix C), and 1 ml of concentrated HCl were added and the mixture was shaken. Tubes were capped, placed in a boiling water bath for 10 min and cooled under running water. The solution was extracted three times with 4 ml of chloroform each time and centrifuged to give a completely clear water phase. The intensity of the yellow color in the

water phase was read with a Unicam spectrophotometer at 490 nm against a reagent blank. The weight of DNA present in each tube was plotted against the corresponding absorbance (Appendix D). The amount of DNA present in 1-2 mg LPS was determined from the standard curve.

(c) Determination of the total carbohydrate content of LPS.

The total carbohydrate content of LPS was measured as described by Dubois et al. (1956). Solution (2 ml) containing 10-100 ug of D-glucose or 0.2 mg of LPS in distilled water was pipetted into a tube and 0.05 ml of 80% phenol was added. Then 5 ml of concentrated H_2SO_4 was added rapidly, with the stream of acid being added directly into the solution to allow for good mixing. The tubes were allowed to stand for 10 min and then placed in a water bath at 25-30°C for 10-20 min. The absorbance of the yellow-orange color was measured at 490 nm against a blank in which distilled water was substituted for the sugar solution. The amount of total carbohydrate present in the LPS sample was determined from the standard curve (Appendix D).

(d) Determination of hexoses in LPS.

The anthrone method described by Scott and Melvin (1953) was used for the determination of hexoses in LPS, using D-glucose as the standard. A wire basket containing test-tubes was placed in a rapidly circulating cold water bath (10-15°C). Then 10 ml of anthrone reagent (2 g of anthrone in 1 litre concentrated H_2SO_4) was added into each tube using a pipet with enlarged orifice. The same drainage time was allowed for each delivery. Tubes were covered with loose-fitting caps and 5 ml of sample containing 10-100 ug/ml of D-glucose, or 0.2 mg of LPS in distilled water, was added into each tube, carefully forming a layer above the anthrone reagent. Two blanks, using 5 ml of distilled water were included in each experiment.

Tubes were shaken vigorously while immersed in the cold water bath, until the contents were thoroughly mixed, and cooled to room temperature. The baskets were placed in the 90°C water bath for 16 min, and then cooled in cold water until contents were at room temperature. The absorbances of the samples were read immediately at 625 nm, against the reagent blank. The concentration of hexoses in LPS was determined from the standard curve (Appendix D).

(e) Heptose determination by the cysteine - sulfuric acid method.

The heptose content of LPS was determined using Dische's cysteine-sulfuric acid method as modified by Wright and Rebers (1972). LPS (0.4 mg) was suspended in 2 ml distilled water and heated for 2 min in a boiling water bath. The LPS solution was cooled to room temperature and clarified by centrifugation at 1,000 xg for 10 min. The opalescent supernatant fraction was assayed for heptose. In the assay, 4.5 ml of H_2SO_4 (6 vol. concentrated H_2SO_4 : 1 vol. H_2O) was added to 1 ml solution containing 5-80 ug of D-glycero-D-guloheptose (Sigma, St. Louis, Mo.) or 1 ml of the LPS solution (0.2 mg/ml) in a tube placed in the ice bath. The mixture was shaken vigorously, and held for 3 min at 0°C and 3 min at 25°. Into the mixture was added 0.1 ml of freshly prepared 3% (w/v) cysteine - HCl. The mixture was heated for 20 min at 100°C in a boiling water bath, and cooled under tap water. The absorbance of the sample was read at 505 and 545 nm after 1h, against a reagent blank. The differential absorbance $A_{505-545}$ was calculated, and plotted against the concentration of D-glycero-D-guloheptose in samples. The concentration of heptose in LPS was calculated from the standard curve (Appendix D).

(f) KDO determination.

The KDO content of LPS preparations was determined by the method described by Karkhanis et al. (1978), using KDO (Sigma, St. Louis, Mo.), as the standard. 1 ml of 0.2N H_2SO_4 was added to a tube containing 1-10 ug KDO or 1-2 mg of LPS in distilled water. The reaction mixture was heated at 100°C for 30 min, cooled and centrifuged at 1,500 x g in a clinical centrifuge (Canlab) for 5 min. The clear supernatant (0.5 ml) was pipetted into another test tube and 0.25 ml of periodic acid (0.04M H_5IO_6 in 0.125N H_2SO_4) was added. The mixture was "vortexed" and left at room temperature for 20 min. Then 0.25 ml of 2.6% $NaAsO_2$ in 0.5 N HCl was added, 'vortexed' and allowed to remain until the brown color disappeared. A 0.5 ml volume of 0.6% thiobarbituric acid (TBA) in water was added and mixed by "vortexing". The mixture was heated in boiling water bath at 100°C, for 15 min, and 1 ml of dimethylsulfoxide (DMSO) was added while the mixture was still hot. The mixture was allowed to cool to room temperature and the absorbance was read at 548 nm against a reagent blank, containing no KDO. The amount of KDO in LPS was calculated from the standard curve (Appendix D) using the formula:

$$\text{Amount of KDO in LPS} = \frac{2 \times \text{Amount of KDO calculated from graph (ug)}}{\text{amount of LPS analysed (ug)}}$$

The method of Brade et al., (1983) was also used in the determination of KDO content of LPS. LPS (0.2-0.5 mg) was hydrolysed in 0.2 ml of 4M HCl for 1h in a sealed ampoule. The ampoule was cooled in ice water and the volume of the LPS suspension was adjusted to 0.5 ml with water. To each tube containing 0.5 ml of sample (2.5-20ug) KDO or hydrolyzed LPS, was

added 125 ul of 0.04M H_5IO_6 in 0.06M H_2SO_4. The mixture was incubated at room temperature for 30 min, and 125 ul of 0.2M $NaAsO_2$ in 0.5M HCl was added to stop the oxidation. Then 0.25 ml of freshly prepared 0.6% aqueous TBA solution was added and the mixture was kept in a boiling water bath for 15 min. DMSO (0.5 ml) was added to each tube while hot. The tube was cooled to room temperature, and the absorbance was measured at 549 nm against a reagent blank. A plot of absorbance versus KDO (ug) is shown in Appendix D.

(g) N-acetylneuraminic acid (sialic acid) determination.

The method of Jourdian et al. (1971) was used to determine the amount of N-acetylneuraminic acid (NANA) present in LPS. NANA purchased from Sigma, was used as the standard. Samples (0.5 ml) containing 0.012-0.20 umol of NANA in 0.5 ml volume, were prepared. LPS (1-2) mg in 0.5 ml distilled water was also prepared. Then 0.1 ml of 0.04M H_5IO_6 solution was added and mixed thoroughly and allowed to stand in an ice bath for 20 min. Into the mixture was added 1.25 ml of resorcinol reagent (Appendix C), mixed, and placed in an ice bath for 5 min. The mixture was heated at 100°C for 15 min in a water bath, and cooled in tap water. After cooling, 1.25 ml of tert-butyl alcohol was added, and mixed vigorously to give a single phase solution. The samples were placed in a 37°C water bath for 3 min to stabilize the colour and cooled to room temperature. The absorbance was measured at 630 nm against a reagent blank without NANA. A plot of the absorbance versus known amounts of NANA is shown in Appendix D. The NANA content of LPS was determined from the standard curve.

(h) Determination of phosphorus.

The phosphorus content of the LPS preparations was determined by the method of Fiske and Subbarow (1925) as cited by Lelor and Cardini

(1957). Samples containing 0.1-1 umol K_2HPO_4 or 1 mg LPS were prepared with distilled water. Then 1 ml of 5N H_2SO_4 was added to each sample, and the mixture was evaporated in the test tube over a free flame. When contents became brown and had cooled, 1 drop of 2N HNO_3 was added and heating was continued until white fumes appeared. After cooling, 1 ml of water was added and the tube was placed in a boiling water bath for 5 min. The mixture was allowed to cool and 1 ml of 2.5% (w/v) ammonium molybdate was added and mixed. Then 0.1 ml of reducing solution (Appendix C) was added, mixed and the volume of the mixture was adjusted to 10 ml with distilled water. About 10 min after mixing, the absorbance of the mixture was measured at 660 nm. The absorbance was plotted against known concentrations of the standard (Appendix D). Phosphorus content of LPS was determined from the standard curve.

(i) Hexosamine determination.

The hexosamine content of LPS was determined by the method of Smith and Gilkerson (1979). Samples containing 4-40 ug of glucosamine or 0.2 mg LPS were prepared in 0.2 ml of 0.5M HCl. The sample was mixed by "vortexing" and then heated at 110°C for 2h. The samples were cooled to room temperature in a water bath, and 0.4 ml of 2.5% $NaNO_2$ was added in the fumehood. The mixture was "vortexed" and allowed to stand at room temperature for 15 min. Then 0.2 ml of 12.5% ammonium sulfamate was added, "vortexed", and allowed to stand at room temperature for 5 min to allow for the liberation of excess $NaNO_2$. After this time period, 0.2 ml of 0.25% 3-methyl-2-benzothiazolone hydrochloride (MBTH) was added, "vortexed" and incubated at 37°C for 30 min. Then 0.2 ml of 0.5% $FeCl_3$ was added and incubated at 37°C for 5 min. The samples were cooled to room temperature and the absorbance was read at 650 nm. The MBTH and $FeCl_3$ used in the

assay were prepared fresh weekly. The standard curve obtained using glucosamine as the standard is shown in Appendix D.

R. Inhibition of serum bactericidal reactions by LPS.

H. ducreyi LPS was suspended in PPS to a final concentration of 4 mg/ml and 'sonicated' at $4^{\circ}C$ until the solution cleared. LPS solution in the concentration range of 0.05-1 mg was added to 0.5 ml of serum in a tube and the volume was adjusted to 0.9 ml with PPS. The mixture was incubated at $37^{\circ}C$ with shaking for 30 min. Then, 0.1 ml of bacterial suspension (5×10^7 CFU) in PPS was added to the tube and incubated at $37^{\circ}C$ for an additional 60 min before dilution plating.

S. Complement titration.

1. Preparation of sheep erythrocytes

Sheep erythrocytes were washed three times and suspended to a concentration of 2.8% in PPS. The concentration of the cell suspension was checked by mixing 1 ml of 2.8% sheep erythrocytes with 14 ml of distilled water and the concentration of lysed cells was read at 540 nm. The target O.D. was 0.32. The volume of 2.8% sheep erythrocytes was adjusted with PPS by using the formula:

$$\text{Final Volume} = \frac{(\text{O.D. of test rbc}) \times (\text{original vol. of test rbc} - 1\text{ ml})}{\text{target O.D. for 2.8\% sheep erythrocyte suspension}}$$

Sheep erythrocytes were standardized each week.

2. Sensitization of sheep erythrocytes.

Sheep erythrocytes were sensitized by adding 1 ml of 1:1500 hemolysin (Appendix C), obtained from Difco Laboratories, Detroit, Mich., to 1 ml of standardized 2.8% sheep erythrocytes, and mixed by constant swirling. The mixture was incubated at 37°C in a water bath for 20 min to sensitize cells.

3. Measurement of complement activation by H. ducreyi LPS.

The anticomplementary activity of LPS was measured by mixing 0.1 ml of serum with 0.005 - 0.1 mg LPS suspended in PPS. Serum mixed with PPS alone represented the control. The mixture was incubated with shaking at 37°C for 30 min. Antibody-sensitized sheep erythrocytes in 0.2 ml PPS were added to a four-fold dilution of treated human serum (0.2 ml) and incubated for an additional 30 min in a 37°C water bath. Ice-cold saline (3 ml) was added to the mixture, the cells were pelleted by centrifugation and the absorbance of the supernatant was measured at 412 nm. The positive control was sensitized erythrocytes plus serum without added LPS; the negative control consisted of LPS plus erythrocytes without added serum.

T. Preparation of antibodies to H. ducreyi.

Whole cell "sonicates" of H. ducreyi strains were prepared by suspending 24h culture in sterile physiological saline. The suspension was "sonicated" with a Biosonik IV (Bronwill Scientific, Rochester, N.Y.) until complete lysis had occurred. The protein content of the suspension was determined by the method of Bradford (1976) as previously described.

Six female 2-3 kg white New Zealand rabbits were bled prior to

immunization. The preimmune sera were tested by double diffusion, and none had antibodies to H. ducreyi. The rabbits were injected intradermally with four 0.1 ml inoculations of 1:1 dilution of cell extract and Freund's complete adjuvant (Difco), and then intravenously with 0.2 ml of cell extract. The total concentration of proteins in the whole cell "sonicate" injected into each rabbit was 1 mg.

A weekly injection of 1 mg of protein in 0.25 ml of whole cell sonicate was given intravenously to each rabbit for five weeks. The rabbits were bled, and the antisera tested for activity. All antisera were reactive by double diffusion. The H. ducreyi whole cell antisera were frozen at -20°C in aliquots until required.

U. Ouchterlony immunodiffusion technique.

Double diffusion was performed using immunodiffusion agar tablets (Oxoid, London, England) melted in 50 ml of 0.025M Oxoid barbitone acetate buffer. The melted agar was cooled to 50°C and 15 ml was poured over the surface of 10 x 10 cm clean glass plate (LKB Products, Bromma, Sweden). Wells were made in the cooled agar. Whole cell sonicate and antisera raised againt whole cells in rabbits were pipetted into the wells. The plates were placed in moist petri dishes, and left for 24h at room temperature and then for 72h at 4°C. The plates were washed in saline for 2h, then in distilled water for 1-2h, and air-dried. These gels were fixed in aqueous 2% acetic acid for 10 min and stained (2g coomasie brilliant blue R250, 10 ml glacial acetic acid and 90 ml ethanol) for 2h. The stained plates were rinsed in tap water and then destained in a solution containing 10 ml glacial acetic acid, 85 ml ethanol and 5 ml distilled water.

V. Electron microscopy.

1. Negative staining.

Negative staining of H. ducreyi cells was performed as previously described by Bertram (1980). A drop of 24h culture of H. ducreyi in liquid broth (Appendix A) was spotted on a formvar 400-mesh copper grid (Polyscience Inc., Warrington, Pa.) and excess liquid was removed with a filter paper. Then 1.5% phosphotungstic acid at pH 8.6 was pipetted onto the grids. These were air-dried and examined in a Phillips 201 electron microscope at 50,000x or 80,000x magnification.

2. Fixation and thin sectioning techniques.

Bacterial cells were prepared for thin sectioning using the fixation technique routinely used in the Medical Microbiology Department. Cells grown in liquid broth (Appendix A) were centrifuged and the pellet was suspended in 3 ml of fixative containing ruthenium red and alcian blue stains (Appendix C). The cells were fixed for 15 min at $4^{o}C$, washed two times in fixative and resuspended in 1% OsO_4 (made up in fixative). They were fixed for 15 min at $4^{o}C$, and washed two times in fixative. The cells were embedded in an equal volume of molten 3% agarose in filtered distilled water, and drawn into cores. The agar cores were placed in fresh fixative containing alcian blue and ruthenium red stains, and fixed for 2h. The cores were dehydrated in a graded series of acetone-water mixtures (Appendix C). The agarose cores were placed in acetone-propylene oxide mixture (Appendix C), and embedded in plastic (Appendix C). The agarose cores were placed in numbered Beem capsules with fresh plastic and polymerized by heat at $48^{o}C$ for 16h followed by 48h at $60^{o}C$.

Specimen blocks were sectioned on a LKB 8800 Ultratome (Broma, Sweden) with glass knives. Sections were placed on 400 mesh copper grids and stained with 1% uranyl acetate, followed by 1% lead citrate. Specimens were examined in a Phillips 201 electron microscope at 100,000x magnification.

R E S U L T S

PART I - Host factors involved in the virulence of *H. ducreyi*.

A. Criteria of virulence applied to *H. ducreyi*: the rabbit intradermal test.

Intradermal injection of 10^9 CFU of virulent *H. ducreyi* strains in separate sites on the back of rabbits resulted in necrosis and eschar formation not later than day 11. Avirulent strains produced no cutaneous lesions in rabbits (Figure 6). Table 4 shows the degree of virulence of strains tested, based on the size of the ulcer produced by the same number of inoculated bacteria. Strains 409, 35000, C148, 78118, BG411 and V1159 were virulent according to the criteria stated while strains A75, A77, A76, 078 and 36-F-2 were avirulent. Virulent strains were recovered from the lesions between days 4-6, but none of the avirulent organisms were recovered from the site of inoculation.

B. Susceptibility of *H. ducreyi* strains to the bactericidal action of normal serum.

1. Survival of strains in various suspending fluids used in serum bactericidal assays.

The survival of *H. ducreyi* strains in various suspending fluids was determined. Results indicated that PPS (pH 7.2) or PPS supplemented with 2% normal human serum supports the viability of the strains for 3h at 35^oC (data shown for strain A77, Figure 7). Hanks balanced salt solution (HBSS) supplemented with 0.1% gelatin did not sustain viability. Strains died quickly in saline and phosphate-buffered saline (0% survival within 60

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Figure 6: Rabbit intradermal test for H. ducreyi. Photograph taken on day 8 post-inoculation. Virulent strains: A, 35000; D, BG411; F, 409; G, V1159; J, 78118; K, C148. Avirulent strains: B, A77; C, 36-F-2; E, A75; H, A76; I, 078.; L, broth Control.

Table 4. Virulence of H. ducreyi strains in the rabbit intradermal test.

Strain	Virulence*
409	+++
35000	+++
BG411	+++
78118	++
V1159	++
C148	+
36-F-2	0
A77	0
A75	0
078	0
A76	0

* Degree of virulence based on size (diameter) of ulcers:

+++ >1 cm

++ 0.5-1 cm

+ <0.5 cm

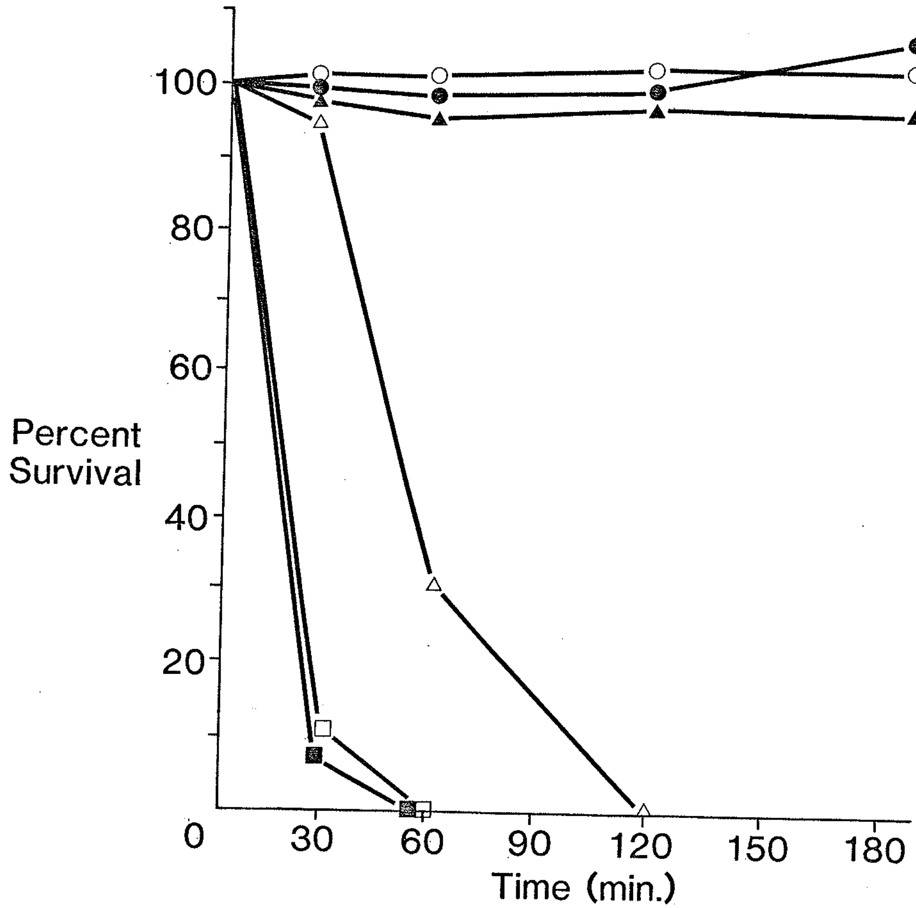

Figure 7: Results of a representative experiment showing the survival curves of H. ducreyi A77 in various suspending fluids (pH 7.2) at 35°C. PPS + 2% NHS (●); PPS (○); HBSS + 0.1% gelatin + 2% NHS (▲); HBSS + 0.1% gelatin (△); Phosphate buffered saline (□) and saline (■).

min). PPS was therefore used as a suspending fluid in all subsequent experiments.

2. Susceptibility of H. ducreyi to human and rabbit serum.

Figure 8 shows the survival curves of virulent strains 35000, 409, C148 and avirulent strains A77, A75 and A76 incubated in 50% human serum at 35°C. Virulent strains positive in the rabbit intradermal test were resistant to the bactericidal activity of human serum (<50% kill in 120 min). Avirulent strains were very sensitive with no survivors after 120 min incubation in serum.

A concentration-dependent bactericidal effect of serum was observed on the avirulent strains. Serum concentrations above 5% were bactericidal for these strains (Figure 9, data shown for strain A77), whereas the survival of the serum-resistant strains improved in fluids with <50% human serum (Figure 10, data shown for strain 35000). All strains were unaffected by heat-inactivated serum.

The bactericidal effect of normal rabbit serum on H. ducreyi strains was similar to that of human serum (Figure 11).

3. Susceptibility of strains to immune rabbit serum.

The susceptibility of H. ducreyi strains to immune and non-immune rabbit sera was compared. Immune sera from patients with chancroid (which had been properly stored to preserve complement), were not available for serum bactericidal assays. Immune sera raised in rabbits were therefore used in the assays. Figure 12 shows the survival curves of serum-resistant

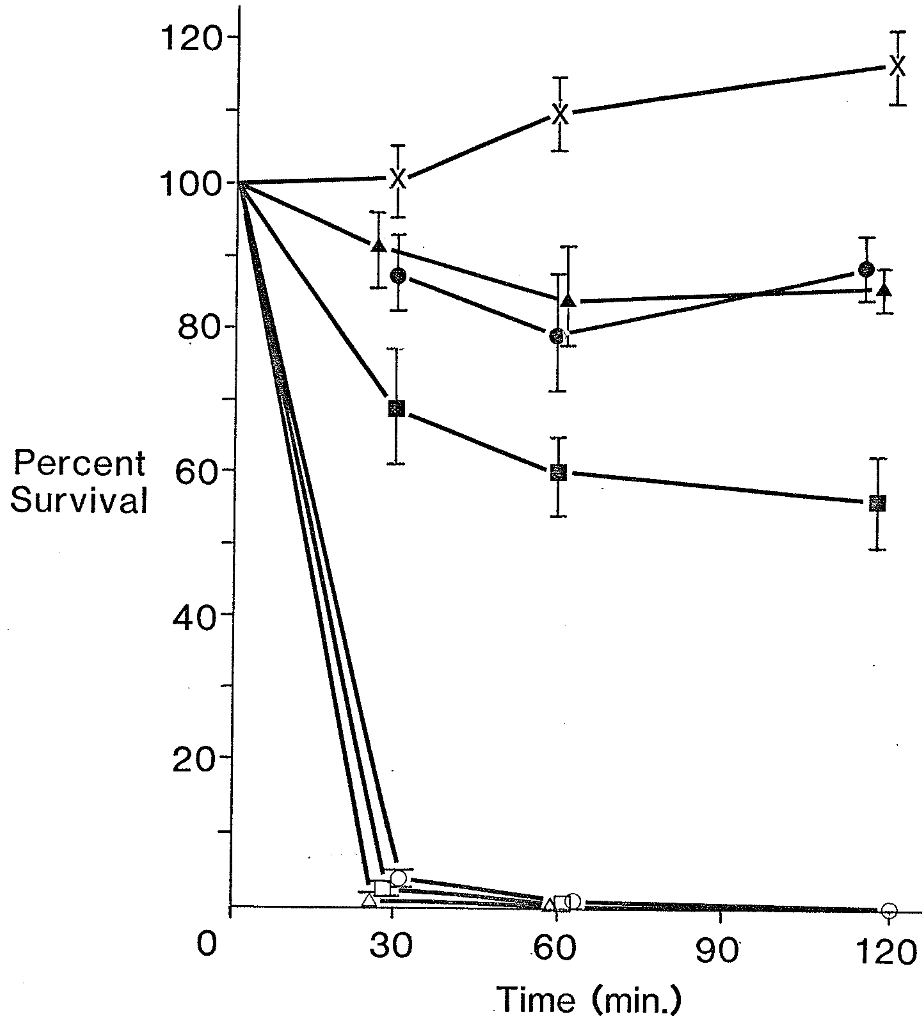

Figure 8: Bactericidal effect of 50% pooled normal human serum on H. ducreyi strains; 35000 (●), 409 (▲), C148 (□), A77 (○), A75 (△), and A76 (□). Heat treatment abolished bactericidal activity of human serum [data shown only for H. ducreyi A76 (X)]. Data points represent the mean ± standard deviations of three separate experiments.

