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SERUM RESISTANCE AND LIPOPOLYSACCHARIDE OF *HAEMOPHILUS DUCREYI*

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A dissertation submitted to the Faculty of Medicine, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree Master of Science.

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ABSTRACT

The pathogenesis of *Haemophilus ducreyi,* the aetiological agent of chancroid is poorly understood. An ability to resist killing by pooled normal human serum (PNHS) appears to contribute to the virulence of several Gram-negative bacteria, influenced by their lipopolysaccharide (LPS) in certain cases. The aim of this investigation is to measure the degree of serum resistance in clinical strains of *H. ducreyi,* and to determine whether their LPS plays a role in this serum resistance.

On exposure of three reference strains of *H. ducreyi* (A75, A76 and A77; Pasteur Institute, France) and two clinical strains (Y155 and Y206; Carletonville, South Africa) to 50 per cent PNHS, the clinical strains were more resistant to complement-mediated killing. Reference strains from the Pasteur Institute are less toxic in intradermal animal inoculations than freshly isolated clinical strains. The finding that clinical strains (Y155 and Y206) were more serum resistant than the reference strains (A75, A76 and A77) in this study suggests that serum resistance may contribute to the virulence of *H. ducreyi.*

LPS analysis using polyacrylamide gel electrophoresis (PAGE) and biochemical tests specifically measuring the carbohydrate markers of LPS did not conclusively reveal a significant difference associated with this variation in serum resistance. On PAGE the pattern produced by the LPS of *H. ducreyi* conforms to rough-type LPS. Biochemically, the LPS contained hexose, heptose, hexosamine and 2-keto-3-deoxyoctonate (KDO).

A76*, an isogenic strain of A76 which was passaged in PNHS, was killed more slowly than its parental strain. Associated with this delayed serum killing was a quantitative increase in LPS components, and the hexose : KDO ratio. Based on the findings that LPS of the isogenic 'serum-resistant' strain A76* and its parental 'serum-sensitive' strain A76 were different, it is possible that the LPS may be partly responsible for the serum resistance of *H. ducreyi.* However, PAGE and the biochemical tests used in this study did not detect differences in the LPS of the serum-sensitive reference strains and the serum-resistant clinical strains.

DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the degree of Master of Science in the faculty of Medicine, University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

Jocelyn Leonie Jardine

JJardine

February 1990

I hereby declare that the Committee for Research on Human Subjects has approved : 'Serum resistance and lipopolysaccharide of Haemophilus ducreyi' on the 31 October 1988 (Protocol number 20/10/88).

JJardine

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LIST OF ABBREVIATIONS

ATCC	American type culture collection
BHI	brain heart infusion
C′	complement
CAT	chloramphenicol acetyltransferase
CFU	colony forming unit/s
CHO	carbohydrate
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetraacetic acid
FCS	foetal calf serum
GC	gonococcal agar
G-C	guanine-plus-cytosine
HIV	human immunodeficiency virus
IFA	immunofluorescent antibody assay
Ig	immunoglobulin
KDO	2-keto-3-deoxyoctonate
LPS	lipopolysaccharide
MAC	membrane attack complex
M-H	Mueller-Hinton
MIC	minumum inhibitory concentration
NCTC	National collection of type cultures
OMP	outer membrane protein/s
PAGE	polyacrylamide gel electrophoresis
PMNL	polymorphonuclear leucocytes
PNHS	pooled normal human serum
RNA	ribonucleic acid
S.A.I.M.R	South African Institute for Medical Research
SDS	sodium dodecyl sulphate
T	doubling time
tris	tris (hydroxymethyl) aminomethane
μ	growth rate

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INTRODUCTION

1.1 HISTORICAL BACKGROUND

Possibly the earliest account of chancroid was given by Hippocrates, who recognised that certain genital ulcers were accompanied by buboes. Celsus divided genital ulcers into two groups : clean and dry, and moist and purulent (Kampmeier, 1979; Bilgeri, 1983a; Morse, 1989). Syphilis was distinguished from chancroid by Bassereau in 1892. He observed that non-indurated (soft sore) lesions did not progress to a generalized infection as did syphilis, and that each resulted from exposure to a clinically similar lesion. Earlier, Ricord and Clerc (1854) were unconvinced that two separate diseases caused these genital ulcers, and proposed that the non-systemic form was due to an attenuation in virulence of the syphilis 'virus'. In the same way as an attenuated form of variola was called varioloid, this type of chancre was called 'chancroid' introducing, for the first time, the term currently used to describe this infection (Bilgeri, 1983a).

In 1889, Augusto Ducrey at the University of Naples, observed bacilli in stained smears from chancroid lesions that were 1.5µm long and 0.5µm wide with rounded edges, occasionally showing indented centres. In tissue they appeared in chains, but in secretions as single organisms. Since he was unable to culture the organism on nutritive gelatin or other artificial media, Ducrey passaged three strains fifteen times by serial inoculation of lesion material into the skin of patients and observed similar lesions as the original genital infection. In this manner, the exudate was cleared of contaminants, and the bacillus retained its virulence (Ducrey, 1889; Kampmeier, 1979; McEntergart *et al,* 1982; Bilgeri, 1983a; Morse, 1989).

Eleven years elapsed before the first successful culture of the aetiological agent of chancroid, *Haemophilus ducreyi,* was achieved in artificial media. Initially media containing rabbit

blood (Teague and Deibert, 1920; Saelhof, 1924; Greenwald, 1943; Satulsky, 1945; Deacon *et al,* 1954; Kerber *et al,* 1969), were used, but cultures grown on these media and their successors were associated with high rates of contamination by associated bacteria inhabiting genital ulcers (Nayyar *et al,* 1979; Kraus *et al,* 1982a; Nobre, 1982; Sturm *et al,* 1987a). In an attempt to develop more selective media, aniline dyes (Saelhof, 1924), penicillin, potassium tellurite and gentian violet (Reymann, 1947) were incorporated into the media but these proved to be inefficient. A significant advance was made with the incorporation of vancomycin as a selective agent by Hammond *et al* (1978a). Results of isolation of *H. ducreyi* obtained after 1978 were more consistent than previously observed, and additional media have since been formulated.

Blood or blood products are a prerequisite for the isolation of *H. ducreyi.* Several sources have been proposed, including rabbit blood (Teague and Deibert, 1920; Hammond *et al,* 1978a; Sottnek *et al,* 1980; Nobre, 1982; Messing *et al,* 1983; Sturm *et al,* 1987a), sheep blood (Reymann, 1947; Messing *et al,* 1983), human blood (Deacon *et al,* 1954; Kerber *et al,* 1969; Borchardt and Hoke, 1970; Sng *et al,* 1982), horse blood (Kilian and Theilade, 1975; Bilgeri *et al,* 1982; Nobre, 1982; Meheus *et al,* 1983; Nsanze *et al,* 1984; Dylewski *et al,* 1986; Kunimoto *et al,* 1986), and haemin (Kinghorn *et al,* 1982a; 1982b). The isolation of African strains has improved with the enrichment of media as developed by Hammond *et al* (1978a) containing gonoccocal agar, haemoglobin, IsoVitaleX and vancomycin, with foetal calf serum (FCS) (Sottnek *et al,* 1980; Nsanze *et al,* 1981; 1984). In support of this finding was a study conducted in Swaziland in which an extremely low isolation rate of *H. ducreyi* with gonococcal agar base, haemoglobin and vancomycin without FCS was reported (Meheus *et al,* 1983).

Optimal conditions for growth of *H. ducreyi* appear to be a neutral pH, an atmosphere containing 5 per cent carbon dioxide and incubation in humid conditions (Satulsky, 1945; Beeson, 1946; Reymann, 1947; Hammond *et al,* 1978a). Most reports indicate good isolation rates at temperatures between 33^0 C to 35^0 C.

A combination of at least two different media has been advised for maximum isolation rates. Currently the medium containing gonococcal agar base, haemoglobin, IsoVitaleX and FCS (Nsanze *et al,* 1981), used in conjunction with media consisting of Mueller-Hinton agar base, horse blood, IsoVitaleX and vancomycin appears to be the most efficient for the isolation of African strains (Bilgeri, 1983b; Nsanze *et al,* 1984; Dylewski *et al,* 1986; Kunimoto *et al,* 1986; Dangor, 1988a). Other recently described media include a modified haemin medium or Sheffield medium (Kinghorn *et al,* 1982a; 1982b; Hafiz *et al,* 1982; 1984; MacDonald *et al,* 1989) containing gonococcal agar base, peptone, gelatin, defined supplement and haemin, and Bielings medium (Sturm *et al,* 1984a; Kunimoto *et al,* 1986) containing Columbia agar, horse blood and yeast dialysate. These have however not proved to be superior to Nsanze's medium. More recently, a medium consisting of gonococcal agar base, horse blood, Fildes extract and vancomycin has been reported which yielded results comparable to Nsanze's medium (Y. Dangor, personal communication).

Evaluation of transport media has been lacking. Cary-Blair medium (Kerber *et al,* 1969), Amies transport medium (Borchardt and Hoke, 1970; Choudhary *et al,* 1982; Mallard *et al,* 1983) and Stuart's medium (Sturm *et al,* 1987a) have been proposed, but with little reproducible success. More recently Dangor (personal communication) has developed a novel haemin-based transport medium. This has yielded primary isolation rates of 86 per cent up to seventy-two hours after collection and storage at 4^0 C. .

Successful storage of *H. ducreyi* by lyophilisation has been reported (Reymann, 1947; Deacon *et al,* 1954; Bilgeri *et al,* 1982). However, this organism is most commonly preserved by freezing at minus 70^0 C or in liquid nitrogen in skim milk (Oberhofer and Back, 1982; Odumeru *et al,* 1984; Taylor *et al,* 1985; Lubwama *et al,* 1986). A storage medium consisting of brain heart infusion broth and rabbit blood at minus 70^0 C is currently being used.

1.2. EPIDEMIOLOGY

Chancroid is generally regarded as a disease of the tropics. However the isolation of *H. ducreyi* has been achieved worldwide as Table 1.1 indicates.

Table 1.1 Occurrence of chancroid.

LOCATION	REFERENCE
Durban / South Africa	Coovadia *et al* 1985
Johannesburg / South Africa	Duncan *et al* 1981
Johannesburg / South Africa	Dangor 1988a
Pretoria / South Africa	Crewe-Brown *et al* 1982
Swaziland	Meheus *et al* 1983
Zimbabwe	Latif 1982
Nairobi / Kenya	Nsanze *et al* 1981
Nairobi / Kenya	Plummer *et al* 1983
Nairobi / Kenya	Ndinya-Achola *et al* 1986
Gambia	Mabey *et al* 1987
Nigeria	Joshi and Lawande 1983
Rwanda	Bogaerts *et al* 1989
Bangkok / Thailand	Taylor *et al* 1984
Singapore	Tan *et al* 1977
Singapore	Thirumoorthy *et al* 1986
India	Choudhary *et al* 1982
India	Sehgal and Prasad 1985
United States	Handsfield *et al* 1981
United States	Schmid *et al* 1987
Orange County, California	Blackmore *et al* 1985
Palm Beach County, Florida	Becker *et al* 1987
Britain	Hafiz *et al* 1984
Sheffield / England	Kinghorn *et al* 1982a; 1982b
Manchester / England	Mallard *et al* 1983
Winnipeg / Canada	Hammond *et al* 1980
Winnipeg / Canada	Diaz-Mitoma *et al* 1987
Rotterdam / Netherlands	Nayyar *et al* 1979
Amsterdam	Sturm 1987b
Greenland	Lykke-Olsen *et al* 1979
Antwerp / Belgium	Piot *et al* 1983
New Zealand / Australia	Anzimt 1982

In temperate countries, the occurrence of chancroid is irregular, with very few cases (Chapel *et al,* 1978; Mallard *et al,* 1983; Diaz-Mitoma *et al,* 1987). Localised outbreaks have been recorded, usually following importation of *H. ducreyi* into a sexually-active community (Hammond *et al,* 1980). Schmid *et al* (1987) reviewed the epidemiology of chancroid in the United States. The incidence peaked in 1947, corresponding to World War II, and decreased steadily until 1981. During 1971 to 1980, there was an average of 878 cases annually. Between 1981 and 1983, the incidence increased, boosted by an outbreak in California in which 923 cases were reported (Blackmore *et al,* 1985). In 1986, 94 per cent of all cases were found clustered in areas of New York, Texas, California, Florida and Georgia. Contact tracing and treatment contributed to the control of these outbreaks.

The incidence of chancroid increased during the major wars. 39 044 cases of chancroid were reported amongst troops of the United States army between 1917 to 1918, accounting for 11 per cent of all sexually-acquired diseases (Greenwald, 1943). In the same population group, chancroid accounted for 38.6 per cent (1942), and 22.5 per cent (1943), of sexually-transmitted diseases (Satulsky, 1945). An increased incidence of chancroid was also reported in the United States military during the Vietnam war (Kerber *et al,* 1969; Marmar, 1972; Carpenter *et al,* 1981).

Whether chancroid occurs in outbreaks, or epidemics, factors such as low socioeconomic status, a highly sexually-active population and frequent contact with prostitutes often determine its prevalence (Meheus *et al,* 1983; D'costa *et al,* 1985; Thirmoorthy *et al,* 1986).

Chancroid is the most common cause of genital ulcers in South Africa and other African countries (Duncan *et al,* 1981; Nsanze *et al,* 1981; Crewe-Brown *et al,* 1982; Latif, 1982; Meheus *et al,* 1983; Plummer *et al,* 1983; Coovadia *et al,* 1985; Ndinya-Achola *et al,* 1986; Mabey *et al,* 1987). Control is constrained because of the high infective rate, where diagnosis and treatment is problematic, and promiscuity - as in the case of migrant workers - inevitable. In addition, an increase in resistance to antimicrobial agents such as penicillin,

sulphonamide and tetracycline has been observed worldwide (Handsfield *et al,* 1981; Sng *et al,* 1982; Fast *et al,* 1983; Nsanze *et al,* 1984; Taylor *et al,* 1985), including South Africa (Bilgeri *et al,* 1982; Dangor *et al,* 1988b). This has important implications for the appropriate treatment.

Worldwide the male : female ratio indicates a predominance of male infection, and varies from 1.6 : 1 (Lykke-Olsen *et al,* 1979), to 27 : 1 (Sehgal and Prasad, 1985). This ratio may be exaggerated due to the longer incubation time in females (average thirteen days) than in males (average four days), fear and difficulty of self-examination by females and stoicism (Hammond *et al,* 1980). In South Africa, the majority of infected individuals are black males.

H. ducreyi is generally believed not to be part of the body's normal flora (Messing *et al,* 1983). Results of older studies that appear to conflict with this belief (Brams, 1924; Saelhof, 1925), could be due to misidentification of the organism.

Asymptomatic carriage of *H. ducreyi* by males (Kunimoto *et al,* 1986) and females (Plummer *et al,* 1983; D'Costa *et al,* 1985; Diaz-Mitoma *et al,* 1987) is rare, and does not appear to be of epidemiological importance. Prostitutes (commercial sex workers) have been incriminated as a major reservoir of sexually-transmitted diseases, including chancroid (Hammond *et al,* 1980; Plummer *et al,* 1983; Blackmore *et al,* 1985). The presence of old healed ulcers (Felman and Nikitas, 1983) or cervical ulcers (Ronald and Plummer, 1985) may contribute to asymptomatic carriage of *H. ducreyi* in females.

Alergant (1972) suggested that different races vary in susceptibility to chancroid e.g. in the United States, the most commonly infected were blacks and hispanics (Hammond *et al,* 1980; Schmid *et al,* 1987). Greenwald (1943) described a population where the ratio of coloureds to whites was 1 : 5, but the admissions for chancroid were in a ratio of 120 : 1. This disposition was not observed for other sexually-acquired diseases.

The age of infected individuals corresponds to the most sexually-active years. In a study of migrant mineworkers in Carletonville, South Africa, 77 percent were between twenty and thirty-two years of age, the average age being 23.4 (Dangor, 1988a).

In Africa, prostitution is an important factor in the transmission of venereal diseases. Chancroid is most prevalent in the lower and middle classes of prostitutes. These two groups have an average of 5.04 and 5.63 sexual partners a day, respectively (D'Costa *et al,* 1985). Prostitution is an economic necessity for unskilled females in poverty-stricken areas. In Nairobi, 70 per cent of all prostitutes have at least one sexually-transmitted disease. Prostitution is also promoted by the constant influx of male migrants into mining areas, resulting in a very high-risk promiscuous group. Of 240 migrant mineworkers studied in Carletonville, Transvaal, 15 per cent implicated prostitutes as the source of infection, 31 per cent casual partners, 51 per cent short-term regular girlfriends and 4 per cent spouses (Dangor, 1988a). In addition to migrant workers having multiple sexual partners, polygamy occurs more frequently in this population.

In a review Cates (1988) concluded that all sexually-transmitted diseases are linked - behaviourally, epidemiologically, biologically, clinically, economically and historically. An association between genital ulcers (herpes, syphilis or chancroid) in both homosexual men and heterosexuals, and human immunodeficiency virus (HIV) infection has been reported (Greenblatt *et al,* 1988; Piot *et al,* 1987; 1989), and this is hardly surprising. Evidence is accumulating indicating that genital ulceration disease increases susceptibility to HIV infection (Stamm *et al,* 1988; Piot and Laga, 1989). It is speculated that loss of epithelial integrity or bleeding as a result of genital ulcer infections could provide the portal of entry or exit for HIV infection. In addition, lymphocytes and macrophages associated with inflammation may present an increased pool of target cells for HIV infection (Cates, 1988; Piot and Laga, 1989). Possibly this association between HIV infection and genital ulceration disease contributes to the rapid spread of HIV in Africa.

1.3 LABORATORY AND PHYSICAL FEATURES

1.3.1 MORPHOLOGICAL AND CULTURAL CHARACTERISTICS

Between 1889 and 1978, most descriptions of *H. ducreyi* were based on smears of lesion material (Greenwald, 1943; Heyman *et al,* 1945; Borchardt and Hoke, 1970). This has been shown to be an inaccurate and variable method. The Ducrey bacillus was described as non-motile, non-sporing, non-acid fast, small Gram-negative streptobacilli appearing in pairs, groups, chains lying parallel, or in a 'school-of-fish' pattern. The dimensions were 0.3µm to 0.5µm wide by 0.6µm to 2µm long with a chain formation of three to twenty-five cells per chain (Ducrey, 1889; Brams, 1924; Saelhof, 1924; Satulsky, 1945; Felman and Nikitas, 1983). Other workers have declined to estimate the dimensions, since pleomorphism was observed. Bipolar staining was also observed (Allison, 1945; Alergant, 1972; Lubwama *et al,* 1986).

More consistent results have been obtained with the introduction of selective culture media. A Gram-stain from colonies generally shows pleomorphic rods varying from coccoid to filamentous, and in clumps or in short chains (Alergant, 1972; Hammond *et al,* 1978a; Nobre, 1982; Nsanze *et al,* 1984; Odumeru *et al,* 1984; Lubwama *et al,* 1986). Occasionally, the typical 'school-of-fish' pattern is observed (Oberhofer and Back, 1982). In liquid media, *H. ducreyi* appears to form longer chains (Brams, 1924; Saelhof, 1924; Hammond *et al,* 1978a; Nobre, 1982; Sng *et al,* 1982; Felman and Nikitas, 1983).

The cellular morphology of *H. ducreyi* thus appears to depend on the strain, the age of the culture, the growth medium and the staining technique. These factors also affect the colony morphology. On a clear modified haemin medium containing gonococcal agar base, peptone, gelatin, defined supplement and haemin (Kinghorn *et al,* 1982a; Hafiz *et al,* 1982; 1984), starch aggregation around the colonies was reported. Deacon *et al* in 1954 described colonies of old stock cultures as small (0.5 - 1mm diameter) and rough, while clinical isolates formed smooth, white, larger (1 - 3mm diameter) colonies.

H. ducreyi colonies are generally round (Brams, 1924; Saelhof, 1924), small (Saelhof, 1924; Nobre, 1982), convex (Brams, 1924; Saelhof, 1924; Nobre, 1982; Oberhofer and Back, 1982; Nsanze *et al,* 1984; Lubwama *et al,* 1986), yellow-grey (Hammond *et al,* 1978a; Nobre, 1982; Oberhofer and Back, 1982; Sng *et al,* 1982; Nsanze *et al,* 1984; Lubwama *et al,* 1986), granular (Oberhofer and Back, 1982; Ronald and Plummer, 1985), non-mucoid (Hammond *et al,* 1978a; Felman and Nikitas, 1983; Odumeru *et al,* 1984) and 1 - 2mm in diameter (Lubwama *et al,* 1986). These colonies are characteristically cohesive and can be pushed across the agar surface with an inoculating loop. The organism auto-agglutinates and forms chains in a liquid medium. This property may result in inconsistent results when a standardised inoculum is required, for example serum bactericidal assays and antibiotic susceptibility testing.

1.3.2 BIOCHEMICAL FEATURES

H. ducreyi has traditionally been assigned to the genus *Haemophilus* because of its X-factor (haemin or certain other porphyrins) requirements, Gram-negative staining and pleomorphic cellular morphology (Kilian and Biberstein, 1984).

Biochemical tests have been applied to the identification of *H. ducreyi*, however some have yielded conflicting results. Table 1.2 lists the results obtained by various investigators.

The result of the oxidase test varied between investigators and differed according to the substrate employed. A positive test results was obtained when using tetra-methyl-p-phenylene-diamine, and a negative result with di-methyl-p-phenylene-diamine (Nobre, 1982; Lubwama *et al,* 1986). Sng *et al* (1982) suggested that the oxidase reaction could be weakly positive due to a non-specific action of haemin.

Sturm and Zanen (1984b) reported that the catalase test was positive by the tube method, but not by the plate method.

Nitrate reduction results were consistent after a six hour incubation period, but varied with prolonged incubation over several days (Sng *et al,* 1982). Several other investigators have reported strains which fail to reduce nitrate (Hammond *et al,* 1978a; Sottnek *et al,* 1980; Sturm and Zanen, 1984b).

Sottnek *et al* (1980) reported three strains of *H. ducreyi* that demonstrated weak urease activity. Urease activity has not been previously reported for *H. ducreyi.*

Sng *et al* (1982) reported utilisation of the carbohydrates glucose, mannose and fructose, by *H. ducreyi* : a finding that has not been corroborated by other workers. This discrepancy could have been due to the method employed. The method used involved impregnating filter paper with the reagent, followed by heavy inoculation of the paper, and incubation. This allowed for more cell-reagent contact than the conventional tube method, and contributed to the positive results obtained. Based on a similar principle, Sturm and Zanen (1984b) suggested that enzymatic tests could be negative as a result of a low inoculum size.

Table 1.2 Biochemical identification tests.

REFERENCES	oxidase	catalase	X factor requirement	nitrate reduction	alkaline phosphate	urease	ornithine decarboxylase	H_2S production	indole production
Hammond *et al* 1978	-	-				-	-	-	-
Carpenter *et al* 1981			+	+		-	-		
Choundary *et al* 1982				+	+				
Kinghorn *et al* 1982a	-		+	+			-	-	-
Nobre 1982	+/-[a]		+						
Oberhofer and Back 1982	-	-		+					
Sng *et al* 1982	+			+/-[b]	+				
Bilgeri 1983b	+	-	+/-[c]	+	+	+/-[c]	+/-[c]	-	-
Felman and Nikitas 1983		-	+	+	+		-	-	-
Becker and Larsens 1984	+	+	+	+	+				
Odumeru *et al* 1984	+	-				-	-	-	-
Nsanze *et al* 1984	+		+	+					
Lubwama *et al* 1986	+	-	+					-	-

[a] Substrate dependent
[b] Incubation period dependent
[c] One or two of eighty-six clinical isolates with uncharacteristic biochemical features.
H_2S hydrogen sulphide.

H. ducreyi has been reported not to ferment various sugars, viz. glucose, sucrose, lactose, xylose, maltose, deoxyribose and mannitol (Hammond *et al,* 1978a; Carpenter *et al,* 1981; Odumeru *et al,* 1984). However, Choudhary *et al* (1982) reported fermentation of glucose and sucrose by *H. ducreyi.*

Commercial identification kits have been applied to the biochemical identification of *H. ducreyi.* These include the Mintek system (Oberhofer and Back, 1982), API ZYM (Casin *et al,* 1985; Van Dyck and Piot, 1987) and Rapid ID NH (Hannah and Greenwood, 1982). These kits did not produce identical results. Sturm and Zanen, (1984b) studied twenty-nine international strains on twenty-eight substrates to determine enzymatic activity. Of the tests, only three were in agreement i.e. alkaline phosphatase, arginine aminopeptidase and lack of glycosidases. He suggested that the differences observed might reflect variations in biotypes so that uniformity in one study might be due to testing of a common pool of isolates. This argument could likewise be applied to the geographical distribution of β-lactamase production.

H. ducreyi may also be characterised on the basis of its outer membrane protein structure. Several workers have grouped strains of *H. ducreyi* into subtypes by comparing their protein profiles on sodium dodecyl sulphate polyacrylamide gels. Results of independent studies (Odumeru *et al,* 1983; Sanson-Le Pors *et al,* 1985; Taylor *et al,* 1985) did not produce similar subtype groupings.

The Gram-stain is a key taxonomic characteristic, as it reflects the structure of the bacterial cell wall. Kilian and Theilade (1975) studied the ultrastructure of three clinical strains isolated by Reymann in 1947, and one control strain. All stained Gram-negatively, but with different intensities. Curiously, transmission electron microscopy studies revealed that the clinical strains had a Gram-positive cell wall structure; as opposed to the control which had a typical Gram-negative cell wall structure. Deacon *et al* (1954) also previously reported a Gram-positive form of *H. ducreyi.* Perhaps the clinical strains were not the *'H .ducreyi'* we know

today, since identification of the organisms was based solely on cultural characteristics. In subsequent studies, electron micrographs have shown that the outer membrane of *H. ducreyi* appears to be similar to those of other Gram-negative bacteria (Morse, 1989).

Most recently, studies of deoxyribonuclease relatedness using twenty-five clinical strains of *H. ducreyi* from diverse geographical origins, revealed that the organism constituted a homogeneous deoxyribonucleic acid (DNA) hybridisation group. Although *H. ducreyi* is classified within the genus *Haemophilus* with respect to biochemical tests and has a guanine-plus-cytosine (G-C) mol ratio of 38 per cent which is similar to *Haemophilus influenzae* (Kilian, 1976), the organism has only a 0 to 6 per cent hybridisation homology with other *Haemophilus* species (Casin *et al,* 1985). The validity of including *H. ducreyi* in the genus *Haemophilus* can be questioned from investigations on the isoprenoid quinones of the organism. Quinones are lipophilic components of the cytoplasmic membrane which are used as markers in taxonomic grouping since strains with approximately 70 per cent DNA homology have the same quinone composition. Carlone *et al* (1988) found two isoprenoid quinone structural types, viz. demethylmenaquinone and menaquinone, in *H. ducreyi.* This represents a major physiological and chemotaxonomic difference between *H. ducreyi* and other *Haemophilus* species.

H. ducreyi appears to have phenotypic and biochemical features that are similar to other members of the genus *Haemophilus* (Kilian, 1976). It is also antigenically cross-reactive with other *Haemophilus* species (Saunders and Folds, 1986; Schalla *et al,* 1986). However DNA homology studies and isoprenoid quinone analysis suggest otherwise (Kilian and Theilade, 1975; Casin *et al,* 1985; Carlone *et al,* 1988). On the basis of genetic transformation studies, Gromkova (personal communication) suggested that *H. ducreyi* was only distantly related to *H. influenzae* and *Haemophilus parainfluenzae.*

For convenience, the traditional nomenclature of *H. ducreyi* is still retained, and this name is applied to pleomorphic Gram-negative bacteria that are X-factor requiring, nitrate reducing, alkaline-phosphatase positive and non-saccharolytic.

Studies of epidemological markers for *H. ducreyi* have been lacking. Plasmid and antigen typing may contribute to the study of the distribution of strains. Identical plasmids were found in strains from patients who had been in contact with one another (Handsfield *et al,* 1981). Plasmids of identical molecular weight (Thomson and Bilgeri, 1982) or similar restriction endonuclease-treated fragments of plasmids (Totten *et al,* 1982) may indicate the geographical origin of strains. Several other techniques have been suggested to differentiate strains on a geographical basis e.g. outer membrane protein typing (Taylor *et al,* 1984), immunoassays (Saunders and Folds, 1986; Schalla *et al,* 1986), enzyme profiles (Van Dyck and Piot, 1987) and lectin agglutination (Korting *et al,* 1988).

1.4 VIRULENCE FACTORS

The indication of virulence of a strain of *H. ducreyi* has not significantly changed with time. Following the discovery by Ducrey in 1889, human inoculation was used for diagnosis (Cole and Levin, 1935; Reymann, 1947; Dienst, 1948), rather than for the purpose of reflecting differences in the virulence of strains. This was discontinued since results obtained were non-specific, and the ethics of the method controversial. Intradermal inoculation of rabbits yielded more consistent results (Dienst, 1948; Deacon *et al,* 1956). A positive result was taken as an induration of greater than 0.5cm four days after the inoculation (Hammond *et al,* 1978a; Odumeru *et al,* 1984). The success of these results depended on several factors i.e. the size of the bacterial inoculum (Choudhary *et al,* 1982), the virulence of the strain, the number of passages *in vitro* and the experimental animal used. In two separate studies, control *H. ducreyi* strains from the Pasteur Instititute, France (A75, A76 and A77) were

avirulent, while local Canadian strains were virulent with respect to intradermal rabbit inoculation studies (Hammond *et al,* 1978a; Odumeru *et al,* 1984).

Virulence, as defined by rabbit inoculation studies and development of skin lesions, is rather presumptive, since it implies comparable reactions in human individuals. Ulcers in rabbits healed spontaneously after two to three weeks (Reymann, 1949; Deacon *et al,* 1954; 1956). Dienst (1948) reported a persistence of skin lesions for seven to ten days in rabbits. This observation suggests there is a difference between the way experimental animals and humans react to intradermal inoculation of *H. ducreyi.* Intradermal inoculations of mice resulted in lesions more closely resembling that in humans than in rabbits (Tuffrey *et al,* 1988), although the lesions also healed spontaneously within thirteen days.

The virulence of *H. ducreyi* may not be a stable trait. Loss of virulence was observed after *in vitro* subculture by Deacon *et al* (1956), but not by Hammond *et al* (1978a).

Several factors account for the pathogenicity of Gram-negative bacteria (Brubaker, 1985). Few studies of the virulence factors in *H. ducreyi* have been reported. Odumeru *et al* (1984) found that virulence was related to resistance to killing by complement in normal human serum, and resistance to phagocytosis and killing by polymorphonuclear monocytes. Resistance to polymyxin B was associated with virulence (Hammond *et al,* 1978a), but disproved in later studies (Odumeru *et al,* 1984). Other aspects affecting the pathogenicity of *H. ducreyi* have been investigated, e.g. cell wall structure (Taylor *et al,* 1985; Odumeru *et al,* 1983; 1987; Abeck *et al,* 1987a; 1987b), antibiotic-resistance mechanisms (MacLean *et al,* 1980; Handsfield *et al,* 1981; Brunton *et al,* 1982; Deneer *et al,* 1982; Totten *et al,* 1982; Anderson *et al,* 1984; Albritton *et al,* 1982; 1984), lectin agglutination (Korting *et al,* 1988) and antigenicity (Hansen and Loftus, 1984; Slootmans *et al,* 1985; Saunders and Folds, 1986; Schalla *et al,* 1986).

1.5 CLINICAL FEATURES

The clinical symptoms of chancroid are not well defined and vary considerably. The appearance of the lesions has been described (Greenwald, 1943; Satulsky, 1945; Alergant, 1972; Gaisin and Heaton, 1975; Felman and Nikitas, 1983; Becker and Larson, 1984; Ronald and Plummer, 1985). It begins as a tender papule, becoming pustular, eroded and ulcerated. It is irregular, undermined, with yellow-grey necrotic material covering its base. The lesions may remain pustular or merge to form large ulcers (Figure 1.1).



Figure 1.1 Typical chancroid ulcer (courtesy of Y. Dangor, S.A.I.M.R, South Africa).

The incubation time from sexual contact to the appearance of ulcers varies in males and females, and between individuals. The average period is seven days, although it may be longer in females (Allison, 1945; Lykke-Olsen *et al,* 1979; Hammond *et al,* 1980). The average size of the lesion is between 5mm (Kerber *et al,* 1969) and 7mm (Thirmoorthy *et al,* 1986) but ranges from 1mm to 30mm (Kerber *et al,* 1969; Gaisin and Heaton, 1975; Becker and Larson, 1984; Thirmoorthy *et al,* 1986). Multiple lesions are more common than single ulcers.

The occurrence of lesions differs in males and females. Hammond *et al* (1980) suggested various factors may be involved e.g. area of sexual trauma, moisture, temperature, epithelial thickness or specificity of tissue. In males, the areas affected are prepuce, frenulum and coronal sulcus, with the shaft, glans and anus being less affected areas (Greenwald, 1943; Satulsky, 1943; Kerber *et al,* 1969; Alergant, 1972; Gaisin and Heaton, 1975; Hammond *et al,* 1980; Nsanze *et al,* 1981; Felman and Nikitas, 1983; Becker and Larson, 1984; Taylor *et al,* 1984). In females, lesions occur on the labia, fourchette, vestibule and clitoris. Less affected areas are the vagina, cervix and anus (Allison, 1945; Alergant, 1972; Gaisin and Heaton, 1975; Hammond *et al,* 1980; Felman and Nikitas, 1983; Becker and Larsen, 1984).

Extragenital lesions are rare, and probably occur as a result of auto-inoculation. Kinghorn *et al* (1983) described three cases where *H. ducreyi* was isolated from the oropharynx following orogenital contact with an infected individual. Lesions have also been found on the hands (Alergant, 1972; Gaisin and Heaton, 1975), on the tibia, and below the navel (Hammond *et al,* 1980), and on the foot (Marckmann *et al* 1989). Two cases of abdominal lesions have been reported in South Africa (F. Exposto, personal communication - Figure 1.2).



Figure 1.2 Extragenital (abdominal) chancroid (courtesy of Y Dangor, S.A.I.M.R, South Africa).

Lymphadenopathy, painful swellings in one or both groins, may form within two to fourteen days after the appearance of the ulcers (Alergant, 1972; Felman and Nikitas, 1983; D'costa *et al,* 1986; Sturm *et al,* 1987a) in 25 per cent to 60 per cent of the infected patients. The lymph nodes are enlarged, discrete and tender. If untreated the nodes increase in size greater than 5cm (Nsanze *et al,* 1984) forming fluctuant buboes finally resulting in suppuration and rupture (Alergant, 1972; Gaisin and Heaton, 1975; D'costa *et al,* 1986). In females

lymphadenopathy is uncommon and usually less severe. The response of buboes to treatment with most antimicrobial regimens varies from individual to individual. The recommended treatment for a fluctuant bubo is aspiration through normal skin and is repeated as often as necessary.

Serum resistance has been implicated as a virulence mechanism especially if the micro-organism invades the blood. Systemic infection by *H. ducreyi* has not been investigated. *H. ducreyi* does not appear to produce bacteraemia in its host. Perhaps serum resistance confers on *H. ducreyi* an ability to invade and multiply in human tissue resulting in necrotic genital ulcers. The clinical features of chancroid reflects the pathogenicity of *H. ducreyi* strains.

1.6 DIAGNOSIS OF CHANCROID

Prior to the discovery of suitable media for the isolation of *H: ducreyi*, diagnosis of chancroid was based largely on clinical features. Other techniques used were Gram-stained smears, skin tests, biopsy and culture. These old techniques were non-specific and insensitive. The descriptions of the Ducrey bacillus in older studies (Brams, 1924; Saelhof, 1925; Deacon *et al,* 1956) suggest that the organisms may not have been the same as present strains. This was confirmed by Kilian and Theilade (1975) who tested *H. ducreyi* strains isolated in 1947 in studies by Reymann and found that these strains had a Gram-positive cell wall structure. These factors should be borne in mind when reviewing older papers.

The accuracy of microbiological diagnosis of chancroid is made difficult by the polymicrobial flora present in genital ulcers (Chapel *et al,* 1978; Sehgal and Prasad, 1985; Thirmoorthy *et al,* 1986). Furthermore the concomitant presence of other sexually-transmitted pathogens, e.g. *Treponema pallidum* and herpes simplex virus may alter, accelerate or modify the progress, clinical characteristics and recoverability of a given infection (Allison, 1945). Often chancroid lesions are confused with ulcers due to other causes (Borchardt and Hoke, 1970;

Alergant, 1972; Kinghorn *et al,* 1982a; Becker and Larsen, 1984; Sehgal and Prasad, 1985). This may be due to mixed infections with herpes simplex virus (Becker and Larsen, 1984; Thirmoorthy *et al,* 1986), syphilis (Coovadia *et al,* 1985), lymphogranuloma venereum (Kraus *et al,* 1982a), septic scabies, or secondary invasion of other bacteria. *H. ducreyi* is sometimes isolated from lesions uncharacteristic of chancroid (Gaisin and Heaton, 1975; Sehgal and Prasad, 1985).

Ducreyi-like organisms have been isolated from genital ulcers of patients in Swaziland (Ursi *et al,* 1982) and the Netherlands (Sturm *et al,* 1987a). Their Gram-negative streptobacillus characteristics allowed for the preliminary classification in the genus *Haemophilus.* Ducreyi-like organisms differ from typical *H. ducreyi* in various respects including colony and cellular morphology, enzyme production, carbohydrate-fermenting ability, optimal growth conditions and viability *in vitro.* Because their isolation rate was low, between 6.5 and 16 per cent, and they were not easily cultured, limited studies have resulted.

1.6.1 MICROBIOLOGICAL DIAGNOSIS

Since the discovery of *H. ducreyi,* the observation of pleomorphic streptobacillus in the lesion exudate has been used for diagnosis (Brams, 1924; Allison, 1945; Kerber *et al,* 1969). Stains employed include Giemsa, Gram, Wright, Sellers and Pappenheimer (Gaisin and Heaton, 1975). Although staining is convenient and rapid, it is also insensitive and non-specific.

In early lesions fewer bacteria are present, and in more advanced lesions, many organisms from secondary infections appeared (Heyman *et al,* 1945). Nsanze *et al,* (1981) could not correlate 'smear' and culture of *H. ducreyi* for the diagnosis of chancroid.

Deacon *et al* (1956) described bacteria that shared common features with *H. ducreyi,* which could lead to misdiagnosis. These included Gram-variable aerobic streptococci; *'Corynebacterium acnes'* (Gilchrist) : a Gram-positive anaerobic rod antigenically related to *H. ducreyi*; and *Bacteriodes melaninogenicus* which has a similar morphology to that of *H. ducreyi* and was isolated from all lesions, but did not require blood for growth, and was not pathogenic for rabbits in the intradermal test.

The sensitivity of diagnosis by examination of Gram-stained smears is shown in Table 1.3.

Table 1.3 Sensitivity of Gram-stained smears in diagnosis of chancroid.

Expressed as % of clinical cases		Expressed as % of culture positive cases	
Greenwald 1943	65	Borchardt and Hoke 1970	87
Heyman *et al* 1945	52	Nsanze *et al* 1981	49
Nayyar *et al* 1979	82	Taylor *et al* 1984	62
Nsanze *et al* 1981	63	Coovadia *et al* 1985	28
Choudhary *et al* 1982	36	Sehgal and Prasad 1985	4.7
		Sturm *et al* 1987a	10

At present, diagnosis of chancroid is based on culture of *H. ducreyi* and confirmation of its identity by biochemical methods. This may be performed in any diagnostic laboratory (Oberhofer and Back, 1982; Lubwama *et al,* 1986). Other tests such as dark-field microscopy for syphilis, culture for herpes simplex virus and urethral or cervical swabs for *Neisseria gonorrhoeae* or *Chlamydia trachomatis* (Duncan *et al,* 1981; Becker and Larsen, 1984; Mabey

et al, 1987) should be performed on all patients suspected of having chancroid to exclude other concomitant pathogens.

Following the discovery of the Ducrey bacillus in 1889, investigators cultured the organism on media containing rabbit blood (Teague and Deibert, 1920; Saelhof, 1924; Greenwald, 1943; Satulsky, 1945; Deacon *et al,* 1954; Kerber *et al,* 1969), although alternative media have been described (Nayyar *et al,* 1979; Nobre, 1982; Sturm *et al,* 1984a; 1987a). The presence of a polymicrobial flora in genital ulcers has invariably resulted in contamination and inaccurate microbiological diagnosis.

A major advance in the isolation of *H. ducreyi* was the development of a selective medium by incorporating vancomycin (Hammond *et al,* 1978a) and enrichment with FCS (Sottnek *et al,* 1980; Nsanze *et al,* 1981; 1984). Media that include gonococcal agar base, haemoglobin and FCS appear to be the most efficient for the isolation of African strains including South African isolates. The use of at least two different media is advised for optimal isolation (Nsanze *et al,* 1984; Dylewski *et al,* 1986; Kunimoto *et al,* 1986). Differences in nutritional requirements (Ajello *et al,* 1956; Nsanze *et al,* 1984), or susceptibility to vancomycin (Hammond *et al,* 1978a) may be responsible for the growth of *H. ducreyi* on some, but not all, isolation media. Manufacture-related differences in media constituents (Coovadia *et al,* 1985) and differences between laboratory strains and fresh clinical strains (Deacon *et al,* 1956; Kilian and Theilade, 1975; Sturm *et al,* 1984a) have been described.

Optimal conditions for growth of *H. ducreyi* appear to be a neutral pH, an atmosphere of oxygen supplied with 5 per cent carbon dioxide, and humid conditions (Satulsky, 1945; Beeson, 1946; Reymann, 1947; Hammond *et al,* 1978a) and an incubation temperature of 33^0 C to 35^0 C.

1.6.2 IMMUNOLOGICAL METHODS FOR DIAGNOSIS

1.6.2.1 Ito-Reenstierna skin test

An antigen for use in an intradermal skin test consisting of killed *H. ducreyi* cells, was produced by Dmelco (Europe) and Lederle (United States), but is no longer commercially available. The procedure for the skin test has been described by Dienst (1948). A positive result presented as a delayed hypersensitive response showing induration of greater than 6cm diameter (Heyman *et al,* 1945). Although the positivity (expressed as a percentage of cases of clinically diagnosed chancroid), was as high as 75 per cent (Greenwald, 1943) and 77 per cent (Heyman *et al,* 1945), several disadvantages of this method have discouraged its use in diagnosis. These include a prolonged delay after initial infection of up to one to two weeks in obtaining an adequate intradermal response and persistence of the response for several years (Greenwald, 1943; Dienst, 1948). Thus one could not determine whether the infection was old or recent. False positive reactions occurred; Heyman *et al* (1945) found 33 per cent of 473 Negro Americans without chancroid were positive on this skin test.

1.6.2.2 Auto-inoculation

Immediately following Ducrey's pioneering work, a diagnosis of chancroid was made using auto-inoculation studies. A loopful of exudate from the ulcer was rubbed onto a scarified area of the forearm and bandaged. After forty-eight hours, small pustules formed, and *H. ducreyi* was identified by stained smears (Cole and Levin, 1935; Heyman *et al,* 1945). The disadvantages are that it is non-specific, since other pyogenic bacteria may be transferred (Heyman *et al,* 1945; Gaisin and Heaton, 1975); the ethics of the procedure is questionable; it necessitates confirmation by smear or culture techniques; it may spread the infection by

auto-inoculation (Sheldon and Heyman, 1946); and if used currently, would delay treatment with an antibiotic for several days.

1.6.2.3 Immunodiagnosis

Immunological studies of *H. ducreyi* have only recently been developed for diagnosis. Indirect immunofluorescent antibody assay (IFA) was first suggested by Denys *et al* (1978) to identify *H. ducreyi*. Results were significantly improved by the development of monoclonal antibodies (Hansen and Loftus, 1984) who used antibodies to detect *H. ducreyi* in smears of lesion material from experimentally-infected rabbits. Dot-immunobinding and IFA were later applied for the diagnosis of human genital ulcer disease (Schalla *et al,* 1986). The sensitivity of these tests was constrained, because a single antigen was used. Further substantiating this, is the observations of geographical differences in the patterns of outer membrane proteins of strains of *H. ducreyi* (Taylor *et al,* 1984). An immunoblot technique and an enzyme immunoassay was also described for immunodiagnosis of chancroid by Saunders and Folds, (1986) and Museyi *et al* (1988) respectively. Recently, DNA probes encoding *H. ducreyi*-specific proteins were selected for the identification of *H. ducreyi* in pure and mixed cultures. The success of these studies introduces a potentially new method for the diagnosis of chancroid (Parsons *et al,* 1989).

1.6.3 HISTOLOGICAL METHODS FOR DIAGNOSIS

Biopsy for the diagnosis of chancroid is not currently used, since it is non-specific and painful especially with respect to early lesions. It was previously used because securing tissue was a simpler and quicker procedure than culture of the organism, and also in cases where contamination was obvious (Sheldon and Heyman, 1946), or malignancy was suspected

(Alergant, 1972). Heyman *et al* (1945) and Sheldon and Heyman, (1946) described biopsy for diagnosis. The histological changes have been documented (Heyman *et al,* 1945; Sheldon and Heyman, 1946; Alergant, 1972). Three zones have been observed, viz. the base of the ulcer which is shallow containing polymorphonuclear leucocytes, fibrin, red blood cells and necrotic tissue; a wide layer of oedematous tissue with proliferating endothelial cells, newly-formed blood vessels and occasionally thrombosis; and a deep zone presented by a dense infiltration of plasma cells and lymphocytes.

