TITLE

COUNTERIMMUNOELECTROPHORESIS IN THE DETECTION OF SURFACE ANTIGENS OF *Neisseria gonorrhoeae*

being a thesis presented by

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to the University of St. Andrews in application for the degree of Master of Science.

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ABSTRACT

The study was initiated to investigate the possibility of developing a new serological method for the diagnosis of infection caused by Neisseria gonorrhoeae. A full description of the organism is given, with regard to its biochemical and serological characteristics, and its pathogenicity and disease pathology. A resumé of the methods used for isolation and identification of the organism is presented together with an evaluation of the reliability of each method. The rationale of the method under investigation, using a counterimmunoelectrophoresis system to detect capsulate Neisseria gonorrhoeae in infectious exudates, is discussed.

A full investigation of capsule-staining methods was undertaken in order to identify reliably capsulate organisms in vivo and in vitro; the most reliable method being found to be the India ink stain.

A number of capsule-promoting media were developed and examined to ascertain their capacity to sustain rapid, luxuriant growth of capsulate N. gonorrhoeae for use in raising antibodies, in rabbits, to the N. gonorrhoeae capsule, using both capsulate whole cells and purified capsular material.

A description of the use of the counterimmunoelectrophoresis system in assessing the reactivity of the antisera raised in rabbits towards capsulate N. gonorrhoeae strains and strains of other bacterial species is given, together with possible reasons for the cross-reactivity of both sera toward the other bacterial species.

A discussion is presented of the possible use of the N. gonorrhoeae capsule in vaccine development and suggested lines of further research into the biochemical and serological make-up of the capsule.

DECLARATION

I hereby declare that the following thesis is based on work carried out by me, that the thesis is my own composition and that no part of it has been presented previously for a higher degree.

The research was conducted in the Department of Microbiology, Fife Area Laboratory, Kirkcaldy and in the Department of Biochemistry and Microbiology, University of St. Andrews, under the joint supervision of Dr. P. N. Edmunds and Dr. Stephen Bayne.

DEDICATION

To my wife Elaine for her perseverance and encouragement which sustained me throughout the course of this work.

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INDEX

2. MATERIALS AND METHODS (cont.)

1.1. Neisseriaceae (1)

The Neisseriaceae are a family of closely related gram-negative organisms. They are either spherical, and found in pairs or masses with their adjacent sides flattened, or rod-shaped, and found in pairs or short chains. None of the family are flagellate, though some species show a twitching motility.

Some species are pigmented with xanthophyll, and some require complex growth factors for primary isolation. All species are aerobic with an optimum temperature for growth of 32 - 37°, and most species produce catalase and cytochrome oxidase.

Neisseria sp., Branhamella sp. and Moraxella sp. are parasitic, whilst Acinetobacter sp. are saprophytes or opportunistic pathogens.

The guanine plus cytosine content of the deoxyribonucleic acid ranges from 39 - 52 moles % and the type genus is Neisseria.

1.2. Neisseria (1,2)

There are six member species of the genus Neisseria; Neisseria gonorrhoeae, N. meningitidis, N. sicca, N. subflava, N. flavescens and N. mucosa.

All six species are gram-negative cocci, 0.6 - 1.0 um in diameter, occurring singly, though often arranged in pairs. Division at right angles to the axis joining the paired cocci often results in the formation of tetrads, and one species, N. subflava, appears

frequently in the form of dense clumps with only occasional isolated organisms.

None of the species form endospores and all are non-motile and produce catalase and cytochrome oxidase.

They possess weak fermentative ability, few carbohydrates being utilized, and are either aerobic or facultatively anaerobic.

The guanine plus cytosine content of the deoxyribonucleic acid ranges from 47.0 - 52.0 moles % and the type species of the genus is Neisseria gonorrhoeae.

Dr. Albert Neisser first described the causal organism of gonorrhoea in 1879 (3), in the pus cells of 35 patients suffering from the disease. He called the organism Micrococcus der Gonorrhoe.

It was, however, several years before the organism could be successfully cultivated, and this was achieved first by Leistikow and Loeffler in 1882 (4), on blood serum gelatin, and then by Bumm (3) on coagulated human serum. Neisseria meningitidis was first isolated by Weichselbaum, in 1887, from several cases of cerebrospinal meningitis (2).

1.3. Neisseria gonorrhoeae and Neisseria meningitidis

Apart from the properties possessed by all six members of the genus, N. gonorrhoeae and N. meningitidis possess a number of characteristics exclusive to these two species.

They are both pathogenic for man, though N. meningitidis

may be carried, asymptomatically, by 10 - 25% of the population, and in this respect may be found implicated in disease in similar situations. Both species have been isolated from blood, conjunctiva, joints, and petechiae in skin, whilst, uncommonly, N. meningitidis has been implicated in cases of venereal disease (5) and N. gonorrhoeae in cases of septic meningitis (6). When implicated as the causal organism of disease, both N. gonorrhoeae and N. meningitidis are found, characteristically, inside the polymorphonuclear cells of the inflammatory exudate.

Cultural morphology of both species is also similar, although N. meningitidis tends to produce slightly larger colonies, and neither species will grow on media lacking in certain growth factors, which are to be found in animal fluids. This may be due rather to their susceptibility to inhibitory substances present in chemically defined media, which are neutralised by the 'growth factors' than to exacting nutritional requirements per se.

When inflammatory exudates, from diseases caused by either organism, are examined using Gram's stain, then N. meningitidis, or N. gonorrhoeae may appear as oval or spherical cells, lacking the characteristic diplococcal arrangement. When the exudates are examined using Neisser's stain, both species may be found to contain blue-black granules. Although these granules may be present in the gonococcus only during the active phase of disease, they are a phenomenon exclusive to these two members of the genus.

Sensitivity to external agents is also similar in both

species; N. meningitidis and N. gonorrhoeae are both susceptible to killing by heat, 55° for 5 min. being sufficient, and both are susceptible to drying. Until the emergence of β-lactamase penicillin-resistant mutants of the gonococcus, both species had similar sensitivities to antibacterial agents.

The most significant feature, however, is that the meningococcus and the gonococcus are genetically very closely related species. This has been shown by transformation experiments, by direct hybridization, and by thermal stability studies of the deoxyribonucleic acid duplexes (7). The guanine plus cytosine content of the deoxyribonucleic acid in the gonococcus is 49.5 - 49.6 moles % and in the meningococcus is 50.0 - 51.5 moles % and this again demonstrates the close relationship between the two species.

The possession of a capsule by N. meningitidis has proved useful as an aid in diagnosing meningococcal disease and in identifying the organism by serological methods (8). If N. gonorrhoeae is shown to possess a capsule then the existing close similarities between the two species would suggest that methods of identifying gonococcal disease, similar to methods already in existence for identifying meningococcal disease, might be feasible.

The possession of a capsule by N. gonorrhoeae has not yet been proven conclusively and text-books define N. gonorrhoeae as being a non-capsulate organism (9).

1.4. Neisseria gonorrhoeae and disease

Neisseria gonorrhoeae is a strict parasite of man and when present in the human is invariably the causal organism of disease, and never takes the role of saprophyte.

Neisseria gonorrhoeae is, as its name suggests, the causative organism of gonorrhoea, a venereal or sexually transmitted disease, though direct transfer from person to person may not be necessary. Gilbaugh and Fuchs (10) have shown that gonococci can survive in pus on toilet seats for up to 2 hr., and have suggested that contaminated toilet paper could be a vehicle of infection. Elmoss and Larsen (11) demonstrated the survival of gonococci for up to 24 hr. on a towel rinsed periodically with physiological saline, indicating that spread of infection could be facilitated by the use of contaminated communal towel facilities in public washrooms and lavatories.

The probability of a man acquiring gonorrhoea from a single exposure has been estimated to be between 20 - 35% (12). In the male the first symptom of the disease is a purulent discharge from inflammation of the urethra. The infection may then spread along the mucous membrane of the urogenital tract and infect the prostate, seminal vesicles, epididymis and the periurethral tissue. A cohort study of United States Navy personnel showed that urethritis developed within two weeks of exposure in 97% of patients (13) and between 1% and 3% of men with five or more episodes of gonococcal disease develop bilateral epididymitis, depending on the treatment given to them (14).

Before effective chemotherapy was available, epididymitis occurred in 17 - 30% of male cases (15), whilst in areas providing full health service cover the incidence is now 0.7% (16).

Estimates as to the prevalence of asymptomatic male carriers varies from 2.3% in one study (13) to 10% in another (17), and these carriers are very important epidemiologically (13). These men will not need to seek treatment and will continue to pass the infection unknowingly to their partners, who may then themselves have to seek treatment. If, however, the symptomless carrier is not treated, then he will continue to re-infect his partner, thereby negating any treatment his partner may have had.

The incidence of infection in females without any genitourinary symptoms has been estimated to be approximately 60% (18, 19). Higher incidences have been reported (4), but these figures have usually been derived from the numbers of patients visiting Special Treatment Clinics (S.T.C.s.) in the U.S.A., where most male patients are seen because of acute symptomatic urethritis, whilst most females attend because they have been named as contacts and have no symptoms or have symptoms which they have ignored. Also it may be that males with genitourinary symptoms would tend to seek advice from S.T.C.s., whilst females would tend to visit their general practitioners (20).

In the female the urethra and cervix are infected but the resultant discharge may be slight and go unnoticed. Bartholinitis, infection of the vestibular glands, may follow and endometritis,

salpingitis and infection of the peritoneal cavity are common complications. Acute salpingitis is estimated to occur in 10 - 15% of women with gonorrhoea (21, 22) and, in the U.S.A., the proportion of acute salpingitis caused by N. gonorrhoeae ranges between 47 - 85% (4). The most serious complication of gonorrhoeal infection in the female is pelvic inflammatory disease which develops in 9 - 20% or more of recently infected women during the first or second menstrual cycle after infection. The disease may be so serious as to cause perihepatitis (23), peritonitis (24), and chronic invalidism (25). Studies by Westrom (25) indicated that sterility, resulting from occlusion of the Fallopian tubes, occurred in 12.8% of patients who had had one attack of salpingitis, in 35% who had had two attacks and in 75% who had had three or more attacks.

Rectal infection is found in approximately 40% of females, as a result of anal coitus or spread from the genitalia, and is generally found in the passive partner of male homosexual relationships.

Infections of the pharynx (26, 27) with associated sore throat, pharyngitis and tonsillitis (28, 29) are now becoming increasingly more common owing to the recent upsurge in the need for unusual acts of sexual expression, such as buccal coitus. Surveys would suggest that 7% of heterosexual men, 12.5% of all females (30) and 25% of homosexual men (31) attending S.T.C.s. with ano-genital infection also have gonococcal throat infections. It is difficult to establish if N. gonorrhoeae is transmitted by mouth to mouth contact,

though Willmott (32) has described one case of transfer of gonococcal pharyngitis by kissing. Transmission would depend on the gonococci lying free in the saliva and not firmly attached to the mucous membranes of the pharynx. Wallin and Siegel (27) suggest that N. gonorrhoeae will colonise the pharynx without producing any complications and that they doubted that mouth to mouth spread was likely.