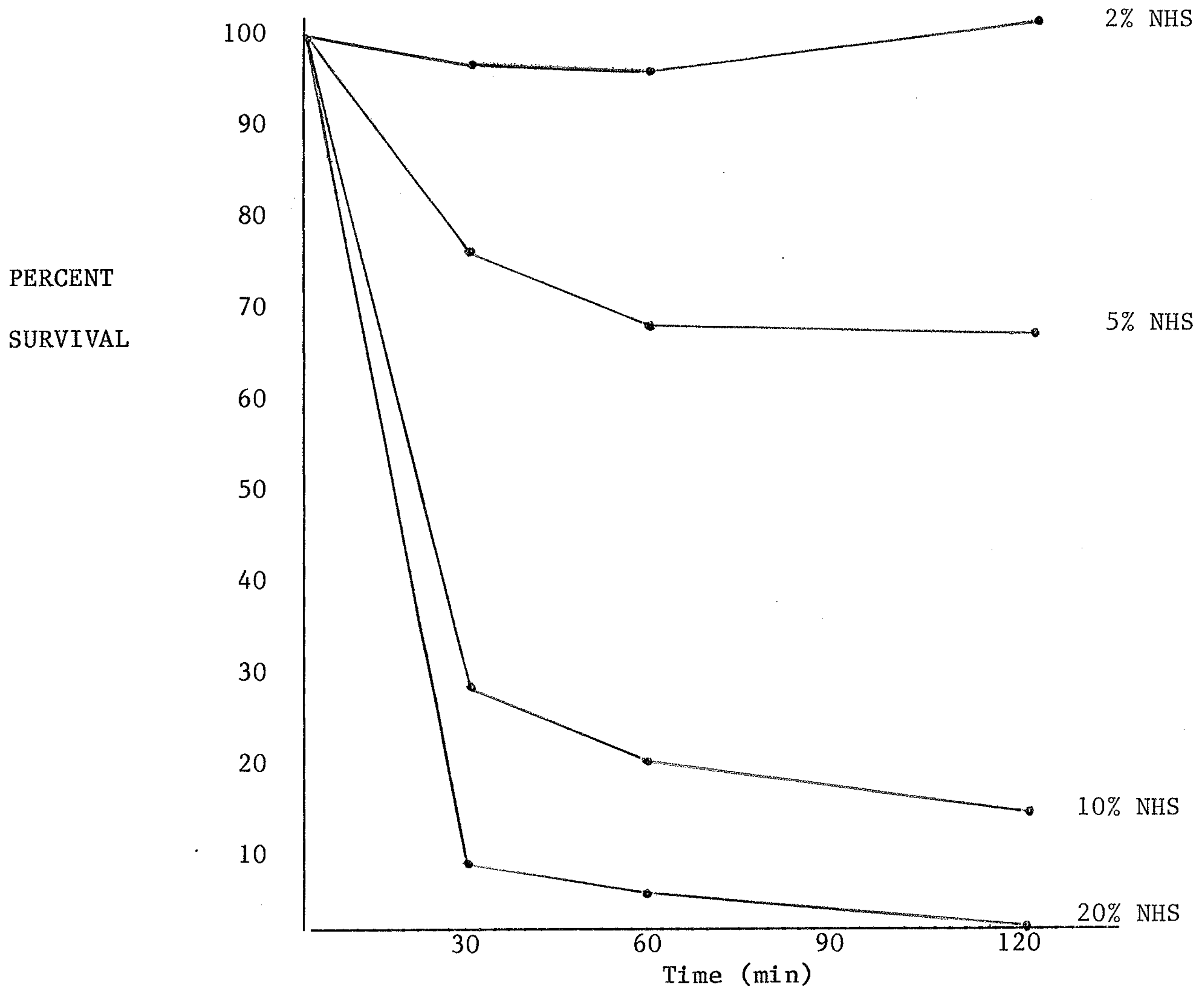

Figure 9: Survival curves of serum-sensitive strain A77 in various concentrations of pooled normal human serum (NHS). Data points represent the mean of two experiments.

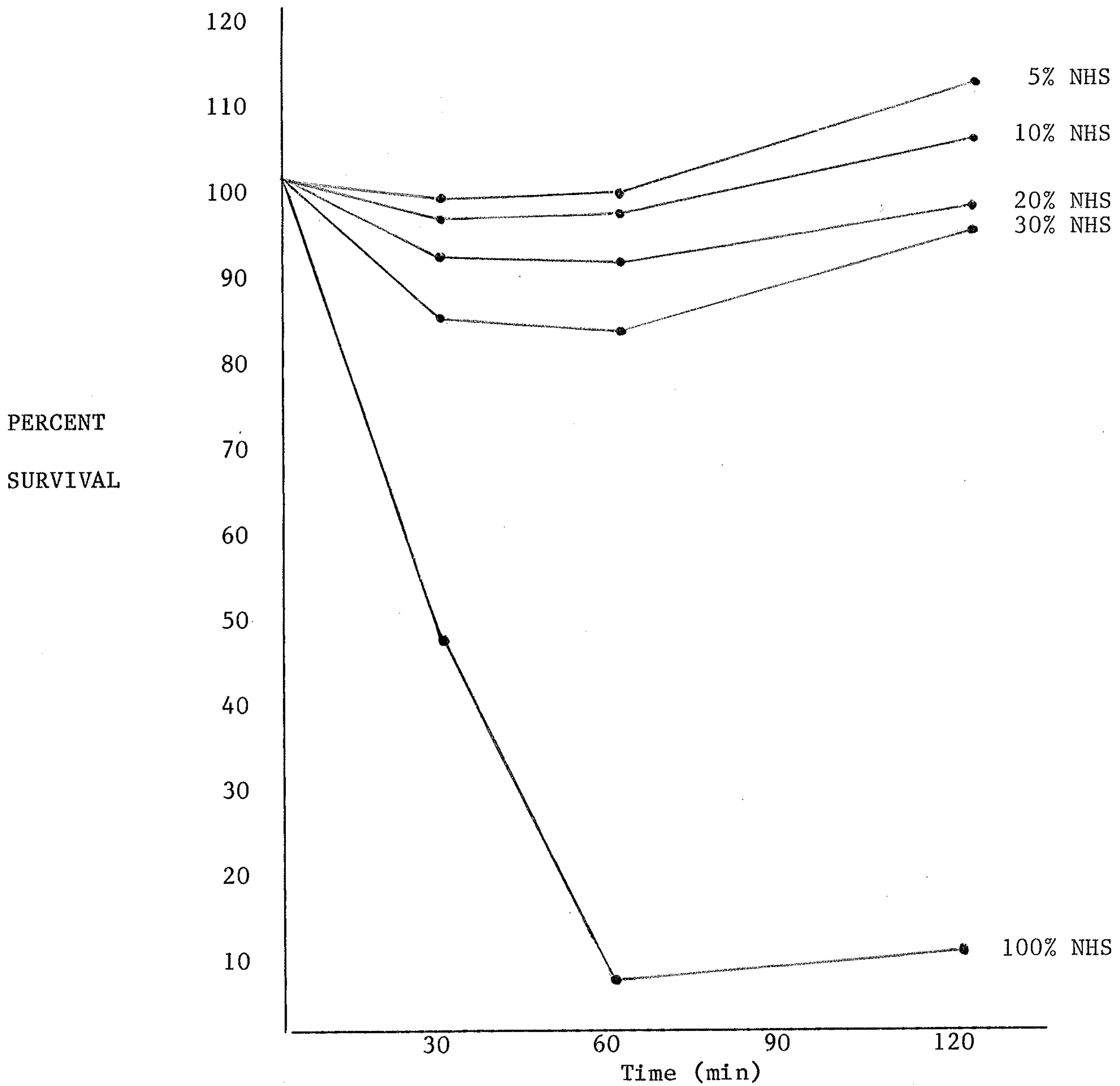

Figure 10: Survival curves of serum-resistant H. ducreyi 35000 in various concentration of pooled NHS. Data points represent the mean of two experiments.

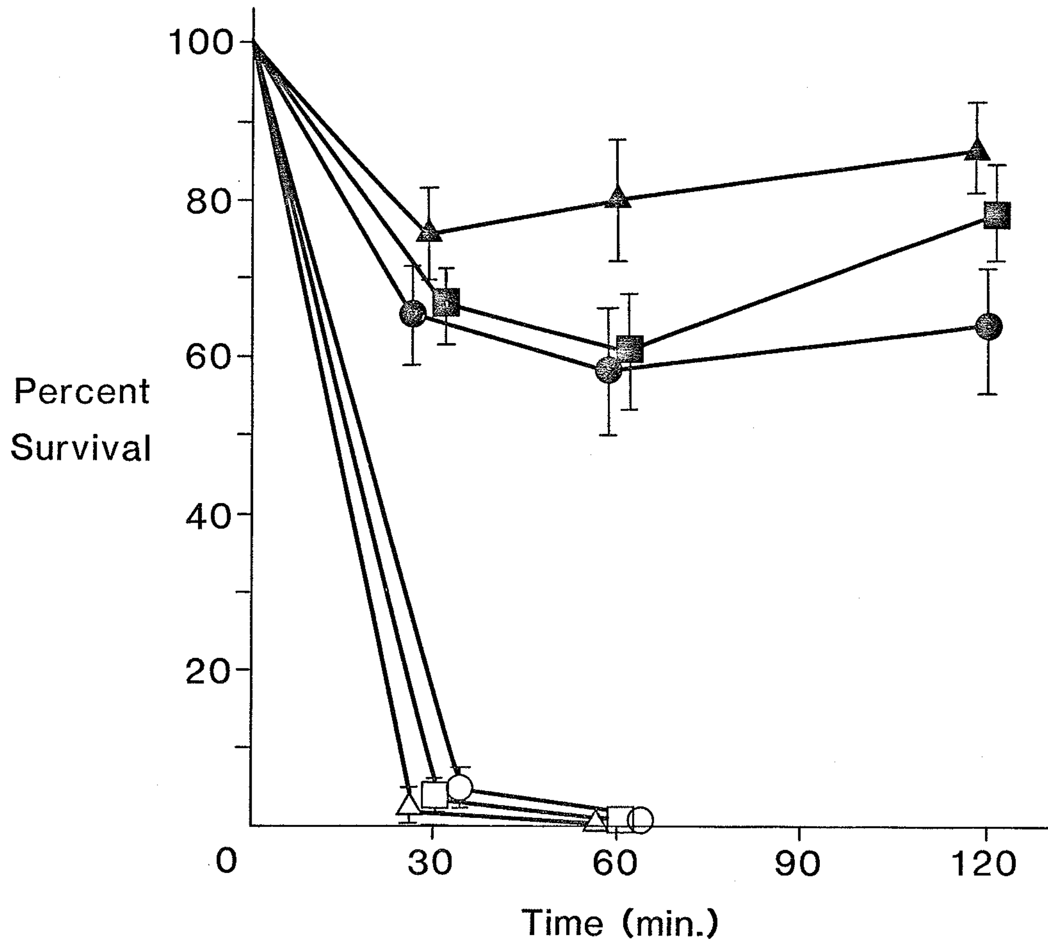

Figure 11: Bactericidal effect of 50% normal rabbit serum on H. ducreyi strains 409 (▲), 35000 (■), C148 (●), A77 (○), A75 (△) and A76 (□). Data points represent the mean ± standard deviation of three experiments.

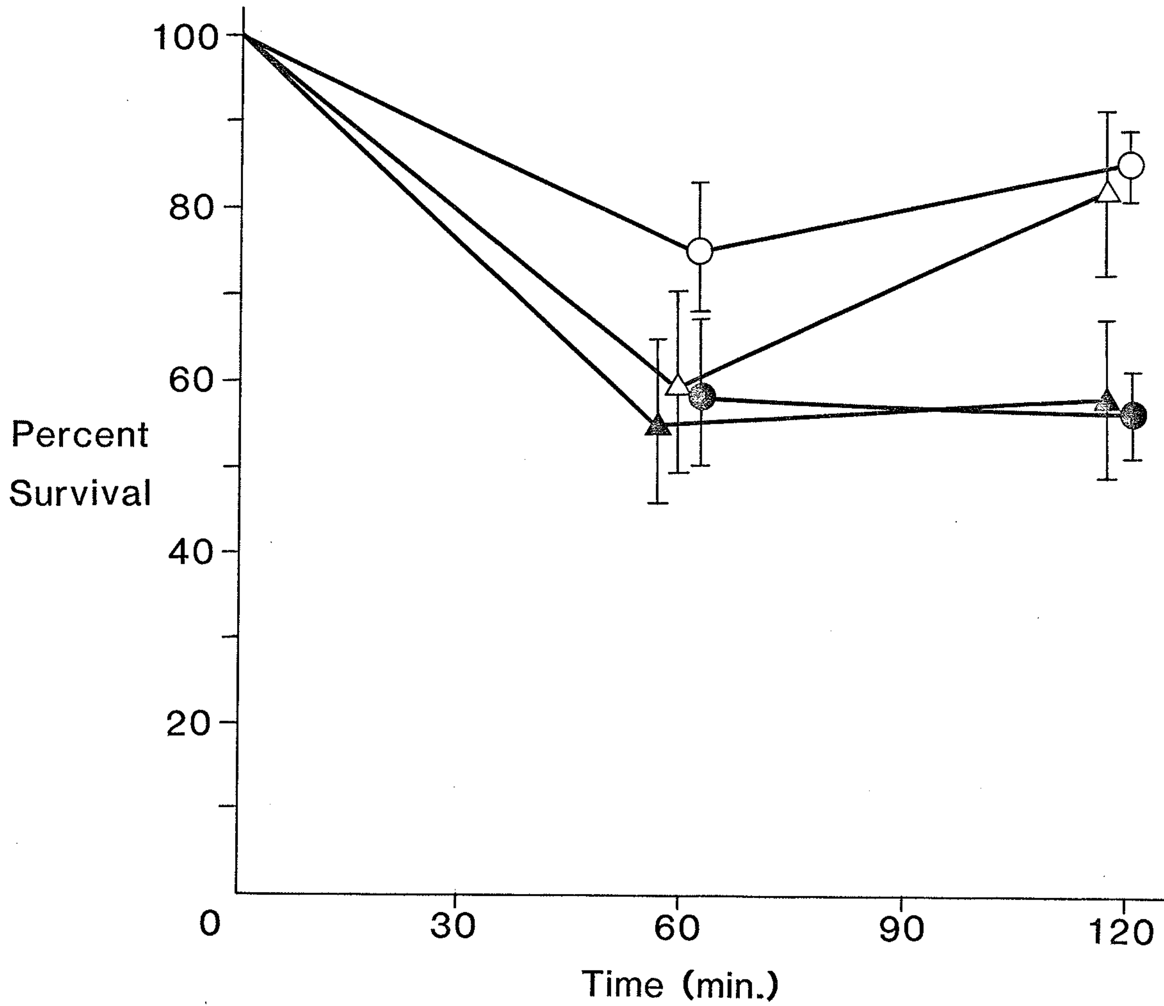

Figure 12: Bactericidal effect of 50% immune rabbit serum on <u>H. ducreyi</u> strains; 409 (●); 35000 (▲); Effect of control non-immune serum on strains 409 (○); 35000 (△).

strains 409 and 35000 in immune and non-immune rabbit sera. The strains were less resistant to immune rabbit sera compared to their resistance to non-immune sera. However, these strains remained resistant to the bactericidal effect of rabbit serum even in the presence of antibodies raised against the organisms.

C. Mechanism of complement (C) activation by *H. ducreyi* strains.

Heating serum at 56°C for 30 min abolished its bactericidal activity against *H. ducreyi* strains. This suggested the involvement of C. As the mechanism of C activation varies depending on the microorganism, the pathway of C activation by serum-sensitive *H. ducreyi* strains was determined. Serum treated with Mg^{2+} EDTA which selectively inhibits the classical C pathway and serum treated with EDTA which inhibits both the classical and the alternative pathway, were non-bactericidal to serum-sensitive *H. ducreyi* strain A77 (Figure 13). The strain also survived well in the control serum heated at 56°C for 30 min to inactivate C. It was however, rapidly killed in untreated serum and in serum pre-treated with inulin or serum heated at 50°C for 20 min (which depleted it of alternative pathway activity). Results obtained with other serum-sensitive strains A76 and A75 were similar to that of strain A77. These data suggest that such killing of *H. ducreyi* strains is mediated by the classical pathway of C activation.

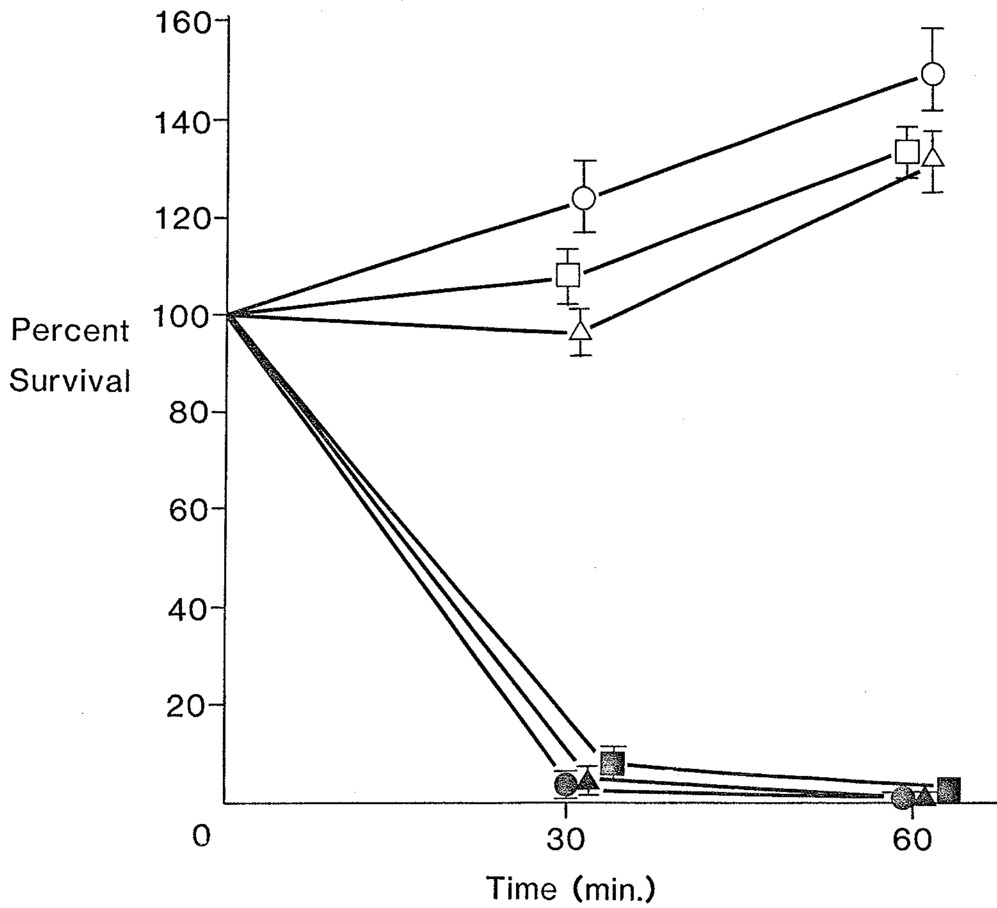

Figure 13: Kinetics of killing of H. ducreyi A77 by 50% human serum. Serum control (●), serum heated at 56°C for 30 min. (○), serum + 20 mM EDTA (□), serum + 20 mM EGTA + 2 mM $MgCl_2$ (△), serum + inulin (2 mg/ml) (▲), serum heated at 50°C, for 20 min. (■).

D. Susceptibility of strains to phagocytosis and killing by human polymorphonuclear leukocytes (PMNL).

Two methods of assessing phagocytosis were used. The first required lysis of the PMNL incubated with bacteria for various time intervals and determination of viable counts. Figure 14 shows the percent survival of each strain after exposure to human PMNL and 2% normal human serum. Serum at this concentration was not bactericidal for any of the strains. Virulent strains 409, 35000 and C148 were more resistant to killing than avirulent strains A76, A75 and A77. Strains were less susceptible to the killing activity of human PMNL when incubated with heat-inactivated serum, an indication that C present in fresh serum is required for optimal phagocytosis of these strains.

The second method of phagocytosis assay required the use of acridine orange and crystal violet, the later quenching fluorescence of organisms not actually ingested by the PMNL. Figures 15a and 15b are photographs of PMNL and H. ducreyi taken at 15 min and 45 min incubation time. Bacteria can be seen in the polymorphs as early as 15 min. Numerous granules were present in the polymorphs at this point, but as more bacteria were ingested, degranulation was observed. This was complete in most polymorphs by 45 min. A time-dependent increase in the number of bacteria found inside the polymorphs was evident. Most virulent and avirulent organisms fluoresce red inside the polymorphs, indicating that H. ducreyi does not survive in PMNL.

A significantly higher percentage of avirulent strains was ingested in relation to virulent strains (Figure 16).

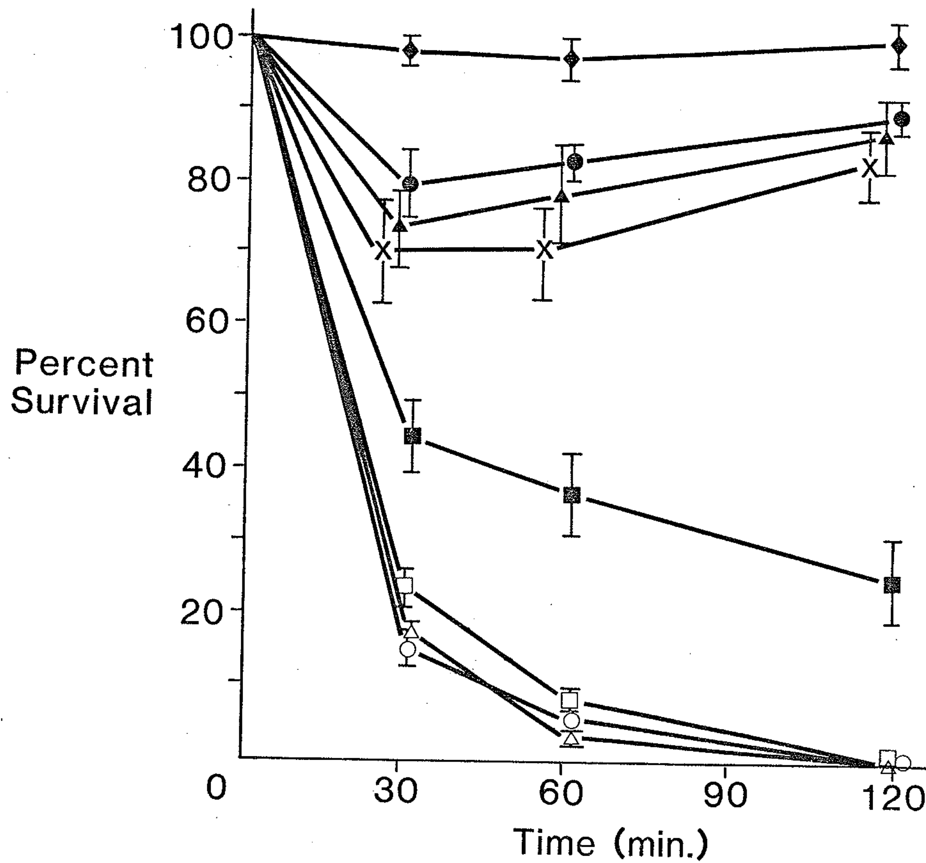

Figure 14: The susceptibility of H. ducreyi strains to bactericidal action of human PMNL. H. ducreyi strains 409 (●), 35000 (▲), C148 (X), A77 (□), A75 (△), and A76 (○), incubated with PMNL + 2% NHS. H. ducreyi strain A77 incubated with PMNL + 2% heated NHS (■). Control: H. ducreyi incubated with 2% NHS only [data shown for H. ducreyi A77 (◆)]. Data points represent the mean ± standard deviation of three separate experiments.

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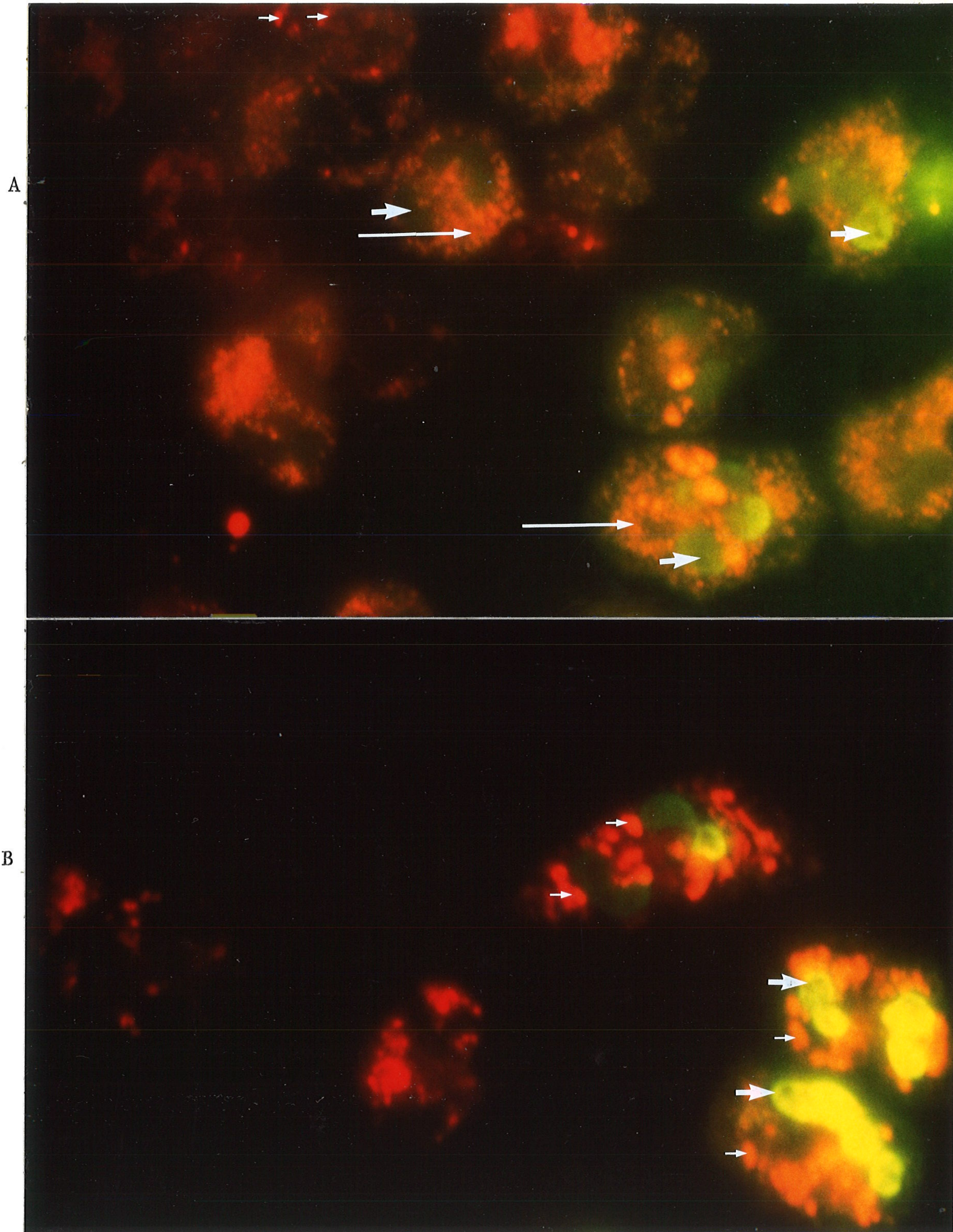
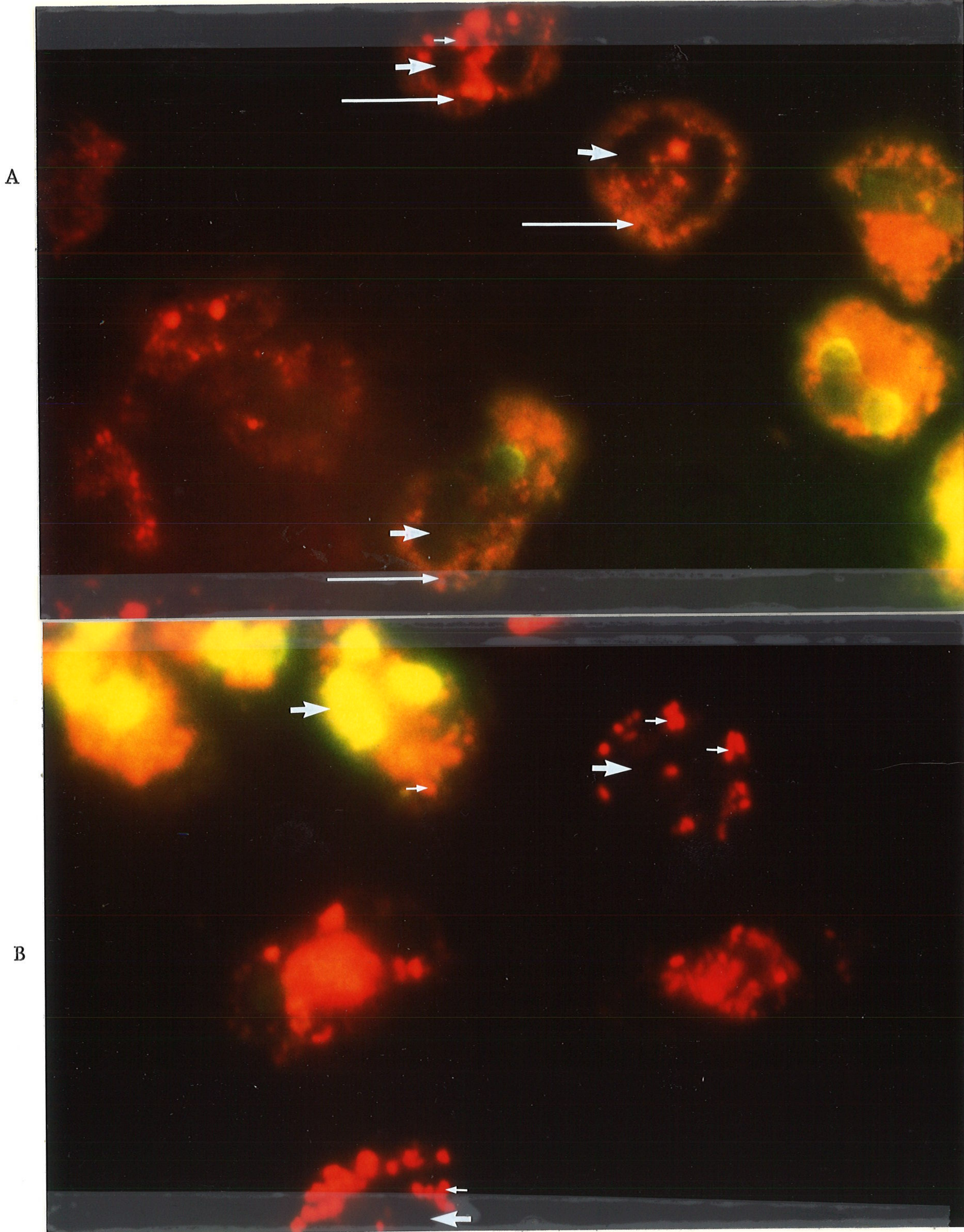


Figure 15a: H. ducreyi A77 incubated with human PMNL at 37°C for A, 15 mins and B, 45 mins. Long arrows indicate cytoplasmic granules and large arrows indicate the nuclei of PMNL. Small arrows indicate ingested bacteria.