1.6.4 CLINICAL DIAGNOSIS

Diagnosis of chancroid is based on clinical features of the ulcer i.e. tender, irregular and deep, with raised red margins and a soft purulent base (Dangor, 1988a; Boyd, 1989). The accuracy of this method is compromised by features that are non-characteristic of chancroid (Gaisin and Heaton, 1975; Sehgal and Prasad, 1985), or mixed infections of other sexually-acquired pathogens e.g. herpes simplex virus (Becker and Larsen, 1984; Thirmoorthy *et al,* 1986), *T. pallidum* (Coovadia *et al,* 1985) or *C. trachomatis* (Kraus *et al,* 1982a). Clinical diagnosis has been reported inconsistent in the United States (Chapel *et al,* 1978) and Kenya (Fast *et al,* 1984). In a study in Carletonville, South Africa, diagnostic accuracy was found to be 79 per cent for chancroid (Dangor, 1988a). The experience of clinicians in sexually-transmitted diseases, and the previously mentioned high incidence of chancroid in the population, may have contributed to this high percentage of diagnostic accuracy.

1.7 TREATMENT OF CHANCROID AND ANTIBIOTIC RESISTANCE IN HAEMOPHILUS DUCREYI

1.7.1 ADJUVANT TREATMENT

Initially, treatment of chancroid varied from surgery to local applications of various medicinal combinations. Many were ineffective, sometimes recommended to satisfy the patient rather than to cure the disease. Perhaps these measures indirectly functioned by removing secondary contaminants.

Hanschell (1938) reported on the one hour immersion of the ulcer/s in coloured water in a wide-mouthed jar every day. "This immersion ritual gives him a much needed something to do for himself. The water from the hot tap in the clinic, when lightly tinted with potassium permanganate, acriflavine or methylene blue, interests him; gives him a ring of colour changes; often provides satisfaction for fancy, and, since 'sea lawyers' are many, something to argue about as to the merits with his clinic mate, equally afflicted but of a different fancy; keeps him happy, and the ulcer immersed".

Hanschell (1938) and Satulsky (1945) recommended washing with soap, but others opposed this (Alergant, 1972; Gaisin and Heaton, 1975). Saline soaks have been recommended to remove the purulent, and necrotic material, and to increase granulation and healing (Alergant, 1972; Kraus *et al,* 1982b).

Other substances applied as local medications include : mercury bichloride, protein silver (Greenwald, 1943); phenol, silver nitrate, gentian violet, zinc peroxide (Allison, 1945); iodine, balsam of Peru, azochloramide in triacetin (Satulsky, 1945); sodium hypochlorite and potassium permanganate (Alergant, 1972).

Since treatment was not effective, lesions often developed complications, in which case surgery followed (Kraus *et al,* 1982b).

Lymphadenopathy was treated with bed-rest, and with ice packs (Satulsky, 1945; Alergant, 1972) instead of hot fomentations to relieve pain. Fluctuant buboes were aspirated (Satulsky, 1945; Alergant, 1972; Kraus *et al,* 1982b).

Other treatments used were X-raying, vaccines made of killed organisms, foreign proteins and arsenicals (Satulsky, 1945). With the introduction of antibiotics for treatment, the time to healing has decreased from four to twelve weeks to less than ten days; costs are lower and hospitalisation is not required.

1.7.2 ANTIMICROBIAL THERAPY

With the introduction of antibiotics, treatment of chancroid became less traumatic, more efficient (cure within ten days), and more specific although the early 'antibiotics' sulphathiazole (Greenwald, 1943), tetracycline and sulphonamide (Hammond *et al,* 1980) were reported as having little effect on bubo formation. *H. ducreyi* was previously found to be sensitive to penicillin (Beeson, 1946; Reymann, 1949), tyrothricin (Beeson, 1946) and streptomycin (Reymann, 1949). Today antibiotic-resistant strains, particularly by virtue of β-lactamase production, are common, and distributed worldwide with increasing incidence. For example in Amsterdam, from 1979 to 1982, more than 30 per cent of the strains were

β-lactamase negative, and the minimum inhibitory concentration (MIC) for tetracycline was 2mg/l or less. In 1982 to 1985, 1.7 per cent were β-lactamase negative, and all were tetracycline resistant. The MIC for cefotaxime, sulphamethoxazole and trimethoprim also increased. No changes were seen for cephradine, erythromycin, kanamycin, gentamicin, chloramphenicol, nalidixic acid and ciprofloxacin (Sturm, 1987b).

This trend has been observed in South Africa. Bilgeri *et al* (1982) studied a population of mineworkers similar to that of Dangor *et al* (1988b). β-lactamase producers increased from 93 per cent to 100 per cent. Previously all strains were moderately susceptible to minocycline. Six years later, 10 per cent were fully resistant. β-lactamase plasmids of *H. ducreyi* have also been isolated from strains in the United States (Anderson *et al,* 1984) and Kenya (McNichol and Ronald, 1984).

Trimethoprim was recommended in Nairobi (Fast *et al,* 1983) and Amsterdam (Slootmans *et al,* 1983); chloramphenicol was recommended in South Africa (Bilgeri *et al,* 1982) and Zimbabwe (Latif, 1982) for the treatment of chancroid. However, only recently Dangor *et al* (1988c) discovered trimethoprim and chloramphenicol resistance in South African *H. ducreyi* strains. *H. ducreyi* strains in Thailand have also been reported to have trimethoprim minimum inhibitory concentrations of greater than 32μg/ml (Taylor *et al,* 1985).

Some clinical failures conceivably reflect antibiotic resistance. This has already been reported for tetracycline (Kerber *et al,* 1969; Fast *et al,* 1983), sulphonamide (Kerber *et al,* 1969; Fast *et al,* 1983) and doxycycline (Fast *et al,* 1983).

There are different antimicrobial susceptibility patterns for each geographical area. The proportion of β-lactamase producing strains varies from 0 per cent to 12 per cent in Britain (Hafiz *et al,* 1986), and 92 per cent to 100 per cent in the Far East (Sng *et al,* 1982; Taylor *et al,* 1985) and Africa (Bilgeri *et al,* 1982; Nsanze *et al,* 1984; Dangor *et al,* 1988b). The susceptibility of a strain to a certain antibiotic is relative to the region the strain originated

from, and the selective pressure exerted by the increased utilisation of certain antibiotics. Even though the use of β-lactam drugs, tetracycline and sulphonamide has been discouraged, they may still be effective in areas where the antimicrobial agent is not commonly administered, or in areas where chancroid occurs infrequently (Lykke-Olsen *et al,* 1979; Nayyar *et al,* 1979; Hammond *et al,* 1980).

The use of penicillin, tetracycline or sulphonamide, previously recommended, has been supplanted by more appropriate regimens. Recent studies in southern Africa indicate that the following antibiotics are available for the treatment of chancroid : erythromycin, ciprofloxacin, sulphamethopyrazine with trimethoprim, sulphametrole with trimethoprim and ceftriaxone (Ballard *et al,* 1989).

The choice of any one of these regimens depends largely on financial considerations, accuracy of diagnosis, and the susceptibility of local *H. ducreyi* strains.

1.7.3 ANTIBIOTIC-RESISTANCE PLASMIDS

Plasmids of *H. ducreyi* have been reviewed by McNichol and Ronald (1984) and Johnson *et al* (1988).

1.7.3.1 β-lactamase production / ampicillin resistance

Penicillin has been used successfully to treat chancroid (Reymann, 1949; Hammond *et al,* 1978a). In 1979 Brunton *et al* studied three strains of *H. ducreyi* from an outbreak in Winnipeg (Hammond *et al,* 1978a) which harboured a 6 MDal plasmid (pJB1). This plasmid had a G-C base of 0.41 mol fraction and transformed into *Escherichia coli* but not *H. influenzae.* Genetic studies showed that pJB1 carried Tn A, the transposable ampicillin-

resistance sequence (MacLean *et al,* 1980). Sanson-Le Pors *et al* (1983) in France found 90 per cent of the strains carried the same type of ampicillin-resistance plasmid.

A 5.7 MDal plasmid found in Seattle, and originally from Mexico (Totten *et al,* 1982), was identical to pJB1 i.e. the Winnipeg strain. This 5.7 MDal plasmid has been found in association with other antibiotic-resistance plasmids (Handsfield *et al,* 1981; Brunton *et al,* 1982). About 40 per cent of strains from Kenya carried a 5.7 MDal plasmid (Brunton *et al,* 1982).

A 7 MDal plasmid was first found in 1982 (Totten *et al,* 1982) in Seattle; in a strain originally introduced from the Philippines. It has also been reported by Deneer *et al* (1982) in Canada, Anderson *et al* (1984) in California and Brunton *et al* (1982) in Kenya (57 per cent).

Other ampicillin-resistance plasmids are 3.9 MDal (Thomson and Bilgeri, 1982), 5.2 M Dal (Thomson and Bilgeri, 1982), and 3.6 MDal (Handsfield *et al,* 1981; Totten *et al,* 1982). A 3.2 MDal plasmid was observed in strains originally from Brazil (Handsfield *et al,* 1981; Totten *et al,* 1982) and California (88 per cent) (Anderson *et al,* 1984), but not in Kenya (Brunton *et al,* 1982).

Study of these plasmids may suggest their origin, or indicate infectivity and resistance within a population. Handsfield *et al* (1981) found that plasmids were identical for isolates from epidemiologically-linked cases, and differed according to geographical location. The use of of restriction endonucleases for fingerprinting plasmid fragments has been suggested (Totten *et al,* 1982). Isolates from Mexico and the Philippines carried Tn A sequence, while the strain from Brazil carried a truncated Tn A fragment. The 3.2 MDal plasmid is uncommon in African, North American and Asian strains.

Brunton *et al* (1982) compared plasmids of *N. gonorrhoeae* and *H. ducreyi.* The 4.4 MDal gonococcal plasmid was homologous to the 7 MDal plasmid of *H. ducreyi* i.e. the *H. ducreyi* plasmid carried the entire Tn A sequence while the *N. gonorrhoeae* plasmid carried only 40 per cent of this sequence. Similar findings were obtained regarding the 3.2 MDal plasmid in *N. gonorrhoeae* and the 5.7 MDal plasmid in *H. ducreyi.* The 5.7 and 7 MDal plasmids in *H. ducreyi* differed from one another only by a 1.3 MDal insertion element. This element is

present in the larger gonococcal plasmid as well. The 3.2 MDal ampicillin-resistance plasmid of *H. ducreyi* was found to be identical to that of *N. gonorrhoeae* (Anderson *et al,* 1984). These results suggested that ampicillin-resistance plasmids were introduced into *N. gonorrhoeae* from *H. ducreyi,* the Tn A sequence being deleted before or during the transfer. Brunton *et al* (1982) showed cryptic plasmids of *H. parainfluenzae* had similar restriction endonuclease patterns to β-lactamase plasmids of *H. ducreyi* and *N. gonorrhoeae.* They speculated that ampicillin resistance was acquired by *Haemophilus* through insertion of the Tn A sequence into these cryptic plasmids, and subsequently mobilised from *Haemophilus* to *N. gonorrhoeae.*

1.7.3.2 Sulphonamide resistance

Sulphonamide resistance was observed in Kenya (Nsanze *et al,* 1981) and is associated with a 4.9 MDal plasmid with or without ampicillin-resistance plasmids (Albritton *et al,* 1982). This plasmid had a G-C ratio of 57 per cent and was transformed into *E. coli.* By hybridisation and electron-microscopy heteroduplex analysis, this plasmid was found to be 79 per cent related to the enteric streptomycin-sulphonamide-resistance plasmid (RSF 1010) found in *Salmonella, Proteus,* and *Pseudomonas aeruginosa.* Genes coding for kanamycin resistance that is not plasmid-mediated is found in other species of *Haemophilus,* and is related to a transposon found in enteric bacteria. It was proposed that kanamycin resistance arose by transposition of resistant determinants from enteric plasmids to cryptic plasmids in *Haemophilus.* This could also explain the origin of sulphonamide-resistance plasmids in *H. ducreyi.* In Kenya, 14 per cent of the strains carried this 4.9 MDal plasmid (MacLean *et al,* 1980). Deneer *et al* (1982) also observed it in their studies. Fast *et al* (1983) found this plasmid in strains from patients who did not respond to sulphomethoxazole.

1.7.3.3 Tetracycline and chloramphenicol resistance

Prior to 1984, no plasmid-related tetracycline resistance was found. Of the forty-one tetracycline-resistant strains carrying other small antibiotic-resistance plasmids (4.9, 5.7 and 7 MDal), ten had a 30 MDal plasmid tranferable by conjugation to other *H. ducreyi* strains. Three tetracycline-resistant strains did not carry this plasmid but still transferred tetracycline resistance to transconjugants, the latter carried the 30 MDal plasmid. There was no difference in the level of tetracycline resistance, whether carried chromosomally or as a stable or unstable integrated plasmid. Using restriction enzyme digests, this plasmid was found to be related to an *H. influenzae* plasmid (Albritton *et al,* 1984). Recently investigations by Johnson *et al* (1989) and Roberts (1989) confirmed that the tetracycline-resistance determinant, Tet M, of *H. ducreyi* was encoded by chromosomal and plasmid-coded genes, respectively. Albritton *et al* (1984) also isolated one strain which was tetracycline and chloramphenicol resistant and carried a 34 MDal plasmid (Albritton *et al,* 1984).

Chloramphenicol resistance has been observed in South Africa (Dangor *et al,* 1988c), France (Sanson-Le Pors *et al,* 1985), United States and the Philippines (Roberts *et al,* 1985). This resistance has been related to a conjugative plasmid. Chloramphenicol acetyltransferase (CAT) of *H. ducreyi* is molecularly related to the enteric type III class and to CAT of *H. influenzae* i.e. a 70 to 80 per cent DNA homology (Roberts *et al,* 1985).

1.7.3.4 Aminoglycoside resistance

Although kanamycin (Marmar, 1972) and streptomycin (Anderson *et al,* 1984) have been used for the successful treatment of chancroid, resistance to aminoglycoside has been reported in Amsterdam (Sturm, 1987b) and Thailand (Taylor *et al,* 1985). Three clinical strains, resistant to streptomycin and kanamycin, were studied in France (Sanson-Le Pors

et al, 1985). This resistance was due to a 4.7 kilobase plasmid coding for aminoglycoside phosphotransferase.

1.7.3.5 Conjugative plasmids

Non-conjugative plasmids can be transferred in the presence of self-transmissible plasmids e.g. ampicillin-resistance and sulphonamide-resistance plasmids are not conjugally self-transmissible to *H. ducreyi* and other *Haemophilus* species, but can be transferred by transformation to *H. influenzae* and *E. coli.* A 23 MDal plasmid was discovered in Kenya (Deneer *et al,* 1982) which mediated transfer of the 4.9 MDal and 7 MDal plasmid of *H. ducreyi.* It occurred with a very low frequency of 3 per cent. Based on the presence of a 24.5 MDal mobilising plasmid for ampicillin-resistance plasmids of *N. gonorrhoeae*, and the fact that ampicillin-resistance plasmids of *N. gonorrhoeae* are homologous to those of *H. ducreyi,* they proposed that gonococcal plasmids allowed for the transfer of antibiotic-resistance plasmids between these genera. In spite of a similiar size and function, DNA hybridisation studies and restriction mapping have shown that the 24.5 MDal gonococcal plasmid and the 23.5 MDal plasmid of *H. ducreyi* differ (McNichol *et al,* 1986). McNicol *et al* (1986) found the Ori-V site (origin of replication) of this mobilising plasmid to be located close to the Ori-T site (origin of mobilisation). Both Ori-V and Ori-T sites map in the region homologous to the β-lactamase-specifying plasmids of *H. ducreyi* and *N. gonorrhoeae,* thus confirming the function of this plasmid, and indicating some homology between the conjugative plasmids of *N. gonorrhoeae* and *H. ducreyi.* The 30 MDal tetracycline-resistance plasmid is self-transmissible but does not mobilise the 7 MDal ampicillin-resistance plasmid (Albritton *et al,* 1984).

Since serum resistance has been implicated as plasmid-mediated in other Gram-negative bacteria (Roantree and Rantz, 1960), it may have some significance in serum resistance of *H. ducreyi*. Similarities between the plasmids of *H. ducreyi* and those of other Gram-negative bacteria have been observed. It is thus possible that serum resistance may have been introduced by this mechanism into *H. ducreyi*.

SERUM RESISTANCE AS A BACTERIAL VIRULENCE FACTOR

2.1 INTRODUCTION

2.1.1 HISTORICAL BACKGROUND

Pasteur, between 1880 and 1889, first directed attention to the practical and theoretical possibilities connected with immunity. Other workers described the occurrence or development of mysterious properties or substances in the bloodstream and body fluids. For example, blood was shown to possess some power of inhibiting putrefaction. Bacteria introduced into the bloodstream rapidly disappeared and apparently did not leave the body by excretion or secretion.

Probably the first person to describe an assay to determine the killing power of blood was Nuttall in 1886. Blood from rabbits, mice, pigeons, sheep and dogs was defibrinated with sterile quartz. Known quantities of the anthrax bacillus were added to the blood *in vitro* and plate cultures were made at intervals thereafter. A profound reduction in the number of bacteria was observed. Nuttall discovered not only that the bactericidal power of blood was lost after a time, but that it was destroyed on heating to 52^0 C in ten to thirty minutes for dog blood; and to 55^0 C for forty-five minutes for rabbit blood.

These results were confirmed by Nissen (1889). In addition, it was found that some types of bacteria were killed by blood, while others were not. Behring (1890) found that whereas the serum of normal guinea pigs was incapable of destroying *Vibrio metchnikovii*, the serum of guinea pigs immunised against this organism could accomplish this easily. In several experiments by Buchner (1850 - 1902) of Munich, the existence of bactericidal substances in

the blood was confirmed. This property was also observed in a cell free serum. These factors were called 'alexines' or 'complement' due to their defensive or protective properties. The historical background of serum resistance has been documented by Bullock (1938).

2.1.2 CONCEPTS OF SERUM RESISTANCE

A micro-organism invading the human body, encounters two types of host defences. 'Innate immunity' consists of physical barriers e.g. skin or mucous membranes; serum proteins e.g. lyzozyme, complement (C'); and phagocytes e.g. macrophages and polymorphonuclear leucocytes (PMNL), and does not depend on prior exposure to micro-organisms. 'Specific immunity' involves a complicated interaction between antigen-antibody complexes and specialised cells involved in the immune system, which include B-cells, T-cells and phagocytes.

The bactericidal activity of serum is mainly due to a C'-mediated lysis of the invading pathogen. C' may be activated by the classical, or the alternative pathway; the former involves binding of antibody to antigen on the pathogen surface, while the latter occurs independently of antibody. Both pathways consist of a stepwise series of interactions between the nine C' proteins. The result is the formation of a membrane attack complex, which inserts into the cell membrane forming circular lesions leading to cell death - Figure 2.1 (Hanson and Wigzell, 1985; Frank *et al,* 1987).

Virulence of a micro-organism depends on its ability to enter and multiply in the host tissue. In addition, it must be able to withstand host defence mechanisms pre-existent in the tissue, or induced or mobilised as a result of the microbial attack (Brubaker, 1985).

A contributing factor is an ability to resist the bactericidal activity of serum. Precise evaluation of the role of resistance to the C'-mediated lysis of certain Gram-negative organisms

is difficult, since distinct components of the host defence act in concert. C′-mediated killing is more important in the pathogenesis of some infections. For example, serum resistance plays a limited role in infections by micro-organisms which survive intracellularly, but is important in infections at sites in the human body inaccessible to phagocytes e.g. bacterial endocarditis (Taylor, 1983b; Brubaker, 1985).

The activity of C′ at various sites of infection in the human body may contribute to the survival of serum-sensitive strains *in vivo*. Establishment of localised gonococcal infection in the genital tract, may be promoted by the inhibition of C′ by proteases in seminal plasma (Martin *et al,* 1982). Inadequate concentrations of antibody and C′ in the cerebrospinal fluid may contribute to the maintenance of bacteria in untreated cases of meningitis. Ammonia in the kidney is also thought to have anti-complementary properties (Taylor, 1983b).

The measure of serum resistance falls between two extremes. Taylor (1983b) suggested that the description 'serum resistant' be restricted to strains that are totally uninjured by serum and may even show an increase in viability when tested in early logarithmic phase in assays containing excess amounts of the components of the bactericidal system. 'Serum-sensitive' strains were classified as those that show a significant reduction in viability, even after lengthy periods of incubation. However many studies have described 'serum-resistant' organisms as those that are less susceptible than other strains within the same study (Binns *et al,* 1979). Taylor (1974) described strains of *Escherichia coli* not killed immediately on exposure to normal human serum (NHS) as 'delayed-serum-sensitive' strains, as opposed to 'promptly-serum-sensitive' strains. A similar observation was made by Traub and Kleber (1976) with *Serratia marcescens*. In addition, serum resistance may be unstable in consequence of environmental factors affecting the cell surface. Variations of the test to measure serum resistance have been reported. The distinction between serum resistance and serum sensitivity is therefore ill-defined when results of independent studies are compared.

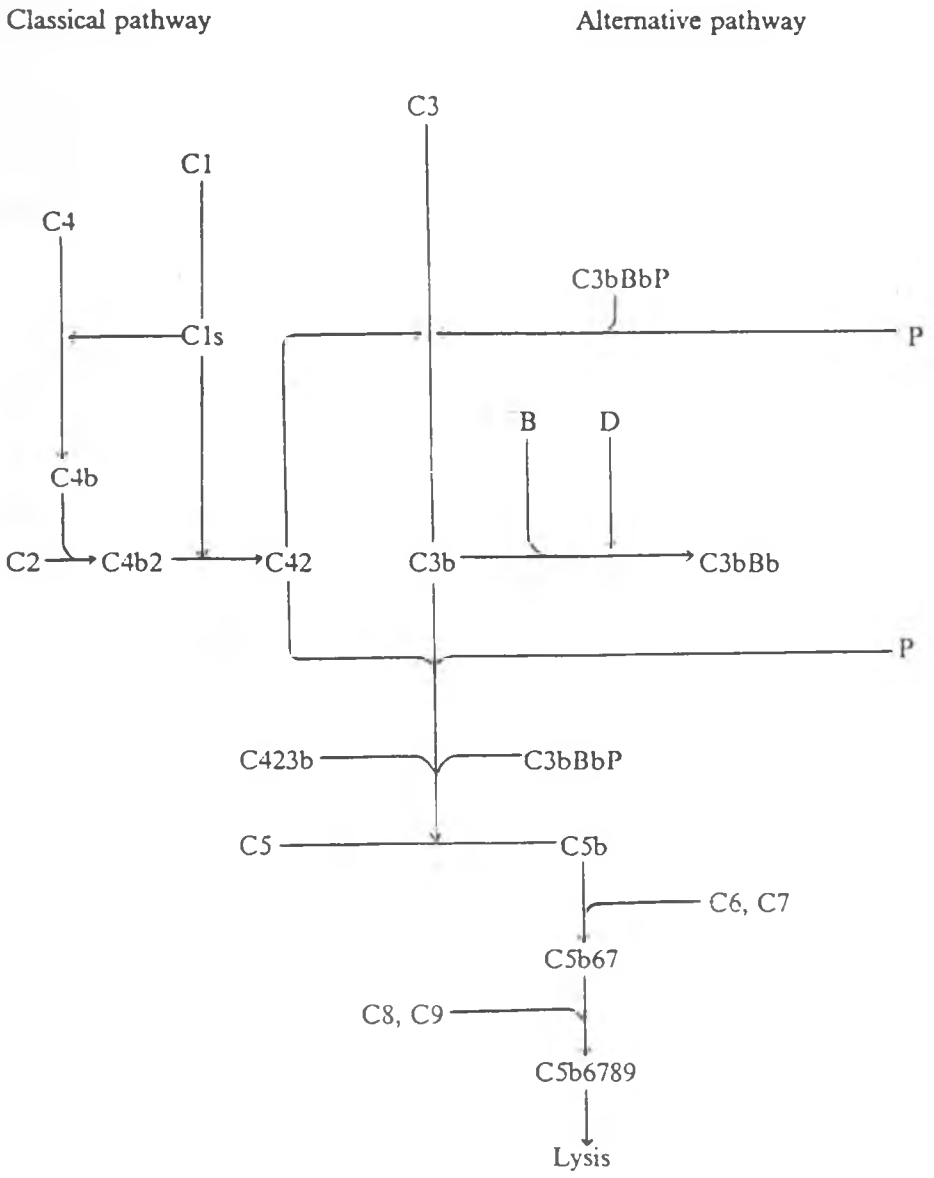


Figure 2.1 The classical and alternative pathway of complement (Hanson and Wigzell, 1985). *Classical pathway:* C1 is activated by binding to the antibody in immune complexes. Activated C1 cleaves C4 into C4a and C4b. Cell-bound C4b binds C2. C2 is split by activated C1. C2a and C4b join, forming a complex C42, which cleaves C3 into C3a and C3b. C5 convertase, or C423b, is formed by C42 and C3b combining. This enzyme cleaves C5 into two. C5b binds to the cell surface where a trimolecular complex of C5b, C6 and C7 is built up. C8 and C9 react with this complex forming C5b-C9 or the membrane attack complex. Channels form through the cell membrane, which finally result in death of the cell.
Alternative pathway: A C3b-like molecule becomes cell bound. Factor B binds to C3b, followed by cleavage of factor B by factor D. The resulting complex C3bBb is stabilised by properdin (P) and cleaves innate C3 into C3a and C3b. The reaction proceeds as in the classical pathway creating C5b-C9 complexes.

2.1.3 CLINICAL RELEVANCE

An extensive body of literature has implicated serum resistance as an important determinant of virulence in certain infections. Much of the data is derived from several broad topics.

2.1.3.1 Epidemiology and incidence of serum-resistant strains.

There is a strong correlation between serum resistance and the ability of a variety of Gram-negative bacteria to invade and survive in the human bloodstream. A higher proportion of strains isolated from the blood of bacteraemic patients, than of those isolated from faeces or urine, were resistant to killing by NHS (Roantree and Rantz, 1960; Fierer *et al,* 1972). A similar relationship has been observed for *Pseudomonas aeruginosa* (Young and Armstrong, 1972), *S. marcescens* (Simberkoff *et al,* 1975), *Bacteriodes fragilis* (Casciato *et al,* 1979), *Neisseria gonorrhoeae* (Cannon *et al,* 1981), *Klebsiella pneumoniae* (Benge, 1988), *Campylobacter jejuni* and *Campylobacter fetus* (Blaser *et al,* 1985). Blood isolates of *Capnocytophaga* were all serum resistant, while oral or non-oral isolates of this organism, were not (Wilson *et al,* 1987).

The location of serum-resistant isolates in the bloodstream suggests that micro-organisms that are serum resistant are likely to cause more serious systemic diseases than those from localised invasion (Blaser *et al,* 1985).

Some of the more severe diseases caused by serum-resistant Gram-negative bacteria are bacteraemia, meningitis and sepsis. Bacteraemia caused by serum-resistant *E. coli* was more likely to be associated with shock and death than if due to serum-sensitive strains (Elgefors and Olling, 1978; McCabe *et al,* 1978; Clumeck *et al,* 1982). Kimball *et al* (1964) also reported that *E. coli* strains isolated from patients with bacteraemia were more serum resistant than organisms responsible for urinary tract infections. Strains of *N. gonorrhoeae* causing

disseminated gonococcal infection were serum-resistant (Ross *et al,* 1985) but isolates from local uncomplicated genital infections varied in their susceptibility to serum (Glynn and Ward, 1970; Tramont *et al,* 1977; Brooks and Inwer, 1978; Rice *et al,* 1980; Ross *et al,* 1985).

Serum resistance is more prevalent amongst certain bacterial genera and species. Roantree and Rantz (1960), tested several genera of bacteria associated with infection. *Shigella* and *Klebsiella* groups showed a higher proportion of serum sensitivity, whereas the highest proportion of strains expressing serum resistance was found in the *Salmonella* group. The majority of Enterobacteriaceae causing bacteraemia were serum resistant compared with other genera or species. *C. fetus* is generally more serum resistant than *C. jejuni* (Blaser *et al,* 1985). Similarly, *K. pneumoniae* is more serum resistant than *Klebsiella oxytoca* (Benge, 1988). Ross *et al* (1985) observed differences in serum susceptibilities *in vitro* between *Neisseria meningitidis* and *N. gonorrhoeae.* A species-dependent factor in serum susceptibility was also reported for strains of the *B. fragilis* group (Casiato *et al,* 1979).

Clinical isolates of *E. coli* (Roantree and Rantz, 1960; McCabe *et al,* 1978), *S. marcescens* (Simberkoff *et al,* 1976), *Yersinia enterocolitica* (Pai and DeStephano, 1982), *Haemophilus influenzae* type b (Sutton *et al,* 1982), *Salmonella typhimurium* and *Salmonella enteritidis* (Nelson and Roantree, 1967) were serum resistant. These observations seems to support the concept of serum resistance as a virulence factor for several micro-organisms.

2.1.3.2 Invasive bacterial infection in experiments using laboratory animals.

Evidence obtained from animal challenge experiments using isogenic strains suggests a clear relationship between virulence and serum resistance. A conversion of virulent strains of *Y. enterocolitica* to avirulence, using the bacteraemic rabbit model, was associated with the loss of several virulence markers including serum resistance (Pai and DeStephano, 1982). Similarly, a serum-sensitive variant of *H. influenzae* obtained from a serum-resistant population

was subsequently proved to be avirulent, while the parent strain was virulent. Virulence was indicated by bacteraemia following intranasal infant rat inoculations (Gilsdorf and Ferrieri, 1986). Acquisition of a plasmid coding for serum resistance in *E. coli* was associated with an increase in pathogenicity in chicks. One-day old chicks were intramuscularly injected and the 50 per cent lethal dose was calculated (Binns *et al,* 1979).

2.1.3.3 The increased susceptibility to certain pathogens of patients with an abnormal C′-system.

Host factors including age, nutritional status, pre-existing disease (especially those affecting the immune system), and corticosteriod treatment (Elgefors and Olling, 1978), are of major importance in the susceptibility of patients to microbial infection. Patients with total or partial congenital deficiencies of one or more C′ protein/s are generally more susceptible to microbial infection, implicating serum bactericidal activity as a host defense mechanism. *P. aeruginosa,* isolated from sputum of a patient suffering from chronic recurrent pulmonary infection, was rapidly killed in NHS, but grew quickly in the patient's serum (Waisbren and Brown, 1966). A deficiency or defect in earlier acting C′ components e.g. C1, does not result in a significant increase in susceptibility to pathogens. It is more likely that the alternative pathway rather than the classical pathway is responsible for preventing serious infections. The success of meningococcal and possibily gonococcal infection of some patients can be explained by a deficiency of one or more late C′ components (C5, C6, C7, C8 and C9). In these patients, the frequency of infection by *N. meningitidis*, and the rate of relapse or re-currence, is higher than that of a normal population (Ross and Densen, 1984). The ability of a pathogen to resist C′-mediated killing of serum offers no survival advantage in immunocompromised patients. Thus, it is not surprising that serum-sensitive campylobacters (Blaser *et al,* 1987) and neisseriae (Ross *et al,* 1985) have been isolated from the bloodstream of these susceptible hosts.

2.1.3.4 Bactericidal activity of serum of newborn babies.

Serum-sensitive *E. coli* K1 strains have been isolated from newborn babies suffering from sepsis and meningitis. Dossett *et al* (1969) reported that the bactericidal capacity of blood from newborn babies against *E. coli* was deficient compared to that of the mother's blood. A difference in serum opsonic activity, rather than in PMNL activity was responsible for this deficiency in the infants.

While serum resistance most likely plays a role in determining bacterial virulence, it is not necessarily the primary determining factor. In several studies, serum-sensitive but invasive strains have possessed other virulence factors, such as the virulence antigen, auto-agglutination and calcium dependency in *Y. enterocolitica* (Chiesa and Bottone, 1983); biotype, association with PMNL and virulence in mice inoculations of *H. influenzae* (Sutton *et al,* 1982); or antibiotic susceptibility and pyocin type in *P. aeruginosa* (DeMatteo *et al,* 1981). Similarly, serum resistance is not the only determinant for virulence amongst *S. typhimurium* and *S. enteritidis* (Nelson and Roantree, 1967) and *Klebsiella* species (Benge, 1988).

Since the definition of 'serum resistance' amongst bacteria has been somewhat inconsistent, comparisons of data in published studies may be misleading. The influence of *in vitro* compared to *in vivo* conditions needs to be considered in the definition of 'serum resistance'. For example serum sensitivity has been associated with the higher metabolic state of Gram-negative bacteria (Michael and Braun, 1959; Melching and Vas, 1971; Morse *et al,* 1983) during the exponential phase *in vitro,* compared to the *in vivo* situation when growth conditions are sub-optimal, resulting in a lower metabolic state and thus increased resistance to serum. Also, subculturing of clinical strains *in vitro* may result in the expression of serum sensitivity of a previously serum-resistant strain. This has been reported for *N. gonorrhoeae* (Rittenberg *et al,* 1977; James *et al,* 1982), *E. coli* (Akiyama and Inoue, 1977) and *Trichomonas vaginalis* (Demes *et al,* 1988).

2.1.4 HAEMOPHILUS DUCREYI

Haemophilus ducreyi has not been reported to invade the bloodstream. However, serum resistance has been reported in clinical isolates. In an attempt to determine the significance of this observation, it was suggested that serum resistance might allow the organism to multiply more readily within human tissue during infection. In addition, this property has been related to virulence by intradermal rabbit inoculations and resistance to phagocytosis by PMNL (Odumeru *et al,* 1984). The susceptibility to polymyxin was correlated with virulence and therefore serum resistance of *H. ducreyi* (Hammond *et al,* 1978b), but this was not observed later by Odumeru *et al* (1984). Lipopolysaccharide (LPS) from serum-sensitive strains of *H. ducreyi* inhibited the bactericidal activity of NHS against other serum-sensitive strains, whereas LPS from serum-resistant strains had little effect except in high concentrations. LPS was thus implicated as a determinant of serum resistance in *H. ducreyi* (Odumeru *et al,* 1985). Further, it was found that the LPS of virulent strains differed from that of avirulent strains when biochemically analysed, the former possessing more carbohydrate associated with the core polysaccharide (Odumeru *et al,* 1987).

Long lasting immunity following *H. ducreyi* infection has not been demonstrated and reinfection of individuals has been reported (Alergant, 1972; Felman and Nikitas, 1983; Saunders and Folds, 1986; Naamara *et al,* 1987). In studies by Schalla *et al* (1986) an immune response to chancroid has nonetheless been suggested. Cross-reaction with other *Haemophilus* species was low. Sera from patients who had neither syphilis nor herpes antibodies reacted strongly with outer membrane proteins (OMP) of *H. ducreyi* CIP 54.2 in a dot-immunobinding assay. Ig G and Ig M to *H. ducreyi* occurred in 47.6 per cent and 34.9 per cent of patients with chancroid respectively, compared with lower levels in serum from healthy controls, and from subjects with *H. influenzae* infection. A higher rate of detection of Ig G to *H. ducreyi* among patients with chancroid was obtained by Museyi *et al* (1988) than in persons without genital ulcer disease. Using an enzyme immunoassay, Ig G in 55 per cent of infected men in Bangkok, and 89 per cent of Kenyan patients was detected. False

positive reactions were due to cross-reacting antibodies detected against other *Haemophilus* species, or due to previous or undetected chancroid infections.

The complexity of *H. ducreyi* antigenicity has been observed by Odumeru *et al* (1983), Slootmans *et al* (1985), Saunders and Folds (1986) and Abeck and Johnson (1987b), who observed heterogeneous outer membrane protein (OMP) profiles and antigen types. The relationship of these with serum resistance has not been clearly elucidated. Similarly, the opsonic properties of anti- *H. ducreyi* antibodies and their role in C′ activation have not been studied.

2.2 BACTERIAL COMPONENTS ASSOCIATED WITH SERUM RESISTANCE

There is convincing evidence that the main barrier to serum killing is associated with the outer membrane of bacterial cells. Cell surface components thought responsible for serum resistance are LPS, outer membrane proteins and acidic polysaccharides. Results of independent studies of these factors have sometimes conflicted. Although this may be a reflection of differences in techniques used to measure serum resistance, it also suggests that serum resistance is multifactorial and complex. There is no evidence that serum-resistant bacteria produce proteases or other cellular products that neutralise or destroy functional C′ in the fluid phase.

2.2.1 LIPOPOLYSACCHARIDE

The structure, biosynthesis and genetics of LPS have been reviewed by Osborn (1969), Wright and Kanegasaki (1971), Lugtenberg and Van Alphen (1983) and Hitchcock *et al* (1986).

LPS is a characteristic component of the cell envelope of Gram-negative bacteria consisting of a lipid (lipid A), which is linked to an oligosaccharide unit (R core), which is in turn linked to the O polysaccharide (O antigen, O-side chain). The three components are covalently joined, forming a single amphipathic macromolecule.

'Rough' strains lack the O antigen or polysaccharide; their LPS consists of lipid A and R core. In 'smooth' strains, the individual LPS molecules are heterogenous, varying between the extremes of lacking the O polysaccharide (rough LPS) to molecules possessing one (smooth-rough LPS), or more (smooth LPS) O-polysaccharide units (Jann *et al,* 1975; Goldman and Leive, 1980; Kropinski *et al,* 1982).

Smooth to rough transitions, as a result of change/s in the cell surface composition which have been associated with serum resistance, involves some or all of the following changes : the formation of colonies of altered appearance; loss of specific smooth-strain antigens and hence loss of agglutinability in specific antiserum; an increase in cell-surface hydrophobicity; increased susceptibility to phagocytosis; increased auto-agglutination in saline; change in susceptibility to particular bacteriophage/s and bacteriocin/s; and loss of, or decrease in, virulence. The description 'smooth' may be applied to a strain that has a glossy colony appearance, is encapsulated or possesses smooth-type LPS (Singleton and Sainsburg, 1978). The term 'smooth/rough' should be used with caution, since it is generally applied to the description of bacteria varying in colony morphology, but may be unrelated to changes in their LPS structure.

Paradoxically, a great deal of systematic knowledge about LPS from a limited number of genera is available, but little about the wide spectrum of micro-organisms has been forthcoming. In addition, the total composition or organisation of the LPS of a single species is poorly understood. Undoubtedly, one of the reasons for this, is the apparent complexity of these molecules.

Since studies have concentrated on the LPS of Enterobacteriaceae, the structure of *S. typhimurium* LPS is given in Figure 2.2, and may be used as a model for describing the structure of LPS.

A basic structural component of lipid A is a diglucosamine unit that is substituted with fatty acids and phosphate groups. The glucosamine units are linked glycosidically to one another and they carry both O- and N- acyl substituents. The sugar components of the LPS are linked ketosidically to lipid A through 2-keto-3-deoxyoctonate (KDO), the innermost component of the R core.

The backbone of the core region consists of a seven-carbon sugar, L-glycero-D-mannoheptose (Adams *et al,* 1967); an eight-carbon sugar, KDO (Droge *et al,* 1970); phosphate; and O-phosphorylethanolamine; to which short oligosaccharide chains containing the amine sugar, N-acetyl-glucosamine; and the hexoses, glucose and galactose are attached. The internal core of the LPS, composed of lipid A and R core is identical throughout the genus *Salmonella*, and closely related structures occur in all enteric bacteria. The O polysaccharide consists of regular repeating sequences of three to six constituent monosaccharides present as straight or branched units. Common components of the O polysaccharide include a number of hexoses : glucose, galactose and mannose; N-acetylated hexosamines : glucosamine and galactosamine; deoxy-hexoses : rhamnose and fucose; and dideoxy-hexoses : abequose, paratose, tyvelose and colitose (Rogers and Perkins, 1968).

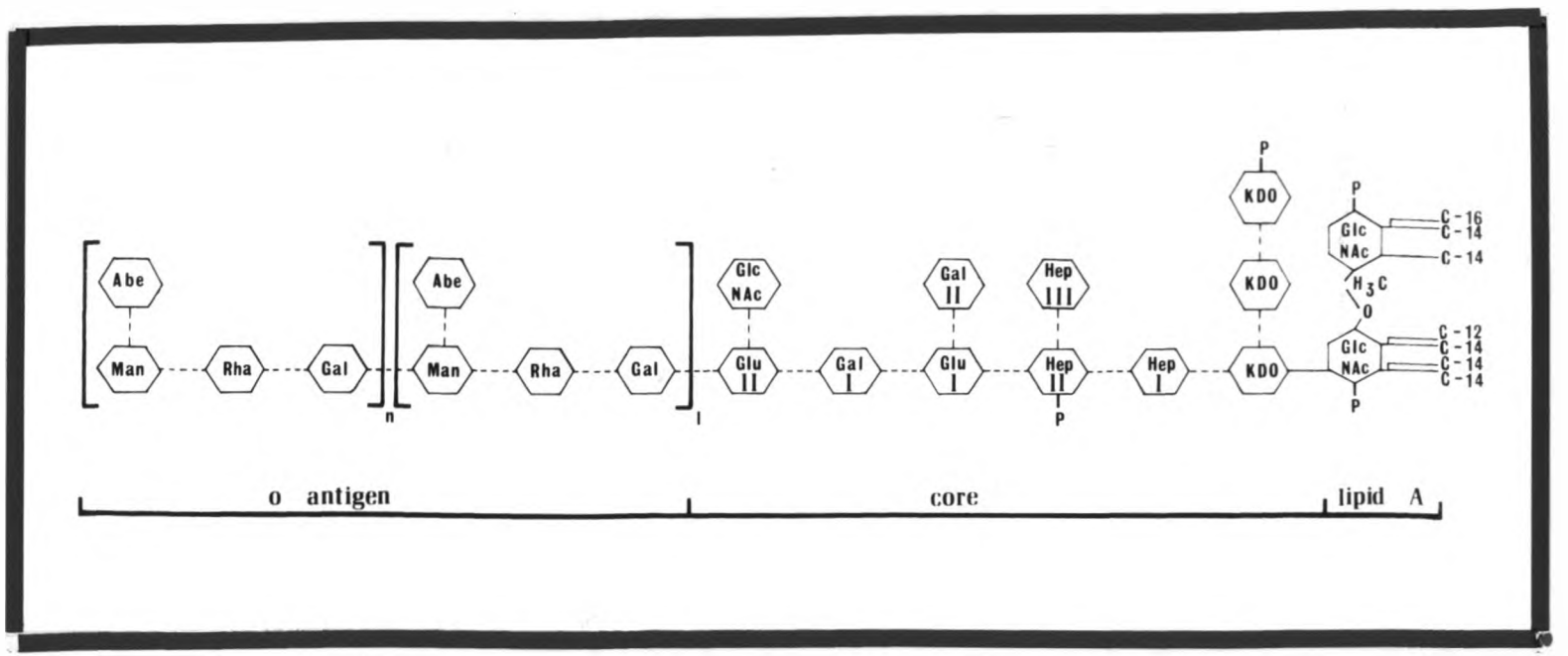


Figure 2.2 Smooth LPS of *S. typhimurium* (Hitchcock *et al*, 1986).

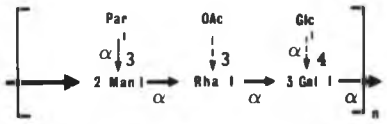
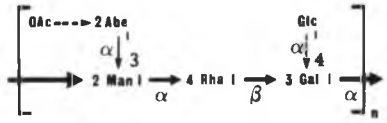
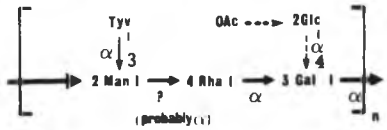
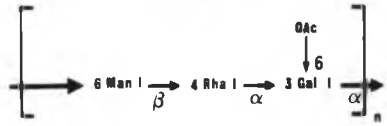
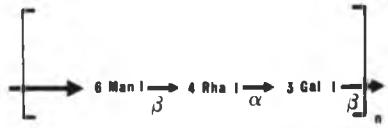
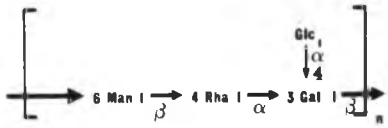
The lipid backbone is composed of β1-6-linked N-acetylated glucosamine monosaccharides (GlcNAc) and seven fatty acids. 2-keto-3-deoxyoctonate (KDO) links the lipid A and the polysaccharide portions of the molecule. The remainder of the core sugars include heptose (Hep), glucose (Glu), galactose (Gal) and N-acetylglucosamine (GlcNAc). Some of the core sugars may be modified by both phosphate and amino groups. The O-antigen sugars are galactose, rhamnose (Rha), mannose (Man), and abequose (Abe). (Hitchcock *et al* 1986).

Much of the complexity of LPS may arise from multiple variations of a single basic structure (repeat units) : the length, linkage and conformation of the O polysaccharide as determined by the nature of the repeat units. These variations in O polysaccharide determine a bacterium's O-antigenic specificity. *Salmonella* have been particularly well characterised on the basis of their surface antigen structures. About forty serological groups (A, B, C, etc.) have been identified as indicated in Table 2.1.

Additional information on the LPS structure of Enterobacteriaceae is available for *Salmonella* (Osborn, 1963; Kasai and Nowotony, 1967; Luderitz *et al,* 1967; Nelson and Roantree, 1967; Holme *et al,* 1968; Rowley, 1968; Valtonen, 1970; Lindberg and Hellergvist, 1980), *E. coli* (Osborn, 1963; Ghalambor *et al,* 1966; Taylor *et al,* 1966; Muller-Seitz *et al,* 1968; Prehm *et al,* 1978; Rosner *et al,* 1979), *Citrobacter freundii* (Raff and Wheat, 1968), *Proteus* (Dmitriev *et al,* 1971), *Yersinia pestis* (Hartley *et al,* 1974), *Shigella* (Reed and Albright, 1974) and *S. marcescens* (Jessop and Lambert, 1986).

