Studies on *Haemophilus ducreyi*

A Thesis

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Master of Science

by

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STUDIES ON HAEMOPHILUS DUCREYI.

by

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

Master of Science

In memory of my mother,

Mrs. Marguerite (Rita) J.S. Bertram

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ABSTRACT

The cell wall ultrastructure of well characterized isolates of Haemophilus ducreyi from a Winnipeg outbreak of chancroid and reference strains was examined by electron microscopy. Both Winnipeg and reference strains were found to have Gram-negative cell walls, consistent with their Gram's staining properties. Staining with the cationic dyes ruthenium red and Alcian Blue revealed the presence of exocellular (capsular) material in all strains. Stabilization of the exocellular material with antisera and subsequent staining with ruthenium red or Alcian Blue allowed visualization of a continuous capsular layer in all strains.

Virulence testing, in the rabbit intradermal model, indicated that all the Winnipeg isolates were virulent, while the reference strains were avirulent. The rabbit model also indicated that virulent strains produced a more acute inflammation in the associated skin lesion than did the avirulent strains. Virulence in selected H. ducreyi strains was associated with an increased resistance to Polymyxin.

Antigenic studies indicated that a complex pattern of precipitating antibodies against H. ducreyi antigens could be produced in rabbits. Among the H. ducreyi antigens, heat labile, heat stable, and protease-heat labile antigens were identified. Column chromatography allowed partial separation of these antigens. The antigenic structure of all H. ducreyi strains appeared homogeneous. However, cross-reacting antigens between H. ducreyi and other Haemophilus species were evident.

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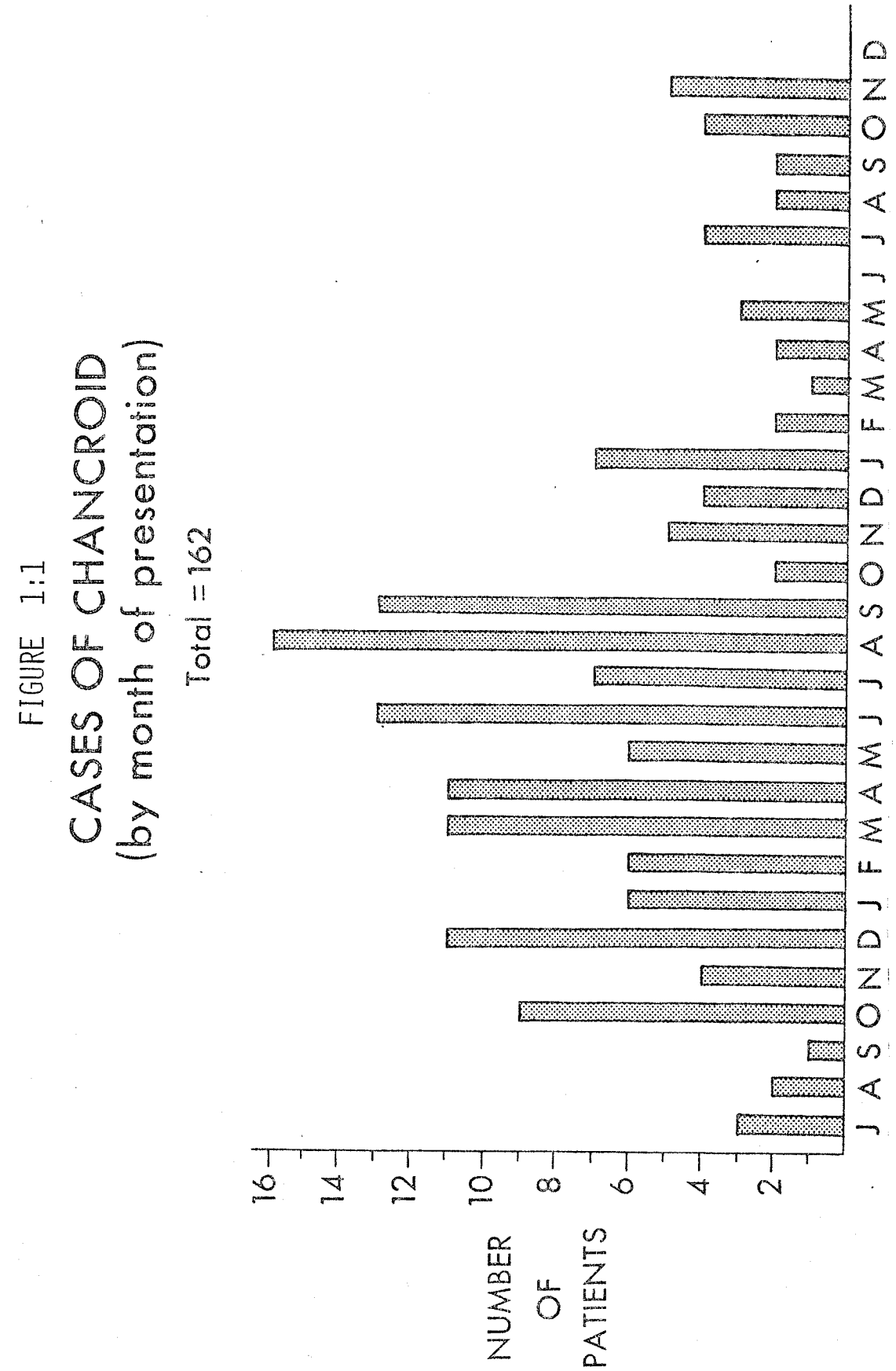
INTRODUCTION

Between July 1975 and November 1977 more than 100 patients with symptoms of the venereal disease chancroid were seen at the Primary Health Care Unit of the Health Sciences Centre in Winnipeg, Manitoba, Canada (Figure 1:1). Twenty-one isolates identified presumptively as Haemophilus ducreyi, the causative agent of chancroid, were cultured from these patients. The isolation of the Winnipeg strains and the acquisition of reference strains of H. ducreyi from the Pasteur Institute allowed Hammond and co-workers (1978 a,b,c) to investigate isolation techniques, nutritional requirements and antimicrobial susceptibility.

H. ducreyi was described in the late 1800's and early 1900's (Ducrey, 1889; Unna, 1892, Davis 1903, etc.). Studies continued sporadically and even now many of the features of H. ducreyi are inadequately defined. Disagreement on the staining and ultra-structural characteristics of the organism has led to confusion with regard to the Gram reaction of the cell wall. Although initially described as a Gram-negative organism (Davis, 1903), some investigators (Nicolau and Bancrou, 1926; Deacon et al, 1954) have reported that the bacillus became Gram-positive on subculture. Recent electron microscopic studies (Cazarre and Barreto, 1974; Ovchinnikov et al, 1976; and Marsch et al, 1978) confirmed the presence of a Gram-negative cell wall, but failed to describe the nutritional or biochemical characteristics of the strains examined.

FIGURE 1:1


CASES OF CHANCROID
(by month of presentation)
Total = 162
NUMBER OF PATIENTS
16
14
12
10
8
6
4
2
J A S O N D J F M A M J J A S O N D J F M A M J J A S O N D
1975
1976
1977

Only Kilian and Theilade (1975) have described the ultrastructure of H. ducreyi strains which were accurately classified by nutritional and biochemical tests. The accumulation of well characterized strains of H. ducreyi from Winnipeg and reference culture collections provided an opportunity to confirm the Gram-negative character of recent isolates of H. ducreyi.

The virulence of H. ducreyi as measured by the skin response in man and animals to an intradermal injection of viable organisms, has been investigated in a series of studies (Greenblatt et al, 1943 a&b; Feiner and Mortara, 1945; Kaplan et al, 1956a, Thayer et al, 1955; Singer and Deacon 1956; and Hammond et al 1978c). (Both virulent and avirulent strains of H. ducreyi exist.) Hammond et al (1978c) recently reported that all of the Winnipeg isolates were virulent whereas the Paris reference strains were avirulent. This permitted a study of the relation of virulence of the Winnipeg isolates to variations in ultrastructure and immunochemistry. Virulence in other bacteria, including H. influenzae, has been associated with capsular layers at the cell surface (Leidy et al, 1960; and Onderdonk et al, 1977). In addition to this, some reports suggested that virulence in H. ducreyi was associated with differences in antimicrobial susceptibility (Thayer et al, 1955; Singer and Deacon, 1956; and Hammond et al, 1978c).

It was postulated that the isolation of Winnipeg strains presented an almost unique possibility to explore the relationships

between virulence, ultrastructure and antimicrobial susceptibility.

Although serological studies on H. ducreyi have been reported, most were carried out before 1950. The identity of the organisms used in these studies is uncertain as exemplified by Reymann's 1950 report (Ph.D. thesis, Copenhagen, 1950). Kilian (1976) has shown that the Reymann organisms did not possess the currently recognized ultrastructural, nutritional or biochemical characteristics for Haemophilus species. None the less, all studies to date, using a variety of immunological techniques, have suggested that H. ducreyi strains are antigenically homogeneous. Deny et al (1977) have also suggested that antibodies directed against H. ducreyi, cross-react with other Haemophilus species.

Based on the foregoing, it was decided to study three aspects of well characterized H. ducreyi strains. These were:

1. To investigate the cell wall ultrastructure of H. ducreyi.
2. To study the virulence properties of Winnipeg and reference strains, and relate this to their ultrastructure and antimicrobial sensitivities.
3. To determine the antigenic profile of H. ducreyi and its relationship to other Haemophilus species.

Chapter 1

Literature Review

1:1 Clinical Aspects of Chancroid

Haemophilus ducreyi is the causative agent of the sexually transmitted disease chancroid. This disease, also called soft chancre, ulcus molle, chancre mou or Ducrey's infection, is described as an acute autoinoculable infectious disease usually affecting the genital region and manifested by painful ulceration (Alergant, 1972). The incubation period of the disease, the time from sexual contact to development of clinical symptoms, is suggested to be between one and 14 days (Asin, 1952). Others report incubation periods of one to five days (Alergant, 1972), two to five days (Gaisin and Heaton, 1975) and three to 12 days (Greenwald, 1943).

The first clinical symptom is the development of a small vesicle or papule with a narrow zone of erythema (Gaisin and Heaton, 1975) which rapidly becomes a pustule (Alergant, 1972; Gaisin and Heaton, 1975). The pustule breaks down to form an ulcer or lesion which is characterized as painful and shallow with a sharply circumscribed saucer shape. The edges of the ulcer are frequently ragged in appearance with an undermined or shelved character. The base of the ulcer is frequently covered by a necrotic, dirty looking, grayish exudate, and once removed reveals a floor consisting of an

uneven layer of vascular granulation. Ulcers range in size from 3 to 20 mm. in diameter and are usually multiple -- a characteristic apparently dependent on the autoinoculability of the disease (Greenwald, 1943). Certain clinical varieties of chancroid have been described based upon the variation in size and destructiveness of the lesion (Alergant, 1972 and Gaisin and Heaton, 1975).

Lesions are usually found on the genitalia in both men and women; extragenital lesions are rare. In the male, the lesions are usually found on the prepuce, coronal sulcus, fraenum, and less often on the shaft of the penis or near the anus. Female lesions usually occur on the labia, forchette vestibule and clitoris (Alergant, 1972).

Along with genital lesions another common manifestation of chancroid is regional lymphadenopathy in the groin (bubo formation). Buboes have been reported to accompany chancroidal lesions in the range from 33 to 56% (Greenwald, 1943; Satulsky, 1943; Alergant, 1972) and are most often seen one to two weeks after the appearance of the ulcer. Buboes are usually unilateral although some authors have noted bilateral involvement (Satulsky, 1943; and Alergant, 1972).

Chancroid is believed to have a world wide distribution, although it is more common in tropical and subtropical areas. Alergant (1972) has also suggested that there exists a close association between chancroid and personal hygiene. The incidence of chancroid has risen sharply during war time. Asin's (1952) report on American servicemen in the Korean War indicates that

chancroid was 14 to 21 times as common as syphilis and two times as common as gonorrhoea. Kerber et al (1969), in a report on chancroid in American servicemen in Vietnam, states that the appearance of chancroid was second only to gonorrhoea among the diagnosed venereal diseases.

Other venereal infections often accompany the appearance of chancroid. Various authors (Greenwald, 1943; Strakosch et al, 1945; and Asin, 1952) have reported the presence of chancroid with simultaneous infections of syphilis, gonorrhoea, granuloma inguinal and lymphogranuloma venereum (LGV).

The introduction of sulpha drugs in the mid 1930's provided the first effective treatment of chancroid. Hanschell (1938) was first to use sulfanilamide to successfully treat chancroid. In 1943 sulfanilamide was replaced with sulfathiazole and Strakosch et al (1945) reported that 90% of patients treated with sulfathiazole were cured within 10 days. Recently Kerber et al (1969) found clinical failure rates of 70% with tetracyclines and 11% with sulfonamides in chancroid patients in Vietnam. However, a combination of both tetracycline and sulfonamides proved effective.

1:2 Causative Agent of Chancroid

Ducreyi (1889) is credited with discovering the causative agent of chancroid. By successive inoculation of patients with pus from chancroidal ulcers and by a series of re-inoculations with the

pus from the induced ulcer, Ducrey was able to show that a bacterium of 1.48μ in length and 0.50μ wide with rounded ends was responsible for the production of the chancroidal ulcer. Kefting, in 1892, confirmed this finding of a short bacillus in the pus from the bubo associated with chancroid. Unna (1892) in tissue sections from chancroidal lesions, also described a similar bacillus occurring in long chains, which he found consistently in the lesions. Other investigators (Peterson, 1893; Nicolle, 1893; and Cheinesse, 1894) also confirmed the identity of the organism from chancroid lesions. As stated by Davis in 1903, it was generally accepted at the time that the bacillus in question was about 1.5μ in length and 0.5μ wide with rounded and deep staining ends and a fainter staining central portion. This bacillus occurred consistently in the pus of chancroidal ulcerations and less frequently in the pus of chancroidal buboes. The bacterium was found both singly and in masses intercellularly as well as intracellularly. This bacillus was also noted to have a characteristic chaining pattern, stained easily with basic aniline dyes, was decolorized by alcohol and therefore appeared Gram-negative.

Early investigators found that ordinary media would not permit the growth of the Ducrey's bacillus. Lenglet (1898), Besancan et al (1901), Himmel (1901) and Davis (1903) were some of the first investigators to grow the organism. They found that agar media with either human or animal blood, sera, or pulverized skin would support the growth of the bacteria at 32-37^{o}C. Lwoff

and Pirosky in 1937 demonstrated a requirement for hemin (X factor), a heat stable component of blood. Although other investigators (Sanderson et al, 1940; and Beeson, 1946) could not show X or V factor (nicotinamide-adenine dinucleotide) (NAD) requirement, Hammond et al (1978b) reaffirmed the requirement for X factor with recently isolated strains of H. ducreyi.

Growth on solid media produced colonies 1-2 mm that appeared as bright globules at 24 hours which became opaque and grayish at 48 hours. After 72 hours growth or longer, a small zone of haemolysis could appear at the periphery of a colony (Beeson and Heyman, 1945). Many authors noted that the colonies remained intact when pushed across the agar surface, a feature described as characteristic of the Ducrey bacillus. The early discovery of the requirement for a blood fraction (factor X), the colonial and the light microscopic morphology prompted investigators to classify the Ducrey bacillus in Haemophilus with the species as H. ducreyi.

1:3 Ultrastructure

Early investigators found that the bacillus they isolated from chancroid lesions varied in its Gram reaction. Nicolau and Bancrou (1926) noticed that on initial isolation their strains gave a negative Gram reaction; however, after subculture the strains appeared Gram-positive. Assis (1926) pointed out that the Gram reaction was dependent on the method used for fixation

prior to staining. Organisms that were affixed to the slide with alcohol stained Gram-positive and those fixed by heat stained Gram-negative. Reymann (Ph.D. thesis, Copenhagen, 1951) in a comprehensive study of H. ducreyi, described his strains as Gram-negative. However, the authenticity of these isolates is in doubt (Kilian, 1976). Deacon et al (1954) reported the isolation of a Gram-positive, smooth phase of an organism classified as H. ducreyi, although subsequently these authors (Deacon et al, 1956) realized they had been working with Corynebacterium acnes and not H. ducreyi. The conflicting results of Nicolau and Bancrou (1926), and those of Deacon et al (1954) might be explained by the isolation of organisms other than H. ducreyi from chancroid lesions.

The polymicrobial flora of genital ulcers (Chapel et al, 1978), the lack of specificity of the clinical diagnoses of chancroid (Chapel et al, 1977), the fastidious nutritional requirements and the absence of unique biochemical characteristics of H. ducreyi are features which could contribute to such a misclassification.

Kilian and Theilade (1975) were among the first to work with well characterized strains of H. ducreyi. In an electron microscopic study they showed that three of Reymann's strains had cell walls characteristic of Gram-positive bacteria. The biochemical and nutritional characteristics of the Reymann strains were also inconsistent with those of the genus Haemophilus. In contrast, the Paris strain (CIP 542) was found to possess a cell wall consisting of an outer membrane and a poorly defined medium

electron dense layer situated between the outer and cytoplasmic membranes. Kilian and Theilade felt that the Paris strain possessed a typical Gram-negative cell wall. This strain was also shown to require X factor and have the biochemical characteristics consistent with Haemophilus species.

Other electron microscopic studies on organisms described as H. ducreyi (Cazane and Barretto, 1974; Ouchinnikov et al, 1976; and Marsch et al, 1978) also report the presence of a typical Gram-negative cell wall; the generic status of the strains examined was not confirmed by other tests. As exemplified by Marsch et al's (1978) report, H. ducreyi was described as a rod 1.25-1.40 μ long and 0.55-0.60 μ broad with rounded ends and a cell wall 115 to 125 A^{o} thick. To date, no other morphological features of H. ducreyi have been reported.

1:4 Clinical Studies

Nicolle (1893) was the first investigator to produce a lesion similar to that found in chancroidal infections in experimental animals. The lesion was produced in a single rhesus monkey by inoculating intradermally with the pus from a chancroid lesion.