Although the usual pathology of gonorrhoea is one of urethral and cervical inflammation with accompanying purulent exudate, there have been a large number of reports of more unusual pathology. Gonococcal bacteraemia is estimated to occur in 1 - 3% of women and 1% of men with gonorrhoea (33, 34) and the most common manifestation of gonococcaemia is the gonococcal arthritis-dermatitis syndrome (35, 36, 37). This has become the leading cause of polyarthritis in adults. Symptoms include a polyarticular tenosynovitis and petechial, papular, pustular and haemorrhagic-pustular skin lesions, particularly over the joints. Bacteraemia is demonstrable by blood culture and gonococci may be seen by smear from the skin pustules. If treatment is delayed purulent synovial effusion involving one or two joints may develop and Angevine (38) has described a case of gonococcal osteomyelitis in a patient suffering from gonococcal arthritis.

Less common manifestations of gonococcal disease which have been described are:- meningitis (6, 39), amnionitis (40), lymphadenitis (41), otitis externa (42), scalp wound infection in a

neonate as a result of transmission from an infected mother (43), blenorrhagia (44) and conjunctivitis (45, 46).

Epidemics of gonococcal conjunctivitis used to be relatively common occurrences, particularly in institutions (47) where there is close personal contact between the 'inmates', the spread of infection usually being carried from the urogenital tract to the eyes via the hands. Infection of the eyes in the newborn, ophthalmia neonatorum, is now virtually unknown in Great Britain and most other countries where adequate peri-natal care of mother and child is provided (48). The administration of silver nitrate at delivery is the best prophylaxis against gonococcal ophthalmia (49) and the risk of the condition developing in a child born to an infected mother is estimated to be less than 2% if silver nitrate is used (50).

Outbreaks of vulvovaginitis in young females in paediatric wards and other children's institutions have also been reported (51, 52, 53), usually as the result of cross-infection caused through the use of infected hand towels etc.. Cohn, Steer and Adler (51) suggest that vulvovaginitis in young females with the gonococcus as the causative organism is now rare and probably only occurs as a result of sexual contact, and this hypothesis is in agreement with Branch and Paxton (54) and Lang (55).

Perhaps the most bizarre presentation of gonococcal disease was reported by Jokl (56) of a case of sudden death during athletic activity due to gonococcal myocarditis.

Undoubtedly there are both organism- and host-related factors which can influence the course of gonococcal infection. Each site of colonization and infection provides a different environment where nutrient availability, pH and competing micro-organisms may differ. In spite of their strict nutritional requirements Neisseria gonorrhoeae can survive in a number of environments within the host, suggesting that sufficient quantities of all the required 'growth factors' are present at each site.

1.5. Methods of isolating and identifying Neisseria gonorrhoeae and gonococcal infection.

1.5.1. Culture and biochemistry

The diagnosis of gonococcal infection in the laboratory has generally centred around the cultivation and subsequent biochemical identification of the organism. One of the major problems associated with the cultivation of the gonococcus from exudates and other material has been the organism's poor viability during transport of the specimen to the laboratory. This is particularly true if there is a comparatively long period between the taking of the specimen and its inoculation onto culture medium (57).

Transport media (58, 59), are used, where available, for the presumed safe carriage of the specimen from the patient to the laboratory. The media should be non-nutrient, so as to minimise

overgrowth of contaminating organisms, and prevent oxidation. Many samples of agar contain inhibitory substances which suppress the gonococcus and these are best neutralised by the addition of charcoal to the swabs, the charcoal binding the offending fatty acids. There have been reports, however, by Khandari et al. (60) and by Shtibel (61) of difficulties encountered with the use of transport media in conditions of extreme heat or cold.

If the specimen is inoculated directly onto culture medium at the 'bedside' then safe transport of the inoculated medium to the laboratory must be effected, in order to minimise conditions which might adversely affect the survival of the organism, for example low temperature and low carbon dioxide concentration. A simple means of transport is the 'candle jar', the inoculated medium being placed inside a glass 'sweet-jar' together with a lighted candle. The lid of the jar is then replaced tightly and the candle allowed to burn until all the available oxygen in the jar is used up and the candle ceases to burn. The burning of the candle provides the necessary carbon dioxide concentration and humidity for the survival of the gonococcus but the jar still has to be transported quickly to the laboratory to minimise any adverse temperature drop.

Commercially available systems (62, 63, 64, 65) for the 'bed-side' inoculation of the specimens onto suitable media are now being assessed. These systems preclude the use of petri-dishes and candle-jars and provide almost ideal nutrient and atmospheric conditions for the transport and isolation of the gonococcus.

Donald (66) found that one such system gave superior isolation figures to a conventional transport medium and laboratory selective agar medium, described below.

The medium most often used for the isolation of the gonococcus is a modified heated blood agar medium, 'chocolate agar', containing antibacterial agents to which the gonococcus is not susceptible but to which any likely contaminants are susceptible. The antibacterial agents used vary from laboratory to laboratory but generally are combinations of vancomycin, polymyxin, nystatin, trimethoprim, lincomycin and amphotericin B (67, 68). Selective media containing antibiotics should not be used alone because a small percentage of gonococci are sensitive to vancomycin or trimethoprim (69, 70) and a more satisfactory, though seldom used, system would be to inoculate specimens onto bi-plates, one half containing selective medium and the other half containing a 'chocolate agar' without added selective agents.

A Gram-stained smear of the exudate or discharge should also be examined, and typically the gonococcus, if present, should be visible inside the polymorphonuclear cells or epithelial cells. If gram-negative diplococci are seen intracellularly in the polymorphs then the smear may be regarded as indicating probable gonococcal infection. Often, however, gram-negative diplococci are seen in the extracellular debris, which may be regarded as sufficient evidence of gonococcal infection in the male but not if the patient is female (71, 72, 73). The female genital tract is a common source of other

members of the Neisseriaceae which are indistinguishable from gonococci by Gram's stain alone. Jacobs and Kraus (74) suggest that microscopic examination of Gram-stained smears of urethral exudates from male patients with gonococcal urethritis allows identification of infection in approximately 98% of cases, whilst Barlow and Philips (75) reported that only 60% of infected females would be detected by Gram's stain microscopy, partly owing to the smaller numbers of gonococci present in cervical and urethral material in women than in urethral secretions from men, and also because specimens from a female which contain material from the vagina are perhaps the most frequent source of gonococcal-like artefacts being seen in Gram-stained smears.

The plate cultures of the exudates or discharges are incubated overnight at 35 - 37° in an environment of at least 70% humidity and a 3 - 7% concentration of carbon dioxide in air (76). If typical colonies are seen then further identification tests should be performed; if the culture is negative after 24 hr. then reincubation for a further 24 hr. is necessary before a negative result can be assumed.

A Gram stain of a typical colony should be performed as the first step in identification with the addition of a drop of a 1% solution of tetramethyl-paraphenylene diamine to the suspect colony to ascertain oxidase production. The isolation of oxidase-producing gram-negative diplococci is not however conclusive evidence of gonococcal infection. Bøvre et al. (77), who relied solely on

colonial morphology and the oxidase test, found two species of bacteria from suspected cases which had typical gonococcal colonial morphology and gave a positive result in the oxidase reaction. One of the organisms was Kingella kingae, a rod-shaped organism and the other was Neisseria elongata. Platt and Snell (78) isolated a number of oxidase-positive, gram-negative cocci not belonging to the genus Neisseria from the urogenital tract of a number of male and female patients over a twelve-month period. The organisms were fortunately sufficiently different in their biochemical and genetic makeup from Neisseria gonorrhoeae to enable the two workers to identify them positively as not being gonococci; this would not have been the case had reliance been put solely on colonial morphology and the oxidase reaction.

Once preliminary identification of the isolate has been made then confirmatory biochemical tests to identify the organism positively have to be performed. The standard procedure relies on the degradation of sugars by the suspect gonococcus. Cysteine trypticase agar containing glucose or maltose or sucrose or lactose is inoculated with the test organism and the cultures incubated as before. If the isolate is Neisseria gonorrhoeae then only glucose will show a colour change in the indicator-containing medium, indicating the production of acid from that sugar, the remaining three sugar media showing growth but no colour change.

There are, however, a number of disadvantages to the use of these biochemical tests in the identification of Neisseria gonorrhoeae.

Gonococci frequently grow poorly on the maltose-containing medium, making it impossible to score a valid negative result in the absence of adequate growth, and as there are meningococci with little or no ability to utilise maltose differential diagnosis is made difficult (79). The sugar degradation tests frequently take forty-eight hours before the gonococcus produces a visible colour change in the medium, which may be as much as ninety-six hours after the taking of the specimen. Maniar (80) did find that the addition of guinea-pig serum to the sugar media gave a more luxuriant growth of the gonococcus; however his cultures still had to be incubated for forty-eight hours before acid production was discernible.

The media for sugar degradation tests must be sterile, which is difficult to achieve satisfactorily without degrading the sugars with the heat necessary to achieve sterilization. The pH of the medium must also be within strictly defined limits in order to allow the gonococcus to grow but also to allow a colour change in the indicator used to exhibit acid production by the organism under examination (81). Another difficulty lies in using inocula for the sugar tests taken directly from the selective medium used for primary isolation. The selective medium may contain contaminating organisms in a dormant state, which will become viable once inoculated onto the antibiotic-free sugar media. Inoculation of the sugar media should be made from pure culture on 'chocolate agar' and this procedure adds a further 24 hr. to the time necessary for identification of the organism, unless both 'selective' and

'non-selective' 'chocolate agar' is used for primary isolation.

A number of rapid carbohydrate assimilation test procedures have been developed which do not require growth of the organism but rely solely on the identification of pre-formed sugar-assimilating enzymes in the isolate (79, 82, 83). Pre-formed enzyme is measured by adding a suspension of the overnight growth of the suspect organism to a buffered, non-nutrient, solution containing the sugar to be tested and a pH indicator. The pre-formed enzyme degrades the appropriate sugar and the indicator exhibits a colour change, in as little as 60 min. in most cases. Young, Paterson and McDonald (79) found that all ninety-seven isolates of Neisseria species from human sources were correctly identified by their rapid carbohydrate utilization test, when compared with conventional sugar degradation tests and immunofluorescence.

A test kit for the identification of Neisseria gonorrhoeae from culture plates is now commercially available (84) and is derived from a technique described by Daniellsson and Kronvall (85) and Menck (86). This identification procedure relies on gonococcal-antibody sensitized staphylococci being co-agglutinated by Neisseria gonorrhoeae from culture media. The test is supposedly specific for Neisseria gonorrhoeae and no cross-reactions are said to occur; however, when the process was in the developmental stage, Barnham and Glynn (87) found that 75% of meningococcal isolates, and 40% of other Neisseria species gave positive reactions due to difficulty in

obtaining sufficiently specific antibody to the gonococcus. Daniellsson and Kronvall (85) also reported positive reactions with meningococci, Moraxella, Haemophilus and Pseudomonas species and suggested that the variable results reflected variations in the staphylococcal protein A- gonococcal antibody reagent and the actual performance of the test. Shankar, Daly and Sorrell (88) compared the co-agglutination technique with the rapid carbohydrate utilization test and found that the co-agglutination gave 4% erroneous results, suggesting that the method could not replace biochemical methods of identification but might replace more time-consuming tests for screening isolates.

Bruckner and Christianu (89) reported that gonococci were seen to be agglutinated by antisera to gonococci raised in horses or rabbits; however their antisera also agglutinated meningococci. Torrey in 1907 (90) and Vannod (91) claimed that it was impossible to differentiate between gonococci and meningococci by this method, owing to the organisms possessing common antigens. Work performed by these same workers over subsequent years failed to unravel the serological make-up of the gonococcus despite exhaustive absorption and re-agglutination studies on large numbers of strains (92).