The LPS of *P. aeruginosa* (Clarke *et al,* 1967; Fensom and Meadow, 1970; Drewry *et al,* 1973; Chester and Meadow, 1973; 1975; Wilkinson and Galbraith, 1975; Kropinski *et al,* 1979; 1982; Riveria *et al,* 1988) is similar to that of the Enterobacteriaceae, but differs in that it contains an unusually high phosphate content, alanine is present in the core region, and high levels of amino sugars or uronic acids are found in the O-side chains (Riveria *et al,* 1988).

Table 2.1 *Salmonella* **serotypes (Wright and Kanegasaki, 1971).**

Sero group	O-determinant	Organism	O-polysaccharide sequence
A	$2,12_2$	*S. paratyphi A*	
B	$4,5,12_2$	*S. typhimurium*	
D_1	$9,12_2$	*S. typhi*	
E_1	3,10	*S. anatum*	
E_2	3,15	*S. newington*	
E_3	3,15,34	*S. minneapolis*	
C_1	$6_2,7$	*S. cholerae-suis*	

Abe	abequose	Man	mannose
Fuc	fucose	Par	paratose
Gal	galactose	Rha	rhamnose
GalNAc	N-acetylgalactosamine	Tyv	tyvelose
Glc	glucose	OAc	O-acetyl
GlcNAc	N-acetylglucosamine	-- >	incomplete substitution

Other unusual observations have been made in the study of LPS of other potential pathogens. No KDO was detected in the LPS of *B. fragilis* (Kasper, 1976), *Vibrio cholerae* (Kabir, 1982) and *Leptospira interrogans* (Vinh *et al,* 1986). In addition, the LPS of *B. fragilis* also lacked heptose (Kasper, 1976). Fructose, a rare component of LPS has been found in studies of *V. cholerae* (Kabir, 1982).

The LPS of some non-enteric clinical strains appears to be rough, lacking the O-antigen component. These include *H. influenzae* (Zamze and Moxon, 1987), *H. ducreyi* (Abeck *et al,* 1987a), *N. gonorrhoeae* (Johnson *et al,* 1975; Stead *et al,* 1975; Inzanza, 1983; Mintz *et al,* 1984), *C. jejuni* and *Campylobacter coli* (Blaser *et al,* 1986). Results obtained by Naess and Hofstad (1984) suggested that *C. jejuni* and *C. coli* have short O-side chains, rather than a completely rough-type LPS. Clearly, these observations indicate that smooth LPS is not a prerequisite for virulence, and many clinical isolates have rough or smooth-rough type LPS.

Further diversification of LPS composition between bacteria of different genera and between individual species or strains of the same genus may result from variation in fatty acid and/or carbohydrate composition of the LPS. The fatty acids are principally the even-numbered carbon fatty acids which vary from C_{10} to C_{20}, often in the two or three hydroxy-form. Galactose and glucose occur frequently in the polysaccharides of Gram-negative bacteria, while mannose, rhamnose and N-acetyl-glucosamine occur less frequently. Other sugars, e.g. arabinose and fucose occur occasionally. A variety of rare sugars are found uniquely in the LPS of Gram-negative bacteria, viz. 3,6-dideoxyaldohexoses, aldoheptose and KDO (Rogers and Perkins, 1968). Just as the O polysaccharide varies, the core oligosaccharide composition and structure may vary considerably. These variations are reflected in differences in serological specifities in rough strains of *S. typhimurium* (Brade *et al,* 1983), *N. gonorrhoeae* (Morse and Apicella, 1982; Mintz *et al,* 1984) and *H. influenzae* (Zamze and Moxon, 1987). LPS are thus heterogeneous, capable of varying in fatty acid, R-core or O-polysaccharide composition.

To determine the role of LPS in serum susceptibility, many investigators have compared the LPS of clinical isolates, or isogenic strains varying in susceptibility to serum. Other evidence has been obtained from *in vitro* techniques which implicate LPS as a serum-resistant determinant. In neutralisation tests, LPS isolated from serum-sensitive strains of *E. coli* (Allen and Scott, 1980), *H. ducreyi* (Odumeru *et al,* 1985) and *N. gonorrhoeae* (Tan *et al,* 1986) protected their respective serum-sensitive strains by adsorbing appropriate antibodies from the serum. Liposomes containing LPS from a serum-sensitive *E. coli* strain were lysed in the presence of serum, in contrast to liposomes containing LPS from serum-resistant strains (Porat *et al,* 1987).

A contribution to serum resistance by lipid A, the component of LPS responsible for an endotoxic reaction, was suggested by the observation that serum-sensitive Enterobacteriaceae possessed less endotoxic activity than serum-resistant strains (Rowley, 1956; Michael and Landy, 1961). Akiyama and Inoue, (1977) found that isogenic strains of serum-resistant and serum-sensitive *E. coli* were comparable in their OMP composition and sensitivity to certain bacteriophages, but not in their fatty acid composition. This was confirmed by Kato and Bito (1978). The role of fatty acids in serum susceptibility has rarely been reported, suggesting that it is of minor importance. Interestingly, there appears to be a correlation between the fatty acyl composition of the more complex erythrocyte-like membrane of *Acholeplasma laidlawii* and C′-mediated killing of this organism (Dahl *et al,* 1979).

Early work implicating the involvement of LPS in serum resistance amongst Enterobacteriaceae, particularly *E. coli* included culture of this organism with an inhibitor of phosphorylation. This correlated with a decrease in serum resistance, suggesting that the phosphate-containing LPS may be involved in the reaction (Rowley and Wardlaw, 1958). Wardlaw (1963) compared *E. coli* strains differing in susceptibility to serum. The serum-resistant strain had nine-fold more LPS than the serum-sensitive strain, but did not differ in the amount of protein or lipid.

Smooth colony forms were associated with serum resistance and vice versa. The smooth colonies consisted of bacterial cells which had a higher content of LPS than cells from the rough colonies. A closer analysis revealed that the smooth-to-rough conversion in colony morphology was as a result of loss of LPS O-side chains (Nelson and Roantree, 1967; Rowley, 1968; Jann *et al,* 1975; Taylor, 1975). Rough mutants of *Salmonella minnesota* were more serum sensitive than the parental smooth strain (Rowley, 1968). Feingold (1969) observed that serum-resistant *E. coli* and *P. aeruginosa* became serum sensitive after growth in broth containing diphenylamine. This was associated with a decrease in the ratio of O-chain sugars to core sugars, when compared to cells grown in normal broth. The LPS composition of a rough serum-sensitive strain, and a smooth serum-resistant strain of *E. coli* was compared. There was a significant quantitative and qualitative difference in the carbohydrate content of the LPS. The LPS of the serum-resistant strain yielded glucose, galactose, rhamnose and two minor sugar components, while the LPS of the serum-sensitive strain yielded glucose only (Wardlaw, 1963). In the abovementioned investigations, a reduction in LPS O-side chains associated with serum resistance was also correlated with the more drastic change in colony morphology.

No significant difference in the amount of LPS from twenty-eight smooth urinary isolates of *E. coli* differing in serum resistance, was observed (Taylor, 1976). Likewise, mutations to serum resistance occurred independently of LPS changes (Taylor, 1975). Also, changes in the yield of LPS was not observed for *E. coli* strains which were phenotypically induced from serum sensitivity to serum resistance (Taylor and Robinson, 1980).

Several questions arose as to why some clinical strains of *E. coli* were smooth by cultural, morphological and serological criteria, indistinguishable from serum-resistant strains : but susceptible to serum exhibiting a delayed response. Colony morphology could possibly be an unimportant determinant of serum resistance. Subsequent results have supported this

statement, indicating that changes in the O-side chain material may occur independently of colony morphology.

In other genera, stable serum resistance has been associated with a change in colony morphology of *N. gonorrhoeae* (McCutchan *et al,* 1976; James *et al,* 1982) and *H. influenzae* (Gilsdorf and Ferrieri, 1986). Transparent colonies of *N. gonorrhoeae* (James *et al,* 1982) and opaque colonies of *H. influenzae* (Gilsdorf and Ferrieri, 1986) were serum resistant; and vice versa. The association between serum susceptibility and colony morphology appears to be a minor determinant of serum resistance, since no difference in colony appearance of strains varying in serum susceptibility was observed in other studies of *E. coli* (Reynard and Beck, 1976), *N. gonorrhoeae* (Tramont *et al,* 1977) and *P. aeruginosa* (Schiller *et al,* 1984).

Two rough variants were obtained from a smooth serum-resistant *E. coli* strain. One rough form had lost its ability to synthesise LPS O-side chains, due to a mutation in the *rbf* gene involving O-repeat unit biosynthesis, the other rough form retained its serological specificity and produced a complete LPS. The former was killed immediately; the latter in a delayed fashion (Taylor, 1975; 1976). Furthermore, the degree of killing was directly related to the ratio of mannose, an O-side chain sugar, to L-glycero-D-mannoheptose, a core sugar (Taylor and Robinson, 1980). Later studies confirmed that a high degree of substitution of LPS core stubs by long O-side chains was responsible for the increased serum resistance of *E. coli* (Joiner *et al,* 1982a; 1982b; 1986). Recently Porat *et al* (1987), reported that LPS from serum-resistant *E. coli* clones had a greater proportion of higher molecular weight and more highly O-antigen-substituted subunits than LPS isolated from their serum-sensitive parent strain.

Although other bacterial species have not been as extensively studied as *E. coli*, the general idea that LPS and in particular its O-side chain, is a major determinant of serum resistance, has been reported.

Serum-sensitive *S. marcescens* carried LPS which was less antigenic, and present in smaller amounts compared to serum-resistant strains (Jessop and Lambert, 1986).

Clinical serum-sensitive strains of *P. aeruginosa* obtained from patients suffering from cystic fibrosis had rough LPS i.e. LPS deficient in O-antigen (Hancock *et al,* 1983). Schiller *et al* (1984) studied isogenic strains of *P. aeruginosa* differing in serum susceptibility. LPS from the serum-resistant strains were heterogeneous, containing O-side chains of variable length, while the serum-sensitive strain had LPS consisting of only lipid A and core oligosaccharide. Serum susceptibility was also associated with LPS lacking heptose, glucose, galactose and N-acetyl hexosamines, components of the O polysaccharide in *P. aeruginosa* (Guymon *et al,* 1982).

Certain LPS mutants of *N. gonorrhoeae* also differed in their response to serum (Shafer *et al,* 1984). By culturing *N. gonorrhoeae* in continuous culture under glucose-limiting conditions, an increase in LPS antigen related to an increase in serum resistance was obtained (Morse *et al,* 1983).

Recent studies of *C. fetus* (Perez *et al,* 1985a) and *C. jejuni* (Blaser *et al,* 1986) showed that the mechanism of serum resistance was similar to that of *E. coli* previously reported. The LPS of serum-resistant *C. fetus* had a high carbohydrate content and a smooth-type LPS on polyacrylamide gel analysis, in contrast to serum-sensitive strains (Perez *et al,* 1985a). Although *C. jejuni* produced only rough or smooth-rough type LPS, serum sensitivity corresponded with the amount of LPS, reflected by the amount of carbohydrate or KDO, and to the length of the LPS O-side chains as determined by the KDO : carbohydrate ratio (Blaser *et al,*1986).

Affirmatory evidence for the involvement of O-side chains in serum resistance, is an association of serum resistance with O-antigen serotypes. This has only been observed for clinical

isolates of *E. coli*. Table 2.1 indicates *E. coli* serogroups corresponding to serum susceptibility.

Table 2.2 *E. coli* **serogroups corresponding to serum susceptibility.**

Serogroups	01	02	04	06	07	09	018	075
Kimball *et al* 1964	SR[a]		SR	SR				SR
Taylor 1974	SS	SS	SS			SS	SR[a]	SS
McCabe *et al* 1978	SS	SR[b]	SR	SS	SR		SR	
Hughes *et al* 1982				SS	SS	SS	SR[b]	SS

[a] > 50 per cent of strains are serum resistant

[b] serum resistance of these strains are significantly different from serum-sensitive strains

Occasionally, serum resistance has not been associated with the LPS of *E. coli* (Pitt, 1978), *P. aeruginosa* (Hancock *et al,* 1983), *C. fetus* and *C. jejuni* (Perez *et al,* 1985a).

At the other extreme, a loss of LPS content was observed when serum resistance of *Capnocytophaga* was expressed (Wilson *et al,* 1987). The LPS of serum-resistant *N. gonorrhoeae* was deficient in a higher molecular weight component of LPS compared with that of serum-sensitive parental strains (Tan *et al,* 1986). This indicates that non-LPS characteristics may also confer serum resistance to certain bacterial species.

In reviewing the literature, it is important to realise that different smooth and rough LPS types may be artefactual, since methods for extraction have differed. The phenol-water method (Westphal and Jann, 1965) efficiently extracts smooth LPS, but only a proportion of rough LPS. Conversely, the method described by Galanos *et al* (1969) preferentially extracts rough LPS, not smooth LPS. These inconsistencies question the validity of differences described between serum-resistant smooth strains and serum-sensitive rough strains.

2.2.2 ACIDIC / CAPSULAR POLYSACCHARIDES

Capsular polysaccharides are heterogeneous polymers differing in antigenicity, molecular size, biophysical properties and in the intimacy of their association with the cell wall. Perhaps it is due to these factors that inconsistent results have been reported regarding the interactions of encapsulated bacterial strains with serum, and especially with its C′ components.

The virulence antigen, an acidic polysaccharide, of *S. typhimurium* (Muschel *et al,* 1958; Osawa and Muschel, 1964) and urinary and intestinal strains of *E. coli* (Muschel, 1960) have been associated with serum resistance. However, the correlation was incomplete since too few strains were studied.

No correlation was observed between serum susceptibility and the K antigen of *E. coli* strains isolated from patients suffering from bacteraemia or meningitis (McCabe *et al,* 1978; Pitt, 1978; Bortolussi *et al,* 1979). Mutations to serum resistance did not result in an increase in exopolysaccharide production (Taylor, 1975). The inheritance of the *his*-linked genes involved in K27-antigen production by *E. coli* did not lead to an altered serum response (Taylor and Robinson, 1980). This lack of contribution to serum resistance by the K27 antigen was confirmed by Opal *et al* (1982). The absence of a relationship between capsular polysaccharides and serum resistance was reported for clinical strains of *Klebsiella, P. aeruginosa, Salmonella* species (Taylor, 1983a) and *H. influenzae* (Gilsdorf and Ferrieri, 1986).

Since *E. coli* strains producing the K1 capsular antigen were isolated from neonates with meningitis and renal infections (Taylor, 1983a), K1 antigen appears to contribute to the pathogenicity of *E. coli.* In animal experiments using mice, the K1 antigen of *E. coli* has been linked with virulence, with (Howard and Glynn, 1971) and without (Bortolussi *et al,* 1979) serum resistance. In another *in vitro* study, loss of a capacity to produce K1 antigen in chemostatically-grown cells was accompanied by an increase in serum sensitivity (Taylor and Robinson, 1980). Opal *et al* (1982) also obtained results comparing isogenic strains of *E. coli*, suggesting that the K1 antigen does protect some rough strains of *E. coli* from serum. The role of the capsular polysaccharide in serum resistance of *E. coli* K1 isolates was confirmed by Leying *et al* (1990). However, no relationship between the K1 antigen and serum resistance was observed in faecal, blood or cerebrospinal fluid isolates of *E. coli* K1 that were tested (Bjorksten *et al,* 1976).

An association of the K21 capsular polysaccharide of *'Klebsiella aerogenes'* with serum resistance has been reported recently. This action was to a certain degree specific, since the colanic acid capsule which was antigenically related to the K21 antigen but chemically distinct produced by *E. coli,* did not confer serum resistance on *E. coli* (Allen *et al,* 1987).

It appears that the acidic or capsular polysaccharides of certain Gram-negative bacteria cannot be universally considered as a mechanism providing protection against serum killing.

2.2.3 OUTER MEMBRANE PROTEINS

Outer membrane proteins (OMP) are thought to be the major serum resistance determinant for *N. gonorrhoeae*. Serum-resistant strains may be obtained by transformation of serum-sensitive strains with deoxyribonucleic acid (DNA) isolated from gonococci causing systemic infection. This conversion is accompanied by the acquisition of a new 36 500 Dal OMP, also known as protein 1 (Hildebrandt *et al,* 1978). This association was observed by Cannon *et*

al (1981), and has been thought to be a result of a close linkage between the serum-resistance and protein-1 genes. McShan *et al* (1987) reported on an influence of a 29 000 Dal protein, antigenically distinct from protein 1, on serum resistance. Recently, Odum *et al* (1987) studied *N. gonorrhoeae* strains which differed in antigenic protein-1 serotypes previously described by Buchanan and Hildebrandt (1981). Generally, serotypes one to three were serum resistant, while serotypes eight and nine were serum sensitive, confirming the role of protein 1, and OMP, in serum resistance. In contrast, James *et al* (1982) found that the absence of protein 1 was associated with an increase in serum resistance.

However, OMP is not a key determinant of serum resistance for other Gram-negative bacteria, including *H. influenzae* (Gilsdorf and Ferrierri, 1986), *S. marcescens* (Jessop and Lambert, 1986), *C. jejuni* and *E. coli* (Blaser *et al,* 1986; 1988).

There have only been two reports of an influence of OMP on serum resistance, provided a full complement of LPS O-side chains (smooth LPS) was present. A serum-resistant smooth mutant of *E. coli* produced more of a 46 000 Dal OMP than its serum-sensitive parent strain (Taylor and Parton, 1977). Similarly, a 100 000 to 125 000 Dal OMP was required to convert smooth strains of *C. fetus* to complete serum resistance (Blaser *et al,* 1987).

2.3 GENETICS OF SERUM RESISTANCE

Genes involved in serum resistance have been encoded on various plasmids in a few enteric bacteria.

In *E. coli* an increased serum resistance was observed when the Col-V plasmid-carrying strains were tested using rabbit and chick serum. The Col-V plasmid conferred increased virulence in experimental chicks. The serum-resistance gene *iss* (increased survival in serum), was mapped to a 5.3kb sequence, closely linked to the genes involving colicin production

(Binns *et al,* 1979). Of eight Col-V plasmids transferred by conjugation into plasmid-lacking serum-sensitive strains of *E. coli* K12, only three conferred serum resistance against rabbit serum (Nilius and Savage, 1984).

Antibiotic-resistance plasmids, R1 and R100 (NR1) conferred a low level of resistance to rabbit serum on *E. coli* K12 (Reynard and Beck, 1976). Subsequent studies of rough *E. coli* K12 strains carrying a wide range of antibiotic-resistance plasmids and colicin-production plasmids, including R1 and NR1, indicated that these plasmids had no significant effect on serum resistance (Taylor and Hughes, 1978). This discrepancy in results was thought to be partly due to a different source of serum. Reynard and Beck (1976) used commercially available lyophilised rabbit serum, whereas Taylor and Hughes (1978) employed fresh rabbit and human serum. Further insights into this matter were gained by the findings of Ogata and Levine (1980) who studied the effects of various concentrations of rabbit, guinea pig and human sera on *E. coli* K12 strains carrying the R100 plasmid. The level of resistance was found to be dependent on the type of serum, the organism used, the bacterial and the serum concentration employed. A difference between serum susceptibility of strains with or without the plasmid was only observed when a high inoculum (10^7 -10^8 CFU/ml) and a concentration of rabbit or human serum less than 10 per cent was used. Previous investigators may thus have failed to detect the protective effects of these plasmids against serum if the assay parameters were not optimised.

There is some evidence suggesting that LPS, the outer membrane component mainly responsible for serum resistance in Enterobacteriaceae, influenced the expression of the plasmid factors which affected susceptibility to serum. The acquisition of the R100 plasmid by a 'smooth' strain of *E. coli* K12 carrying the O polysaccharide, correlated with a slight increase in serum resistance (Taylor and Hughes, 1978; Ogata and Levine, 1980). The R1 and R100 plasmid affected the serum sensitivity of strains exhibiting a delayed serum-killing kinetics, but had no effect on strains which were killed immediately by serum. This difference in response to serum was associated with a change in LPS (Taylor and Robinson, 1980).

A large, conjugative, multiple antibiotic-resistance plasmid R6-5 carried a gene involved in resistance against rabbit serum, *tra T,* in *E. coli* K12. This gene coded for an OMP (25 000 Dal) participating in surface exclusion in conjugal transfer processes, and very possibly in preventing C′ interactions at the cell wall. The mechanism of this protein appeared to be dose unrelated and specific, since point mutations in the *tra T* gene no longer conferred protection for the micro-organism against serum (Moll *et al,* 1980).

The plasmids R100 and R6-5 shared some homology with each other, but not with the Col-V plasmid, suggesting that the latter involved a different gene product (Taylor and Hughes, 1978). The gene products of the serum-resistance genes, *iss* (Col-V plasmid) and *tra T* (R6-5 plasmid) were antigenically distinct, although a similar level of serum resistance was conferred by both. The effect on serum resistance of both gene products was not affected by an increased gene copy number (Binns *et al,* 1982).

Although plasmid-determined serum resistance has been described, it appears to be a contributing rather than a primary factor in *E. coli.* The serum resistance it conferred is insufficient to account for the complete resistance to NHS observed in many clinical isolates. The protection conferred by plasmids in *E. coli* was expressed in chicken serum (Binns *et al,* 1979), guinea pig serum (Ogata and Levine, 1980) and rabbit serum (Reynard and Beck, 1976; Taylor and Hughes, 1978; Moll *et al,* 1980; Ogata and Levine, 1980). However, concentrations of greater than 10 per cent NHS killed strains with or without these plasmids, implying that plasmid-determined serum resistance plays a minor role in increased pathogenesis in human infections. In support is a lack of correlation observed between serum resistance and antibiotic resistance or colicin production (R or Col-V plasmids) in *E. coli* strains isolated from patients with urinary tract infection, where the serum concentration was greater than 10 per cent in the serum bactericidal test (Hughes *et al,* 1982).

Serum resistance has also been correlated with plasmid carriage and virulence in *Y. enterocolitica* (Pai and DeStephano, 1982) and *S. typhimurium* (Hackett *et al,* 1987; Van den Bosch *et al,* 1987). A 11 000 Dal OMP coded by a 100 000 Dal *S. typhimurium* plasmid, conferred a higher level of serum resistance to *S. typhimurium* than the *tra T* protein conferred on *E. coli* strain (Hackett *et al,* 1987).

Serum resistance is not plasmid mediated in *N. gonorrhoeae*. The predominant determinant of serum resistance in *N. gonorrhoeae* has been identified as protein 1, a 35 000 to 37 000 Dal OMP. This was confirmed by genetic studies, where serum-sensitive strains were transformed by DNA isolated from strains causing disseminated gonococcal infection, to serum resistant (Hildebrandt *et al,* 1978). However, Cannon *et al* (1981) found that the production of protein 1 and the expression of serum resistance in *N. gonorrhoeae* could be genetically separated. The genetic locus affecting susceptibility to NHS, *sac-1*, was closely linked to the gene coding for protein-1 synthesis, *nmp-2*. Other loci affecting susceptibility to serum have been described as *sac-2* (Spratt *et al,* 1980) or *sac-3* (Shafer *et al,* 1982). *Sac-1* or *sac-3* are closely linked, and when present in combination, result in an accumulated level of serum resistance, suggesting that serum resistance is a genetically-dominant characteristic (Shafer *et al,* 1982). The *'sac'* genes are also positioned near to genes involving auxotype (Spratt *et al,* 1980) and OMP (Cannon *et al,* 1981) production; both properties have been associated with virulence of *N. gonorrhoeae*. The varying sensitivity to serum of clinical gonococcal isolates may be explained by the fact that serum resistance is encoded by closely-linked multiple chromosomal genes.

2.4 FACTORS AFFECTING SERUM RESISTANCE

The outer membrane composition determines the outcome of serum killing of Gram-negative bacteria. Environmental factors affecting the outer membrane including the LPS, would indirectly influence the organism's susceptibility to serum. These factors are frequently related to a change in metabolism and are described below.

The composition of the media on which bacteria are grown is important in the determination of LPS structure. *P. aeruginosa,* grown in minimal medium with succinate, produced an LPS which was deficient in heptose, glucose, alanine, galactosamine and glucosamine; this deficiency was not observed when the bacterium was grown in nutrient broth (Chester and Meadow, 1975). Rough and serologically non-typable strains of *P. aeruginosa* became smooth and typable when maintained in a soft agar containing 0.5 per cent peptone in distilled water. In addition to these changes, a change in pigment production and growth rate was also noted (Liu, 1988). Polyacrylamide gel electrophoresis analysis of LPS from *S. typhimurium* strains grown on complete medium and on minimal medium were similar except for a difference in the intensity of the bands on staining (Palva and Makela, 1980). Different media altered the LPS, but not outer membrane proteins, of *H. ducreyi.* In addition small variations in LPS were observed when different batches of the same medium were used for culture of *H. ducreyi* (Abeck *et al,* 1987c).

The growth rate of a microbial population in a continuous culture may be adjusted by varying the dilution rate of the energy source. As the growth rate of *S. enteritidis* increased, total nitrogen and protein content, mannose, rhamnose and tyvelose content decreased. The fastest growing cells underwent a partial smooth to rough form conversion which could be reversed when the growth rate was decreased (Collins, 1964). Conversely, Morse *et al* (1983), found that gonococcal LPS isolated from rapidly growing cells contained more O-serotype antigen viz. a three- to four-fold increase in total carbohydrate content, than LPS from

slower growing cells. Similarly, *Neisseria sicca,* cultured at a high aeration rate, grew faster and contained a greater proportion of LPS than did cells grown at a lower aeration rate. A difference between the two LPS populations was found with respect to hexosamine, KDO, galactosamine and fatty acids content (McDonald and Adams, 1971).

Generally, Gram-negative bacteria are more susceptible to serum in the early logarithmic phase compared to the lag or the stationary phase (Rowley and Wardlaw, 1958; Davis and Wedgwood, 1965; Tramont *et al,* 1977; Allen and Scott, 1980; DeMatteo *et al,* 1981). The metabolic state of a cell contributes to the efficiency of C′-mediated killing. In support of this was the finding that optimal killing of *E. coli* and *Y. pestis* by C′ was energy requiring (Griffith, 1974; Taylor, 1983b). The use of glycerol or acetate as a carbon source by *E. coli* was associated with serum susceptibility (Melching and Vas, 1971). High concentrations of broth protected *S. typhimurium* (Maaloe, 1948) and *Shigella dysenteriae* (Michael and Braun, 1959) from the bactericidal activity of serum. The utilisation of various sugars and vital amino acids increased susceptibility to serum; metabolic inhibitors decreased susceptibility to serum (Maaloe, 1948; Michael and Braun, 1959). This substantiates an association between serum resistance and metabolism.

The effect of temperature on LPS has mainly been studied in Enterobacteriaceae. When *Y. pestis* was cultivated at 25^0 C, the cell walls were 'smooth', and became 'rough' when grown at 37^0 C as reflected by a lower proportion of KDO, a lower phosphate : KDO ratio and an increased gel mobility on polyacrylamide (Darveau *et al,* 1983a). Similarly, *P. aeruginosa* at 15^0 C contained a higher content of long-chain LPS than that extracted from cells grown at 45^0 C (Kropinski *et al,* 1987). Temperature also affected the fatty acid content of lipid A in *Salmonella* species (Wollenweber *et al,* 1983) and *P. aeruginosa* (Kropinski *et al,* 1987).

Chemical manipulations affecting the cell wall have been reported to influence the organism's response to serum killing. *N. gonorrhoeae*, grown at different pH's have varying requirements for glucose, produce a modified cell wall, and have altered surface properties. pH in-

directly contributes to serum resistance of *N. gonorrhoeae* (Veale *et al,* 1981). Hypertonic sucrose inhibited the bacterial activity of lysozyme against rough strains of *E. coli* (Feingold *et al,* 1968), and incubation of *E. coli* with diphenylamine converted serum-sensitive strains to serum resistant (Feingold, 1969). Ethylene diamine tetraacetic acid (EDTA) and/or Tris, chemicals which remove components from the cell wall of serum-resistant *Salmonella* strains, rendered them serum sensitive. The presence of magnesium or calcium ions appeared to have the opposite effect of stabilising the cell wall and thus serum resistance (Reynolds and Rowley, 1969).

Induced serum resistance has been reported in particular for *N. gonorrhoeae*. However, the mechanism of the induction or the factors involved are unknown. Serum resistance was induced by passage of *H. influenzae* in rats (Gilsdorf and Ferrieri, 1986), and *N. gonorrhoeae* in subcutaneously implanted chambers in guinea pigs (Rittenberg *et al,* 1977; Goldner *et al,* 1979). In the latter study, the chamber was slightly alkaline, not anaerobic (Goldner *et al,* 1979), and had a C′ concentration one tenth that of blood serum (Rittenberg *et al,* 1977). After the gonococcal infection was established the chamber fluid was analysed. Rittenberg *et al* (1977) reported that glucose concentration decreased, while alkaline phosphatase, asparate aminotransferase activity and uric acid concentration, increased. Lactate, glutamine and proline decreased by 60 to 100 per cent; glucose decreased by 0 to 30 per cent in the study by Goldner *et al* (1979). These changes suggested that serum resistance induction may be as a result of utilisation of a specific substrate. In both studies, acquisition of serum resistance was not only due to absorption of factors, but also required metabolism. The conversion did not occur at sub-optimal growth temperatures.

Other fluids have been reported to induce serum resistance to *N. gonorrhoeae*. These include urethral pus (Ward *et al,* 1970), vaginocervical secretions and seminal plasma (Martin *et al,* 1982).

A 'serum resistance inducing factor' for *N. gonorrhoeae* has been postulated to occur in human serum (McCutchan *et al,* 1976; Veale *et al,* 1981; Martin *et al,* 1982), guinea pig serum and foetal calf serum (Veale *et al,* 1981). The conversion to serum resistance *in vitro* of *N. gonorrhoeae* was again related to metabolism and not just absorption, and was lost after prolonged incubation (twenty-four hours), as observed in *in vivo* studies. However, *in vitro* alteration of glutamine, proline, lactate or iron concentration had little effect on serum resistance induction. Guinea pig serum proved most efficient in inducing serum resistance, followed by foetal calf serum and NHS. Swine, sheep and horse serum did not contain this inducing factor. Both non-diffusable and diffusable fractions of dialysed guinea pig serum promoted serum resistance induction, although the lower molecular weight diffusable fraction was most efficient. A low molecular weight fraction in guinea pig red blood cell lysate was also found to induce gonococcal serum resistance (Tan *et al,* 1986).

Serum-resistant strains have been isolated from serum-sensitive populations of *E. coli* (Akiyama and Inoue, 1977) and *N. gonorrhoeae* (Goldner *et al,* 1979; James *et al,* 1982), after repeated exposure to NHS in serum bactericidal assays.

Generally, the environmental factors resulting in phenotypical cell wall changes are reversible. The conversion to serum resistance was too rapid to be attributed to selection or mutation.

2.5 MECHANISMS OF SERUM RESISTANCE

The normal bactericidal property of human serum depends on the presence of C′ precursors and their activation in specific ways. C′ may be activated by the so-called classical pathway, which involves antigen and Ig M and Ig G antibody interaction. Conversely, the alternative pathway may be activated by LPS, and is more immediately available to the host albeit with a lower efficiency of bacterial killing.

The bacterial cell wall (Winkelstein and Tomasz, 1977), especially LPS (Galanos *et al,* 1971; Fine, 1974) plays a role in C′ activation. In the study of C′ activation, isolated LPS instead of whole bacterial cells were used. This removed possible variables created by the presence of several other cellular components (Morrison and Kline, 1977; Grossman and Leive, 1984). Similarly LPS-coated red blood cells (Fine, 1974) or LPS-containing liposomes (Porat *et al,* 1987) identified LPS as a site mediating C′ activation.

LPS is capable of activating both pathways, although killing via the classical pathway appears to be more rapid than by the alternative pathway (Traub and Kleber, 1976; Taylor, 1983b). The LPS component involved in activating the classical pathway is lipid A (Galanos, 1971; Morrison and Kline, 1977). 'Smooth' strains activated the alternative pathway via the O-polysaccharide LPS component (Morrison and Kline, 1977; Joiner *et al,* 1983b; Liang-Takasaki *et al,* 1983a; 1983b; Goldman *et al,* 1984; Grossman and Leive, 1984). Morrison and Kline (1977) reported that two LPS preparations which were antigenically and chemically identical, activated C′ differently, implying that the carbohydrate composition was not the primary determining factor. In contrast, C′ activation via the alternative pathway was found to be sensitive to variations in chemical structure, but not to large variations in the length of the O-side chains (Grossman and Leive, 1984). Thus enteric bacteria viz. *E. coli, Salmonella, Shigella* (Reed and Albright, 1974) and *S. marcescens* (Traub and Kleber, 1976) are able to activate C′ via either pathway, depending on the heterogeneity of their LPS composition.

As previously mentioned, non-enteric bacteria commonly have rough-type LPS, i.e. they lack long O-polysaccharide chains. One would thus expect the classical pathway to be activated by these bacteria. Indeed this has been observed for *N. gonorrhoeae* (Shafer *et al,* 1984a; Joiner *et al,* 1985a), *C. jejuni* (Blaser *et al,* 1985) and *H. influenzae* (Gilsdorf and Ferrieri, 1986).

Whether activated via the classical or alternative pathway, the C′ cascade ultimately results in the formation of a polymeric C5b-C9 protein known as the membrane attack complex (MAC). This cylindrical or channel-like structure inserts through the bacterial cell membrane, with a hydrophobic surface providing a lipid anchor and a hydrophilic centre, a region through which water and small ions move (Hanson and Wigzell, 1985; Frank *et al,* 1987).

The action of C′ on bacterial cells appears to gradually disrupt essential metabolic processes thus contributing to cell death. When a smooth strain of *E. coli* was exposed to NHS, certain biochemical changes were observed. Ribonucleic acid (RNA) and DNA synthesis decreased after five minutes and fifteen minutes respectively. The cell membranes were functionally and physically disrupted after approximately thirty minutes. Permeability control and protein synthesis were also affected at this time following exposure to serum (Melching and Vas, 1971). Taylor (1983b) also observed that macromolecular synthesis was altered only late in the reaction sequence while inhibition of RNA, DNA and protein synthesis occurred only after total loss of bacterial colony-forming ability.

There are several schools of thought regarding the site of action and mechanism of C′ killing. There is evidence to suggest that outer membrane damage may be non-lethal. Wright and Levine (1981) reported that EDTA, which resulted in an increased outer membrane permeability, did not affect the viability or growth rate of *E. coli*; mutants of *E. coli* that synthesised leaky outer membranes grew well in nutrient-rich medium; and spheroplasts were capable of growing in an osmotically protective medium.

Similarly, outer membrane damage due to MAC insertion was also thought to be non-lethal, since osmotically-shocked (plasmolysed) *E. coli* cells were not immediately killed by NHS (Feingold *et al,* 1968),

Wright and Levine (1981) proposed that the MAC inserted at the junction between the inner and outer membrane of the bacterial cell wall (Bayer's junction), since inner membrane and

outer membrane damage occurred with identical kinetics and dose response, indicating they were closely coupled events. Inner membrane damage differed from outer membrane damage in that only small molecules traversed the damaged inner membrane, whereas macromolecules traversed the damaged outer membrane. This proposed mechanism resembled colicin killing of bacteria, where cell death was a result of the formation of ion-permeable channels in the inner membrane, which permitted a rapid efflux of intracellular potassium ions. In a later study, Taylor (1983b) found that MAC insertion did not simultaneously disrupt the inner and outer membrane as previously reported (Wright and Levine, 1981). Taylor's results were obtained from radiolabelling, and analysis of intracellular enzyme release.

Several workers have emphasised a positive contribution of lyzozyme to C′ killing (Glynn and Milne, 1967; Feingold *et al,* 1968; Taylor, 1983b). Lyzozyme, which degrades peptidoglycan, a cell wall component, gained access via the 'holes' created by the MAC. This resulted in further degradation of the cell wall and eventually cell lysis.

A change in fatty acid composition has been correlated with an altered serum susceptibility of *E. coli* strains. It was thought that an increased rigidity of the cell membrane repelled the insertion of the MAC preventing C′ killing of serum-resistant strains (Akiyama and Inoue, 1977).

Following observations that serum-resistant strains were commonly of 'smooth' colony morphology as opposed to serum-sensitive 'rough' strains; and that serum-resistant strains had quantitatively more LPS relative to serum-sensitive strains, several mechanisms of serum resistance had been proposed (Reynolds and Pruul, 1971, Binns *et al,* 1982; Frank *et al,* 1987) prior to detailed studies by Joiner *et al* (1982a; 1982b). However these are not well established :

- C′ was not activated by serum-resistant strains.

- The thick polysaccharide layer of serum-resistant strains blocked the cell receptors to which C′ binds to the bacterial cell wall.

- C′ was activated, but the sequence to the formation of the MAC was blocked. Plasmid-mediated serum resistance observed in *E. coli* was thought to be due to disruption of the C′ pathway subsequent to the C5 step (Ogata and Levine, 1980).

- C′ was activated by serum-resistant strains but one or more C′ components did not bind to the organism. Jessop and Lambert (1986) observed that serum-resistant strains of *S. marcescens* bound less C3 than serum-sensitive strains.

- C′-mediated damage of serum-resistant strains occurred, but was non-lethal due to the presence of a supportive LPS layer.

- C′ was activated by serum-resistant strains, but the MAC was bound too far from the cell membrane due to the polysaccharide component of LPS. Rowley (1971) mathematically considered the physical dimensions of a bacterial cell in relation to its interaction with C′, and reasoned that the concentration of C′ required to reach the underlying cell membrane was inversely proportional to the cube of the thickness of the endotoxin layer. Thus after a certain thickness was reached, an organism could become totally resistant to serum, due to the failure to attain the required concentration of attached C′.

In extensive studies, Joiner *et al* (1982a) found that serum-resistant and serum-sensitive *Salmonella minnesota* strains activated C′ in NHS. C3 deposited on both serum-resistant and serum-sensitive bacterial surfaces; twice as much C3 was deposited on the serum-resistant strains than on the serum-sensitive organisms. Previously, quantitative C3 deposition was not related to serum susceptibility of *E. coli* and *S. typhimurium* (Reynolds *et al,* 1975; Ogata and Levine, 1980). Serum-resistant strains of *S. minnesota* also depleted C5,

C7 and C9 components from NHS to a greater extent than serum-sensitive strains. Deposition of C5, C7 and C9 was stable on the surface of serum-sensitive but not serum-resistant organisms. In the latter, C5, C7 and C9 were progressively lost ten minutes after being cell bound. These results suggested that binding of C′ was stable until the completion of the MAC. Subsequently, the MAC was not successfully inserted into the outer membrane conferring serum resistance on smooth strains of *S. minnesota* (Joiner *et al,* 1982b). Buffers of high ionic strength, and trypsin eluted the MAC from serum-resistant strains, but not from serum-sensitive strains, suggesting firstly, that hydrophilic interactions held the MAC to the cell surface, and secondly, that the MAC was surface bound and not inserted into the lipid (Joiner *et al,* 1982b).

The role of LPS in this mechanism was investigated. The length of the LPS O-side chain, as determined by the ratio of mannose (O-polysaccharide sugar) to galactose (R-core sugar), was compared with the quantity of C3 bound to the cell wall. It appeared that the longest O-side chain was the site of attachment for C3. Since C3 attachment leads to the activation of the MAC, the MAC would be less likely to insert successfully if the C3 component was bound too far from the lipid part of the cell membrane, resulting in serum resistance (Joiner *et al,* 1986; Frank *et al,* 1987).

Goldman *et al* (1984) characterised the O antigen of *E. coli* LPS. The results obtained supported the mechanism proposed by Joiner *et al* (1986) i.e. the presence of the O-side chains prevented successful insertion of the MAC, although C′ was activated and the C′ sequence was completed.

Serum resistance of *P. aeruginosa*, a non-enteric bacterium, was also found to be due to a failure of the MAC to stably insert into the outer membrane; rather than a failure to activate C′ efficiently, as confirmed by trypsin treatment of serum-resistant and serum-sensitive strains following exposure to NHS (Schiller and Joiner, 1986). This new finding suggested

that the mechanism of serum resistance proposed by Joiner *et al* (1986) may not necessarily be confined to Enterobacteriaceae.

However, an alternative mechanism of serum resistance was sought for genera of bacteria with rough-type LPS, in particular *N. gonorrhoeae*. In these cases, C′ activation follows an antibody-antigen interaction.

Initially, serum resistance was thought to be a result of an absence of an antigen-binding site (LPS) for the antibody. Hildebrandt *et al* (1978) suggested that the large OMP responsible for serum resistance in *N. gonorrhoeae*, protein I, blocked the site of antigen-antibody reaction in LPS, thus preventing cell lysis. A decrease in accessibility of a monoclonal antibody directed to a core LPS determinant was associated with an increase in serum resistance (Morse *et al,* 1983).

Although rough LPS lacks the O-polysaccharide component, variations have been found in the core oligosaccharide of *N. gonorrhoeae*. These variations have been implicated in the determination of serum susceptibility (Shafer *et al,* 1984).

The attachment of late C′ components to serum-resistant and serum-sensitive strains of *N. gonorrhoeae* was studied. C′ activation, deposition and stability on the cell surface were similar in both cases (Harriman *et al,* 1982; Joiner *et al,* 1983a). Treatment with a high molarity salt solution had little effect on the MAC, although trypsin treatment released more MAC from the serum-resistant strains than the serum-sensitive strains.

Results obtained from degradation of the exposed gonococcal cells with detergent, and centrifugation of the components, suggested that the fragments to which the MAC was attached, were larger in the serum-resistant than in the serum-sensitive organism. The molecular configuration of the C5b-C9 complex, or the cellular proteins to which this MAC was associated, varied with serum susceptibility (Joiner *et al,* 1983a). Monoclonal antibodies

directed against identical or closely associated surface-exposed epitopes of protein 1 differed markedly in bactericidal activity despite leading to deposition of nearly equivalent amounts of C3 and C9 molecules per organism (Joiner *et al,* 1985a).

Exposure of a serum-resistant strain to immune rabbit antisera resulted in increased killing and C9 uptake on subsequent incubation in serum. It was suggested the C9 may have altered the site and distribution of the MAC resulting in cell lysis (Joiner *et al,* 1983a). Subsequent studies have shown that monomers of C9 incubated at 37^0 C polymerised to form more stable MAC complexes. These findings suggest that multimeric C9 polymerisation may have an important role in optimising bacterial cell lysis (Joiner *et al,* 1985b).

A blocking agent, thought to be an antibody in NHS has been reported to contribute to serum resistance in *N. gonorrhoeae* (Tramont *et al,* 1977; McCutchan *et al,* 1978; Harriman *et al,* 1982; Rice and Kasper, 1982; Joiner *et al,* 1985c). However, this is not always the case. Tramont *et al* (1977) tested several gonococcal strains on specimens of NHS and consistently found at least one strain that was serum sensitive.

In a study of serum-resistant strains of *N. gonorrhoeae* isolated from patients suffering from disseminated gonococcal infection, Rice and Kasper (1982) reported that seven of ten specimens of NHS inhibited killing of these serum-resistant strains by immune sera. The blocking agent was identified as Ig G. Its action was specific, since F $(ab)_2$ fragments prepared from the Ig G also prevented killing by immune serum. The binding site of this blocking antibody (Ig G) appeared to be an outer membrane protein of serum-resistant strains. The presence of protein I has been associated with serum resistance in *N. gonorrhoeae.* The blocking antibody bound to an OMP identified as protein III, which was closely located to protein I. These results were confirmed by Joiner *et al* (1985c) who concluded that blocking antibody Ig G competes with the bactericidal antibody for binding to the organism, but enhances rather than blocks C′ activation, and leads to C′ deposition at new sites that do not result

in serum killing. Later Rice *et al* (1986) verified the role of protein III antibodies in human serum as blocking antibodies i.e. a gonoccocal strain possessing protein III has the potential to generate antibodies directed against it (blocking antibodies), thus creating an environment where these strains appear 'serum resistant'.

In addition, bactericidal Ig G molecules directed against the LPS of *N. gonorrhoeae* have been reported. The blocking antibody is thus capable of interfering with natural (Ig M) and immune (Ig G) serum killing of both serum-sensitive and serum-resistant organisms, by virtue of cross-reacting outer membrane components, either LPS or OMP (Rice and Kasper, 1982; Frank *et al,* 1987).

The mechanism of serum resistance observed in Enterobacteriaceae differs to that of *N. gonorrhoeae* in several ways. Enterobacteriaceae have smooth or rough LPS, activating both classical and alternative pathway, whereas *N. gonorrhoeae* is characterised by rough LPS and activates the classical pathway. The serum-resistant determinant in the former is the O-polysaccharide chain; in the latter it is due to an interaction of OMP, core oligosaccharide, bactericidal antibody and blocking antibody. In both, C′ is activated. Serum-resistant strains of *Salmonella* consumes more terminal components than its serum-sensitive counterpart. With regard to C′ consumption, serum-resistant *N. gonorrhoeae* did not differ from serum-sensitive strains. Unlike serum-resistant strains of *Salmonella,* serum-resistant *N. gonorrhoeae* strains bind C5 and C9 stably, i.e. for longer than one hour, and to the same extent as serum-sensitive gonococcal strains. The MAC remains on the surface of *Salmonella* and *N. gonorrhoeae*, although it is gradually lost from serum-resistant *Salmonella* bacteria (Harriman *et al,* 1982; Joiner *et al,* 1983a; Frank *et al,* 1987).