However, Himmel (1901) reported that in the guinea pig intraperitoneal, subcutaneous or intradural injections with H. ducreyi cultures produced no response. Himmel reported that 48 hours after injection he could recover viable H. ducreyi from the

peritoneum of a guinea pig although no lesion was apparent. Stressing the animal by placing it in a 4-5°C environment extended the survival of H. ducreyi in the peritoneum for five days. Further attempts to stress the guinea pig by injecting with lactic acid or antialexin prior to the inoculation with H. ducreyi resulted in their death. Himmel postulated that decreasing cellular responses of guinea pigs to H. ducreyi resulted in increased pathogenicity of H. ducreyi organisms.

Later investigators (Davis, 1903; and Saelhof, 1924) confirmed the observation of Nicolle (1893) that small pustules or ulcers could be produced by inoculating the skin of monkeys with cultures of H. ducreyi. In 1911 Fontana was able to produce keratitis in rabbits using the pus from chancroidal ulcers and Reenstierna (1921) also reported the production of lesions in four out of ten rabbits by puncturing their scrotum with a knife dipped in a culture of H. ducreyi.

Saelhof (1924) failed to infect rabbits by subcutaneous injections of H. ducreyi or by injecting the organism directly into lymph glands. However, Saelhof produced lesions in monkeys following intradermal injections of H. ducreyi.

Maximowa (1936) was the first investigator to use intradermal injections of H. ducreyi to produce lesions in rabbits. He found that 48-72 hours post inoculation a necrotic pustule developed with induration limited to the area around the injection site. He also noted that rabbits previously infected by this

method were not immune to reinfection. Reinfections often resulted in larger and more necrotic lesions.

Another animal model which was shown to be successful in demonstrating a reaction to H. ducreyi were the extra embryonic membranes of chicks. Anderson and Snow (1940) produced "first generation" lesions in the chorioallantoic and amnionic membranes of chick embryos but were unable to transfer the infection for more than two passages.

During a series of experiments on the prophylaxis of chancroid disease, Greenblatt et al (1943a) produced typical local chancroidal skin lesions in human volunteers by scarifying the skin and applying one or two drops of an inoculum of H. ducreyi. Only cultures of recently isolated strains produced skin lesions. Stock cultures kept by repeated transfer for three years were incapable of producing lesions, although one of these cultures had previously produced lesions in volunteers. These workers also noted that a heavy inoculation of H. ducreyi was required to produce lesions. Although actual numbers of bacteria were not given, Greenblatt et al (1943a) noted that an inoculation dilution greater than 1:50 substantially decreased the number of successful lesions. Intravenous, intraperitoneal and intratesticular inoculations of H. ducreyi cultures into mice, rabbits, rats, hamsters and dogs failed to produce a reaction. In addition, inoculation of organisms suspended in mucin failed to produce any lesions.

Maximowa's experiments with rabbits and Greenblatt et al's

work with human volunteers was confirmed in 1945 by Feiner and Mortara. They also found that injections of large doses of viable H. ducreyi subcutaneously, intraperitoneally or intravenously were without effect in the rabbits. Similarly, intracerebral and intraperitoneal injections of mice with H. ducreyi cultures suspended in mucin were also ineffective. However, Feiner and Mortara (1945) were successful in producing lesions in the rabbit by intradermal injections of viable H. ducreyi. Viable H. ducreyi could be recovered from these lesions up to 72 hours after the inoculation. In common with previous authors, Feiner and Mortara reported that large numbers of organisms were needed to initiate lesions and that a 1:100 dilution of their inoculum seldom produced a response.

Examination of the immunity produced in rabbits after injection confirmed the observations of Maximowa (1936). Reinfection was possible and more extensive lesions were produced. Extending these observations Feiner and Mortara demonstrated skin hypersensitivity in infected animals using a suspension of heat killed H. ducreyi. Hypersensitivity to heat killed organisms developed seven to 14 days after the initial response and lasted for at least three months. Control uninfected rabbits did not respond to intradermal injections of heat killed organisms. Feiner and Mortara (1945) also reported that their stock strains of H. ducreyi, like those of Greenblatt et al (1943a) in the human, failed to produce lesions when injected intradermally in rabbits.

Mortara and Saito (1947) reported that they were able to protect against the development of the H. ducreyi lesion in rabbits by giving an intramuscular injection of streptomycin hydrochloride within 24 hours of the intradermal injections.

Deacon and co-workers (Deacon et al, 1956; Kaplan et al, 1956a, 1956b) confirmed previous reports with regard to the production of lesions in rabbits by intradermal injections but they were initially unable to produce lesions in these animals by skin scarification. Scarification of male or female rabbit genitalia also produced no lesions and experiments using female rabbits with the vagina contaminated with H. ducreyi showed that the infection was not transferred to males during copulation. Only in previously sensitized rabbits could lesions be provoked by an inoculation on broken skin. Deacon and co-workers (Kaplan et al, 1956b) also reported that rabbits in which diabetes mellitus was induced with alloxan, were no different in their reaction to live H. ducreyi cultures than control animals. These investigators also found no difference in lesions produced in the rabbit's abdomen or back. However, Kaplan et al (1956b) did show that the virulence of H. ducreyi strains could be increased by passage through fresh blood clots. This increase in virulence resulted in lesions of greater size and intensity after inoculation of the passaged strains onto scarifications on the backs of previously sensitized rabbits.

An important observation in the early literature was the variation in the ability of H. ducreyi to produce lesions in the

human or rabbit model. It was also found that the avirulent strains usually developed as a result of multiple subculturing. Other studies noted that virulent and avirulent strains of H. ducreyi could be distinguished by other characteristics. Thayer et al (1955) suggested that there might be some relationship between the in vitro antibiotic sensitivity of H. ducreyi strains and their ability to produce lesions in rabbits. These authors found that a virulent strain of H. ducreyi was more resistant to polymyxin than avirulent strains. Singer and Deacon (1956) also noted that their avirulent strains of H. ducreyi differed from virulent strains in their sensitivity to penicillin G and other antibiotics.

Hammond et al (1978c) compared the antibiotic sensitivities of stock cultures and recent isolates of H. ducreyi. They reported that four avirulent stock cultures were more sensitive to a total of 13 antimicrobial agents than 19 virulent H. ducreyi isolates. One of the most striking differences was the high level of resistance to polymyxin shown by the virulent strains as compared to that shown by the avirulent strains.

1:5 Histology of Clinical and Experimental Chancroid Lesions

Reenstierna (1921) reported that the histological picture of chancroid lesions in rabbits was similar to that seen in man. Chancroidal lesions in man have been studied extensively and Pund

et al (1938) classified the histology and showed that the lesions could be differentiated histologically from other genital ulcers. The chancroidal lesion was described (Pund et al, 1938) as being characterized by superficial necrosis infiltrated with polymorphonuclear leukocytes (PMN's). In the area surrounding the lesion plasma cells, lymphocytes and endothelial leukocytes were seen. It was proposed that the necrosis seen in the lesion was due to an acute perivasculitis and endovasculitis producing swelling of the endothelium, which frequently resulted in occlusion of the lumen of the associated capillaries. Pund et al (1938) felt that the vascular changes seen were characteristic of chancroid lesions.

Sheldon and Heyman (1946) reported on the biopsies of 45 lesions diagnosed as chancroid. They describe the general picture of lesions of about two to three week's duration which rarely extended to more than two or three millimeters in depth. Under low magnification, the histological sections could be divided into three zones. The surface of the sections or surface zone, represented the base of the ulcer. This zone was composed of necrotic tissue, red blood cells, fibrin and large numbers of neutrophilic PMN's. Below the base of the ulcer a mid-zone could be detected which was formed from oedematous tissue with numerous blood vessels which were prominent and dilated. The vessel walls in this zone were thin and newly formed. Endothelial cells in various stages of proliferation were the dominant cell type in this region. At the union of the midzone and surface layer these endothelial cells

were necrotized whereas in other areas they developed into capillaries. Pre-existing and newly formed vessels often showed fibrinoid degeneration of the walls, with margination and infiltration by PMN's. Little proliferation of fibroblasts could be detected in the mid-zone.

Below the mid-zone a third layer (the deep zone) displayed a diffuse infiltration by numerous plasma cells together with some lymphocytes. No degeneration of the vessel walls, leukocytic infiltration or thrombus was present.

Sheldon and Heyman (1946) felt that the histological character of the chancroid infection as viewed in their study was sufficiently distinct to permit diagnosis and to differentiate this condition from other genital lesions.

Summarizing the findings above, the histological features of the chancroid lesion are:

(1) the zonal character of the inflammatory reaction;

(2) the marked endothelial proliferation in the mid-zone which leads to vascular changes;

(3) the meager fibroblastic response in the mid-zone;

(4) the dense infiltration with plasma cells and lymphocytes in the deep zone.

1:6 Serological Studies

Reports of the serological examination of *H. ducreyi* began with Gallia's (1907) studies of human chancroid. He concluded

from complement fixation using a whole cell antigen that specific antibody production occurred in response to infection. In 1922 Teague and Diebert tried to induce an antibody response in rabbits to H. ducreyi. They found that rabbits injected with varying doses of H. ducreyi suspensions and inoculated at different time intervals produced sera that exhibited variation in activity when tested by agglutination of suspensions of H. ducreyi. Teague and Diebert also found that some normal rabbit sera produced agglutination. However, from their results these authors did feel "there was no serological difference between the 10 or 15 strains of H. ducreyi used". Further attempts by these authors to immunize chickens produced sera that gave similar results to those obtained from the rabbits.

Saelhof (1924) also attempted to serogroup strains of H. ducreyi using rabbit antisera and obtained results essentially in accord with those of Teague and Diebert (1922). Both of these reports mentioned that agglutination tests were inconsistent with normal sera often producing agglutination. These authors both noted the difficulty in obtaining a homogenous suspension of H. ducreyi in saline suspensions due to the spontaneous clumping of the organism. Saelhof also determined the opsonic index and point of opsonic extinction in serum from chancroid patients. The point of opsonic extinction of the sera from chancroid patients varied from 1:48 to 1:384 with an average of 1:96. Normal sera had an average point of extinction of 1:16. No definite serogrouping of

H. ducreyi was obtained by this method.

Greenblatt and Sanderson (1938b) studied the response of chancroid patients to an intradermal injection of a heat killed preparation of whole cell H. ducreyi (the Ito-Reensterina test). On the basis of the reactions obtained in these patients using a variety of H. ducreyi strains these authors concluded that the various strains used had a homogeneous antigenic structure.

Dienst (1948) tested patients and sensitized rabbits by injecting heat killed virulent and avirulent strains intradermally. He showed that the reactions in rabbits and man were identical. This was confirmed by Kaplan et al (1956b).

Reymann (1950a) also found that two chancroid patients reacted identically to four strains of H. ducreyi when tested by the intradermal skin test. In an examination of a single H. ducreyi strain using an agglutination test he confirmed the autoagglutination of H. ducreyi. In addition, Reymann examined several H. ducreyi strains by complement fixation and precipitant reactions. Complement fixation tests suggested that there was only one serological type. A similar result was obtained from direct precipitation tests which indicated that there was complete serological identity between the H. ducreyi strains.

The work of Denys et al (1977) is the most recent report of the serological homogeneity of H. ducreyi. These authors used an indirect fluorescent antibody test to examine strains from varying geographical locations. All strains gave similar reactions with a

rabbit antiserum against a single strain of H. ducreyi.

This review of H. ducreyi has noted the current state of knowledge of isolation, antimicrobial susceptibility, virulence, ultrastructure and serology of this unique organism. However in some instances, the literature is confusing and much of this confusion can be related to the doubtful identity of the isolates used. The more recent studies, those of Kilian (1976), and Hammond et al (1978 a,b,c) and Denys et al (1977) did use well characterized isolates. However confirmation of some characteristics, particularly ultrastructure, virulence and serology are needed. The results of studies of virulence and serology could have a direct relationship to clinical investigation as skin tests and the direct measurement of antibody levels could aid in diagnosis. In addition, the production of fluorescein-labelled antibodies (specific for H. ducreyi) could enable the identification of the pathogen in smears from lesions. Denys et al (1977) have suggested that this technique is possible.

Further studies of the H. ducreyi antigens are essential to refine methods for serological procedures.

Chapter 2

Materials and Methods

2:1 Origin of Bacterial Strains

Haemophilus species and strains were acquired from various sources as indicated in Table 2:1. All Winnipeg strains were isolated from patients at the Primary Health Centre of the Health Sciences Centre (HSC) in Winnipeg, Manitoba, Canada. *Escherichia coli* Y10, an F^+ pilated strain was obtained from J. Brunton of the University of Manitoba. *Staphylococcus aureus* ATCC 2493 and *E. coli* ATCC 25992 type strains were obtained from the American Type Culture Collection (ATCC), Rockville, Maryland, U.S.A.

Table 2:1

Origin of Haemophilus Species and Strains

Haemophilus Species	Strain No.	Origin And/Or Source
H. ducreyi	35000	HSC, Winnipeg, Man.
H. ducreyi	35199	HSC, Winnipeg, Man.
H. ducreyi	78118	HSC, Winnipeg, Man.
H. ducreyi	54211	HSC, Winnipeg, Man.
H. ducreyi	54239	HSC, Winnipeg, Man.
H. ducreyi	A75	Institute Pasteur, Paris, France
H. ducreyi	A77	Institute Pasteur, Paris, France
H. ducreyi	CIP.542	Institute Pasteur, Paris, France

H. ducreyi	6V	Center for Disease Control, Atlanta, Georgia
H. ducreyi	4391	Univ. Goteborg, Goteborg, Sweden
H. ducreyi	36-F-2	Received from M. Kilian (originally lyophilized by M. Pittman, 1948)
H. influenzae	B031	HSC, Winnipeg, Man.
H. influenzae	1-637	HSC, Winnipeg, Man.
H. influenzae	1-567	ATCC
H. influenzae	3-545	HSC, Winnipeg, Man.
H. parainfluenzae	2-004	HSC, Winnipeg, Man.
H. pleuropneumonia	0-1	ATCC
H. paraphrophilus	0-1	ATCC
H. gallinarum	17551	U.S. Dept. of Agriculture

2:2 Methods Used to Grow and Characterize *H. ducreyi* Strains

2:2:1 Cultural Characteristics

Previous workers (Hammond et al, 1978c) had suggested that *H. ducreyi* strains required CO_2 and a moist atmosphere for growth. Consequently, all cultures were grown in a moist atmosphere with 5-10% CO_2. Unless otherwise indicated, all *H. ducreyi* strains were grown at 35°C for 24 hours prior to use.

2:2:1 a Solid Media

Growth was tested in the following solid media: (a) blood agar, (b) 5% defibrinated sheep's blood chocolate agar (SBCA),

(c) GC agar base, (d) nutrient agar, (e) chocolate agar (CA). (See Appendix 1 for media formulae.)

2:2:1 b Liquid Media

All strains of H. ducreyi were tested for growth in three liquid media: (a) Mueller Hinton broth (MHB), (b) Eugon broth, and (c) brain heart infusion broth, with and without 0.1% hemoglobin. (See Appendix 2 for broth formulae). A 24-hour culture of H. ducreyi from CA was suspended in each broth and diluted to a concentration of 10^9 CFU/ml. Two ml of this suspension was inoculated in 150 ml of the respective broth. Flasks were agitated in a 5% CO_2 incubator at 35°C. Each broth was examined at 48 hours for any visual increase in turbidity.

2:2:1 c Biphasic Media

As indicated in the techniques for electron microscopy (2:3:3), H. ducreyi strains were grown in biphasic media, with CA as the solid phase overlain with 200 ml of MHB. Biphasic media were inoculated with a 1.0 ml MHB suspension (approximately 10^8 CFU/ml) from a 24-hour CA culture. Organisms could be removed from the liquid phase of the biphasic media with reduced manipulation, thereby preventing disruption of cells.

2:2:2 Morphological Characteristics

2:2:2 a Gram Stain

A saline suspension of H. ducreyi was heat-fixed to a glass slide and Gram stained by Hucker's modification (Hucker & Conn, 1923).

2:2:2 b Light Microscopic Capsular Staining

H. ducreyi colonies were stained by a wet-film India ink method according to Duguid (1951).

2:2:2 c Colonial Morphology

Colonial morphology of H. ducreyi was recorded after 24, 48 and 72 hours growth on CA. Colonies were viewed by hand lens (x5) under normal laboratory lighting.

2:2:3 Physiological and Biochemical Characterization

Tests for a requirement for X (Hemin) or V (Nicotinamide adenine dinucleotide, or other definable coenzymes) factors were made using a routine satellite growth test on supplemented GC agar base (Gibco) as described by Hammond et al (1978b). Confirmation of X factor requirement was sought using the porphyrin test (Kilian, 1974).

Attempts were made to test the activities of H. ducreyi in routine carbohydrate fermentation broths. In addition, tests were done in the API-20E^{R} (Analytab Products, Plainview, N.Y.). Twenty-four hour colonies from CA were suspended in physiological saline and inoculation of the API-20E strips followed procedures as outlined by the manufacturer.

2:2:4 Polymyxin Minimum Inhibitory Concentrations

The minimum inhibitory concentrations (MIC) for polymyxin B (Pbx) (Sigma Chemical Co., St. Louis, Mo.) was determined following the agar dilution technique (Ronald et al, 1969) as used by Hammond

et al (1978c). S. aureus ATCC 24923 and E. coli ATCC 25992 were used as the resistant and sensitive controls respectively. The agar dilution was carried out in duplicate on all H. ducreyi strains except 4391 and 36-F-2 which were not tested. This technique measured the growth on CA plates containing from 0.025 to 256 μg/ml Pbx in a $\log_2$ dilution series. The MIC of Pbx was considered to be the lowest concentration that allowed growth of three colonies or less or that resulted in a haze that did not appear to be elevated above the surface of the agar when a hand held lens (x5) was used. MIC's were read after 48 hours incubation.