A



B

Figure 15b: H. ducreyi 409 incubated with human PMNL at 37°C for A, 15 min and B, 45 min. Long arrows indicate cytoplasmic granules and large arrows indicate the nuclei of PMNL. Small arrows indicate ingested bacteria.

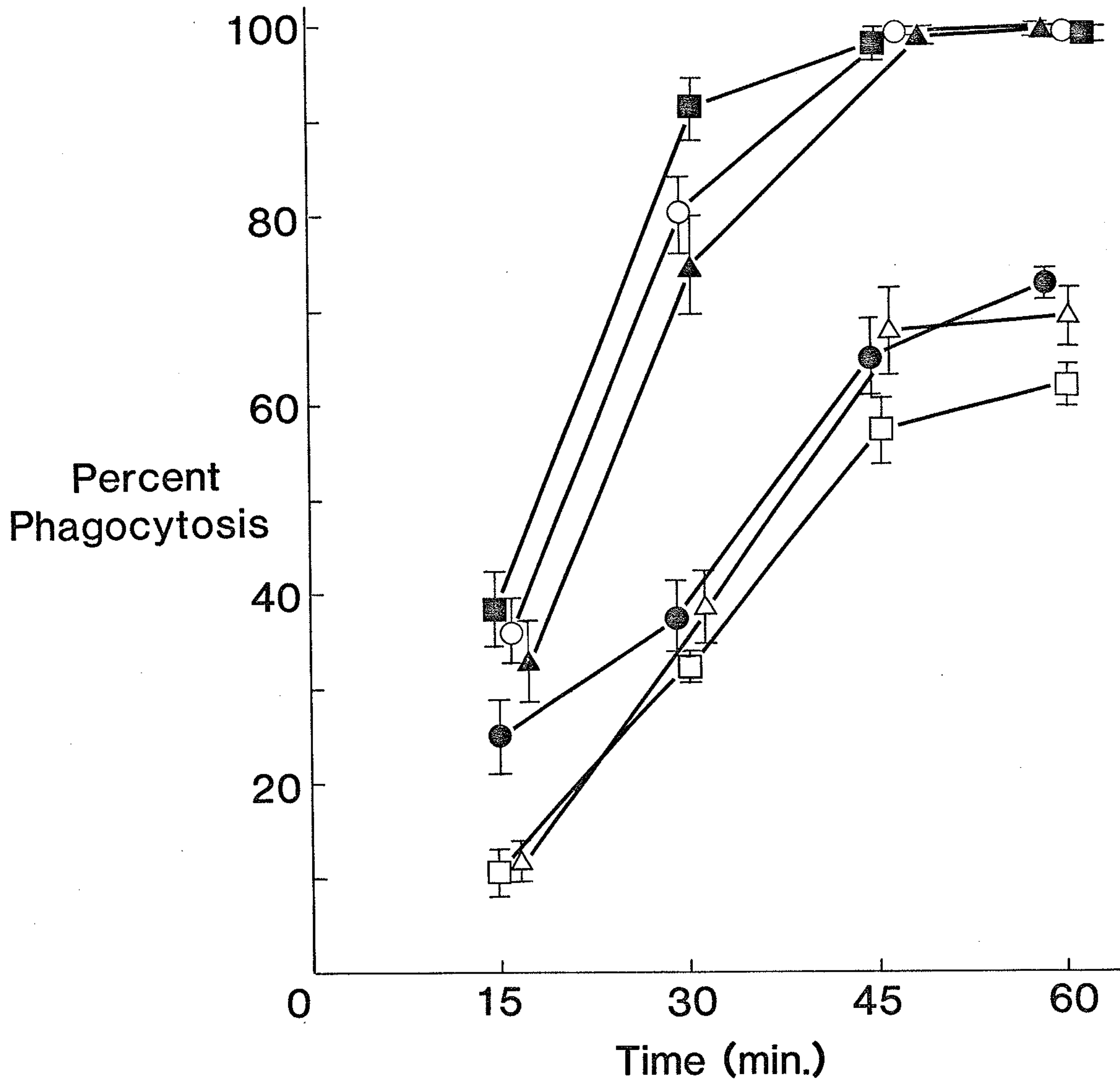


Figure 16: Kinetics of phagocytosis of H. ducreyi strains by human PMNL. H. ducreyi strains 409 (□), C148 (●), 35000 (△), A77 (○), A75 (■), and A76 (▲). Data points represent the mean ± standard deviation of three separate experiments. Polymorphs and bacteria were stained with acridine orange.

PART II - Bacterial factors involved in the virulence of *H. ducreyi*.

A. Relationship between polymyxin resistance and virulence of *H. ducreyi*

Hammond *et al*. (1978) and Bertram (1980) observed a correlation between polymyxin resistance and virulence of *H. ducreyi* as defined by the rabbit intradermal test. The results of the present study confirm this observation (Table 5). The minimum inhibitory concentration (MIC) of polymyxin B for virulent strains was $\geq$32 ug/ml, while the MIC of polymyxin for avirulent strains was <32 ug/ml, except for avirulent strain 36-F-2 with an MIC of 64 ug/ml. In order to determine whether polymyxin resistance is a true virulence marker for *H. ducreyi*, polymyxin-sensitive strains 409^s and $C148^s$ were obtained from polymyxin-resistant parent strains 409 and C148, respectively, as described in Materials and Methods. Strain $C148^r$ lost its resistance to polymyxin when the strain was made resistant to rifampin. These strains were tested for their sensitivity to the bactericidal action of human serum and PMNL and their virulence in rabbits. Results (Table 6) showed that the virulence of these strains for rabbits and their resistance to the bactericidal effect of serum and phagocytosis were unaffected by conversion from polymyxin resistance to polymyxin sensitivity. However, isogenic strains $C148^s$ and $C148^r$ were less resistant to serum and phagocytosis than was the parent strain C148.

In other experiments, polymyxin-resistant strains obtained from polymyxin-sensitive parents by adaptive resistance to polymyxin, remained avirulent for rabbits and susceptible to the bactericidal action of serum and to phagocytosis as did the parent strains (Table 6). These results indicate that resistance to polymyxin and virulence can be dissociated.

Table 5. Virulence of H. ducreyi and resistance to polymyxin B.

Strain	Rabbit Intradermal Test[a]	MIC of Polymyxin B ug/ml
35000	+	>128
409	+	>128
5439	+	>128
3019	+	>128
54211	+	>128
78118	+	>128
557	+	>128
78226	+	>128
C148	+	>128
BG411	+	32
54213	+	>128
36-F-2	0	64
A77	0	1
A75	0	0.5
A76	0	0.5
078	0	8

[a] Central reaction based on the presence of hard central eschar at 11 days: +, eschar present; 0, eschar absent.

Table 6. Relationship between polymyxin resistance and virulence of *H. ducreyi* strains.

Strain[a]	MIC (ug/ml) of polymyxin	Rabbit Intradermal Test	% Survival[b] after 2h incubation in:	
			NHS	PMNL + 2% NHS
409*	>128	+	86.7 ± 2.8	90.8 ± 2.3
409^{s}	<8	+	82.4 ± 3.2	88.7 ± 2.9
$C148^{*}$	>128	+	57.1 ± 4.1	84.6 ± 4.9
$C148^{s}$	<16	+	47.5 ± 2.8	76.9 ± 3.5
$C148^{r}$	1	+	40.2 ± 4.6	84.8 ± 4.1
$A77^{*}$	1	-	0	0
$A77^{p}$	250	-	0	0
$A75^{*}$	0.5	-	0	0
$A75^{p}$	250	-	0	0
078^{*}	8	-	3.2 ± 1.5	7.2 ± 1.6
078^{p}	250	-	2.8 ± 1.2	6.8 ± 2.9

[a] Strains with ≥50% survival in 50% NHS or in PMNL + 2% NHS after 2h of incubation were considered resistant. Intermediate resistance was 10 to 49% survival. Strains with <10% survival were considered susceptible. *, parent strains; p, polymyxin-resistant strains; s, polymyxin-sensitive strains; r, rifampin-resistant strain.

[b] Average of three separate experiments ± standard deviation.

B. Outer membrane protein (OMP) and lipopolysaccharide (LPS) composition of H. ducreyi.

Preliminary data (Figure 17) on serum absorption experiments with heat-killed whole cells of H. ducreyi suggest that heat-stable envelope components are involved in serum susceptibility of these strains. Absorption of serum with whole cells of strain A77 removed bactericidal activity against serum-sensitive strains A77, A75 and A76 (data shown for strain A77) all of which are avirulent. Similar results were obtained with strain A75 as the absorbing isolate. Absorption of serum with virulent serum-resistant strain 409 or serum-resistant isogenic strain A77* resulted in partial reduction in the bactericidal activity against strain A77, suggesting that these strains differ in the heat-stable cell components involved in the serum bactericidal reaction. Absorbed sera had $\geq$70% complement activity in relation to unabsorbed samples.

Absorption of serum with a serum-sensitive H. influenzae NCTC 8143 also inhibited serum-killing of H. ducreyi (data not shown).

1. SDS-PAGE analysis of OMP.

Sarcosinate-insoluble OMP composition of virulent and avirulent strains of H. ducreyi was analysed by SDS-PAGE in order to determine whether there is a relationship between OMP composition of these strains and virulence. The OMP profiles of virulent strains in Figure 18 lanes A,B,C and K are heterogenous, but differ from those of avirulent strains in lanes D,E,F, H and J which appear similar. A 47,000 molecular weight protein indicated by the short arrow is present in all avirulent strains but absent in the

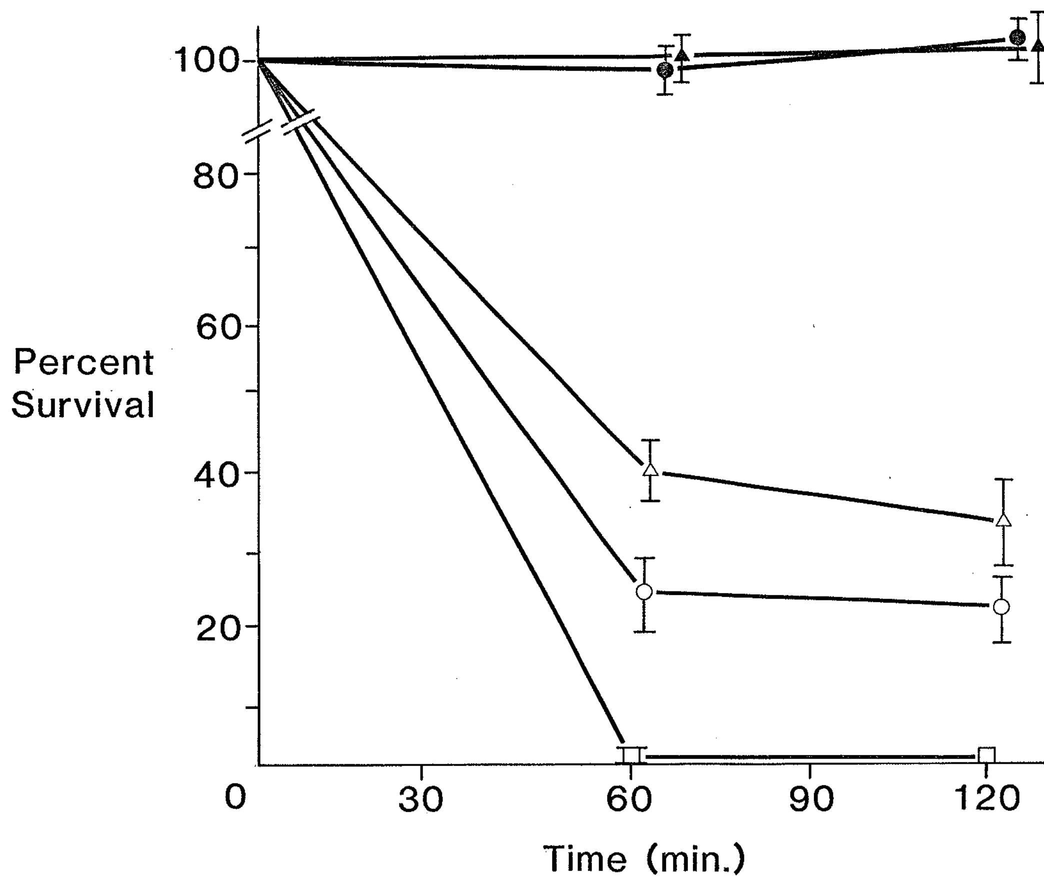


Figure 17: Bactericidal activity of 50% human serum on H. ducreyi A77 after absorption with heat-killed whole cells of strains A75 (●), A77 (▲), A77* (△), 409 (○), and proteose peptone saline (□) control.

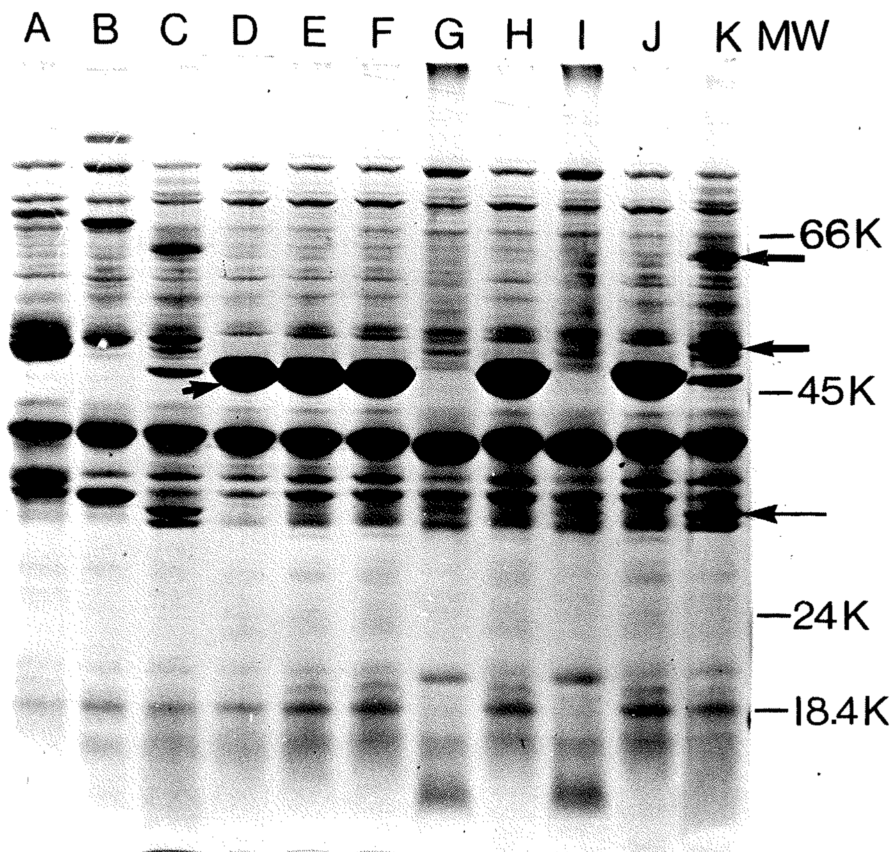


Figure 18: Electrophoretic profiles of the OMP of H. ducreyi. Virulent strains: lanes A, 409; B, BG411; C, 35000; K, A77*. Avirulent strains: lanes D, 36-F-2; E, 078; F, A76; G, A75P, H, A75; I, A77P; J, A77. Arrows indicate 62,000, 50,000 and 30,000 molecular weight proteins in isogenic strain A77*.

protein profiles of virulent strains 409 (lane A) and BG411 (lane B), and found in small amounts in virulent strains 35000 (lane C) and A77* (lane K). Although this 47,000 molecular weight protein is found mostly in the avirulent strains, the loss of this protein as a result of adaptive resistance to polymyxin as demonstrated by strains $A75^p$ (lane G) and $A77^p$ (lane I) did not result in acquisition of serum resistance or virulence (Table 6). All H. ducreyi examined share a 40,000 molecular weight OMP in common. This protein can be regarded as the principal outer membrane protein (POMP) of H. ducreyi.

Strain $A77^*$ (lane K), is a serum-resistant isogenic strain obtained by passage of strain A77 (lane J) in the presence of increasing concentrations of serum, until a 70% survival rate was reached. This isogenic strain was positive in the rabbit intradermal test and was considered virulent. The OMP profiles of these isogenic strains differ. Acquisition of serum resistance was accompanied by the appearance of proteins indicated by the arrows (lane K) and a reduction in the 47,000 molecular weight protein. Two of these newly acquired proteins (62,000 and 30,000 molecular weights) are absent in virulent strains 409, lane A, and BG411, lane B. The third protein (50,000 molecular weight) is present in the avirulent strains. Figure 19 is an expanded version of the OMP profiles of serum-resistant isogenic strain A77* (lane A) and serum-sensitive parent strain (lane B).

2. SDS-PAGE analysis of LPS.

The LPS composition of virulent and avirulent strains was analysed by SDS-PAGE and a silver staining technique. Two methods of LPS isolation were used, the rapid isolation method (RIM) described by Inzana (1983), and

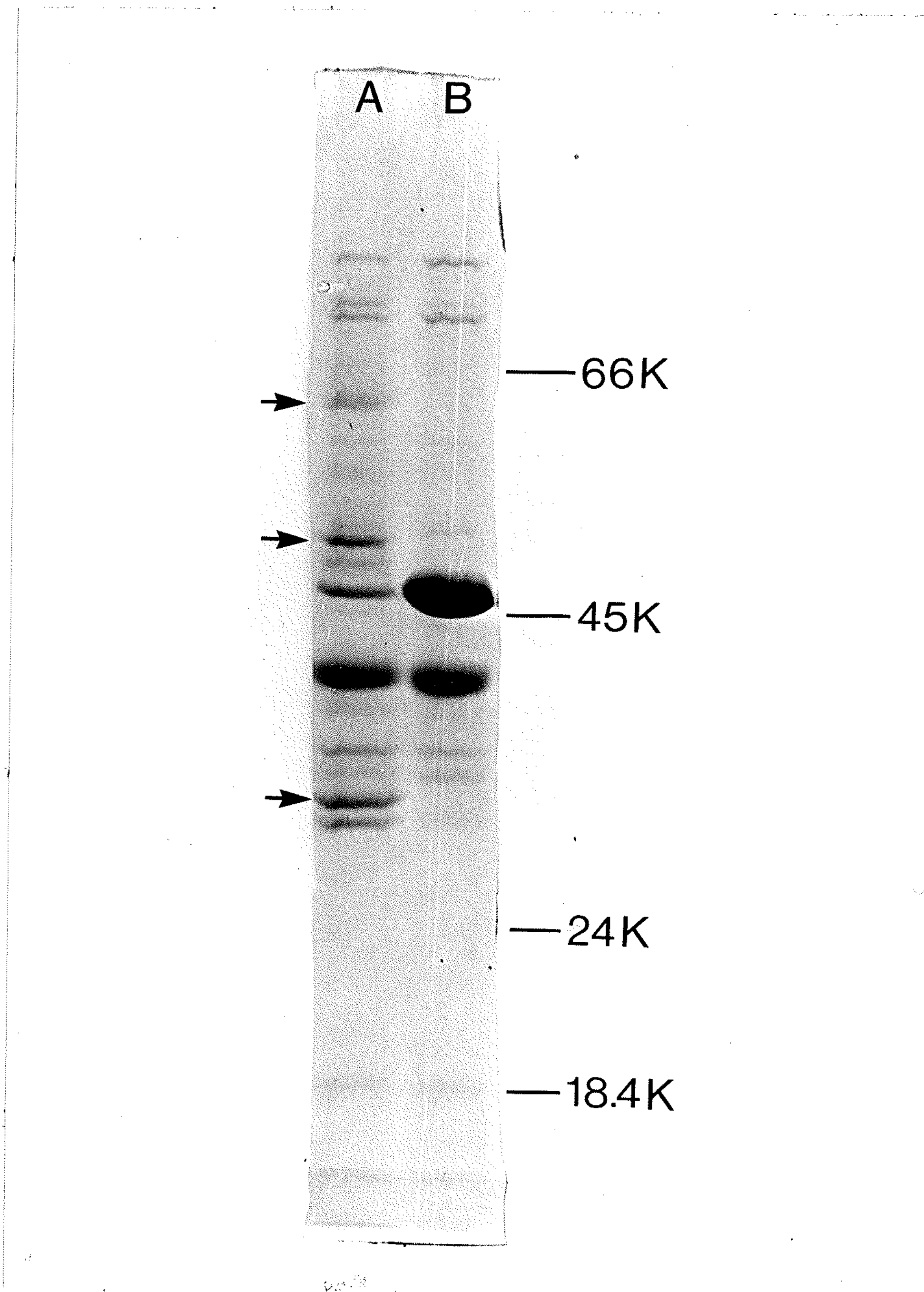


Figure 19: Electrophoretic profiles of the OMP of H. ducreyi. Lane A, serum-resistant isogenic strain A77* and lane B, serum-sensitive parent strain A77. Arrows indicate newly acquired proteins by strain A77*.

the hot phenol-water procedure described by Westphal and Jann (1965). The electrophoretic mobility of the LPS obtained by these methods was similar (Figure 20). The RIM required only 4 ml of 10^9 CFU H. ducreyi as opposed to the Westphal method which requires 10-18g (wet-weight) of cells. The RIM LPS is a crude preparation containing large amounts of nucleic acids and traces of phenol, but this preparation was found suitable for LPS analysis by SDS-PAGE. LPS obtained by the Westphal method is a purified preparation suitable for chemical analysis. About 60-100 mg of LPS per 18g (wet-weight) of cells was obtained by this method.

The electrophoretic profiles of the LPS of virulent and avirulent strains of H. ducreyi are shown in Figure 21. Those of avirulent strains in lanes A-D are similar and differ from those of virulent strains in lanes E-K which appear heterogenous. About 3-6 LPS bands can be seen in these profiles. The LPS profile of serum-resistant isogenic strain A77* in lane E differs from that of the serum-sensitive parent strain A77 in lane D, and appears similar to the LPS profiles of serum-resistant virulent strains 35000 and 409 (lanes F and K). This strain (A77*) was positive in the rabbit intradermal test and therefore considered virulent. Thus acquisition of serum resistance and virulence by this strain was accompanied by alteration in its LPS composition. The LPS profiles were stained with coomasie blue stain, and no proteins were detected.

In an attempt to determine whether strains with similar LPS profiles also had similar OMP composition, the LPS profiles of strains which had been shown to belong to the same OMP subtypes by Odumeru et al. (1981), were compared. Some of the strains with similar OMP profiles had similar LPS profiles as demonstrated by paired strains in lanes C, D, G, H, I and J (Figure 22), while paired strains in lanes A, B, E and F differed.