Recent work by Angelsen and Maeland (93) using antisera raised in rabbits against three strains of N. gonorrhoeae suggested that prior heat treatment of the organisms under test to 100° for 2 hr. prevented problems arising, owing to inagglutinability hyperagglutinability or autoagglutinability, which had hampered

agglutination reactions previously. They were, however, working with only three laboratory strains of gonococci and further work using a more diverse source of organisms would be of value in giving a true assessment of the test's capability.

1.5.2. Serological methods of diagnosis and identification

1.5.2.1. Assessment of bacterial antigens in exudates

Although co-agglutination and slide agglutination techniques, described above, can be regarded as serological methods, they do require isolation of the causative organism as a prelude to their use, whereas the techniques described below may be used to detect either whole organisms or antigenic components of those organisms directly in exudates or other body fluids.

Deacon *et al.* in 1959 (94) reported a technique for the identification of *N. gonorrhoeae* in exudate smears using a direct fluorescent antibody technique (DFA). The technique relies on smears of exudate being allowed to react with gonococcal antiserum previously labelled with a fluorescein dye. The antiserum reacts with any gonococci in the smear and these fluoresce when viewed under ultra-violet light. Commercial antisera are available from various sources though some workers prefer to produce their own antisera using the method of Peacock (95).

The DFA technique however has suffered from the problem of cross-reactivity with organisms other than the gonococcus.

Lind (96) found cross-reactions with N. meningitidis, N. catarrhalis, N. flava, N. subflava and also with Staphylococcus aureus and Streptococcus pyogenes.

Peacock (95), and Simon et al. (97) reported that non-specific staining could be removed by absorption of the fluorescent antiserum with bovine bone marrow, whilst Lind (96) diluted out the non-specific staining, and like Peacock (95), found that absorption with N. meningitidis minimised cross-reactions with other Neisseria species. Care must be taken that absorption of the antiserum with N. meningitidis does not absorb out all the reactivity of the antiserum with the gonococcus and a delicate balance exists between the appearance of non-specific staining and failure to produce any staining at all.

Many other attempts have been made to minimise the effects of non-specific staining in the DFA technique. Thin (98) treated the smears with pooled human serum and then counterstained with naphthalene black, reporting that he had no difficulty with non-specific staining when employing this method. Somerville (99) also used naphthalene black as a counterstain and also reported that this improved the sensitivity of the method. Schmid et al. (100) combined the DFA stain with Gram's stain and was able to demonstrate intracellular gonococci, by which means he hoped to differentiate between gonococcal and non-specific infection. Gaafar (101) used a variation of the established DFA technique in which he heated the antisera and smear at 59°. He used the method

as a screening test and found it had 95% sensitivity and 97% specificity when compared with established methods of culture and biochemical characterization.

Doubt still remains however about the sensitivity and specificity of the DFA technique. Thin, Williams and Nicol (102) found that their DFA method identified 126 of 144 culture-positive females and that the commercial conjugate used also stained meningococci. Barteluk et al. (103) found that only 59% of their culture-positive cases were identified by DFA, while Gilliet (104) found the DFA method positive in 40 of 46 males and 14 of 25 females who were found positive by other means. Daniellsson and Kronvall (85) also suggest that the DFA technique is not specific enough to use as the sole means of identifying gonococcal disease, particularly in rectal and tonsillo-pharyngeal gonorrhoea where other neisseriae may be present and cause cross-reaction, and like Enfors et al. (105), suggest that the DFA method be used as an adjunct to other more reliable methods.

A method of detecting gonococcal antigens by solid-phase radioimmunoassay with radioactively-labelled antibody has been described by Thornley et al. (106). They identified 74% of 42 males and 70% of 14 females who were proved infected by other methods; however they also found that 16% of 18 non-infected females gave positive results. They suggest, however, that the method is as yet in the developmental stage and that with refinement could be useful. The two major drawbacks to this method would be the complicated

technique and need for expensive ancillary equipment which may preclude its use widely.

Several methods have been evolved to allow the rapid diagnosis of gonococcal infection by the detection of gonococcal growth products in exudates. Spagna, Prior and Perkins (106a, 106b) have described the use of Limulus lysate to detect gonococcal endotoxin. The test, however, is non-specific, particularly in females where there may be a preponderance of other bacterial flora giving false positive results.

Takeguchi et al. (106c) have described the presence of an enzyme, 1,2-propanediol oxidoreductase, in large amounts in N. gonorrhoeae. This enzyme catalyses the conversion of 1,2-propanediol into an unknown product in the presence of NAD and this reaction may be used in an NAD-linked fluorimetric assay after bacterial cell lysis. The enzyme is however found in large amounts in N. meningitidis and in smaller amounts in other members of the genus and this lowers the specificity of the test.

In Neisseria the major hydroxy fatty acid is 3-hydroxydodecanoic acid and this exceeds 3-hydroxytetradecanoic acid, the major fatty acid of most gram-negative bacteria, by the ratio of 20:1. The detection of this fatty acid in secretions by thin layer chromatography and gas liquid chromatography has been described by Sud and Feingold (106d). The method is tedious and the presence of this enzyme in other members of the genus precludes its use for the

specific detection of gonococcal disease.

1.5.2.2. Assessment of gonococcal antibodies in exudates and other fluids

Gonorrhoea is principally an infection of mucosal surfaces and causes stimulation of the local immune response on the mucous membranes at the infected sites, this antibody response antedating any systemic response (107). Antibodies produced at these sites are detectable in secretions and Tomasi (107) found that IgA predominated, as did McMillan, McNeillage and Young (108), who also demonstrated IgG in 90% of urethral exudates and IgM in 49% of urethral exudates from 132 men. They found increased levels of IgA in secretions from the uterine cervix in 95% of 75 women, in rectal secretions from 17% of 18 male homosexuals and in the saliva of 100% of 12 men and 66% of 6 women, in all cases associated with IgG and IgM. They found that the IgA and IgG cross reacted with N. lactamica and N. meningitidis in an indirect fluorescent antibody test and also that IgA and IgG, but not IgM, could be detected in secretions from some non-infected males and females.

Following successful treatment, titres of IgA in secretions decline rapidly, becoming undetectable, in most cases, in 14 days. IgM similarly decreases but IgG decreases more gradually and can be detected in 80% of men after 28 days (108).

Ison and Glynn (109) using an enzyme-linked immunosorbent

assay (ELISA) technique to detect antibody response in patients to envelope antigens of N. gonorrhoeae found that IgG antibodies predominated in the serum, but that IgM and IgA were also present. They suggested that the presence of IgA in the circulation could be explained by its absorption from sites of local production.

The most satisfactory confirmation of a diagnosis of gonorrhoea, or its complications, is the isolation and identification of the causative organism. As we have seen above, however, there are a number of problems associated with the isolation and biochemical identification of the gonococcus which make it worthwhile to look at serological methods of diagnosis by identifying anti-gonococcal antibodies in body fluids. This is particularly true where cultivation of the gonococcus is unlikely to succeed, when, for example, the patient has already received antibiotics.

One of the more simple serological tests is the 'Gonorrhoea Card Test' devised by Kamei, Chen and Gaafar (110). The test relies on charcoal particles, previously sensitized with an antigen extract of N. gonorrhoeae, being used to indicate gonococcal infection in an agglutination assay with human serum. The test is simple and detected 81% of infected men and 85% of infected women; however, the specificity is reported as being only 83% and this would need to be improved before the test could be used routinely. A similar test procedure using the flocculation of sensitized bentonite particles as the indicator complex was shown

by Lee and Schmale (111) to detect 68% of infected males and 86% of infected females but with 12% of false positive results, and Wallace et al. (112) found that their system cross-reacted with N. catarrhalis.

Using sensitized sheep red blood cells in a microhaemagglutination test Logan, Cox and Norins (113) detected only 35% of infected males and 47% of infected females with 10 - 19% false positives, Ward and Glynn (114) detected 33% and 60% of infected men and 27% and 84% of infected women in two series with 10% false positives, while Fletcher, Miller and Nicol (115) had similar findings with a 51% detection of infected males and 82% of infected females.

The problems associated with slide and tube agglutination, and flocculation techniques, is their lack of sensitivity and specificity. Although there is a need for a simple rapid test, the reliability of the test should not be in doubt.

A more popular serological method for the detection of gonococcal antibodies in secretions or in serum is the indirect fluorescent antibody (IFA) test. Gaafar (116) reported an IFA technique using strains of gonococci with heat-labile trypsin-sensitive antigen (L-antigen). This L-antigen was used to detect human IgG -anti-L-antigen in persons suspected of having gonococcal infection. Gaafar reported the test to be 94.6% sensitive in females and 86.9% sensitive in males with 96.7% and 100% specificity respectively, and he commented that the lower specificity in females

was caused by the carriage of non-gonococcal Neisseria species in the female urogenital tract with subsequent non-specific antibody response.

Many other workers, however, disagree with Gaafar's findings. Wilkinson (117) detected only 24 of 119 infected males and 52 of 85 infected females with 7 false positives in 182 patients and Toschack (118), investigating the effect that meningococcal carriage would have on the results of the IFA technique, found that 27% of 122 males, only one of whom had gonococcal infection, gave positive results, and 68% of 127 females gave positive results although only 50% were infected. McMillan (119) suggests that the sensitivity and specificity of the Gram-stained smear in the detection of gonococci in urethral exudates is similar to the sensitivity and specificity of the IFA technique, but significantly cheaper. In the detection of antibodies in anorectal secretions and in saliva the IFA test is not sensitive or specific enough and only in the detection of IgA in cervical secretions did he think the results encouraging.

The most widely used test for the sero-diagnosis of gonorrhoea is the complement-fixation test (CFT). In an evaluation of the CFT, Ratnatunga (120) found that, in cases of uncomplicated gonorrhoea, he detected 34% of infected females and 18% of infected males while in complicated gonorrhoeal infections the findings were 41% and 25% respectively. Peacock (121), using both his own preparation of a gonococcal protoplasm antigen, isolated by agar-gel

and Sephadex gel filtration from disrupted N. gonorrhoeae cells, and a commercial antigen, detected 80% of males and 50% of females with the 'home-made' preparation, and 72% and 45% respectively with the commercial preparation. His own antigen gave a false positive rate of 4% and the commercial antigen a false positive rate of 10%.

Danielsson et al. (122) had similar findings to Peacock with slightly higher detection rates in females than in males as did also Young, Henrichsen and McMillan (123), Reyn (124) and Rodas and Ronald (125). All these workers, however, found positive results in patients with meningococcal pharyngeal carriage or meningococcal disease, and the test therefore cannot be relied upon by itself for screening purposes to diagnose or exclude gonorrhoea but would have to be used in conjunction with more reliable methods.

Radioimmunoassay (RIA) using pili antigen from gonococci to detect gonococcal antibody has been reported (126). Luoma, Cross and Rudbach (127), however, found the system at best only 75% and 36% efficient in two trials involving male patients, whilst Buchanan et al. (126), who suggested that using a purified antigen decreased the chance of detecting cross-reacting antibody, found good correlation between RIA and other methods, although, again, the proportion of detectable females was greater than detectable males. Although preliminary results with RIA seem encouraging, it seems unlikely that its use will become widespread as a screening tool, owing to its need for technical expertise and expensive

equipment.