2:3 Electron Microscopy

2:3:1 Negative Staining

One, two and three day old cultures of H. ducreyi were removed from CA surfaces and suspended in sterile physiological saline (approximately 10^8CFU/ml). A drop of the suspension was spotted on a Formvar (Polysciences Inc., Warrington, PA.) 400 mesh copper grid and excess liquid was removed with a filter paper. Grids were then stained with 1.5% phosphotungstic acid, pH 8.6, air dried and examined in a Philips 201 electron microscope.

2:3:2 Fixation Techniques

All fixation procedures followed standard electron microscopic techniques and are essentially those used by Mackie et al (1979). (These procedures were initially described by J.W. Costerton,

University of Calgary in personal correspondence.)

2:3:2 a Normal Fixation

Cells were suspended in MHB and swabbed onto CA. After 18 hours growth, colonies were prefixed by adding 0.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (SC buffer), pH 6.6, for one hour at room temperature. Colonies were removed from the CA by pipetting off the prefix solution and gently spun in an Adams Rynac (Clay Adams Inc.) desk top centrifuge at 6000 rpm for ten minutes ($\sim$ 720 g). Pelleted bacteria were embedded in 4% Bacto agar (Difco) and drawn into cores. The agar cores were then fixed in 5% glutaraldehyde in SC buffer for two hours, washed in SC buffer (five times) and post fixed in osmium tetroxide for two hours. They were then washed in SC buffer (five times) and dehydrated in a graded series of acetone/water mixtures (30,50,70,90,100%). Cores were placed twice in propylene oxide for one-half hour and then left overnight in a 3:1 propylene oxide/Vestophal W (Polyscience Inc.) solution. The next day, after the propylene oxide had evaporated, the cores were removed and placed in fresh Vestophal W in agitated tubes. After four hours the cores were embedded in fresh plastic and cured for 14 hours at 60°C.

2:3:2 b Fixation Combined with Stains

Ruthenium red (RR) and Alcian Blue (AB) (Polyscience Inc.), two polyanionic stains, were used to demonstrate the presence of capsular substances which contain negatively charged acidic groups (Springer & Roth, 1973; Cassone & Garaci, 1977; Mackie et al, 1979).

In the RR and AB staining methods, the stains were incorporated into the normal fixative. Both stains were added to the fixing fluid SC buffer at a concentration of 0.15% (wt/vol). The stains were present in the fluids during the fixation process from prefixation in 0.5% glutaraldehyde until the first (10%) step of dehydration in acetone.

2:3:3 Pretreatment of H. ducreyi and H. influenzae with Antisera

Pretreatment of bacteria with homologous antibodies has been used to stabilize surface components (Baker and Loosli, 1966; Doern and Buckmire, 1976). In the present study this method was used to attempt to retain capsular material on Haemophilus species.

H. influenzae B031 and H. ducreyi from an eight hour and 24 hour culture respectively, were pretreated by adding type b (H. influenzae) specific Burro and H. ducreyi whole cell rabbit antisera (see section 2:5:2 a) diluted 1:1 with distilled water. The antisera was added directly to the CA plate for one hour before prefixation.

H. ducreyi grown in the biphasic media was separated from the liquid phase by mild centrifugation and also treated with H. ducreyi antisera diluted 1:1 with water. Prefixation in both cases (CA or biphasic growth) was continued as usual or in some cases, with the addition of RR or AB.

2:3:4 Thin Sectioning Techniques

Ultra thin sections were cut from the plastic specimen blocks on a LKB 8800 Ultratome (Broma, Sweden) with glass knives.

Sections were placed on 400 mesh copper grids and post stained with 1% uranyl acetate, followed by 1% lead citrate. Grids were examined in a Philips 201 electron microscope.

2:4 Routine Histological Techniques

Experimental lesions produced as described in section 2:5 d were biopsied to determine the histological changes that had occurred in these lesions, compared to control sites in normal skin. At days 2,4,6,8 and 11 post inoculation, the response produced by each strain (H. ducreyi 54211 and A77) was biopsied and fixed in 10% formaldehyde and processed to paraffin wax (by Pathology Laboratory, Health Sciences Centre). Sections were stained with hematoxylin-eosin and the histological features described (Dr. W. Curry, Dept. of Pathology, University of Manitoba). Photomicrographs were taken using a Leitz Ortholux II (Germany) microscope.

2:5 Experimental Animal Studies and Antisera Production

2:5:1 Rabbit Virulence Model

The virulence of H. ducreyi strains was tested by the intradermal injection of 0.20 ml of a MHB suspension of viable organisms (Hammond et al, 1978c). These suspensions contained approximately 10^9CFU/ml (as indicated by a No. 4 McFarland Barium sulphate opacity standard) and were made by scraping colonies from a 24-hour culture on CA and suspending in MHB. In one experiment,

the suspensions were diluted in $\log_{10}$ dilutions from 10^9 to 10^6 CFU/ml. All injections were made in separate sites on the backs of previously shaved 2-3 kg, one year old female New Zealand white rabbits. Uninoculated MHB and heat killed (60° for 30 minutes) suspensions served as controls and were inoculated intradermally into each of the rabbits used for virulence testing.

Induration and necrosis were determined daily from day 1 until day 11 post inoculation. The criteria for virulence were as follows: by day 4, induration measuring $\geq$ 0.5 cm which progressed to an eschar by day 11.

In one series of experiments, samples of pus from the lesions were swabbed onto CA and CA plus 3 µg/ml Vancomycin (CAV) (Eli Lilly & Co., Toronto, Ontario). Lesions were swabbed once daily until cultures for H. ducreyi were negative.

Biopsy samples (sect. 2:4) were also cultured. A portion of the biopsy was placed in 2.0 ml of MHB and vortexed for two minutes. The broth was removed from the tube and plated out onto CA and CAV and examined for growth of H. ducreyi at 24, 48 and 72 hours.

2:5:2 Preparation of Antisera

2:5:2 a Antigen Preparation

Whole cell H. ducreyi antigen preparations were collected by scraping 24-hour culture of H. ducreyi 54211 from CA. Bacteria were suspended in 0.5% formalized saline and diluted to 1.5 - 2.0 x 10^9 CFU/ml. A cell extract of H. ducreyi 54211 was also prepared

according to Buckmire's (1976) method for crude capsular antigen.

DEAE Sephadex pooled fractions (sect. 2:6:4) were also used as antigen preparations where indicated.

2:5:2 b Immunization Schedule

All rabbits' antisera was tested (by double diffusion - 2:6:2) against whole cell sonicates of H. ducreyi 54211 prior to immunization to ensure the nonreactivity of baseline antisera.

2:5:2 bI Antisera Against Whole Cells

Antisera against whole cell H. ducreyi 54211 was prepared by injecting two, female, 2-3 kg white New Zealand rabbits with a formalized whole cell saline suspension according to the schedule of Alexander et al (1946). Antisera were tested for activity and after three complete schedules of immunization, the activities remained low. Consequently, one rabbit was injected intradermally with viable organisms as described in section 2:5:1 to initiate a lesion. The second rabbit was injected intradermally with four 0.1 cc inoculations of a 1:1 dilution of cell extract and Freund's Complete Adjuvant (Difco). This rabbit was rested for eight weeks and then injected intravenously with cell extract in three doubling doses weekly, starting with 0.1 ml. These two rabbits were bled and antisera was collected two weeks after the initiation of the lesion and two weeks after the last cell extract injection respectively. All sera was stored at -70°C until used. The sera from the rabbit that received the cell extracts was found to be more reactive by double diffusion. This sera is referred to as H. ducreyi

whole cell antisera and was used for the electron microscopic studies (2:3:3) and testing column fractions (2:6:4).

2:5:2 bII Pooled Fraction Antisera

Pooled fractions three to six from the DEAE Sephadex A50 column (see sect. 2:6:4 b) were freeze dried and diluted in distilled H_2O to a concentration of 2.0 µg/ml. Female New Zealand white rabbits, approximately 1.5 kg in weight, were used for production of antisera to the pooled fractions. Two rabbits were used for each fraction and the antisera tested against a cell sonicate (2:6:1) range of antigens. The antisera from different rabbits were not pooled. Following essentially the schedule of Bowden et al (1976), a 1:1 mixture of Freud's Complete Adjuvant (Difco), and each pooled fraction was emulsified by passage between two 10.0 ml hyperdermic syringes connected by a double headed needle. Four areas on the back of a rabbit, just above the limbs, were clipped and shaved. Approximately 0.15 ml of each antigen emulsion was injected intradermally into each site on two rabbits. In addition, approximately 0.5 ml was injected intramuscularly into each hind limb.

After this initial injection, the animals were rested for three weeks. At this time, each rabbit was given an 0.25 cc intravenous booster of antigen without adjuvant. Prior to this injection, animals were given an intraperitoneal injection of Phenergan (Poulenc Ltd., Montreal, P.Q., Canada), 2.5 mg/kg to avoid harmful allergic reactions. One week later, a second 0.25 cc

intravenous booster injection of antigen was given to each rabbit.

Two weeks after the second intravenous injection, the animals were bled from the central ear artery and sera collected and stored at -70°C until used.

2:5:2 c Antisera from Other Workers

H. influenzae type b antiserum was graciously provided by J.B. Robbins, National Institute of Child Health and Human Development, Bethesda, Md. The antiserum was produced in burro against H. influenzae type b, strain Rab (Alexander et al, 1946).

2:6 Antigenic Analysis of H. ducreyi

2:6:1 Preparation of Whole Cell Sonicates

It has been shown that whole cell sonicates provide a comprehensive spectrum of the antigens recognized on bacterial cells (Smith et al, 1976 and Hoff & Høiby, 1978). For this reason whole cell sonicates were used to test all sera for the range of antibody response. Whole cell sonicates were prepared by taking colonies of Haemophilus species (Table 2:1) and suspending them in 0.1 M tris-HCl buffer (tris buffer) at pH 7.0. These suspensions were centrifuged and pellets resuspended in tris buffer. Sonicates were then produced by sonicating a 1.0 ml aliquot in a Biosonik IV (Bronwill Scientific, Rochester, N.Y.) until complete lysis (checked by phase contrast microscopic examination) had occurred. Whole cell sonicates were diluted to a protein concentration of between

3.5 and 5.0 mg/ml as determined by the Micro Biuret method (Zamenoff, 1957) using bovine serum albumin (Sigma Chemicals) as a standard.

2:6:2 Double Diffusion

Double diffusion was carried out using ID Agar tablets (Oxoid, London) melted in 50 ml of 0.025 M Barbitone Acetate Buffer prepared by diluting Oxoid Buffer (Oxoid, London, England) 1:4 with distilled water. The melted agar was cooled to 50°C and 10 ml was allowed to flow over the surface of a 3¼ x 3¼ inch clean glass plate (Kodak Ltd., London, England). Wells of standard pattern were cut in the agar after the gel had hardened.

Antigens and antisera for test were placed in the wells, the arrangement depending upon the reactants used. Plates were left in a moist petri dish for 24 hours at room temperature and for an extended period of up to 96 hours at 4°C and then for four to six hours in distilled water at room temperature. Drying was carried out by placing filter paper (Whatman No. 54) over the surface of the plate and allowing the water to evaporate until the paper and plate were just damp. The paper was then removed and the plates allowed to dry in the air. Finally the plates were placed at 37°C for one hour.

A permanent record was made by staining the dried gels. Dried gels were fixed in 2% aqueous acetic acid for three to five minutes and then stained in the following stain for one hour:

Glacial acetic acid	10 ml
Ethanol	90 ml
Comassie Brilliant Blue R 250 (BDH, Poole, England)	2 gms

The stained plates were rinsed rapidly in tap water and then decolorized in:

Glacial acetic acid	10 ml
Ethanol	85 ml
Distilled water	5 ml

Differentiation was continued until the precipitin lines showed up clearly on a clear background.

2:6:3 Immunoelectrophoresis

Immunoelectrophoresis was carried out using a multiphor (LKB Products, Bromma, Sweden) and an LKB power pack. The techniques employed followed those outlined in the manual supplied by LKB (Axelsen, 1973). In general, throughout this study, immunoelectrophoresis in a single direction was made at a voltage of 10 volts/cm for 30 minutes at 5°C. With two-way and rocket immunoelectrophoresis, the second dimension into the antisera containing gel was run at 2 volts/cm for 16 hours at 5°C. The optimum level of reactants was determined by trial runs and the figures (3:15 & 3:16) show the best results obtained. Drying and staining of the gels was done in the same way as described for double diffusion (sect. 2:6:2).

2:6:4 Separation of H. ducreyi Whole Cell Sonicates

Separation of the antigenic components of extracts of H.

ducreyi54211 was attempted by chromatography using molecular sieve and ion exchange chromatography.

2:6:4 a Chromatography on Sepharose 4B

A whole cell sonicate of H. ducreyi 54211 was fractionated on Sepharose 4B (Pharmacia, Upsala, Sweden). The column was 1.0 x 80 cm, the column volume 78 ml, with a Vo of 24.0 ml (measured by the exclusion of blue dextran) (Pharmacia, Sweden). Buffer was gravity fed at a rate of 0.1 ml/min. The fractions were collected by a Model 270 fraction collector (ISCO, Lincoln, Nebraska, USA). Columns were loaded with 5.0 ml of a 200 mg/ml (wet weight) whole cell sonicate of H. ducreyi 54211 in tris buffer. The absorbance at 280Å of each fraction was determined in a Unicam SP1800 spectrophotometer (Pye Unicam, Cambridge, England). Antigens in the fractions were detected by double diffusion (sect. 2:6:2). All fractions were stored at -20°C until used.

2:6:4 b Chromatography on DEAE Sephadex

A whole cell sonicate of H. ducreyi 54211 was gently spun in a desk top centrifuge. The supernant contained 22.5 mg/ml of protein and was used as the antigenic preparation separated on DEAE Sephadex A50 (Pharmacia, Sweden).

The column had a total volume of 18.0 ml and was packed with the DEAE Sephadex which had been equilibrated with 0.1 M tris/HCl buffer pH 8.0 at 4°C for 48 hours. Two ml of the WCS supernant was applied to the column and eluted in 0.1 M tris/HCl buffer pH 8.0 in a NaCl gradient (0.0 + 0.8 M NaCl pH 8.0). The electrical

conductance of each 0.5 ml fraction was measured on a CDM3 conductivity meter (Radiometer, Copenhagen, Denmark). Fractions were collected and tested for antigenic activity by rocket immunoelectrophoresis (sect. 2:6:3). Fractions containing antigens were pooled, dialyzed and freeze dried.

2:6:5 Heat and Enzyme Treatments

H. ducreyi whole cell sonicates and pooled fractions from the DEAE Sephadex (2:6:4 b) were heat treated at 100°C for 15 minutes. Whole cell sonicates were also treated with protease (No. P-5130 Sigma, St. Louis, Mo.) for 16 hours at 37°C. These protease treated samples were then heat treated, as previously mentioned, to inactivate the protease. Heat treated and protease treated samples were tested for their antigenic activity by double diffusion or rocket immunoelectrophoresis as indicated.

Results

3:1 Growth on Various Media

The growth of H. ducreyi appears limited to solid media which contains hemin. As indicated in Table 3:1, this includes sheep's blood chocolate agar and chocolate agar. The different broth media (MHB, Eugon and Brain Heart Infusion broths) did not support the growth of H. ducreyi even after the addition of hemin. It was only possible to maintain strains over long periods of time on CA.

3:2 Morphology Characteristics

3:2:1 Colonial Appearance

Colonies (of H. ducreyi) grown on CA after 24 to 48 hours incubation appeared as non-mucoid, yellow-gray globules. The 7-8 mm colonies were very cohesive and remained intact when pushed across the agar surface. This feature is considered characteristic of H. ducreyi (Beesen & Heyman, 1945 and Hammond et al, 1978c). After 72 hours growth, a green (alpha) zone of hemolysis was present on the CA directly under most colonies. This zone of hemolysis extended no further than 1-2 mm past the diameter of the colony.

3:2:2 Cellular Appearance

H. ducreyi appeared as a pleomorphic Gram-negative rod when viewed by the light microscope after Gram staining. The rods averaged approximately 1.5 x 0.5 μ with rounded ends. Organisms

Table 3:1

Growth of H. ducreyi on Various Media

Solid Media[1]	Growth	Number of Subcultures Possible
Blood Agar	$\pm$	≤ 2
Sheep Blood Chocolate Agar	+	∞
GC Agar Base	-	0
Nutrient Agar	-	0
Chocolate Agar	+	∞

Liquid Media[1]	Plus 0.1% Hemin		
Mueller Hinton Broth	-	-	0
Eugon Broth	-	-	0
Brain Heart Infusion Broth	-	-	0

[1] Composition of the various media is found in Appendix 1 and 2.

would often align themselves in parallel chains (the classical "school of fish" pattern), a feature characteristic of all of the H. ducreyi strains used in this study.

Duguid's (1951) wet-film India ink method for staining capsules did not reveal the presence of capsular layers in any of the H. ducreyi strains.

3:3 Physiological and Biochemical Characteristics

Nutritionally all H. ducreyi strains exhibited a requirement for X-factor (hemin) and were independent of V factor (Table 3:2). A negative porphyrin test confirmed the need for an exogenous hemin source in the growth media. The porphyrin test measures the ability of bacteria to utilize δ-aminolaevulinic acid in the biosynthesis of porphobilinogen and porphyrins. As the strains did not grow in broth, it was not possible to test the activities of H. ducreyi in routine biochemical and carbohydrate fermentation broth.

However, tests performed on the API-20E indicated that H. ducreyi is relatively inactive biochemically (see Table 3:2). The only difference noticed between strains in the API system was that CIP542 failed to ferment glucose and strain 54211 did not ferment glucose or arabinose. Nitrate reductase could only be demonstrated in strains 54211 and 78118. All strains were Voges-Proskauer and Oxidase positive.