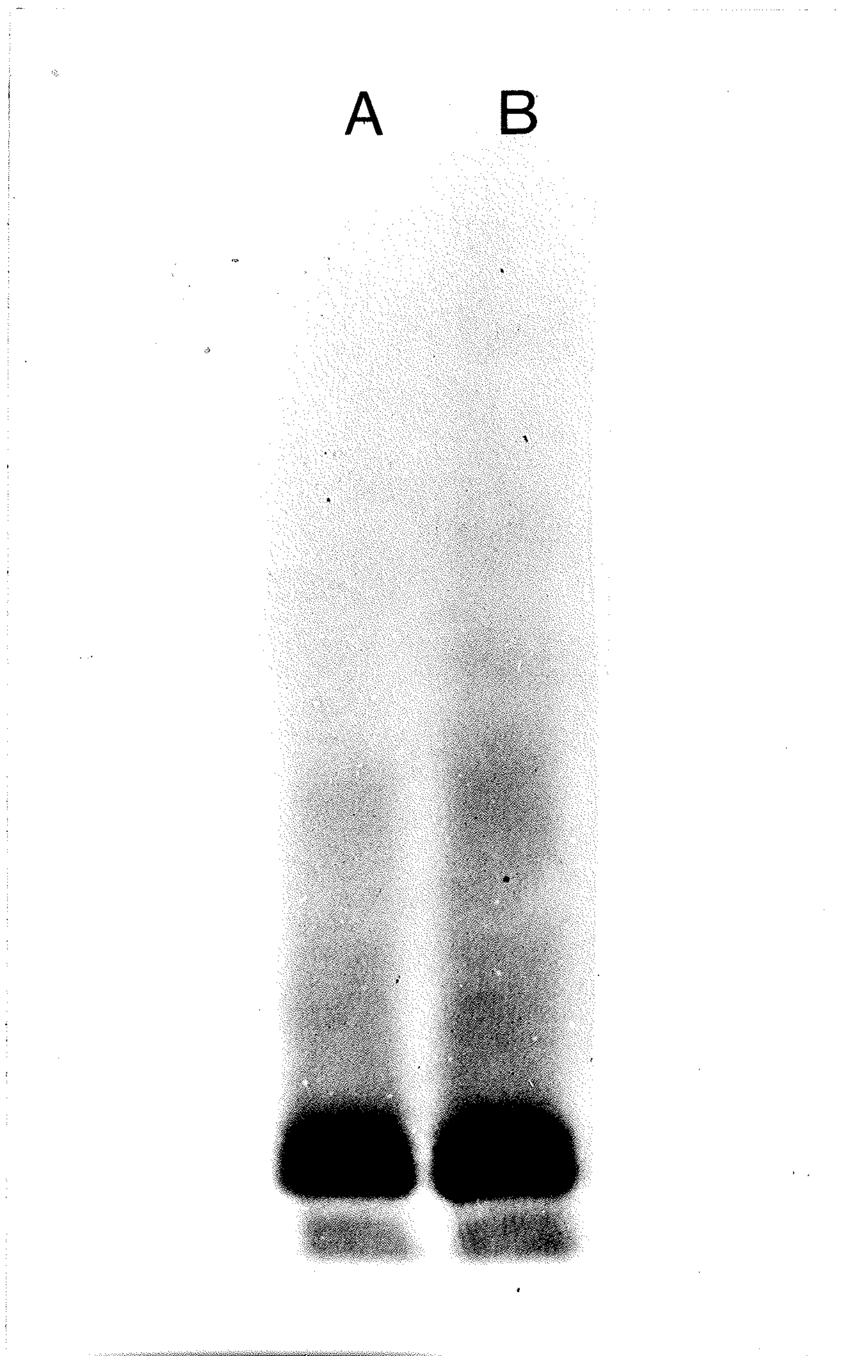


Figure 20: Electrophoretic profiles of H. ducreyi LPS isolated by Westphal method or by rapid isolation method (RIM). Lanes A, 5 ul of RIM LPS; B, 2.5 ug of LPS purified by Westphal method.

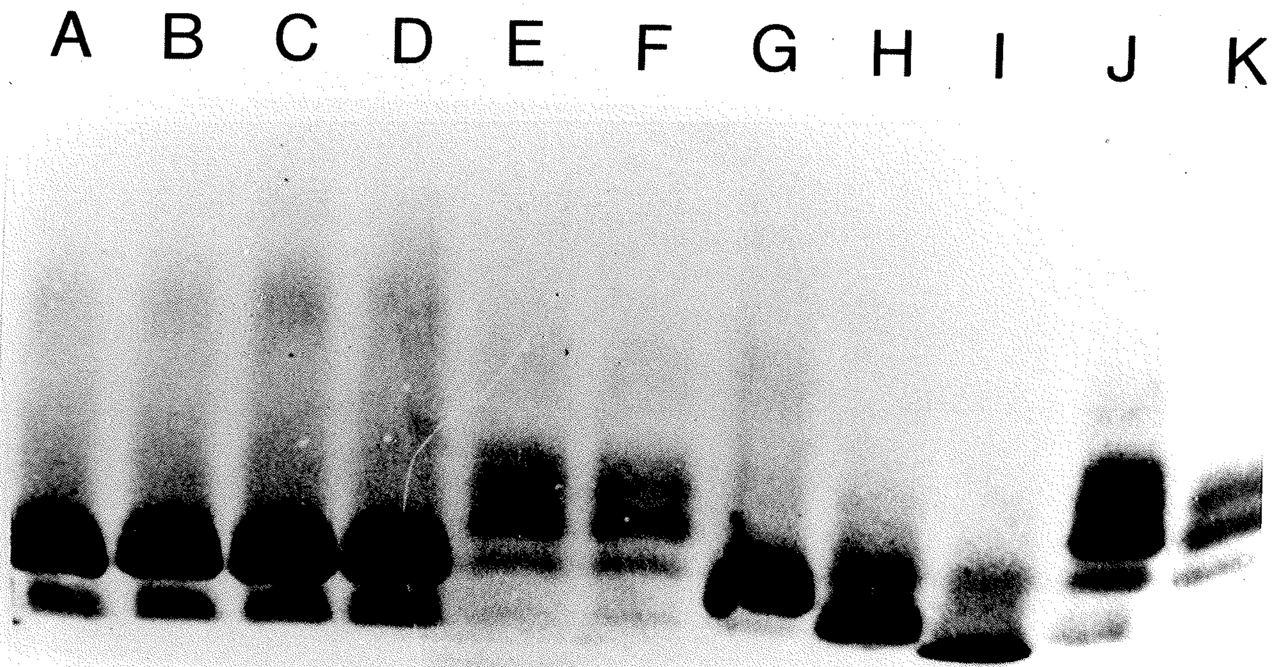


Figure 21: Electrophoretic profiles of the LPS of H. ducreyi. Avirulent strains: lanes A, 36-F-2; B, A76; C, A75; D, A77. Virulent strains: lanes E, A77*; F, 35000; G, 557; H, CH39; I, BG411; J, 78226; K, 409.

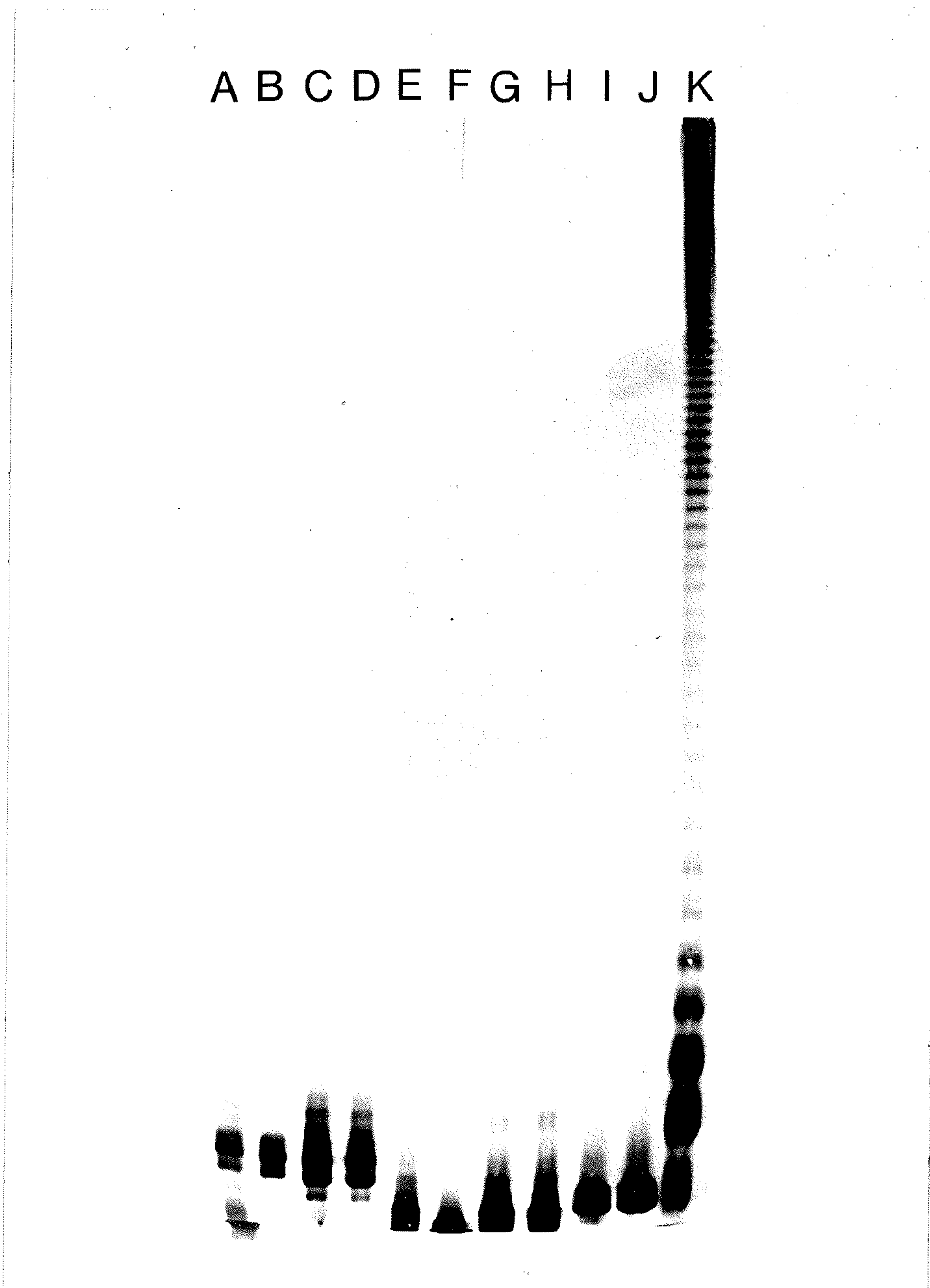


Figure 22: LPS profiles of H. ducreyi and S. typhimurium. H. ducreyi strains: lanes A, C148; B, 409; C, 78226; D, CIP542; E, CH39; F, BG411; G, V1159; H, V1157; I, 557; J, C147. S. typhimurium: lane K.

Also some strains with different OMP composition have similar LPS patterns as demonstrated by strains 35000, lane F and strain 409, lane K (Figure 21). Thus there is no absolute correlation between the OMP and LPS composition of H. ducreyi strains.

In lane K (Figure 22) is the LPS profile of a smooth strain of S. typhimurium containing a "ladder" of closely spaced high molecular weight LPS bands. These bands, characteristic of smooth strains of Salmonella spp. and E. coli, were not found in LPS of H. ducreyi strains, prepared either by the RIM method or the phenol-water method.

3. Physicochemical composition of H. ducreyi LPS.

(a) Infrared (IR) Spectra.

The IR spectra of four H. ducreyi LPS examined were identical and are similar to that of E. coli $0111B_4$ and N. gonorrhoeae P^-6472 (Figure 23). Strains 35000, 409 and A77* are virulent and strain A77 is avirulent. The spectra contained broad absorption peaks at $3350cm^{-1}$ attributed to hydroxyl radicals characteristic of carbohydrates. Absorption bands at $1650cm^{-1}$ and 1510 to $1550cm^{-1}$ are due to monosubstituted amide groups ($-NH.CO.CH_3$). The presence of an ester carbonyl band at 1720 cm^{-1} to 1200 cm^{-1} characterizes the LPS preparation as containing fatty acid esters. IR scans of H. ducreyi LPS lack the sharp absorption bands at 1275 cm^{-1} which are present in E. coli. The overall characteristics of the spectra of the four H. ducreyi LPS indicate that their composition is qualitatively alike and similar to that found in other gram-negative bacteria such as the E. coli reference strain and N. gonorrhoeae p^-6472.

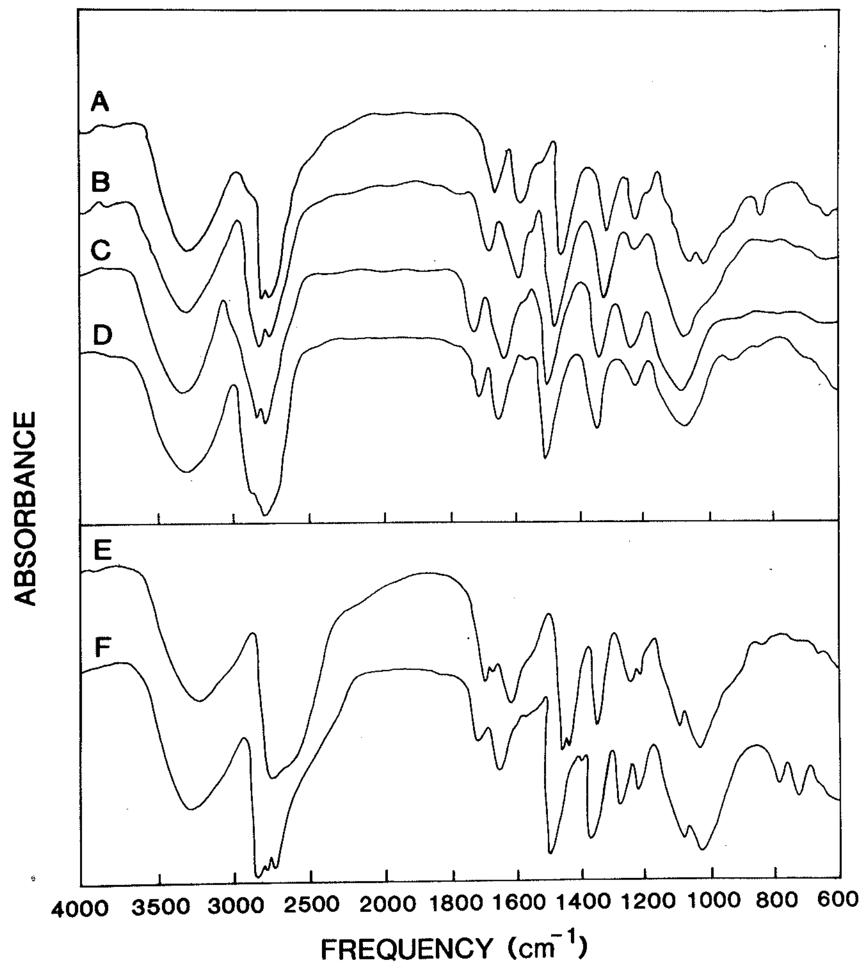


Figure 23: Infrared spectra of the LPS of H. ducreyi strains: A77* (A), A77 (B), 35000 (C), 409 (D); N. gonorrhoeae P^{-}6472 (E); E. coli 0111B$_4$ (F).

(b) Chemical analysis of LPS.

The chemical composition of preparations from eight H. ducreyi strains (four virulent and four avirulent) was determined. The LPS of E. coli 0111B$_4$ and N. gonorrhoeae p$^-$6472 were included for comparison. Qualitatively, the major components of all LPS preparations examined were similar (Table 7). These LPS preparations were characterized by a nucleic acid content of <1% and a protein content of 2% or less. The total carbohydrate, hexose, and NANA contents of virulent strains were relatively higher than those of the avirulent strains in contrast with lipid A content which was the reverse. Other LPS components varied among strains and there was no correlation between the amount of these and virulence.

The concentration of the various components of the LPS of E. coli 0111B$_4$ was much higher than those of H. ducreyi strains and N. gonorrhoeae P$^-$6472, except for lipid A, NANA and phosphate content of E. coli which was lower than that of H. ducreyi.

The KDO content of the LPS of H. ducreyi strains and N. gonorrhoeae P$^-$6472 was less than 1% when LPS was hydrolysed with 0.2N H_2SO_4 (Table 8). Hydrolysis of LPS with 4N HCl released more KDO from the LPS in amounts similar to that of E. coli 0111B$_4$. The amount of KDO released from N. gonorrhoeae LPS by this method was substantially higher than from E. coli and H. ducreyi.

(c) Identification of glycoses.

Qualitative and quantitative analysis of the trimethysilyated (TMS) derivatives of the glycoses of the LPS of H. ducreyi strains, E. coli 0111B$_4$ and N. gonorrhoeae P$^-$6472 were performed by GLC. Those identified were glucose, galactose, glucosamine, galactosamine, fucose and ribose (Figure 24a-c). Mannose and rhamnose were not detected in the H. ducreyi

Table 7. Chemical composition of lipopolysaccharides isolated from H. ducreyi, E. coli, and non-pilated N. gonorrhoeae.

	[a] Percentage (dry wt) of lipopolysaccharide								
Organism	Total Carbohydrate	Total Hexose	Hexosamine	Heptose	Protein	Nucleic Acid	Phosphate	[b]NANA	Lipid A
H. ducreyi									
Virulent:									
35000	25.6	20.3	7.3	7.1	2.0	0.3	4.5	3.7	41.3
409	26.8	20.0	5.4	6.5	1.8	0.4	4.8	3.7	32.6
C148	24.5	16.8	5.5	5.2	1.7	0.5	4.7	2.7	35.8
A77*	25.9	19.7	6.3	7.8	1.9	0.8	4.6	3.1	32.5
Avirulent:									
A77	20.7	11.8	5.8	8.0	1.8	0.3	4.8	2.8	47.5
A75	22.8	13.5	7.0	7.4	0.9	0.3	5.1	1.2	43.6
36-F-2	19.0	13.0	4.1	8.0	1.1	0.2	4.8	1.2	42.5
A76	20.3	12.6	5.6	7.8	1.3	0.3	5.3	2.2	48.5
E. coli									
$0111{:}B_4$	44.5	34.7	23.3	12.5	0.8	0.9	3.5	0.6	25.1
N. gonorrhoeae									
$P^{-}6472$	30.0	21.8	5.5	4.5	2.1	0.6	4.3	0.9	15.5

[a] Mean of three separate determinations.

[b] N-acetylneuraminic acid.

Table 8. KDO[a] content of LPS preparations from H. ducreyi, E. coli, and N. gonorrhoeae.

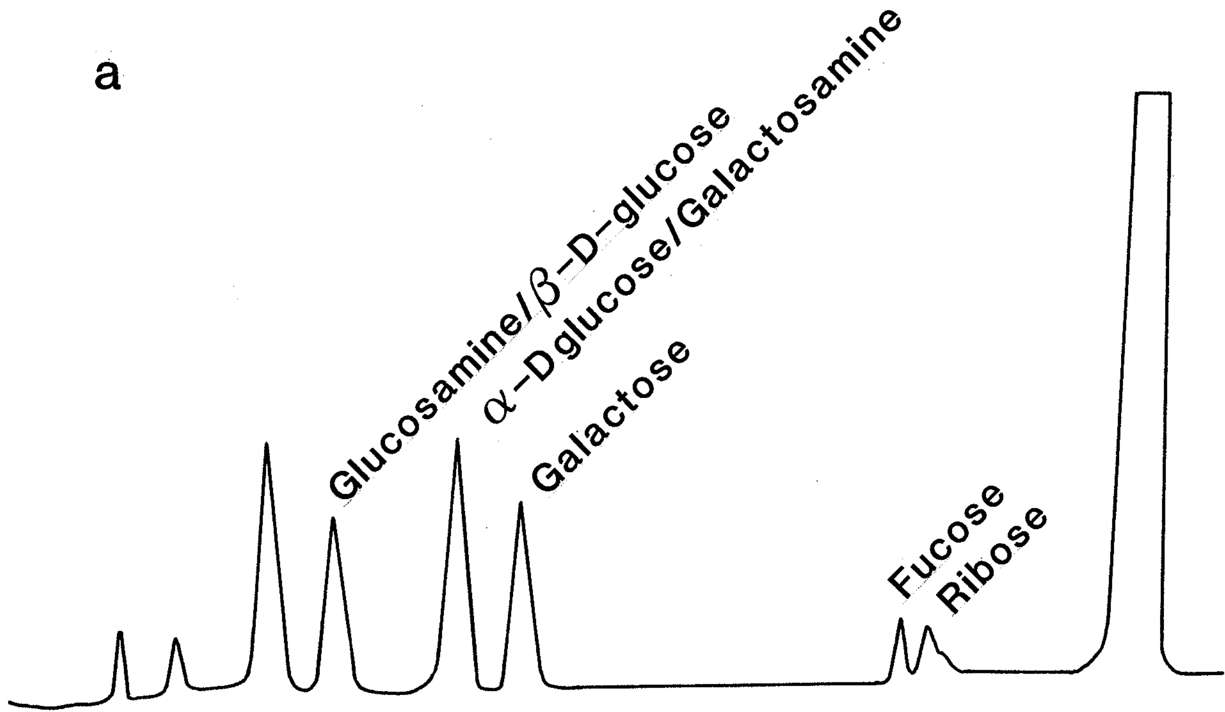
Strain	Karkhanis Method[b]		Brade Method[c]	
	Percent KDO	Ratio Glycose:KDO	Percent KDO	Ratio Glycose:KDO
H. ducreyi				
Virulent:				
35000	0.7[d]	2.8	4.0	0.49
409	0.6	3.7	4.0	0.57
C148	0.4	3.5	3.8	0.37
A77*	0.7	3.1	4.4	0.50
Avirulent:				
A77	0.7	1.3	4.4	0.20
A75	0.6	1.9	4.3	0.27
36-F-2	0.6	1.6	3.8	0.31
A76	0.6	1.9	4.2	0.23
E. coli				
$0111B_4$	3.4	0.9	4.3	0.71
N. gonorrhoeae				
P 6472	0.9	1.8	7.6	0.21

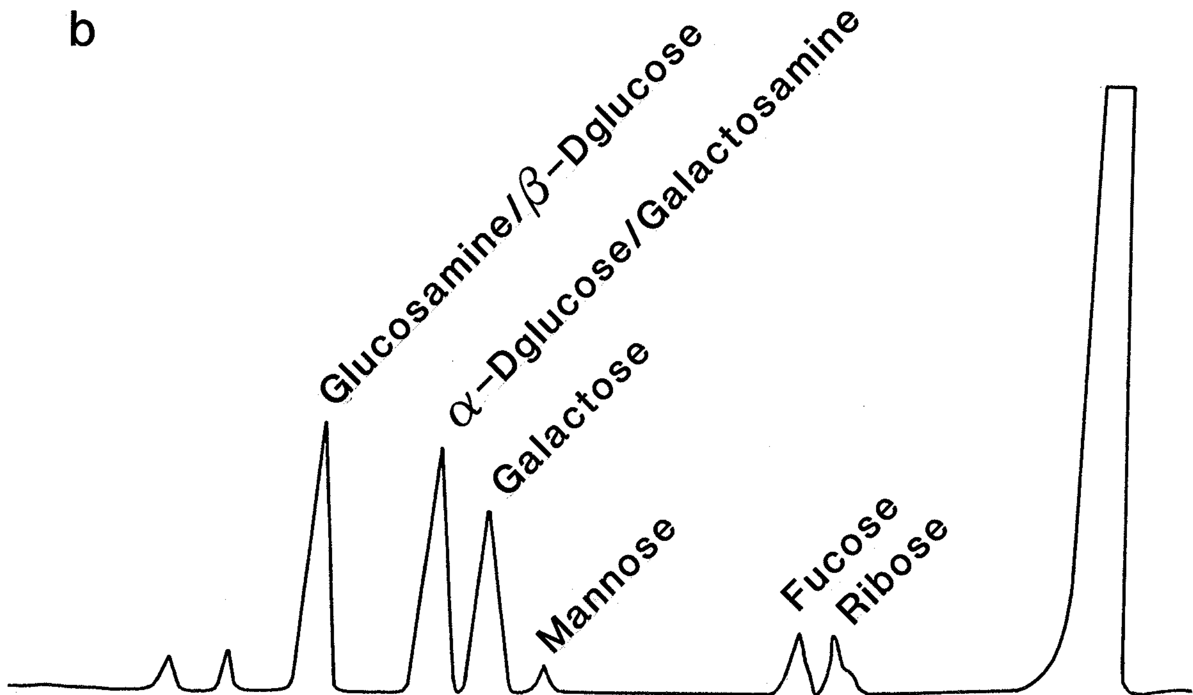
[a] 2-Keto-3-deoxyoctulosonic acid.

[b] KDO released by 0.2N H_2SO_4.

[c] KDO released by 4N HCl.