From the abovementioned literature review, it is clear that the mechanisms of serum resistance are multiple and complex involving several outer membrane components of Gram-negative bacteria.

PROBLEMS IN DETERMINING SERUM RESISTANCE

3.1 INTRODUCTION

Determination of the bactericidal activity of serum involves exposure of a suspension of viable organisms to a suitable concentration of serum, incubation at an optimum temperature for complement (C′) activity, and determination of the number of surviving organisms after a suitable period of time. This test, the serum bactericidal assay, was also used as a guide of antibiotic therapy by measuring the effect of the patient′s serum against his own infecting organism, during antimicrobial therapy (Pien and Vosti, 1974; Reller and Stratton, 1977; Stratton and Reller, 1977; 1988). Landy *et al* (1962) modified the serum bactericidal assay in order to measure the amount of antibody in normal human serum (NHS); the sensitivity of this test was comparable to passive haemagglutination.

Many variations of the serum bactericidal assay have been employed making significant comparisons between studies impossible. A number of influencing parameters will be discussed.

3.2 INOCULUM PREPARATION

The growth conditions of a bacterium affects its metabolic state and the biosynthesis of its cell surface structures.

Generally, Gram-negative bacteria are more susceptible to serum in early exponential phase as previously mentioned (Rowley and Wardlaw, 1958; Davis and Wedgwood, 1965; Allen

and Scott, 1980; DeMatteo *et al,* 1981). Serum-sensitive strains may thus appear completely refractory to NHS if the inoculum is not in a rapidly dividing state (Davis and Wedgwood, 1965). Most investigators use inocula consisting of rapidly growing cells (Glynn and Ward, 1970; Fierer *et al,* 1972; Goldner *et al,* 1979; Taylor, 1972; 1983a; Nilius and Savage, 1984; Porat *et al,* 1987; Ross *et al,* 1985; 1987; Benge, 1988). However, bacterial inocula obtained from plate cultures have also been used (Michael and Braun, 1958; Waisbren and Brown, 1966; Young and Armstrong, 1972). *Escherichia coli* cells were more readily killed when grown in a simple salts medium than in a nutritionally complex medium (Melching and Vas, 1971). Maaloe (1948) reported that *Salmonella typhimurium* grown in diluted broth was more susceptible to serum than that grown in undiluted broth. It is thus advisable to employ cells in a defined metabolic state in media with minimal batch variation.

Optimum temperatures for growth are important, since deviations may affect serum killing of the inoculum (Osawa and Muschel, 1964; Davis and Wedgwood, 1965; Kato and Bito, 1978).

Serial passage of *E. coli* (Pitt, 1978) and *Campylobacter* species (Blaser *et al,* 1985) did not affect its serum susceptibility. However, conflicting results were reported for *E. coli* (Akiyama and Inoue, 1977), *Neisseria gonorrhoeae* (Ward *et al,* 1970; McCutchan *et al,* 1976; Rittenberg *et al,* 1977; Goldner *et al,* 1979) and *Trichomonas vaginalis* (Demes *et al,* 1988)

Harvesting and washing of the cells may influence the outcome of the assay (Michael and Braun, 1958). Centrifugation or resuspension at 0^0 C to 4^0 C could result in a temperature shock for the cells. (Young and Armstrong, 1972; Wright and Levine, 1981). Although washing of cells was generally not applied, several investigators washed the inoculum in buffer (Taylor, 1972; Young and Armstrong, 1972; Fierer *et al,* 1974; Reynard and Beck, 1976; Binns *et al,* 1982; Gilsdorf and Ferrieri, 1986; Allen *et al,* 1987). Whether this procedure affects the cells is not known, but may depend on the nature of the buffer.

3.3 SOURCE AND CONCENTRATION OF SERUM

C′ activation occurs via the classical or alternative pathway. Both may occur simultaneously (Reed and Albright, 1974; Taylor and Kroll, 1983a). Early colonisation of the intestinal tract by commensal bacteria ensures that small quantities of antibody directed against surface antigens of many types of Gram-negative bacteria are present in the blood and tissue fluids of humans (Mason and Richardson, 1981). Thus Ig M and Ig G antibodies directed against certain neisseriae, *E. coli* (Cohen and Norins, 1966) and other Enterobacteriaceae (Michael and Rosen, 1963) can be detected in the serum of adults in the absence of any history of immunisation or specific infection i.e. NHS. In these cases, antibody need not be added to the serum bactericidal assay. Antibodies against primary pathogens e.g. *Vibrio cholerae* and *S. typhimurium* are generally absent from non-immune sera. In these cases, an external source of antibody is supplied. However, natural and immune antibodies differ in physicochemical properties which may affect their behaviour in the serum bactericidal assay (Michael and Braun, 1964).

Another factor to consider is that immune sera rich in Ig G (blocking antibody) may inhibit the C′-mediated killing (Rice and Kasper, 1982; Joiner *et al,* 1985b), or function poorly in C′ activation compared to Ig M (Normann *et al,* 1972). One should thus be aware of the constituents of the serum employed in the serum bactericidal assay.

Possible sources of error may materialise when serum bactericidal assay systems contain heterogeneous sources of antibody and C′. Use of exogenous C′ is discouraged, since it may complicate the system by adding antibodies which may act synergistically or by blocking the relevant antibodies (Tramont *et al,* 1977); or result in contamination by other bacterial antigens (McCutchan *et al,* 1976) resulting in a deviation of C′ in the serum bactericidal assay.

Sera from different species of vertebrates viz. human, horse, cow, rabbit, guinea pig, pig, kangaroo, chicken, lizard, toad, eel and shark expressed variable bactericidal activity against Gram-negative bacteria (Landy *et al* 1962; Schwab and Reeves, 1966). Mouse serum appeared to have the least bactericidal activity (Landy *et al,* 1962; Wardlaw, 1963; Gilsdorf and Ferrieri, 1986). Within a given species, there was little variation of serum from different healthy individuals, reflecting the ubiquity of the C′ system and a shared spectrum of antibody specificities. This has been particularly well established with human subjects (Wardlaw, 1963; Reed and Albright, 1974). Human, rabbit and guinea pig serum have been used to demonstrate plasmid-mediated serum resistance in *E. coli* (Reynard and Beck, 1976). 1 to 3 per cent of human serum, 5 to 20 per cent rabbit serum and 10 to 30 per cent guinea pig serum was required to demonstrate equivalent plasmid-mediated serum resistance, suggesting that NHS is more bactericidal than rabbit or guinea pig serum (Ogata and Levine, 1980; Binns *et al,* 1982).

Many investigators have used a high serum concentration to ensure that killing is not limited by the availability of essential serum components. It also better simulates *in vivo* conditions (Roantree and Rantz, 1960; McCutchan *et al,* 1976; Corbeil *et al,* 1978). The bactericidal activity of the alternative pathway of C′ was insignificant at a concentration equivalent to a 1 : 16 dilution of NHS (Schreiber *et al,* 1979). However, antibodies are required in such small amounts that they are rarely limiting (Glynn and Milne, 1967).

The rate of serum killing is proportional to the serum concentration present in the serum bactericidal assay (Muschel *et al,* 1958; Rowley, 1956). Strains of *Serratia marcescens* were not killed by 1 per cent NHS. A decrease of four logarithms took one hour in 25 per cent serum but only fifteen minutes in 50 per cent serum (Simberkoff *et al,* 1976). Similar observations were made with *Bacteriodes* (Casciato *et al,* 1979), *Campylobacter* (Blaser *et al,* 1985), *Legionella* (Plouffe *et al,* 1985), *N. gonorrhoeae* (Ross *et al,* 1985) and *Klebsiella* (Allen *et al,* 1987). In the above investigations, less than 10 per cent NHS appeared to be

inefficient in identifying all serum-sensitive strains, whereas serum concentrations 50 per cent or greater sufficed.

Although the most direct way to measure serum sensitivity is the use of a high serum concentration, some workers have estimated the survival of bacteria over a range of serum dilutions (Hildebrandt *et al,* 1978; Branefors and Dahlberg, 1980; Shafer *et al,* 1982). Possible differences in bactericidal titres may reflect different amounts of C′ present. C′components may be diluted to such an extent that the serum becomes non-functional.

3.4 BACTERIAL INOCULUM SIZE

The size of the bacterial inoculum in conjuction with the serum concentration may be an influencing factor in serum bactericidal assay. If the bacterial inoculum is too large, the organism may appear more serum resistant than when a smaller inoculum is used (Stratton, 1988). An inoculum may be too small so that the survivors can not be sucessfully demonstrated. No variations in results were observed when inocula of 10^4 to 10^{10} CFU/ml (Olling, 1977) and 10^2 to 10^6 CFU/ml (Davis and Wedgwood, 1965) were used.

3.5 DILUENTS USED IN THE SERUM BACTERICIDAL ASSAY

The buffer and diluent used in the serum bactericidal assay may have significant and unpredictable effects on the efficiency of C′ killing. Saline (Landy *et al,* 1962; Clas and Loos, 1980; Blaser *et al,* 1985) and distilled water (Taylor *et al,* 1972; Blaser *et al,* 1985) were reported to have a deleterious effect on Gram-negative bacteria, enhancing the serum sensitivity of the test organism. Nevertheless, saline (Fierer *et al,* 1972; Binns *et al,* 1979; Hughes *et al,* 1982; Pai and DeStephano, 1982; Jessop and Lambert, 1986; Hackett *et al,* 1987; Benge, 1988),

phosphate buffer with magnesium chloride (Rowley, 1968) and water (Bortolussi *et al,* 1979) have been employed. Reynard and Beck (1976) also criticised the use of Tris buffer, since divalent cations necessary for C′ activity (Mayer *et al,* 1946) may interact with this buffer. Tris buffer was nevertheless found suitable for serum bactericidal assays in other studies (Taylor *et al,* 1972; Clas and Loos, 1980).

The optimum pH for serum killing in the serum bactericidal assay was found to be 8.4 (Wardlaw, 1963) although Taylor *et al* (1972) and Casciato *et al* (1979) found no significant difference between systems varying in pH from 7.2 to 8.23 and 6.5 to 7.4, respectively. The use of a relatively high serum concentration may limit the effect of pH in the serum bactericidal assay due to its buffering capacity (Landy *et al,* 1962; Taylor *et al,* 1972).

To avoid a variation due to buffering, nutrient media have been used occasionally viz. trypticase soy broth (Kimball *et al,* 1964) and nutrient broth (Taylor *et al,* 1972). Unfortunately, the addition of complex nutrients (Young and Armstrong, 1972) or nutrient media (Davis and Wedgwood, 1965) may have undesirable effects on the outcome of serum bactericidal assay (DeMatteo *et al,* 1981). High concentrations of nutrient broth were reported anticomplementary (Michael and Braun, 1959). Complex nutrient media protected *Shigella dysenteriae* from sera, whereas concentrations of nutrient media less than 0.1 per cent enhanced its susceptibility (Michael and Braun, 1959). Glucose and amino acids known to stimulate metabolic activity enhanced serum killing in serum bactericidal assays (Maaloe, 1948; Michael and Braun, 1959). It is thus recommended that the addition of complex nutrient solutions to the serum bactericidal assay should be avoided, since it may modify the kinetics of serum killing.

3.6 INCUBATION CONDITIONS OF THE BACTERIA-SERUM MIXTURE

Occasionally the mixture of serum and bacteria is chilled prior to (Rowley, 1968; Clumeck *et al,* 1982), or after use (Gilsdorf and Ferrieri, 1986), assuring that each assay in a battery of assays proceeds for a constant time period. This 'cold-shock' may influence the susceptibility of the test micro-organism to serum.

Incubation of the serum bactericidal assay in 10 per cent carbon dioxide may be necessary to maintain the required pH, since the pH of NHS increases to nine in two hours if incubated in air. This may influence the action of C′ (Van den Bosch *et al,* 1987).

Melching and Vas (1970) suggested that agglutination of bacteria during the antibody-antigen reaction, particularly with immune serum, may lead to an underestimation of the survival rate. It has been suggested that shaking of the serum bactericidal assay mixture increases contact between the micro-organisms and the serum (Stratton, 1988) but relatively few investigators have used shaking in their technique. However, DeMatteo *et al* (1981) reported that agglutination of bacteria did not appear to significantly influence the serum bactericidal assay.

3.7 TIME OF EVALUATION

It is important to define serum resistance with a time parameter e.g. serum killing of *Haemophilus influenzae* (Branefors and Dahlberg, 1980) and *N. gonorrhoeae* (Odum, 1982) only attained a maximum after at least one hour incubation in the serum bactericidal assay. Also, smooth *E. coli* strains showed little change during the first hour of exposure to serum, but the number of survivors after three hours was less than 1 per cent (Taylor, 1974). Furthermore, a difference between serum-resistant and serum-sensitive (plasmid-mediated) *E.*

coli strains was only observed after a two hour incubation with serum, and not after one hour (Binns *et al,* 1979). A difference in serum susceptibility of *Legionella pneumophila* was only observed after six hours. After a twenty-four hour incubation period, the number of surviving organisms of the serum-resistant strain decreased ten-fold, while with strain RH1 the numbers decreased by five logarithms (Plouffe *et al,* 1985). Cell death of *Pasteurella septica* only occurred forty minutes after exposure to serum (Griffiths, 1974). Obviously, the time of evaluation selected largely depends on the test strain, the inoculum size and the concentration of serum present, making comparisons between independent studies unacceptable. To demonstrate the diversity of time limits selected, the following time parameters have been chosen for serum bactericidal assay of enteric bacteria viz. thirty minutes (Fierer *et al,* 1972), sixty minutes (Reed and Albright, 1974; Allen and Scott, 1980; Opal *et al,* 1982; Hackett *et al,* 1987; Van den Bosch *et al,* 1987), ninety minutes (Kimball *et al,* 1964; Allen *et al,* 1987) and 120 minutes (Roantree and Rantz, 1960; Nelson and Roantree, 1967; Reynard and Beck, 1976; Chiesa and Bottone, 1983; Benge, 1988).

3.8 MODIFICATIONS AND ALTERNATIVE METHODS

Plate counting is the conventional method of viable counting of the survivors of the serum bactericidal assay. A micromethod was introduced by Miles and Misra (1938). Volumes as small as 10µl have been suitable for use (Taylor and Kroll, 1983a; Ross *et al,* 1985). Brooks and Ingwer, (1978) compared a macro (1ml) and a micro (0.25ml) assay; the latter proved more sensitive and reproducible.

Disadvantages of plate counting have led to the development of alternative and better methods for viable counting. Several investigators have added growth media to the mixture after the test period, followed by a second incubation period. The resulting bacterial concentration was measured spectrophotometrically, reflecting the number of organisms surviv-

ing the initial serum bactericidal assay (Muschel and Treffers, 1956; Osawa and Muschel, 1964; Hancock *et al,* 1983; Nilius and Savage, 1984). This method assumed that C′ was inactivated by the presence of the nutrients. Michael and Braun (1964) compared a similar photometric assay with the conventional plating method. The results suggested that C′-mediated killing continued after addition of the nutrient broth. A new colorimetric assay was developed by Moll *et al* (1980) who incorporated bromothymol blue into the peptone glucose broth added after the serum bactericidal assay. The level of survival was determined by the extent of a colour change in the medium from green to yellow.

For screening large numbers of bacterial strains with respect to serum susceptibility, more rapid assays have been developed. Plaque assays involved spotting of NHS onto seeded agar plates and observing the inhibition of growth at these sites (Fierer *et al,* 1972; Corbeil *et al,* 1978; Odum, 1982). This method depends on factors such as minimal batch-to-batch variation of media and optimum inoculum size; it may thus be difficult to control. Relatively high correlations between the plaque assay results and the serum bactericidal assay results have been obtained (Fierer *et al,* 1972; Odum, 1982).

Indirect assays not based on viability have been used to quantify susceptibility to serum. Kato and Bito (1978) reported a loss of protein synthesis as an index of the bacteria killed. Fierer *et al* (1974) documented a correlation between chromium-labelled lipopolysaccharides release and serum susceptibility.

3.9 CONCLUSION

Clearly, the degree of serum sensitivity of Gram-negative bacteria varies extensively. Prolonged incubation in the presence of serum may result in an increase in viable count, or a rapid decline of the total bacterial inoculum.

Even though several authors have emphasised that if independent investigations are to be comparable, the standardisation of factors which significantly influence the serum bactericidal assay, viz. bacteria inoculum size, serum concentration, time of assay, etc., would be necessary (Davis and Wedgwood, 1965; Pien and Vosti, 1974; Branefors and Dahlberg, 1980); parameters have yet to be standardised. DeMatteo *et al* (1981) compared three methods to test twenty-nine strains of *Pseudomonas aeruginosa*. According to the different definitions of serum resistance, the number of serum-resistant strains varied from 3.45 per cent to 96 per cent. Agar-grown organisms exposed to 10 per cent serum in one method, survived better than broth-grown bacteria exposed to 50 per cent serum of another method.

Not surprisingly, 'serum resistance' has been defined according to the method employed and it varies extensively.

Results may be expressed relative to a control without serum (Binns *et al,* 1982), or inactivated serum (Veale *et al,* 1981; Martin *et al,* 1982; Odum, 1982), or the initial inoculum size (Rice *et al,* 1980; Opal *et al,* 1982; Shafer *et al,* 1982; Gilsdorf and Ferrieri, 1986; Hackett *et al,* 1987; Porat *et al,* 1987; Ross *et al,* 1987). Furthermore 'serum resistance' may be expressed as titre of a bacterial or serum dilution resulting in a certain level of killing if the serum or bacterial inoculum is serially diluted. In the conventional serum bactericidal assay, the level of 'serum resistance' alters with changes in bacterial inoculum size, time of assay, and serum concentration.

In conclusion, certain Gram-negative bacteria that are considered serum resistant, are merely less susceptible than other bacteria, and special experimental conditions are required for their destruction by serum. Instead of an absolute distinction between serum-sensitive and serum-resistant organisms, the susceptibility of Gram-negative bacteria may be normally distributed between these two extremes (Osawa and Muschel, 1964).

PRELIMINARY LABORATORY INVESTIGATIONS OF HAEMOPHILUS DUCREYI AND DETERMINATION OF ITS GROWTH KINETICS

4.1 INTRODUCTION

Haemophilus ducreyi, the aetiological agent of chancroid, has been isolated worldwide, most commonly in tropical countries and also in South Africa. The micro-organism was first described in 1889 as a Gram-negative bacillus 0.5µm wide and 1.5µm long (Ducrey, 1889).

Culture of *H. ducreyi* on suitable media is essential for accurate diagnosis of chancroid. Also, culture *in vitro* has greatly contributed to the development of research on this micro-organism (Johnson *et al,* 1988). Because *H. ducreyi* is fastidious, the exact nutritional requirements have not been well defined. Generally, it is agreed that blood or blood products are needed. Furthermore, a requirement for haemin as nutrient is a criterion contributing to the classification of *H. ducreyi* into the genus *Haemophilus* (Kilian, 1976).

Two major advances were recently made in the search for improved cultivation media for *H. ducreyi.* The first was the inclusion of vancomycin (Hammond *et al,* 1978a). This antibiotic inhibits the growth of secondary organisms present in the genital ulcer which could interfere with the diagnosis of chancroid (Chapel *et al,* 1978; Seghal and Prasad, 1985; Thirmoorthy *et al,* 1986). Secondly, the addition of foetal calf serum (FCS) to the solid medium increased the isolation rate of African strains (Sottnek *et al,* 1980; Nsanze *et al,*

1984). In support, a study in Swaziland reported an extremely low isolation rate of *H. ducreyi* on media consisting of gonococcal agar, haemoglobin, IsoVitaleX and vancomycin without FCS (Meheus *et al,* 1983). The specific growth factors in FCS remain unspecified.

A medium described by Nsanze *et al* (1984) consisting of gonococcal agar, haemoglobin, IsoVitaleX, FCS and vancomycin, appears to be the most efficient for the isolation of African strains. Another recommended medium consists of Mueller-Hinton (M-H) agar base, horse blood, IsoVitaleX and vancomycin (Bilgeri, 1983b; Nsanze *et al,* 1984; Dylewski *et al,* 1986; Kunimoto *et al,* 1986; Dangor, 1988a). The colonies are round, convex, small (1-2mm diameter) and yellow-grey in colour. The use of at least two different media is recommended in obtaining maximum isolation rates of *H. ducreyi* from patients suspected of chancroid (Hannah and Greenwood, 1982; Nsanze *et al,* 1984; Dylewski *et al,* 1986; Kunimoto *et al,* 1986; Dangor, 1988a). This perhaps implies that clinical strains vary in their nutritional requirements.

The slow development of a liquid medium has been due to the fastidious nature of *H. ducreyi,* auto-agglutination resulting in unpredictable turbidities of the culture, and contamination by other organisms present in the genital ulcer. Only recently have liquid media containing M-H or brain heart infusion broth (BHI), haemin, FCS or horse serum, IsoVitaleX and vancomycin been employed (Sng *et al,* 1982; Slootmans *et al,* 1983; Anderson *et al,* 1984; Schalla *et al,* 1986; Abeck *et al,* 1987; Carlone *et al,* 1988).

A micro-organism in a batch culture utilises the nutrients in its surrounding medium to produce cellular constituents. After a period of time (generation / doubling time), the cell biomass doubles resulting in cell division. The population thus increases exponentially, which can be expressed arithmetically by $X = X_0 2^{t/T}$, where X_0 is the size of the inoculum, T is the doubling time and X is the size of the bacterial population after an incubation period of time t.

The growth of a bacterium in the closed system of a batch culture follows a characteristic pattern. Initially, in the lag phase, the cells are synthesising enzymes and nuclear material, i.e. cellular components. Following this adaptation, the growth rate increases to a maximum for a short period. This is called the exponential or logarithmic phase. When the concentration of nutrients becomes limiting, and toxic metabolites accumulate, the rate of cell division decreases. This stationary phase is followed by death of the culture, represented by a decrease in viable count (Koch, 1981).

Knowledge of an organism's growth characteristics is also important when studying bacterial properties which may be affected by the surrounding media, or the metabolic state of the organism. For example, some Gram-negative bacteria, including *Salmonella typhimurium* (Maaloe, 1948), *Escherichia coli* (Melching and Vas, 1971) and *Neisseria gonorrhoeae* (Morse *et al,* 1983), were more serum sensitive during the exponential phase of growth than those in the lag or stationary phase of growth. Similarly, the composition of the culture medium for *Pseudomonas aeruginosa* (Chester and Meadow, 1975; Liu, 1988), *S. typhimurium* (Palva and Makela, 1980) and *H. ducreyi* (Abeck *et al,* 1987); or the metabolic phase of *Salmonella enteritidis* (Collins, 1964), *Neisseria sicca* (McDonald and Adams, 1971) and *N. gonorrhoeae* (Morse *et al,* 1983) resulted in changes in their lipopolysaccharide composition.

Furthermore, a technique for efficient homogenisation of the colonies of a micro-organism allows for the production of a standardised inoculum and accurate enumeration. This is particularly true for *H. ducreyi*, whose typically coherent colonies are a criterion for a presumptive identification.

Following the discovery of the Ducrey bacillus (Ducrey, 1889), two techniques for homogenisation of colonies were used. Colonies off solid media were placed in a small globe of a ball mill, and run continuously for forty-eight hours resulting in an emulsion which was centrifuged and the supernatant removed (Saelhof, 1924). Colonies were also crushed between two sterile glass slides (Reymann, 1949). Two techniques are currently used. The one

involves either mechanical agitation by vortexing for three seconds (Hammond *et al,* 1978a; Girourard *et al,* 1981; Bilgeri *et al,* 1982), or for thirty seconds (Slootmans *et al,* 1983; Taylor *et al,* 1985), or for five minutes (Hafiz *et al,* 1984). Tween 80, a detergent, has also been included in the suspending medium (Sturm, 1987a). The other more recently developed technique is ultrasonication of the colonies (Hafiz *et al,* 1986; Jones *et al,* 1986; Dangor *et al,* 1988b).

Mechanisms for adherence of *H. ducreyi* are poorly understood. Under electron microscopy, Kilian (1975) observed no capsular material around individual cells. In contrast, McEntergart *et al* (1982) noticed that colonies of *H. ducreyi* had a smooth surface, suggesting the presence of some interstitual matrix bonding the organisms together.

Successful storage of cultures of *H. ducreyi* has been acheived by using lyophilisation (Reymann, 1947; Deacon *et al,* 1954; Bilgeri *et al,* 1982), or freezing at minus 70^0 C in skim milk (Oberhofer and Back, 1982; Odumeru *et al,* 1984; Taylor *et al* 1985; Lubwama *et al,* 1986).

In this study, media were selected which supported the growth of all reference and clinical strains of *H. ducreyi*, and the effect of FCS (Highveld Biological) was more closely investigated. Growth curves were constructed to better understand the growth kinetics, and the doubling times were subsequently calculated. A novel technique for homogenisation was also introduced, which appeared to be more efficient than methods previously reported. The morphology of negatively-stained *H. ducreyi* cells was observed under an electron microscope. The results of this investigation provided assistance for further 'in vitro' manipulations of *H. ducreyi.*

4.2 MATERIALS AND METHODS

4.2.1 BACTERIAL STRAINS AND SOURCE OF STRAINS

H. ducreyi strains A75, A76 and A77 are reference strains obtained from the Pasteur Institute in Paris.

Clinical strains, including D11, $Y56R_2$, Y168, Y155 and Y206 were isolated by Mr Y. Dangor, from black male migrant workers attending the sexually-transmitted disease clinic in Carletonville, South Africa (Dangor, 1988a). After a clinical diagnosis was made, material from the base of the ulcer was collected with a swab and directly inoculated onto selective media containing gonococcal agar (GC), haemoglobin, IsoVitaleX, FCS and vancomycin (Nsanze *et al,* 1984). *H. ducreyi* was identified by its typical yellow-grey, non-mucoid cohesive colony characteristics and according to biochemical reactions proposed by Kilian (1976).

Reference strains of *Staphylococcus aureus* (ATCC 25923, ATCC 29213), *Escherichia coli* (ATCC 25922), *'Klebsiella edwardsii'* (NCTC 10896), *Pseudomonas aeruginosa* (ATCC 27853) and *Bacillus subtilis* (ATCC 6633) were supplied by Ms J. Saunders, S.A.I.M.R.

4.2.2 INCUBATION CONDITIONS, STORAGE AND MEDIA

All strains were incubated at 33^0 C to 35^0 C under 5 per cent carbon dioxide and humidity for forty-eight to seventy-two hours. They were stored at minus 70^0 C in BHI (Difco) broth with 25 per cent defibrinated rabbit blood. For all experiments, clinical strains were low passage cultures. The most suitable diluent for all manipulations was found to be BHI broth.

Lyophilised cultures of the reference strains A75 and A76, were reconstituted onto a range of media in order to determine which was optimal for recovery. These included BHI agar (Difco) and 10 per cent rabbit blood (rabbit blood agar); GC agar (Gibco), 1 per cent haemoglobin (Difco) and CVA (Gibco) enrichment; and Oxoid agar (Columbia) and 5 per cent chocolated horse blood (chocolated agar).

The seven media used for recovery of a lyophilised culture of A77 were : GC agar, 1 per cent IsoVitaleX (BBL, Microbiological), 1 per cent haemoglobin, 5 per cent FCS (Highveld Biological) and 3mg/l vancomycin (enriched gonococcal medium); GC agar, 1 per cent IsoVitaleX, 1 per cent haemoglobin, 1 per cent haemin and 5 per cent FCS; GC agar, 1 per cent haemin, 1 per cent IsoVitaleX and 5 per cent FCS; BHI agar, 1 per cent haemin, 1 per cent IsoVitaleX and 3mg/l vancomycin; BHI agar, 1 per cent haemin, 1 per cent IsoVitaleX, 5 per cent FCS and 3mg/l vancomycin; BHI agar, 1 per cent haemin, 1 per cent IsoVitaleX and 5 per cent FCS; M-H agar (BBL,Microbiological), 10 per cent chocolated horse blood, 1 per cent IsoVitaleX and 3mg/l vancomycin; chocolated agar; and BHI broth, 1 per cent IsoVitaleX and 0.5 per cent haemin.

A duplicate set of reference strains was obtained from the Pasteur Institute, Paris on blood agar slopes and were reconstituted on or into the following six media : GC agar, 1 per cent haemoglobin, 1 per cent IsoVitaleX and 5 per cent FCS; GC agar, 1 per cent haemoglobin, 1 per cent CVA enrichment and 5 per cent FCS; chocolated agar; BHI agar and 15 per cent horse blood (horse blood agar); rabbit blood agar; and BHI broth, 1 per cent IsoVitaleX and 0.5 per cent haemin.

The efficiency of the media was confirmed by the growth of clinical strain $Y56R_2$ on all formulations. All the investigated strains, including the clinical strains were subsequently cultivated on the medium which best supported the growth of all reference strains.

The final medium selected for culture of strains proved to be cost effective, and produce an adequate yield of growth. This medium consisted of BHI agar, 1 per cent haemoglobir 1 per cent IsoVitaleX and 3mg/l vancomycin. For liquid culture, BHI broth, 0.5 per cer haemin and 1 per cent IsoVitaleX was equally suitable (APPENDIX 1). This was a slightl modified version of the medium previously reported by Abeck *et al* (1987). The modifica tion was a decrease in the concentration of haemin from 1 per cent to 0.5 per cent, since thi did not affect the yield and allowed some visible assessment of turbidity in the liquid me dium.

4.2.3 THE INHIBITORY FACTOR IN ENRICHED GONOCOCCAL MEDIUM

To determine what component in the enriched gonococcal medium was inhibitory to th reference strains but not the clinical strains, the different components of the medium wer investigated. GC agar was replaced by BHI or Columbia agar bases; or vancomycin, or FC was excluded from the medium. Preliminary results suggested FCS (Highveld Biological was the inhibitory factor in the enriched gonococcal medium. Confirmatory experiment were performed using FCS (Highveld Biological) and inactivated FCS (Highveld Biological) compared to FCS (Microbiological Associates) and BHI broth as controls.

Approximately thirty colonies of *H. ducreyi* strains A75, A77, Y56R$_2$, Y168 and Y20 grown on supplemented BHI agar plates were suspended in 2ml diluent and homogenise using a thin needle and syringe. 100µl of the suspension was plated out resulting in an ever lawn of bacterial growth on subsequent incubation. Sterile filter paper discs were impregnated with the different sera and controls, and gently placed on the inoculated aga surface. The plates were allowed to dry slightly, and incubated as usual.

Similarly, inhibitory activity was determined against reference strains of *S. aureus* (ATCC 25923, ATCC 29213), *E. coli* (ATCC 25922), *'K. edwardsii'* (NCTC 10896) and *P. aeruginosa* (ATCC 27853).

To determine whether the inhibitory effect was bactericidal, the reference strains of *H. ducreyi* were grown overnight in BHI broth supplemented with 10 per cent FCS (Highveld Biological), and viability was determined by subculturing onto solid medium.

To determine whether the inhibitory agent in the FCS was penicillin, three penicillin-sensitive reference strains were used, viz. *B. subtilis* (ATCC 6633) and *S. aureus* (ATCC 25923, ATCC 29213). M-H agar plates were seeded with each reference strain from a bacterial suspension 0.5 McFarlane standard. One drop of penicillinase (Wellcome) was added to 0.5ml FCS (Highveld Biological). Sterile filter paper discs were dipped into this mixture, or FCS, and placed on the agar surface. Control experiments included the use of penicillin discs (Abteck Biologicals), and penicillin discs inactivated with penicillinase.

4.2.4 HOMOGENISATION AND ENUMERATION

To homogenise *H. ducreyi* approximately thirty colonies of strain A76 and Y206 incubated for seventy-two hours were suspended in 2ml BHI broth and sucked through a needle and syringe (Microfine 28g 12.7mm 0.45mm - Figure 4.4) eight to ten times. This technique was compared both with mechanical agitation by vortexing for three seconds or three minutes, followed by a fifteen minute period to allow larger clumps to settle (Hammond *et al,* 1978a); and sonication at six microns for ten seconds (SoniPrep, 150 MSE) (Jones *et al,* 1986). For the inoculum, the number of colonies sampled and their age were kept constant for all experiments.

The turbidity of the suspension after homogenisation through the needle and syringe was measured at 560nm (Bausch and Lomb Spectronic 20, Spectrophotometer) at several time intervals, to determine whether it was stable.

The method used for viability count of the bacterial suspensions was the microtechnique of Miles and Misra (1938) - Figure 4.5. Serial dilutions were made in 1ml diluent in duplicate and 10µl of each dilution was dropped onto the surface of supplemented BHI agar plates, which were incubated at 33^0 C in a humid atmosphere containing 5 per cent carbon dioxide for seventy-two hours.

Experiments were performed in duplicate. The Duncan t-test was used to determine the level of significance of difference between the different methods.

4.2.5 ELECTRON MICROSCOPY

Bacterial suspensions of strains A77, Y155 and $Y56R_2$ were produced as previously described. A drop was placed on G300 copper grids (Wirsam products) coated with 0.8 per cent formvar (Merck) in chloroform, blotted dry and negatively stained with 1 per cent uranyl acetate (BDH Chemicals) for ten to thirty seconds. The grids were rinsed with distilled water, dried and viewed in a JEM 100S transmission electron microscope.

4.2.6 GROWTH CURVE

The strains A75, A76, D11, $Y56R_2$ and Y206 were inoculated into 2ml supplemented BHI broth and incubated overnight. This was then homogenised using a thin needle and syringe, and 1ml thereof was used to inoculate 100ml liquid medium in cottonwool-stoppered conical flasks. The flasks were incubated at 33^0 C in a candle jar. Samples of 1ml were removed after several time intervals and homogenised as above. Serial dilutions were made in 1ml

volumes in duplicate; 10µl of each was dropped onto supplemented BHI agar and the plates were incubated (Miles and Misra, 1938) - Figure 4.5.

The concentration of viable organisms in the growth culture was expressed as the logarithm to the natural base of the colony forming units (CFU) per millilitre. This concentration was plotted against time (hours), and the gradient, representing the growth rate was calculated (APPENDIX 2).

4.3 RESULTS

4.3.1 MEDIA

Lyophilised cultures of the reference strains were not viable on reconstitution despite several media being used. The different media were quality controlled by concurrent culture of a clinical strain $Y56R_2$.

The transport of reference strains of *H. ducreyi* on blood agar slopes was however successful and the strains remained viable. Although no growth was observed when the reference strains were subcultured onto the enriched gonococcal media, and only poor growth was obtained on chocolated agar, the reference strains grew better on BHI agar with horse or rabbit blood.

The medium (Figure 4.4) containing BHI agar or broth, haemoglobin or haemin, IsoVitaleX and vancomycin, was chosen to culture *H. ducreyi* for further experiments as it resulted in a suitable yield of growth, and was more cost effective than rabbit or horse blood agar. Significant growth on solid media of reference strains required three days incubation at 33^0 C. Clinical strains grew faster (two days) incubated at 33^0 C and were not as temperature

sensitive as the reference strains i.e. were also able to grow at 36^0 C. Viability on the proposed medium was observed for at least seven days.

The colony morphology of the reference strains on the BHI medium was greyish, mucoid, small and round. Clinical strains appeared yellower, drier and larger than the reference strain colonies.

4.3.2 THE INHIBITORY FACTOR IN ENRICHED GONOCOCCAL MEDIA - REFERENCE STRAINS

An inhibitory effect of FCS (Highveld Biological) was noticed in batches of enriched gonococcal media consisting of GC agar, IsoVitaleX, haemoglobin, FCS and vancomycin. With both reference and clinical strains, the use of different bases e.g. GC agar, Columbia agar and BHI agar, made no significant difference. The inhibition was observed with or without the presence of vancomycin. When IsoVitaleX was replaced with CVA enrichment, and haemoglobin with haemin, the inhibition was still observed. Subsequently, it was found that the constituents of IsoVitaleX and CVA are identical thus explaining the lack of difference observed. Replacement of haemoglobin by haemin rendered colony forming units of the clinical strains difficult to visualise, while the reference strains still failed to grow on this medium.

The effect of inactivation of FCS was also evaluated. In liquid medium and solid medium, both containing either normal or inactivated (heating for thirty minutes at 56^0 C) FCS (Highveld Biological), growth of the reference strains A75 and A77 (Figure 4.1) - but not the clinical strains Y168, Y206 and $Y56R_2$ (Figure 4.2) - was inhibited. Growth inhibition was also noted with *S. aureus* and *E. coli* strains, but not with *'K. edwardsii'* and *P. aeruginosa.* This property was bactericidal since subculture on solid media from an overnight broth culture in liquid medium containing 10 per cent FCS (Highveld Biological), indicated an ab-

sence of survivors. This inhibitory quality was not observed when FCS from other suppliers . (Microbiological Associates, Flow laboratories and Delta Bioproducts) was used.



Figure 4.1 Growth of *H. ducreyi* reference strain A75 inhibited by FCS.



Figure 4.2 FCS has no effect on the clinical strain of *H. ducreyi* Y206.

The possibility that the inhibitory substance in the Highveld Biological brand of the FCS might have been penicillin (the reference strains were penicillin-sensitive while the clinical strains were resistant to this antibiotic) was investigated. Penicillinase inactivated penicillin on the antibiotic discs as indicated by the selective inhibition of growth of the penicillin-sensitive reference strains. In contrast, the inhibition of the FCS was not affected by penicillinase suggesting that penicillin is absent from the FCS supplied by Highveld Biological (Figure 4.3).

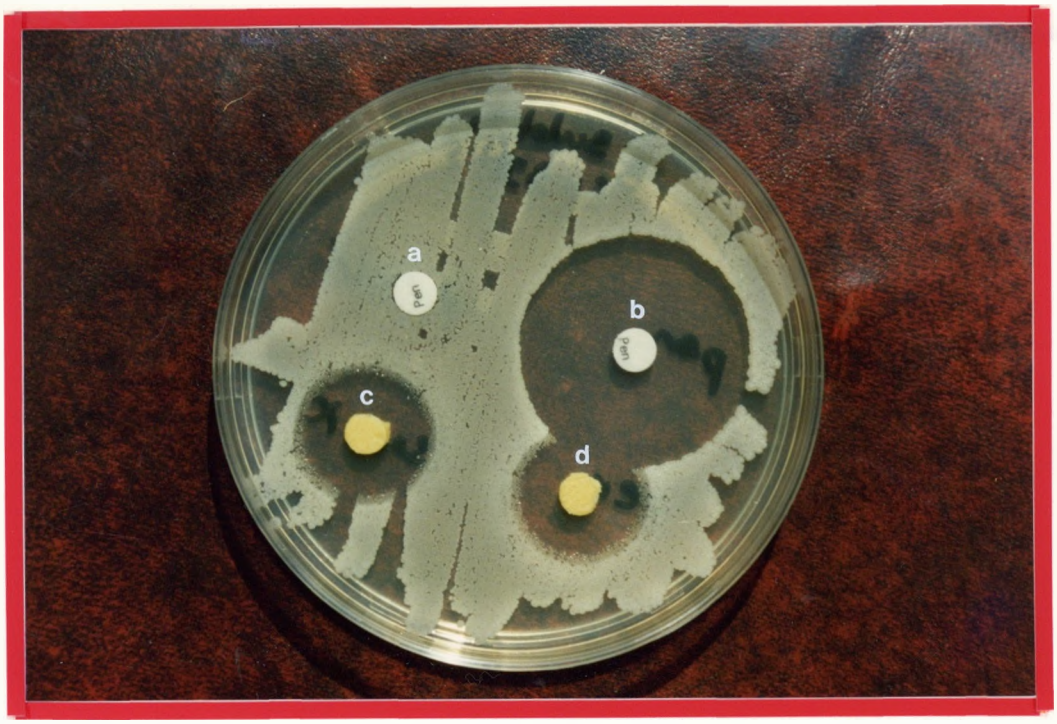


Figure 4.3 Effect of penicillinase on FCS (Highveld Biological) using the reference strain *B. subtilis* (ATCC 6633); a) penicillin and penicillinase, b) penicillin, c) FCS and penicillinase, and d) FCS only.

4.3.3 HOMOGENISATION AND ENUMERATION

The comparative efficacy of the different homogenisation techniques is given in Table 4.1. In the case of both A76 and Y206 strains, homogenisation by vortexing for three seconds or three minutes or by sonification did not yield significantly different results ($p > 0.05$; Duncan t-test). However, when the novel needle-syringe method was employed, the number of CFU/ml was two to four logarithms greater. Homogenisation of the clinical strain Y206 yielded significantly lower CFU/ml than the reference strain A76.

The turbidity of the suspension after the syringe method was used was relatively stable, decreasing to 88 per cent of the original turbidity, after one to two minutes, and then reaching a stable level of 84 per cent after ten minutes.

Table 4.1 Comparison of cell counts obtained using different techniques for homogenisation of *H. ducreyi* strains of A76 and Y206.

Treatment	Vortexing				Sonication		Syringe	
	3 secs.		3 mins.					
	x[a]	SD[b]	x	SD	x	SD	x	SD
A76	3.54	0.085	3.51	0.085	2.82	0.43	7.95	0.33
Y206	1.14	0.66	1.5	0.1	1.62	0.14	3.95	0.42

[a] Average $\log_{10}$ CFU/ml

[b] Standard deviation



Figure 4.4 Supplemented BHI broth containing 0.5% haemin and 1% IsoVitaleX, with the diluent used (BHI broth) in bottles. The needle and syringe (Microfine 28g 12.7mm 0.45mm) for homogenisation of *H. ducreyi* is also shown.



Figure 4.5 Enumeration of *H. ducreyi* using the Miles and Misra (1938) technique.

4.3.4 ELECTRON MICROSCOPY

Electron microscopy of *H. ducreyi* confirmed that this micro-organism is a rod-shaped bacterium. The width of each cell is consistently 0.6µm, but the length varies viz. 1.8µm, 2.6µm and 3.5µm (Figure 4.6). The pleomorphic nature of the cells is clearly indicated in Figure 4.7. Auto-agglutination is apparent in Figure 4.8; a bonding matrix was observed between the cells. Occasionally, ghost cells were noticed in the bacterial suspensions of both reference and clinical strains (Figure 4.9). Under electron microscopy, strain A77 and strain Y155 did not appear to differ in their cellular morphology.

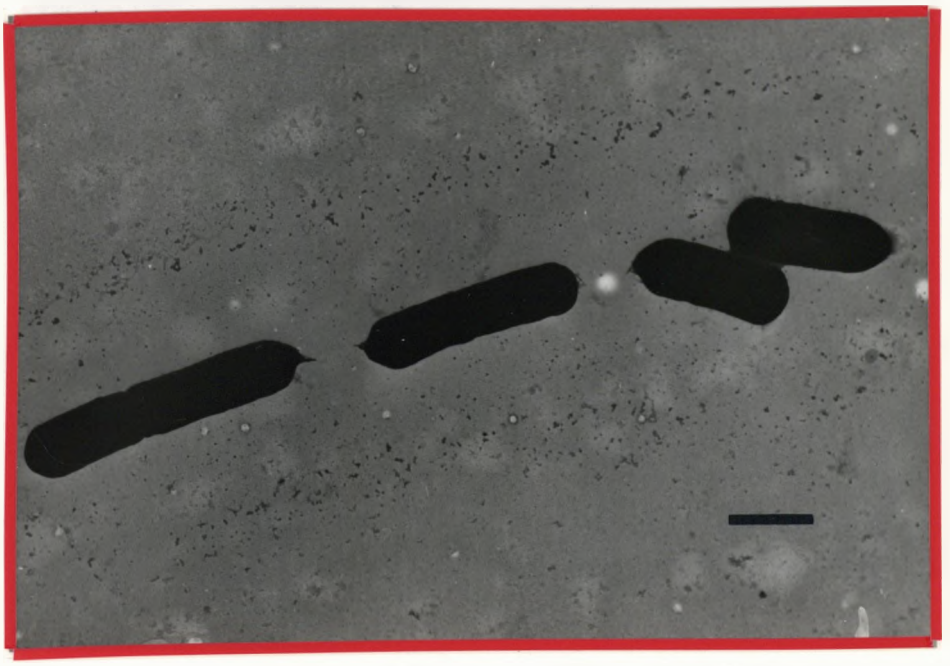


Figure 4.6 *H. ducreyi* **(Y155) is a rod-shaped bacillus. Scale bar equals 1µm.**



Figure 4.7 Pleomorphic nature of *H. ducreyi* **(Y155). Scale bar equals 4µm.**

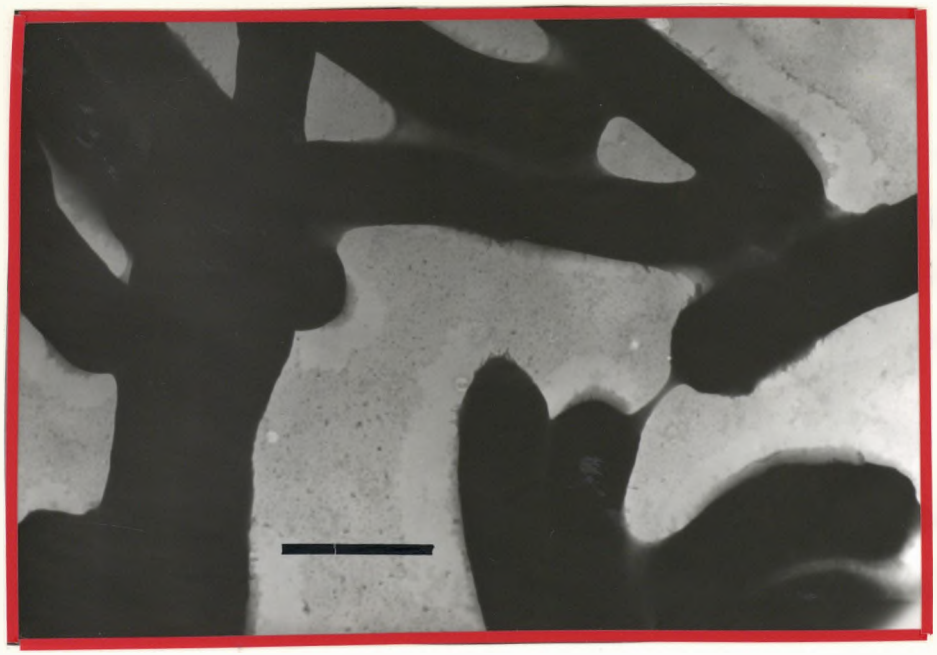


Figure 4.8 Auto-agglutination of *H. ducreyi* (Y155) due to interstitual bonding matrix. Scale bar equals 1µm.