Table 3:2

Nutritional and Biochemical Activities of H. ducreyi

H. ducreyi strain	o-galactosidase	Arginine dihydrolase	Lysine decarboxylase	Ornithine decarboxylase	Citrate	Hydrogen sulfide	Tryptophane deaminase	Indole	Voges-Proskauer	Gelatin liquefaction	Glucose	Mannitol	Inositol	Sorbitol	Rhamnose	Sucrose	Melibiose	Amygdalin	(+) Arabinose	Nitrate Reduction	Catalase	Oxidase	Urea	Growth on Media[1] Containing X Factor	V Factor	XV Factor
35000	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	+	-	-	+	-	+	-	+
35199	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	+	-	-	+	-	+	-	+
78118	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	+	+	-	+	-	+	-	+
54211	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-	+	-	+	-	+
54239	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	+	-	-	+	-	+	-	+
A75	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	+	-	-	+	-	+	-	+
A77	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	+	-	-	+	-	+	-	+
CIP542	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	+	-	+	-	+
6V	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	+	-	-	+	-	+	-	+
4391	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	+	-	-	+	-	+	-	+
36-P-2	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	+	-	-	+	-	+	-	+

[1]Media was GC Agar Base (Gibco) supplemented with 0.1% Glucose, 0.01% Glutamine and 0.01% Cystine. Strips of Hemin (1,000 µg), NAD (100 µg), and hemin with NAD (2000 µg and 100 µg respectively) (BBL Taxo stript) were placed aseptically on the agar surface to test for growth factor requirements.

3:4 Polymyxin Minimum Inhibitory Concentration

Table 3:3 demonstrates the sensitivity of selected H. ducreyi strains to polymyxin by using the agar dilution test. The reference strains of H. ducreyi (A75, A77 and CIP542) were sensitive to polymyxin with MIC's of ≤ 1.0 µg/ml. The Winnipeg strains 35000, 35199, 78118, 54211 & 54239 demonstrated resistance of up to 128 µg/ml. E. coli and S. aureus control strains exhibited sensitivity (< 1.0 µg/ml) and resistance (> 256 µg/ml) to polymyxin respectively.

3:5 Cell Wall Ultrastructure

3:5:1 Examination for Pili

As a control of the negative staining method, pili were demonstrated on E. coli Y10, a F^+ piliated strain (Figure 3:1 a). The pili were spread evenly over the surface of the bacteria and were present on the majority of cells. No pili could be seen on the surface of any of the H. ducreyi strains using the same negative staining technique (Figure 3:1 b). Pili could not be demonstrated in H. ducreyi after 24, 48 or 72 hours growth on agar media.

3:5:2 Cell Wall Characteristics

Past workers have reported the presence of a Gram-negative cell wall in H. ducreyi. As seen in Figures 3:2 and 3:3, all H. ducreyi strains used in this study possess the tripartite outer membrane characteristic of the Gram-negative cell wall. (This includes all strains in Table 2:1, except 6V, 4391 and 36-F-2

Table 3:3

Sensitivity of H. ducreyi to Polymyxin B

H. ducreyi Strain No.	Polymyxin B MIC (ug/ml)
35000	128
35199	128
78118	128
54211	>256
54239	>256
A75	<0.25
A77	<0.25
CIP 542	1.0
Controls	
E. coli ATCC 25992	1.0
S. aureus ATCC 24923	>256

FIGURE 3:1a. Electron Micrograph of Negatively Stained E.coli Y10

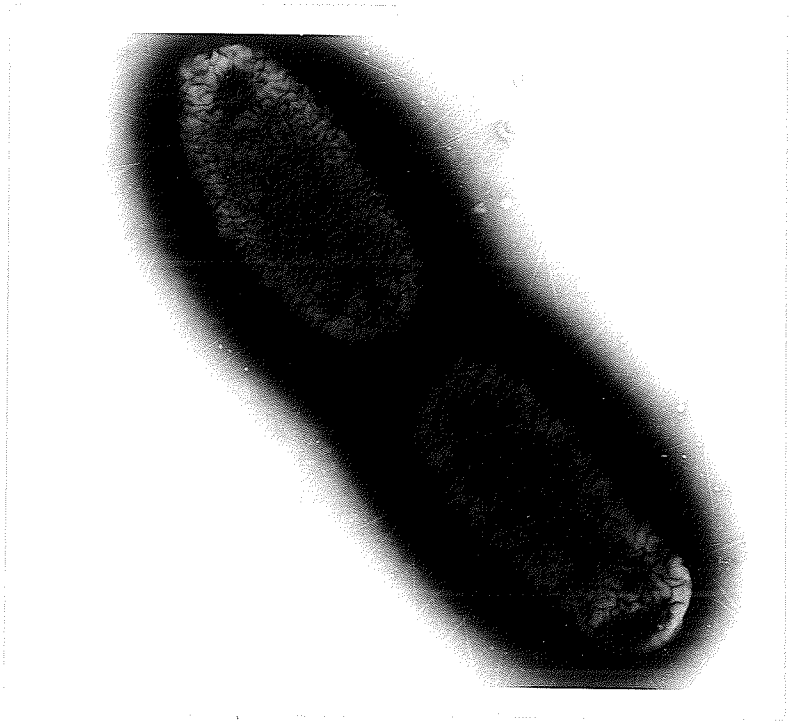


FIGURE 3:1b. Electron Micrograph of Negatively Stained H.ducreyi 54211

FIGURE 3:2 Electron Micrograph of Thin Sectioned H.ducreyi 35199 after Normal Fixation.
Bar represents 0.1μ; Arrows delineate area of Gram-Negative cell wall.

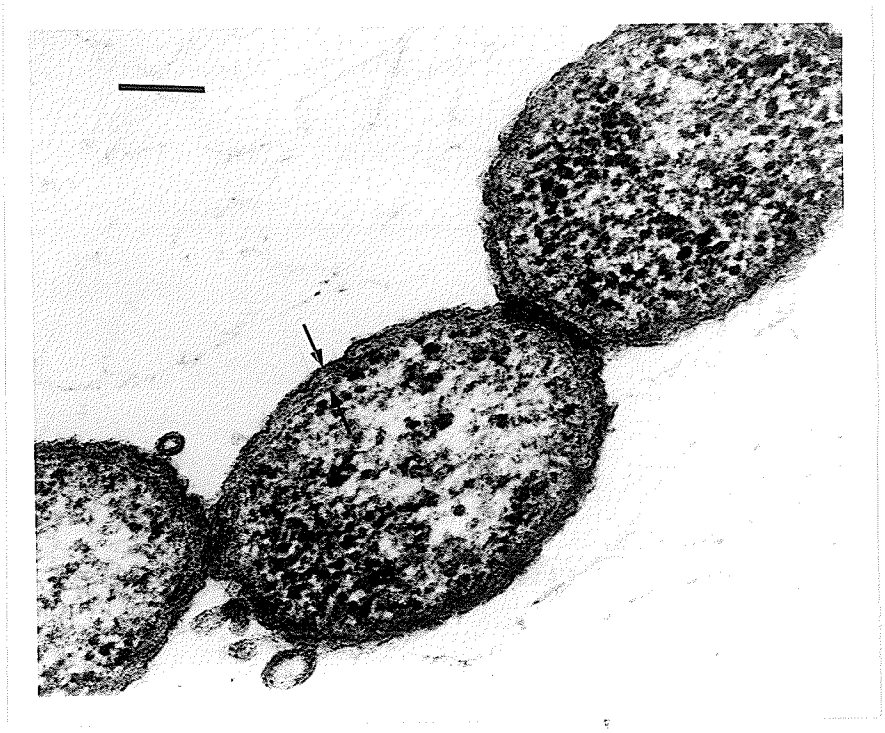
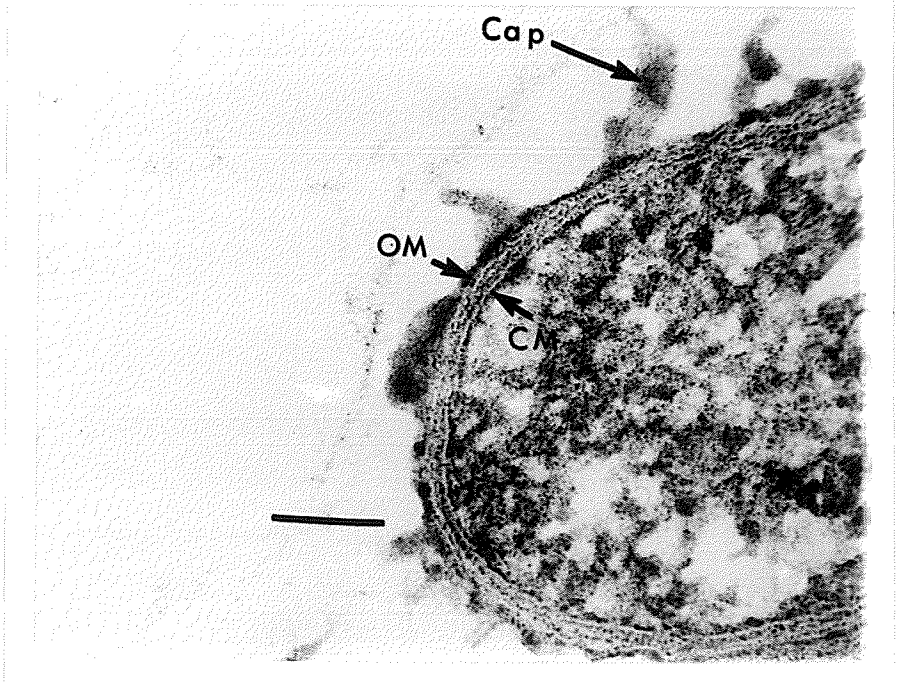


FIGURE 3:3 Electron Micrograph of Thin Sectioned H.ducreyi 35199 after Normal Fixation and Ruthenium Red Staining.
Arrows indicate Outer Membrane(OM),Cytoplasmic Membrane(CM), and Exocellular(Capsular)Material(CaP) extending outward from the cell envelope; Bar represents 0.1μ.



which were not examined.) The cell envelope is approximately 100 nm thick in which there are three separate layers: the inner cytoplasmic membrane, the outer membrane and between these, the periplasmic space and peptidoglycan layer.

3:5:3 Demonstration of a Capsular Layer

The use of ruthenium red as a capsular stain during the normal fixation procedures revealed the presence of exocellular material, distributed randomly along the outer membrane of H. ducreyi (Figures 3:3 and 3:4). This densely stained material is seen clumped along the outer membrane with occasional strands extending outward from the cell and often connecting two or more cells. Ruthenium red staining demonstrated surface material on all strains mentioned in section 3:5:1. Similar exocellular material to that seen in Figure 3:3 and 3:4 could be detected after staining two strains (54211 and 54239) with Alcian Blue (Figure 3:5). As with ruthenium red, Alcian Blue showed a discontinuous layer of exocellular material.

In order to improve visualization of this exocellular material, rabbit antisera against whole cell H. ducreyi was used to stabilize the material on cells from colonies of H. ducreyi grown on CA or in biphasic media (2:3:3). Strains A75, CIP542, 54211 and 54239 were all tested by this technique. Figure 3:6 demonstrates the presence of a continuous exocellular layer varying from 0.01 μ to 0.05 μ after stabilization with antisera and reaction with ruthenium red. The use of Alcian Blue as a stain in this procedure

FIGURE 3:4 Electron Micrograph of Thin Sectioned H.ducreyi 35199 after Normal Fixation and Ruthenium Red Staining. Arrow indicates exocellular material around and between cells; Bar reprsents 0.1μ.

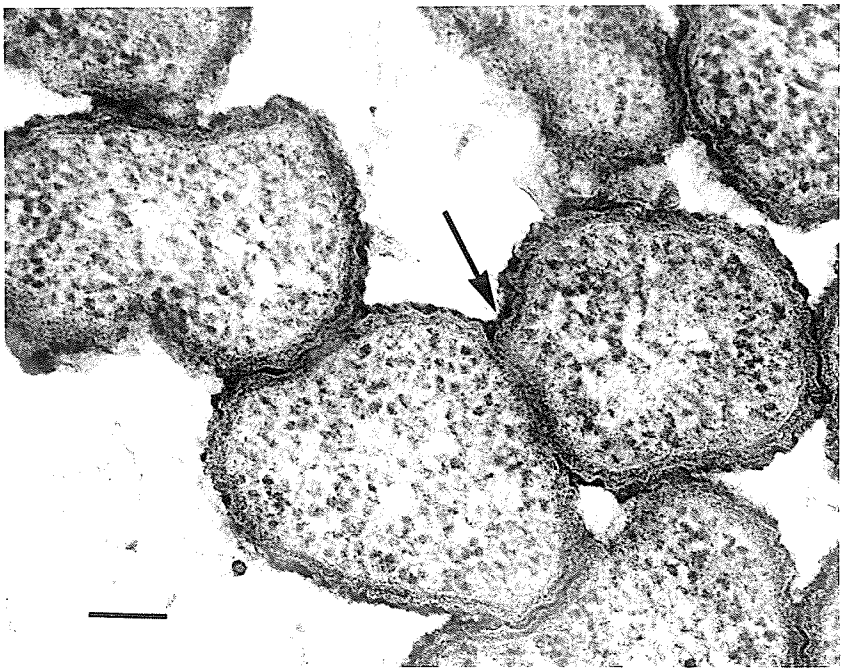


FIGURE 3:5 Electron Micrograph of Thin Sectioned H.ducreyi 54211 after Normal Fixation and Alcian Blue Staining. Discontinuous Capsular Material(Cap) extends outward from cell envelope; Bar represents 0.1μ.

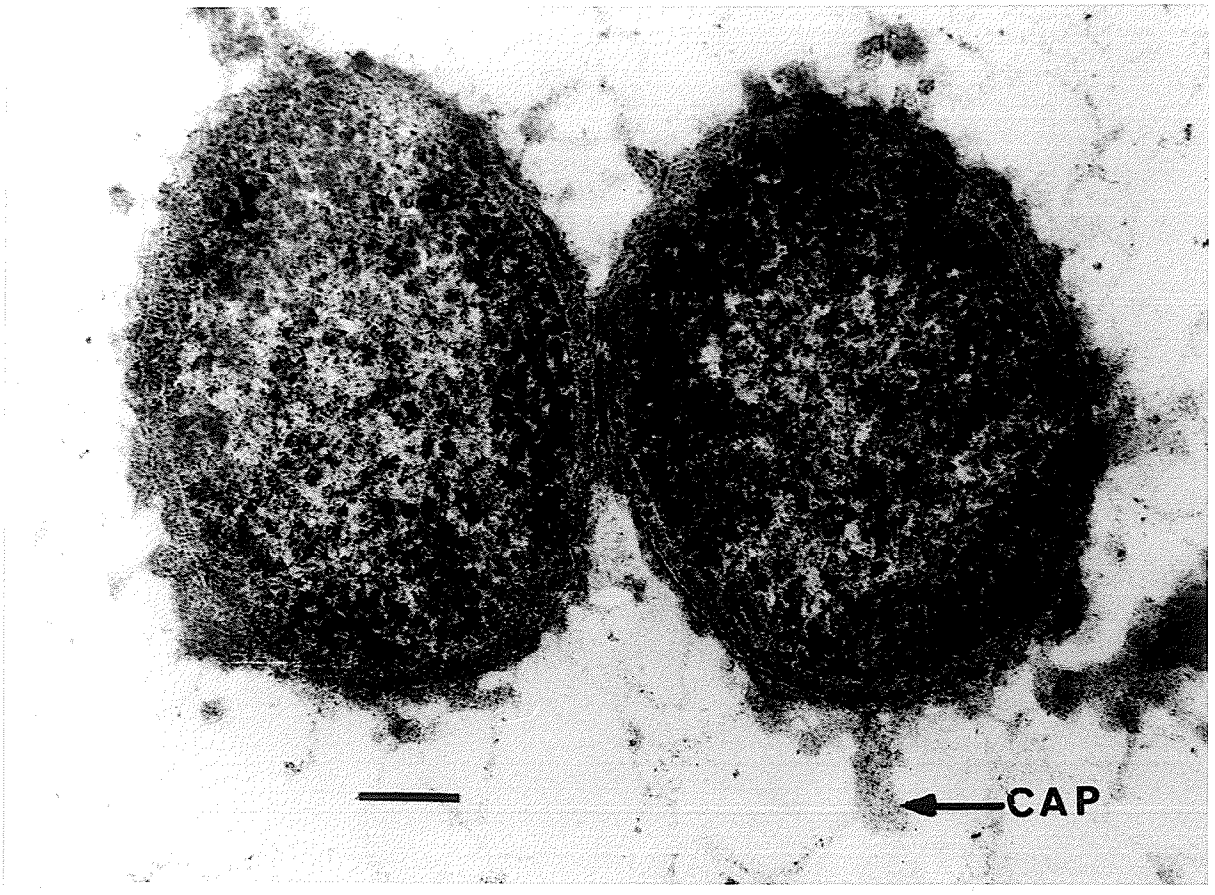
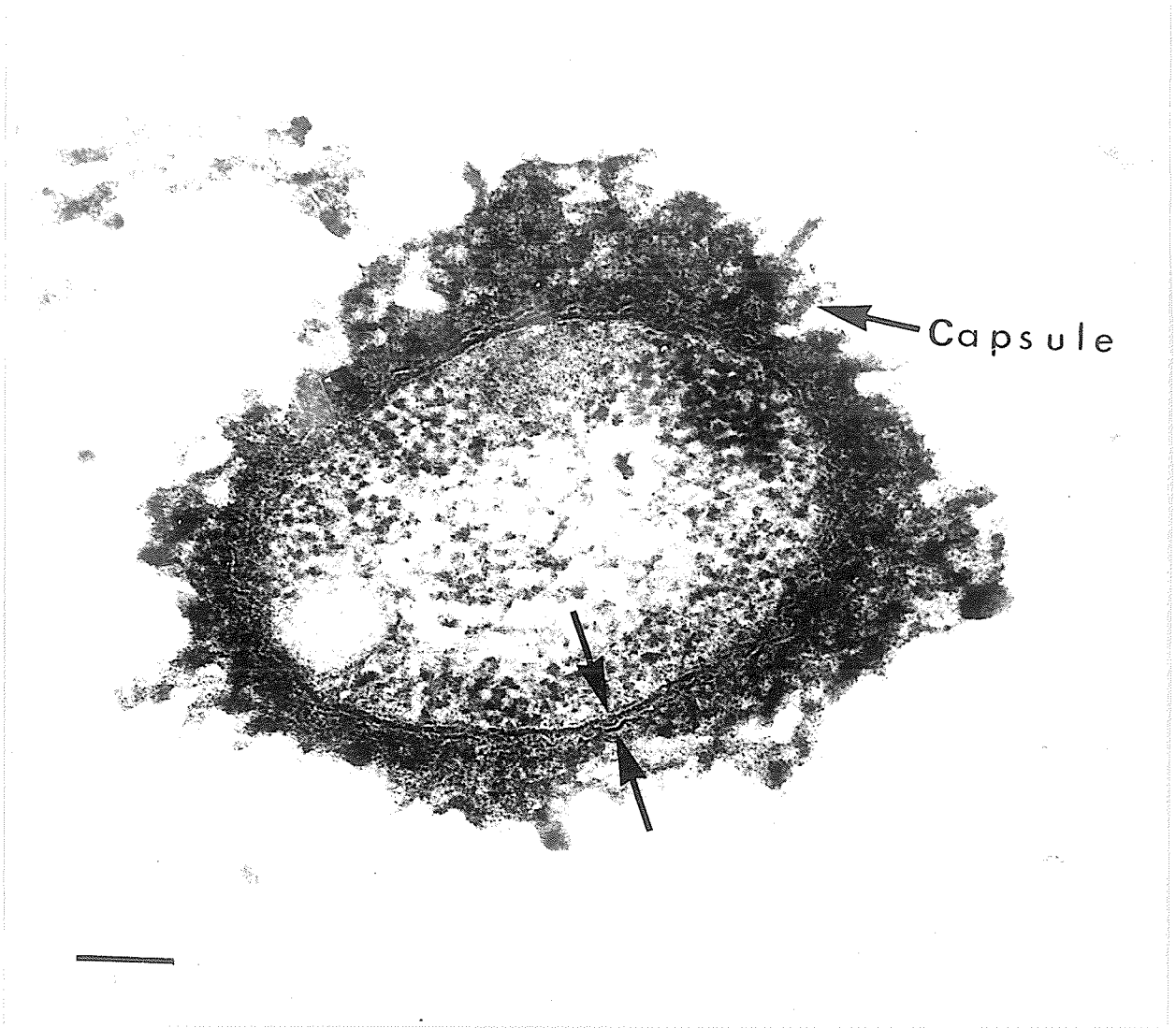