[d] Mean of three separate determinations.


a
Glucosamine/β-D-glucose
α-Dglucose/Galactosamine
Galactose
Fucose
Ribose


b
Glucosamine/β-Dglucose
α-Dglucose/Galactosamine
Galactose
Mannose
Fucose
Ribose

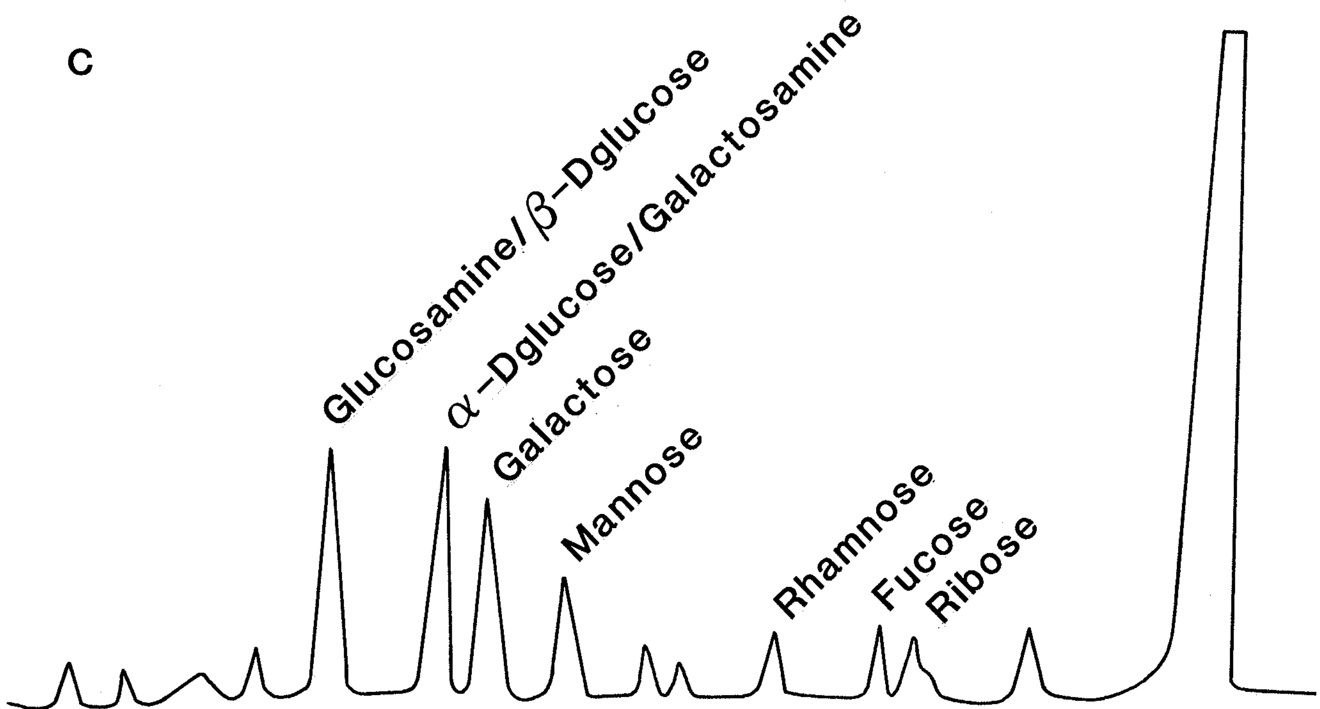


Figure 24: Schematic representation of gas liquid chromatogram of trimethysilyl (TMS) derivatives of glycoses obtained after hydrolysis of LPS of a) H. ducreyi strains, b) N. gonorrhoeae P^{-}6472 c) E. coli $0111B_4$.

LPS (Figure 24a). Rhamnose was also absent in the LPS of N. gonorrhoeae (Figure 24b). These sugars were identified in the LPS of E. coli $0111B_4$ (Figure 24c). Three other monosaccharide peaks in the GLC trace of H. ducreyi LPS were not identified. Also 2 monosaccharide peaks in the GLC tracings of N. gonorrhoeae LPS and 6 small peaks in that of E. coli LPS were detected but unidentified. The large number of peaks suggests greater complexity of E. coli LPS.

Quantitative estimations of individual monosaccharides are recorded in Table 9. The total hexose/hexosamine concentration of the LPS of virulent strains 35000, 409, C148, and A77* exceeded that of avirulent strains A76, 36-F-2, A77 and A75. Total glycose-KDO ratios of virulent strains were also higher than those of avirulent strains (Table 8). This ratio for nonpilated N. gonorrhoeae P^-6472 was in the same range as those of the avirulent H. ducreyi strains.

(d) Identification of fatty acids.

Fatty acid methyl esters identified by GLC in the lipid A moiety of H. ducreyi strains were C14, C16, C18, and C20 acids (Figure 25a). OH - substituted C14 (β-hydroxymyristic) acid was tentatively identified based on the retention time of this acid relative to the corresponding C14 fatty acid. No standard was available at the time of analysis to confirm this identification. C_{12} (lauric) acid identified in E. coli $0111B_4$ (Figure 25b) was absent from H. ducreyi strains. A number of peaks remained unidentified.

Quantitatively, there was little difference in the fatty acid content of virulent and avirulent strains (Table 10). The values for the fatty acids C14, C16 and C18 varied from strain to strain. C20, however, appeared to be somewhat higher in avirulent strains A77, A75, A76 and

Table 9. Monosaccharide content of the LPS from H. ducreyi strains, N. gonorrhoeae and E. coli

Strain	Glu-cose	Galac-tose	Man-nose	Rham-nose	Fucose	Gluco-samine	Galacto-samine	Total
	[a]Glycose concentration (nmol/ug of LPS)							
H. ducreyi								
Virulent:								
C148	0.41	0.30	0	0	0.04	0.42	0.25	1.42
409	0.81	0.53	0	0	0.04	0.51	0.38	2.27
35000	0.71	0.48	0	0	0.01	0.47	0.30	1.97
A77*	0.79	0.41	0	0	0.06	0.52	0.41	2.18
Avirulent:								
A77	0.27	0.19	0	0	0.01	0.32	0.12	0.91
A75	0.48	0.33	0	0	0.01	0.20	0.16	1.18
A76	0.34	0.21	0	0	0.005	0.24	0.16	0.95
36-F-2	0.40	0.26	0	0	0.01	0.31	0.21	1.19
N. gonorrhoeae								
P 6472	0.64	0.44	0.02	0	0.15	0.31	0.17	1.58
E. coli								
$0111B_4$	1.10	0.69	0.36	0.03	0.04	0.56	0.31	3.05

[a] Determined by GLC as TMS derivatives.

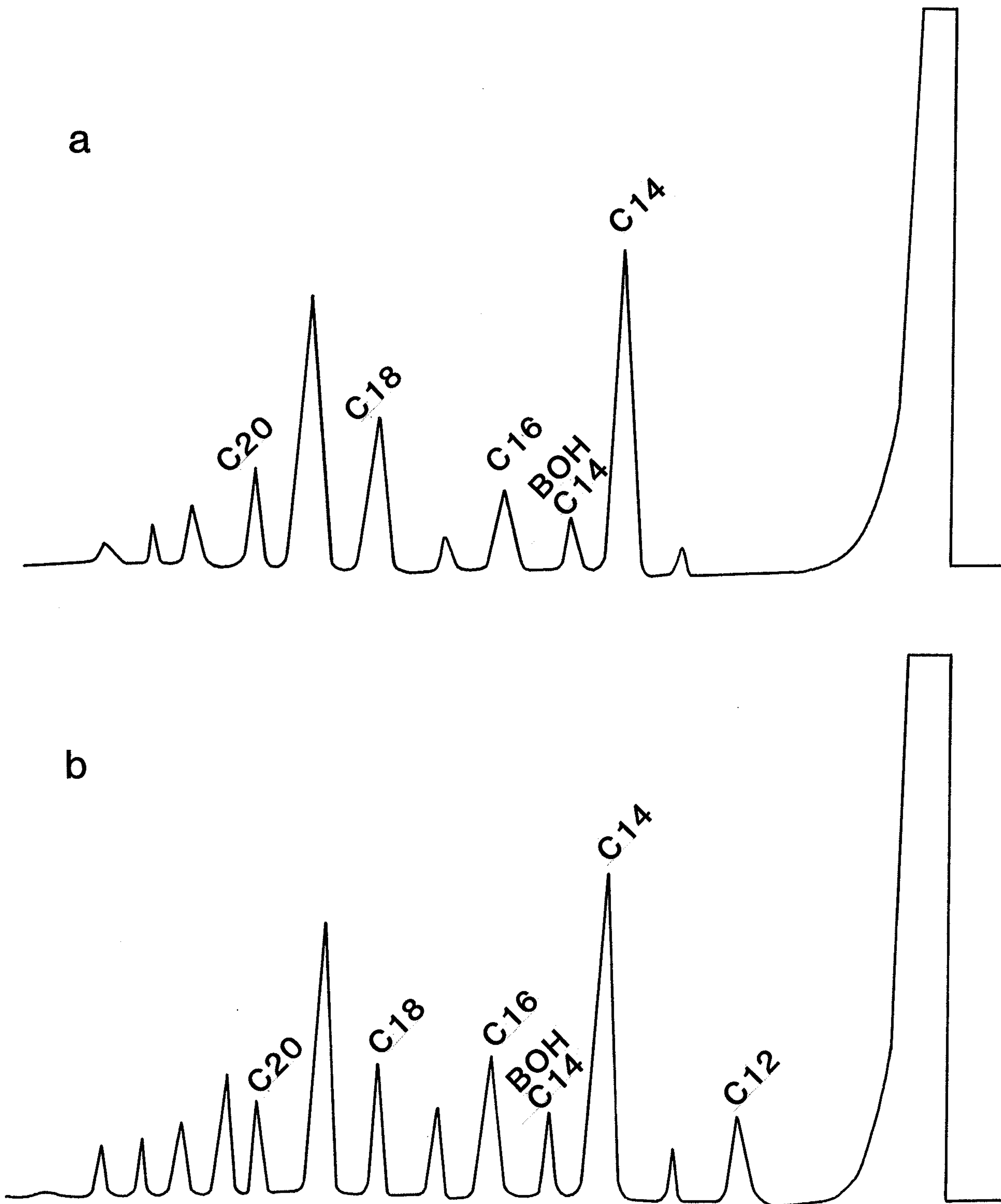


Figure 25: Schematic representation of the gas liquid chromatogram of fatty acid methyl ester derivatives of a) H. ducreyi LPS

b) E. coli LPS

Table 10. Lipid A fatty acid methyl ester content of H. ducreyi strains and E. coli $O111B_4$.

	Fatty Acid Chain Length				
Strains	C12	C14	C16	C18	C20
H. ducreyi					
409	0[a]	0.61	0.06	0.10	0.05
35000	0	0.65	0.04	0.12	0.05
A77*	0	0.72	0.03	0.14	0.03
A77	0	0.68	0.04	0.10	0.07
A75	0	0.70	0.04	0.14	0.07
36-F-2	0	0.75	0.06	0.16	0.07
A76	0	0.61	0.04	0.16	0.08
E. coli					
$111B_4$	0.17	0.58	0.22	0.09	0.05

[a] Values represent the fatty acid concentrations (nmol/ug lipid A), as determined by GLC.

36-F-2 than that of virulent strains 409, 35000 and A77*.

4. The role of H. ducreyi LPS in serum sensitivity.

(a) Inhibition of serum bactericidal activity by H. ducreyi LPS.

In order to determine whether LPS mediated the serum sensitivity of strains, normal human serum was pretreated with LPS and incubated at 37°C for 30 min prior to its use in serum bactericidal assays. H. ducreyi A77 LPS in the concentration range 0.1-1 mg/ml completely inhibited the bactericidal activity of serum against strain A77 (Table 11). An inhibitory effect was also observed even at low concentrations (0.05 mg/ml).

Table 12 shows the percent survival of H. ducreyi strains in normal human serum treated with 0.1 mg/ml LPS from serum-resistant and serum-sensitive strains. Serum-sensitive strains A76, A75 and A77 did not survive in serum during the 60 min incubation time, but these strains survived well in serum preincubated with LPS obtained from any of the three serum-sensitive strains. LPS from serum-resistant strains 409, 35000 and serum-resistant isogenic strain A77* did not inhibit serum bactericidal activity against the serum-sensitive strains (<5% survival, 60 min.). The viability of serum-resistant strains improved in serum treated with homologous and heterologous LPS.

High concentrations of LPS (1 mg/ml) of serum-resistant strains 35000 and 409 did not inhibit serum bactericidal activity against serum-sensitive strains A77, A75 and A76 (Table 13). However, LPS from serum-resistant isogenic strain A77* at this concentration was inhibitory. It is possible that the LPS structural site involved in serum sensitivity is retained in the LPS of this strain.

Table 11. Inhibition of the bactericidal activity of 50% human serum against serum-sensitive *H. ducreyi* A77 by different concentrations of homologous LPS.

Concentration of LPS (mg/ml)	Percent survival[a] of *H. ducreyi* A77 in serum + LPS after 60 min. incubation at 35°C
0	0
0.05	57
0.1	112
0.2	110
1.0	108

[a] Mean of three experiments

Table 12. Inhibition of serum bactericidal activity against *H. ducreyi* strains by homologous and heterologous LPS[a].

		Percent survival[b] of strains after 60 min. incubation in Serum + LPS from strains:					
		ser^s			ser^r		
Strains	Control Serum	A76	A75	A77	A77*	409	35000
A76	0	136	140	132	0.1	0.1	0.3
A75	0	115	108	116	1.7	0.7	0.5
A77	0	128	107	132	3.7	1.1	0.5
A77*	70	112	108	104	122	132	108

[a] Concentration of LPS was 0.1 mg/ml

[b] Mean of three experiments

Ser^s, serum-sensitive

Ser^r, serum-resistant

A77* is a Ser^r isogenic strain obtained from Ser^s strain A77

Table 13. Inhibition of serum bactericidal activity against serum-sensitive *H. ducreyi* strains by LPS from serum-resistant strains[a].

	Percent Survival[b] of strains after 60 min. incubation in: Serum + LPS from strains		
Strains	35000	409	A77*
A77	3.7	0.7	125
A75	3.2	0.5	118
A76	2.7	1.2	122

[a] Concentration of LPS was 1 mg/ml

[b] Mean of three experiments

(b) Anticomplementary activity of H. ducreyi LPS.

The anticomplementary activity of H. ducreyi LPS was measured in order to determine whether its inhibitory effect on serum bactericidal activity was due to its ability to activate and deplete C. Figure 26 shows the inhibitory effect of the LPS of serum-resistant and serum-sensitive strains on C-mediated hemolysis of sensitized sheep erythrocytes. The anticomplementary activity of LPS of serum-sensitive strains A76, A75 and A77 was dose-dependent. Complete inhibition of C-mediated hemolysis of erythrocytes was observed when serum was preincubated with LPS (1 mg/ml) from serum-sensitive strains. The LPS of serum-resistant strains on the other hand had low anticomplementary activity even at this concentration. However, LPS of serum-resistant isogenic strain A77*, unlike other serum-resistant strains, exhibited high anticomplementary activity when high concentrations of LPS (0.5-1mg/ml) were used to treat the serum. The LPS of strain A77* at these concentrations inhibited serum bactericidal activity (Table 12). Thus, there is a correlation between anticomplementary activity of LPS and its inhibitory effect on serum bactericidal activity.

Incubation of serum with 10^{10} CFU of H. ducreyi at $37^{o}C$ for 30 min resulted in inhibition of C-mediated hemolysis of sensitized sheep erythrocytes (Table 14). Serum-sensitive strains exhibited higher anticomplementary activity than serum-resistant strains at this concentration. However, the inhibitory effect of serum-resistant strains on hemolytic activity of serum was comparable when serum was incubated with $>10^{10}$ CFU (Table 13).

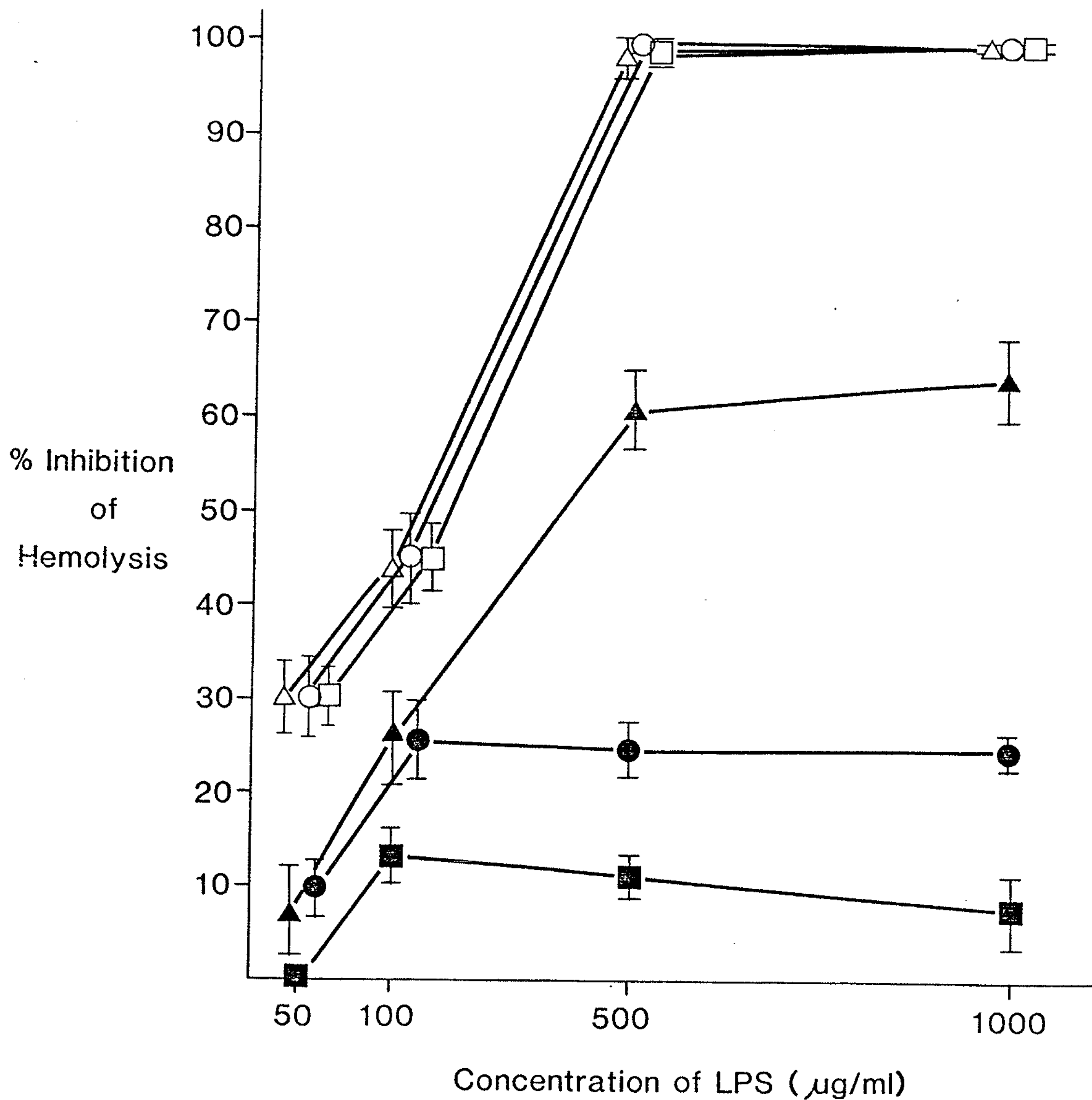


Figure 26: Inhibition of the C-mediated hemolysis of sensitized sheep erythrocytes after 30 min incubation of human serum with purified LPS from H. ducreyi strains 409 (■), 35000 (●), A77* (▲), A77 (□), A75 (△), and A76 (○).

Table 14. Inhibition of the complement-mediated hemolysis of sensitized sheep erythrocytes after 30 min incubation of human serum with whole cells of *H. ducreyi*.

Strain	[a]Percent inhibition of hemolysis by 10^{10} CFU	$>10^{10}$ CFU
Ser^r		
409	35	73
C148	35	65
35000	30	58
A77*	42	77
Ser^s		
A77	63	77
A76	74	81
A75	68	79
36-F-2	79	82

[a] Mean of two separate determinations.

Ser^r, serum-resistant

Ser^s, serum-sensitive

C. LPS components involved in serum sensitivity.

The LPS of most gram-negative bacteria consists of two parts: the lipid A and the carbohydrate moiety. In order to determine which of these LPS components are involved in serum sensitivity, the LPS of serum-sensitive strain A77 was treated with mild alkali (0.5N NaOH), which selectively destroys lipid A activity by hydrolyzing the lipid A ester-linked fatty acids and phosphates (Neter *et al.*, 1956). The LPS was then incubated with human serum at 37°C for 30 min, prior to the use of serum in bactericidal assays. Untreated LPS was used as control. Figure 27 shows the percent survival of serum-sensitive strain A77 in serum preincubated with increasing concentrations of homologous LPS and LPS treated with 0.5N NaOH. The LPS of this strain lost its ability to inhibit the serum bactericidal reaction after treatment with mild alkali, whereas untreated LPS retained its inhibitory effect.

D. Role of capsular polysaccharide, and pili in the virulence of *H. ducreyi*.

Electron microscopic examination of thin sections of *H. ducreyi* strains revealed the presence of a microcapsule (Figures 28a, and b) with ruthenium red staining. Virulent strain 35000 and avirulent strain A75 both have microcapsules. Similar pictures were obtained for virulent strain 409 and avirulent strain A77. These microcapsules could not be seen under the light microscope. The trilaminar appearance of the cell wall characteristic of the cell envelope of gram-negative bacteria is evident in the electron micrograph of the thin sections of *H. ducreyi* cells.

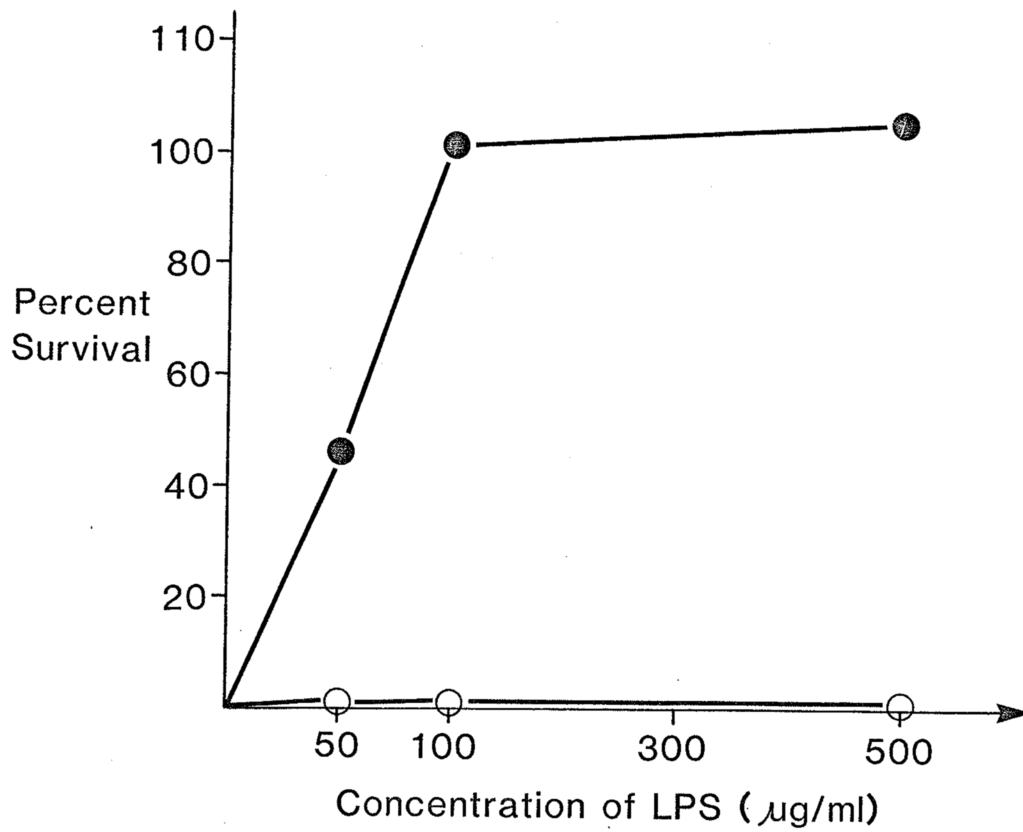


Figure 27: Percent survival of serum-sensitive H. ducreyi in normal human serum preincubated with increasing concentrations of homologous LPS (●); LPS treated with 0.5N NaOH to destroy lipid A (0).

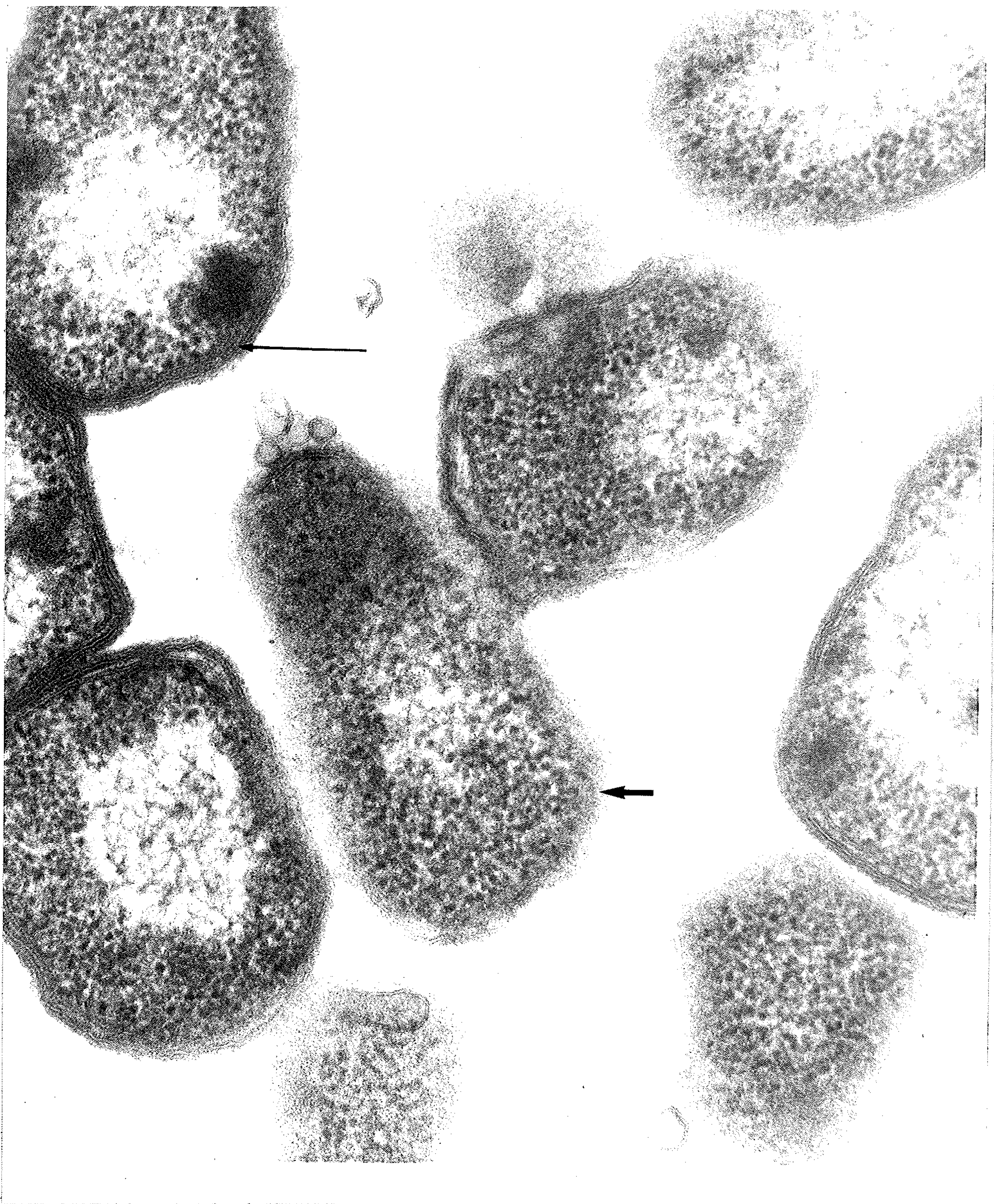


Figure 28a: Electron micrograph of thin sectioned H. ducreyi 35000 after fixation and ruthenium red staining. Short arrow indicates a microcapsule around the cell, and long arrow indicates cell wall layers. Magnification 100,000x.