Enzyme linked immunosorbent assay (ELISA) to detect gonococcal antibodies directed against outer membrane complex antigens (128, 129) or envelope antigens (109) is the latest development in the search for a serological diagnostic test for gonococcal infection. Ison and Glynn (109) using ELISA to investigate the antibody response to gonococcal infection and then develop its use into a diagnostic tool (129). They found that the incidence of false positive reactions was unacceptably high at 16% in males and 11% in females, whilst Brodeur, Ashton and Diena (128) found a high level of specificity, with N. meningitidis serogroup A giving the only cross-reactions. Brodeur et al. obtained their results using rabbit sera raised against purified outer-membrane complex antigens derived from laboratory strains and have yet to evaluate the method in the field where they may find widely different results.

The problems associated with most of the above serological tests have been their lack of sensitivity, particularly in males, their lack of specificity, with many cross-reactions, and their capacity to detect small amounts of antibody from sites of local infection. The cross-reactivity is not only due to the complexity of the antigens used but also as a result of infection or carriage of other Neisseria spp. or even other bacterial genera.

Unfortunately the heterogeneity of antigens from the gonococcus and associated heterogeneity of antibody response by

different people may necessitate the preparation of antigens from several antigenic types of gonococcus, in order to demonstrate an immune response in all patients, and this will compound the problems associated with cross-reactions.

We can see, therefore, from the above that in spite of a great deal of intensive research being carried out no reliable sero-diagnostic test has been developed. It is the intention of this current work to devise a test system for the detection of genitally acquired gonococcal infection which would prove significantly more reliable than those previously described.

1.6. Detection of a capsule on *Neisseria gonorrhoeae*

Many bacteria, particularly those giving mucoid growths, produce extra-cellular material generally of a gelatinous or gummy consistency. This material may remain firmly adherent to the cell as a distinct covering layer, and is described as a capsule, or may be lying partly or wholly free from the cell and be described as loose slime or free slime. Typical capsule-forming bacteria show capsules on most or all of the cells in culture and may produce loose slime in addition to capsules. Most capsules are composed mainly of complex carbohydrates or polysaccharide gums though others may contain nitrogen and phosphorus and be more in the nature of polypeptides, mucus or mucins.

In 1959 Deacon *et al.* (94) in their attempt to produce an immunofluorescent test for the detection of *N. gonorrhoeae* described the presence of a 'K-antigen' on gonococci recently isolated from urethral exudate. They noticed that this antigen disappeared progressively over a period of 30 hr. when the organism was grown *in vitro* and described it as being a possible capsule surrounding the gonococcus. Previously Wolfgang Caspar in 1939 (130), while investigating the carbohydrate content of *N. gonorrhoeae*, had mentioned the possibility of the organism possessing a capsule similar to that possessed by *N. meningitidis*. Both Deacon and Wolfgang Caspar, however, failed to pursue the matter further and interest in the possibility of a gonococcal capsule diminished.

The matter rested until 1976 when a number of workers

simultaneously 're-discovered' that N. gonorrhoeae may, under certain conditions, produce a capsule-like organelle. Richardson and Sadoff (131), using a commercially available medium for the growth of N. gonorrhoeae supplemented with growth factors, noticed that in one of their cultures, subsequently found to be contaminated with a strain of Streptococcus viridans, a number of gonococci were surrounded by large capsules. The same strains, when grown in pure culture in the same medium either produced very small equivocal capsules or failed to produce capsules at all. They postulated that the Str. viridans produced, as a by-product of its growth, either a substance which the gonococcus needs for capsule production, or caused the gonococcus to produce a capsule as a defence mechanism against this hypothetical factor.

Hendley et al. (132) found that N. gonorrhoeae could produce a capsule when grown in an enriched medium containing casein hydrolysate when the organisms were freshly isolated from infectious exudates, though they were only capable of demonstrating the phenomenon in four or five strains. De Hormaeche, Thornley and Glauert (133) have demonstrated that gonococci recently grown in vivo, in subcutaneous chambers in guinea-pigs, did, in two cases, produce capsules on some cells but not on others. It was the re-discovery of the capsule on the gonococcus which prompted this work.

It is now undeniable that, given certain strict conditions for growth, the gonococcus does produce a capsule, but, so far existence of capsulate gonococci in exudates has not been proven.

However, it would seem probable that the capacity of the gonococcus to become capsulate *in vitro* must reflect a need for the organism to produce a capsule *in vivo*. If the gonococcus is shown to be capsulate when present in exudates, and the capsular antigens shown to be specific to the gonococcus, then a more reliable method of gonococcal detection using specific anti-capsular sera might be developed.

1.7. Detection of antigens and antibodies using counterimmunoelectrophoresis

As has been described above, serological monitoring of bacterial infections fall into two basic categories. One category relies on the use of specific antibodies, raised in animal models and directed against whole cells, or their component organelles, being used to react in an indicator system with suspected infectious fluids in order to assess the presence or absence of those cells or component organelles in those fluids. The second category relies on the use of whole cells or specific organelles being used in an indicator system to monitor changes in the immunological make-up of the host which would indicate an infectious process. We have seen from the above that several classes of antibody are produced in response to a gonococcal infection, and these antibodies are directed against whole organisms, outer-membrane complex, pili and other sub-cellular components. It would therefore seem reasonable to suppose that antibodies would be produced which would be directed against gonococcal capsular material, if the gonococcus causing the

disease is capsulate. A system for the detection of these anti-capsular antibodies would therefore seem feasible.

For the purpose of this study it was decided however to raise antibodies, to gonococcal capsules, in rabbits, and use these antibodies to react in an indicator system with suspected infectious urethral and cervical exudates, in order to detect the presence or absence of gonococcal capsular material, either in combination with intact cells or lying free in the fluid matrix of the exudate.

The many and various serological indicator systems used previously are described above and include haemagglutination, co-agglutination, complement-fixation, radioimmunoassay, enzyme linked immunosorbent assay and fluorescent antibody techniques, and all the systems have exhibited, to a lesser or greater degree, disadvantages either in their practice or in their results. It was therefore decided to use a system which had proven its reliability in other disciplines and yet was simple to use and did not need excessively expensive equipment.

Ouchterlone in 1949 (134) used a system of double diffusion in agar gel to detect antigen-antibody precipitation, whereby the two reactants are placed in opposing wells, cut in the agar, and allowed to diffuse towards each other. Disadvantages of this method are, that only low concentration of the two reactants meet owing to their radial diffusion from the wells, and also the process is, in most cases, very slow. If the two reactants could be concentrated into a smaller reaction area then the sensitivity of the process would be

increased. This can be achieved in the process of counterimmunoelectrophoresis (CIE), by subjecting the reactants to the effects of an electric field such that they are forced together more rapidly and in greater concentrations.

The phenomenon of CIE depends on the ratio of carboxyl to amino groupings present in the reactants. If proteins have excess amino groups they can be positively charged, if they have excess carboxyl groups they can be negatively charged, and if they have equal amino and carboxyl groups they can be electrically neutral. The electrical charge on the antigen may be selected by using a buffering system which allows either the amino groups or carboxyl groups to predominate. Molecules with no net charge, and therefore at their iso-electric point, will not move under the influence of an electric field.

A typical CIE system is shown in Figure 1.

Counterimmunoelectrophoresis is most often performed using agar gel at pH 8.2 - 8.6 supported on a glass plate. At this pH, antigens are negatively charged because of the neutralization of the amino groups by the alkaline buffer. The antigen will therefore move towards the anode (+) and as the antibody or immunoglobulin has its iso-electric point at this pH it will remain stationary with respect to the imposed electric field. There is, however, a hydraulic flow of hydronium ions (H_3O^+) through the agar from the anodal well toward the cathode (-) and this flow, known as endosmosis, tends to carry the antibody molecules toward the cathode (-).

Antigen is therefore placed in the cathodal well and antibody in the anodal well. The antigen molecules migrate towards the anode (+) under the influence of the electrical field and antibody migrates towards the cathode (-) under the influence of endosmosis. The antigen and antibody meet between the opposing wells and form a zone of precipitation in the agarose gel.

CIE has only recently emerged as a diagnostic tool in microbiology in the detection of bacterial antigens in cerebrospinal fluid in cases of suspected bacterial meningitis. In 1974 Smith and Ingram (135), Shackleford, Campbell and Feigen (136), Myhre (137) and Higashi et al. (138) independently realised the significant role that CIE could play in the early diagnosis of bacterial meningitis. They found it reliable, quick and easy to perform and, after the modest initial capital outlay on equipment, reasonably economic.

Many other workers found it useful, not only as a tool for the rapid diagnosis of disease but also as an alternative aid in the laboratory for the identification of organisms or their metabolites (139, 140, 141, 142, 143, 144).

The proven reliability and sensitivity of CIE in the diagnosis of bacterial meningitis prompted me to use this system as the means of identifying and assaying gonococcal capsular material in exudates with a view to using this method as a rapid and reliable means of diagnosing gonococcal infection in man.

MATERIALS AND METHODS

2.1. Assessment of capsule stains

Three organisms were used as capsule stain controls:- Klebsiella aerogenes, Streptococcus pneumoniae and Staphylococcus aureus. The three organisms were all isolated from routine culture of sputum in the Microbiology Department at Victoria Hospital, Kirkcaldy. The Kl. aerogenes and Str. pneumoniae served as the capsulate controls and the Staph. aureus as a non-capsulate control, and all three were maintained on freshly-poured 0.2% glucose blood agar.

2.1.1. Anthony's method (145)

2.1.1.1. Materials:

Distilled water

1% (w/v) crystal violet (BDH, Poole, England) in water

20% (w/v) copper sulphate (BDH) in water

2.1.1.2. Method

Each organism was emulsified in distilled water and used to make smears on glass microscope slides, which were then allowed to dry in air. The smears were then stained with the 1% aqueous solution of crystal violet for 1 min., washed with 20% copper sulphate solution and then blotted dry.

The smears were then viewed under oil immersion at 1000 times magnification.

2.1.2. Hiss's method (145)

2.1.2.1. Materials: Pooled human serum

Saturated alcoholic solution of basic fuchsin (BDH)

Distilled water

20% (w/v) copper sulphate (BDH) in water

2.1.2.2. Method

Each organism was emulsified in pooled human serum and used to produce smears on glass microscope slides which were then allowed to dry in air at room temperature, before being fixed by passing through a bunsen flame. The films were then stained with a mixture of 2 vol. of saturated alcoholic solution of basic fuchsin in 19 vol. of distilled water, while being slowly heated, until steam was seen to be rising from the slide. The slide was then washed with 20% copper sulphate and blotted dry.

The smears were then examined as before.

2.1.3. Richard Muir's method (146)

2.1.3.1. Materials: Solution 'A' - dilute carbol fuchsin

Powdered basic fuchsin (BDH)	10 g
Absolute ethanol (BDH)	100 ml
Phenol (BDH)	50 g
Distilled water	1000 ml

The phenol and basic fuchsin were mixed with heat in a boiling water bath for 5 min. The alcohol was added and mixed well, and then the distilled water and mixed well. The working solution was prepared by diluting with 20 vol. of distilled water.

Solution 'B' - mordant

Saturated solution of mercuric chloride (BDH) in water	200 ml
20% solution of tannic acid (BDH) in water	200 ml
Saturated solution of aluminium potassium sulphate (BDH) in water	500 ml

The mercuric chloride, tannic acid and aluminium potassium sulphate solutions were mixed.

Distilled water

0.5% solution of methylene blue (BDH) in water

Absolute ethanol (BDH)

Xylol (BDH)

74 O.P. Methylated spirit (BDH)

DePeX (Gurr, London, England) mounting fluid

2.1.3.2. Method

Each organism was emulsified in distilled water and used

to produce a very thin smear on clean glass microscope slides. Each slide was then allowed to dry in air without fixing. The films were then stained with filtered dilute carbol fuchsin, washed in spirit and then in distilled water. They were then placed in mordant for 30 sec., washed well with distilled water, placed in the methylated spirit for 1 min. and again washed well in distilled water. Finally the films were counterstained with 0.5% methylene blue for 30 sec. dehydrated in the absolute alcohol for 1 min., cleared with xylol and mounted under coverslips using DePeX mounting fluid.