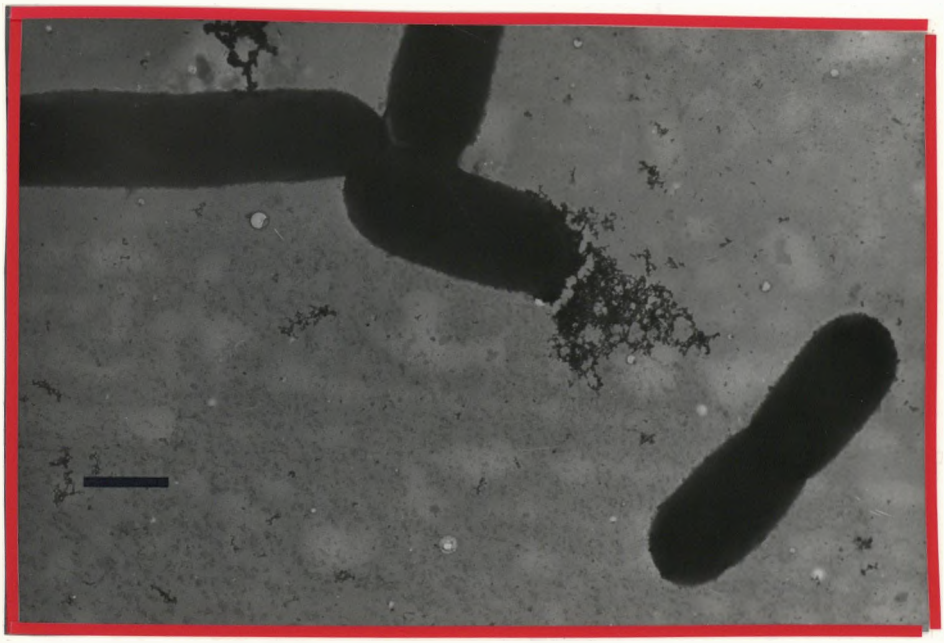


Figure 4.9 Ghost cells of *H. ducreyi* (A77). Scale bar equals 1µm.

4.3.5 GROWTH RATE

The equation for an exponential growth curve is $X = X_0 2^{t/T}$, which may be written as $\ln X = \ln X_0 + \mu t$ where X_0 is the inoculum size, μ is the growth rate and X is the number of CFU/ml at time t. The doubling time T is the time it takes for the population to double, and is equal to $\ln 2/\mu$. A plot of ln X versus time t (hours) will produce a line whose gradient is μ (APPENDIX 2).

Table 4.2 and Figure 4.10 show that the reference strains grow more slowly in supplemented BHI broth than the clinical strains, having doubling times of greater than two hours.

Table 4.2 Growth rate and doubling time of *H. ducreyi* in supplemented BHI broth.

Strain	μ [a]	T (hours) [b]
A75	0.237	2.9
A76	0.325	2.13
D11	0.386	1.8
$Y56R_1$	0.374	1.85
Y206	0.348	1.99

[a] μ is the growth rate when $\mu = (\ln X - \ln X_0)/t$ and ln is the logarithm to the natural base (ln) of the growth at time t (in hours) and $\ln X_0$ is the logarithm to the natural base (ln) of the initial inoculum.

[b] Doubling time T (hours) = $\ln 2/\mu$

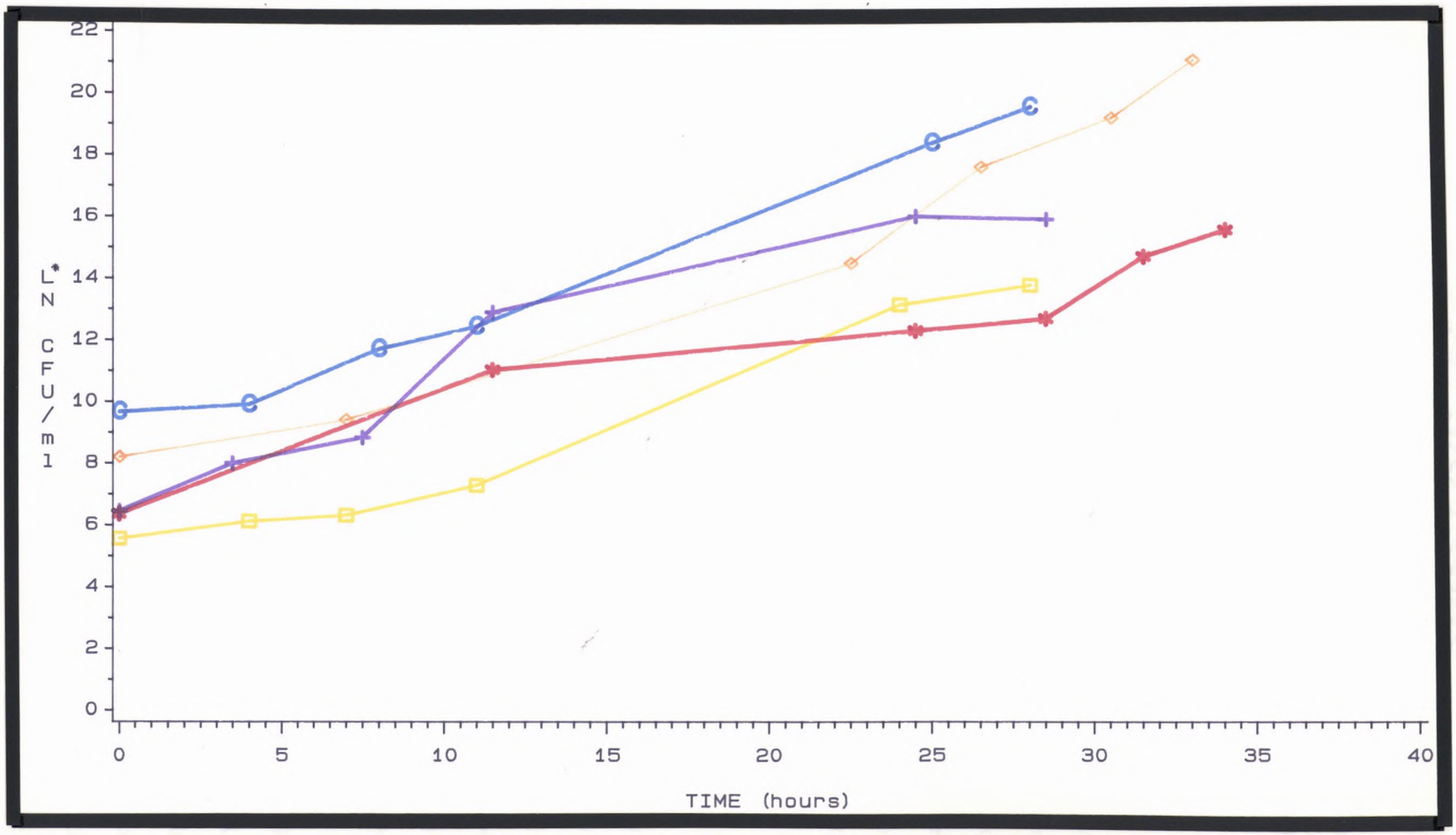


Figure 4.10 Growth curves of *H. ducreyi* strains in supplemented BHI broth: A75 A76 D11 $Y56R_1$ Y206

* The number of viable organisms were expressed as the logarithm to the natural base of the colony forming units per millilitre.

4.4 DISCUSSION

The study of *H. ducreyi in vitro* depends largely on a predictable or consistent growth pattern of the strains on suitable media, the ability to produce a homogeneous suspension for inoculation, accurate enumeration and a suitable means of storage of strains.

Successful lyophilisation of clinical strains has been reported by Deacon *et al* (1954), Reymann (1947) in a semifluid agar and defibrinated sheep blood, and Bilgeri *et al* (1982) in horse serum, neopeptone and sucrose. In this study, all three lyophilised reference strains of *H. ducreyi* were not viable on reconstitution on several different media. A possible explanation for this non-viability could be an increased susceptibility of the reference strains as opposed to clinical strains, to the adverse effects of lyophilisation. Alternatively, this specific batch of reference strains was not viable at the time of lyophilisation. All strains of *H. ducreyi* were stored successfully in BHI broth and defibrinated rabbit blood at minus 70^0 C.

The reference strains despatched on blood agar slants in enclosed glass vials, were viable on reconstitution on rabbit blood agar and horse blood agar, and less efficiently on chocolated agar. In addition they also grew in supplemented BHI broth. These results confirm a requirement for blood or blood products for growth of *H. ducreyi.* The incorporation of haemin instead of haemoglobin or whole blood into solid media supported growth of clinical strain $Y56R_2$ - however the colonies were not easily visible. An interesting observation was the better yield of *H. ducreyi* on horse blood agar compared with chocolated agar. The only difference between the two media is that the latter is heated. In previous studies, the factor/s in serum required by *H. ducreyi* for growth was stable at 100^0 C (Beeson, 1946; Ajello *et al,* 1956). However the nutritional factor/s in red blood cells was inactivated by heating to 65^0 C (Beeson, 1946). Thus the heating process performed in the production of chocolated agar could have resulted in the destruction or increased inaccessibility of certain nutrients available

to *H. ducreyi*, possibly in the red blood cell content. This may provide a valuable clue in the understanding of the fastidious nature of *H. ducreyi*.

The choice of media containing BHI agar or broth, haemoglobin or haemin and IsoVitaleX was appropriate for several reasons. Since all strains grew on/in this medium, a comparative study could be performed. It is relatively cost efficient compared with rabbit blood agar, horse blood agar and enriched gonococcal medium. Also, the absence of serum in the media excluded the possibility of inducing serum resistance on the strains.

The colonies of the reference strains incubated with medium for seventy-two hours were generally smaller than those of the clinical strains. This was due to the difference in growth rate, since further incubation resulted in larger colonies similar to that of the clinical strains. Deacon *et al* (1954) previously reported colonies of old stock cultures which were 0.5mm to 1mm in diameter, compared to colonies of fresh strains which were 1mm to 3mm in diameter when incubated on rabbit blood agar for seventy-two hours.

The inhibitory effect of FCS (Highveld Biological) in enriched gonococcal medium, which is recommended for isolation of *H. ducreyi* in Africa, on the reference strains but not the clinical strains, was unexpected. Although enriched gonococcal medium has resulted in comparatively higher isolation rates of 71 per cent (Nsanze *et al,* 1984), 67 per cent (Dylewski *et al,* 1986), 84 per cent (Kunimoto *et al,* 1986) and 82 per cent (Dangor, 1988a), compared to other media, a small proportion of strains of *H. ducreyi* do not grow on this medium. It is possible that FCS inhibition factor/s which may be present in different concentrations in different batches or brands of FCS could play a contributing role in the lack of complete efficiency of enriched gonococcal medium. In general however, media without serum are not recommended since FCS increases the rate of primary isolation of most clinical strains of *H. ducreyi* (Sottnek *et al,* 1980; Nsanze *et al,* 1984).

Several possible explanations for this bactericidal activity of FCS was considered. *Haemophilus* infections have been reported in cattle, including aborted foetuses (Pritchard and MacLeod, 1977). Cross-reacting antibodies to *H. ducreyi* in FCS may, in the presence of complement be bactericidal to the reference strains. However, this hypothesis is not favoured, since inactivation of FCS (Highveld Biological) did not abolish its inhibitory effect. Secondly, low concentrations of selenium ions in different batches of serum, previously reported to enhance growth of *H. ducreyi*, may have played a contributing role in discouraging growth of the reference strains (Van den Berghe, 1987). However this type of effect would be bacteriostatic rather than bactericidal. Thirdly, since FCS is most commonly supplied pooled from approximately eighty to a hundred foetuses, a more likely explanation for the difference of inhibition between reference and clinical strains may be their antibiotic susceptibility. Antimicrobial susceptibility testing revealed that reference strain A76 was much more susceptible to penicillin, tetracycline, doxycyline and chloramphenicol than South African clinical strains (Y. Dangor, personal communication). Cattle are commonly fed with low doses of antibiotics especially penicillin and tetracycline, which may accumulate and be transferred to offspring. By this route, the resulting FCS may contain levels of antibiotic/s to which reference strains are susceptible, but the clinical strains are not. Subsequently penicillin was not found in the FCS, since penicillinase had no affect on its bactericidal activity. Finally, different strains of *H. ducreyi* may vary in their nutritional requirements and growth characteristics (Sturm and Zanen, 1984a).

Although the inhibitory factor/s of FCS (Highveld Biological) were not identified, this observation is relevant for laboratories engaged in research on *H. ducreyi.* Routine quality control of new batches of FCS is recommended. Serum-impregnated filter paper discs overlayed onto a lawn of *H. ducreyi* proved to be a simple and reliable method for this purpose.

There are confusing opinions on the most suitable source of sera from different animals, as a supplement to solid media. Initially, Reymann (1947) found that sheep and rabbit blood resulted in better growth compared to human, ox or horse serum. Ajello *et al* (1956) found

that rabbit and human serum was better than bovine or porcine serum at concentrations of 0.2 per cent to 50 per cent. FCS was better than rabbit or human serum in a study by Sottnek *et al* (1980).

The difference in growth rates between reference and clinical strains on solid media was confirmed by growth in liquid media. The doubling time of *H. ducreyi* varied from 108 to 174 minutes. Low rates of growth may also imply that the cultivating medium for *H. ducreyi* is not optimal, and cell division is slow, followed by rapid cell death. Under electron microscopy, ghost cells were observed in bacterial suspensions made from colonies. These 'ghosts' may be a result of autolysis (Figure 4.9). Perhaps the rate of growth of *H. ducreyi* may have been increased by the addition of factors which have been reported to improve the isolation of *H. ducreyi* e.g. FCS (Sottnek *et al,* 1980), selenium ions (Van der Berghe, 1987). Another contributing factor may be due to the physical characteristics of *H. ducreyi.* The pleomorphic nature and cohesiveness between cells may deter accurate enumeration; thus the increase in cell count may have been more rapid than that reported.

The finding that clinical strains grew faster than reference strains was surprising. One would expect stock strains which have been passaged *in vitro* for several generations to be well adapted to that environment. However, clinical strains have been reported to be less fastidious than stock strains (Deacon *et al,* 1956; Reymann, 1947); this was also observed in this study. Sturm and Zanen (1984a) compared nineteen recently isolated strains and ten laboratory strains, and recorded differences in requirements for carbon dioxide and moisture, growth on chocolated agar, and sensitivity to low and high temperatures of 22^0 C and 37^0 C respectively. Thus differences in growth rates may be a reflection of inherent differences in the ability of strains to grow under *in vitro* conditions.

The macro-physical features with respect to the cohesive nature of *H. ducreyi* colonies have been well documented. However, electron microscopy results are confusing. Kilian and Theilade (1975) observed no capsular material around cells of *H. ducreyi.* However,

McEntegart *et al* (1982) observed a smooth surface of *H. ducreyi* colonies suggesting the presence of some interstitial matrix bonding the organisms together. Attempts to disrupt the colonies with trypsin, papain, cellulase, 20 per cent sulphuric acid and 20 per cent potassium hydroxide had no effect. In this study using negative staining, no individual capsule per cell was observed, but binding material was observed between cells of a clinical strain of *H. ducreyi* (Figure 4.8). The constituents of this material have yet to be elucidated. These results confirm Kilian's findings, and are supportive of Mc Entergart's observations.

Electron microscopy also confirmed the pleomorphic nature of *H. ducreyi* and its cellular dimensions. The dimensions of the Ducrey bacillus were described as 0.3µm to 0.5µm wide by 0.6µm to 2µm long with a chain formation of three to twenty-five cells per chain (Ducrey, 1889; Brams, 1924; Saelhof, 1924; Satulsky, 1945; Felman and Nikitas, 1983). Other workers have stressed the pleomorphic nature of the organism which made estimation of the dimensions of *H. ducreyi* very difficult.

The needle and syringe method for homogenisation of *H. ducreyi* colonies recorded in this dissertation produced a significantly higher count than other techniques previously described, viz., sonication and mechanical agitation by vortexing ($p < 0.05$; Duncan t-test). The syringe technique is a modification of a method used in tissue culture technology. Clumps of tissue are suspended in media, and gently pipetted up and down until the tissue is completely dispersed into a single cell suspension (Freshney, 1988). A similar method was also employed for the homogenisation of *Gardnerella vaginalis* (Boustoller and Johnson, 1986). For both the clinical and the reference strain (A76, Y206), the viable count produced after the syringe method of homogenisation was used was approximately a 10^3 -fold increase compared with other methods. In this study, sonication did not produce yields significantly higher than mechanical agitation. Using the new method, a count of 10^4 CFU/ml was obtained for clinical strain Y206 and 10^8 CFU/ml for the reference strain A76. Possible factors contributing to this difference are firstly, that the clinical strains are inherently more cohesive than the reference strains; this characteristic was confirmed by the physical properties of the col-

onies on the solid medium; or secondly, that the clinical strain grew more rapidly than the reference strain, and could therefore have been in a later stage of growth. This could have resulted in an increase of bonding matrix. A third possible explanation is that a late decrease in viability resulted from an earlier high growth rate of strain Y206. If the first explanation proves to be correct an interesting analogy with the cord factor of *Mycobacterium tuberculosis* would exist. Cord factor, which is a trehalose mycolate has virulence properties (Bloch, 1950), and binding substances of similar chemistry have been found in pathogenic corynebacteriae and nocardiae (Ioneda *et al,* 1963; 1970).

Mechanical agitation by vortexing of the inoculum from a forty-eight hour plate culture for three seconds, followed by a fifteen minute settling period has been reported to yield at least 10^8 CFU/ml (Hammond *et al,* 1978a; Girouard *et al,* 1981; Bilgeri *et al,* 1982). Yields obtained by vortexing an inoculum from an overnight broth culture for a longer period of thirty seconds have been lower; in the order of 10^5 CFU/ml (Taylor *et al,* 1985) or 10^7 CFU/ml (Slootmans *et al,* 1983). Similarly, ultrasonication of inocula from a forty-eight hour plate culture and a ninety-six hour plate culture, yielded 10^8 CFU/ml (Dangor *et al,* 1988b) or 10^9 CFU/ml (Hafiz *et al,* 1986; Jones *et al,* 1986) respectively. These various factors which could affect the efficiency of a homogenisation technique, including the age of the culture, the physical state and size of the inoculum, the strains or the culture medium used, the type and volume of diluent and the time the homogenised suspension remained in the diluent before use.

Since there are multiple factors influencing the results, and these have not been well documented, it would be fruitless to compare results of different investigators before every step of the homogenisation technique is standardised. It should be emphasised however, that in the present study the same conditions including inoculum size, age of culture and type of medium prevailed when the various homogenisation techniques were compared.

In addition to the advantage of obtaining a higher yield using this new method, it is also easy to perform in any routine microbiological laboratory, and does not require expensive equipment. Also, the production of a bacterial suspension whose turbidity remains stable even after ten minutes - only decreasing to 84 per cent of the original turbidity - may lend itself to the development of more rapid techniques for the estimation of total counts of *H. ducreyi* using spectrophotometry or nephelometry. The results obtained in this study are of particular relevance in antimicrobial susceptibility testing, where a standardised bacterial inoculum is essential in producing accurate and consistent results.

The findings described in this chapter provide evidence for differences between the reference strains and the clinical strains. This was clearly observed in the following features : growth rate, inhibition by FCS (Highveld Biological), cohesive properties and colony morphology. In the next chapter further evidence of differences relating to the virulence properties will be described.

4.5 CONCLUSION

Lyophilised cultures of the reference strains A75, A76 and A77 (Pasteur Institute, Paris), were not viable on reconstitution; however, importation on blood agar slants was successful. The recommended medium for African strains, enriched gonococcal medium, did not support the growth of these reference strains. The inhibitory component in enriched gonococcal medium which affect the reference strains but not the clinical strains was identified as FCS (Highveld Biological). The doubling times of five strains of *H. ducreyi* in a liquid supplemented BHI medium were determined, and found to vary between 1.8 hours to 2.9 hours. Reference strains grew more slowly than clinical strains.

Using a thin-needle and syringe technique, colonies of *H. ducreyi* were more efficiently homogenised than by mechanical agitation through vortexing, or ultrasonication. A bonding matrix between cells was observed under electron microscopy. Differences between clinical and reference strains were noted with respect to FCS inhibition, growth rate and cell-to-cell adherence.

DETERMINATION OF SERUM RESISTANCE OF HAEMOPHILUS DUCREYI

5.1 INTRODUCTION

Chancroid, a genital ulcer disease, is caused by *Haemophilus ducreyi*. The ulcer is typically ragged with undermined edges; the base is covered with a purulent exudate.

The virulence factors contributing to the pathogenicity of *H. ducreyi* are poorly understood. Virulence of strains is commonly indicated by rabbit or rat intradermal inoculations; variations in the ability of strains to produce skin lesions have been assumed to reflect variations in virulence (Hammond *et al,* 1978b; Odumeru *et al,* 1984). In one study, this virulence was associated with resistance to the bactericidal activity of human serum and human polymorphonuclear leucocytes (Odumeru *et al,* 1984).

Serum resistance is an important virulence factor of many different Gram-negative bacteria, particularly those causing systemic infections e.g. *Pseudomonas aeruginosa* (Young and Armstrong, 1972), Enterobacteriaceae (Simberkoff *et al,* 1976; Benge, 1988), *Neisseria gonorrhoeae* (Brooks and Ingwer, 1978; Tramont *et al,* 1977; Rice *et al,* 1980), *Bacteriodes fragilis* (Casciato *et al,* 1979), *Campylobacter fetus* (Blaser *et al,* 1985) and *Capnocytophaga* (Wilson *et al,* 1987). *H. ducreyi* does not cause systemic disease and its isolation from the bloodstream of patients with clinical chancroid has not been described. Whether transient bacteraemia occurs is unknown as its demonstration has probably not yet been attempted. However, a frequent sign of chancroid is the swelling of one or both lymph nodes in the groin. The relationship between the formation of lymphadenopathy and *H. ducreyi* infection is not known, but implicates some involvement of the immune system.

Since reinfection by *H. ducreyi* is commonly observed, early investigators assumed that no host immunity was conferred against chancroid (Alergent, 1972). However, in two independent studies, an elevation of the levels of antibodies specifically directed against certain antigens of *H. ducreyi*, was reported in the sera of patients with chancroid (Schalla *et al,* 1986; Museyi *et al,* 1988). However such antibodies may not necessarily be protective. Although the immunology of *H. ducreyi* infection is a growing field of interest in research, relatively little is known at present about humoral and cell-mediated mechanisms related to this organism. Details of the interaction of this organism with serum factors in the host have also yet to be fully elucidated.

In this study, the sensitivity to human serum of two clinical strains isolated in South Africa, and three reference strains (Pasteur Institute, France) of *H. ducreyi* was measured. The effect of passaging a serum-sensitive reference strain in human serum *in vitro* several times to induce serum resistance is also reported. The efficiency of the methodology in serum susceptibility testing, and the relevance of the results obtained are discussed.

5.2 MATERIALS AND METHODS

5.2.1 BACTERIAL STRAINS AND MEDIA

Strains A75, A76 and A77 are reference strains from the Pasteur Institute, Paris. Y155 and Y206 were obtained from ulcers of patients with clinical chancroid (supplied by Mr Y Dangor, S.A.I.M.R). A76* is an isogenic strain of A76, which was passaged through human serum several times.

All strains were cultured on brain heart infusion (BHI) agar (Difco) with 1 per cent haemoglobin (Difco), 1 per cent IsoVitaleX (BBL, Microbiological systems) and 3mg/l vancomycin. The liquid medium contained BHI broth (Difco), 0.5 per cent haemin (Sigma)

and 1 per cent IsoVitaleX (APPENDIX 1). Cultures were incubated in candle jars at 33^0 C. The diluent employed was BHI broth without additives.

5.2.2 PREPARATION OF POOLED NORMAL HUMAN SERUM (PNHS)

Blood was collected from at least eight healthy volunteers who had no previous history of *H. ducreyi* infection. The blood was allowed to clot at 4^0 C overnight, centrifuged at 2000g for ten minutes, and the supernatant serum was removed. The serum was pooled, filter sterilised, dispensed in 1ml aliquots, and stored at minus 70^0 C until required.

5.2.3 SERUM BACTERICIDAL ASSAY

An overnight culture of *H. ducreyi* in supplemented BHI broth was used. A bacterial suspension was produced by homogenisation using a thin needle and syringe, and the bacterial concentration was adjusted to approximately 10^6 colony forming units (CFU)/ml in BHI broth as determined by previous Miles and Misra (1938) counts. At time zero, 200µl of this bacterial suspension was added to 1000µl PNHS and 800µl diluent (BHI broth). This mixture was incubated at 36^0 C. At time intervals zero, thirty, sixty and 120 minutes, 10µl was removed and serially diluted in BHI broth. Duplicate volumes of 10µl of each dilution were dropped onto supplemented BHI agar to determine the viable count. Two controls were used for a comparison of killing, viz. inactivated PNHS (heated for thirty minutes at 56^0 C) and no serum (BHI broth was used to make up the volume). Each assay was performed at least four times. The results were analysed statistically using a Duncan t-test.

5.2.4 INDUCTION OF SERUM RESISTANCE

Two methods were employed to induce serum resistance in the reference strains of *H. ducreyi*.

In the first method, bacteria which had survived exposure to 50 per cent PNHS after sixty or 120 minutes, as indicated by the viable count on solid media, were collected and inoculated into 2ml supplemented BHI broth. This was incubated overnight in a candle jar, and the resulting suspension was used for another serum bactericidal assay. This procedure was repeated serially at least thirteen times in an attempt to select for clones expressing greater serum resistance.

In the second method, colonies of the reference strains were heavily inoculated into 1ml 50 per cent PNHS and 50 per cent supplemented BHI broth. This was incubated overnight. A sample was removed and streaked onto supplemented BHI agar as a test for viability. 1ml supplemented BHI broth was added and the mixture, now containing 25 per cent PNHS was further incubated. If viability was detected after the initial overnight incubation with 50 per cent PNHS, 0.5ml of the bacterial suspension was again exposed to a new supply of 50 per cent PNHS. This procedure was repeated at least six or seven times; at each step the concentration of serum was increased slightly. The degree of induced serum resistance was determined by assessing the bactericidal activity of 50 per cent PNHS against the experimental strain.

5.3 RESULTS

5.3.1 SERUM RESISTANCE

The values plotted in Figures 5.1 to 5.5 are mean values obtained from at least four repeated experiments, expressed as a percentage of the bacterial inoculum at time zero. The results shows that clinical strains Y155 and Y206 are more serum resistant than the reference strains with significantly different viable counts after 120 minutes incubation in 50 per cent PNHS ($p < 0.05$; Duncan t-test). The reference strains were extremely serum sensitive with a percentage survival in 50 per cent PNHS less than 10 per cent of the original inoculum after sixty minutes incubation. Of the reference strains, A77 is the most serum resistant. It also appears to be killed less rapidly. The inocula of A75 and A76 were reduced to less than 10 per cent survival by thirty minutes incubation with PNHS, compared to 43 per cent in the case of A77.

In all cases, there was a decrease in CFU/ml with PNHS. An exception was Y206, which declined in number up to sixty minutes incubation, but then increased slightly after 120 minutes incubation. This increase in CFU/ml was not significant and may reflect growth of a more serum-resistant subpopulation in the initial inoculum.

When *H. ducreyi* was suspended in BHI broth, there was a slight loss of viability, with the exception of strain Y206.

For all strains in 50 per cent inactivated PNHS, the percentage survival was greater than 100 per cent of the inoculum, indicating that growth had occurred.

5.3.2 INDUCTION OF SERUM RESISTANCE

The first method to induce reference strains to become serum resistant, failed. The degree of serum sensitivity remained less than 10 per cent survival after sixty minutes, despite at least eleven consecutive serum bactericidal assays.

The second method involved direct subculture of reference strains through 50 per cent PNHS in liquid medium seven times. An isogenic strain of A76 (A76*), was produced. In a measurement of its serum resistance, 24 per cent of the inoculum survived after sixty minutes in 50 per cent PNHS. The inoculum was however decreased to approximately 2 per cent after two hours incubation (Figure 5.2). Passaged strains of A75 and A77 which were treated in the same way remained serum sensitive.

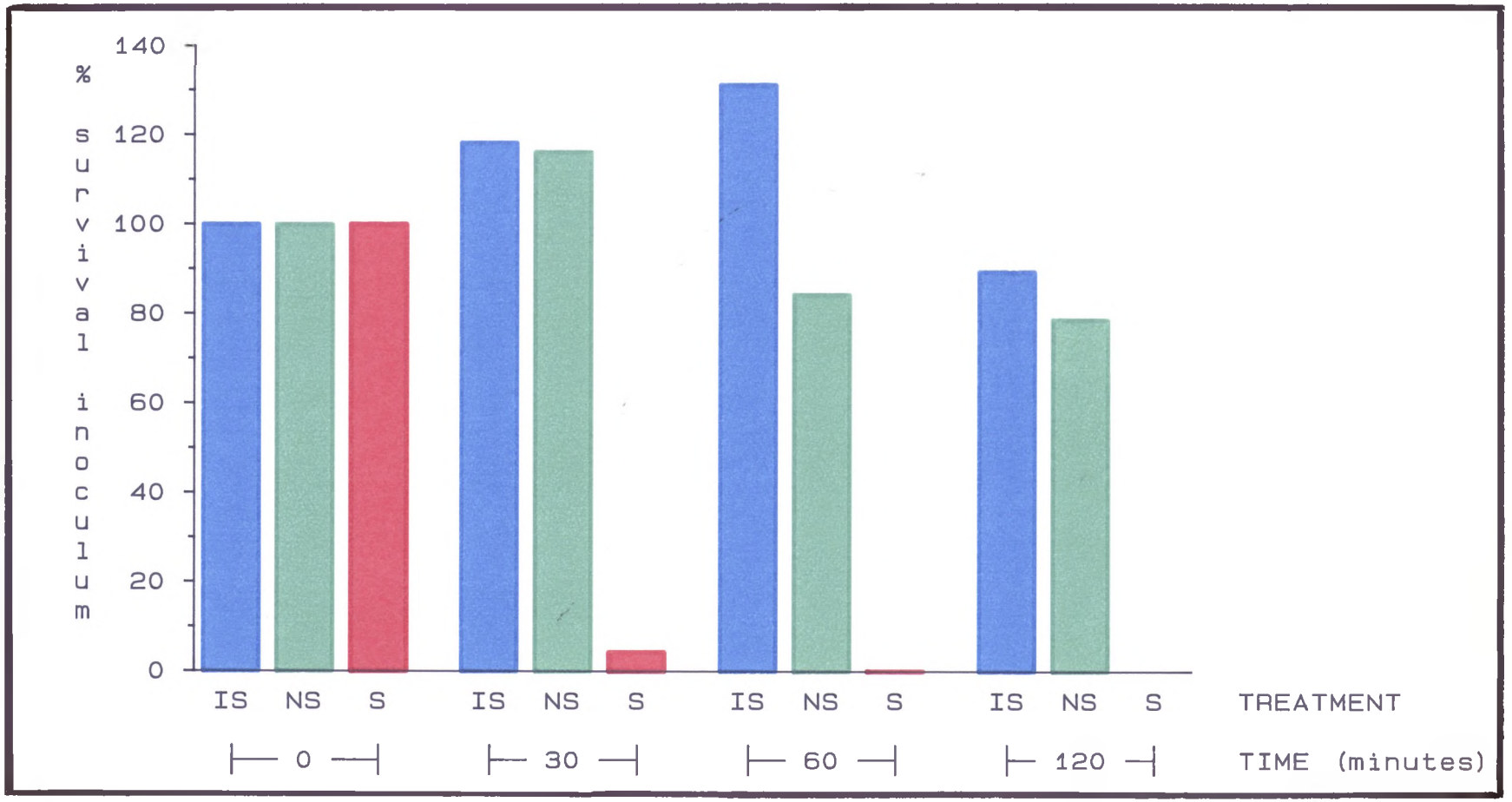


Figure 5.1 Survival of serum-sensitive reference strain of *H. ducreyi* A75 on exposure to 50% PNHS at 37^0 C after time intervals 0, 30, 60 and 120 minutes, expressed as a percentage of the bacterial inoculum at time zero (S). Two control experiments included inactivated PNHS (IS) and no PNHS (NS).

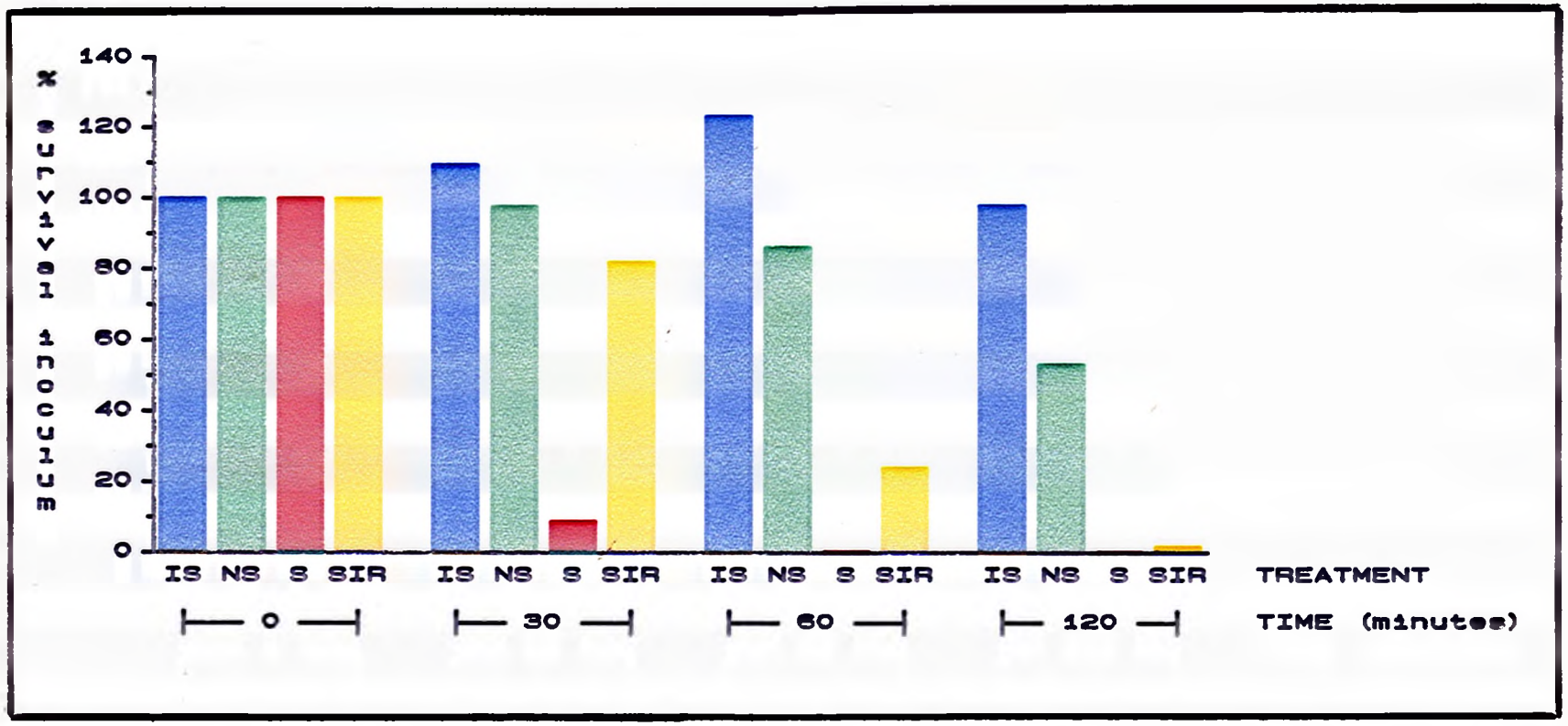


Figure 5.2 Survival of serum-sensitive reference strain of *H. ducreyi* A76 on exposure to 50% PNHS at 37^0 C after time intervals 0, 30, 60 and 120 minutes, expressed as a percentage of the bacterial inoculum at time zero (S). Two control percentage of the bacterial inoculum at time zero (S). Two control experiments included inactivated PNHS (IS) and no PNHS (NS). The graph also indicates the serum sensitivity of the isogenic strain A76* passaged in PNHS (SIR).

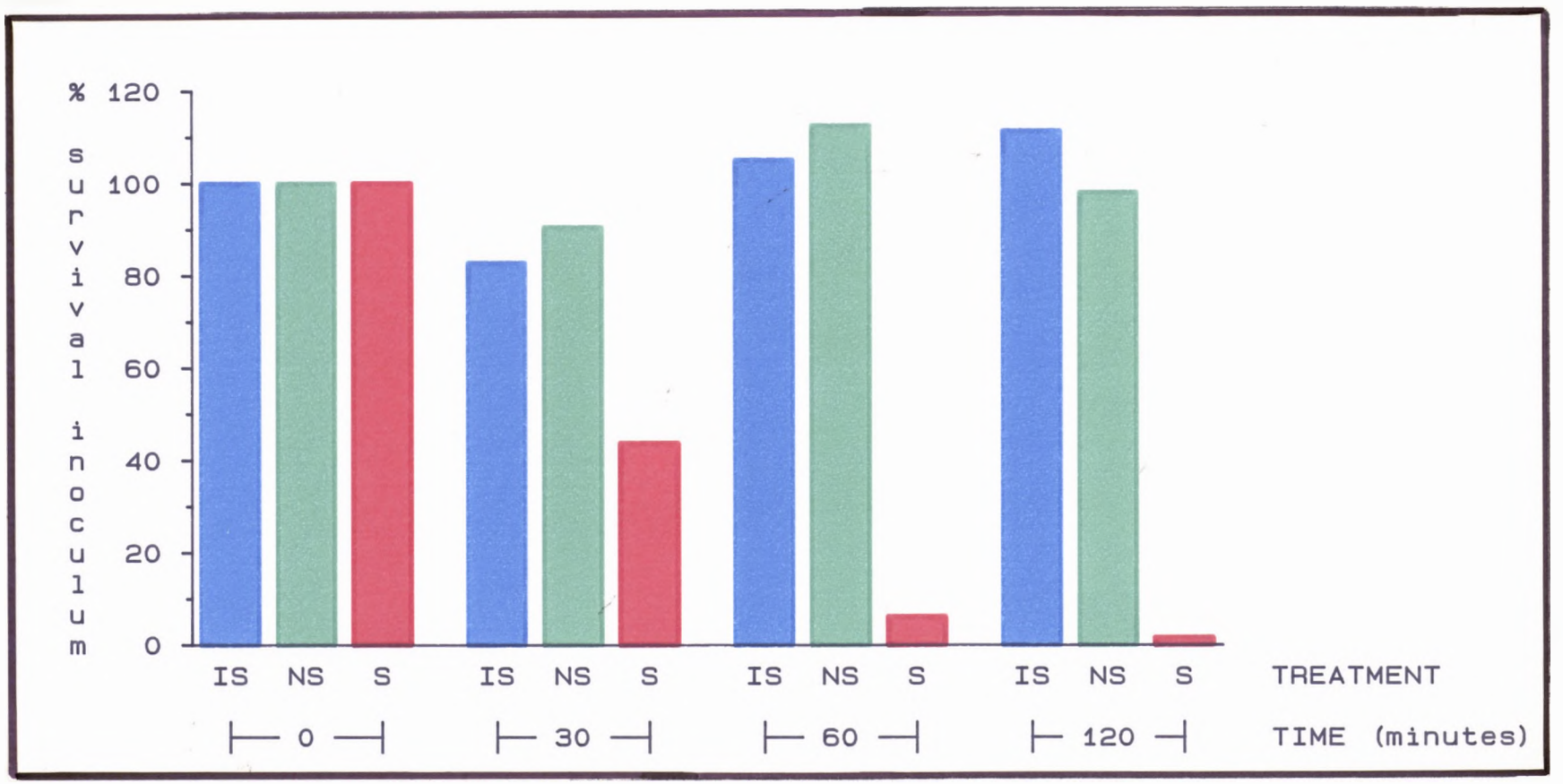


Figure 5.3 Survival of serum-sensitive reference strain of *H. ducreyi* A77 on exposure to 50% PNHS at 37^0 C after time intervals 0, 30, 60 and 120 minutes, expressed as a percentage of the bacterial inoculum at time zero (S). Two control experiments included inactivated PNHS (IS) and no PNHS (NS).

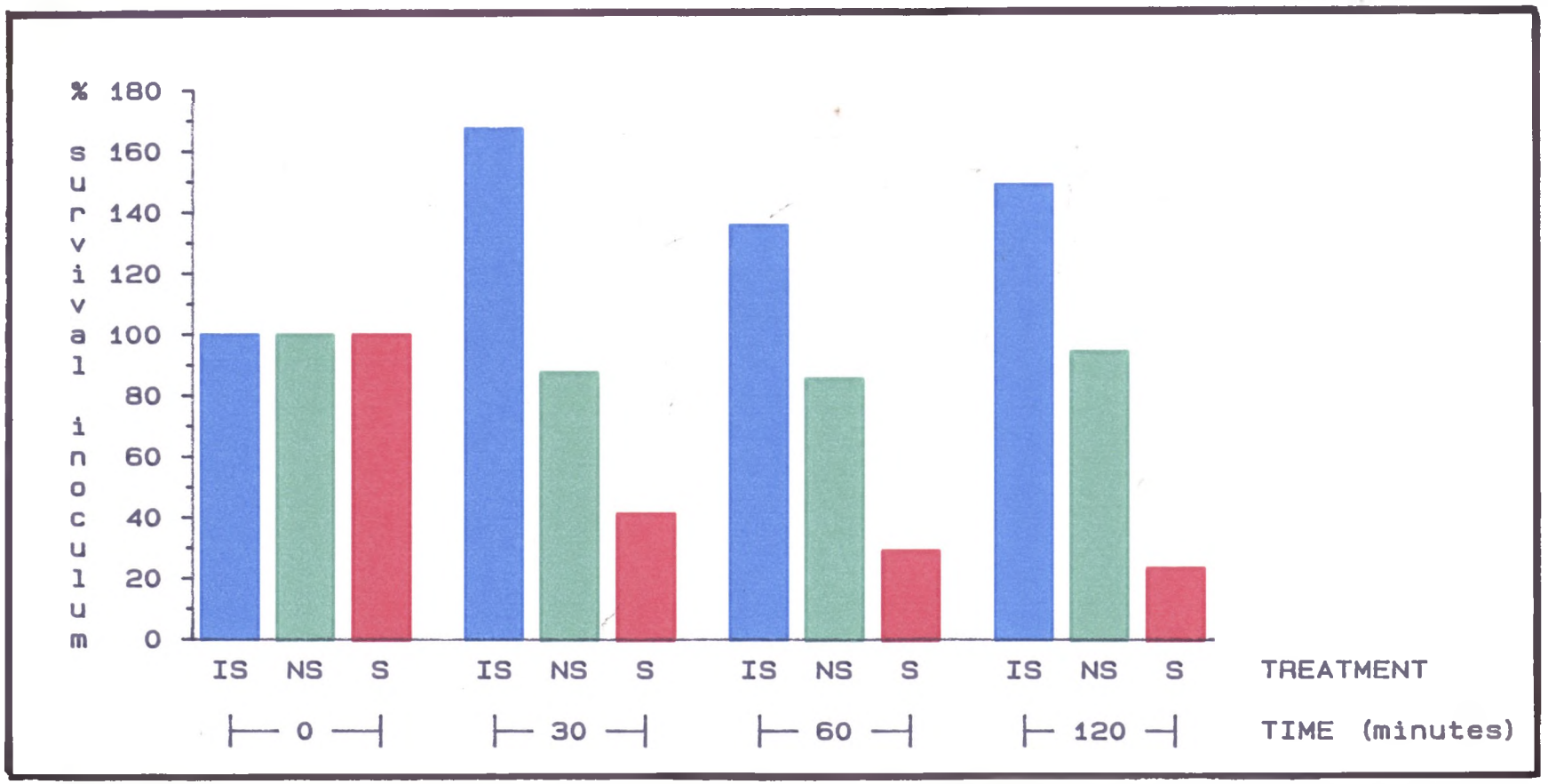


Figure 5.4 Survival of serum-resistant clinical strain of *H. ducreyi* Y155 on exposure to 50% PNHS at 37^0 C after time intervals 0, 30, 60 and 120 minutes, expressed as a percentage of the bacterial inoculum at time zero (S). Two control experiments included inactivated PNHS (IS) and no PNHS (NS).