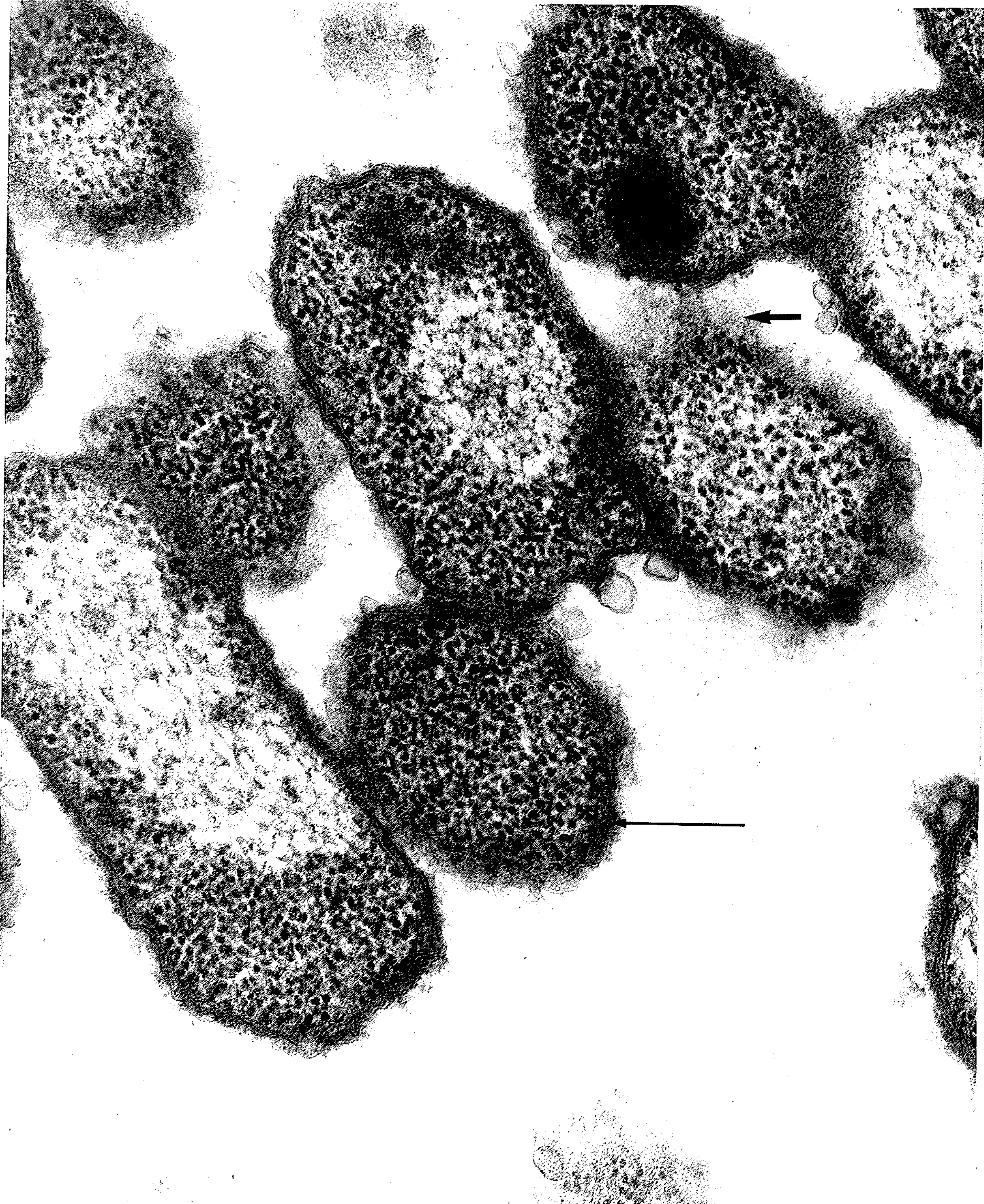


Figure 28b: Electron micrograph of thin sectioned H. ducreyi A75 after fixation and ruthenium red staining. A microcapsule is indicated by the short arrow. Long arrow indicates cell wall layers. Magnification: 100,000x.

No pili could be seen on the surface of any of the H. ducreyi strains after negative staining. Thus the presence of a capsular layer or pili may not contribute to the virulence of H. ducreyi.

E. Plasmid carriage and virulence of H. ducreyi.

In Table 15 is a list of H. ducreyi strains and the molecular weight of plasmids harboured by these strains, as reported by Ronald and Maclean (personal communication). Plasmids were absent in virulent strains 35000, 78118, 78226, and avirulent strains A77, A75, A76, 36-F-2. Plasmids coding for antibiotic resistance were present in other strains. A 7.0 Mdal plasmid coding for ampicillin resistance is present in virulent strains BG411, V1159, C147, 78118 and in avirulent strain 078. This plasmid is absent in virulent strain C148. Virulent strains C148 carry a 4.9 Mdal plasmid coding for sulfonamide resistance. The 34 Mdal in virulent strain V1159 codes for tetracycline and chloramphenicol resistance. A 23 Mdal plasmid present only in virulent strain C147 is a mobilizing plasmid.

An overall summary of the comparative properties of virulent and avirulent strains investigated is listed in Table 16.

Table 15. Plasmid carriage in virulent and avirulent H. ducreyi.

Strain	Antibiotic Resistance	Plasmid (Mdal)
Virulent		
35000	--	--
C148	TS	4.9
BG411	AT	7.0
V1159	ATC	7.0, 34
C147	ATS	7.0, 4.9, 23
78118	--	--
78226	--	--
Avirulent		
36-F-2	--	--
A77	--	--
A75	--	--
A76	--	--
078	AT	7.0

--, No detectable plasmid

T, Tetracycline

A, Ampicillin

C, Chloramphenicol

S, Sulfonamide

Table 16. Summary of properties of virulent and avirulent H. ducreyi strains investigated.

Strains	% Survival in human serum	% Phago- cytosis in human PMNL	MIC Polymyxin ug/ml	47K Protein	LPS ratio Glycose:KDO	Inhibition of serum bactericidal effect by LPS	% Reduction in complement activity by LPS (0.5 mg/ml)	% Reduction in complement activity by 10^{10} CFU
Virulent								
409	86.7	90.8	>128	--	3.7	--	12.5	35
35000	75.8	78.4	>128	±	2.8	--	26.6	30
C148	57.1	84.6	>128	--	3.5	--	ND	39
A77*	70.0	72.5	64	±	3.1	--	59.5	41
C148^{r}	47.5	84.8	1	--	ND	ND	ND	ND
Avirulent								
A77	0	0	1	+	1.3	+	98.6	63
A75	0	0	0.5	+	1.9	+	100	74
36-F-2	8.8	9.5	64	+	1.6	+	ND	79
A76	0	0	0.5	+	1.9	+	98.8	75
078	3.2	6.8	8	+	ND	ND	ND	ND

+ Present

-- Absent

± Trace

ND not done

D I S C U S S I O N

A. Host factors and virulence of *H. ducreyi*.

Serum bactericidal activity and phagocytosis are important first line defences against microbial pathogens, especially gram-negative bacteria (Schultz, 1980; Quie, 1980). Virulent strains of *H. ducreyi* in this study were resistant to serum bactericidal activity of normal human or rabbit serum in contrast with those strains considered avirulent in the rabbit intradermal test. Although the survival of serum-resistant strains improved in fluids with less than 50% normal human serum, this concentration was used in most of the experiments because differences in the sensitivity of virulent strains were most readily observed at this concentration. More importantly, this serum concentration is close to that found in the blood. A number of investigators however have successfully used serum in the concentration range 2.5-10% in serum bactericidal assays (Taylor and Kroll, 1983; Musher *et al*., 1983; Schiller *et al*., 1984) while others have used 20-100% serum in bactericidal assays (Taylor, 1983; Pluschke and Achtman, 1983; Pitt, 1978; McCuthan *et al*., 1978). In most of these studies, the rate of bacterial killing generally increases with increasing serum concentration. The dose-dependant killing of virulent *H. ducreyi* strains was observed at serum concentrations above 30%, while avirulent strains were rapidly killed at serum concentrations above 5%. Both virulent and avirulent strains were susceptible to the bactericidal activity of undiluted serum. The sensitivity of *H. ducreyi* to normal serum is an important observation, since previous studies (Deacon *et al*., 1956; Kaplan *et al*., 1956b) have shown that virulent strains could be cultivated in fresh human blood or rabbit blood clots, whereas avirulent strains failed to grow in blood-containing media. The sensitivity of avirulent

strains to serum may account for the lack of growth of these organisms in such media.

The bactericidal activity of normal human serum on H. ducreyi was C-dependent since serum-sensitive strains incubated in heat-inactivated serum survived well within 2h incubation at 35°C. Thus, inactivation of serum C by heating serum at 56°C for 30 min prior to its use in artificial medium is essential, in order to abolish its inhibitory effect, especially on sensitive strains. Resistance to bactericidal action of serum has been associated with virulence in several gram-negative bacteria (Muschel, 1960; Pai and DeStephano, 1982; Sutton et al., 1982) most notably as a marker for bacteremic spread. H. ducreyi does not produce bacteremia, but resistance to bacteriolysis by normal human serum may allow successful extracellular multiplication of the organism within tissues.

Virulent strains were found to be resistant to rabbit serum even in the presence of specific antibodies. This is unusual since one would expect C-fixing antibodies to enhance the bactericidal activity of serum against the organism. Many serum-resistant gram-negative bacteria are known to be sensitive to the bactericidal effect of serum containing antibodies directed against cell surface components (Goldschneider et al., 1969; Bjornson and Michael, 1970; Taylor, 1983), but some strains of E. coli are known to be resistant to the bactericidal effect of immune serum (Taylor, 1976). N. gonorrhoeae responsible for disseminated gonococcal infection (DGI) are resistant to killing by antibody and C in normal human serum (Schoolnik et al., 1976). The resistance of DGI strains to antibody and C has been attributed to the IgG blocking antibody that competes with bactericidal antibody, mainly IgM in human serum (McCutchan et al., 1978). IgG functions poorly in C activation in comparison to IgM, and at

high concentrations, IgG may inhibit C-mediated killing mechanisms by competing with IgM (Norman et al., 1972). Since immune serum is particularly rich in IgG, it is possible that the resistance of H. ducreyi to immune serum may be due to the presence of blocking antibodies which inhibit IgM-mediated C activation and bacterial killing in serum. Avirulent strains however, were sensitive to immune serum. The resistance of virulent H. ducreyi to the bactericidal activity of immune serum may be a contributing factor in the recurrence and chronic state of the disease. The fact that autoinoculation may occur at any time during the course of the disease suggests that antibodies produced against the organism do not protect against reinfection. Thus host response may have a minimal effect in altering the course of the disease.

The bactericidal effects of serum are mediated by activated components of the classical or alternative C pathway (Taylor, 1983). Our results suggest that the classical pathway is involved in serum killing of susceptible H. ducreyi strains. Serum treated with Mg^{2+}EGTA (which chelates Ca^{2+} essential for the classical pathway) was non-bactericidal, but serum depleted of the alternative pathway by treatment with inulin, was bactericidal. Thus, alternative pathway activation is probably not involved in the serum killing of sensitive H. ducreyi strains. Most gram-negative bacteria can activate either of the C pathways or both pathways simultaneously (Taylor, 1983). For example H. influenzae (Quinn et al., 1977), Salmonella spp. (Joiner et al., 1982), and E. coli (Taylor, 1983) have been shown to activate both pathways, but N. gonorrhoeae (Shafer et al., 1984) and P. aeruginosa (Schiller et al., 1984) activate mainly the classical pathway (Table 17). There are some strains of P. aeruginosa

Table 17. Activation of complement pathways by gram-negative bacteria.

Species	Pathway Activated	Reference
E. coli	Classical, alternate	Taylor (1983)
H. influenzae	Classical, alternate	Quinn *et al*. (1977)
Salmonella spp.	Classical, alternate	Joiner *et al*. (1982)
N. gonorrhoeae	Classical	Shafer *et al*. (1984)
P. aeruginosa	Classical	Schiller *et al*. (1984)
H. ducreyi	Classical	*

* in the present study

however, which activate both pathways (Meshulam et al., 1982). Joiner et al. (1984a) suggested that the mechanism of C activation by bacteria might be a property of a given strain and not a species. Regardless of the pathway being activated, the end result is the production of C5b-9 complex (the membrane attack complex) which disrupts both the outer and inner membranes of gram-negative bacteria, causing cell death.

Virulent H. ducreyi strains were also resistant to phagocytosis and killing by human PMNL. Avirulent strains were readily killed, but they were less susceptible to the killing activity of human PMNL when heat-inactivated serum was substituted for fresh serum in the phagocytosis assay. Thus C was required for the efficient killing of H. ducreyi strains by human PMNL. Complement has been shown to be one of the principal heat-labile opsonin systems in serum (Joiner et al, 1984a). Activated C component C3b enhances phagocytosis via C3b receptors on the phagocyte, and results in activation of the membrane attack pathway of C (Pangburn, 1983). Kinetic studies of phagocytosis of H. ducreyi strains by human PMNL as measured by the use of the acridine orange technique showed that a higher percentage of avirulent strains was ingested in relation to virulent strains. This suggests that the resistance of virulent strains to killing by human PMNL was due to the resistance of the organisms to ingestion. Generally the virulent and avirulent organisms which were ingested fluoresced red, an indication that H. ducreyi does not survive inside polymorphs, and it therefore can be considered an obligate extracellular pathogen.

B. Polymyxin resistance and virulence of H. ducreyi.

Analysis of H. ducreyi strains for sensitivity to polymyxin B indicated that virulent strains were resistant in contrast with avirulent strains which were sensitive. These results confirm those reported by Hammond et al. (1978). In order to determine whether polymyxin resistance was a virulence marker of H. ducreyi, polymyxin-sensitive strains were obtained from polymyxin-resistant parent strains by a one-step mutation that resulted in the loss of resistance. Polymyxin-sensitive derivatives from polymyxin-resistant virulent strains remained virulent for rabbits and resistant to the bactericidal activity of normal human serum and to phagocytosis. Also, a strain which lost its resistance to polymyxin when made resistant to rifampin remained virulent for rabbits. Acquisition of polymyxin resistance by polymyxin-sensitive avirulent strains did not result in the appearance of virulence or resistance to bactericidal activities of serum and phagocytosis. These results suggest that polymyxin resistance is not directly linked to virulence and confirm the observation of Thayer et al. (1955) who succeeded in making one strain avirulent in vitro after several months of repeated laboratory passages without changing its susceptibility to polymyxin.

The only change observed following the acquisition of polymyxin resistance was the loss of a 47,000 molecular-weight protein. The biologic function of this protein is unknown but it may act as a porin, facilitating uptake of polymyxin B. This antibiotic is an amphipathic molecule composed of D- and L- amino acids and a fatty acid attached to the peptide through an amide bond (Storm et al., 1977). The inner and outer membranes appear to be the target of action of polymyxin in gram-negative cells. Disruption

of these membranes leads to leakage of cell contents and cell death. Ultrastructural and chemical studies (Gilleland and Murray, 1976; Gilleland and Lyle, 1979) support this mechanism of action.

C. Relationship between cell envelope components and virulence of H. ducreyi.

SDS-PAGE analysis of sarcosinate-insoluble OMP of H. ducreyi strains revealed differences in the OMP composition of virulent and avirulent strains. The OMP of avirulent strains appeared similar, differing from those of virulent strains which appeared heterogeneous. Avirulent strains shared the 47,000 molecular weight protein alluded to above. It is possible that this protein in serum - sensitive avirulent strains is a receptor for C components as well as a porin. However, acquisition of polymyxin resistance resulting in the loss of this protein, did not result in acquisition of serum resistance and virulence. Therefore, the 47,000 molecular weight protein may not be related to the serum sensitivity or the avirulent state of strains. The OMP profiles of serum-resistant isogenic strain A77* differed from those of the parent strain. Three newly-acquired proteins were seen in the OMP profile of the isogenic strain. However, two of these proteins were not found in some of the virulent strains, while the other protein was present in strains which acquired resistance to polymyxin but remained avirulent. Although the serum resistant isogenic strain was virulent in the rabbit model, the newly acquired proteins may not be related to virulence, but could serve as the basis for a study of protective antigens.

The OMP composition of gram-negative bacteria such as N. gonorrhoeae (Hildebrandt et al., 1978), N. meningitidis (Stephens and McGee, 1983), E. coli (Moll et al., 1980) and S. salmonicida (Munn et al., 1982) has been correlated with serum resistance and virulence. In these studies, individual proteins related to virulence were identified. Although virulent and avirulent H. ducreyi strains differ in their OMP composition, none of the proteins appeared to be distinctly associated with virulence. Since the differences reside mainly in the 47,000 molecular weight protein, there is really no strong evidence suggesting a relationship between OMP composition of H. ducreyi and virulence.

The LPS composition of virulent and avirulent strains was also examined to determine whether it contributed to virulence of H. ducreyi. SDS-PAGE analysis revealed differences in the electrophoretic mobility of the LPS from virulent and avirulent strains. The LPS profiles of avirulent strains were identical and differed from those of virulent strains which appeared heterogeneous. The LPS of serum-resistant isogenic strain A77* differed from that of the serum-sensitive parent strain as in the case of their OMP. Thus acquisition of serum resistance appears to affect both the LPS and OMP composition of this strain. An absolute correlation was not observed between LPS and OMP profiles of strains, since some of the strains with similar OMP differed in their LPS profiles while some strains with similar LPS profiles differed in their OMP profiles. Inzana (1983), in his analysis of the composition of H. influenzae strains, also found that some with identical OMP subtypes differed in the LPS subtype, but none with identical LPS band mobility differed in their OMP subtype. Heterogeneity in LPS electrophoretic mobility is not unique to H. ducreyi. Other

gram-negative bacteria such as S. typhimurium (Goldman and Leive, 1980), H. influenzae (Inzana, 1983), Campylobacter spp. (Logan and Trust, 1984), N. gonorrhoeae (Mintz et al., 1984), N. meningitidis (Tsai et al., 1983) and E. coli (Jann et al., 1975) all exhibit heterogeneity in their LPS composition.

The electrophoretic profiles of "smooth" LPS from S. typhimurium and E. coli consist of regularly spaced bands with a wide range of molecular weight. Previous studies (Palva and Makela, 1980; Jann et al., 1975) have shown that the high molecular weight bands correspond to terminal O-polysaccharide side chains of the LPS. All H. ducreyi strains examined contained LPS of low molecular weight, suggesting that the O-side chains typical of "smooth" LPS of Salmonella spp., E. coli and Shigella spp. (Jann et al., 1975; Luderitz et al., 1971) are absent from these strains.

Some studies have demonstrated a correlation between SDS-PAGE profiles of bacterial LPS and its biochemical composition (Goldman and Leive, 1980; Palva and Makela, 1980; Mintz et al., 1984). In the present study, the chemical composition of the LPS of H. ducreyi strains was determined. The LPS of virulent and avirulent strains was subjected to infrared (IR) spectophotometric analysis to detect the various functional groups present. E. coli 0111B_4 and N. gonorrhoeae P^-6472 LPS were included for comparison. The IR spectra of virulent and avirulent strains were similar to that of E. coli and N. gonorrhoeae with minor differences. The IR spectra contained absorption peaks representative of fatty acid esters, hydroxyl and amide groups characteristic of the structure of gram-negative bacterial LPS. Thus, H. ducreyi LPS has a structural composition similar to that of other gram-negative bacteria. Chemical analysis of LPS from virulent and avirulent strains revealed no qualitative differences in the various

components identified but there were quantitative differences in some of the components. The most striking were found in total carbohydrate, hexose and lipid A contents of these strains. The concentrations of the first two components were higher in the virulent strains while that of lipid A was lower in the virulent strains. The higher lipid A content of avirulent strains is relative to the lower carbohydrate content of their LPS. The values for phosphate and heptose contents in all strains and the hexose content of virulent strains were close to that reported for H. influenzae type b (Flesher and Insel, 1978). However the lipid A concentration was higher in H. ducreyi strains than those reported for H. influenzae (29.3%) while total carbohydrate concentration of H. ducreyi was slightly less when compared to H. influenzae (30%). Total carbohydrate concentrations as high as 40% has been reported for H. influenzae isolates (Parr and Bryan, 1984). The concentrations of all LPS components identified were much higher in E. coli 0111B$_4$ than in H. ducreyi strains except for lipid A, phosphate and N-acetylneuraminic acid, which were lower than those of H. ducreyi strains. Again, the low lipid A content of E. coli probably reflects only the high carbohydrate content of the smooth LPS.

Hydrolyses of H. ducreyi LPS with 0.2N H_2SO_4 as described by Karkhanis et al. (1978) yielded KDO in amounts less than 1% of LPS (dry weight). Low KDO content (<1%) has been reported for LPS of H. influenzae types a - f (Flesher and Insel, 1978; Zoon and Scocca, 1975). In fact, Raichvarg et al. (1979) detected no KDO in their strain of H. influenzae. It has been suggested that such LPS may be characteristic of the genus Haemophilus (Flesher and Insel, 1978). However, Parr and Bryan (1984) detected KDO in three strains of S. typhimurium (approx. 3%), by hydrolysing LPS with 4N HCl as previously described by Brade et al. (1983). In our study, this

method released more KDO from the H. ducreyi LPS in amounts similar to that of E. coli 0111B$_4$. It has been shown that heptosyl-substituted KDO residues are resistant to degradation by mild acid hydrolyses and that strong acid hydrolysis frees C-4 and C-5 heptosyl substituted KDO from LPS. The high KDO levels obtained by strong acid hydrolysis compared to low levels detected after weak acid hydrolysis suggest the presence of highly substituted KDO in H. ducreyi LPS. The same may be true of P^- N. gonorrhoeae 6472 LPS in which KDO at levels higher than E. coli and H. ducreyi was detected following strong acid hydrolysis.

Qualitative and quantitative analyses of glycoses present in H. ducreyi LPS revealed the presence of glucose, galactose and their amino derivatives glucosamine and galactosamine, fucose and ribose. Ribose is presumably a contaminant from traces of nucleic acid in the LPS since it is not part of LPS structure. Glycoses such as rhamnose and mannose which are characteristic of the O-specific chains of the LPS of many gram-negative bacteria were not detected in the H. ducreyi strains. Although there were no qualitative differences in the glycose content of the LPS of virulent and avirulent strains, the total glycose:KDO ratios of virulent strains exceeded that of avirulent strains. The glucose content of virulent strains with the exception of strain C148 was also higher than those of avirulent strains. The fact that glycoses characteristic of O-side chains of smooth LPS were not detected in the LPS of virulent and avirulent strains, suggests that H. ducreyi has a "rough" type LPS. Other gram-negative bacteria such as N. meningitidis (Tsai and Frasch, 1982), N. gonorrhoeae (Mintz et al., 1984), B. pertussis (Peppler, 1984), H. influenzae (Inzana, 1983), Chlamydia spp. (Caldwell and Hitchcock, 1984) also have a "rough" type LPS, indicating that "smooth" type LPS is not a

not a prerequisite for virulence of these organisms, as well.

Fatty acids identified in the LPS of H. ducreyi strains were similar qualitatively, having no apparent correlation with virulence. C20 fatty acid however appeared slightly higher in avirulent strains than in virulent strains, but is probably not significant. Lauric acid (C12) identified in E. coli lipid A was not detected in H. ducreyi. Heterogeneity in the lipid A component of bacterial LPS with respect to the ester-linked fatty acid composition has been reported (Rosner et al., 1979; Mattsby-Baltzer et al., 1984). However such heterogeneity in fatty acid composition has not been related to virulence, but is more likely related to LPS toxicity.

D. Role of LPS in serum susceptibility of H. ducreyi.

Serum absorption experiments with heat-killed whole cells of H. ducreyi suggest that heat-stable components of the bacterial cell surface are involved in serum susceptibility of H. ducreyi strains. Absorption of serum with whole cells of a serum-sensitive strain removed the bactericidal effect of serum against the absorbing strain, whereas absorption of serum with serum-resistant strains resulted in partial removal of the bactericidal effect. This suggests that these two groups of strains differ in heat-stable cellular components involved in serum susceptibility. There is a possibility that antibody present in human serum is involved in the killing of these strains since the sera used in the assays had $\geq$70% C activity after absorption with whole cells at 4°C. This must, however, be non-specific or cross-reacting antibody directed against the heat-stable components of the organism, since the serum used in these assays was obtained from persons with no history of chancroid, and absorption with a

serum-sensitive H. influenzae also abrogated killing of H. ducreyi. Natural antibodies directed against the surface antigens of a number of gram-negative bacteria have been demonstrated in non-immune serum (Schoolnik et al., 1979; Skaines, 1978; Schwab and Reeves, 1966). Their production is believed to be initiated by commensal bacteria in the intestinal tract (Kunin, 1962).

LPS from serum-sensitive avirulent strains inhibited serum-bactericidal activity, whereas LPS from serum-resistant virulent strains was not inhibitory even at high concentrations. These findings correlated with the data on the SDS-PAGE and LPS composition for virulent and avirulent H. ducreyi groups. The ability of H. ducreyi LPS to inhibit serum bactericidal activity is not unique to this organism. Purified LPS from serum-sensitive strains in E. coli (Allen and Scott, 1981), and N. gonorrhoeae (Shafer et al., 1984) has also been shown to inhibit serum-bactericidal activity in vitro as a result of anticomplementary activity of the LPS (Shafer et al., 1984) or by binding to the bacterial cells such that they were protected from C-mediated bactericidal reactions (Allen and Scott, 1980, 1981). The ability of H. ducreyi LPS to inhibit serum-bactericidal activity against serum-sensitive strains correlated with anticomplementary activity of the LPS. Thus, the inhibitory effect of the LPS of serum-sensitive H. ducreyi strains on the bactericidal activity of serum may be due to their ability to activate and deplete serum C. The fact that high concentrations (0.5-1 mg/ml) of the LPS of serum-resistant isogenic strain A77 inhibited serum bactericidal activity, suggests that part of the LPS structural site involved in serum-sensitivity is retained in the LPS of this isogenic strain.