The smears were then examined as before.

2.1.4. Kirkpatricks' method (146)

2.1.4.1. Materials: Fixing solution

Formalin (BDH)	10 ml
Chloroform (BDH)	30 ml
Absolute ethanol (BDH)	60 ml

The three ingredients were mixed.

Borax methylene blue

Methylene blue (BDH)	1 g
Borax (BDH)	2 g
Distilled water	100 ml

The borax was dissolved in the water over heat, the methylene blue added and the mixture allowed to cool. For use the

solution was diluted with 5 vol. of distilled water.

74 O.P. Methylated spirit (BDH)

Distilled water

2.1.4.2. Method

Each organism was emulsified in distilled water and used to produce smears on clean glass microscope slides which were then allowed to dry in air at room temperature. The smears were then placed in the fixing solution for 3 min., washed with the methylated spirit and stained with the dilute borax methylene blue for 3 min. The smears were then washed in distilled water and blotted dry.

The smears were then examined as before.

2.1.5. Eosin negative stain method (146)

2.1.5.1. Materials: Staining solution

10% aqueous solution of eosin (BDH)

Pooled human serum

1 crystal of thymol (BDH)

To 4 vol. of eosin solution were added 1 vol. of pooled human serum and the crystal of thymol. The mixture was stored in the dark for 4 days, centrifuged, and the supernatant stored at room temperature.

Materials (cont.)

Cell stain

Basic fuchsin (BDH)	1 g
Absolute ethanol (BDH)	10 ml
Phenol (BDH)	5 g
Distilled water	500 ml

The phenol and the fuchsin were heated in a boiling water bath for 5 min. until thoroughly mixed. The alcohol was then added, the solution mixed, and then the water added with a final mixing.

2.1.5.2. Method

Each organism was emulsified in distilled water and one drop of each emulsion mixed with one drop of cell stain on a clean glass slide. The stain was allowed to act for 1 min. and then one drop of staining solution was added and again allowed to act for a further min. before the mixture of stain and organism was spread thinly into a smear using the edge of another microscope slide. The smears were allowed to dry and viewed in the same manner as before.

2.1.6. Nigrosin negative stain (146)

2.1.6.1. Materials: 1% aqueous solution of nigrosin (BDH)
25% aqueous solution of eosin negative stain carbol fuchsin (2.1.5.1.)

2.1.6.2. Method

Each organism was emulsified in distilled water to give an

opacity equivalent to Brown's opacity 1 (147), and one drop of this emulsion mixed with one drop of the staining solution, comprised of equal parts of nigrosin and dilute carbol fuchsin. The stain was allowed to act for 1 min. and then the mixture smeared thinly over the surface of a glass slide using the edge of another slide, and then allowed to dry.

The smears were examined as before.

2.1.7. Wet India Ink method (148)

2.1.7.1. Materials: Higgin's India ink (Faber-Castell, New Jersey, U.S.A.)

2.1.7.2. Method

A loopful of India ink was placed on a previously well-cleaned glass microscope slide and one loopful of the organism under test, emulsified in physiological saline to Brown's opacity 1, was placed in the ink and mixed. A previously well-cleaned coverslip was placed on the ink-organism mixture and pressed flat underneath a blotting paper pad to absorb excess fluid.

The films were viewed as before.

2.2. Incidence of capsulate Neisseria gonorrhoeae on primary isolation

2.2.1. Materials: Selective chocolate agar

Blood agar base No. 2 (Oxoid, Basingstoke, England)	40 g
Horse blood, defibrinated (Gibco, Paisley, Scotland)	100 ml
Distilled water	1000 ml

The blood agar base was mixed thoroughly with the distilled water and the mixture autoclaved at 15 lb pressure for 15 min. The agar was allowed to cool to 80° and then the horse blood added and mixed thoroughly, before holding at 45°.

Nystatin (Squibb, Hounslow, England)	4.2 mg
Vancomycin hydrochloride (Eli Lilly, Basingstoke, England)	3 mg
Colistin sulphate (Pharmax Ltd. Dartford, England)	7.5 mg

The three antibiotics were added to the molten chocolate agar at 45° and plates poured with the mixture.

Sugar utilization media : Base

G.C. Medium base (Difco, Detroit, U.S.A.)	36 g

Materials (cont.)

Distilled water		1000 ml
0.2% phenol red (BDH)		10 ml

Sugar utilization media : Supplement

Solution A :	L-glutamine (BDH)	1	g
	Distilled water	90	ml
Solution B :	Ferric nitrate (BDH)	0.1	g
	Distilled water	20	ml

90 ml of solution A was added to 10 ml of solution B.

Sugar utilization media : Sugars

10% aqueous solution of sucrose (BDH)

10% aqueous solution of maltose (BDH)

10% aqueous solution of glucose (BDH)

The basal agar and the distilled water were mixed and then steamed to dissolve the agar. The phenol red was added while the agar was still molten at 45° and the pH adjusted to 7.6 using IN sodium hydroxide. 20 ml of supplement was then added to the molten base and the mixture distributed in 45 ml aliquots which were then autoclaved at 15 lb pressure for 10 min. The media were allowed to cool and 5 ml of the respective sugar, previously sterilised by filtration, was added to 45 ml of agar and the mixture dispensed in 3 ml. amounts in glass Bijou bottles.

2.2.2. Method

50 strains of N. gonorrhoeae were isolated on 'selective chocolate agar' from 40 patients, both male and female, attending the Special Treatment Clinic at either Victoria Hospital, Kirkcaldy or Dunfermline West Fife Hospital, Dunfermline. The organisms were isolated from both the urogenital and upper respiratory tract and were identified using the sugar utilization media described above. Each strain was then assessed for the presence of capsules using the wet India ink method (2.1.7.), by emulsifying several colonies from the chocolate agar plate in one loopful of physiological saline and mixing with one loopful of India ink. 100 microscope fields were examined on each slide and the ratio of capsulate:non-capsulate cells calculated.

2.3. Incidence of capsulate Neisseria gonorrhoeae in vivo

2.3.1. Materials: 25% strength Ringer solution (Oxoid) in 0.5 ml aliquots

2.3.2. Method

The Special Treatment Clinic at the Victoria Hospital, Kirkcaldy was provided with Bijou bottles containing 0.5 ml aliquots of the Ringer solution.

The staff in the clinic agreed to take swab samples of exudate from the urogenital tract of patients presenting with gross polymorphonuclear exudates consistent with gonorrhoeal infection. The swabs were then immersed in the Ringer solution and the exudate

washed off into solution. The resultant suspensions of exudate in Ringer solution were then examined by India ink stain (2.1.7.).

Exudates from 40 patients (15 male, 25 female) were examined for the presence of capsulate organisms and all 40 exudates were also subjected to routine culture and biochemical identification of isolates.

2.4. Assessment of media for capsule production

2.4.1. Materials: 10 strains of *N. gonorrhoeae* isolated from patients attending the Special Treatment Clinic at Victoria Hospital, Kirkcaldy or Dunfermline West Fife Hospital, Dunfermline

5 capsule-producing media, described below

2.4.1.1. Capsule promoting medium of Hendley *et al.* (132)

Materials:

Proteose peptone No. 3 (Difco)	15	g
Casein hydrolysate-acid (Oxoid)	7.5	g
Soluble starch (BDH)	1	g
Haemoglobin (Difco)	10	g
Isovitalex enrichment (B.B.L. Baltimore, U.S.A.)	10	g
Distilled water	1000	ml

Method

The ingredients, with the exception of the starch, were added to the water while stirring over heat. The starch was suspended in 100 ml of cold distilled water and heated to boiling to form a transparent starch solution. The starch solution was then added to the other ingredients and the whole heated to dissolve the dry ingredients.

The broth was centrifuged at 3,000 g to remove any particulate matter and then sterilized by filtration and dispensed in 100 ml amounts in glass screw-capped bottles. The bottles were incubated at 37° for 24 hr. as a check of their sterility.

2.4.1.2. Capsule promoting medium of Richardson and Sadoff (131)

Materials: Agar base : G.C. medium base (Difco) 36 g

Haemoglobin (Difco)	10 g
Isovitalex (B.B.L.)	10 g
Distilled water	1000 ml

The agar base was added to the water and sterilized by autoclaving at 15 lb pressure for 15 min. The agar was allowed to cool to 56° and the haemoglobin and Isovitalex added aseptically. The agar base was then dispensed in 20 ml amounts in 50 ml capacity flat sided glass bottles and allowed to solidify into a wedge on the side of the bottle.

Materials (cont.) Broth : Nutrient broth base No. 2 (Oxoid) 25 g

Casein hydrolysate (Oxoid) 7.5 g

1% p-aminobenzoic acid (BDH) 5 ml

Glucose (BDH) 5 g

Distilled water 1000 ml

All the ingredients, with the exception of the glucose, were added to the distilled water and mixed to suspension. The broth was then autoclaved for 10 min. at 10 lb pressure and allowed to cool. The glucose was dissolved in 10 ml of distilled water, sterilized by filtration, and added aseptically to the cooled broth. The glucose-broth was then dispensed aseptically in 10 ml amounts into each of the agar wedge containing bottles. The resultant media were then incubated at 37° for 24 hr. as a check of their sterility.

2.4.1.3. Capsule promoting medium of Jephcott and Reyn (149)

Materials: Agar base : G.C. medium base (Difco) 36 g

Distilled water 1000 ml

The base was suspended in the water while cold and then sterilized by autoclaving at 15 lb pressure for 15 min. and then allowed to cool.

Materials: (cont.) Agar Base

Supplement : L. glutamine (BDH) 1 g

Dextrose (Oxoid) 40 g

Ferric nitrate (BDH) 0.05 g

Co-carboxylase (BDH) 0.02 g

Distilled water 100 ml

The ingredients were suspended in the distilled water and sterilised by filtration. 20 ml of supplement was added to 1000 ml of cooled agar base and the resultant supplemented agar dispensed aseptically in 20 ml amounts in 50 ml capacity flat sided bottles and allowed to cool to form a wedge of agar on one side of the bottle.

Broth : As in Richardson and Sadoff medium above (2.4.1.2.)

The broth was dispensed aseptically in 10 ml amounts into the bottles containing the agar wedge.

2.4.1.4. Capsule promoting medium of Hafiz, McEntegart and Jephcott (1st modification) (150)

Materials: Broth : Proteose peptone No. 3 (Difco) 15 g

Soluble starch (BDH) 1 g

Sodium chloride (BDH) 5 g

Di-potassium hydrogen phosphate (BDH) 4 g

Materials: (cont.)

Potassium di-hydrogen phosphate (BDH)	1	g
Sodium bicarbonate (BDH)	0.15	g
Glucose (BDH)	5	g
Casein hydrolysate (Oxoid)	7.5	g
Distilled water	1000	ml

All the dry ingredients were added to the water while heating and stirring to dissolve. The resultant broth was then allowed to cool to 56°.

Supplement : As in Jephcott et al. medium above (2.4.1.3.)

20 ml of supplement was dispensed aseptically into 1000 ml of broth base and the resultant supplemented broth dispensed in 100 ml aliquots in glass screw-cappsed bottles. The medium was then steamed for 30 min. on 3 consecutive days to sterilize.