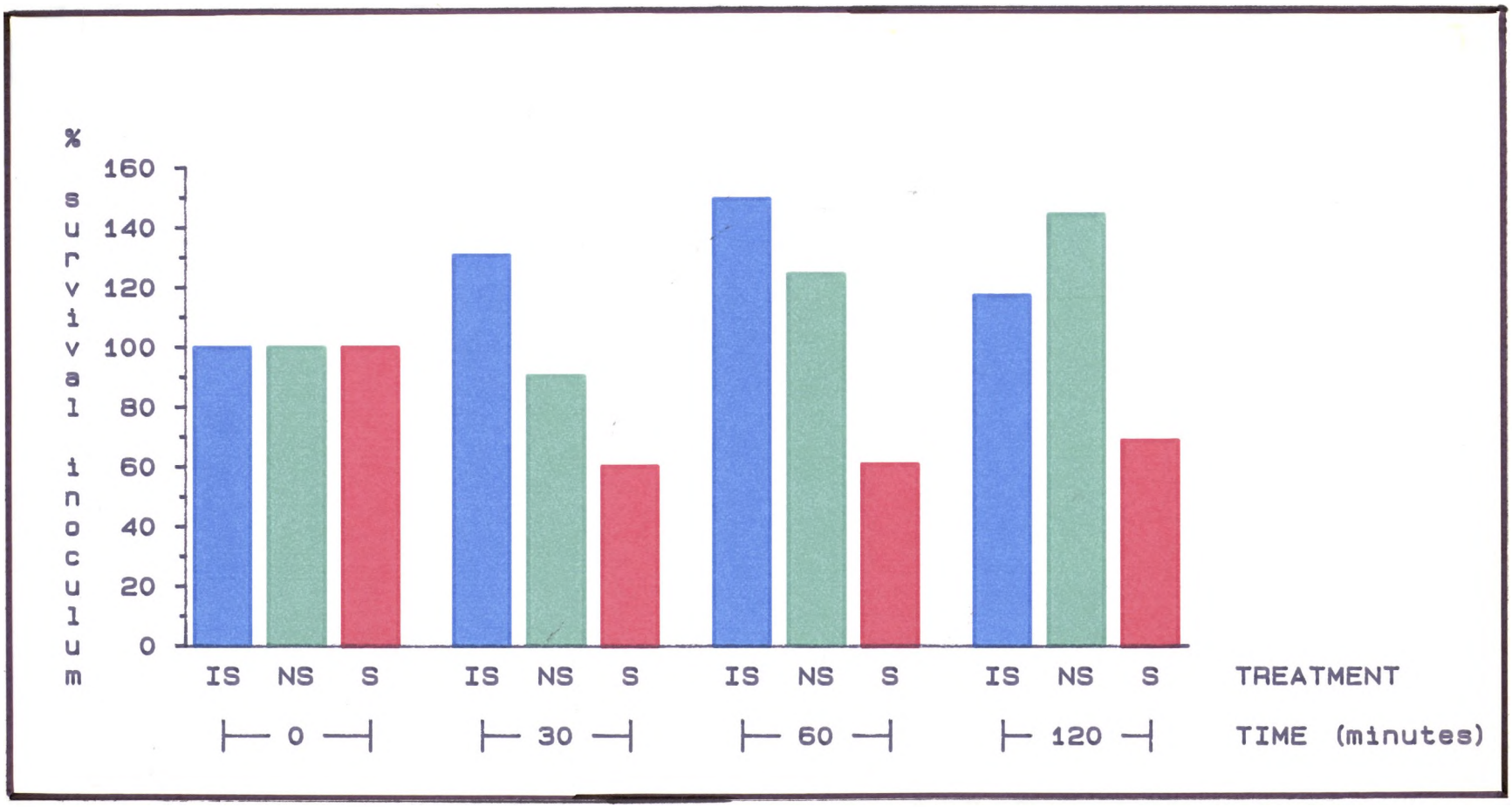


Figure 5.5 Survival of serum-resistant clinical strain of *H. ducreyi* Y206 on exposure to 50% PNHS at 37^0 C after time intervals 0, 30, 60 and 120 minutes, expressed as a percentage of the bacterial inoculum at time zero (S). Two control experiments included inactivated PNHS (IS) and no PNHS (NS).

5.4 DISCUSSION

5.4.1 SERUM RESISTANCE DEFINED

The bacteriolytic activity of serum is a well-defined host defence mechanism (Frank *et al,* 1987). Resistance to this serum killing contributes to the virulence of certain Gram-negative bacteria (Brubaker, 1985; Finlay and Falkow, 1989). It confers a survival advantage to micro-organisms invading the blood (Roantree and Rantz,1960; Fierer *et al,* 1972). Serum resistance has been observed in clinical strains of *Escherichia coli* (Roantree and Rantz, 1960; McCabe *et al,* 1978), *Serratia marcescens* (Simberkoff *et al,* 1976), *Yersinia enterocolitica* (Pai and DeStephano, 1982), *Haemophilus influenzae* type b (Sutton *et al,* 1982), *Salmonella typhimurium* and *Salmonella enteritidis* (Nelson and Roantree, 1967).

Taylor (1983b) defined 'serum-resistant' *E. coli* strains as those totally refractory to serum killing. Strains which showed any decrease in the viable inoculum size were 'serum sensitive'. By this definition of serum resistance, all the strains in this study and probably other bacteria in several other studies, would be serum sensitive. Conversely, Ogata and Levine (1980) described plasmid-mediated serum resistance of *E. coli*, where resistance was not detectable in serum concentrations greater than 3 per cent PNHS. Although, these plasmids did reduce the rate of serum killing by a small amount, they hardly conferred a level of 'serum resistance' equivalent to that observed in clinical strains of *E. coli* mentioned above. Consequently, all the strains in this study, would by this definition be serum resistant. Serum resistance is thus defined relative to the construction of an experiment and its parameters arbitrarily chosen by the experimentor.

Another consideration is that serum resistance is a property of certain bacterial genera and species. Roantree and Rantz (1960) showed that of several Gram-negative bacteria of different genera e.g *Shigella* and *Klebsiella,* had a higher proportion of serum-sensitive strains;

whereas the highest proportion of strains expressing serum resistance was found in the *Salmonella* group. Similarly differences between different species of the same genus were reported for *Campylobacter* (Blaser *et al,* 1985), *Klebsiella* (Benge, 1988) and *B. fragilis* (Casciato *et al,* 1979). Serum resistance of *Shigella* would not be equivalent to serum resistance of *Salmonella* by the same definition i.e. the level of serum sensitivity of one type of bacteria may not be equivalent to serum sensitivity of another micro-organism.

It is necessary to take into account the *in vitro* parameters which influence serum resistance of a bacterium. Manipulations before and during the serum bactericidal assay could drastically alter its susceptibility to serum killing. There are several factors influencing the outcome of the serum bactericidal assay - the metabolic state of the bacterial inoculum, the source and concentration of the serum, the size of the inoculum, the diluent used, the incubation conditions and the time of evaluation (Osawa and Muschel, 1964; Taylor, 1972).

5.4.2 SERUM RESISTANCE AND VIRULENCE

A relationship between virulence as determined by intradermal rabbit inoculation and serum resistance of *H. ducreyi* was first observed by Deacon *et al* (1954). Avirulent stock strains grew best in inactivated (non-functioning complement) human or rabbit blood clot media, while virulent cultures grew equally well in either fresh or inactivated blood clot media, suggesting that the bactericidal property of fresh blood was inoperative for newly-isolated virulent cultures. In other words, clinical strains of *H. ducreyi* were more serum resistant than stock cultures. These early observations were confirmed by Odumeru *et al* (1984) and this present study. Clinical strain Y206 was more serum resistant than Y155; A75 was the most serum sensitive of the three reference strains.

The finding that clinical strains are more resistant to killing by serum provides circumstantial evidence implicating serum resistance as a virulent factor of *H. ducreyi.* Several more strains

for testing are required to indicate a significant difference between the serum susceptibility of reference and clinical strains of *H. ducreyi.*

More direct evidence for an association of serum resistance with virulence might be the ability of a strain to cause 'disease' in experimental animals. This approach, however, is not without its difficulties. In a controversial review, Isenberg (1988) describes the problems associated with defining and measuring virulence of pathogens *in vitro* using experimental animals. Factors such as a lack of suitable animal models, unnatural introduction of laboratory-grown cultures by subcutaneous or intradermal injection, infective dose dependency and an absence of the influence of a polymicrobial flora, are mentioned. These have particular relevance to the virulence assay of *H. ducreyi* i.e. the development of subcutaneous lesions on intradermal inoculation of rabbits (Dienst, 1948; Deacon *et al,* 1954; Hammond *et al,* 1978b; Odumeru *et al,* 1984) or rats (Tuffrey *et al,* 1988).

The suitability of these animal models is also questionable since animal infections differ from human chancroid in several aspects. In both cases the lesions are self-limiting, healing within several weeks of inoculation (Deacon *et al,* 1954; 1956; Tuffrey *et al,* 1988). A minimum infective dose of about 10^6 organisms has been documented (Choudary *et al,* 1982; Tuffrey *et al,* 1988). An additional variable in the rat model is an apparent lack of bactericidal activity of mouse serum against several Gram-negative bacteria that are serum sensitive on exposure to sera from other animals (Rowley and Wardlaw, 1958). In a recent study, Tuffrey's group (R. Ballard, personal communication) reported that lesion development was not dependent on the viability of *H. ducreyi.* Also, intradermal inoculation of viable and non-viable *N. gonorrhoeae*, extracted lipopolysaccharides of *H. ducreyi* and *N. gonorrhoeae* also resulted in 'virulent'-type lesions indicating a crucial lack of specificity of this assay.

Reference strains A75, A76, A77 and CIP 54.2 were proved 'avirulent' in rabbits by this assay (Hammond *et al,* 1978b; Odumeru *et al,* 1984). However, CIP 54.2 produced skin lesions in the rat model (R. Ballard, personal communication). The use of laboratory

primates in virulence studies may more closely resemble human chancroid infection. Coincidently, Saelhof (1924) documented the first properly controlled animal inoculation with *H. ducreyi* by Nicolle in 1893 using the scalp of a Rhesus monkey. Human intradermal inoculation of lesion material was common following the discovery of *H. ducreyi* by Ducrey in 1889 (Ducrey, 1889; Cole and Levin, 1935; Heymann *et al,* 1945; Dienst, 1948).

The assessment of 'virulence' of *H. ducreyi* is thus not well defined and requires clarification before other factors such as serum resistance can be fully appreciated. More likely it contributes to, but does not determine, pathogenicity of *H. ducreyi.*

5.4.3 IMMUNOLOGY

The external and localised site of infection of *H. ducreyi* on the host, further questions the contribution of serum resistance to the pathogenicity of *H. ducreyi.* Moreover, there is no reported evidence of this micro-organism invading and surviving in the bloodstream of infested patients. However, the formation of lymphadenophy in approximately 50 per cent of patients, implicates an involvement of *H. ducreyi* infection with the immune system of its host. The concentration of antibodies in blood may be significantly higher than those in the lymph tissue. Drinker *et al* (1935) reported that intravenous injection of anti-pneumococcal antiserum, followed by intravenous injection of pneumococci rendered the blood sterile but the number of organisms in the thoracic duct was only slightly reduced. The lymphatic system may thus sequester organisms including *H. ducreyi*, independently of their susceptibility to the bactericidal activity of serum.

Killing of *H. ducreyi* by PNHS is probably complement (C′) mediated in this study, since inactivation significantly reduced its bactericidal activity. By treating PNHS with ethylene glycol-bis(aminoethyl ether) N N N′N′ tetraacetic acid and inulin, which inhibits the classical and the alternative pathway of C′-mediated killing respectively, Odumeru *et al* (1985) found

that serum killing of *H. ducreyi* followed the classical pathway. The classical pathway requires antibody and C′ for initiation. Antibodies specific for *Haemophilus* antigens have been reported in PNHS, and may be cross-reactive with *H. ducreyi* (Schalla *et al,* 1986). Antisera raised in immunised laboratory animals against *H. ducreyi* antigens, cross-reacted with bacteria of other *Haemophilus* species (Slootmans *et al,* 1983; Saunders and Folds, 1986; Abeck *et al,* 1988), further indicates that certain antigens are shared.

While PNHS is bactericidal for *H. ducreyi*, inactivated PNHS appears to have the opposite effect. An increase in the inoculum size was observed on incubation with inactivated PNHS, suggesting that growth, particularly of the clinical strains, was encouraged. The necessity for blood or blood products for nourishing growth of *H. ducreyi*, has been satisfied by human serum for primary isolation of this micro-organism from patients (Reymann, 1947; Ajello *et al,* 1956; Borchardt and Hoke, 1970; Sottnek *et al,* 1980) - although rabbit serum (Reymann, 1947) and FCS (Sottnek *et al,* 1980) appear to be more efficient than PNHS in supporting growth of *H. ducreyi.*

5.4.4 THE SERUM BACTERICIDAL ASSAY AND INDUCTION OF SERUM RESISTANCE

The influence of passaging serum-sensitive reference strains of *H. ducreyi* through PNHS, was unclear. In the first method, *H. ducreyi* was repeatedly passaged through consecutive serum bactericidal assays (120 minutes in 50 per cent PNHS), interrupted by growth on agar and in broth. In the second method, the bacteria were passaged only in liquid medium containing at least 50 per cent PNHS at each step. It was hoped that a spontaneous mutation to serum resistance would occur, and be selected by the presence of human serum. There are possible reasons accounting for the failure of the first method to induce serum resistance. Firstly, the time of contact with the serum may have been too short i.e. two hours every third passage. Secondly, if serum-resistant mutants were selected by exposure to serum, a serum-

sensitive subpopulation may have overgrown these mutants during the two subcultures in the absence of serum.

Spontaneous mutation of *H. ducreyi* appears to be infrequent, and induced serum resistance is produced with difficulty. Possibly the use of higher concentrations of serum, an absence of subculture without serum, and a greater number of passages could have resulted in a higher level of serum resistance, or a greater number of serum-resistant mutants being obtained. Unfortunately, the exact method which Odumeru *et al* (1984) used to obtain an isogenic serum-resistant mutant of strain A77 was not clarified. In that study, serum-sensitive A77 was passaged in increasing concentrations of PNHS. In the same study, clinical strains of *H. ducreyi* were more serum resistant (greater than 50 per cent survival of the inoculum in 50 per cent PNHS after 120 minutes) than that reported in this study. Odumeru *et al* (1984) reported the use of a sixteen hour culture for the serum bactericidal assay, but it is unclear whether *H. ducreyi* was grown in broth or on solid media, since only the latter form of media were mentioned. If *H. ducreyi* strains were cultured on solid media for sixteen hours, sufficient bacterial growth for harvest is not likely to be obtained. Moreover, a bacterial suspension of $5x10^8$ CFU/ml was produced. Generally, at least twenty-four hours incubation of *H. ducreyi* on solid media is required for visible colonies. A higher level of serum resistance of the clinical strains observed by Odumeru *et al* (1984) could be attributed to an older bacterial inoculum, or agar-grown *H. ducreyi,* compared with the overnight broth-grown bacteria in this study.

To prevent serum-resistance induction, the medium selected for maintainance of the strains, lacked any source of serum. In several studies the sensitivity of some Gram-negative bacteria has been altered after subculture in the presence of serum (Akiyama and Inoue, 1977; Tee and Scott, 1980; Martin *et al,* 1982; Schiller *et al,* 1984).

Since serum resistance may also be lost by *in vitro* passaging (Akiyama and Inoue, 1977; Demes *et al,* 1988), the frequent passage of fresh isolates of *H. ducreyi* was avoided.

Serum from rabbits, mice and guinea pigs (Landy *et al,* 1962; Ogata and Levine, 1980; Binns *et al,* 1982; Pai and DeStephano, 1982; Odumeru *et al,* 1984; Gilsdorf and Ferrieri, 1986) have been used in serum bactericidal assays against Gram-negative pathogens. However, the use of human serum reveals results of more clinical relevance. It appears that PNHS is more bactericidal than serum from other animal sources (Roantree and Rantz, 1960). Odumeru *et al* (1984) reported that 'similar' results against *H. ducreyi* were obtained using rabbit and human serum in the serum bactericidal assay. Quantitative differences between the two sera were not reported. It is likely that a difference in bactericidal activity does exist, since fresh rabbit blood is commonly used in the isolation medium of clinical *H. ducreyi.* In present observations (Y. Dangor, personal communication), growth of reference strains on BHI agar and 10 per cent fresh rabbit blood produced a good growth yield. This suggests that rabbit blood has minimal inhibitory properties against reference strains of *H. ducreyi.*

An observation made was that serum resistance induction was incomplete, and killing of the isogenic strain A76* in the serum bactericidal assay was delayed. This phenomenon of delayed killing by serum has been reported for a rough mutant (Taylor, 1975) and smooth (Taylor, 1974) strains of *E. coli.* In those experiments there was little change in the viable count of *E. coli* during the first hour of exposure to PNHS, but the number of survivors decreased to less than 1 per cent after three hours incubation. Generally, most strains of serum-sensitive *E. coli* were rapidly killed during the first thirty minutes of exposure to serum.

Serum resistance of *E. coli* has been induced by several investigators. The methodology varies from a single three hour incubation in 75 per cent PNHS (Taylor *et al,* 1972), to a one to three day incubation in 80 per cent PNHS, followed by an overnight incubation without serum, five to fifteen times (Akiyama and Inoue, 1977). Tee and Scott (1980) subcultured *E. coli* directly from 100 per cent PNHS to 100 per cent PNHS forty times. Similarly, Schiller *et al* (1984) induced serum resistance in *P. aeruginosa* by passaging

serum-sensitive strains overnight in increasing concentrations of serum until growth was visible in 40 per cent PNHS. The technique to alter serum susceptibility of micro-organisms *in vitro* clearly requires standardisation.

Most serum-sensitive Gram-negative bacteria are not easily mutated to serum resistance. *N. gonorrhoeae* appears to have a unique mechanism of serum resistance acquisition. Various substances appear to induce gonococcal serum resistance e.g. human serum (Martin *et al,* 1982), urethral exudate (Ward *et al,* 1970), guinea pig chamber fluid (Goldner *et al,* 1979) and the lysate of guinea pig red blood cells (Tan *et al,* 1986). The acquisition of serum resistance required only a one to three hour incubation in one of the above fluids at 37^0 C (McCutchan *et al,* 1976; Martin *et al,* 1982; Tan *et al,* 1986) and was rapidly lost on subculture in normal media (Ward *et al,* 1970; McCutchan *et al,* 1976; Rittenberg *et al,* 1977; Goldner *et al,* 1979). This loss and gain of serum resistance was too rapid to be attributed to mutation or selection, the conversion was thought to be phenotypically controlled, possibly as a result of a change in growth limiting nutrients (Rittenberg *et al,* 1977).

Serum resistance has been encoded by Col-V plasmids (Binns *et al,* 1979; Nilius and Savage, 1984) and antibiotic-resistant plasmids - R plasmids - (Reynard and Beck, 1976; Moll *et al,* 1980; Ogata and Levine, 1980) in *E. coli*; and by virulence plasmids in *S. typhimurium* (Hackett *et al,* 1987; Van den Bosch *et al,* 1987). The R plasmids responsible for serum resistance were R1, R100 (Reynard and Beck, 1976) and R6-5 (Moll *et al,* 1980). These plasmids conferred resistance against chloramphenicol, streptomycin, sulphonamide, ampicillin, kanamycin and tetracycline.

The possibility that serum resistance of *H. ducreyi* is plasmid encoded was considered for several reasons. Clinical strains of *H. ducreyi,* including South African strains, have expressed resistance to many antibiotics including those mentioned above, except for streptomycin (Handsfield *et al,* 1981; Bilgeri *et al,* 1982; Taylor *et al,* 1985; Dangor *et al,* 1988b). Resistance to ampicillin, sulphonamide, tetracycline and chloramphenicol has been shown to be

plasmid mediated in *H. ducreyi* (McNicol and Ronald, 1984; Johnson *et al,* 1988). Although none of the plasmids encoding serum resistance of *E. coli* is found in *H. ducreyi*, it is possible that certain *H. ducreyi* plasmids have an enteric origin. By hybridisation and heteroduplex analysis, the sulphonamide-resistance plasmid of *H. ducreyi* was found to be 79 per cent related to an enteric streptomycin-sulphonamide-resistance plasmid. Also, the genes encoding kanamycin resistance of *H. ducreyi* are thought to originate from a transposon of enteric derivation (Albritton *et al,* 1984). More recently, it has been shown that the chloramphenicol acetyltransferase which confers chloramphenicol resistance to *H. ducreyi* is molecularly related to the enteric type III class of this enzyme (Roberts *et al,* 1985).

Furthermore, the behaviour of the reference strains passaged in PNHS could be accounted for if serum resistance of *H. ducreyi* was plasmid mediated. The passaging of the survivors from a serum bactericidal assay, without serum, may have resulted in selection for cells lacking the plasmid.

Alternatively, serum resistance of *H. ducreyi* could be encoded chromosomally as observed for *N. gonorrhoeae* (Hildebrandt *et al,* 1978; Spratt *et al,* 1980; Cannon *et al,* 1981; Shafer *et al,* 1982). The induction of serum resistance might thus be a function of the expression and regulation of the chromosomal genes.

5.5 CONCLUSION

Clinical strains *H. ducreyi* were more serum resistant than the reference strains. The association between this phenomenon and virulence for man remains unclear. Further investigations are required to determine the level of serum resistance and its prevalence in a statically suitable number of clinical strains of *H. ducreyi.* The results of a serum bactericidal assay should be applied with caution, since *in vitro* conditions may differ significantly from *in vivo* conditions.

PNHS was bactericidal for all the strains. Killing by serum was probably C mediated since heating the serum for thirty minutes at 56^0 C destroyed C′ and thus its bactericidal activity.

Inactivated PNHS had no killing effect, and even promoted growth especially of the clinical strains. This confirms the requirement by *H. ducreyi* for growth-promoting factors in serum.

Serum resistance was only induced in one of the three reference strains when directly passaged seven times through at least 50 per cent PNHS. Serum resistance was not induced when survivors of a serum bactericidal assay were consecutively subjected to another such assay with interruptions of two subcultures. The mode of expression of serum resistance is uncertain, but is thought to require exposure to serum for prolonged periods of time. This could suggest plasmid mediation, or reflect a function of gene regulation and expression. The acquisition of serum resistance of the isogenic A76* strain appeared to be delayed serum killing rather than achievement of total serum resistance.

LIPOPOLYSACCHARIDE OF HAEMOPHILUS DUCREYI

6.1 INTRODUCTION

Lipopolysaccharide (LPS) is a major component of the cell wall of Gram-negative bacteria. However thorough investigations of LPS composition have focused on members of the family Enterobacteriaceae, and knowledge of the detailed structure of the LPS of bacteria in other genera is lacking. Polyacrylamide gel electrophoresis (PAGE) has proved to be a practical and rapid method for comparative analyses of LPS. The relationship between LPS structure and silver-stained bands on polyacrylamide gels has been thoroughly investigated using LPS mutants of *Salmonella*, which differ in their O-antigen chemotypes (Palva and Makela, 1980; Hitchcock and Brown, 1983; Mintz and Deibel, 1983).

Three regions of LPS molecules have been described as the O antigen which is a long-chain polysaccharide consisting of repeating units containing one to seven monosaccharides; an oligosaccharide core composed of approximately ten monosaccharides; and lipid A, a lipid backbone consisting of diglucosamine and β1-6-linked fatty acids. All these components are present in smooth LPS. In rough LPS, the O-antigen component is missing. Semirough LPS is defined as lipid A, a complete core, and one covalently bound O-antigen unit (Hitchcock *et al,* 1986).

Generally, it is believed that enteric bacteria express both rough and smooth LPS while non-enteric bacteria have only rough LPS in their cell walls (Hitchcock *et al,* 1986).

In previous studies, the LPS of clinical strains of *Haemophilus ducreyi* was analysed by PAGE, and found to be rough i.e. appearing as a single low molecular weight band on silver-stained gels. Some degree of interstrain heterogeneity in this LPS pattern was also observed (Abeck *et al,* 1987a; 1987c; Odumeru *et al,* 1987).

The biochemical composition of the LPS of *H. ducreyi* was investigated by Odumeru *et al* (1987) using gas liquid chromatography and several biochemical tests. Fatty acids of chain length C_{14}, C_{16}, C_{18} and C_{20}, heptose and 2-keto-3-deoxyoctonate (KDO) were present. Glycoses identified were glucose, galactose and their amino derivatives, glucosamine and galactosamine. Fucose was present in trace amounts, but mannose and rhamnose were absent.

In this study, the LPS of three serum-sensitive reference strains, two serum-resistant clinical strains, and an isogenic serum-resistant strain of A76 (A76*) of *H. ducreyi,* were analysed. The LPS was extracted using the method described by Darveau and Hancock (1983b). The structure and biochemical composition was determined by PAGE, and several colorimetric assays, respectively.

6.2 MATERIALS AND METHODS

6.2.1 STRAINS AND MEDIA

H. ducreyi strains Y155 and Y206 were isolated from patients with chancroid in Carletonville, South Africa (Dangor, 1988a). Three reference strains A75, A76 and A77 were obtained from the Pasteur Institute, France. A76* is an isogenic strain of A76 which ex-

pressed a relatively higher level of serum resistance than its parent strain, following several direct passages in 50 per cent pooled normal human serum.

Media consisting of brain heart infusion (BHI) agar (Difco) supplemented with 1 per cent haemoglobin (Difco), 1 per cent IsoVitaleX (BBL, Microbiological Systems) and 3mg/l vancomycin, was used for culture of the strains. The plates were incubated in candle jars at 33^0 C.

Commercial preparations of phenol-extracted LPS isolated from *Salmonella typhimurium* (L6511) and *Escherichia coli* (L2630) were obtained from Sigma.

6.2.2 SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS- PAGE)

Clinical and reference strains of *H. ducreyi* were cultured for two and three days respectively as described above. Two loopfuls of each strain were suspended in 100µl of solubilising buffer as described by Abeck *et al* (1987a) (APPENDIX 3). After boiling for five minutes, a solution of 100µg Proteinase K (Boehringer Mannheim) in 20µl of solubilising buffer was added. The mixture was incubated at 60^0 C in a waterbath for sixty minutes. Without shaking, 70µl to 80µl of each sample was analysed by PAGE using a discontinuous gel system (Laemmli, 1970) with a resolving gel containing 15 per cent polyacrylamide, and a stacking gel containing 4.5 per cent polyacrylamide (Hoeffer Scientific Instruments, SE600) (APPENDIX 3). The gels were run at a constant current of 30mA and fixed and stained with silver as described by Tsai and Frasch (1982) (APPENDIX 4).

The two LPS control preparations of *S. typhimurium* and *E. coli* were treated in the same way as viable *H. ducreyi* cultures.

6.2.3 EXTRACTION AND ISOLATION OF LIPOPOLYSACCHARIDE

For analytical purposes the LPS of *H. ducreyi* was isolated by the method described by Darveau and Hancock (1983b). The details of the extraction process are given in APPENDIX 5.

Clinical cultures were harvested from solid media after two days growth; reference strains were incubated for three days before harvest. Briefly, whole cells of *H. ducreyi* were suspended in Tris buffer containing magnesium chloride, DNase and RNase. After sonication and incubation at 37^0 C, the mixture was treated with ethylene diamine tetraacetic acid (EDTA) and sodium dodecyl sulphate (SDS). Centrifugation removed unwanted peptidoglycan. The resulting supernatant was treated with pronase overnight. Crude LPS was precipitated in a magnesium chloride-ethanol solution at 0^0 C, and separated by ultracentrifugation. This preparation was again exposed to EDTA, SDS and pronase, precipitated and subsequently collected as previously described. After washing the pellet in Tris buffer, a final ultracentrifugation concentrated the extracted LPS.

The phenol-extracted LPS of *E. coli* 0111 : B4 (L2630) and *S. typhimurium* (L6511) were obtained from Sigma.

6.2.4 ANALYTICAL METHODS

Total carbohydrate (CHO) was measured by treating the LPS samples with phenol and sulphuric acid as described by Dubois *et al* (1956). The colour reaction was measured at 490nm (APPENDIX 6).

The method of Scott and Melvin (1953) was used to quantify total hexose content. Solutions of LPS were added to anthrone, mixed and then boiled. The absorbance of the samples were read to 640nm (APPENDIX 7).

Glucose (Sigma) was used as a standard in both the abovementioned assays.

Heptose was determined by the method described by Wright and Rebers (1972) with glucoheptose (Sigma) as a standard. Cysteine-hydrochloride was added to the LPS which had been previously boiled in sulphuric acid. The difference in absorbance at 490nm and 550nm reflected the amount of heptose in the sample (APPENDIX 8).

Glucosamine (Sigma) was the standard for the hexosamine assay (Smith and Gilkerson, 1979). After hydrolysis of the LPS at 100^0 C for two hours in hydrochloric acid, the samples were cooled to room temperature. The following solutions were added in sequence with the appropriate incubation conditions : sodium nitrite (room temperature, five minutes), 3-methyl-2-benzo-thiazolinone (37^0 C, thirty minutes) and ferric chloride (37^0 C, five minutes). The amount of glucosamine was determined by measuring the absorbance at 640nm following cooling to room temperature (APPENDIX 9).

The final component of LPS measured was KDO. The LPS samples were hydrolysed by boiling for one hour in acetate buffer, and cooled to room temperature. The LPS was oxidised with periodic acid; the reaction was terminated by the addition of sodium arsenite. Thiobarbituric acid was added and the mixtures were boiled. Dimethyl sulphoxide was added before the absorbance was read at 550nm (Brade *et al,* 1983; APPENDIX 10).

All the results were measured by a Leitz photometer at the appropriate wavelengths. All the values were calculated and described as per mg of dry weight LPS. The two commercial preparations of LPS were analysed in the same manner.

6.3 RESULTS

6.3.1 POLYACRYLAMIDE GEL ELECTROPHORESIS

Polyacrylamide gel electrophoresis is a rapid and relatively sensitive technique for the analysis of different forms of LPS molecules. The larger the molecule, the slower its migration through the polyacrylamide gel (Hitchcock and Brown, 1983; Peterson and McGroarty, 1985). Smooth LPS presents as a ladder-like pattern, each step representing molecules of increasing molecular weight. This is clearly indicated in Figure 6.1 by the LPS profiles of *E. coli* (lanes one and two) and *S. typhimurium* (lanes nine and ten). Rough LPS which consists of lipid A and core oligosaccharide appears as low molecular weight band/s.

All strains of *H. ducreyi* produced LPS profiles consisting of a large low molecular weight band, and two higher molecular weight bands (Figure 6.1). This pattern is quite unlike that of smooth LPS derived from the control strains *E. coli* 0111 : B4 and *S. typhimurium.*

The LPS profiles of the serum-sensitive reference strains and the two serum-resistant clinical strains were much the same. Y155 differed in that the low molecular weight band appeared to be larger compared with the other strains of *H. ducreyi* (Figure 6.1).

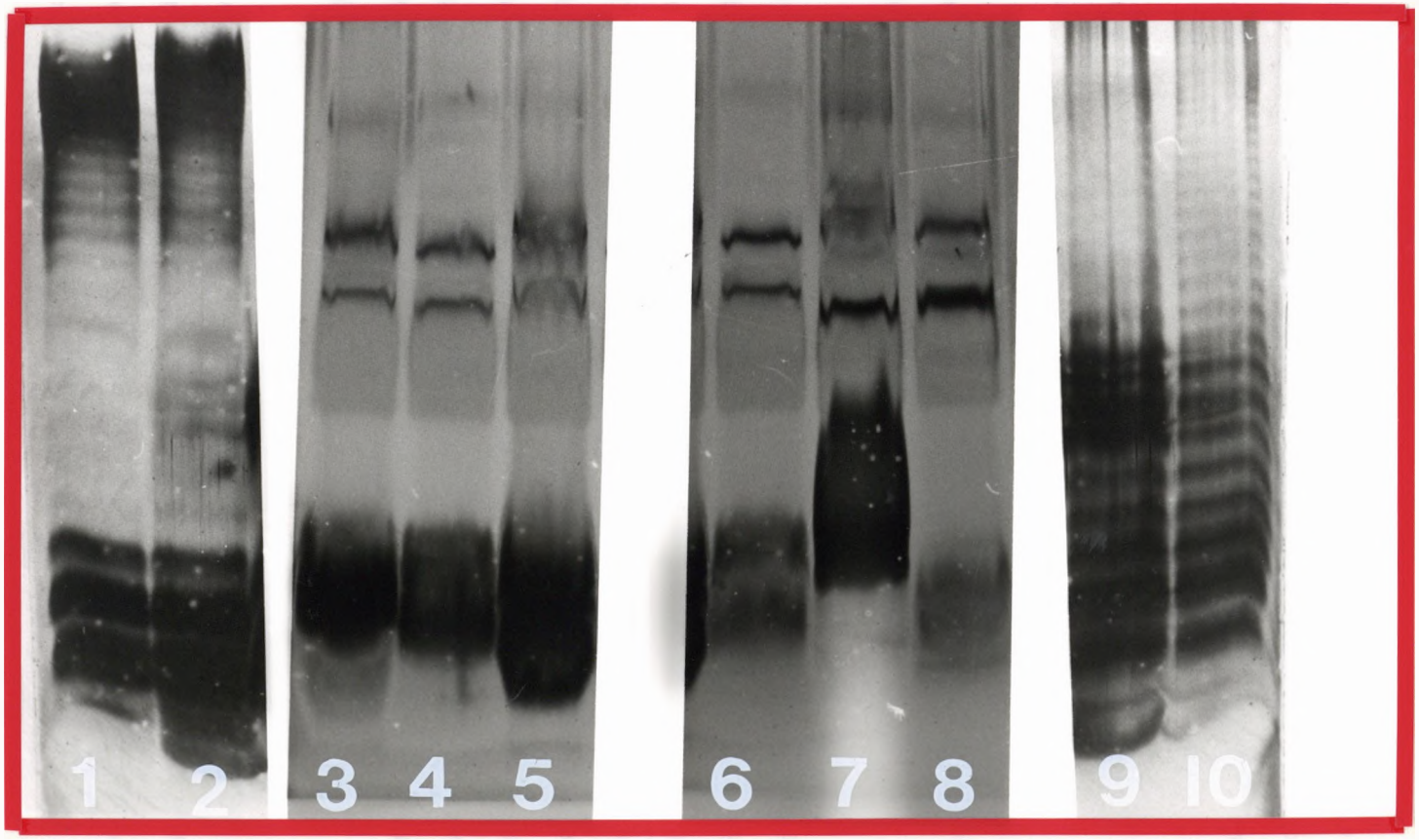


Figure 6.1 LPS profiles of *H. ducreyi* strains on SDS PAGE : A75 - lane 3; A76 - lane 4; A77 - lane 5; A76* - lane 6; Y155 - lane 7 and Y206 - lane 8. Commercial preparations of LPS is shown in lanes 1 and 2 (*E. coli* 0111 : B4) and in lanes 9 and 10 (*S. typhimurium*).

6.3.2 BIOCHEMICAL ANALYSIS OF LIPOPOLYSACCHARIDE

Sugar components common in the LPS of most Gram-negative bacteria viz. hexose, heptose, KDO and hexosamine, were also detected in the LPS of *H. ducreyi.* Heptose appeared to be present in the highest concentration; while KDO was only present in minute quantities.

The induction to serum resistance of reference strain A76 was accompanied by an increase in all the sugar constituents tested except for the KDO component.

The LPS of commercially prepared LPS (*E. coli* 0111 : B4 and *S. typhimurium*) contained more carbohydrate than that of *H. ducreyi* (Table 6.1).

Commonly, the ratio CHO : KDO is thought to reflect the length of the variable polysaccharide region (O antigen) in relation to the core region. The ratios CHO : KDO, hexose : KDO, hexose : heptose and hexose : hexosamine are listed in Table 6.2, where KDO, heptose and hexosamine are constituents of the core oligosaccharide of the LPS of enteric bacteria. The concentration of 'total CHO' in the LPS of strain A77 was significantly lower compared with that of other strains of *H. ducreyi* (Table 6.1). This result does not appear to be spurious since the assay was repeated, and also performed concurrently with the LPS of the other strains and commercial preparations of LPS from *S. typhimurium* and *E. coli* 0111 : B4.

There was no significant difference between the ratios of O-antigen sugar : core oligosaccharide sugar of the reference and clinical strains of *H. ducreyi.* These ratios for *S. typhimurium* were significantly higher than those of the other strains tested, suggesting the presence of O-antigenic side chain/s.

Table 6.1 Biochemical composition of the LPS isolated from *H. ducreyi;* and commercial preparation of the LPS of *S. typhimurium* and *E. coli* 0111 : B4.

Strain	µg / mg dry weight LPS[a]					
	Total Carbohydrate (CHO)	Hexose	Heptose	Hexosamine	KDO	Total of LPS components assayed
H. ducreyi						
Λ75	25.6	14.9	31.5	22.7	0.765	95.5
Λ76	19.3	14.5	21.5	22.5	0.82	78.6
Λ77	8.7	18.0	20.5	17.9	0.74	65.8
Λ76	24.6	19.1	29.7	25.7	0.57	99.0
Y155	18.0	16.3	32.0	28.7	0.925	95.0
Y206	26.1	15.9	30.1	23.4	0.84	95.6
E. coli						
0111 : B4	180.0	90.2	151.7	45.2	3.8	471.0
S. typhimurium	177.6	191.2	176.2	19.5	3.4	568.0

[a] The assays were repeated at least in duplicate. The values presented are mean values.

Table 6.2 Ratios of total carbohydrate or hexose concentration to the concentration of core oligosaccharide markers (heptose, KDO and hexosamine) of the LPS of *H.ducreyi*, *E. coli* 0111 : B4 and *S. typhimurium*.

Strain	CHO : KDO	Hexose : KDO	Hexose : heptose	Hexose : hexosamine
H. ducreyi				
A75	33.5	19.5	0.47	0.66
A76	23.5	17.7	0.64	0.64
A77	(11.76)	24.3	0.88	1.0
A76*	43.2	33.5	0.64	0.74
Y155	19.5	17.6	0.51	0.54
Y206	31.1	18.9	0.53	0.68
E. coli				
0111 : B4	47.3	23.74	0.59	2.0
S. typhimurium	52.2	56.2	1.1	9.8

6.4 DISCUSSION

6.4.1 POLYACRYLAMIDE GEL ELECTROPHORESIS

Since PAGE separates LPS molecules on the basis of their mass, LPS which consists of lipid A and the core oligosaccharide only, migrate faster than LPS molecules carrying additional carbohydrate O-side chains. Rough LPS thus appears as low molecular weight band/s in silver-stained gels. A ladder-like appearance of smooth LPS on similar gels represents collection of LPS molecules varying in length of O-side chains (Hitchcock *et al,* 1986). Goldman and Leive (1980) proposed that each band has one repeat unit more than the band beneath it. The absence of a fast-leading band indicates a complete substitution in the core by the O-antigenic polysaccharides (Krauss *et al,* 1988). The ladder-like pattern is evident in the LPS profile of *S. typhimurium* in Figure 6.1 lanes one and two. The LPS of *E. coli* 0111 : B4 is similar to that of *S. typhimurium,* but the bands present midway in the gel is lacking (Figure 6.1 lanes nine and ten).

On silver-stained polyacrylamide gels, smooth LPS has been isolated from the cell wall of *E. coli* (Goldman and Leive, 1980; Munford *et al,* 1980), *S. typhimurium* (Palva and Makela, 1980; Tsai and Frasch, 1982; Tolan *et al,* 1986), *Serratia marcescens* (Jessop and Lambert, 1986; Gaston *et al,* 1988), *Pseudomonas aeruginosa* (Chester and Meadow, 1973; Hancock *et al,* 1983; Riveria *et al,* 1988) and *Legionella pneumophila* (Barthe *et al,*, 1988).

Rough LPS has been reported in several other Gram-negative bacteria including *Haemophilus influenzae* type b (Inzana and Pichichero, 1983; 1984; Zwahlen *et al,* 1983; Gilsdorf and Ferrieri, 1986; Kimura and Hansen, 1986; Tolan *et al,* 1986; Zamze and Moxon, 1987), *Campylobacter jejuni* (Blaser *et al,* 1986), *Nesseria meningitidis* (Tsai and Frasch, 1982; Hitchcock *et al,* 1986), *Neisseria gonorrhoeae* (Guymon *et al,* 1982; Morse *et al,* 1983; Mintz *et al,* 1984; Tan *et al,* 1986), *Bordetella pertussis* (Tsai and Frasch, 1982;

Hitchcock *et al,* 1986) and *Yersinia pestis* (Darveau *et al,* 1983a). Interstrain heterogeneity, represented by changes in the mobility of the low molecular weight band appears to be common in these micro-organisms, thought to be due to changes in the carbohydrate content of their LPS (Zamze and Moxon, 1987). Previous studies on the LPS of *H. ducreyi* by this method also indicated that it is rough and varies from strain to strain (Abeck *et al,* 1987a; 1987c; Odumeru *et al,* 1987).

In this study, the presence of rough LPS in *H. ducreyi* was confirmed by a densely-staining fast migrating band. The two higher molecular weight bands have not been previously described. The LPS profiles of all the strains were similar, except for the low molecular weight band of Y155 (Figure 6.1) which migrated more slowly suggesting that interstrain variation may occur in clinical strains, although several more strains are required for testing to confirm the extent of this phenomenon.

In a review by Hitchcock *et al* (1986), the LPS of non-enteric bacteria was thought to be limited to the rough form, while smooth LPS was observed more frequently in enteric bacteria. There is however evidence contesting this trend. For example, in two independent studies, different clinical isolates of *P. aeruginosa* (Darveau and Hancock, 1983; Hancock *et al,* 1983) and *Campylobacter fetus* (Perez and Blaser, 1985b) expressed either smooth or rough LPS on polyacrylamide gel electrophoresis analysis. Neither pseudomonads nor campylobacters are classified into the family Enterobacteriaceae. Also, only rough LPS was isolated on polyacrylamide gels from the enteric bacteria, *Y. pestis* (Darveau *et al,* 1983a) and *Vibrio cholerae* (Hitchcock *et al,* 1986).

Interestingly, rough LPS of enteric bacteria always appear as single densely-staining bands, while rough LPS of non-enteric bacteria often appear as several closely spaced bands in the low molecular weight area, independently of the isolation method used. Between two and four bands are commonly found in these cases. The non-enteric group includes *P. aeruginosa* (Hancock *et al,* 1983); *C. jejuni* and *C. fetus* (Logan and Trust, 1982; Perez and

Blaser, 1985b; Blaser *et al,* 1986), *H. influenzae* (Inzana, 1983; Zwahlen *et al,* 1983; Kimura and Hansen, 1986; Zamze and Moxon, 1987), *N. gonorrhoeae* (Guymon *et al,* 1982; Morse *et al,* 1983; Mintz *et al,* 1984; Tan *et al,* 1986) and *H. ducreyi* (Odumeru *et al,* 1987).

A confusing artefact of rough LPS analysis by PAGE is thought to be due to LPS aggregation. Inzana (1983) observed up to nine faint bands extending half way up the gel on increasing the concentration of LPS isolated from *H. influenzae* loaded onto the gel. A similar pattern for *H. influenzae* was later reported by Zamze and Moxon (1987). A profile of twenty high molecular weight bands of aggregated LPS of *N. gonorrhoeae* was reduced to two to three bands after the LPS was treated with 0.1M sodium hydroxide, or 4M urea was incorporated into the gel. These chemicals disrupt hydrophobic interactions (Mintz *et al,* 1984). Since LPS patterns of *E. coli* and *Salmonella* did not correlate with peaks off filtration columns, Peterson and McGroarty (1985) proposed that SDS dissociated the LPS incompletely resulting in monomers and multimers stained on the polyacrylamide gels.

Although the LPS profile of *H. ducreyi* strains differs in this study from other investigations (Abeck *et al,* 1987a; 1987c; Odumeru *et al,* 1987) as a result of the two extra high molecular weight bands, it is likely that these additional bands are not comparable to the O-antigenic side chains of enteric bacteria, but may be artefacts of the constituents of R-type non-enteric LPS. Most probably they represent aggregates of rough LPS that migrate as high molecular weight bands.

This difference in the rough LPS profiles of enteric and non-enteric bacteria may indicate subtle differences in structure. The LPS of certain enteric bacteria have been well characterised, and they are commonly used as controls in experiments where LPS is analysed by polyacrylamide gel electrophoresis. However, it is important to realise that one may be comparing similar yet distinct molecular structures. For example, the core oligosaccharide of enteric bacteria maintains its integrity throughout the genus. This trend however cannot be assumed true of non-enteric bacteria. Using electroblotting techniques after PAGE, no

cross-reaction between the LPS of *C. fetus* and *C. jejuni* was observed, suggesting that campylobacter possesses species-specific but not genus-specific common core LPS structures (Perez *et al,* 1985a). Therefore, interpretation of LPS isolated from non-enteric bacteria on polyacrylamide gels should be approached with caution. Investigations comparable to that executed in distinguishing the components and structure of enteric LPS, are thus indicated for LPS of non-enteric origin.

Interpretation of LPS patterns on PAGE also depends on the culture conditions of a micro-organism which affects its LPS. The size of the band of *H. ducreyi* (Abeck *et al,* 1987a); or the intensity of LPS staining of *H. influenzae* (Inzana and Pichichero, 1984) and *S. typhimurium* rough mutants (Palva and Makela, 1980) changed with culture in different media. The addition of glucose to the culture medium of *P. aeruginosa* was associated with an alteration of its LPS profile on PAGE (Bailey and Apirion, 1977).

Electrophoretic mobility or intensity of LPS staining was also slightly altered by changes in the growth phase of *H. influenzae* (Inzana, 1983) and *S. typhimurium* (Palva and Makela, 1980). Changes in the incubation temperature of *P. aeruginosa* (Cadieux *et al,* 1983; Kropinski *et al,* 1987) and *Y. pestis* (Darveau *et al,* 1983a) affected the intensity of staining but not the distribution of bands.