FIGURE 3:6 Electron Micrograph of Thin Sectioned H.ducreyi 54211 after Antisera Stabilization of Capsular Material. The preparation was stained with Ruthenium Red. Single arrow shows capsule and double arrows outline cytoplasmic and outer membranes; Bar represents 0.1μ.



also revealed the presence of a continuous capsular layer as seen in Figure 3:7.

Figure 3:8 of H. influenzae B031, a known capsulated organism stained by the same technique (ruthenium red staining and Burro type b antisera stabilization), is included for comparison to the H. ducreyi strains.

3:6 Animal Studies

3:6:1 Virulence Testing

H. ducreyi strains were found to differ in their ability to produce lesions in rabbit's skin. Table 3:4a shows the degree of virulence of the strains tested. Reference strains A75, A77 and CIP542 were avirulent by the criteria stated. In contrast, all of the Winnipeg strains tested were virulent.

Following the intradermal injection of the broth suspensions of H. ducreyi, an area of swelling approximately the size of the inoculum bleb was present. By 24 hours after injection of the virulent organisms, an area of induration and erythema between 1.0 to 2.0 cm in diameter developed. Injections with control broth, avirulent and heat killed organisms, produced a transitory redness with an area of raised induration not exceeding 1.0 cm. The swelling produced by the broth controls and heat killed organisms usually disappeared within 24-48 hours with no residual scars or markings. The avirulent lesions remained pinkish for approximately

FIGURE 3:7 Electron Micrograph of Thin Sectioned H.ducreyi 54239 after Antisera Stabilization of Capsular Material. The preparation was stained with Alcian Blue. Arrow indicates capsular material; Bar indicates 0.1μ.

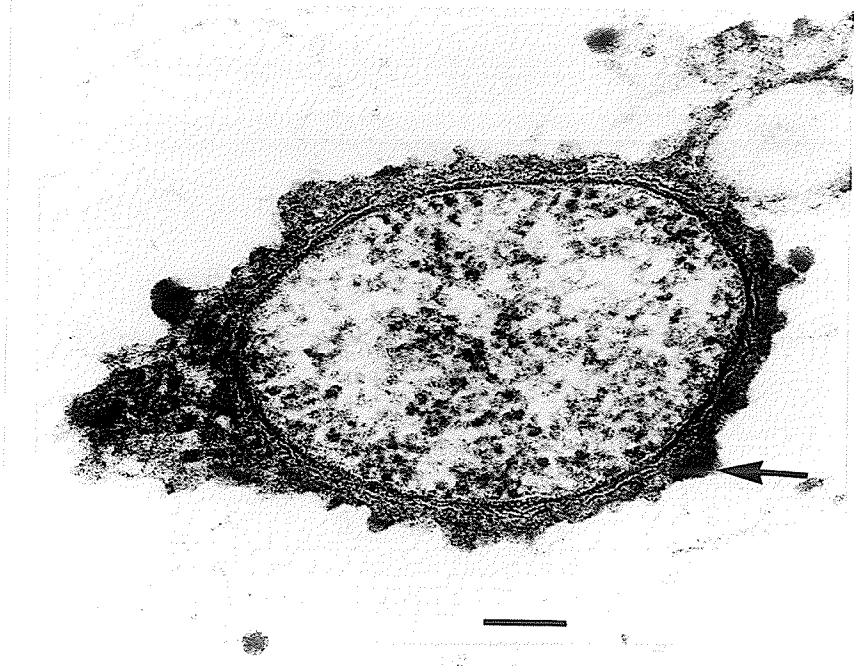
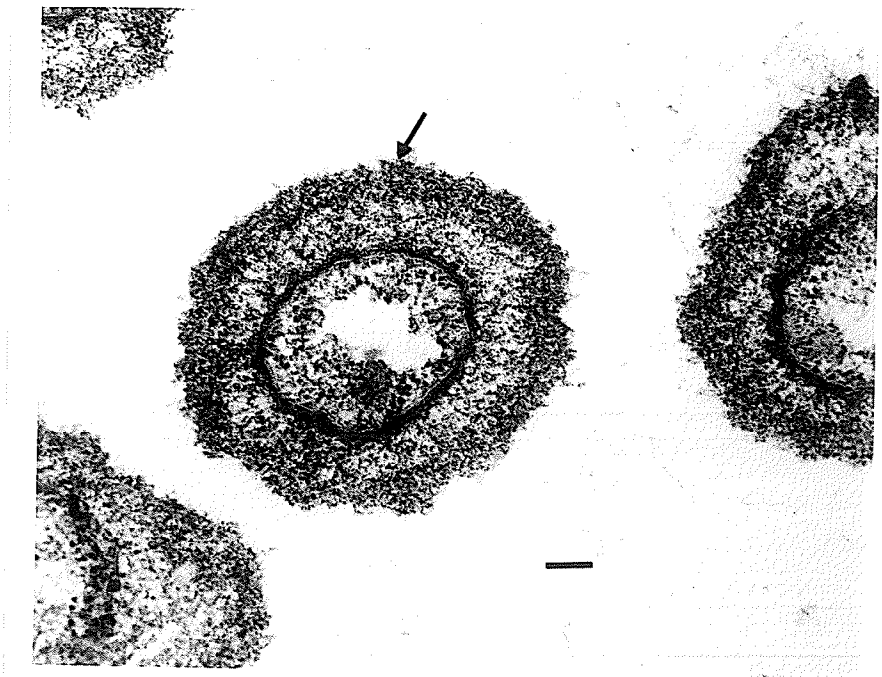


FIGURE 3:8 Electron Micrograph of Thin Sectioned H.influenzae B031 after Antisera Stabilization. The preparation was stained with Ruthenium Red. Arrow indicates capsular material; Bar represents 0.1μ.



72 hours, at which time swelling and induration had subsided to approximately 0.5 cm or less (see Figure 3:9a). This lesion gradually lost its induration and raised appearance over the next four to seven days. At no time was the skin broken or could pus be obtained from the lesion.

Conversely,at 48 hours the virulent lesion was pustular with a distinct zone of erythema extending 0.5 to 1.0 cm beyond the lesion. By 72 hours one could detect evidence of tissue breakdown in the centre of the papule. The breakdown and necrosis progressed to a black eschar by day 11 (see Figure 3:9b). Pus could be excised from the lesion from 48 hours until eight days post inoculation. H. ducreyi was isolated from the pus of the virulent lesion up to 72 hours post inoculation. Virulent lesions began to heal by 14 to 20 days post inoculation with healing complete by days 21 to 28. Scaring was usually evident after healing had taken place.

The effect of $\log_{10}$ dilutions of the inoculum on the expression of virulence by selected H. ducreyi strains is given in Table 3:4b. A dilution of more than one $\log_{10}$ in the inoculum of the Winnipeg strains (7118 and 54239) inhibited their expression of virulence by the stated criteria. As previously mentioned, reference strains A77 and CIP542 were avirulent at all dilutions tested.

3:6:2 Histology of the Biopsied Lesions

The histology of lesions produced by H. ducreyi 54239 and A77 is illustrated in Figures 3:10a) to d). For a comparison, normal skin was biopsied and is shown in Figure 3:10e).

FIGURE 3:9a) Avirulent H.ducreyi A77 Rabbit Lesion.
Rabbit was inoculated with 0.20ml. of a Mueller Hinton broth suspension of viable organisms(approximately 10^9 CFU/ml.)
Photograph was taken at Day 5 post-inoculation.

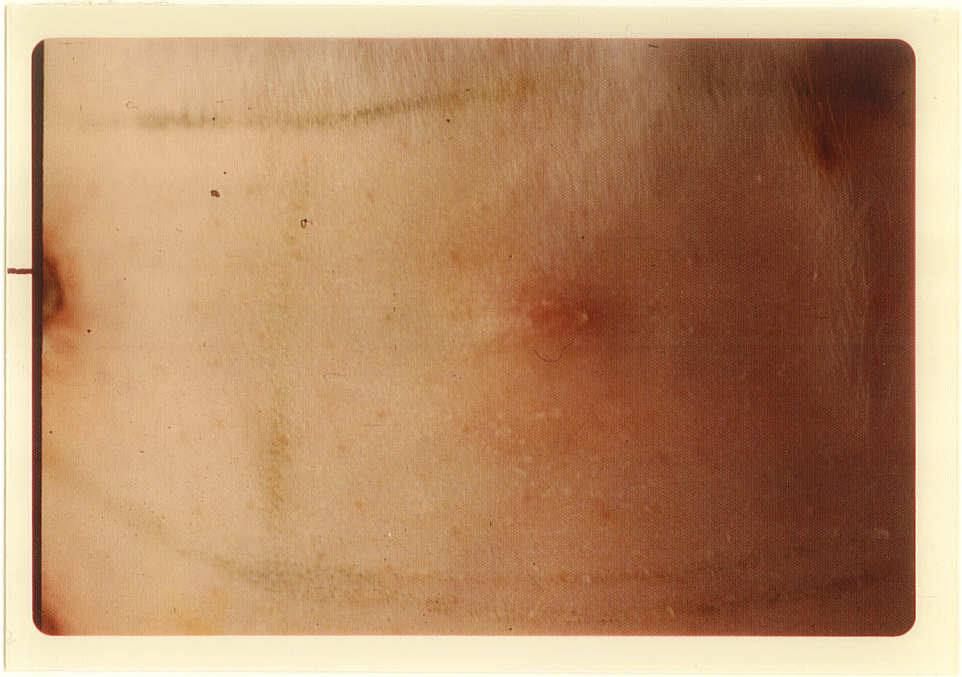


FIGURE 3:9b) Virulent H.ducreyi 54239 Rabbit Lesion.
Rabbit was inoculated with 0.20ml. of a Mueller Hinton broth suspension of viable organisms(approximately 10^9 CFU/ml).
Photograph was taken at Day 5 post-inoculation.

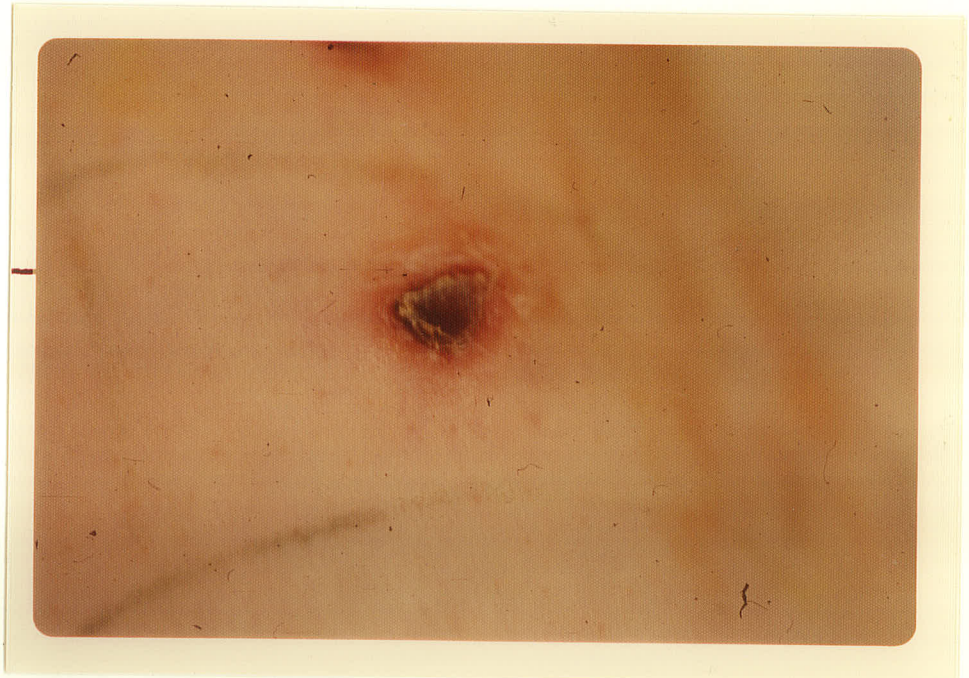


Table 3:4a

Virulence of H. ducreyi Strains

H. ducreyi Strain No.	Virulence[1]
35000	+
35199	+
78118	+
54211	+
54239	+
A75	-
A77	-
CIP 542	-

Table 3:4b

Effect of Log_{10} Dilution of the Inoculum on

H. ducreyi's Expression of Virulence

H. ducreyi Strain	Virulence[1] of Inoculum[2]			
	10^9 CFU[3]	10^8 CFU	10^7 CFU	10^6 CFU
78118	+,+	+,-	-,-	-,-
54239	+,+	+,+	-,-	-,-
A77	-,-	-,-	-,-	-,-
CIP 542	-,-	-,-	-,-	-,-

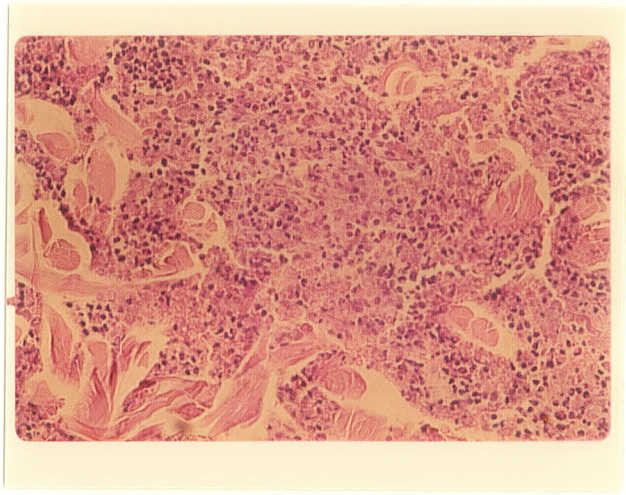
[1]The criteria for virulence in the rabbit model was as follows:
- by day 4 postinoculation, induration measuring $\leq$ 0.5 cm which progressed to an eschar by day 11. (i.e. virulent = +; avirulent = -).

[2]Inoculum was 0.20 cc of a MHB suspension of viable organisms injected into the shaved backs of test rabbits.

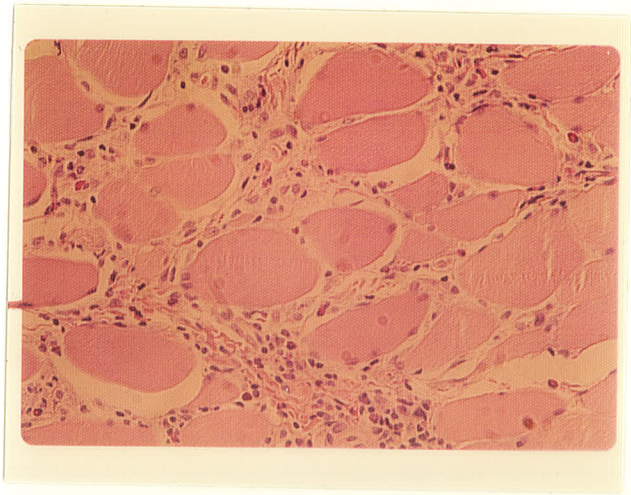
[3]Colony Forming Units per ml of inoculum.

FIGURE 3:10 Histology of the Biopsied Rabbit Lesion

a) Acute Inflammatory Response in the virulent lesion(Day 2 post-inoculation). Polymorphonuculear leucocytes have infiltrated the dermis, with eosinophils as the predominate cell.