2.4.1.5. Capsule promoting medium of Hafiz, McEntegart and Jephcott (2nd modification) (150)

Materials:

Proteose peptone No. 3 (Difco)	15	g
Soluble starch (BDH)	1	g
Sodium Chloride (BDH)	5	g
Di-potassium hydrogen phosphate (BDH)	4	g

Materials (cont.)	Potassium di-hydrogen phosphate (BDH)	1	g
	Sodium bicarbonate (BDH)	0.15	g
	Glucose (BDH)	5	g
	Casein hydrolysate (Oxoid)	7.5	g
	Yeast extract (Oxoid)	2	g
	Distilled water	1000	ml

All the dry ingredients were added to the water while heating and stirring to dissolve. The resultant broth was dispensed in 100 ml amounts in glass screw cap bottles and then steamed for 30 min. on 3 consecutive days to sterilize.

2.4.2. Method

One loopful of each of the 10 strains of gonococci isolated was inoculated from the chocolate agar into each of 4 bottles of each media. The cultures were incubated at 37° in an atmosphere of 5 - 10% CO_2 in air and shaken at intervals over a 48 hr. period.

A loopful of each medium strain combination was taken and subjected to Gram-staining to check purity, and also to an India ink stain to assess presence of capsulate organisms in the broth.

2.5. Effect on gonococcal capsule size and incidence, caused by an increase in carbohydrate : nitrogen ratio of the capsule-promoting media

2.5.1. Ratio altered by increase of glucose content

Materials: 3 l of capsule medium (Hafiz, McEntegart and Jephcott 2nd modification 2.4.1.5.) deficient in glucose and proteose peptone

10 glucose-proteose peptone solutions with altered glucose : proteose peptone ratios of between 1:3 to 1:1.2; by keeping the proteose peptone content stable at 1.5 g in 10 ml and altering the glucose content from 0.5 - 1.25 g in 10 ml Table 2, Media 1 - 10).

Neisseria gonorrhoeae - strain G.C.A., isolated from a patient attending the Special Treatment Clinic, Victoria Hospital, Kirkcaldy.

2.5.1.2. Method

100 ml of capsule medium was dispensed in screw-capped glass bottles. 10 ml of each glucose-peptone ratio solution was then added to 3 bottles of the capsule medium, to give 10 groups of media

in triplicate, each group containing a different carbohydrate : nitrogen ratio, the ratios ranging from 1:3 to 1:1.2.

Neisseria gonorrhoeae strain G.C.A., known to produce large capsules, was incubated overnight in 100 ml of unmodified capsule-promoting broth at 37° and in an atmosphere of 5 - 10% CO_2 in air. 1 ml of this overnight culture was then added to each of the altered broths and the broths incubated at 37° in an atmosphere of 5 - 10% CO_2 in air for 48 hr., with shaking at intervals.

A loopful of each broth culture was taken for Gram-staining to check for growth purity and another loopful taken for India ink stain to assess capsule incidence.

2.5.2. Ratio altered by decrease of proteose peptone content

2.5.2.1. Materials: 3 l of capsule medium (2.4.1.5.) deficient in glucose and proteose peptone

10 glucose-proteose peptone solutions with altered glucose : proteose peptone ratios of between 1:3 to 1:1.2; by keeping the glucose content stable at 0.5 g in 10 ml and altering the proteose peptone content from 1.5 - 0.6 g in 10 ml

(Table 2 Media 11 - 20)

Neisseria gonorrhoeae strain G.C.A.

2.5.2.2. Method

The capsule medium was dispensed in 100 ml aliquots and 10 ml of each glucose : peptone ratio solution was then added to 3 bottles of medium to give 10 groups of media in triplicate, each group containing a different ratio of carbohydrate : nitrogen. 1 ml of the overnight culture of the N. gonorrhoeae was added to each broth and the broths incubated at 37^{o} in an atmosphere of 5 - 10% CO_2 in air for 48 hr., with shaking at intervals.

The broths were then assessed, as before, for purity of growth and capsule incidence. One hundred cells were viewed and the presence or absence of a capsule on each cell noted, together with an estimation of increase or decrease in capsule size, using an eyepiece graticule as the measuring apparatus.

2.6. Production of antisera to capsulate gonococci

2.6.1. Preparation of capsulate organisms

2.6.1.2. Materials: Neisseria gonorrhoeae strain G.C.A.

Capsule-promoting medium, prepared as Hafiz et al. (2nd modification) (2.4.1.5.) but with only 10 g l^{-1} proteose peptone

Physiological saline

2.6.1.3. Method

Six colonies of Neisseria gonorrhoeae strain G.C.A., grown on a chocolate agar plate for 24 hr. were inoculated into 100 ml of capsule medium in a 1 l Roux flask, stoppered with cotton wool. The flask was then incubated at 37° for 48 hr. in an atmosphere of 5 - 10% CO_2 in air, with shaking at intervals. After 48 hr. a Gram's stain and India ink stain were made of the culture as a check of purity and capsule formation.

The flask was then immersed in a water bath at 56° for 15 min. in order to kill the culture (2), a check of culture viability being made by pipetting 1 ml of the heated broth onto a chocolate agar plate and incubating for 24 hr. at 37° in 5 - 10% CO_2 in air. After heating the culture was again submitted to an India ink stain to check capsule integrity.

The culture was centrifuged at 3,000 g and the supernatant discarded. The pellet of organisms was resuspended in 10 ml of physiological saline, respun at 3,000 g and the supernatant discarded. The pellet was resuspended in 2 ml of physiological saline, rechecked for capsule integrity and stored at -20°.

The above procedure was repeated 15 times, in order to provide 30 ml of capsulate Neisseria gonorrhoeae suspension.

The 15 separate aliquots of suspended organisms were defrosted and pooled and the resultant suspension adjusted to an opacity corresponding to Brown's opacity 1 (147), equivalent to 10^9 cells ml^{-1}, using physiological saline as diluent. The

suspension was again checked for capsule integrity then dispensed in 1 ml amounts in glass Bijou bottles and stored at -20°. As approximately 70% of the cells were capsulate then each aliquot contained approximately 7.0×10^{8} capsulate gonococci ml^{-1}.

2.6.2. Administration of antigen material

2.6.2.1. Materials: 4 kg Dutch lop-eared rabbit
15 x 1 ml aliquots of capsulate *N. gonorrhoeae*

2.6.2.2. Method

Over a 42-day period, at 3-day intervals, the 1 ml aliquots of gonococcal suspension were adminstered into the rabbit through an ear vein. Ten days after the last injection 20 ml of blood was taken from the rabbit, allowed to clot, and then spun at 1,000 g for 10 min. The serum was taken off the packed cells and then dispensed in 0.5 ml aliquots in glass Bijou bottles and stored at -20°.

2.6.3. Detection of anticapsular antibodies

2.6.3.1. Materials: Rabbit pre-immune sera
Rabbit immune sera
Capsulate *Neisseria gonorrhoeae* strain G.C.A. 10^{9} cells ml^{-1}.

2.6.3.2. Method

One loopful of gonococcal suspension was mixed with one loopful of suspected immune rabbit sera on a clean glass slide. After the addition of a coverslip onto the mixture the slide was placed into a moist chamber at 4^{o} for 30 min., in order that any antibody present would become attached to capsular material on the gonococci. A control slide was also prepared using sera obtained from the same rabbit prior to the administration of the capsulate gonococcal suspension.

The two slides were then examined at 1,000 times magnification with the microscope condenser sub-stage lowered to allow illumination better suited to the differentiation of the capsule boundary.

2.6.4. Titre of anticapsular antibodies

2.6.4.1. Materials: Rabbit immune serum

Capsulate Neisseria gonorrhoeae strain

G.C.A. 10^{9} cells ml^{-1}

Physiological saline

Glass. W.H.O. tray

2.6.4.2. Method

Two-fold serial dilutions of the rabbit immune serum from 1:2 to 1:256, using physiological saline as diluent, were made. 0.1 ml of undiluted immune serum was added to each of 3 wells in the

glass tray and 0.1 ml of each dilution also added to each of 3 wells in the tray. 0.1 ml of the gonococcal suspension was then added to each well, the tray then sealed with Sellotape and left at 4^{o} for 30 min. to allow attachment of the antibody to the capsule.

After 30 min. one loopful from each well was taken and submitted to an examination at 1,000 times magnification to check for improvement of capsule boundary definition.

2.6.5. Reactivity of rabbit immune serum towards capsulate *Neisseria gonorrhoeae* strain G.C.A. in a counterimmunoelectrophoresis system

2.6.5.1. Materials: Rabbit immune serum

Neisseria gonorrhoeae strain G.C.A. 10^{9} cells ml^{-1}

Counterimmunoelectrophoresis system (Eurolab A/S Copenhagen)

Hole punch template

3 mm diameter well cutters

$2\frac{1}{2}$ cm x $2\frac{1}{2}$ cm glass slides

Ion-agar No. 2 (Oxoid) in barbitone buffer

Tissue paper wicks

Buffer: Barbitone sodium 13.38 g

Sodium acetate (trihydrate) (BDH) 8.83 g

Materials (Cont.)	Buffer (Cont.)	Distilled water	3000 ml
		pH	8.2

2.6.5.2. Method

7 ml of the 1% ionagar, in barbitone buffer, was pipetted, while molten, onto a clean $2\frac{1}{2}$ cm x $2\frac{1}{2}$ cm glass slide resting on a levelled surface. The agar was allowed to cool and solidify. Using the hole punch template and well cutter, four pairs of opposing wells were cut into the agar 3 mm apart. Four of the wells were filled with the gonococcal suspension and the four opposing wells were filled, three with rabbit immune serum and the other with rabbit pre-immune serum as a control. The slide was then subjected to counterimmunoelectrophoresis (CIE). The arrangement of the CIE equipment is shown in Figure 1.

The agar slide containing the reactant filled wells was placed on the edges of the two reservoirs containing the barbitone buffer and the edges of the agar connected to the buffer in the wells using tissue paper wicks. The edge nearest to the wells containing the four sera was connected to the buffer in the anodic reservoir and the edge nearest to the four wells containing the capsulate gonococcal suspension was connected to the buffer in the cathodic reservoir.

The slide was then submitted to a voltage of 4 volts cm^{-1} of slide for 45 min. and the agar was then examined for lines of precipitation between the four pairs of opposing wells.

The slide was dried between blotting paper overnight at 37^{o},

washed in running tap-water for 12 hr., stained with 1% naphthalene black (BDH) and photographed.

2.6.6. Titration of rabbit immune serum against capsulate Neisseria gonorrhoeae strain G.C.A. in the C.I.E. system

2.6.6.1. Materials: as above

2.6.6.2. Method

Two agar slides were prepared as before using Ionagar No.2 and $2\frac{1}{2}$ cm x $2\frac{1}{2}$ cm clean glass slides on a levelled surface. The agar was allowed to solidify and then two opposing rows of wells were cut in the agars 3 mm apart, each row comprised of 5 wells on each slide.

Two-fold serial dilutions of the rabbit immune serum were made from 1:2 to 1:256 using physiological saline as diluent. On one slide the five wells on one row were filled with undiluted immune sera and the diluted serum 1:2 to 1:16; on the other slide four of the wells were filled with dilutions 1:32 to 1:256 plus an undiluted pre-immune control serum. All 9 opposing wells were filled with the capsulate gonococcal suspension and both slides submitted to CIE at 4 volts cm^{-1} for 45 min.

The slides were dried, washed and stained as before.