As mentioned before, artefacts may arise as a result of methodologies employed. The method employed for the extraction of LPS is an important factor influencing PAGE profiles. This point is clearly illustrated in a study of the LPS of *C. fetus* and *C. jejuni* (Perez and Blaser, 1985b). Three methods of LPS extraction were compared. The first was the hot-phenol method (Westphal and Jann, 1965) which preferentially extracts smooth LPS. In constrast rough LPS is favoured by the petroleum ether-chloroform and phenol method (Galanos *et al,* 1969). Lastly the cold-ethanol method previously described by Darveau and Hancock (1983b) effectively extracts both smooth and rough LPS in high yields. Using PAGE analysis the cold-ethanol method extracted the smooth LPS of *C. fetus* more effi-

ciently than the hot-phenol method, while no higher molecular weight bands were observed when the petroleum ether-chloroform and phenol method was employed for the same strain. All three methods produced similar rough LPS profiles of LPS extracted from *C. jejuni* in the same study (Perez and Blaser, 1985b).

The quickest method of LPS isolation for PAGE analysis is digestion with Proteinase K. This method appears to be sensitive for both smooth and rough LPS (Hitchcock and Brown, 1983; Perez and Blaser, 1985b). The results produced by this technique are comparable to more conventional isolation methods i.e. the hot-phenol method (Hitchcock and Brown, 1983), the cold-ethanol method and the petroleum ether-choloroform and phenol method (Perez and Blaser, 1985b). The digestion method has been applied to several Gram-negative bacteria including *Salmonella* (Hitchcock and Brown, 1983a), *E. coli* (Goldman *et al,* 1984; Weiss *et al,* 1986), *C. fetus* and *C. jejuni* (Perez and Blaser, 1985b), *N. gonorrhoeae* (Tan *et al,* 1986; Palmero *et al,* 1987), *H. influenzae* (Kimura and Hansen, 1986) and *H. ducreyi* (Abeck *et al,* 1987a) with acceptable success.

A modification of the hot-phenol method was developed by Inzana (1983), which was less time consuming and more practical for PAGE analysis of LPS. This method has only been used for the study of LPS of *H. influenzae*, but has produced similar results to the Proteinase-K technique (Tolan *et al,* 1986), and the hot-phenol method (Zwahlen *et al,* 1983).

Different methods of LPS extraction have been used by various investigators, and a comparison of the results may be misleading. The ability to detect smooth LPS, or additional high molecular weight bands may thus be partially dependent on the method of LPS isolation.

The constituents of the polyacrylamide gel vary considerably from one investigator to the next, and have a minor effect on the stained LPS profiles. Differences between the LPS of

rough mutants of *Salmonella* were not detected using a 12 per cent polyacrylamide gel. However, a 15 per cent gel revealed the structural differences in the LPS of these mutants indicated by variations in the mobility of the silver-stained bands (Hitchcock and Brown, 1983a). In numerous studies, the percentage of polyacrylamide in a separating gel varies from 10 per cent (Kropinski *et al,* 1982) to 15 per cent (Progulske and Holt, 1984; Perez *et al,* 1985a; 1985b; Peterson *et al,* 1985; 1986; Odumeru *et al,* 1987; Zamze and Moxon, 1987; Riveria *et al,* 1988), to occasionally as high as 20 per cent (Guymon *et al,* 1982). Sometimes urea has been included in the gel (Tsai and Frasch, 1982; Inzana and Pichichero, 1984; Schiller *et al,* 1984; Jessop and Lambert, 1986; Tan *et al,* 1986; Tolan *et al,* 1986; Odumeru *et al,* 1987; Zamze and Moxon, 1987). If a stacking gel was present, the percentage polyacrylamide in it varied from 2.5 per cent (Tolan *et al,* 1986) to 8 per cent (Guymon *et al,* 1982). Gels of various gradients of polyacrylamide have also been employed in several studies (Morse *et al,* 1983; Goldman *et al,* 1984; Grossman and Leive, 1984; Mintz *et al,* 1984).

As previously mentioned, the inclusion of sodium hydroxide into the gel decreased the aggregation of LPS, and thus affected the LPS profile. In contrast EDTA had a negligible effect (Mintz *et al,* 1984; Perez and Blaser, 1985b).

Other steps which may lead to variations on PAGE of LPS are the type and the concentration of detergent used in the solubilising buffer. Concentrations of SDS varied from 1 per cent (Le Dur *et al,* 1980; Palva and Makela, 1980; Morse *et al,* 1983; Mintz *et al,* 1984) to 5 per cent (Perez and Blaser, 1985b). Sodium deoxycholate has also been substituted for SDS in PAGE analysis of LPS (Krauss *et al,* 1988). The duration of boiling the LPS sample in the solubilising buffer also varies from two to three minutes (Perez and Blaser, 1985b; Peterson and McGroarty, 1985) to ten minutes (Schiller *et al,* 1984), although five minutes seems to be the most common time period employed (Jann *et al,* 1975; Goldman and Leive, 1980; Le Dur *et al,* 1980; Palva and Makela, 1980; Tsai and Frasch, 1982; Darveau and Hancock, 1983b; Morse *et al,* 1983; Mintz *et al,* 1983; 1984; Progulske and Holt, 1984).

The concentration of LPS loaded onto the gel appears to play a significant role in the outcome of PAGE analysis of LPS. Generally, smaller amounts of LPS are required to detect rough LPS than smooth LPS, although this does not appear to dictate the amount loaded onto the gel. The concentration of LPS per lane in a gel varies from 50ng (Hancock *et al,* 1983) to 150µg (Cadieux *et al,* 1983). By increasing the concentration of LPS from 5µg to 40µg, additional multiple bands of *S. typhimurium* LPS were observed (Darveau and Hancock, 1983b). Similarly, when Inzana (1983) loaded 1µg to 3µg LPS of *H. influenzae*, three distinct but closely spaced bands were seen. However, on loading 10µg, the three separate low molecular weight bands were indistinguishable, and up to nine faint bands extending half way up the gel were detected.

In conjunction with the concentration of LPS loaded onto the gel, the sensitivity of the staining technique used may also affect the LPS of profile on PAGE. The silver stain (Tsai and Frasch, 1982) appears to be the most sensitive stain available, equivalent to autoradiographic studies. In comparison with the periodic acid-Schiff stain (DiRenzo *et al,* 1978), it detects 5ng of LPS compared with 5µg LPS; and requires two hours as opposed to twenty-four hours for completion (Guymon *et al,* 1982). The exact chemistry of the silver stain is unknown, but is thought to reflect the high affinity of the aldehyde groups in periodate-oxidised LPS for silver (Hitchcock and Brown, 1983). The intensity of the silver stain may not represent the amount of LPS present, but may only indicate a difference in the concentration of carbohydrate in the different LPS samples (Mintz *et al,* 1984). An increase in the developing time of the silver stain increased the visibility of additional LPS bands of *C. fetus* (Perez and Blaser, 1985b) and *S. typhimurium* (Palva and Makela, 1980).

A modification of the Commassie-blue protein stain, also detects LPS. However, this stain appears specific for smooth LPS of *P. aeruginosa*, since it did not stain rough LPS of *P. aeruginosa*, and smooth and rough LPS of enteric origin (Kropinski *et al,* 1982; Cadieux *et*

al, 1983). Thus, although control LPS of non-pseudomonad origin is suitable for such experiments, the technique for staining the LPS may lead to partial detection.

6.4.2 SENSITIVITY OF POLYACRYLAMIDE GEL ELECTROPHORESIS

Although there are several factors which affect LPS profiles on polyacrylamide gels, the technique appears to be sensitive for comparative LPS analysis. Differences in the biochemistry of the LPS isolated from LPS mutants of both enteric (Hitchcock and Brown, 1983) and non-enteric (Guymon *et al,* 1982) origins have been correlated with changes in LPS profiles on PAGE. Goldman and Leive (1980) also reported that the LPS profiles of *E. coli* and *S. typhimurium* corresponded with the peaks eluted from a Sephadex column when isolated LPS was filtered through it. The use of PAGE was recommended for the study of LPS biosynthesis in *E. coli* (Bailey and Apirion, 1977).

In a previous study, LPS isolated from *H. ducreyi*, which had reduced mobility on PAGE, also contained slightly more carbohydrate, and a higher CHO : KDO ratio. This suggests that the electrophoretic mobilities of the LPS of *H. ducreyi* strains may reflect differences in the amount of carbohydrate associated with the core oligosaccharide (Odumeru *et al,* 1987).

In this study, differences in the biochemical composition of the LPS of *H. ducreyi* strains (Table 6.1) did not manifest in altered LPS profiles on PAGE (Figure 6.1). All the strains produced similar LPS profiles. Only the clinical strain Y155 possessed a visibly slower-migrating band. However, this strain did not possess more carbohydrate or a higher CHO : KDO ratio than the other strains which were tested. The LPS of Y155 did nevertheless have the most heptose, KDO and especially hexosamine, relative to the other strains. These three components are unique to the core oligosaccharide of enteric bacteria. Thus the difference

in mobility of Y155 may be due to a larger core oligosaccharide, rather than an increase in the total carbohydrate moeity. Also, the isogenic strain A76*, which was passaged in human serum, possessed higher concentrations of all the LPS components except for the KDO component compared with A76, but its LPS profile was identical to its parent strain A76, on PAGE. PAGE did not detect small biochemical variations in the LPS of *H. ducreyi* in this study.

The LPS profiles of *E. coli* and *S. typhimurium* were typically smooth, and this characteristic was supported by the high concentration of all the LPS constituents tested.

Generally, PAGE is sensitive enough to detect differences between rough and smooth LPS, and is capable of detecting the differences in length and the distribution of LPS molecules carrying the O antigen. However, the results of biochemical analysis may be deceptive and lead to an apparent lack of correlation with PAGE results. For example, strains of *C. jejuni* and *Campylobacter coli* varying in their CHO : KDO ratios, which suggests changes in the length of the O antigen, produced uniform rough-type LPS profiles (Blaser *et al,* 1986).

A high CHO : KDO ratio is indicative of the presence of the O antigen or smooth LPS. However, this ratio does not indicate whether the majority of the core oligosaccharides are substituted with only long 0-side chains (*E. coli* 0111 : B4), or only short O-side chains, or both (*S. typhimurium*). Furthermore, a high concentration of glucose or galactose in the core oligosaccharide of rough Gram-negative bacteria, relative to the KDO component may contribute to a high CHO : KDO ratio. All the abovementioned forms of LPS may produce the same CHO : KDO ratio. The relevance of this ratio will be discussed later.

Similarly, a high carbohydrate content per mg LPS may also be misleading.

6.4.3 BIOCHEMICAL ANALYSIS

LPS, a large class of macromolecules, is a constitutive part of the cell wall of Gram-negative bacteria. Each molecule consists of lipid A and core oligosaccharide; an O-antigenic polysaccharide may or may not be present. The many serotypes amongst different species of Gram-negative bacteria are a result of varying specific LPS molecules. Certain LPS components are species dependent, while others are more gregarious, influenced by external factors such as cultural conditions (Chester and Meadow, 1975; Chatterjee *et al,* 1976; Weiss *et al,* 1986).

The LPS of the Enterobacteriaceae, and especially *Salmonella* have been most intensively investigated probably due to the existence of a well-defined scheme for serological O-antigen classification (Ashwell and Hickman, 1971). The internal core region is consistently identical within the *Salmonella* genus, and is closely related in enteric bacteria (Holme *et al,* 1968; Luderitz *et al,* 1971; Rosner *et al,* 1979). The *Salmonella* core region consists of two parts. The inner backbone, through which the polysaccharide is linked to lipid A, is made up of heptose, KDO, phosphate and ethanolamine. To this backbone are attached short oligosaccharide chains, the outer core, which contains glucose, galactose and glucosamine. The detection of additional carbohydrates are indicative of the presence of the O antigen.

Heptose was isolated from the LPS of seven species of Gram-negative bacteria, including Enterobacteriacae, Pseudomonadaceae and Neisseriaceae (Adams, 1967). In another study, Ellwood (1970) detected KDO in the LPS of sixty-six of seventy-three Gram-negative bacteria from a broad range of genera. This suggests that the core oligosaccharides of most Gram-negative bacteria are similiar in composition.

Studies of the LPS of less well-known enteric bacteria and non-enteric bacteria have supported the suspicion that the enteric core oligosaccharide components are common. The

occurrence of one or more of these recognizable components is often used as a marker to indicate the amount of LPS present (Chester and Meadow, 1975; Goldman *et al,* 1984).

The biochemistry of the LPS of a wide range of Gram-negative bacteria have been investigated, including *N. gonorrhoeae* (Stead *et al,* 1975; Wiseman and Caird, 1977; Guymon *et al,* 1982; Tan *et al,* 1986), *S. marcescens* (Tsang *et al,* 1974; Jessop and Lambert, 1986), *P. aeruginosa* (Clarke *et al,* 1967; Fcmson and Meadow, 1970; Chester and Meadow, 1975; Wilkinson and Galbraith, 1975; Kropinski *et al,* 1982), *H. influenzae* (Inzana and Pichichero, 1985; Zamze and Moxon, 1987), *Campylobacter* species (Naess and Hofstad, 1984; Perez and Blaser, 1985b; Blaser *et al,* 1986), *Y. pestis* (Hartley *et al,* 1974; Darveau *et al,* 1983a), *Klebsiella pneumoniae* (Cuirana and Tomas, 1987), *V. cholerae* (Kabir, 1982) and *Bacteriodes fragilis* (Kasper, 1976).

In this study, and in a previous study (Odumeru *et al,* 1987), the LPS of *H. ducreyi* was found to contain hexose, hexosamine, KDO and heptose; all typical of the LPS of Gram-negative bacteria. Heptose and hexosamine were the main constituents, present in comparable concentrations. KDO was present in minute amounts. Odumeru *et al* (1987) also reported small concentrations of KDO. However, in their study the hexose concentration as determined by the anthrone-sulphuric acid method (Scott and Melvin, 1953), and the total CHO concentration assayed by the phenol-sulphuric acid method (Dubois *et al,* 1956) was higher than the heptose and hexosamine concentration. The results of the biochemical analysis of the LPS of *H. ducreyi* in this study differs quantitatively from that reported by Odumeru *et al* (1987).

Quantitative differences in LPS analysis may occur for several reasons as discussed in this chapter, making comparisons between different investigations difficult and inaccurate.

The cultural conditions of a micro-organism affects its LPS structure. The growth rate of *Salmonella enteriditis* was manipulated by changing the dilution of the energy source. The

fastest-growing cells underwent a partial smooth-to-rough form conversion, which could be reversed if the growth rate was reversed (Collins, 1964). Conversely Morse *et al* (1983) found that gonococcal LPS isolated from rapidly-growing cells contained more O antigen i.e. a three- to four-fold increase in total carbohydrate, than LPS from slower-growing cells at a lower dilution. A similar observation was made with *Neisseria sicca* (McDonald and Adams, 1971).

The growth phase of *H. ducreyi* in Odumeru's study (Odumeru *et al,* 1987) is uncertain. They claim stationery phase cells were used for the analysis of LPS; however the culture plates were only incubated for twenty-four hours. This is unlikely since colonies of *H. ducreyi* are only visible after at least a forty-eight hour incubation period. In this study, *H. ducreyi* was incubated until sufficient growth for harvesting visible colonies was obtained. Reference strains required a longer incubation time, since they grew slower than the clinical strains.

The composition of the culture medium would obviously affect the biosynthesis of LPS. *P. aeruginosa* grown in minimal medium with succinate, instead of in nutrient broth, was deficient in heptose, glucose, alanine and galactosamine (Chester and Meadow, 1975).

The medium used by Odumeru (Odumeru *et al,* 1987) was similar to that used in this study, differing only in the agar base and the absence of vancomycin. The effect of vancomycin on the cell surface is unknown, although it is unlikely that it would have a significant effect on the LPS components.

A contributing variable in the analysis of LPS is the appearance of artefacts as a result of different LPS extraction methods. This has been previously discussed with regard to PAGE analysis. The same principles can be applied to the biochemical analysis of LPS (Ghalambor *et al,* 1966; Morrison and Leive, 1974). The commonly used phenol-water method (Westphal and Jann, 1965), which preferentially extracts smooth LPS, incompletely extracted

the LPS from the cell wall of *Salmonella minnesota* (Kasai and Notwotny, 1967) and *Citrobacter freundii* (Raff and Wheat, 1968). The cold-ethanol method was used in this investigation, since it extracts both smooth and rough LPS without bias (Darveau and Hancock, 1983b).

Quantitative analysis of LPS depends largely on the method used for measuring the different LPS components. For example, Brade *et al* (1983) reported that the KDO released from the LPS of *S. minnesota* was dependent on the concentration of acid present during hydrolysis. Over a sixty minute period of hydrolysis 4M hydrochloric acid, 1M hydrochloric acid, 10mM sulphuric acid and 0.1M acetate buffer, released 90 per cent, 55 per cent, 60 percent and 15 per cent of the KDO, respectively. This was confirmed on the LPS of *H. ducreyi* (Odumeru *et al,* 1987). 4M hydrochloric acid (Brade *et al,* 1983) released four-fold more KDO than 0.2N sulphuric acid (Karkanis *et al,* 1978). The use of a weak acid (0.1M acetate buffer) for the hydrolysis of KDO in this study is the most likely reason why such minute quantities of KDO were detected.

Interestingly, the commonly used thiobarbituric acid assay (Karkanis *et al,* 1978; Brade *et al,* 1983) was not suitable for the detection of KDO in the LPS of *H. influenzae*, no matter what strength of acid was used for hydrolysis. An alternative method, the semicarbizide assay, has been employed (Inzana *et al,* 1985).

Another example which further illustrates the importance of the method used, is the biochemical breakdown of the LPS of *E. coli* 0111 : B4. Several investigators have employed this strain as a control. However the results produced in independent studies are not comparable. Colitose was the largest constituent of this LPS in one study (Leive and Shovlin, 1968), but not in another study (Morrison and Leive, 1974). The KDO fraction was larger than the heptose fraction in two investigations (Ribi *et al,* 1966; Morrison and Leive, 1974), but the reverse was reported by Leive and Shovlin (1968).

Clearly, the biochemical analysis of the LPS of a single strain from different investigations is inconsistent due to the influence of many varying extraneous factors.

A lack of knowledge of the sensitivities of different tests has lead to some confusion. The phenol-sulphuric acid method (Dubois *et al,* 1956) employed in this study measured the 'total CHO concentration'. However, this method was also used for the LPS of *S. marcescens* (Jessop and Lambert, 1986), but measured 'hexose concentration'; and for the LPS of *Nesseria* species (Johnson *et al,* 1975) and *V. cholerae* (Kabir, 1982), but measured 'glucose concentration'. This is only acceptable if the only carbohydrate present in these cases, are 'hexose' or 'glucose'. Similarly, 'hexose concentration' was evaluated by the anthrone method (Scott and Melvin, 1953). This method was also used by Wiseman and Caird (1976) in the determination of 'total CHO concentration' in the LPS of *N. gonorrhoeae.*

A comparison of the results where different methods of measuring the same carbohydrate/s were employed should be undertaken with caution. In the LPS of *H. ducreyi* (Odumeru *et al,* 1987), the 'hexose' (Scott and Melvin, 1953) and 'total CHO' concentration (Dubois *et al,* 1956) was 20.3 per cent and 25.6 per cent dry weight of LPS respectively, compared with a 'hexosamine' concentration (Smith and Gilkerson, 1979) of 7.3 per cent dry weight LPS. However, using gas liquid chromatography, the total glycose (glucose, galactose, fucose) concentration was 0.75 nmol/µg LPS; the total hexosamine (glucosamine, galactosamine) concentration was 0.76 nmol/µg LPS. Therefore using colorimetric biochemical assays, the hexosamine content of the LPS of *H. ducreyi* was significantly different from the hexose and total carbohydrate content. In contrast, gas liquid chromatography detected almost equivalent concentrations of these LPS components.

It appears that the most far-reaching supposition made in the analysis of LPS is that all lipopolysaccharides have a similar structure to that of the familiar enteric bacteria. As mentioned previously, the common core-oligosaccharide sugars are often used as markers of the

amount of LPS present. This assumption is unreliable when unusual lipopolysaccharides are present. No KDO was detected in the LPS of *Bordetella* species (Ellwood, 1970), *B. fragilis* (Kasper, 1976), *V. cholerae* (Kabir, 1982), and *Leptospira interrogans* serovar *copenhageni* (Vinh *et al,* 1986). Heptose was absent in the LPS of *B. fragilis* (Kasper, 1976), *Neisseria sicca* (Limjuco *et al,* 1978), *Agrobacterium tumifaciens* (Salkinoja-Salonen and Boeck, 1978) and *Acinetobacter calcoaceticus* (Brade and Galanos, 1982).

Techniques for the analysis of LPS may detect all the carbohydrate components in the LPS of enteric bacteria, but fail to identify those in the LPS of less commonly studied bacteria.

With these considerations in mind, it is not surprising that the significance of the CHO : KDO (O-antigen sugar : core region sugar) is questioned. Generally the larger the ratio, the longer the O-antigenic chain, and vice versa. This is only correct if the KDO component of the core oligosaccharide is relatively constant from strain to strain. The same is true for other core components such as heptose.

In addition, the amounts of these KDO or heptose are also affected by the growth conditions, independently of the bacterial species (Ellwood, 1970, Chester and Meadow, 1975). Core oligosaccharide heterogeneity is indicated by the occurrence of different serological specificities of rough strains of *N. gonorrhoeae* (Morse and Apicella, 1982; Mintz *et al,* 1984; Palmero *et al,* 1987) and *H. influenzae* (Zamze and Moxon, 1987).

Although glucose, galactose and glucosamine are also found in the core region of the LPS of *S. typhimurium*, they are never used as specific core region indicators, since they may also occur in the O antigen of the same LPS molecule.

Since an unexpectedly low concentration of 'total CHO' was obtained for A77, the quantity of hexose was the best indicator of the amount of O polysaccharide present. From Table 6.2, it is evident that the use of the KDO or the heptose moeity could significantly alter the in-

terpretation of the ratios calculated. From the hexose : KDO ratio calculated for A76 and its isogenic strain A76*, at first glance it would appear that the latter strain possessed LPS with an extended O-antigen; while interpretation of the ratio hexose : heptose for the same strains gives no indication of this.

Another misconception interposed by the CHO : KDO ratio, is that it lacks the ability to demonstrate the distribution of the carbohydrate units relative to the KDO elements. In this study, the LPS of *E. coli* 0111 : B4 consisted of low and high molecular weight bands on PAGE; intermediate bands were absent. This ladder-like pattern is characteristic of smooth LPS. The ratio of hexose : KDO or hexose : heptose for this LPS sample in Table 6.2 is in accordance with this concept.

Thus the interpretation of the results obtained in the analysis of LPS of any micro-organism is complicated. Several factors require concurrent consideration.

6.5 CONCLUSION

Proteinase-K digests of LPS isolated from three reference and two clinical strains of *H. ducreyi*, appeared as low molecular weight bands representing rough LPS. Two additional higher molecular weight bands thought to be LPS aggregates were observed. Commercially prepared LPS from *E. coli* and *S. typhimurium* appeared as ladder-like patterns, typical of smooth LPS.

Biochemical analysis of purified LPS from these *H. ducreyi* strains indicated the presence of constituents commonly found in other Gram-negative bacteria i.e. hexose, heptose, hexosamine and KDO.

The induction to serum resistance of A76 was associated with an increase in carbohydrate constituents per mg (dry weight) LPS, except for the KDO constituent. However, the ratios of total sugar to core sugar did not increase significantly. This suggests that there was an increase in core-oligosaccharide constituents, rather than an increase in O-antigen units.

The sensitivity and suitability of PAGE and the biochemical assays used for the analysis of LPS was discussed, including the possible artefacts which could result.

A comparison of the LPS profiles and composition of the serum-sensitive reference strains with that of the serum-resistant clinical strains of *H. ducreyi* will be addressed in the following chapter.

THE ROLE OF LIPOPOLYSACCHARIDE IN SERUM RESISTANCE OF HAEMOPHILUS DUCREYI

7.1 INTRODUCTION

The development of skin lesions following intradermal inoculation in rabbits (Reymann, 1949; Deacon *et al,* 1954; 1956; Hammond *et al,* 1978b; Odumeru *et al,* 1984) or rats (Tuffrey *et al,* 1988) is the most commonly used indicator of virulence of *Haemophilus ducreyi,* the aetiological agent of chancroid. However the factors contributing to the pathogenicity of this micro-organism are poorly understood. The two abovementioned animal models are not completely suitable in the determination of serum resistance of *H. ducreyi,* since they differ from human chancroid by healing spontaneously after two to three weeks (Dienst, 1948; Reymann, 1949; Deacon *et al,* 1954; 1956; Tuffrey *et al,* 1988). Furthermore, lipopolysaccharide (LPS) isolated from *Neisseria gonorrhoeae* resulted in a virulent-type lesion in rat inoculations, suggesting that the reaction was non-specific (R. Ballard, personal communication).

Several aspects of *H. ducreyi* which may be partly responsible for its virulence include lectin agglutination (Korting *et al,* 1988), antigenicity (Hansen and Loftus, 1984; Slootmans *et al,* 1985; Saunders and Folds, 1986; Schalla *et al,* 1986), antibiotic-resistance mechanisms (MacLean *et al,* 1980; Handsfield *et al,* 1981; Brunton *et al,* 1982; Deneer *et al,* 1982; Totten *et al,* 1982; Anderson *et al,* 1984; Albritton *et al,* 1982; 1984), including polymyxin resistance (Hammond *et al,* 1978b; Odumeru *et al,* 1984) and cell wall structure (Taylor *et al,* 1985; Odumeru *et al,* 1983; 1987; Abeck *et al,* 1987a; 1987b).

Since the ability to resist serum killing is a frequent characteristic of clinical isolates of several Gram-negative bacteria (Roantree and Rantz, 1960; Nelson and Roantree 1967; Simberkoff *et al,* 1976; McCabe *et al,* 1978; Pai and DeStephano, 1982; Sutton *et al,* 1982), especially those causing systemic infections (Kimball *et al,* 1964; Elgefors and Olling, 1978; McCabe *et al,* 1978; Clumeck *et al,* 1982; Blaser *et al,* 1985; Ross *et al,* 1985), it has been implicated as an important determinant of virulence in certain infections. However, some investigators also report that invasive Gram-negative strains possessing other virulence markers remain serum sensitive (DeMatteo *et al,* 1981; Sutton *et al,* 1982; Chiesa and Bottone, 1983).

The bacterial component associated with serum resistance in Gram-negative bacteria appears to be LPS in enteric bacteria (Rowley and Wardlaw, 1958; 1968; Wardlaw, 1963; Feingold, 1969; Taylor and Robinson, 1980; Joiner *et al,* 1982a; 1982b; Porat *et al,* 1987), *Pseudomonas aeruginosa* (Guymon *et al,* 1982; Schiller *et al,* 1984), *Campylobacter jejuni* (Blaser *et al,* 1986) and *N. gonorrhoeae* (Morse *et al,* 1983; Shafer *et al,* 1984). LPS consists of three parts : lipid A, core polysaccharide and O-antigen side chains. The last component is absent from rough-type LPS, but present in smooth-type LPS. Certain outer membrane proteins (OMP) of *N. gonorrhoeae* (Hildebrandt *et al,* 1978; Cannon *et al,* 1981), *Campylobacter fetus* (Blaser *et al,* 1987; 1988) and *Escherichia coli* (Taylor and Parton, 1977); and the capsular polysaccharide of *E. coli* (Howard and Glynn, 1971; Taylor and Robinson, 1980; Leying *et al,* 1990) and *'Klebsiella aerogenes'* (Allen *et al,* 1987) are also involved in the determination of serum resistance. Obviously the mechanisms of serum resistance for certain species of bacteria are not clearly defined, and results of independent investigations are often inconsistent.

In a previous study, the ability of *H. ducreyi* strains to resist complement-mediated killing and killing by polymorphonuclear leucocytes was associated with virulence, as determined by intradermal rabbit inoculation studies (Odumeru *et al,* 1984). The cell wall component involved appeared to be LPS, since isolated LPS from serum-sensitive strains but not serum-resistant strains inhibited the bactericidal activity of pooled normal human serum

(PNHS) (Odumeru *et al,* 1985). Furthermore, biochemical differences in the LPS of serum-resistant virulent and serum-sensitive avirulent strains were reported.

In this study, the LPS profiles on polyacrylamide gel electrophoresis (PAGE), and the biochemical indicators of the LPS of *H. ducreyi* strains varying in serum sensitivity were compared. The experimental results documented in this chapter is the same as that reported in chapter 6.

7.2 MATERIALS AND METHODS

7.2.1 BACTERIAL STRAINS, MEDIA AND INCUBATION CONDITIONS

A75, A76 and A77 are three reference strains of *H. ducreyi* (Pasteur Institute, France). Strain A76* is genetically similar to its parent strain A76, but is relatively more resisant to killing by PNHS. It was obtained by passaging A76 through 50 per cent PNHS several times. Local clinical isolates employed are Y155 and Y206 (Carletonville, South Africa).

All strains were cultured on media consisting of brain heart infusion (BHI) agar (Difco), 1 per cent haemoglobin (Difco), 1 per cent IsoVitaleX (BBL, Microbiological Systems) and 3 mg/l vancomycin at (APPENDIX 1) 33^0 C in candle jars.

7.2.2 SERUM BACTERICIDAL ASSAY

The concentration of an overnight broth culture was adjusted to approximately 10^6 colony forming units (CFU)/ml in BHI broth. 200µl of this suspension was added to 1000µl PNHS and 800µl BHI broth, and incubated at 36^0 C. At time intervals zero, thirty, sixty and 120 minutes, a viable count was performed using the Miles and Misra (1938) technique. Control

experiments included the use of inactivated PNHS (heating at 56^0 C for thirty minutes), and BHI broth (no PNHS) instead of 50 per cent PNHS. The experiment was repeated at least four times, and the results are expressed as a mean percentage of the bacterial inoculum size at time zero.

7.2.3 POLYACRYLAMIDE GEL ELECTROPHORESIS

Two loopfuls of agar-grown bacteria were suspended in 100µl of solubilising buffer (Abeck *et al,* 1987b), boiled for five minutes and treated with Proteinase K (Boehringer Mannheim). 70µl to 80µl of the sample were analysed on a 15 per cent resolving gel (APPENDIX 3), and silver stained using the method described by Tsai and Frasch (1982) (APPENDIX 4).

7.2.4 BIOCHEMICAL ANALYSIS OF LIPOPOLYSACCHARIDE

The cold-ethanol method (Darveau and Hancock, 1983b) was used to isolate the LPS from the *H. ducreyi* strains. Lyophilised cells of *H. ducreyi* were sonicated in Tris buffer. Nucleic acids were removed by treatment with DNase and RNase. Exposure of the mixture to ethylene diamine tetraacetic acid (EDTA) and sodium dodecyl sulphate (SDS) followed by ultracentrifugation removed peptidoglycan. Precipitation of the LPS by a magnesium chloride-ethanol solution at 0^0 C followed pronase digestion at 37^0 C. The crude LPS extract was treated again with EDTA, SDS, pronase and cold magnesium chloride-ethanol as mentioned above. The final LPS preparation was washed in Tris buffer and concentrated by ultracentrifugation. Further details of this method are given in APPENDIX 5.

The biochemical composition of *H. ducreyi* LPS was determined using colorimetric assays (Leitz photometer, USA).

The total carbohydrate (CHO) and the hexose content were determined by the phenol-sulphuric acid method (Dubois *et al,* 1956, APPENDIX 6) and the anthrone-sulphuric acid method (Scott and Melvin, 1953, APPENDIX 7), respectively. Cysteine hydrochloride was added to the LPS samples in sulphuric acid for the determination of heptose (Wright and Rebers 72, APPENDIX 8). After hydrolysis of the LPS by boiling in acid, the concentration of hexosamine and KDO were also determined. The colour changes produced by the reaction of hexosamine with 3-methyl-2-benzo-thiazolinone hydrazone hydrochloride (Smith and Gilkerson 79, APPENDIX 9), while thiobarbituric acid detected KDO (Brade *et al,* 1983, APPENDIX 10).

Commercial preparations of glucose, glucoheptose, glucosamine and KDO were obtained from Sigma and used as standards for the abovementioned assays.

7.3 RESULTS

7.3.1 SERUM RESISTANCE

Table 7.1 Differences in serum susceptibility of reference strains A75, A76 and A77 compared to the clinical strains Y155 and Y206 or the isogenic strain A76*.

	Serum-sensitive strains			Serum-resistant strains		
	A75	A76	A77	A76*	Y155	Y206
60 mins.	0.01	0.3	6	24.4	29	61
120 mins.	0	0.1	1.5	1.7	23	68

Values are expressed as a percentage of the initial bacterial inoculum size after 60 minutes and 120 minutes exposure to 50% PNHS at 37^0 C.

In the absence of serum, there was a slight, insignificant loss of viability. In contrast, a small increase in the viable count was observed when the strains were incubated with inactivated PNHS.

The clinical strains Y155 and Y206 were significantly more serum resistant than the reference strains A75, A76 and A77 after 120 minutes incubation at 36^0 C in 50 per cent PNHS ($p < 0.05$, Duncan t-test). The isogenic strain A76* was more serum resistant than its parental strain A76. This was more evident after sixty minutes incubation, rather than after 120 minutes incubation in 50 per cent PNHS.

7.3.2 GEL ELECTROPHORESIS OF LIPOPOLYSACCHARIDE

The Proteinase-K digests of all the strains of *H. ducreyi* appeared as a large low molecular weight band with the same migration rate except for the LPS of clinical strain Y155, which migrated more slowly than the other samples (Figure 7.1 lane five). This pattern is indicative of rough-type LPS. In addition two minor higher molecular weight bands were observed in all the samples. These bands were thought to be a result of LPS aggregation. The LPS profiles of A76 and A76* were similar, even though the strains differed in their susceptibility to PNHS (Figure 7.1 lanes two and four).

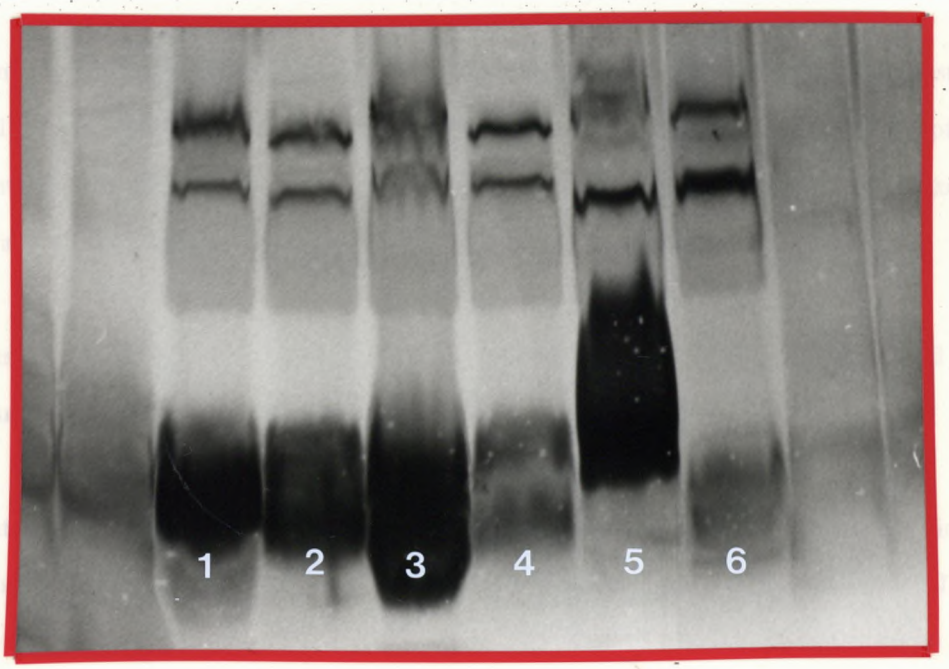


Figure 7.1 LPS profiles of serum-sensitive *H. ducreyi* strains on PAGE - A75, A76 and A77 in lanes 1, 2 and 3, respectively; LPS of serum-resistant stains Y155 and Y206 in lanes 5 and 6, respectively. Lane 4 is the LPS of isogenic strain A76*.

7.3.3 BIOCHEMICAL ANALYSIS

Carbohydrates detected in the LPS of *H. ducreyi* were typical of those found in the LPS of other Gram-negative bacteria.

Of the LPS constituents assayed, the serum-sensitive strains were not qualitatively different from the serum-resistant strains (Table 7.2).

A comparison of the three serum-sensitive reference strains with the two serum-resistant clinical strains revealed no quantitative difference in the total carbohydrate and heptose concentration, although the serum-sensitive strain appeared to have slightly less hexose, KDO and hexosamine per mg LPS than the serum-resistant strains.

In constrast, the LPS of the isogenic serum-resistant strain A76* contained more of all the LPS-sugar constituents assayed, except for KDO.

The ratio of an O-polysaccharide sugar to a core-oligosaccharide sugar is thought to indicate the length of the polysaccharide region of the LPS molecule. The ratios hexose : KDO, hexose : heptose and hexose : hexosamine of the LPS of the reference strains were not significantly different from those of the clinical strains. However, the ratios hexose : KDO and hexose : hexosamine of A76*'s LPS were greater than those of its serum-sensitive parental strain.

Table 7.2 Biochemical analysis of the LPS of *H. ducreyi*. Values are expressed as µg / mg LPS dry weight.

		Total	Total CHO	Hexose	Heptose	KDO	Hexosamine
Serum-sensitive strains	A75	95.5	25.6	14.9	31.5	0.765	22.7
	A76	78.6	19.3	14.5	21.5	0.82	22.5
	A77	65.8	8.7	18.0	20.5	0.74	17.9
Serum-resistant strains	A76*	99.0	24.6	19.1	29.7	0.57	25.7
	Y155	95.0	18.0	16.3	32.0	0.925	28.7
	Y206	95.6	26.1	15.9	30.1	0.84	23.4

Table 7.3 Ratios of hexose to core-oligosaccharide sugars in the LPS of serum-sensitive and serum-resistant strains of *H. ducreyi*.

		Hexose: KDO	Hexose: heptose	Hexose: hexosamine
Serum-sensitive strains	A75	19.5	0.47	0.66
	A76	17.7	0.64	0.64
	A77	24.3	0.88	1.0
Serum-resistant strains	A76*	33.5	0.64	0.74
	Y155	17.6	0.51	0.57
	Y206	18.9	0.53	0.68

7.4 DISCUSSION

There is convincing evidence that serum resistance of a bacterium is determined by its cell wall composition viz. LPS, outer membrane proteins and capsular polysaccharide. Amongst the Gram-negative bacteria, the mechanisms of serum resistance in only a selected few species have been thoroughly investigated.

Pioneer research centred on the enteric bacteria (Wardlaw, 1963; Rowley and Wardlaw, 1968; Feingold, 1969; Taylor and Robinson, 1980; Joiner *et al,* 1982a; 1982b; 1986; Porat *et al,* 1987) particularly *E. coli* and *Salmonella minnesota.* Following C′ activation by both serum-resistant and serum-sensitive strains on exposure to PNHS the entire complement (C′) cascade is completed. The C′ component responsible for the membrane attack complex (MAC) insertion into the cell membrane is C3, and C3b attaches to the longest O-side chain of the LPS. When the C3b component is attached too far from the main part of the cell

membrane as a result of long O-polysaccharide chains of the LPS (smooth-type LPS), the resulting cell-bound MAC is unstable and is gradually lost from the cell surface. Serum resistance is thus determined by a high degree of substitution of the LPS core oligosaccharides by long O-side chains, or smooth LPS (Joiner *et al,* 1982a; 1982b; 1986).

LPS has been implicated as the serum resistance determinant of other bacteria including *P. aeruginosa* (Schiller and Joiner, 1986), *Serratia marcescens* (Jessop and Lambert, 1986) and *C. fetus* (Perez *et al,* 1985a). However difficulty in the determination of the events leading to serum resistance reflects the lack of knowledge about the detailed structure of LPS in species other than *E. coli* and *Salmonella.*

A different mechanism of serum resistance is described for *N. gonorrhoeae,* since its LPS is typically rough (Johnson *et al,* 1975; Stead *et al,* 1975; Inzana, 1983; Mintz *et al,* 1984).

As observed in enteric bacteria, C′ is activated and deposited on both serum-resistant and serum-sensitive strains of *N. gonorrhoeae.* The molecular configuration of the C5b-C9 complex, or the cellular proteins with which the MAC is associated varies with serum susceptibility (Joiner *et al,* 1983a). The relevant OMP is thought to be protein I (Hildebrandt *et al,* 1978). Further studies indicate that a blocking antibody also contributes to gonococcal serum resistance. This antibody is directed against another OMP, protein III, which is located near protein I, the site of attachment for the antigen-antibody complex. The blocking antibody competes with the bactericidal antibody for binding to the micro-organism, thus conferring serum resistance on *N. gonorrhoeae* (Rice *et al,* 1982; 1986; Joiner *et al,* 1985c).

Even though these theories of serum resistance of *E. coli, Salmonella* and *N. gonorrhoeae* have been demonstrated and are well accepted (Joiner *et al,* 1986; Rice *et al,* 1986; Frank *et al,* 1987), other investigations have not confirmed them. Variations in serum susceptibility of *E. coli* occurred independently of LPS changes (Taylor and Robinson, 1975; 1976; 1980), and also in association with the capsular polysaccharide (Howard and Glynn, 1971) and an

OMP (Taylor and Parton, 1977). Similarly alterations in the LPS antigen of *N. gonorrhoeae* were related to changes in its susceptibility to serum (Morse *et al,* 1983; Shafer *et al,* 1984). Serum resistance determinants of a particular micro-organism are probably multi-factorial.

In other studies, serum-resistant strains of *P. aeruginosa* (Guymon *et al,* 1982; Schiller *et al,* 1984), *H. ducreyi* (Odumeru *et al,* 1987), *S. marcescens* (Jessop and Lambert, 1986), *C. fetus* (Perez *et al,* 1985a) and *C. jejuni* (Blaser *et al,* 1986) possessed more LPS than the serum-sensitive strains. Although the mechanism of serum resistance of *P. aeruginosa* appears to be similar to that proposed by Joiner *et al* (1986), i.e. failure of the MAC to stably insert into the outer membrane confers serum resistance on the bacterium, it would be misleading if one assumed that this mechanism is always applicable when serum resistance is associated with a change in LPS. The validity of the abovementioned assumption will be discussed later. Similarly, a large surface protein of *C. fetus* contributed to its ability to resist serum killing, but the mechanism of this property was due to a deficiency in C3 binding (Blaser *et al,* 1988), unlike that of *N. gonorrhoeae.*

The way enteric bacteria avoid killing by serum (Joiner *et al,* 1982a; 1982b; 1986), cannot be applied to Gram-negative bacteria that only possess rough LPS including *H. influenzae* (Zamze and Moxon, 1987), *H. ducreyi* (Abeck *et al,* 1987b), *N. gonorrhoeae* (Johnson *et al,* 1975; Stead *et al,* 1975; Inzanza, 1983; Mintz *et al,* 1984), *C. jejuni* and *Campylobacter coli* (Blaser *et al,* 1986). Nevertheless, the rough LPS of *N. gonorrhoeae* (Morse *et al,* 1983; Shafer *et al,* 1984; Tan *et al,* 1986), *C. jejuni* (Blaser *et al,* 1986) and *H. ducreyi* (Odumeru *et al,* 1985) does somehow contribute to their serum resistance. In all three cases, serum resistance was related to the amount of LPS and the CHO : KDO ratio i.e. the serum-resistant strains possessed more LPS and a larger CHO : KDO ratio than the serum-sensitive strains.

Shafer *et al* (1984) studied the response of LPS mutants and other clinical strains of *N. gonorrhoeae* to normal human serum. He proposed that variations in the core oligosaccharide of the gonococcal LPS contributed to its serum resistance. This proposal can

be applied to other Gram-negative bacteria whose rough LPS plays a role in serum susceptibility, including *H. ducreyi*. The LPS of serum-sensitive and serum-resistant strains of *H. ducreyi* only differed quantitatively. Glycoses characteristic of the O antigen were absent in the LPS from all the strains, supporting the possibility that serum resistance of *H. ducreyi* may be associated with the amount of carbohydrate present in the core region of its LPS (Odumeru *et al,* 1987).

In this study, the inherent serum resistance of the clinical strains Y155 and Y206 (Table 7.1) was not consistently associated with the quantity of certain LPS components (Table 7.2), the hexose to core-oligosaccharide sugar ratio (Table 7.3) or its silver-stained LPS profiles on PAGE (Figure 7.1). The possibility of other cellular components acting as serum-resistance determinants should be considered and requires further investigation.

In contrast, when the serum-sensitive strain A76 was passaged in NHS several times, the resulting strain was relatively more serum resistant (Table 7.1), and possessed more LPS (Table 7.2) and hexose in relation to the other core-oligosaccharide components (Table 7.3). This change in LPS was not detected by PAGE (Figure 7.1).

Even though an increase in the concentration of LPS components was associated with the serum resistance of A76, the quantity of these LPS components in innate strains of *H. ducreyi* is not indicative of serum resistance. The constitution of the LPS from A75 and Y206 are comparable to that of the isogenic strain A76*, although they differ considerably in their susceptibility to serum (Table 7.2).

It appears that the determinant of serum resistance of clinical strains differs from that of strains induced to serum resistance *in vitro*. However, several more strains should be tested before any conclusions can be made. The relationship between LPS composition and serum resistance of *H. ducreyi* is not clearly indicated from the results of this study.

Odumeru *et al* (1987) previously reported an association of serum resistance of *H. ducreyi* with the biochemical composition of its LPS. The reason for this discrepancy between the results of this study and Odumeru's work is not known, but is thought to reflect differences in the methodologies of the investigations. For example, different media for the maintainance of the strains were used, the age of the bacterial cultures was not the same and different clinical isolates were employed.