In order to determine the LPS components involved in the serum sensitivity of H. ducreyi strains, LPS was treated with mild alkali (0.5N NaOH) and its use in serum bactericidal assays was compared with untreated LPS. Such treatment abolished the inhibitory effect of LPS on the serum bactericidal reaction. Hydrolysis of bacterial LPS by alkaline treatment has been shown to selectively hydrolyze the lipid A ester-linked fatty acids and phosphates, leading to loss of toxicity, pyrogenicity (Neter et al., 1956) and also to loss of the ability of lipid A to activate C via the antibody-independent C - activation mechanism (Cooper and Morrison, 1978). Thus the inhibitory effect of H. ducreyi LPS on serum-bactericidal reactions may be due to its lipid A moiety. The anticomplementary activity of the LPS of Salmonella spp. and E. coli smooth and rough mutant strains has been attributed to their lipid A component (Galanos and Luderitz, 1976). However, the polysaccharide portion of the LPS molecule has a modifying influence on its potential anticomplementary activity which decreases with an increase in the length of the polysaccharide moiety (Morrison and Kline, 1977). These latter authorities proposed that the polysaccharide moiety of LPS inhibits the anticomplementary activity of lipid A by "masking" its functionally active groups. Thus, differences in the inhibitory effects of LPS of serum-resistant and serum-sensitive H. ducreyi strains on serum-bactericidal reactions may be due to the amounts of polysaccharide associated with the lipid A portion of the LPS.

Although the LPS of serum-resistant strains was less anticomplementary than that of serum-sensitive strains, the whole cells of serum-resistant strains depleted C activity of serum to a level comparable to those of serum-sensitive strains when a heavy suspension of cells ($>10^{10}$) was used. The whole cells of serum-resistant strains however, were less

anticomplementary than those of serum-sensitive strains when 10^{10} CFU or less were incubated with serum. The fact that whole cells of serum-resistant strains activate C suggests that serum resistance of *H. ducreyi* is not due to the complete absence of C activation. Joiner *et al.* (1984a) proposed three different mechanisms for bacterial resistance to C-mediated killing: (a) shedding of the completed membrane-attack complex (C5b-9) from the bacterial surface, (b) the formation of non-covalent interactions that prevent the C5b-9 complex from inserting into the bacterial outer membrane and (c) the presence of a thick peptidoglycan layer (in gram-positive bacteria) shielding the vulnerable cytoplasmic membrane from attack. The first mechanism has been shown to operate in the resistance of *E. coli* and *Salmonella spp.* to C-mediated bactericidal activity of serum (Joiner *et al.*, 1984a), while the second mechanism operates in serum resistance of *N. gonorrhoeae* (Joiner *et al.*, 1983; Joiner *et al.*, 1985). The latter operates mainly in gram-positive bacteria. In gram-negative bacteria, the LPS is the site to which the C5b-9 complex binds. It is believed that the smooth LPS of *E. coli* and *Salmonella spp.* with long O-side chains sterically hinder access of C3b to the lipid A and core region of the LPS, whereas rough LPS provides a focus of attachment for C5b-9 near the outer membrane leading to C-mediated killing (Joiner *et al.*, 1984b). In *N. gonorrhoeae* however, the C5b-9 complex is bound to the LPS of both serum-resistant and serum-sensitive strains. The C5b-9 complex bound to serum-resistant strains forms high molecular weight complexes which are nonbactericidal while the C5b-9 complexes bound to serum-sensitive strains form low molecular weight complexes which are bactericidal (Joiner *et al.*, 1983). Either of these mechanisms of serum resistance may be operating in *H. ducreyi*. However, one may speculate that

serum-resistance is due to lack of binding of the C5b-9 complex to the LPS whereas the complex is bound to the LPS of serum-sensitive strains, resulting in the disruption of the cell membranes and death. This speculation is based on the fact that LPS of serum-resistant strains was less anticomplementary than that of serum-sensitive strains. Perhaps the LPS of serum-resistant strains failed to bind these C components.

The data presented in this study suggest that LPS plays a role in the serum resistance and virulence of H. ducreyi and may be considered a virulence factor.

E. Role of pili, capsules and plasmids in virulence of H. ducreyi.

Electron microscopic examination of thin sections of H. ducreyi after ruthenium red staining revealed the presence of a microcapsule on the cell of virulent strains. No differences in the size of microcapsules on these cells were apparent. Therefore, the presence of capsular polysaccharides may not contribute to virulence. Encapsulation has been related to the virulence of a number of gram-negative bacteria (Zwahlen et al., 1983; Guerina et al., 1983), however, the virulence of microorganisms such as N. gonorrhoeae and S. typhimurium has not been related to a capsular polysaccharide.

H. ducreyi cells were also examined for the presence of pili under the electron microscope following negative staining. Pili were not detected in any of H. ducreyi cells examined regardless of their virulence status. These observations are in keeping with those of Bertram (1980).

Many strains of H. ducreyi harbor plasmids coding for antibiotic resistance. In the present study, plasmid carriage among virulent and

avirulent strains was examined. Plasmids were absent in three virulent strains and four avirulent strains. Although most of the virulent strains and one avirulent strain have plasmids, none of the plasmids are shared by all virulent strains with plasmids. Based on plasmid carriage among the small number of strains examined, there is no evidence to suggest a relationship between presence of plasmids and virulence of H. ducreyi.

S U M M A R Y

The conclusions derived from this study are summarized as follows:

1. Virulent *H. ducreyi* strains positive in the rabbit intradermal test were resistant to the C-mediated bactericidal action of human and rabbit sera, in contrast with avirulent strains. Also, virulent strains were relatively resistant to phagocytosis and killing by human PMNL *in vitro*, in comparison to avirulent strains.

2. The serum killing of serum-sensitive strains involved mainly the classical pathway of C activation.

3. Resistance to polymyxin does not constitute a reliable marker for virulence in *H. ducreyi*.

4. SDS-PAGE profiles of the OMP of avirulent strains were similar, but differed from those of virulent strains which appeared heterogeneous. A 47,000 molecular-weight protein, which constitutes the major difference between the OMP profiles of virulent and avirulent strains, was associated with polymyxin resistance, but not virulence. There was no strong evidence to suggest a relationship between OMP composition and virulence of *H. ducreyi*.

5. The infrared spectra of the LPS of *H. ducreyi* strains were identical and were similar to those of *E. coli* 0111B_4 and *N. gonorrhoeae* P^-6472. The LPS contained fatty acid esters, hydroxyl and amide groups characteristic of the structure of LPS from other gram-negative bacteria.

6. The electrophoretic mobility of the LPS of virulent H. ducreyi strains differed from those of avirulent strains which appeared similar. The LPS lacked high molecular weight subunits indicative of the presence of variable polymerized O-side-chain repeating units.

7. The LPS of virulent and avirulent strains differed in the amounts of sugars associated with the core polysaccharide moiety of the LPS. The glycose:KDO ratio of virulent strains exceeded that of avirulent strains. Glycoses characteristic of O-side-chains were not detected.

8. LPS of avirulent strains inhibited the bactericidal activity of serum whereas LPS from virulent strains did not. This inhibitory effect correlated with the anticomplementary activity of LPS. Although the anticomplementary activity of LPS resides in the lipid A region, the polysaccharide moiety may have a modifying effect on its activity.

9. Both virulent and avirulent strains appeared to activate C but to a different degree. Serum-resistance of virulent strains may be due to the ability of LPS to block the bactericidal activity of the terminal complexes (C5b-9) produced during C activation, in a manner analogous to that reported for Enterobacteriaceae or N. gonorrhoeae.

10. Virulence of H. ducreyi was not associated with possession of a microcapsule which was found on both virulent and avirulent organisms. Pili were not found in any of the strains examined.

11. Although most of the virulent strains examined carried plasmids, none of the plasmids was associated with virulence of _H. ducreyi_.

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APPENDICES

APPENDIX A

Culture media

Solid media

(a) Hemoglobin agar

36 g GC Agar base (Gibco) in 500 ml distilled H_2O (autoclaved)

10 g of hemoglobin (Gibco) in 500 ml distilled H_2O (autoclaved)

1% sterile CVA (Gibco)

(b) Special transparent media

36 g GC Agar base in distilled H_2O

50 g Hemin

Autoclave for 15 min

Add 1% sterile CVA

25% sterile sheep serum (heat at 56°C for 30 min).

Liquid media

2.1 g PPLO (Difco) in 44 ml distilled H_2O (autoclaved)

20 ml of sterile fetal calf serum

36 ml of sterile Minimal Essential Medium (Difco)

APPENDIX B

Buffers

(a) Phosphate buffered saline pH 7.2

0.0077M Na_2HPO_4

0.0023M $NaH_2PO_4 \cdot H_2O$

0.146M NaCl

(b) Hanks balanced salt solution

Solution A

NaCl	8.0 g
KCl	0.4 g
$MgSO_4$ $7H_2O$	0.2 g

Solution B

Na_2HPO_4 (anhydrous)	0.048 g
KH_2PO_4	0.06 g

Solution C

$CaCl_2$	0.14 g

1. Dissolve salts completely for each solution in about 300 ml of distilled water.
2. Autoclave solutions A,B, and C separately.
3. Solutions A and B are combined with constant stirring and then solution C is added.
4. Add 1 ml of 10% gelatin to every 100 ml of HBSS (0.1% final concentration) to ensure bacterial viability in HBSS.

(c) Proteose Peptone Saline (pH 7.2)

10 g Proteose peptone (Difco) in 1 litre of 0.15M NaCl.

(d) Geys balanced salt solution (pH 7.2)

NaCl	7.00 g
KCl	0.37 g
$CaCl_2$	0.17 g
$MgSO_4 \cdot 7H_2O$	0.07 g
$MgCl_2 \cdot 6H_2O$	0.21 g
$Na_2HPO_4 \cdot 2H_2O$	0.15 g
$KHPO_4$	0.03 g
Glucose	1.00 g
$NaHCO_3$	2.27 g
Water	1000 ml

LPS solubilization Buffer

0.1 M Tris-HCl buffer pH 6.8

2% SDS (w/v)

20% sucrose (w/v)

1% 2-mercaptoethanol (v/v)

0.001% bromophenol blue (w/v)

Protein solubilization buffer

0.0625M Tris-HCl, pH 6.8

2% SDS (w/v)

10% glycerol (v/v)

5% 2-mercaptoethanol (v/v)

0.001% bromophenol blue (w/v)

APPENDIX C

Reagents

Silver staining reagents

Silver stain

2 ml Conc. NH_4OH

28 ml 0.1N NaOH

5 ml 20% Silver nitrate (w/v)

Note: Discard reagent immediately after use because it may become explosive when dry.

Fixative

40% ethanol in 5% acetic acid (v/v)

Oxidative solution

0.7% periodic acid in fixative solution

Formaldehyde developer

50 mg citric acid in

0.5 ml formalin

1 litre of double distilled H_2O

Protein reagent

50 ml 95% ethanol

100 mg coomassie brilliant blue G-250

100 ml 85% (w/v) phosphoric acid

Adjust volume to 1 litre with distilled water

Protein reagent can be purchased from Bio-Rad

Indole reagent

0.04% Indole in distilled H_2O

Resorcinol reagent

0.6 g resorcinol

60 ml 28% HCl

40 ml distilled H_2O

25 umoles $CuSO_4$

Reducing reagent for phosphorus determination

0.2 g 1-amino-2-naphthol-4-sulfonic acid

1.2 g sodium bisulfite ($Na_2S_2O_5$)

1.2 g sodium sulfite (Na_2SO_3)

Dissolve 0.2 g of the above mixture in 10 ml of water prior to use in the assay.

SDS-PAGE reagents

Stock A (acrylamide solution)

Acrylamide	30 g
Bis-acrylamide	0.8 g
Distilled H_2O,	100 ml

Stock B (discontinuous gel buffer)

Tris	36.3 g
1N HCl	48 ml
Distilled H_2O	100 ml (pH 8.8)

Stock C (stacking gel buffer)

Tris	6 g
1N HCl, titrate to pH 6.8	
Distilled H_2O, dilute up to	100 ml

Electrode buffer

Tris	6 g
Glycine	28.8 g
Distilled H_2O,	1 litre (pH 8.3)

Reagent	Stacking gel 5%[a]	Final acrylamide concentration in separating gel[b] 15%	12.5%	10%	Reservoir Buffer[c]
Stock A	1.67	15	12.5	10	--
Stock B	--	3.75	3.75	3.75	--
Stock C	2.50	--	--	--	--
10% SDS (w/v)	0.1	0.30	0.30	0.30	10
TEMED[d]	0.01	0.03	0.03	0.03	--
Dist. H_2O	5.22	9.42	11.92	14.42	490
1.5% $(NH4)_2S_2O_8$[e]	0.50	1.50	1.50	1.50	--
Electrode Buffer	--	--	--	--	500

[a] Amounts of reagents (ml) required to make 10ml of stacking gel solution

[b] Amounts of reagents required to make 30ml of separating gel solution

[c] Amounts of reagents (ml) required to make 1 litre of reservoir buffer

[d] N,N,N',N'-Tetramethylethylenediamine

[e] Ammonium persulfate

Stock 1:100 hemolysin solution

5% (v/v) phenol solution	4 ml
Proteose peptone saline (PPS)	94 ml
Glycerinized hemolysin	2 ml

Mix by swirling in a flask.

Prepare 1:1000 hemolysin solution by adding 9 ml PPS to 1 ml (1:100) hemolysin stock.

Hemolysin dilutions

Final hemolysin dilution	PPS, ml	1:1000 hemolysin dilution, ml.
1:1000	0	1.0
1:1500	0.5	1.0
1:2000	1.0	1.0
1:2500	1.5	1.0
1:3000	2.0	1.0
1:4000	3.0	1.0
1:8000	7.0	1.0

Hemolysin titration

PPS	0.4 ml
Serum with complement (at optimum dilution)	0.4 ml
Sheep erythrocytes sensitized with hemolysin diluted as shown above	0.2 ml

Mixed and incubated in 37°C waterbath for 30 min, prior to determination of percent hemolysis.

Fixative with ruthenium red or alcian blue stain.

Glutaraldehyde (fixative)	0.5% (v/v) in 0.1M sodium cacodylate, pH 6.6
Alcian blue	
or ruthenium red	0.15% (w/v) in fixative

Acetone-Water mixtures for dehydration of agar cores.

Step	Acetone concentration (%)	Time (min)
1	10	Rinse
2	30	3
3	50	5
4	70	5
5	80	10
6	90	10
7	100	15 repeat (2x)

Acetone-Propylene oxide mixture.

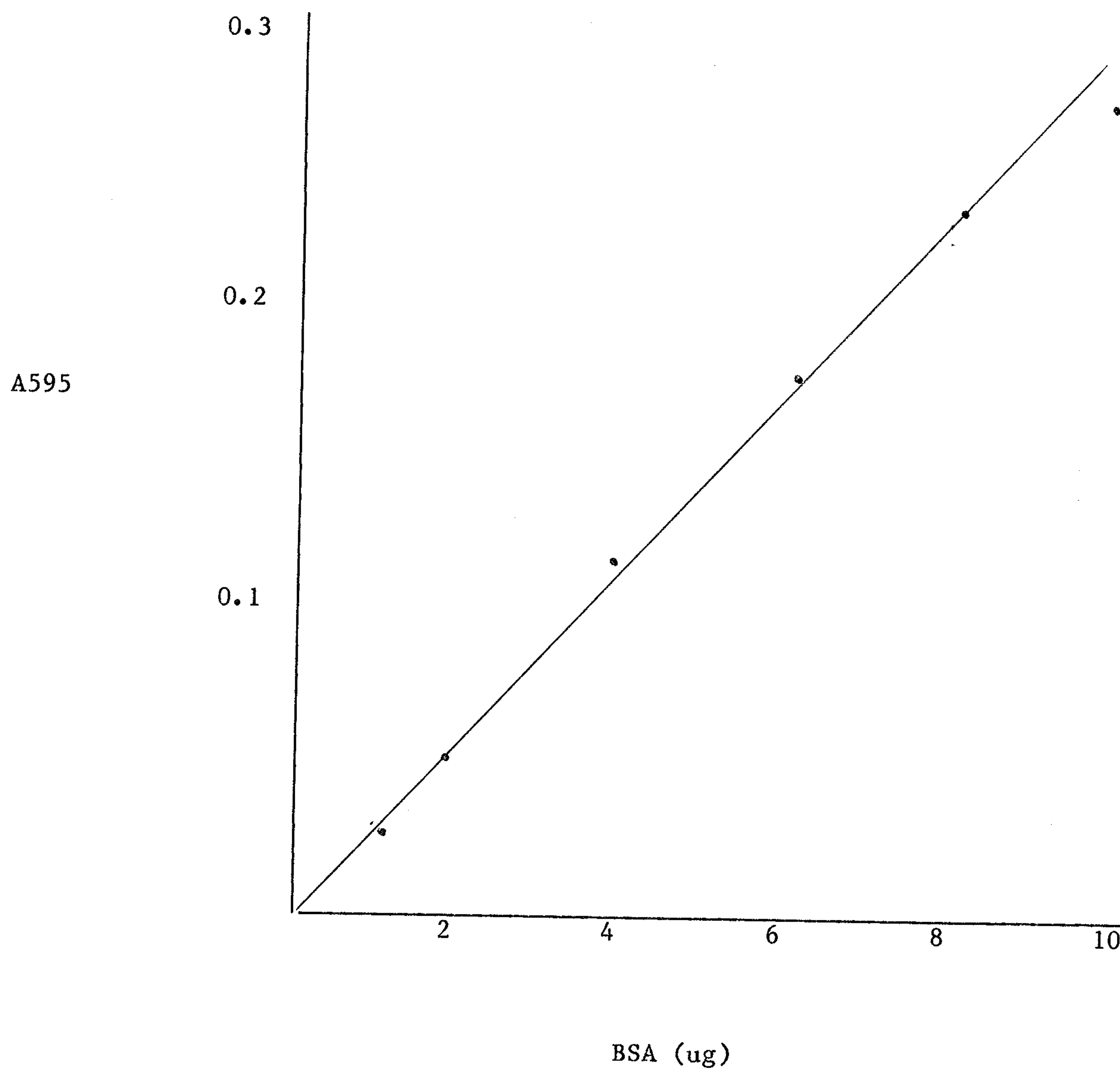
Step 1	Acetone concentration (%)	Propylene concentration (%)	Time (min)
1	75	25	10
2	50	50	10
3	25	75	10
4	---	100	10 repeat(2x)

Propylene oxide - Plastic mixture

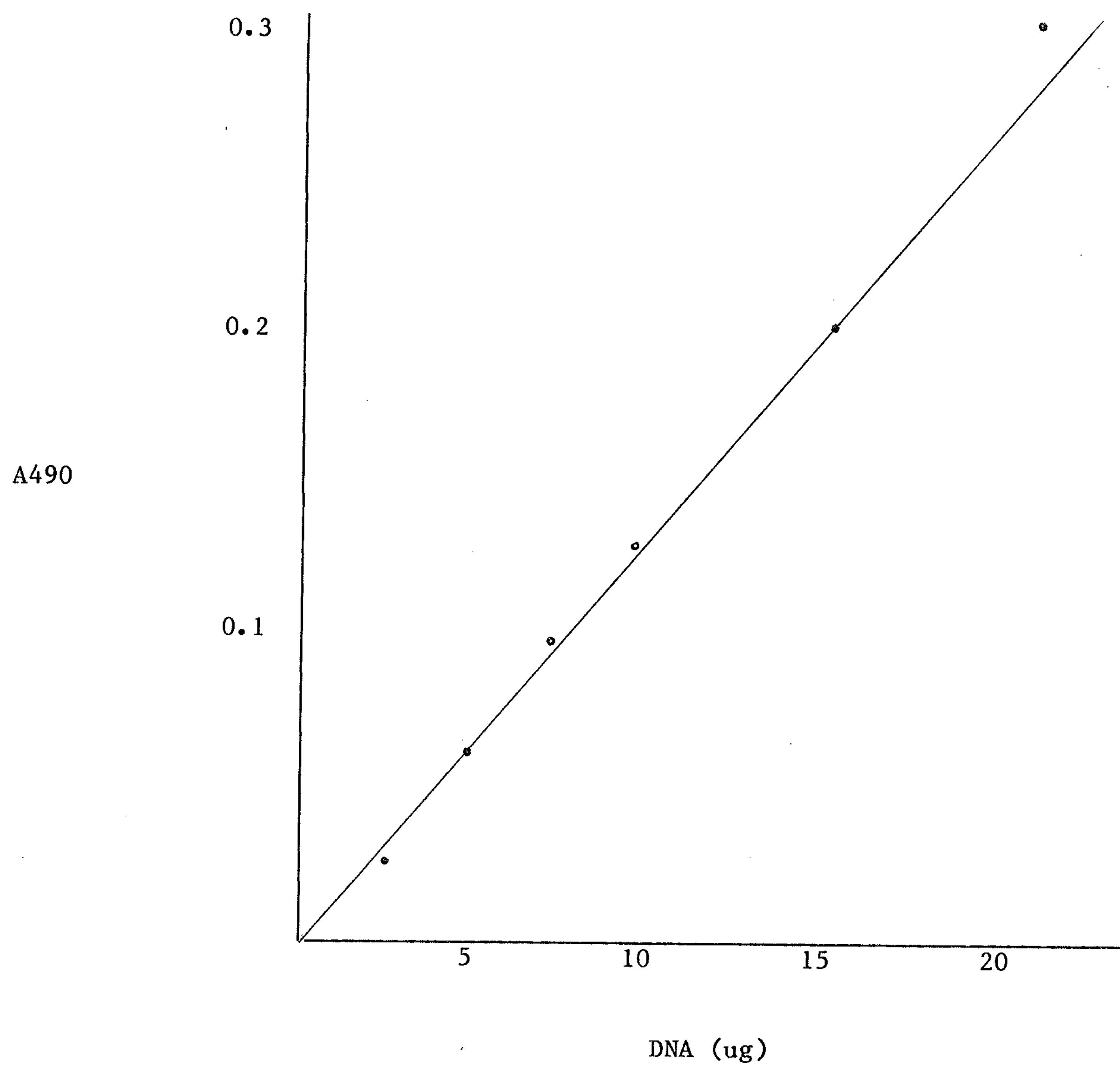
Step	Propylene oxide concentration (%)	Plastic concentration (%)	Time
1	75	25	10 min
2	50	50	10 min
3	25	75	6h
4	--	100	overnight at 4°C
5	--	100 (fresh solution)	8h at 37°C

APPENDIX D

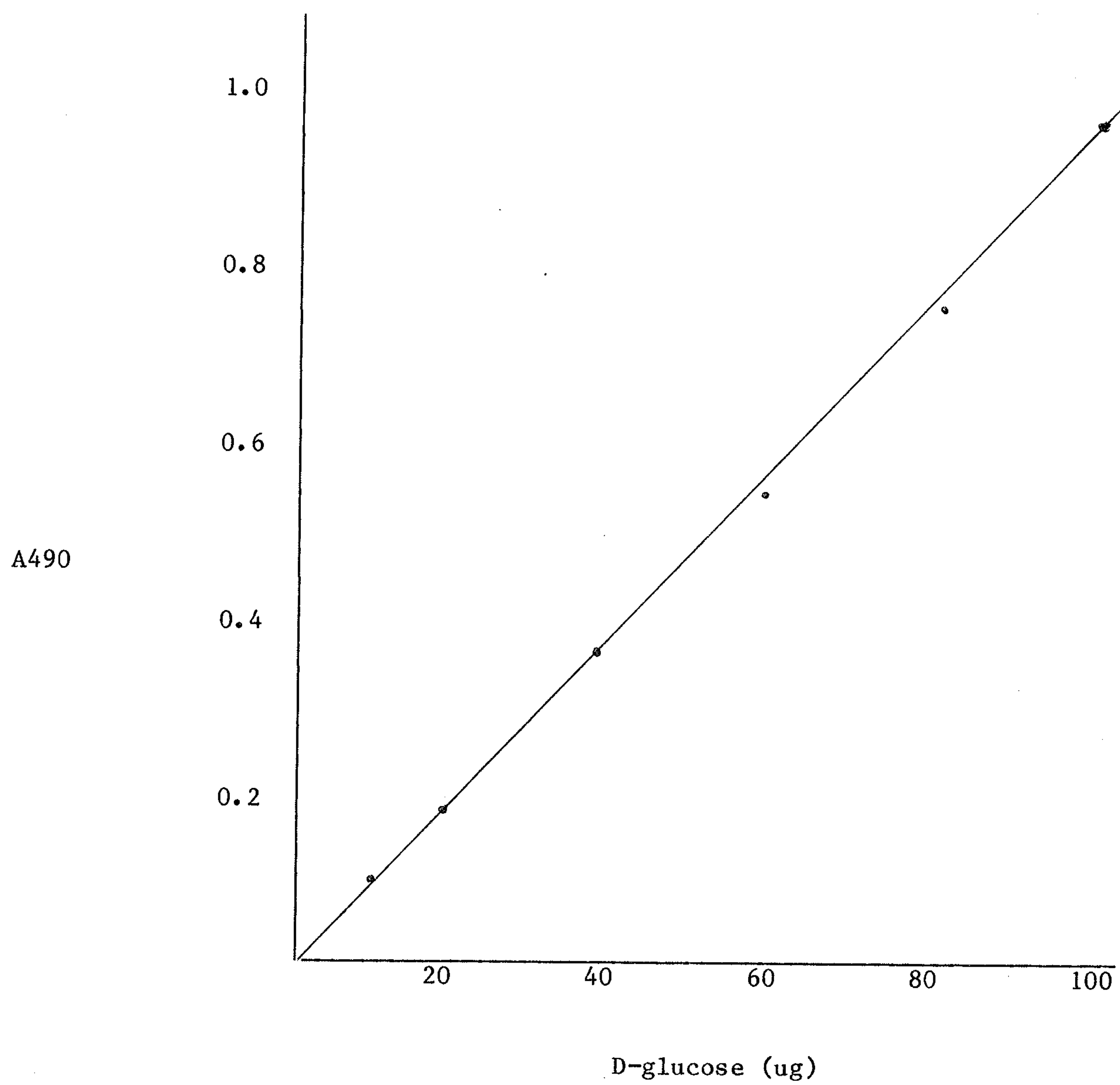
Standard Curves



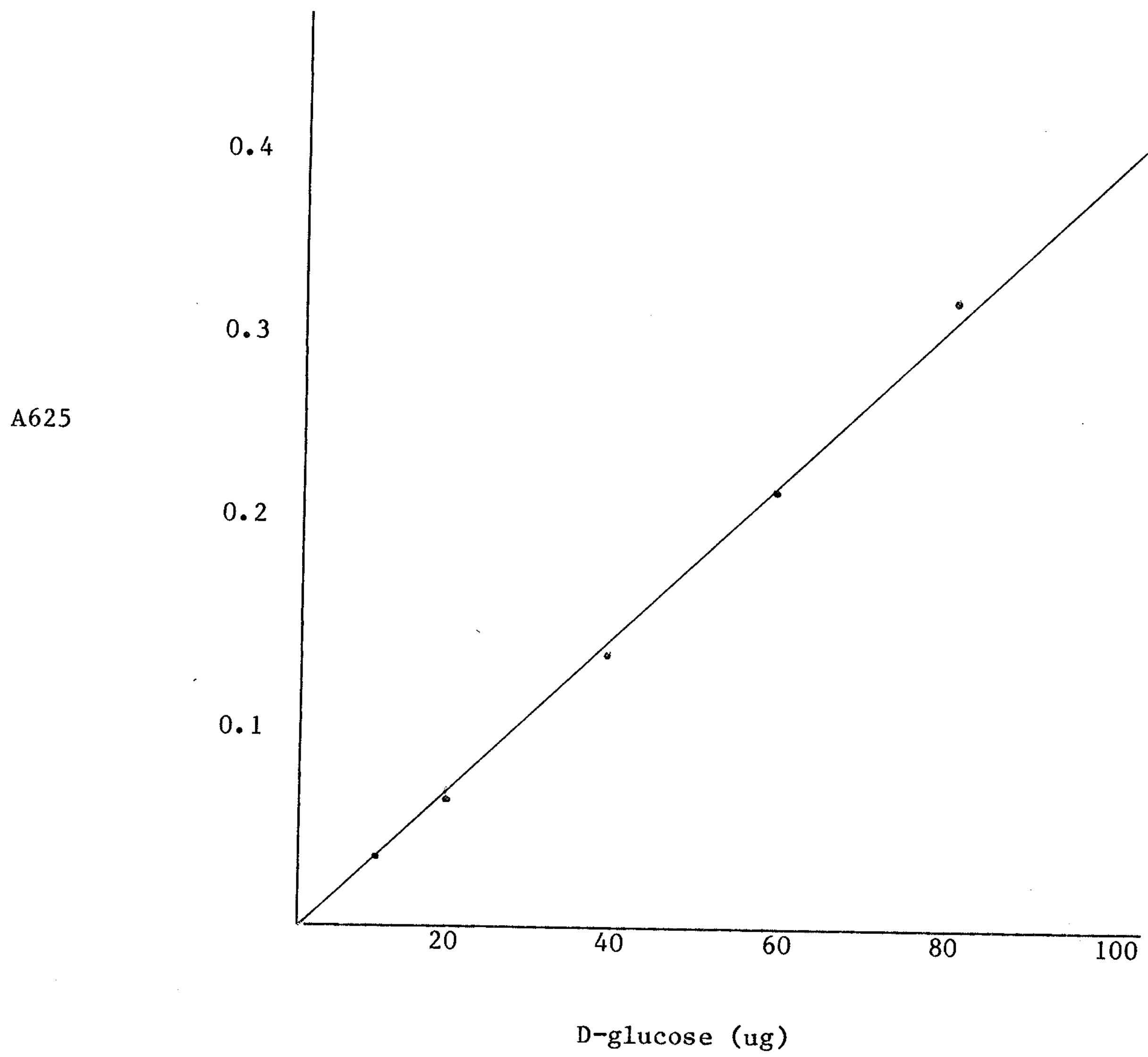
(i) Standard curve for the determination of the protein content of LPS