2.6.7. Reactivity of rabbit immune serum towards 10 strains of capsulate Neisseria gonorrhoeae

2.6.7.1. Materials: As above, also,

10 strains of Neisseria gonorrhoeae, isolated from patients attending the Special Treatment Clinic, Victoria Hospital, Kirkcaldy

Capsule-promoting medium (2.6.1.2.)

2.6.7.2. Method

N. gonorrhoeae strain G.C.A. and 10 other strains of N. gonorrhoeae were incubated for 48 hr. in capsule-promoting broth at 37° in an atmosphere of 5 - 10% CO_2 in air. The cultures were killed as before (2.6.1.3.) and subjected to an India ink stain to ensure that the organisms were capsulate. The cultures were then centrifuged at 3,000 g for 20 min., washed with saline and re-centrifuged, and then adjusted to an opacity equivalent to Brown's opacity tube No. 1 using physiological saline as diluent.

Four agar slides were prepared as above using Ionagar No.2 and $2\frac{1}{2}$ cm x $2\frac{1}{2}$ cm glass slides. Two rows of 6 wells were cut into each agar as before, each row being 3 mm from its opposing row. On two slides, 6 and 5 wells were filled with immune serum and the opposing wells filled with the 11 capsulate gonococcal suspensions. On the other two slides 6 and 5 wells were filled with rabbit pre-immune serum and the opposing wells filled with the 11 capsulate

gonococcal suspensions.

All four slides were subjected to CIE as before at 4 volts cm^{-1} for 45 min. after which the slides were dried, washed and stained as before.

2.6.8. Reactivity of rabbit immune serum towards strains of Neisseria meningitidis groups B and C, Branhamella catarrhalis, Neisseria subflava and Neisseria sicca in a CIE system

2.6.8.1. Materials: Rabbit immune serum

Neisseria meningitidis serogroup B

Neisseria meningitidis serogroup C

Branhamella catarrhalis

Neisseria subflava

Neisseria sicca

Capsule-promoting medium (2.6.1.2.)

CIE system, as above

2.6.8.2. Method

The five organisms were all isolated from routine specimens submitted to the Fife Area Laboratory and identified using the methods of Bergey's Manual of Determinative Bacteriology (1).

Each organism was grown in 100 ml of capsule-promoting media at 37° and in an atmosphere of 5 - 10% CO_2 in air, for 48 hr. with shaking at intervals. The cultures were then killed as before (2.6.1.3.)

centrifuged, washed with physiological saline, centrifuged again and resuspended in saline to give an opacity equivalent to Brown's opacity tube No. 1.

Each organism was then submitted to an India ink stain to assess the presence or absence of capsulate cells.

The five species together with N. gonorrhoeae strain G.C.A. were then reacted in the CIE system against the rabbit immune serum and against rabbit pre-immune serum as control. The method and conditions for the CIE run were as previously stated, (2.6.5.1.). The slides were dried, washed and stained as before and then photographed.

2.7.1. Absorption of rabbit immune serum with bovine bone marrow (Peacock, W.L., 1970 (95))

2.7.1.1. Materials:

Rabbit immune serum	5 ml
Bovine bone marrow	1 g

2.7.1.2. Method

The bovine bone marrow was added to the rabbit immune serum and mixed well. The mixture was incubated at 50° for 2 hr. and then centrifuged at 3,000 g for 30 min. The supernatant was pipetted off into a sterile glass universal bottle and the deposit discarded.

2.7.2. Reactivity of absorbed immune serum towards N. gonorrhoeae strain G.C.A., N. meningitidis serogroups B and C, N. subflava and N. sicca in a CIE system

2.7.2.1. Materials: Rabbit immune serum (absorbed as above)

N. gonorrhoeae, strain G.C.A.

N. meningitidis group B

N. meningitidis group C

B. catarrhalis

N. subflava

N. sicca

CIE system (2.6.5.1.)

2.7.2.2. Method

The six bacterial strains were prepared as before (2.6.8.2.) and reacted in the CIE system against the previously absorbed immune serum. The method and conditions for the CIE run were as previously (2.6.5.1.) and the slide was dried, washed, stained and photographed as before.

2.7.3. Absorption of rabbit immune serum with strains of B. catarrhalis, N. subflava and N. sicca (95)

2.7.3.1. Materials: Rabbit immune sera (absorbed with bone marrow) 5 ml

Branhamella catarrhalis

Materials (cont.) Neisseria subflava

Neisseria sicca

2.7.3.2. Method

Each organism was grown on chocolate agar overnight and the resultant growth harvested by scraping with a glass rod into saline. The resultant suspensions were centrifuged at 3,000 g for 15 min. and the deposit resuspended in 5 ml of saline. This suspension was again centrifuged at 3,000 g for 15 min. and the deposited cells collected. The above procedure was repeated several times to give 1 ml of packed cells of each organism.

1 ml of packed B. catarrhalis cells was mixed into the 5 ml of immune serum previously absorbed with bone marrow. The resultant suspension was incubated at 50° for 2 hr. with mixing and then centrifuged at 3,000 g for 30 min. The supernatant was pipetted into a clean glass universal container and the deposit discarded. The above procedure was then repeated with the 1 ml of packed N. subflava cells and the 1 ml of packed N. sicca cells in turn.

2.7.4. Reactivity of rabbit immune serum absorbed as above towards N. gonorrhoeae strain G.C.A., N. meningitidis serogroups B and C, B. catarrhalis, N. subflava and N. sicca in a CIE system

2.7.4.1. Materials: Rabbit immune serum (absorbed as above)

Materials (cont.) Bacterial strains (as above)

2.7.4.2. Method

The six bacterial species were prepared as previously (2.6.8.2.) to give suspensions containing approximately 10^9 cells ml^{-1} in saline. The suspensions were then reacted against the absorbed immune serum in the CIE system, the method and conditions for which have been described (2.6.5.1.). The resultant slide was dried, washed and stained according to established procedure and was then photographed.

2.7.5. Absorption of rabbit immune serum, previously absorbed with bovine bone marrow, B. catarrhalis, N. subflava and N. sicca, with N. meningitidis serogroup B (95)

2.7.5.1. Materials: Rabbit immune serum (absorbed as above) 5 ml
N. meningitidis serogroup B

2.7.5.2. Method

The meningococcus was grown for 48 hr. in capsule-promoting broth (2.6.1.2.) at 37° in an atmosphere of 5 - 10% CO_2 in air, with shaking at intervals. The culture was centrifuged at 3,000 g for 10 min., the cells washed in saline and re-centrifuged. The above was repeated several times to obtain a packed meningococcal cell volume of 1 ml. The 1 ml of packed meningococcal cells was added to the absorbed serum and incubated at 50° for 2 hr. with frequent

stirring. The mixture was then centrifuged at 3,000 g for 30 min. and the supernatant taken off into a clean glass universal container.

2.7.6. Reactivity of rabbit immune serum absorbed as above towards N. gonorrhoeae strain G.C.A., N. meningitidis serogroup B and N. meningitidis serogroup C in a CIE system

2.7.6.1. Materials: Rabbit immune serum (absorbed as above)
N. gonorrhoeae strain G.C.A.
N. meningitidis serogroup B
N. meningitidis serogroup C

2.7.6.2. Method

The three bacterial strains were processed as previously (2.6.8.2.) to give cell suspensions in saline of 10^9 cells ml^{-1}. The three suspensions were then reacted in the CIE system against the absorbed serum, the method and conditions for which have been previously described (2.6.5.1.). The slide was dried, washed and stained and then photographed.

2.8.1. Bulk culture of capsulate Neisseria gonorrhoeae

2.8.1.1. Materials: Capsule-promoting media (2.6.1.2.) 8,500 ml
Neisseria gonorrhoeae strain G.C.B. - a strain of N. gonorrhoeae isolated from a

Materials (cont.)

patient attending the Special Treatment Clinic at Victoria Hospital, Kirkcaldy and known to produce large capsules

New Brunswick MA114 14000 ml fermenter

2.8.1.2. Method

8 l of capsule-promoting medium was prepared as before (2.6.1.2.) and dispensed into the previously sterilized glass fermentation vessel. The glass vessel and contents were then autoclaved at 10 lb pressure for 2½ hr. and allowed to cool. Neisseria gonorrhoeae strain G.C.B. was grown overnight in 500 ml of capsule-promoting medium at 37° in an atmosphere of 5 - 10% CO_2 in air with constant stirring. The culture was then assessed for purity by Gram-staining and by plating out on a chocolate agar plate.

The fermentation vessel was connected to its heating, gas and mechanical agitation supplies and the medium allowed to attain a steady state of 37° with a 5 - 10% CO_2 in air through-put of 1 litre min^{-1}. The 500 ml of N. gonorrhoeae strain G.C.B. was added to the medium in the fermentation vessel and the bulk culture was agitated at 100 revs min^{-1}. After a 24 hr. incubation period the culture was assessed for purity by Gram-staining. The culture was then reincubated under the same conditions for a further 24 hr. and re-assessed by Gram-staining.

2.8.2. Bulk culture of *Neisseria gonorrhoeae* strain G.C.B.

2.8.2.1. Materials: as above

2.8.2.2. Method

Essentially the method of bulk culture was as above (2.8.1.) except that the medium in the bulk vessel was autoclaved for $3\frac{1}{2}$ hr. at 10 lb pressure instead of $2\frac{1}{2}$ hr., and the agitation seals on the bulk vessel where they connect to the agitation spindle were sealed with a phenolic disinfectant to ensure that contaminating organisms could not gain entry to the vessel through the seal.

The culture was incubated as before for 24 hr. and a sample taken for Gram-staining for assessment of purity. Again the Gram-staining revealed no contaminating organism, only gram-negative diplococci, whilst an Indian ink stain revealed few capsulate organisms. The culture was again incubated for a further 24 hr. and subjected to Gram-staining.

2.8.3. Bulk culture of *Neisseria gonorrhoeae* strain G.C.B.

2.8.3.1. Materials:

Capsule-promoting medium (2.6.1.2.)	8,600 ml
Trimethoprim lactate (Burroughs Wellcome Ltd.)	27.5 mg
Vancomycin hydrochloride (Eli Lilly & Co. Ltd.)	34.4 mg
Colistin sulphate (Pharmax Ltd.)	68.8 mg

Materials (cont.) Distilled water 107.5 ml

Neisseria gonorrhoeae strain G.C.B.

2.8.3.2. Method

The three antibiotics were dissolved in the distilled water and 1.25 ml of the antibiotic solution added aseptically to 100 ml of capsule-promoting medium in a Roux flask. The medium was inoculated with the gonococcus and incubated at 37° for 48 hr. in an atmosphere of 5 - 10% CO_2 in air with frequent shaking. After 48 hr. a Gram's stain of the culture was made to assess purity and an India ink stain to assess capsule formation.

6.25 ml of the antibiotic solution was added to 500 ml of capsule-promoting medium in a wide necked glass flask and the medium inoculated with the gonococcus. The culture was incubated for 24 hr. at 37° in an atmosphere of 5 - 10% CO_2 in air with constant stirring. After 24 hr. a Gram's stain of the culture was made to assess purity.

8 l of capsule-promoting medium was prepared as before (2.6.1.2.) in the bulk culture vessel of the fermenter and the whole sterilized by autoclaving at 10 lb pressure for 3½ hr. The medium was allowed to cool and the remaining 100 ml of antibiotic solution added aseptically. The medium was then allowed to achieve a steady state as before and then inoculated with the 500 ml 24 hr. old culture of gonococci.