Interestingly, the serum resistance acquired by A76 was 'unstable' i.e. the induction to serum resistance of A76* delayed killing by serum for up to sixty minutes relative to its parent strain A76. However, after an incubation period of 120 minutes, more than 90 per cent of the original bacterial inoculum of A76* was destroyed by C'-mediated killing. In contrast the serum resistance of the clinical strains were more stable, and it remained relatively constant after sixty minutes in 50 per cent PNHS. This supports the suspicion that the mechanism of serum resistance of clinical strains differs from that of strains passaged in serum *in vitro*. 'Promptly serum-sensitive' and 'delayed serum-resistant' patterns of serum killing were also reported for *S. marcescens* (Traub and Kleber, 1976) and *E. coli* (Taylor and Parton, 1977). In both cases, LPS appeared to be indirectly involved (treatment with polymyxin B, which dissociates LPS increased the serum sensitivity of *S. marcescens*), or directly involved (LPS *E. coli* mutants varying in O-antigen biosynthesis also varied in serum susceptibility). The different serum-killing kinetics of *S. marcescens* have been investigated (Traub and Kleber, 1976). The 'delayed-serum-sensitive' strains activated the slower-acting alternative pathway, while the 'promptly-serum-sensitive' strain activated the faster-acting classical pathway.

The possibility that the activation of different pathways may be associated with the kinetics of serum killing of A76 and A76* of *H. ducreyi* in this study, was considered. This theory is unlikely since the alternative pathway is generally activated by the O antigen of smooth strains of enteric bacteria (Morrison and Kline, 1977; Joiner *et al,* 1983b; Liang-Takasaki *et al,* 1983a; Goldman *et al,* 1984; Grossman and Leive, 1984). Lipid A is responsible for the activation of the classical pathway (Galanos *et al,* 1971; Morrison and Kline, 1977) and re-

sults in serum killing of bacteria with both smooth and rough LPS. Gram-negative bacteria with rough LPS including *N. gonorrhoeae* (Shafer *et al,* 1984; Joiner *et al,* 1985a), *C. jejuni* (Blaser *et al,* 1986), *H. influenzae* (Gilsdorf and Ferrieri, 1986) and *H. ducreyi* (Odumeru *et al,* 1985), activated the classical pathway of C′ on exposure to PNHS.

The differences in serum resistance of the clinical strains of *H. ducreyi* and A76* may be compared to that of *N. gonorrhoeae*, although the main cellular components determining serum resistance of the two micro-organisms are not the same. Inherent serum resistance of *N. gonorrhoeae* is stable, while serum resistance induced by incubation in PNHS (McCutchan *et al,* 1976; Veale *et al,* 1981; Martin *et al,* 1982), guinea pig serum and foetal calf serum (Veale *et al,* 1981) is unstable. Acquired serum resistance of *N. gonorrhoeae* and *H. ducreyi* differ in several aspects. Although the inherent serum resistance of both strains is stable, the induced serum resistance of *N. gonorrhoeae* is lost after several generations *in vitro* in the absence of serum. The killing of the induced serum-resistant strain of *H. ducreyi* is merely delayed i.e. is lost after prolonged incubation (120 minutes) in the serum bactericidal assay. Also, A76 required at least six or seven direct passages in PNHS, while gonococcal-induced serum resistance required a single three hour incubation in a serum-containing medium at 37^0 C. The increase in serum resistance of *H. ducreyi* was associated with an increase in its LPS constituents. However no changes in the cell wall of these 'unstable' serum-resisant gonococcal strains were observed.

An understanding of the pathogenesis of *H. ducreyi* may be better obtained from a comparison of the LPS of 'avirulent' reference strains and 'virulent' clinical strains. Since *H. ducreyi* has not previously been located in the bloodstream of patients suffering from chancroid, the change in LPS of A76 due to passage in NHS may be of less clinical significance. However, the use of isogenic strains differing in serum susceptibility allows for the identification of the cellular component/s responsible for this property. Theoretically, the isogenic strains A76 and A76* should differ only in a single respect : their serum susceptibility. Different clinical strains may vary in several fields e.g. antibiotic susceptibility, nu-

tritional requirements, antigenicity and serum susceptibility etc. Therefore differences in the cell wall components of clinical and reference strains may be a manifestation of several innate features, rather than a consequence to an increase in serum resistance.

Interpretations of investigations regarding serum resistance, virulence and LPS structure of any micro-organism should be approached critically and with caution, since serum resistance may be associated with virulence and / or the chemical composition of its LPS. However, serum resistance is not always associated with virulence, or the composition of its LPS. Furthermore Gram-negative bacteria with either smooth or rough LPS are capable of causing disease i.e. virulence may be independent of LPS structure.

The discrete study of serum resistance, virulence and LPS structure of any Gram-negative bacterium including *H. ducreyi,* is complicated by influences which may occur in different combinations. Within the serum bactericidal assay several parameters vary from one investigation to the next, viz. the metabolic state of the bacterial inoculum, the source and concentration of the serum used, the bacterial inoculum size, the diluent used, the incubation conditions of the bacteria-serum mixture and the time of evaluation. Similarly, the composition of the LPS of a micro-organism is affected by the metabolic state of the bacterium, the method used for its isolation and analysis, etc.

It is thus essential that one is aware of these influencing factors when a relationship between serum resistance and LPS composition is sought.

7.5 CONCLUSION

Clinical strains of *H. ducreyi* were more serum resistant than the reference strains, suggesting that serum resistance may play a role in the virulence of *H. ducreyi*. The serum resistance of the clinical strains was not entirely dependent on the composition of the LPS or the length of its polysaccharide portion. In contrast, the serum-resistant isogenic strain A76* possessed more LPS than its serum-sensitive parental strain. Biochemical variations of the LPS of different strains were not revealed by PAGE.

Furthermore, the kinetics of this serum resistance of the isogenic strain differed from that of the clinical strains. In the latter case, serum resistance was stable after sixty minutes incubation in 50 per cent PNHS, whereas the acquired serum resistance of A76* dissipated after 120 minutes incubation.

This study suggests that the serum resistance of *H. ducreyi* is not necessarily dependent on the LPS *in vivo*. However, serum-resistance induced *in vitro* did show LPS alteration. It is speculated that the rough LPS of *H. ducreyi* does not play a major role in serum resistance, as observed in enteric bacteria. Variations in the core-oligosaccharide moiety may contribute to the serum resistance of *H. ducreyi*.

CONCLUSIONS

Haemophilus ducreyi, a Gram-negative bacillus is the aetiological agent of chancroid, a sexually-transmitted disease common in South Africa.

Virulence determinants of *H. ducreyi* are poorly understood. Since the ability of several Gram-negative bacteria to resist killing by pooled normal human serum (PNHS) appears to contribute to their serum resistance, the purpose of this investigation was to measure the level of serum resistance of clinical isolates of *H. ducreyi* and to determine if their lipopolysaccharide (LPS) plays a role in this feature. Three reference strains with reduced virulence based on failure to produce a skin lesion on intradermal inoculation, were obtained from the Pasteur Institute, France, for comparison purposes.

As *H. ducreyi* is a fastidious organism, a medium was sought which would support the growth of both reference and clinical strains allowing for a comparative analysis of the serum susceptibilities and LPS composition of these strains. In addition, preliminary investigations were performed to overcome problems in the *in vitro* manipulations of *H. ducreyi,* such as auto-agglutination.

Lyophilised cultures of strains A75, A76 and A77 obtained from the Pasteur Institute, France, were not viable on reconstitution onto various media, suggesting that the effects of freeze-drying of *H. ducreyi* should be more closely investigated and an alternative method of storage developed.

A medium consisting of gonococcal agar, haemoglobin, FCS, IsoVitaleX and vancomycin is recommended for the isolation of African strains of *H. ducreyi.* However, this medium did not support the growth of the reference strains (A75, A76 and A77) in this study. The inhibitory component was the FCS supplied by Highveld Biological. Although the inhibitory factor/s of the FCS were not identified, this observation is relevant for laboratories engaged

in research on *H. ducreyi;* and it may also contribute to the lack of complete efficiency of this enriched gonoccal medium.

A novel method of homogenisation of clumps of *H. ducreyi* cells was introduced for accurate enumeration and production of bacterial inocula. This method involves drawing clumps of cells in a diluent through a thin needle and syringe several times. Results produced by this method produced significantly higher viable counts than other techniques viz. sonication and mechanical agitation by vortexing.

Using this novel homogenisation technique, the growth rate of reference and clinical strains of *H. ducreyi* in liquid medium was determined. The doubling times varied between 1.8 to 2.9 hours; reference strains grew more slowly than clinical isolates.

With a better understanding of the growth kinetics of *H. ducreyi,* and an increased ability to manage this organism in the laboratory, the next step was to measure the serum resistance of the reference and clinical strains using a serum bactericidal assay. This involved exposing a standardised bacterial inoculum to 50 per cent PNHS at 37^0 C and measuring the number of surviving bacteria after several time intervals. Difficulties were experienced when one attempted to compare the results of this study with other investigations, since the variable parameters of this assay has not been standardised. Because the definition of 'serum resistance' is based on the results of such an assay, standardisation of the serum bactericidal assay is urgently indicated.

In this study, the finding that clinical strains (Y155 and Y206) are more resistant than avirulent reference strains (A75, A76 and A77) to killing by PNHS, provides circumstantial evidence implicating serum resistance as a virulence factor. The serum susceptibility of a larger number of clinical strains of *H. ducreyi* requires testing for a definite association to be established. In addition, because experimental animal skin lesions may be non-specific for

H. ducreyi, and different from human chancroid in several aspects, the assessment of virulence of *H. ducreyi* in animal models requires clarification.

The LPS of all the strains were analysed by polyacrylamide gel electrophoresis (PAGE) and biochemical tests specifically measuring its carbohydrate components. The pattern of Proteinase-K treated LPS of *H. ducreyi* analysed by PAGE conformed to rough-type LPS. The possible artefacts introduced by different methodologies questions the sensitivity of PAGE in the analysis of LPS. Differences in the biochemical composition of the LPS of different strains of *H. ducreyi* determined by several colorimetric assays were not associated with changes in the silver-stained LPS patterns on PAGE.

The LPS of *H. ducreyi* contained components commonly found in the LPS of other Gram-negative bacteria, viz. hexose, hexosamine, heptose and 2-keto-3-deoctonic acid (KDO). The ratios of the O-antigen sugar to core oligosaccharide sugar, as in carbohydrate concentration to KDO, are generally thought to reflect the length of the O-polysaccharide side chains. However, they do not indicate whether short chains, long chains or both are present in a single LPS sample. This problem was evident from this study.

The biochemical composition of the LPS of serum-sensitive reference strains of *H. ducreyi* (A75, A76 and A77) and their LPS profiles on PAGE, was not significantly different to that of the serum-resistant clinical strains (Y155 and Y206), suggesting that the LPS of *H. ducreyi* is not solely responsible for its serum resistance. The possibility of other cellular components acting as serum-resistant determinants should be considered and requires further investigation.

It is unlikely that the mechanism of serum resistance of *H. ducreyi* is similar to that of enteric bacteria where serum resistance is a direct result of long O-polysaccharide side chains of the LPS. *H. ducreyi* appears to possess only rough-type LPS, and this has also been reported

by other investigators. If LPS does contribute to the serum resistance of *H. ducreyi* in some way, variations in the core oligosaccharide are more likely to be responsible.

The kinetics of the serum resistance induced *in vitro* differ from the innate serum resistance of clinical strains, probably as a result of different mechanisms of serum resistance. By directly passaging reference strain A76 through PNHS several times, an isogenic strain (A76*) was produced which expressed a relatively higher serum resistance than its parental strain. This induced serum resistance was short-lived since the difference between the serum susceptibility of A76 and A76* was not as distinct after 120 minutes incubation in 50 per cent PNHS, compared with the sixty minute incubation. This delayed serum killing of A76* correlated with an increase in the quantity of the LPS components assayed and an increase in the carbohydrate to KDO ratio, substantiating a small but possible role of LPS in the serum resistance of *H. ducreyi.*

APPENDIX 1

Supplemented brain heart infusion (BHI) agar / l

A. BHI agar (Difco) 52g

Distilled water 600ml

Autoclave 15 minutes.

B. Haemoglobin powder (Difco) 10g

Distilled water 400ml

Mix fast (magnetic stirrer).

Autoclave 15 minutes.

Keep in waterbath.

Final medium

Mix solutions A and B. Cool to 50^0 C.

Add IsoVitalex (BBL, Microbiological systems) 10ml

vancomycin 3mg.

Mix well and distribute into sterile petri dishes.

Brain heart infusion (BHI) haemin broth / l

A. BHI broth powder 37g

Distilled water 1000ml

Autoclave 15 minutes.

B. Haemin (Sigma) 5g

Histidine (Sigma) 5g

Triethanolamine (Merck) 10ml

Distilled water 250ml

Dispense into 10ml aliquots. Sterilise by heating at 60^0 C for 30 minutes. Store at minus 20^0 C till required.

Final medium

Add to BHI broth (solution A) / l

Haemin stock solution 5ml (0.5%)

IsoVitaleX (BBL, Microbiological Systems) 10ml (1%)

Mix well.

APPENDIX 2

Calculation of growth rate, μ

The following SAS program was used to plot the logarithm to the natural base (ln), of the colony forming units (CFU) / ml versus time (hours), and to calculate the best fitting line (a set of data is used as an example) :

```
DATA EXP;
INPUT T COUNT1;
LCOUNT1 = LN(COUNT1);
CARDS;
0 4.1E2
11.5 6.6E4
28.5 2E4
31.5 4.15E6
34 5.6E6;
PROC REG;
MODEL LCOUNT1 = T;
OUTPUT OUT = NEWDATA P = PRED;
SYMBOL1 C = RED I = JOIN V = NONE;
PROC GPLOT;
TITLE1 C = YELLOW H = 3 F = XSWISS 'GROWTH CURVE A751';
PLOT LCOUNT1*T = '+' PRED*T = 1 / OVERLAY CAXIS = BLUE
                                  CT = GREEN;
LABEL T = 'TIME'
      LCOUNT = 'LN(COUNT)';
```

The gradient of this line was calculated; this represents the growth rate μ.

The equation for an exponential growth curve is $X = X_0 2^{t/T}$, which may be written as $\ln X = \ln X_0 + \mu t$ where X_0 is the inoculum size, μ is the growth rate and X is the number of CFU/ml at time t.

A plot of ln X versus t (hours) will produce a line whose gradient is μ. The doubling time T is the time it takes for the population to double, and is equal to $\ln 2/\mu$.

APPENDIX 3

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The following solutions and buffers were required for SDS-PAGE :

A. Acrylamide 30% w/v

1. Acrylamide (Isophore electrophoresis) 29.2g
2. Bis-acrylamide (Isophore electrophoresis) 0.8g
3. Dissolve acrylamide and bis-acrylamide in distilled water with stirring. Use a 100ml beaker. When the acrylamide is in solution adjust the final volume to 100ml. Store in amber bottle at 4^0 C.

B. Sodium dodecyl sulphate (SDS) 10% w/v

1. SDS (Holpro analytics) 10g
2. Dissolve in distilled water and adjust the final volume to 100ml. Store at room temperature.

C. Upper (Hydroxymethyl) aminomethane (Tris) 0.5M (stacking gel tris)

1. Tris base (Associated Chemical Enterprises) 6.06g
2. 10% SDS 4ml
3. Adjust to 90ml with distilled water. pH this solution to 6.8 with concentrated hydrochloric acid. Adjust the final volume to 100ml. Store at 4^0 C.

D. Lower Tris 1.5M (separating gel tris)

1. Tris base 36.6g
2. 10% SDS 8ml
3. Adjust volume to 180ml with distilled water. pH this solution to 8.8 with concentrated hydrochloric acid. Adjust the final volume to 200ml. Store at 4^0 C.

E. Ammonium persulphate 10% (made up fresh daily)

1. Ammonium persulphate (Isophore electrophoresis) 0.1g
2. Add 0.9ml distilled water. Use within 4 hours.

F. N,N,N′,N′, - tetramethyl ethylene diamine (TEMED) (Isophore electrophoresis), use directly with no dilution.

G. Solubilising buffer (Abeck *et al,* 1987a)

1. SDS 0.2g
2. Mercaptoethanol (Biorad) 0.4ml
3. Glycerol 1ml
4. Tris buffer pH 6.8 8.4ml
5. Bromothymol blue (Merck) till coloured.

H. Running buffer / 4l

1. Tris base 12.12g
2. Glycine (BDH Chemicals) 57.6g
3. SDS 4g
4. Bring to volume with distilled water.

Assemble the glass plates forming a sandwich as indicated in the manual (Hoeffer Instruments SE 600).

Prepare the following resolving gel solution per gel (15% polyacrylamide) -

7.5ml lower tris

4.5ml water

15ml acrylamide solution (30%)

3ml glycerol

Mix with a magnetic stirrer.

Add 75µl ammonium persulphate and 20µl TEMED just before pouring the gel.

Allow at least 1cm for the stacking gel above the resolving gel.

Overlay with water and wait for the gel to polymerise, until a sharp band at the water gel interface is observed.

Remove the water by blotting dry with filter paper.

The stacking gel (4.5%) consists of

2ml upper tris buffer

1.2ml distilled water

4.8ml acrylamide per gel.

Just before pouring the solution, add 30µl ammonium persulphate and 8µl TEMED while stirring continuously.

Place the comb between the two glass plates, and pour the stacking gel on top of the resoving gel, and allow for polymerisation.

Gently remove the combs and fill the wells with running buffer.

Load the samples into the wells with a Hamilton syringe (Dynatech instruments) by under-laying.

Connect the upper buffer chamber to the glass plates with the gel, and gently immerse it into the lower buffer chamber which contains running buffer and the cooling unit.

Connect this entire unit to the power supply.

Run each gel at a constant current of 30mA until the marker dye reaches the bottom of the gel.

APPENDIX 4

Silver stain for detecting lipopolysaccharide (LPS) on polyacrylamide gels (Tsai and Frasch, 1982)

Fix LPS in the polyacrylamide gel by placing the gel in 40% ethanol-5% acetic acid solution (200ml) in a clean dish overnight.

Replace the fixing solution with 0.7% periodic acid (Analytics) in 40% ethanol-5% acetic acid solution and oxidise the LPS for 5 minutes.

Perform three 15 minute washes in a second dish with 500ml to 1000ml distilled water.

Drain water, pour in fresh staining reagent (150ml) and agitate vigourously for 10 minutes.

The staining reagent : Add 2ml concentrated ammonium hydroxide (BDH Chemicals) to 28ml 0.1N sodium hydroxide (BDH Chemicals). Add 5ml 20% silver nitrate (Holpro Analyticals) w/v to the solution while stirring continuously. A transient brown precipitate will form, but will disappear within seconds. Add water (115ml) to make 150ml staining reagent. Discard after use.

Perform three 10 minute washes with distilled water.

Replace water with formaldehyde developer (200ml) containing 50mg citric acid (BDH Chemicals) and 0.5ml 37% formaldehyde/l (Merck). The LPS in the gel will stain dark brown. Terminate the development when the clear gel background begins to discolour.

Place in stopbath (200ml distilled water and 10ml 7% acetic acid) for 1 hour (Hitchcock and Brown, 1983).

Store in distilled water.

APPENDIX 5

Method of lipopolysaccharide (LPS) isolation (Darveau and Hancock, 1983b)

Suspend 500mg of bacterial cells (dry weight) in 15ml (hydroymethyl) aminomethane (Tris) - hydrochloric buffer (Associated Chemical Enterprises) (pH 8) containing 2mM magnesium chloride, 100µg DNase (Sigma) per ml, and 25µg RNase (Sigma) per ml.

Sonicate (Soniprep, 150 MSE) for three 1 minute bursts at an intensity of 20 microns.

Add DNase and RNase to a final concentration of 200µg/ml and 50µg/ml respectively, to ensure efficient nucleic acid digestion.

Incubate at 37^0 C for 2 hours.

Add 5ml 0.5M tetrasodium ethylene diamine tetraacetic acid (EDTA) (Merck) dissolved in 10mM Tris - hydrochloride (pH 8) to 2.5ml 20% sodium dodecyl sulphate (SDS) dissolved in 10mM Tris - hydrochloride (pH 8) to give a final volume of the sample of 25ml containing 0.1M EDTA, 2% SDS, and 10mM Tris - hydrochloride.

Vortex.

Centrifuge at 50 000g for 30 minutes (Beckman L5-65 ultracentrifuge) at 20^0 C to remove peptidoglycan.

Decant supernatant and add pronase (Sigma) to give a final concentration of 200µg/ml.

Incubate overnight at 37^0 C.

Add 2 volumes of 0.375M magnesium chloride (Merck) in 95% ethanol, mix and cool in an icebath.

Centrifuge at 12 000g for 15 minutes (Beckman JA 21) at 0^0 C to 4^0 C.

Resuspend the pellet in 25ml 2% SDS - 0.1M tetrasodium EDTA, dissolved in 10mM Tris - hydrochloric (pH 8) buffer and vortex.

Incubate the solution at 85^0 C for 30 minutes to denature protein.

Precipitate LPS with 2 volumes 0.375M magnesium chloride in 95% ethanol at 0^0 C as described before.

After centrifugation, wash pellet in 25ml 10mM Tris - hydrochloride (pH 8) by vortexing . and centrifugation using a bench centrifuge at 1000rpm for 5 minutes.

Centrifuge the supernatant at 200 000g for 2 hours (Beckman L5-65 ultracentrifuge) at 15^{0} C in the presence of 25mM magnesium chloride.

Resuspend the pellet in distilled water and lyophilise extracted LPS.

APPENDIX 6

Total carbohydrate assay (Dubois *et al,* 1956)

Place 2ml of sugar solution containing between 0µg/ml to 70µg/ml glucose (Sigma), or 2mg extracted LPS of *Haemophilus ducreyi* or 800mg commercial LPS preparations of *Escherichia coli* 0111 : B4 and *Salmonella typhimurium* (Sigma) in 0.1N sulphuric acid in glass test tubes.

Add 0.05ml 80% phenol (SMM Chemicals) to each sample.

Add 5ml concentrated sulphuric acid to each sample.

Leave at room temperature to 10 minutes; then shake.

Keep in a 30^0 C waterbath for 10 to 20 minutes.

Measure absorbance of the yellow-orange colour at 490nm (Leitz photometer, USA).

Construct a standard curve of glucose concentration versus absorbance at 490nm.

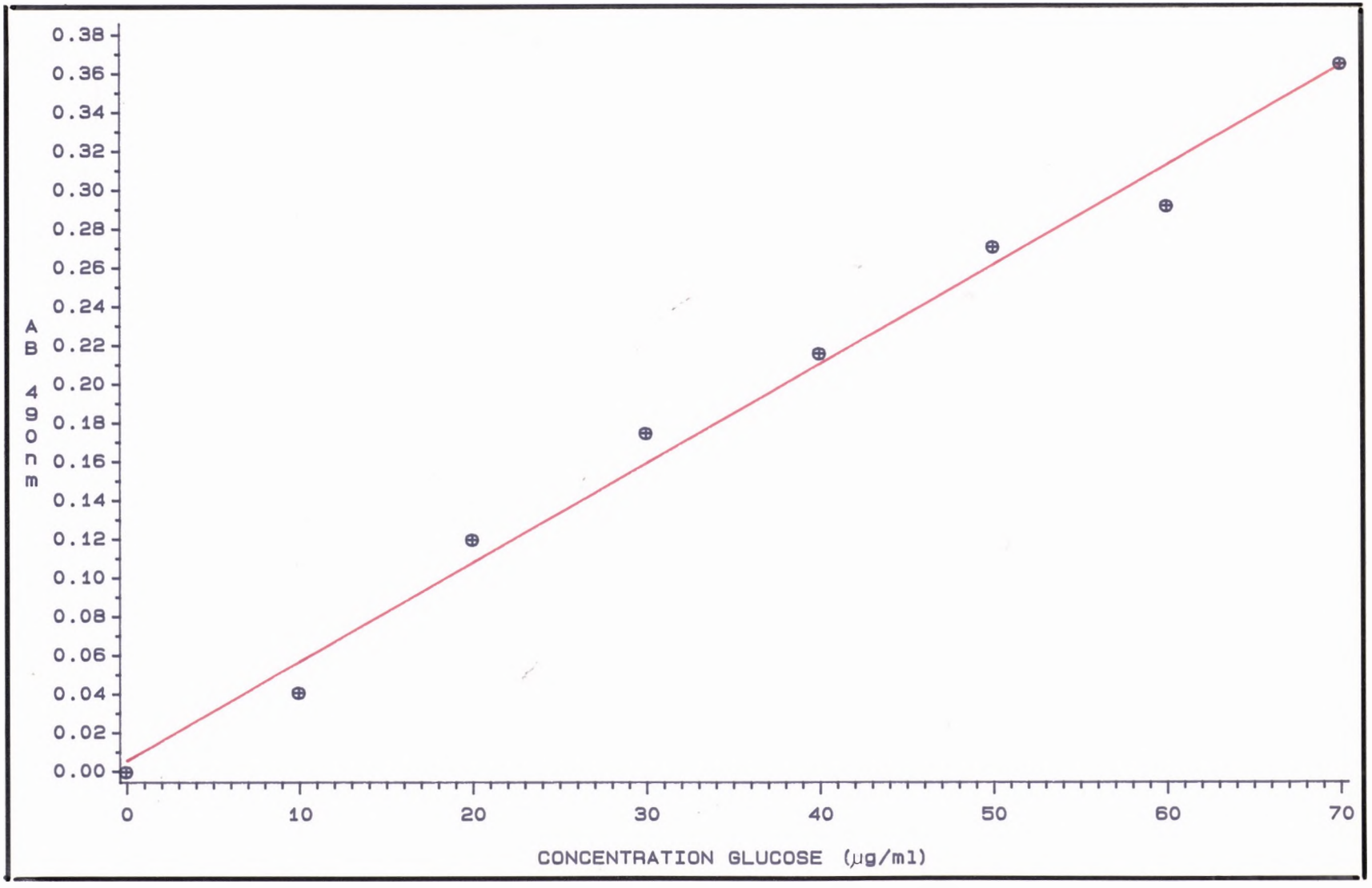


Figure 9.1 Standard curve for total carbohydrate assay (Dubois *et al*, 1956).

APPENDIX 7

Hexose assay (Scott and Melvin, 1953)

Deliver 5ml of anthrone reagent (0.4g anthrone (Riedel-deHaen) in 200ml sulphuric acid) into test tubes standing in cold water (10^0 C to 15^0 C).

Prepare increasing concentrations of glucose (Sigma) in 0.1N sulphuric acid in 2.5ml volumes. 2mg extracted LPS (*Haemophilus ducreyi*) and 800mg of commercial LPS (*Escherichia coli* 0111 : B4, *Salmonella typhimurium* - Sigma) were included in the assay.

Overlay each sample onto the anthrone solution in different test tubes, carefully forming a separate layer.

Shake each tube vigourously, while keeping it immersed in the cold waterbath, until the contents are thoroughly mixed and at room temperature.

Boil for 16 minutes.

Cool to room temperature in a cold waterbath.

Read the absorbance at 640nm (Leitz photometer, USA), and plot the required standard curve.

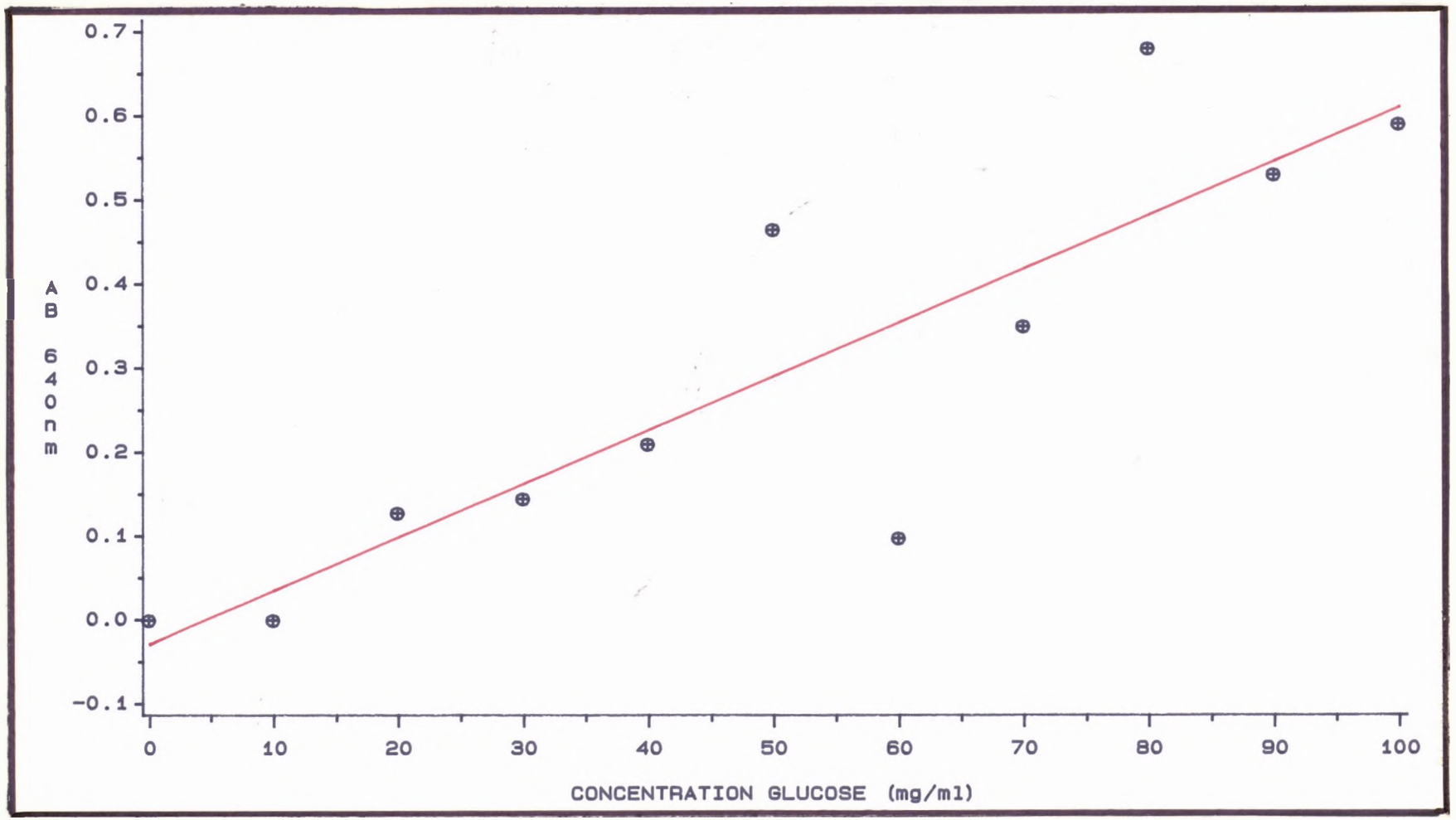


Figure 9.2 Standard curve for hexose assay (Scott and Melvin, 1953).

APPENDIX 8

Heptose assay (Wright and Rebers, 1972)

Add 4.5ml of sulphuric acid (6 volumes acid : 1 volume water) to 0.5ml solutions containing 0µg/ml to 70µg/ml sugar (glucoheptose was obtained from Sigma) in an ice waterbath. 800mg of extracted LPS (*Haemophilus ducreyi*) and 800mg of commercial LPS preparations (*Escherichia coli* 0111 : B4, *Salmonella typhimurium* - Sigma) were suspended in 0.5ml 0.1N sulphuric acid.

Shake mixture vigourously.

Hold in an ice waterbath for 3 minutes.

Hold at room temperature for 3 minutes.

Heat for 20 minutes in a boiling waterbath.

Cool in tap water.

Add 0.1ml 3% cysteine-hydrochloride (Sigma).

Samples were read at 490nm and 550nm (Leitz photometer, USA) after 1 hour.

Construct a standard curve of glucoheptose concentration and the difference in absorbance read at 490nm and 550nm.

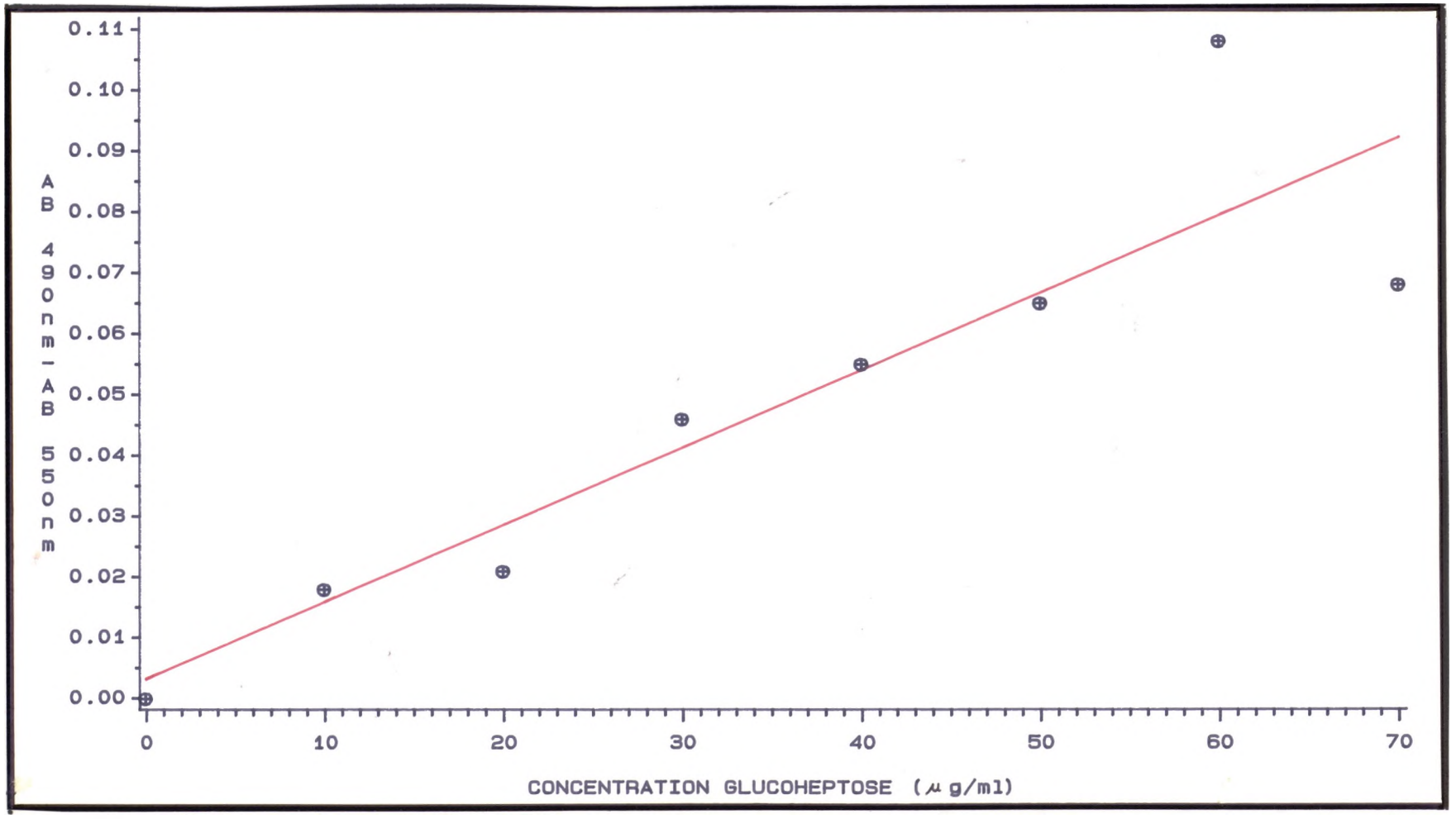


Figure 9.3 Standard curve for heptose assay (Wright and Rebers, 1972).

APPENDIX 9

Hexosamine assay (Smith and Gilkerson, 1979)

Prepare solutions diluted from 0µg to 50µg glucosamine (Sigma) in a total volume of 400µl of 0.5M hydrochloric acid. 500mg of commercial (*Escherichia coli* 0111 : B4, *Salmonella typhimurium* - Sigma) and extracted LPS (*Haemophilus ducreyi*) were employed.

Boil all samples in 0.5M hydrochloric acid for 2 hours.

Cool to room temperature in a waterbath.

Add 800µl 2.5% sodium nitrite (Holpro analytics), vortex, and stand at room temperature for 5 minutes.

Add 400µl 0.25% 3-methyl-2-benzo-thiazolinone (Sigma), vortex, and incubate at 37^0 C for 30 minutes.

Add 400µl 0.5% ferric chloride (Sigma) and incubate at 37^0 C for 5 minutes.

Cool samples to room temperature and read the absorbance of the samples at 640nm (Leitz photometer, USA).

Plot the standard curve of concentration glucosamine versus absorbance at 640nm.

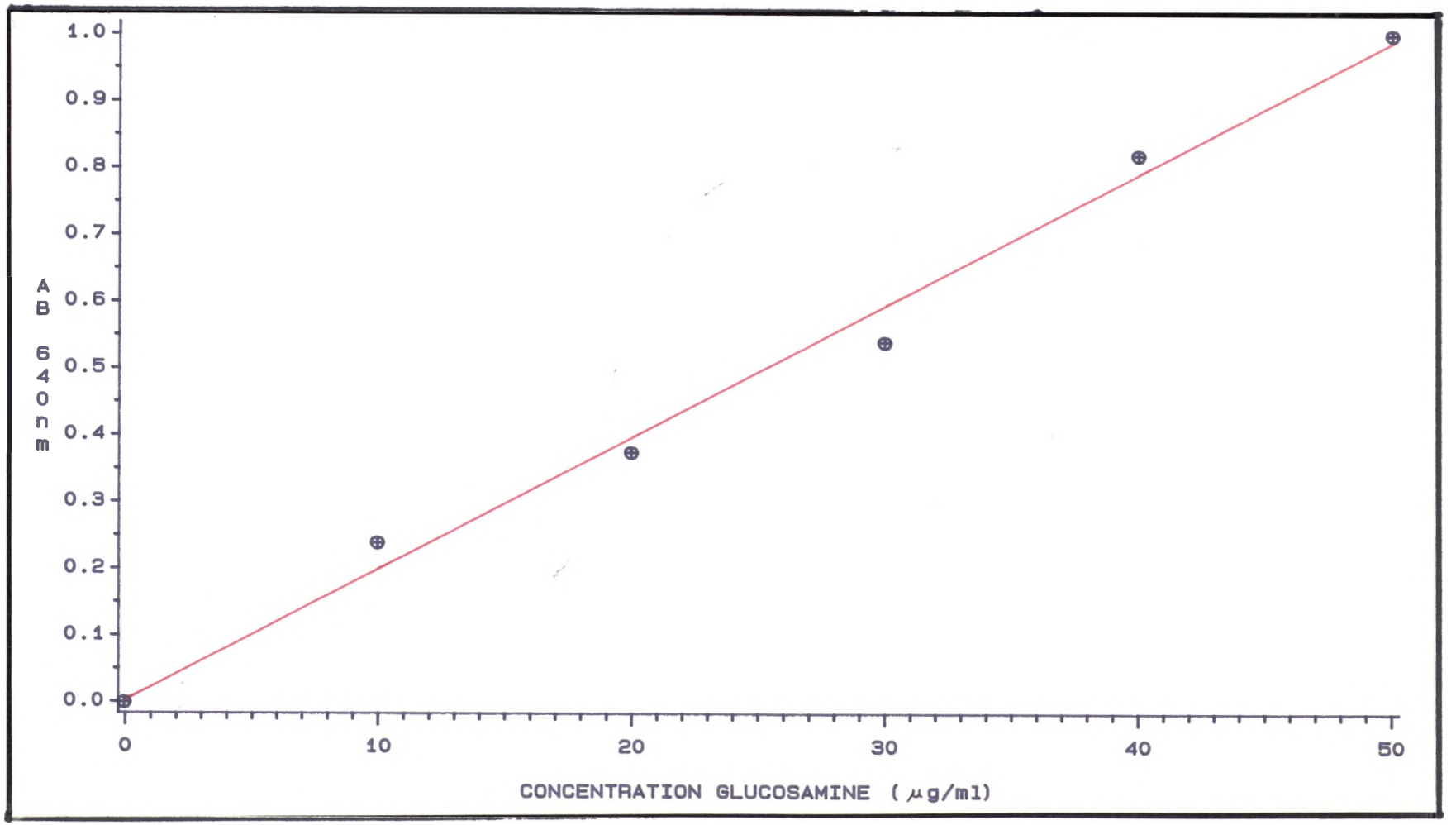


Figure 9.4 Standard curve for hexosamine (Smith and Gilkerson, 1979).

APPENDIX 10

2-keto-3-deoxyoctonate assay (Brade *et al,* 1983)

Hydrolyse solutions of KDO (Sigma) varying from 0µg to 4µg by boiling for 1 hour in 500µl of 0.1M acetate buffer pH 4.4. 2mg extracted LPS (*Haemophilus ducreyi*) and 1.5mg commercial LPS (*Escherichia coli* 0111 : B4, *Salmonella typhimurium* - Sigma) were treated in the same way.

Cool to room temperature.

Add 250µl 40mM periodic acid (Analyticals) in 60mM sulphuric acid, and incubate at room temperature for 30 minutes.

Add 250µl 0.2M sodium arsenite (Sigma) in 0.5M hydrochloric acid to stop the oxidation.

After the reduction of periodate (the disappearance of the brown colour), add 500µl freshly prepared 0.6% aqueous thiobarbituric acid (BDH Chemicals) solution.

Boil for 15 minutes.

Add 1ml dimethyl sulphoxide (Merck) while the samples are still hot.

Cool to room temperature.

Read absorbance at 550nm (Leitz photometer, USA).

Plot the relevant standard curve.

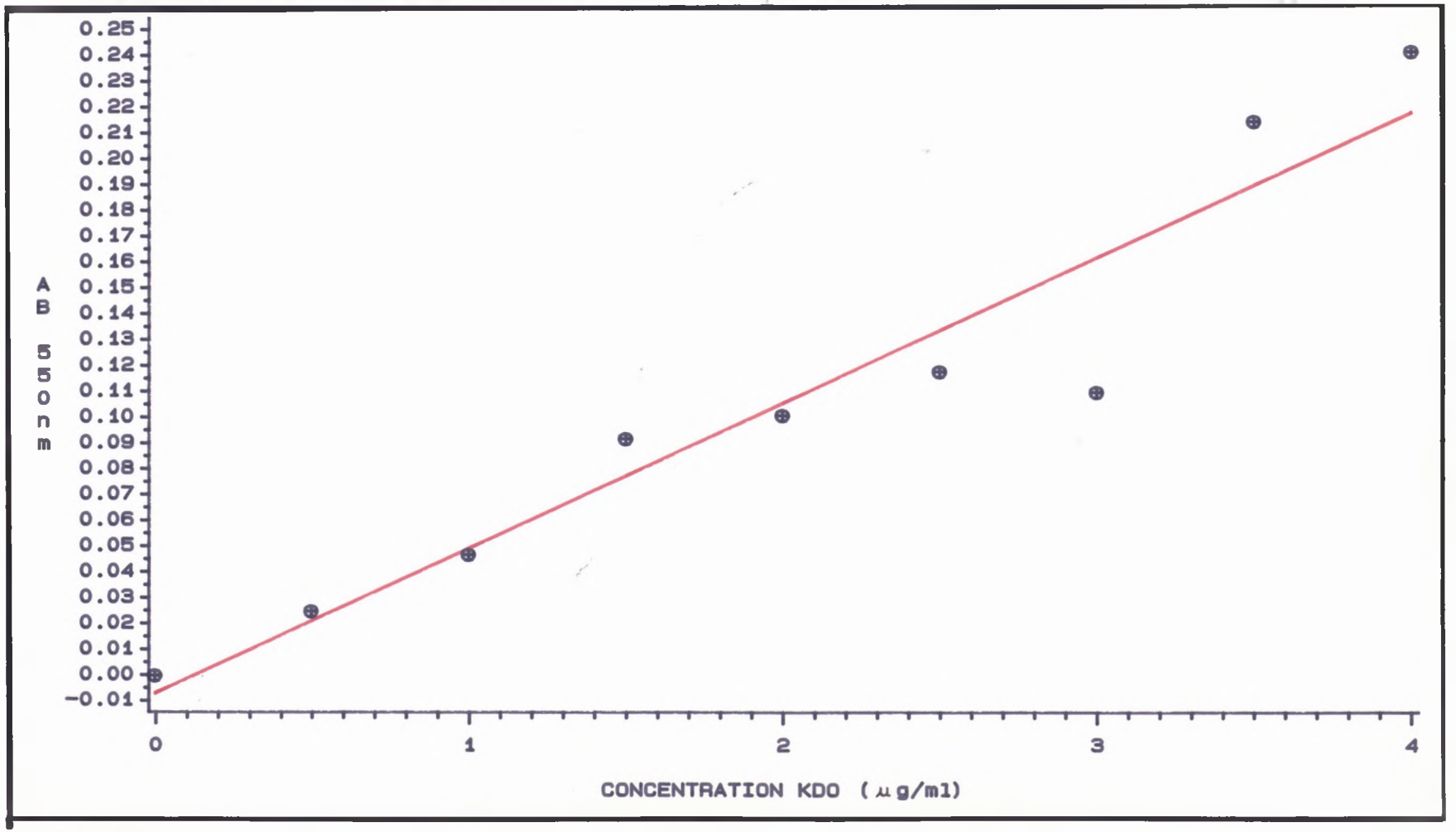


Figure 9.5 Standard curve for keto-deoxy-octulosonic acid (KDO) (Brade *et al*, 1983).

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