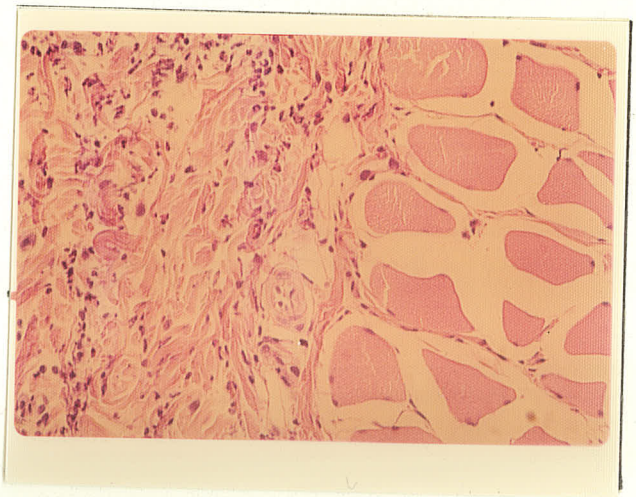


b) Infiltration of Deep Muscle Layers in the virulent lesion (Day 2 post-inoculation).

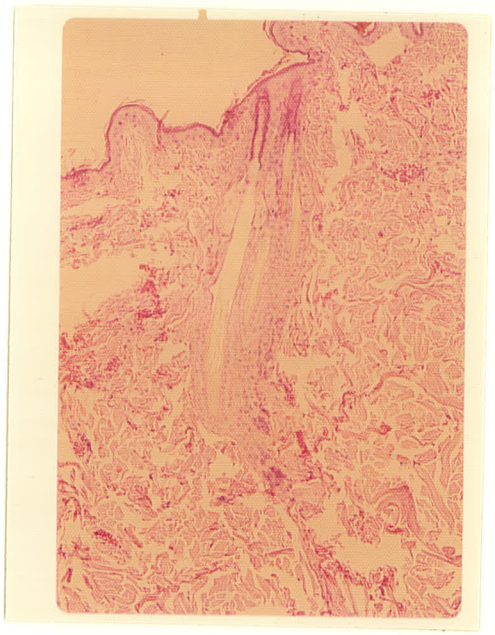


c) Day 8 Cross-section of Virulent Lesion.
Dermis is totally destroyed with a concurrent destruction of the associated collagen fibres.

d) Inflammatory Response in the Avirulent Lesion(Day 2 post-inoculation). The amount of inflammation was less than that in the virulent lesion(see Fig. 3:10a&b), with little infiltration of the lower dermis and muscle areas.



e) Normal Skin.



Lesions produced by a virulent H. ducreyi strain (54239) showed an acute inflammatory reaction by day 2 (Figure 3:10 a & b). This included an intense infiltration of polymorphonuclear leukocytes (PMN's) into the superficial dermis with eosinophils as the predominate cell. The lower dermis and muscle tissue also showed evidence of inflammation. By days 4 to 6 after injection, an ulcer had developed with the surface dermal layers showing increased necrosis. In addition, collagen fibres became necrotic and edema and PMN infiltration was present in the lower muscle layers.

On day 8 the dermis was totally destroyed with a concurrent destruction of the associated collagen fibres. Early fibroblastic proliferation could also be seen at the base of the ulcer. Vasculization of the muscle layer was evident by the presence of macrophages, dilated capillaries and a shift to 100% eosinophilic PMN's in the infiltrate (Figure 3:10c). Healing had started by day 11 with a corresponding decrease in inflammation.

The lesions produced by an avirulent strain of H. ducreyi (A77) by day 2 also demonstrated inflammation, but unlike that of 54239, the inflammation was limited to the dermis with little, if any, in the deeper muscle layers (Figure 3:10d). Although a dense infiltrate did develop in the dermal layers, the amount of collagen degeneration and the number of PMN's were greatly reduced from that evident in the 54239 virulent lesion. By day 8 to 11 the A77 lesion appeared as a small focal necrosis in the dermis but at no time was the surface layer broken. Macrophages were also evident

in the lesion at this time, together with a restricted area of chronic vasculitis.

Portions of the biopsied lesions were also cultured in an attempt to isolate H. ducreyi (see 2:5:1). H. ducreyi could not be isolated from any of the A77 biopsied lesions. In contrast H. ducreyi was isolated from the biopsies of the 54239 lesions up to the sixth day post inoculation; subsequent cultures were negative.

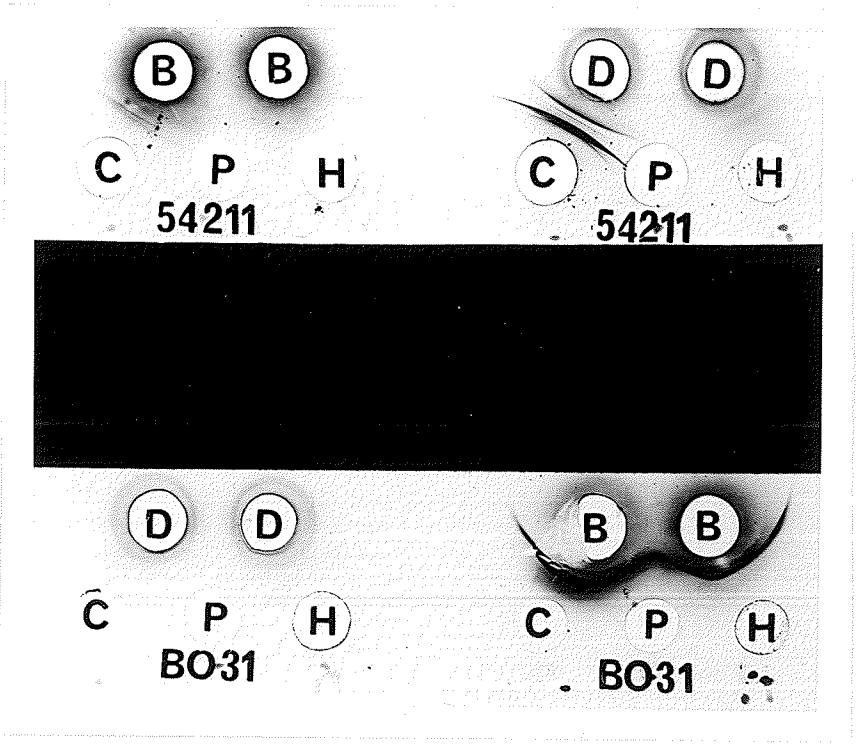
3:7 Initial Characterization of H. ducreyi Antigens

Initial work with the H. ducreyi strains showed that a complex pattern of precipitin lines could be demonstrated by both immunodiffusion and crossed immunoelectrophoresis. Cell sonicates were run against H. ducreyi whole cell antiserum (see Figures 3:11 a&b and 3:12), and these whole cell antigens also reacted weakly with Burro H. influenzae type b antisera. H. influenzae B031 also showed weak activity to H. ducreyi whole cell antisera.

Protease treatment and subsequent heating of the H. ducreyi antigens at 100°C for 15 minutes removed all reactivity with the H. ducreyi whole cell and the H. influenzae type b Burro antisera. Heat treatment alone reduced the activity of H. ducreyi antigens with the H. ducreyi whole cell antisera and destroyed all the reactivity with Burro type b antisera (Figures 3:11 a&b).

Type b H. influenzae B031 lost all reactivity to H. ducreyi

FIGURE 3:11a&b Immunodiffusion of Control, Enzyme and Heat Treated H.ducreyi and H.influenzae Whole Cell Sonicates; B(H.influenzae type b, burro antisera),D(H.ducreyi Whole Cell, rabbit antisera).
Whole cell sonicates of H.influenzae(B031) and H.ducreyi (54211,A75,35199&CIP542) were either untreated(C), heat-treated(H) or protease-heat treated(P) and run against B or D antisera.



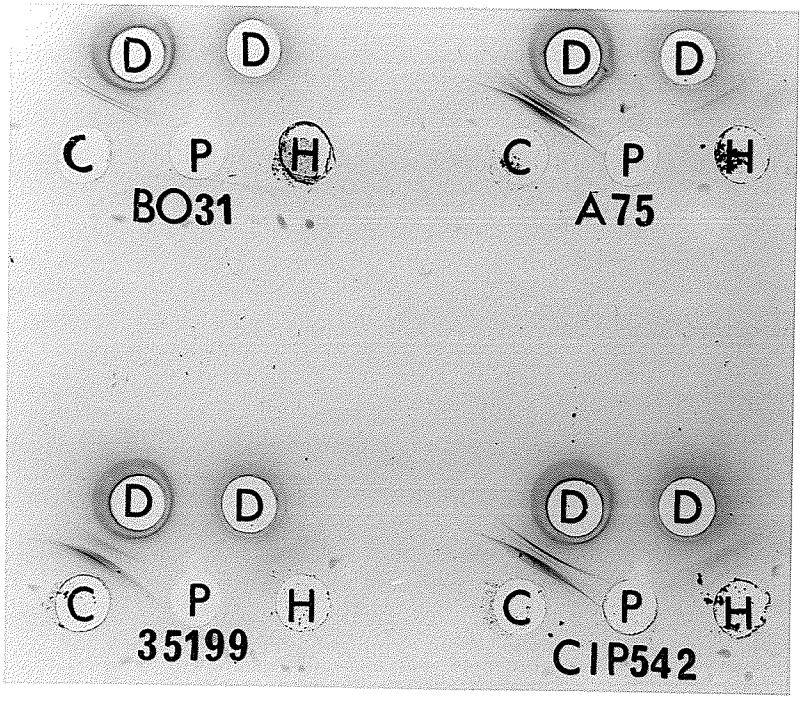
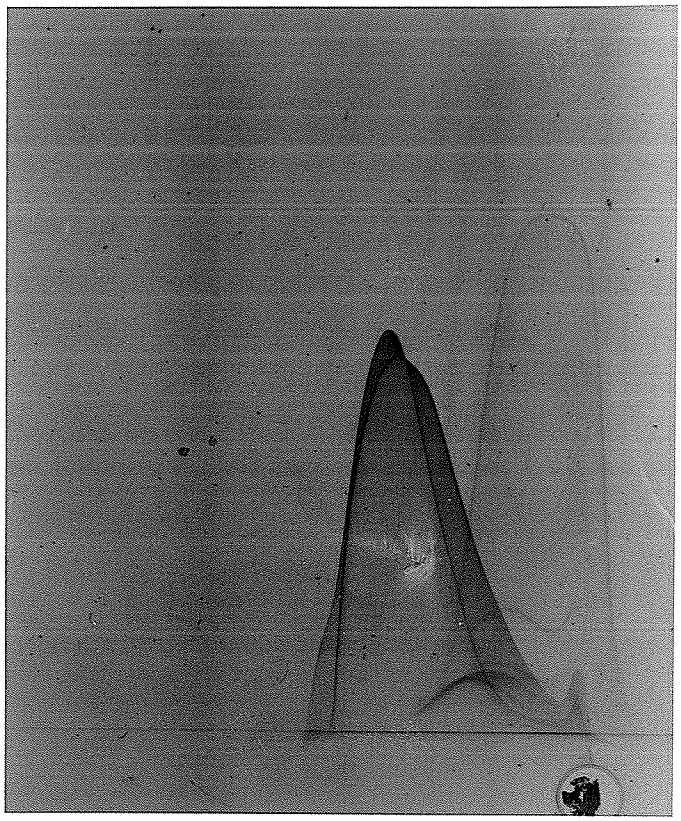


FIGURE 3:12 Crossed Immunoelectrophoresis of H.ducreyi 54211 Whole Cell Sonicate(WSC). The WSC(10ul) was run in the first direction at 10 V/cm. for 30 mins. at 5°C. The second direction was run at 2V/cm. for 16 hrs. at 5°C. into agarose containing 10%(vol./vol) H.ducreyi whole cell antisera



whole cell antisera after both protease-heat treatment and heat treatment alone. However, type b H. influenzae B031 still showed strong reactivity to the homologous Burro type b antisera after both treatments (Figure 3:11a).

In an attempt to separate H. ducreyi antigens, 5.0 ml of a 200 mg/ml (wet-weight) whole cell sonicate of H. ducreyi 54211 was applied to a Sepharose 4B column in 0.1 M tris buffer pH 7.0 and 2.0 ml fractions collected. When these fractions were tested against H. ducreyi whole cell antisera, two groups of fractions with activity against the antisera could be demonstrated (Figure 3:13). Three further groups of fractions containing protein were negative. Inoculation of both the positive and negative fractions intradermally into the back of a rabbit previously sensitized to H. ducreyi produced transitory redness after two days. No difference could be detected between the fractions and only a whole cell sonicate inoculated intradermally in the same manner progressed to a lesion with scab by day 2.

It was felt that the reactivity of these pooled samples was insufficient for further analysis, so an attempt to separate the antigens of the whole cell sonicate by charge was initiated. A DEAE Sephadex A50 column in 0.1 M tris buffer at pH 8.0 in a NaCl gradient from 0 to 0.8 M at pH 8.0 was used to separate the H. ducreyi antigen extracts into 80 fractions. Fractions 50 to 78 produced precipitin peaks when tested by rocket electrophoresis against H. ducreyi whole cell antisera (Figure 3:14). These

Figure 3:13 Elution Profile of H.ducreyi 54211 Whole Cell Sonicate from Sepharose 4B

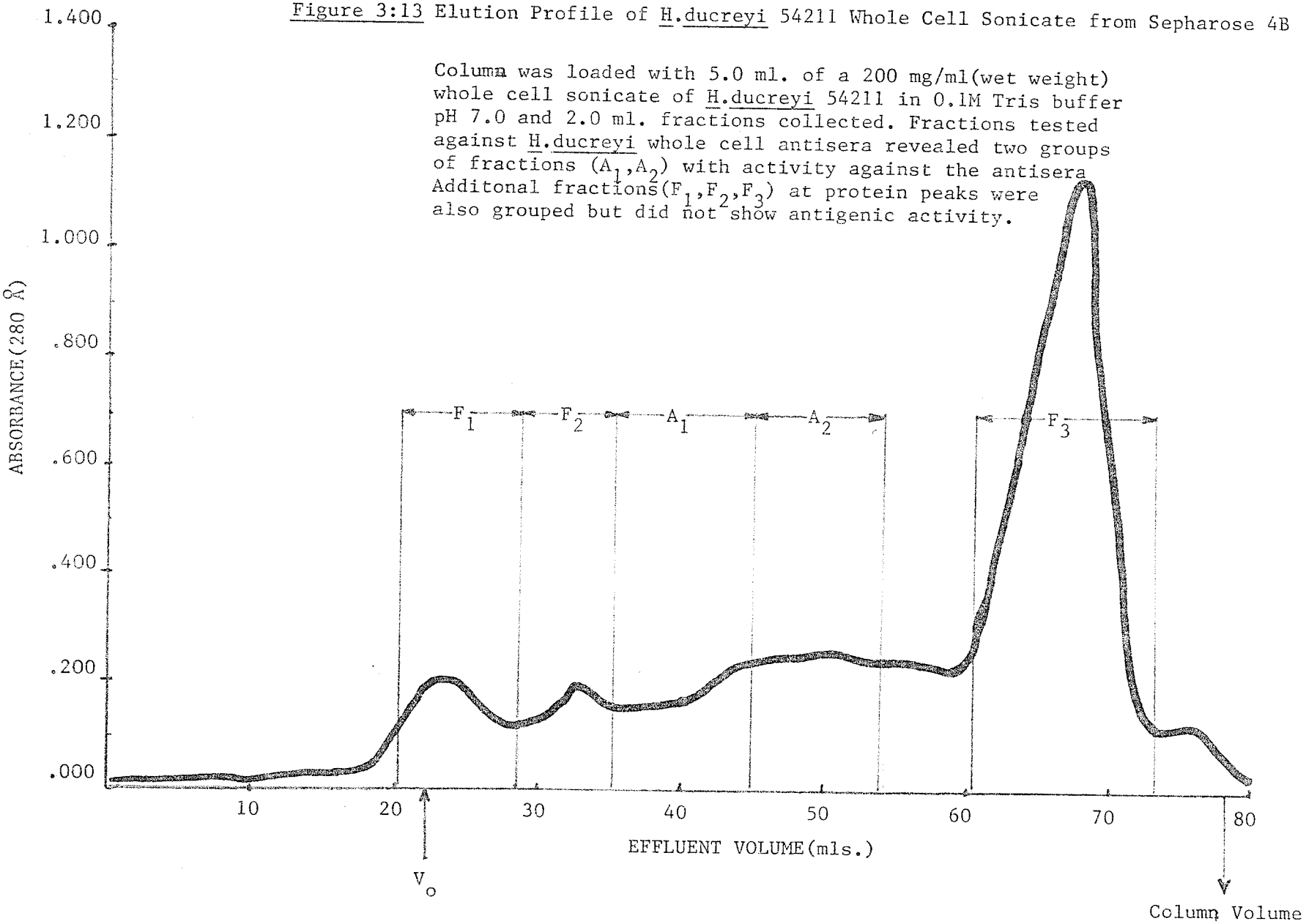
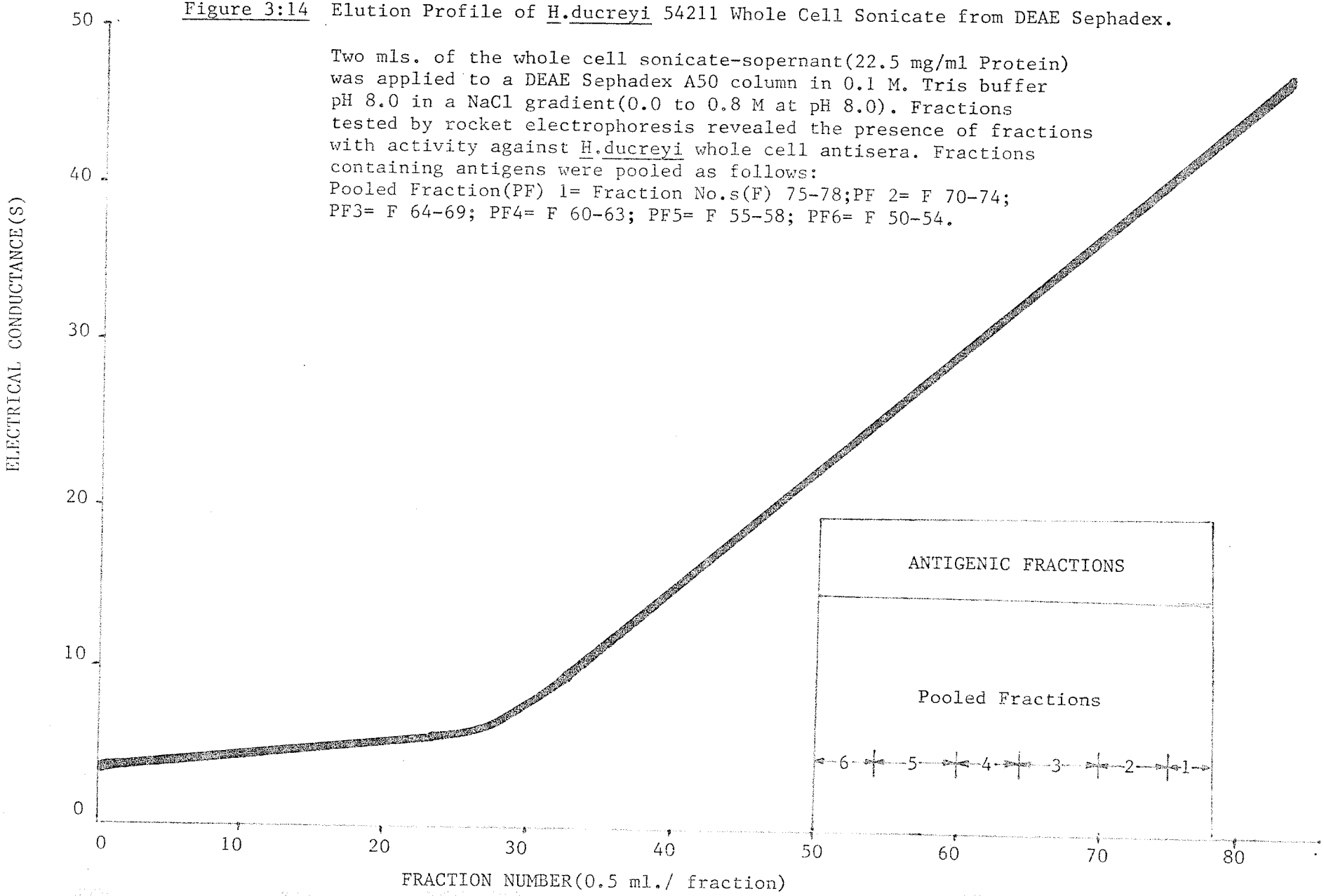