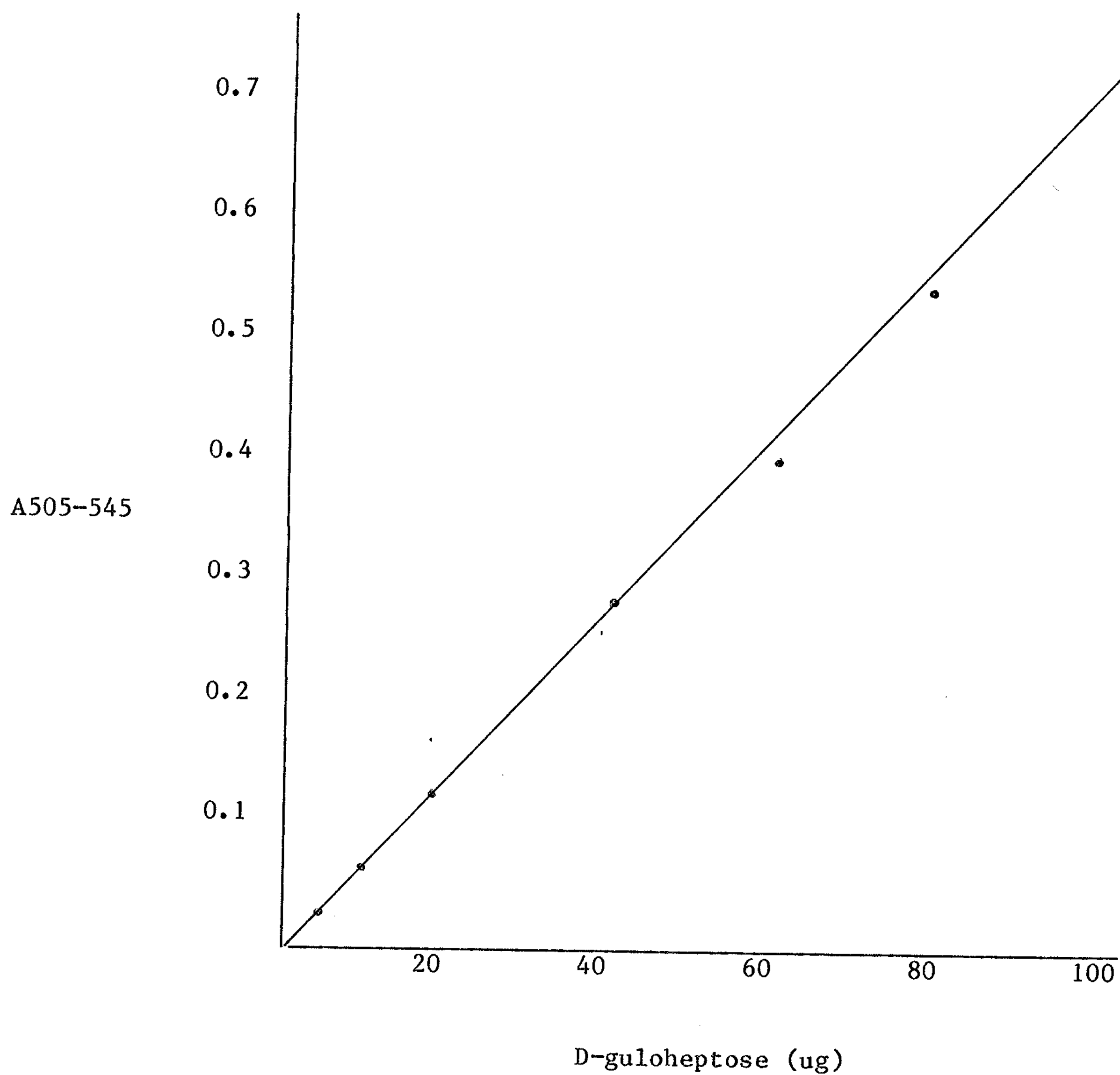
(ii) Standard curve for the determination of DNA in LPS



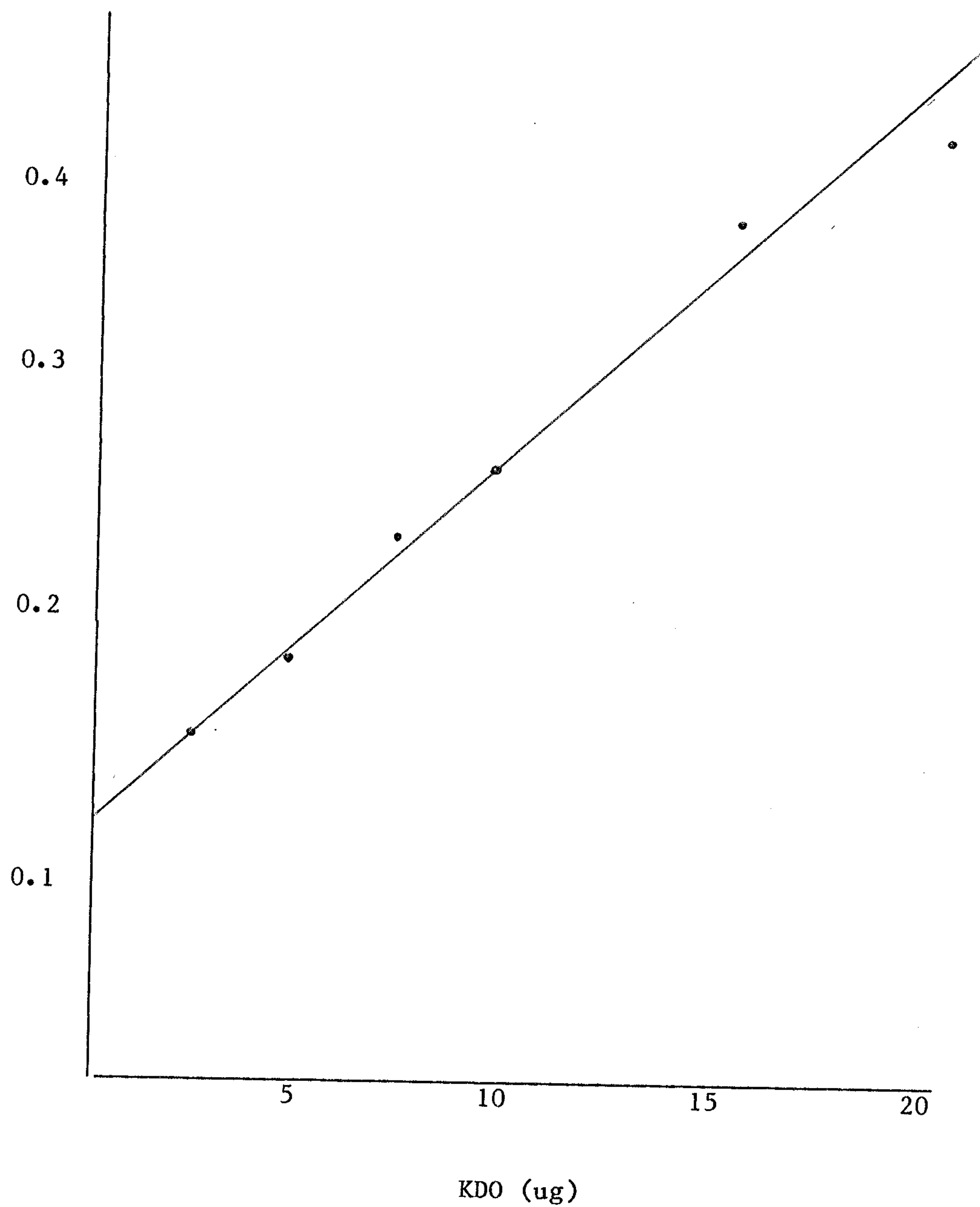
(iii) Standard curve for the determination of the total carbohydrate.



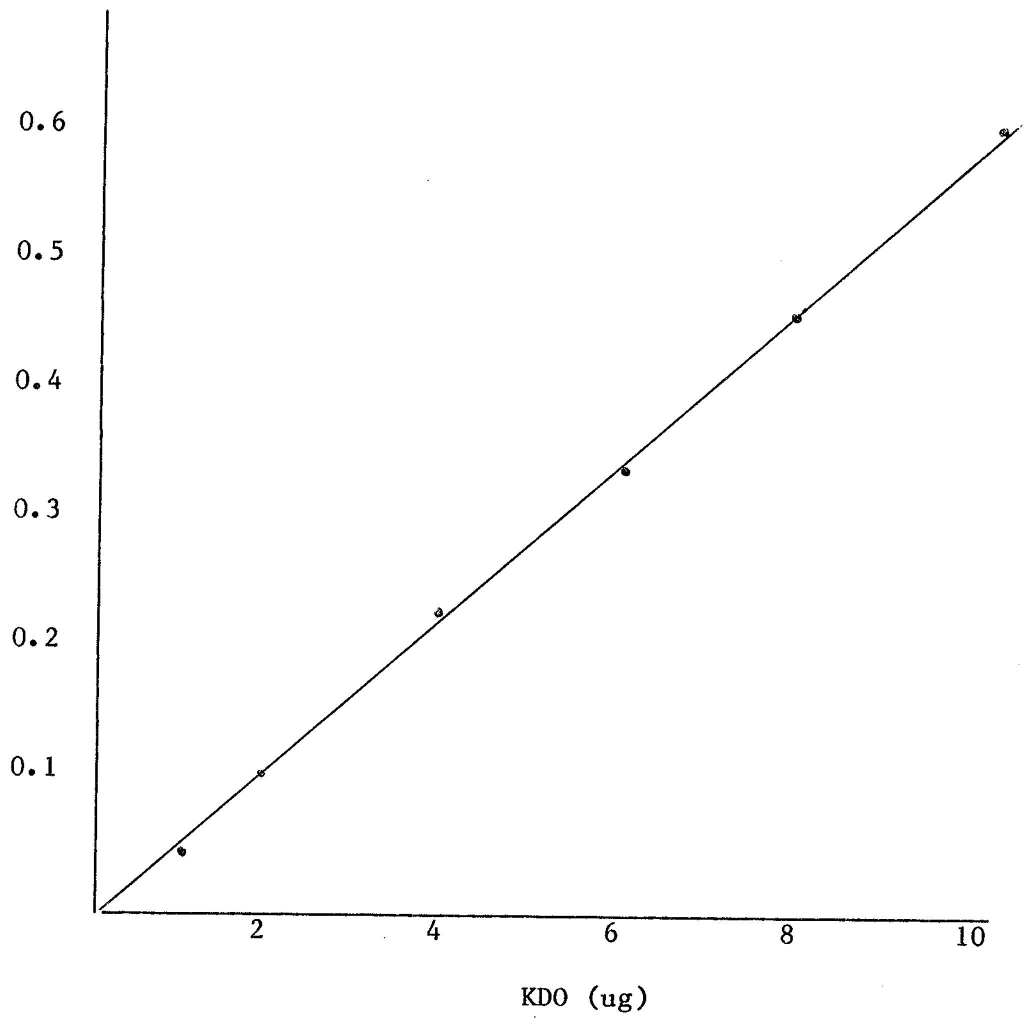
(iv) Standard curve for the determination of hexoses in LPS.



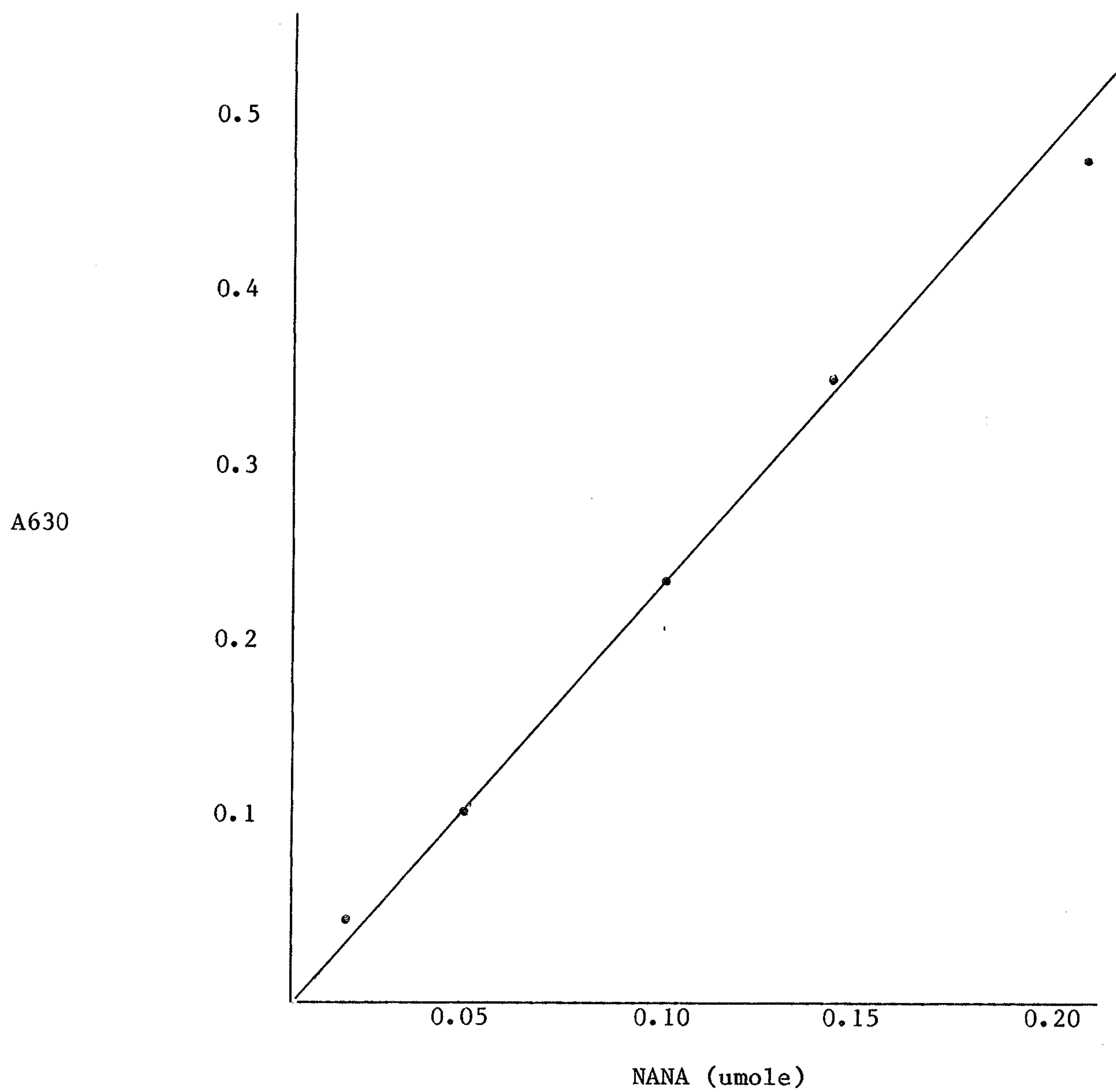
(v) Standard curve for heptose determination.



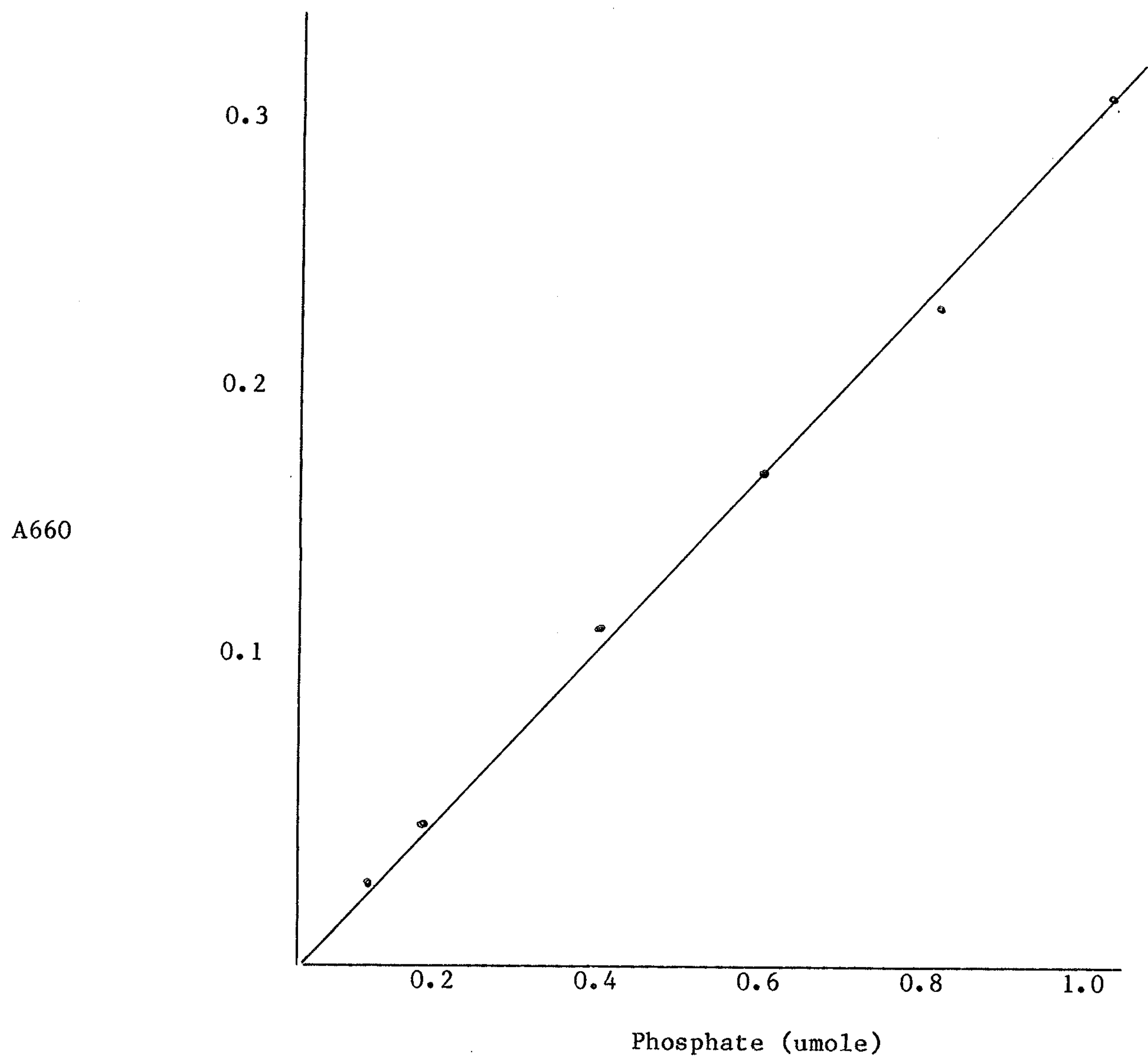
(vi) Standard curve for KDO determination (method of Brade *et al.*, 1983)



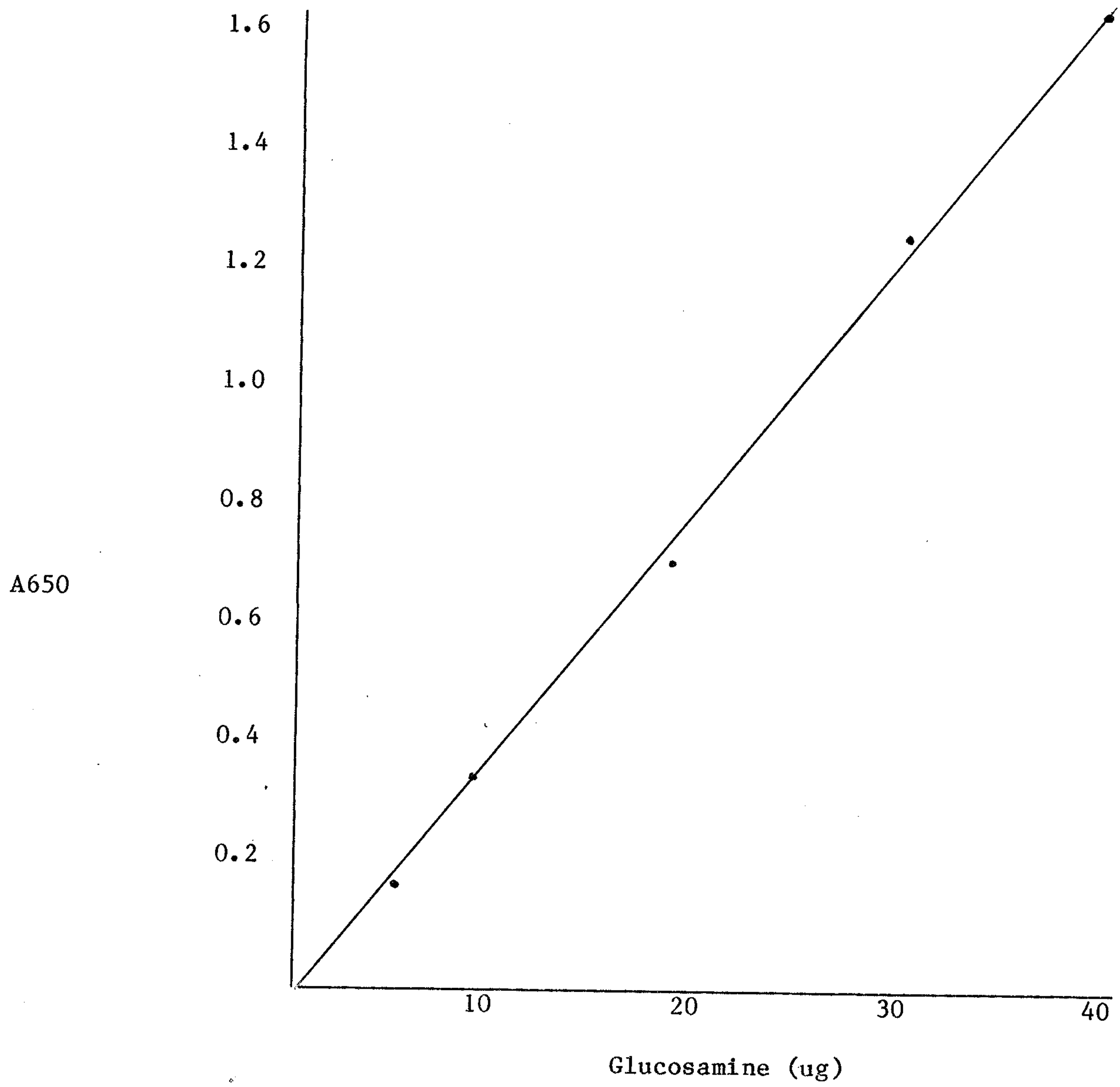
(vii) Standard curve for KDO determination (method of Karkhanis *et al.*, 1978).



(viii) Standard curve for N-acetylneuraminic acid (NANA) determination.



(ix) Standard curve for phosphate determination.



(x) Standard curve for hexosamine determination.