Figure 3:14 Elution Profile of H.ducreyi 54211 Whole Cell Sonicate from DEAE Sephadex.

Two mls. of the whole cell sonicate-sopernant(22.5 mg/ml Protein) was applied to a DEAE Sephadex A50 column in 0.1 M. Tris buffer pH 8.0 in a NaCl gradient(0.0 to 0.8 M at pH 8.0). Fractions tested by rocket electrophoresis revealed the presence of fractions with activity against H.ducreyi whole cell antisera. Fractions containing antigens were pooled as follows:
Pooled Fraction(PF) 1= Fraction No.s(F) 75-78;PF 2= F 70-74;
PF3= F 64-69; PF4= F 60-63; PF5= F 55-58; PF6= F 50-54.



fractions were pooled into six groups and freeze-dried. Pooled fractions and heat-treated pooled fractions were tested by rocket electrophoresis as seen in Figure 3:15. Pooled fractions two to five contained heat stable antigens.

Injection of the pooled fractions three to six from the DEAE A50 column into rabbits as described (2:5:2 bII), produced active antisera. These antisera were tested against a variety of Haemophilus species and H. ducreyi strains by rocket electrophoresis. Table 3:5 illustrates the degree of cross-reactivity between the antisera and whole cell sonicates of Haemophilus species. All H. ducreyi strains reacted similarly (i.e. the same number of precipitin peaks), varying only in the height of the respective lines. The other Haemophilus species showed a reduced activity (i.e. fewer peaks), with the strongest cross-reactions found with H. pleuropneumonia, H. parainfluenzae and H. gallinarum. Figure 3:16 illustrates a typical gel demonstrating the reactivity of the Haemophilus species and H. ducreyi strains with antisera against pooled fraction four.

FIGURE 3:15 Rocket Electrophoresis of DEAE Sephadex Pooled and Heat-Treated Pooled Fractions. Control(c) and Heat-Treated(h) Pooled Fractions (Fractions 1-6) were tested against H.ducreyi Whole Cell Antisera. Gels contained 10% antisera and were run at 2V/cm for 16 hrs. Heat stable antigens are demonstrated in Fractions 2 through 5.

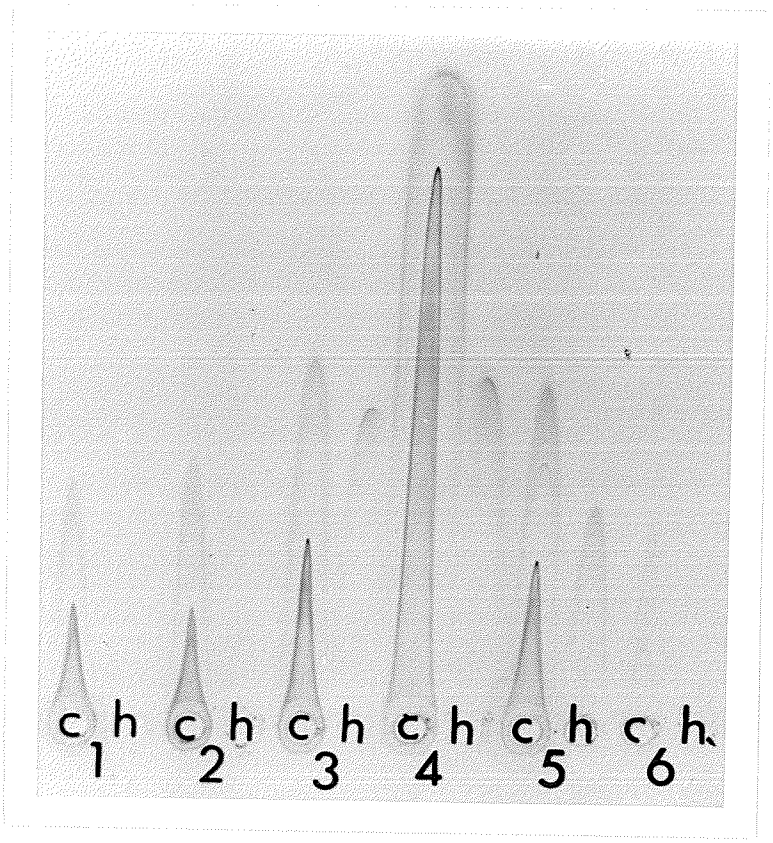


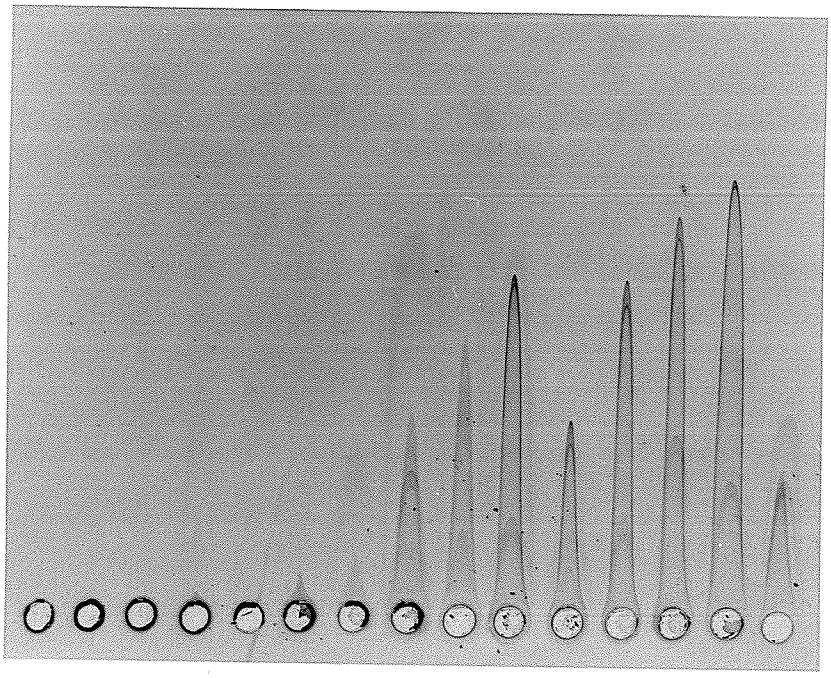
Table 3:5

Activity[1] of H. ducreyi and Haemophilus species to Pooled Fraction Antisera

H. ducreyi strain	Number of Precipitin Peaks in Rabbit Antisera			
	Fraction 3	Fraction 4	Fraction 5	Fraction 6
78118	5	4	5	4
54211	5	4	5	4
54239	5	4	5	4
A75	5	4	5	4
CIP 542	5	4	5	4
6V	5	4	5	4
4391	5	4	5	4
36-F-2	5	4	5	4
H. influenzae 1-637	0	0	1	0
H. influenzae 1-567	0	0	1	0
H. influenzae 3-545	0	1	1	0
H. parainfluenzae 2-004	2	2	2	1
H. pleuropneumonia 0-1	3	3	3	1
H. paraphrophilus 0-1	0	0	1	0
H. gallinarum 17551	2	2	3	1

[1] Activity was determined by the number of precipitin peaks produced when whole cell sonicates were run by rocket electrophoresis against pooled fraction antisera.

FIGURE 3:16 Rocket Electrophoresis of H.ducreyi strains and Haemophilus species run against Pooled Fraction Four Antisera.
Ten microlitres of the bacterial whole cell sonicates were placed in the wells of a rocket electrophoresis gel containing 10% Fraction Four Antisera and run at 2V/cm for 16 hrs.
Wells from Left to Right: H.influenzae strains 1-637,1-567,&3-545; H.pleuropneumonia 0-1; H.paraphrophilus 0-1; H.parainfluenzae 2-004; H.gallinarum 17551; H.ducreyi strains A75, CIP542, 6V, 54239,54211,78118,4391,& 36-F-2.
H.pleuropneumonia, H.parainfluenzae, and H.gallinarum demonstrate definite cross-reactivity with the H.ducreyi Fraction Four antisera (see table 3:5), as indicated by the precipitant peaks.



Discussion

4:1 Introduction

Today, venereal disease remains a constant threat to the health and welfare of mankind. Although antibiotics, effective in treating the majority of venereal infections, were introduced in the 1930's and 1940's these diseases are an increasingly common problem. Chancroid is one such venereal disease and although its incidence has presently decreased in developed countries, the disease is a major problem in underdeveloped areas, especially the tropical regions (Willcox, 1951; Kerber et al, 1969; personal communication with Dr. Causse, W.H.O., 1979). Although research on the causative agent of chancroid, Haemophilus ducreyi, was initiated in the late 1800's, our understanding of the aetiology of this infection remains incomplete.

The complexity of the organism's growth requirements has, from the earliest reports, hampered attempts to characterize H. ducreyi in the laboratory. Therefore, few, if any, of H. ducreyi's features are well described. In addition, due to the complex microflora associated with penile ulcerations (Chapel et al, 1978) confusion over the nature of the isolates described as H. ducreyi (Nicolau & Bancrou, 1926: Reymann, Ph.D. thesis, Copenhagen, 1951) is prevalent throughout past literature.

Therefore, the present study has sought to resolve some of

the disorientation generated by previous studies. The areas examined were: (1) the cell wall ultrastructure of H. ducreyi, (2) the virulence properties of H. ducreyi, (3) H. ducreyi's sensitivity to Polymyxin, (4) characterization of H. ducreyi antigens and their relation to other Haemophilus species. This work has attempted to avoid any confusion surrounding the identity of the organism by establishing a number of characteristics of the H. ducreyi strains used.

4:2 The Identity of Strains Used in this Study

The description of H. ducreyi has changed little from early accounts. It is generally reported that H. ducreyi was a small, Gram-negative rod (0.4 μ x 1.5 μ) with rounded ends, occurring singly and in short chains (Davis, 1903; Saelhof, 1924). The colonies grown on media containing blood were described as small, grayish and glistening with a slight zone of hemolysis around the colony. The organism grew best on media containing blood in aerobic conditions at 37°C. The present study confirmed that these characteristics were indeed typical of H. ducreyi.

However, studies on the biochemical and nutrional characteristics of H. ducreyi are sparse and often contradictory. One example is the conflicting reports on H. ducreyi's fermentation of carbohydrates. Assis (1926) noted the fermentation of glucose by H. ducreyi, but Reymann (1949b), Ajello et al (1956) and Kilian

(1976) were unable to demonstrate the fermentation of any carbohydrates by H. ducreyi. In the present study the majority of strains fermented arabinose and glucose. Kilian's (1976) work on the genus Haemophilus revealed that all Haemophilus species fermented glucose except H. ducreyi. It would seem that the fermentation of glucose is a common characteristic of Haemophilus and the inability of previous studies to demonstrate this feature in H. ducreyi may have been a result of the test method. The method used in this study (API-20E) did not require growth of the bacteria in order to express their capacity to ferment glucose. All of the other studies have used tests which required growth to demonstrate any metabolic activity. In these cases a negative result may have been due to the inability of strains to grow in the media.

Another area of confusion is the requirement of H. ducreyi for growth factors. Several studies (Sanderson, 1940; Beeson, 1946; Reymann, 1951; Ajello et al, 1956) failed to demonstrate a hemin requirement of their H. ducreyi strains. In contrast, Lwoff and Pirosky (1937) and Hammond et al (1978b) demonstrated H. ducreyi's need for exogenous hemin (X factor) in the growth media. The lack of some of the enzymes involved in the hemin biosynthesis pathways accounts for the dependence of some Haemophilus for exogenous X factor. The porphyrin test enables one to demonstrate the presence of these enzymes in bacteria (Kilian, 1974). Kilian (1976) and Hammond et al (1978b) both demonstrated a negative porphyrin test for some of the H. ducreyi strains examined in this study. The

present work has confirmed that H. ducreyi requires exogenous hemin in order to grow on artificial media and gives a negative porphyrin test.

Discrepancies also exist with regard to the presence of oxidase in H. ducreyi. Kilian (1976) and Hammond et al (1978c) both reported that their strains were oxidase negative. In contrast, this study, using the API-20E oxidase test, found all H. ducreyi strains oxidase positive. Some of the strains used in this study were also present in Kilian's (1976) and Hammond et al's (1978c) study and the difference in results could be due to the variation in test methods. The methods used to test for a specific characteristic of H. ducreyi obviously have a significant influence on the result observed. One illustration of the importance of methodology is shown in nitrate reduction. Using the API-20E test for nitrate reduction the majority of our H. ducreyi strains were negative (see Table 3:2). However, subsequent work in our laboratory using the method employed by Kilian (1976) (Cowan & Steel, 1965) showed that all these strains reduced nitrate.

Summarizing the biochemical characteristics of the H. ducreyi strains used in this study one can conclude that they all conform to the features attributed to members of the genus Haemophilus (Kilian, 1976). Although there is some discrepancy, even among tests done on the same strains, the differences may only reflect variations in testing methods. It is apparent that work on the biochemical and nutritional features of H. ducreyi, using a larger number of

strains is still required. Future studies may contribute new tests and characteristics which will enable a more precise identification of *H. ducreyi* strains.

4:3 Ultrastructure

Pili have been proposed as a structure involved in the transfer of chromosomal or plasmid DNA during conjugation (Ottow, 1975). The negatively stained electron micrograph, Figure 3:11a) demonstrates pili in *E. coli* Y10, an organism which contains a conjugable F plasmid. The ability to transfer plasmid DNA by conjugation has also been shown for *H. ducreyi* (personal communication, I. Maclean, 1978). However, no extracellular appendages, such as pili, could be seen in the negatively stained electron micrographs of *H. ducreyi* (Figure 3:1b). This lack of extracellular appendages might be expected, since in the available literature pili or other appendages have never been reported in the genus *Haemophilus*. At this time, the mode of DNA transfer during conjugation by *Haemophilus* species is unknown.

In another area of ultrastructural research, the information on the cell wall of *H. ducreyi* is ambiguous. Although originally described by Ducrey (1889) as a Gram-negative organism, other investigators (Nicolau & Bancrou, 1926) found their *H. ducreyi* strains became Gram-positive after a number of subcultures. Assis (1926) found that the Gram-reaction was dependent on the method used

for fixing the specimen and Reymann (Ph.D. thesis, Copenhagen, 1951) found all his strains consistently Gram-negative. The variation in the reports on the Gram-reaction might have been explained when Deacon et al (1954) reported on a Gram-positive, non-pathogenic, smooth phase of H. ducreyi. However, subsequent work by these authors (Deacon et al, 1956) revealed that they had originally been working with Corynebacterium acnes.

The structure of the cell wall seen under the electron microscope has revealed the physical structures upon which the decision between Gram-negative and Gram-positive bacteria is based (Costerton, 1979). The Gram-negative cell wall, seen in cross-section by electron microscopy, consists basically of an inner cytoplasmic membrane, an outer membrane and between these two membranes, the periplasmic space and peptidoglycan layer. In contrast, the Gram-positive cell wall lacks the outer membrane and usually has a thicker peptidoglycan layer (Costerton et al, 1974; Costerton, 1979).

All H. ducreyi strains examined in this study by electron microscopy, contained the tripartite outer membrane characteristic of the Gram-negative cell wall. These results, using strains previously described by Kilian (1976) and recent Winnipeg isolates, confirm the classification of H. ducreyi as a Gram-negative organism.

The electron microscopic studies of Cazarre and Barretto (1974), Ovchinnikov et al (1976) and March et al (1978) also characterize H. ducreyi as Gram-negative. However, unlike Kilian

and Theilade's (1975) study, these reports failed to describe any biochemical or nutritional features of their isolates. Kilian and Theilade's detailed study (1975) of Reymann's H. ducreyi strains also revealed that they had been incorrectly identified as H. ducreyi. Although Reymann's strains appeared Gram-negative by light microscopy, Kilian (1976) excluded them from the genus Haemophilus, as under electron microscopy they showed a Gram-positive cell wall. In addition, the Reymann strains did not possess the nutritional and biochemical features of Haemophilus.

Recent advances in electron microscopy, involving the cationic dyes ruthenium red and Alcian Blue, have enabled the visualization of capsular layers in a variety of bacteria (Jones et al, 1969; Springer & Roth, 1973; Cassone & Garaci, 1977; Mackie et al, 1979). Alcian Blue and ruthenium red appear electron-dense in electron micrographs because of their reaction with polyanionic substances, and the subsequent catalytic reduction of OsO_4 to a lower insoluable oxide (Cagle, 1974). It has been suggested that ruthenium red-positive material most likely represents acidic mucopolysaccharide (Woo et al, 1979). Chemical analysis of the ruthenium red-positive capsular material of H. influenzae type b (Rodriques et al, 1971) and Bacteroides fragilis (Kasper, 1976) supports this suggestion. The ruthenium red and Alcian Blue stained exocellular material seen in H. ducreyi is, therefore, probably a polyanionic polymer composed of acidic mucopolysaccharide.

Observation of capsules by electron microscopy can be

facilitated by stabilizing the capsular material prior to embedding (Springer & Roth, 1973). In past studies, antisera to capsular antigens has been one of the most effective methods employed to confer stability on bacterial cell surface components (Baker & Loosli, 1966; Doern & Buckmire, 1976). The application of antibody appears to prevent the collapse of the capsular fibrils during dehydration (Birdsell et al, 1975; Bayer & Thurrow, 1977). It is suggested that the stabilization of capsular material results from cross-linking between the capsular polysaccharide molecules producing a more rigid structure.

In the present study a continuous exocellular layer (capsule) in H. ducreyi has been demonstrated by treating cells with antisera prior to fixation and embedding. This capsular layer was present in all of the strains examined, which included both reference and Winnipeg isolates of H. ducreyi. Kilian and Theilade (1975) had previously examined one of these reference strains (CIP542) by electron microscopy. The staining and fixation methods employed by these workers would not reveal any exocellular material after embedding. This could explain the apparent absence of a capsular layer in their electron micrographs of H. ducreyi.

The wet-India ink method has previously been a standard means of defining large bacterial capsules, such as those of pneumococci (Duguid, 1951). However, this technique is not effective for demonstrating smaller capsules of surface layers associated with bacteria. Most organisms, whether encapsulated or not, appear

to have a refractile halo between the cell outline and the India ink particles. Capsules less than 0.25 μ in width are not readily demonstrated by this technique and are often indistinguishable from the refractile halo. The application of this method on H. ducreyi did not reveal the presence of any capsular layers. If the capsular layer in H. ducreyi, as shown by electron microscopy, represents a close approximation of the natural capsule, one would not expect the wet-India ink method to demonstrate it.

Exocellular layers (capsules) have been demonstrated in other Haemophilus species (Robinson et al, 1972; Buchanan & Gibbons, 1974; Buckmire, 1976). H. influenzae type b was included in this study as an example of a known capsulated organism (Figure 3:8).

The importance of capsules on the interaction of an organism with its environment has been well documented (Luderitz et al, 1968; Cheng et al, 1977; Smith, 1977; Mackie et al, 1979). Capsules can influence the ability of an organism to attach to surfaces and its resistance to phagocytosis and antimicrobial agents. Capsules are also important in preventing the reaction of complement and specific antibodies with antigens located beneath the capsule (Schwarzmann & Boring, 1971). It is because of these properties that the virulence of an organism has often been associated with the presence of a capsular layer (Leidy et al, 1960; Onderdonk et al, 1977). In the present study, both virulent (Winnipeg) and avirulent (reference) strains of H. ducreyi appear to possess capsular layers. This would suggest that virulence, as measured

in the rabbit, is not associated with the presence of a capsular layer. However, the virulence of H. ducreyi could be associated with the type or quantity of capsular material, a possibility not examined in this study.

4:4 Virulence

The production of lesions in man or animals, has been used from earliest reports as a criteria for virulence in H. ducreyi. It is apparent from these studies that many of the failures in demonstrating this property were due to improper technique. As exemplified by Feiner and Mortara (1945) only intradermal injections of viable H. ducreyi would produce a response. However, even with intradermal injections some H. ducreyi strains were avirulent. Both Greenblatt et al (1943) and Feiner and Mortara (1945) reported that on repeated transfer their stock strains became avirulent in the human and rabbit models respectively.

In the present study the virulence of recently isolated Winnipeg strains of H. ducreyi could be demonstrated, but reference strains were avirulent. It is assumed that continued subculturing of the reference H. ducreyi strains produced organisms that were avirulent. It has been reported in work on Neisseria gonorrhoeae (Kellogg et al, 1968) and H. influenzae type b (Doern & Buckmire, 1976) that avirulent forms arise after in vitro subculturing. The virulence of the Winnipeg isolates confirms the work of Hammond

et al (1978c). Subsequent work in this laboratory has demonstrated the virulence property in these H. ducreyi strains was consistent even after they had been subcultured more than 200 times.

In the animal model the dose dependent effect on the results of the virulence test had been shown previously (Greenblatt et al, 1943; Feiner & Mortara, 1945). Dilutions of the test inoculum greater than 1:100 (10^7 cells/ml) produced a similar result in the present study.

Although this study could not correlate the virulence of H. ducreyi with the presence of a capsular layer, virulence may be associated with another property. As seen in Table 3:3, the avirulent reference strains (CIP542 and A75) were sensitive to polymyxin (i.e. MIC $\leq$1.0 μg/ml), whereas the virulent Winnipeg isolates were resistant (i.e. MIC $\geq$128 μg/ml). These results are in agreement with the previous findings of Hammond et al (1978c). Polymyxin exerts its action by altering bacterial membrane structure in an analogous fashion to simple cationic detergents (Storm et al, 1978). Teuber and co-workers (Miller et al, 1977) have suggested that the ability to resist the action of polymyxin is dependent on the type of negatively charged lipids in the membranes of Gram-negative bacteria. It has also been demonstrated that the composition of membrane lipids, especially the nature of the lipopolysaccharide (LPS) of the outer membrane, can influence the virulence of an organism (Wiseman & Caird, 1976; Kreutzer et al, 1978). Work with H. influenzae suggests that its LPS may also influence the type

of infection caused by the organism (Flesher & Insel, 1978).

One can, therefore, postulate that the virulence properties of H. ducreyi could be a property of its membrane lipids, which in turn might reflect its resistance to polymyxin. However, only subsequent work on the nature of H. ducreyi's lipids in both the virulent and avirulent strains will answer this question.

The histology of the virulent lesion in rabbits coincides well with past reports on human lesions (Pund et al, 1938; Sheldon & Heyman, 1946). An interesting observation of the present study, was that the avirulent organism produced a tissue response, although little superficial change was evident. The difference between the tissue response to the virulent and avirulent organism was one of degree. Both strains of H. ducreyi produced an inflammatory reaction but because of the more acute response to the virulent organism, superficial tissue layers were destroyed resulting in necrosis and the formation of the black eschar.

The observation that virulent H. ducreyi remained viable in the lesion up to six days post inoculation is also important. The increased ability to survive within the host indicates the more virulent nature of these strains. In contrast, at no time after the inoculation was the avirulent organism recoverable from either the pus or the lesion itself.

4:5 Antigenic Analysis

Previous work on the antigenic analysis of H. ducreyi had

shown that an antibody response to the organism was produced in chancroid patients and could be induced in some experimental animals. Complement fixation (Gallia, 1917; Reymann, 1950a), agglutination (Teague & Diebert, 1922; Saelhof, 1924; Reymann, 1950a), precipitation (Reymann, 1950a) and intradermal skin tests (Greenblatt & Sanderson, 1938b; Dienst, 1948; Kaplanetal, 1956b) all employed whole cells of H. ducreyi as antigen and in some of these studies the autoagglutination of H. ducreyi in saline suspensions gave confusing results. In addition, most of the past studies did not attempt to analyze even the simple antigenic structure of their H. ducreyi strains. Watanabe (1939) had initiated studies on the characteristics of H. ducreyi antigens by using sonicated whole cells as antigen in an intradermal skin test. Watanabe was able to demonstrate that the antigenicity of H. ducreyi was reduced by heat treatment, increased by alkali treatment and unaffected by freezing or acid treatment.

The present study has avoided the problem of autoagglutination by using whole cell sonicates in gel diffusion analysis. The current work shows that an antibody response to antigens of H. ducreyi can be produced in rabbits. In addition, a complex pattern of antigens could be demonstrated by immunodiffusion and immunoelectrophoresis (Figures 3:11 and 3:12) when cell sonicates were run against antisera to whole cells of H. ducreyi.

The precipitin pattern produced by all H. ducreyi strains tested (N = 11) appears to be similar, suggesting that the

collection of strains was relatively homogeneous. This agrees with the past observations of Teague and Diebert (1922), Saelhof (1924), Greenblatt and Sanderson (1938b) and Denys et al (1978).

As demonstrated by Watanabe (1939), heat stable (100°C, 15 min.) antigens were detected in the present study. However, the majority of the H. ducreyi antigens were heat labile and all of these antigens were protease-heat labile. Heat treatment (100°C, 30 min.) of H. influenzae strains has been shown to reduce the number of antigens which can be demonstrated (Clarke, 1977; Branefors, 1979). Branefors (1979) stated that only the capsular and O antigens of H. influenzae produced precipitates after heat treatment and these antigens were considered heat stable. The present study demonstrates that the capsular antigens of H. influenzae type b were resistant to heat and protease treatment. The possibility exists, therefore, that the heat stable antigens of H. ducreyi are either capsular or O antigens. However, the ability of protease heat treatment to destroy H. ducreyi's antigenic activity suggests that the antigenic moieties or determinants are protein in nature. The exact location and chemical nature of these heat stable and protease-heat labile antigens has to be determined.

Additional examination of H. influenzae type b sonicates by gel diffusion suggested that this organism shared common antigens with H. ducreyi. However, it is assumed that these antigens are not heat stable since heat treatment of either H. influenzae or H. ducreyi antigen preparations resulted in the loss of cross-

reactivity with sera against the other organism. The cross-reactivity of H. ducreyi with other Haemophilus species was further demonstrated by rocket electrophoresis (Figure 3:16). H. parainfluenzae, H. pleuropneumonia and H. gallinarum cross-reacted with groups three through six of the pooled fraction antisera, while H. influenzae and H. paraphrophilus strains reacted with groups four and five.

Cross-reactivity of capsular and noncapsular antigens among Haemophilus species and strains has been previously reported. Branefors (1979) showed by gel diffusion that H. influenzae, H. parainfluenzae and H. haemoglobinophilus shared common antigens. Denys et al (1978) also stated there was cross-reactivity between H. influenzae, H. parainfluenzae and H. parahaemolyticus with sera directed against H. ducreyi. These reports and the results of the current study establish an antigenic relationship among these Haemophilus species and further supports the inclusion of H. ducreyi in this genus. The characterization, both chemical and structural, of the antigens that are common among H. ducreyi and other Haemophilus species is a worthwhile goal of future investigation.

This study has also demonstrated that the antigens of H. ducreyi (heat stable and heat labile) can be separated by column chromatography (Figure 3:14). The ability to separate these antigens could be useful in determining whether there are serotypes among various strains of H. ducreyi. Although H. ducreyi appears serologically homogenous, other Haemophilus species have demonstrated up

to six serotypes (Alexander et al, 1961). Establishing strain, species and genus specific antigens would enable more complete epidemiological studies. In addition, determining the distribution of antibodies among the general population that are either specific for, or cross-react with H. ducreyi antigens, would be useful in diagnosing chancroid via serological tests. If antigens can be found which are specific for H. ducreyi, preparation of a fluorescent labelled antisera of high activity is a reasonable objective for further studies. The production of such fluorescent labelled antisera against a specific H. ducreyi antigen would provide a valuable aid in the diagnosis of chancroid infections.

5:1 Summary

The main features of the thesis are summarized as follows:

1. Haemophilus ducreyi strains from a recent outbreak of chancroid in Winnipeg and reference strains were limited to growth on solid media containing hemin. Growth could not be demonstrated in broth cultures using various media, with and without added hemin.

2. Colonies of H. ducreyi grown on chocolate agar after 24-48 hours incubation appeared as nonmucoid, yellow-gray globules which produced a small zone of alpha hemolysis after 72 hours growth.

3. H. ducreyi appeared as a Gram-negative, pleomorphic rod approximately 1.5 x 0.5 μ with rounded ends when viewed by light microscopy after Gram staining.

4. Nutritionally, all strains of H. ducreyi exhibited a requirement for X factor (hemin) and were independent of V factor (NAD). This requirement was confirmed by a negative porphyrin test.

5. Tests performed on the API-20E indicated H. ducreyi is relatively inactive biochemically. All strains were Voges-Proskauer, and oxidase positive, with the majority of strains fermenting glucose and arabinose. Methods for testing biochemical activity gave varying results and nitrate reductase activity could not be demonstrated in the API-20E by the majority of strains. However, all strains were nitrate reductase positive using the

method of Cowan and Steel (1965).

6. No pili or other bacterial appendages could be demonstrated on negatively stained H. ducreyi by electron microscopy.

7. H. ducreyi exhibited a tripartite outer membrane, characteristic of the Gram-negative cell wall, when examined by electron microscopy.

8. Ruthenium red and Alcian Blue staining revealed the presence of exocellular material in electron micrographs of thin sectioned H. ducreyi. Visualization of the exocellular material was improved by treatment with antisera against whole cell H. ducreyi prior to fixation and staining with ruthenium red or Alcian Blue. This technique demonstrated a continuous exocellular (capsular) layer 0.01 to 0.05 μ.

9. Virulence testing in the rabbit intradermal model indicated that all Winnipeg H. ducreyi strains were virulent and reference strains were avirulent. This model also indicated that virulent strains produced a more acute inflammation in the associated skin lesion than did the avirulent strains. Viable H. ducreyi could be isolated from the virulent lesion up to the sixth day post inoculation but at no time after the inoculation could H. ducreyi be isolated from the avirulent lesion. The production of lesions by virulent H. ducreyi strains was found to be dose dependent.

10. Virulence in H. ducreyi could not be associated with any morphological or structural feature of the organism. However, polymyxin resistance was associated with the capacity for virulence in selected H. ducreyi strains.

11. Antigenic studies indicated that a complex pattern of precipitating antibodies against H. ducreyi antigens could be produced in rabbits. The majority of H. ducreyi antigens were heat labile (100°C, 15 min.) but all antigens were protease-heat labile. Heat stable and heat labile antigens could be separated by column chromatography on DEAE Sephadex.

12. The antigenic structure of all H. ducreyi strains appeared homogeneous by gel diffusion and rocket electrophoresis. Cross-reacting antigens between H. ducreyi and H. influenzae, H. parainfluenzae, H. pleuropneumonia, H. paraphrophilus and H. gallinarum were also demonstrated, establishing an antigenic relationship between these Haemophilus species. The cross-reacting antigens of H. influenzae were also shown to be heat labile.

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A P P E N D I X

Appendix I

Solid Media (see Table 3:1)

(a) Blood Agar

Trypticase Soy Agar (Baltimore Biological Laboratories, Baltimore, Md.) (BBL) is overlaid with 5% Sheep Red Blood Cells in the Trypticase Soy Agar.

Trypticase Soy Agar (BBL) (components per litre distilled water)

Trypticase Peptone	15.0 g
Phytone™ Peptone	5.0 g
Sodium chloride	5.0 g
Agar	15.0 g

(b) GC Agar Base (BBL) (components per litre distilled water)

Peptone	15.0 g
Corn starch	1.0 g
Potassium Phosphate Dibasic	4.0 g
Potassium Phosphate Monobasic	1.0 g.
Sodium chloride	5.0 g
Agar	10.0 g

(c) Chocolate Agar

To 1 litre GC Agar Base (BBL) add 10 g Hemoglobin (BBL) and 1% CVA (Gibco).

(d) Sheep's Blood Chocolate Agar

To 1 litre of GC Agar Base (BBL) add the following supplements:

4% Sheep's Red Blood Cells

1% Kellogg's supplement (per 100 ml distilled water)

Glucose	4.0 g
Glutamine	0.5 g
Ferric nitrate	50.0 mg
Thiamine Pyrophosphate	1 ml of 0.2% solution

0.6% Nicotinamide Adenine-dinucleotide (NAD)

Appendix II

Liquid Media (see Table 3:1)

(a) Mueller Hinton (BBL) (components per litre distilled water)

Beef extract	3.0 g
Acidicase™ Peptone	17.5 g
Starch	1.5 g

(b) Eugon (Difco) (components per litre distilled water)

Bacto - Tryptone	15.0 g
Bacto - Soytone	5.0 g
Bacto - Dextrose	5.0 g
Bacto - L-cystine	0.2 g
Sodium chloride	4.0 g
Sodium sulfite	0.2 g
Sodium citrate	1.0 g

(c) Brain Heart Infusion (BBL) (components per litre of distilled water)

Brain Heart Infusion	8.0 g
Thiotone™ Peptone	5.0 g
Trypticase® Peptone	16.0 g
Sodium chloride	5.0 g
Dextrose	2.0 g
Disodium Phosphate	2.5 g