The culture was incubated as previously stated (2.8.1.2.). After 24 hr. a sample of culture was taken for Gram-staining to assess

purity and India ink staining to assess capsule formation. The culture was then incubated under the same conditions for a further 24 hr. and then again assessed for purity by Gram's stain and for capsule formation by India ink stain.

2.9. Extraction of capsular material (from the method of Sutherland and Wilkinson (151)

2.9.1. Precipitation of polysaccharide material

2.9.1.1. Materials:

40% Cetavlon (I.C.I. Macclesfield, England)	2 ml
Distilled water	100 ml

2.9.1.2. Method

After the purity of the bulk culture and the presence of capsulate gram-negative diplococci were established, 2 ml of 40% Cetavlon in 100 ml of distilled water was added to the culture in the fermentation vessel and the culture agitated for 10 min. The culture was then centrifuged at 20,000 g using a continuous flow rotor on an M.S.E. super 18 centrifuge and the supernatant was discarded. The precipitate was homogenized in an Omnimixer with 150 ml of distilled water and re-centrifuged at 13,000 g for 10 min., the supernatant being discarded.

2.9.2. Dissociation of the detergent-polysaccharide complex

2.9.2.1. Materials: 0.9 M Calcium chloride (BDH) 4 x 100 ml

Absolute ethanol (BDH)

2.9.2.2. Method

The precipitate was homogenized in an Omnimixer with 100 ml of 0.9 M calcium chloride and then centrifuged at 13,000 g for 15 min. This procedure was repeated four times and the resultant supernatants pooled and the bacterial residue stored at 4°.

To the pooled supernatants was added absolute ethanol to give a final concentration of 25% (v/v). A fibrous precipitate formed immediately, which consisted of nucleic acid material, and this was removed by spooling on a glass rod. The remaining suspension was held at 4° for 3 hr. and then centrifuged at 2° for 15 min. at 13,000 g. The clear supernatant was removed and absolute ethanol added to give a final concentration of 80% (v/v); this resulted in the formation of another precipitate, this time polysaccharide material. The suspension was centrifuged for 10 min. at 2,000 g, leaving the detergent and calcium chloride in solution. The precipitate was then added to the bacterial residue held at 4°.

2.9.3. Precipitation of bacterial residue and polysaccharide material

2.9.3.1. Materials:

0.9% Sodium chloride (BDH)	500 ml
Phenol (BDH)	10 g
96% Ethanol (BDH)	1600 ml
Saturated solution of sodium acetate (BDH) in a water/alcohol mixture (7/43; w/v)	35 ml
Acetone (BDH)	

2.9.3.2. Method

The bulked precipitates were suspended in 500 ml of 0.9% sodium chloride containing 10 g of phenol. The mixture was stirred overnight at 37° and then 1600 ml of 96% ethanol containing 35 ml of the sodium acetate solution was added to it. After addition of the alcohol the mixture was allowed to sediment by standing for 20 hr. at room temperature in a 2000 ml measuring cylinder, to permit separation of the bacterial residue from extraneous soluble material. The clear supernatant was removed by decanting and the precipitate centrifuged at 4,000 g for 15 min.

The precipitate was washed in acetone and allowed to dry at room temperature for 24 hr.

2.9.4. Purification of exopolysaccharide material

2.9.4.1. Materials:

Distilled water	44 ml
90% aqueous phenol solution (BDH)	44 ml

2.9.4.2. Method

The 4 gm of dried bacterial residue was suspended in 44 ml of distilled water and warmed to 65°. An equal volume of 90% (w/v) phenol in aqueous solution at the same temperature was then added and the mixture stirred vigorously for 5 min. The mixture was then cooled rapidly in ice and centrifuged at 3,000 g for 30 min. at 0° to permit phase separation. The upper aqueous layer was removed and dialysed against running tap water for 24 hr. to remove any contaminating phenol. The solution contained non-diffusible exopolysaccharide, RNA, glycogen and lipopolysaccharide. The lipopolysaccharide was removed by centrifugation at 100,000 g for 4 hr. when it formed a clear viscous gel at the bottom of the centrifuge tube. The supernatant was pipetted off into a clean glass Bijou bottle.

2.9.5. Removal of RNA and glycogen

2.9.5.1. Materials:

5% streptomycin sulphate (Glaxo labs. Ltd. Greenford, England)	1.5 ml
5% α-amylase (Sigma Chemical Co. Poole, England)	1.5 ml

2.9.5.2. Method

The streptomycin solution was added dropwise at 4^{o} for 20 min. to the supernatant of the ultracentrifugation procedure, with continuous stirring. The amylase solution was then added and the solution subjected to a repeat phenol extraction and dialysis. The dialysis was continued until the solution, of pure exopolysaccharide, was reduced to 2 ml in volume, the extracted capsular material forming a viscous aqueous solution in the bottom of the dialysis bag.

2.10 Preparation of capsular antigen (152)

2.10.1. Materials:

Extracted capsular material (2.9.)	2 ml
Mineral oil (Dr. Sommerville, Zoology Dept. St. Andrews University, St. Andrews)	2 ml
0.9% aqueous sodium chloride (BDH)	4 ml

2.10.2. Method

1 ml of the aqueous solution of capsular material was drawn up into a 5 ml syringe and then projected, with force, through a 25G gauge needle into 1 ml of mineral oil in a Bijou bottle. The resultant emulsion was repeatedly drawn up into, and expelled from the syringe through the needle into the Bijou bottle until an extremely viscous water in oil emulsion was formed.

The water in oil emulsion was then taken up into the syringe

and expelled into 2 ml of physiological saline contained in a Bijou bottle. The resultant emulsion was repeatedly drawn up into and expelled from the syringe through the needle into the Bijou bottle until a homogenous water in oil in saline emulsion was formed.

The above was repeated with the remaining 1 ml of capsular material, 1 ml of mineral oil and 2 ml of saline, the two procedures providing two lots of 4 ml of antigenic material for antibody production in a rabbit.

2.11. Production of antisera to capsular material

2.11.1. Materials:

Dutch white lop-eared rabbit	4 kg
Capsular antigen (2.10.)	8 ml

2.11.2. Method

The rabbit was prepared for inoculation by having its flanks shaved and disinfected with iso-propyl alcohol. 4 ml of capsular antigen was then inoculated subcutaneously at multiple sites on both flanks. After four weeks the remaining 4 ml of capsular material was administered in the same manner.

At 8, 12 and 16 weeks after inoculation, blood was taken from the rabbit by means of ear vein slit. The blood was allowed to clot and was then centrifuged at 1,000 g for 15 min. The serum was removed and used in capsule definition and CIE procedures against *N. gonorrhoeae* strain G.C.B. and *N. meningitidis* serogroup B.

2.12. Reactivity of rabbit immune serum towards capsulate *N. gonorrhoeae* strain G.C.B. and *N. meningitidis* serogroup B in a capsule definition reaction

2.12.1. Materials: Rabbit immune serum 8, 12, 16 weeks post-inoculation

Capsulate *Neisseria gonorrhoeae* strain G.C.B. suspension 10^9 cells ml^{-1}

Capsulate *Neisseria meningitidis* serogroup B suspension 10^9 cells ml^{-1}

2.12.2. Method

The bacterial suspensions were prepared and reacted against each of the rabbit sera in a capsule definition reaction as described previously (2.6.3.2.)

2.13. Reactivity of rabbit immune sera towards *N. gonorrhoeae* strain G.C.B. and *N. meningitidis* serogroup B in a CIE system

2.13.1. Materials: Rabbit immune sera 8, 12, 16 weeks post-inoculation

Capsulate *N. gonorrhoeae* strain G.C.B. 10^9 cells ml^{-1}

Capsulate *N. meningitidis* serogroup B 10^9 cells ml^{-1}

CIE system

2.13.2. Method

The bacterial suspensions (2.6.8.2.) and CIE system (2.6.5.1.) were prepared as described previously and the suspensions reacted against each of the 3 sera using the same method and conditions as before (2.6.5.1.)

2.14. Absorption of rabbit immune serum with N. meningitidis serogroup B

2.14.1. Materials: Rabbit immune serum 5 ml

N. meningitidis serogroup B

Capsule-promoting media (2.6.1.2.)

2.14.2. Method

The meningococcus was grown for 48 hr. in capsule-promoting broth at 37° in an atmosphere of 5 - 10% CO_2 in air, with shaking at intervals. The culture was centrifuged at 3,000 g for 10 min., the cells washed in saline and re-centrifuged. The above was repeated several times to obtain a packed meningococcal cell volume of 1 ml. The 1 ml of packed cells was then added to the serum and the mixture incubated at 50° for 2 hr. with frequent stirring. The mixture was then centrifuged at 3,000 g for 30 min. and the supernatant taken off into a clean glass universal container.

2.15. Reactivity of rabbit immune serum absorbed with *N. meningitidis* serogroup B towards *N. gonorrhoeae* strain G.C.B. and *N. meningitidis* serogroup B in a CIE system

2.15.1. Materials: Rabbit immune serum, absorbed as above

N. gonorrhoeae strain G.C.B. 10^9 cells ml^{-1}

N. meningitidis serogroup B 10^9 cells ml^{-1}

CIE system

2.15.2. Method

The preparation of the bacterial suspensions (2.6.8.2.) and the method and conditions for the CIE (2.6.5.1.) were those described previously, except that the run was extended to 8 hr. with frequent 'topping up' of both sets of wells.

RESULTS

3.1. Capsule stain assessment

Bacterial capsules are sometimes visible in smears of routine specimens stained by the usual method of Gram's stain, although there are more specialized techniques available for their demonstration. This present work relies heavily on the reliable demonstration of capsules on gonococci, an organism hitherto not generally believed to possess a capsule, and so it was regarded as of paramount importance to assess a number of specialized capsule staining methods in order that the most reliable and efficient method be used in the study.

Seven methods of capsule stain were assessed, these being a fairly representative cross-section of the methods available. They were:- Anthony's method, Hiss's method, Richard Muir's method, Kirkpatricks's method, Eosin negative stain, Nigrosin negative stain, and wet India ink method.

3.1.1. Anthony's method : though simple in its technique and its use of readily available materials, it proved to be unreliable for the demonstration of capsules. It was found that, on occasions, even the large capsules of the Kl. aerogenes were so reduced in size as to be indistinguishable when compared with phase-contrast microscopy observations of the same culture. The decrease in capsule size was probably due to shrinkage of the capsule during the drying phase of the stain technique.

3.1.2. Hiss's method : also involves a drying and heat-fixing

stage, which inevitably results in the shrinkage of the organism and the capsule. The phenomenon of the shrinkage of the organism away from the surrounding serum vehicle tended to show the organism surrounded by a large clear halo against a pink background. This large halo was difficult to differentiate from the shrunken pale-blue-stained capsule and tended to make the Staph. aureus appear as capsulate organisms.

3.1.3. Richard Muir's method : involved the use of a mordant, which enhances the stain retention of the capsule while preventing stain reaching the bacterial cell. The method is, however, tedious, both in the preparation of the reagents and also in the performance of the staining procedure. The results were adjudged poor, in that the capsules, although stained, were not easily visible owing to severe shrinkage during the drying phase.

3.1.4. Kirkpatrick's method : as with Richard Muir's method, involved tedious preparation of complex reagents. Here too the capsules experienced gross shrinkage and were virtually indeterminable in both capsulate species; also the method caused the capsule to stain light blue on a dark blue stained bacterial cell and this made differentiation of the cell wall-capsule interface difficult.

3.1.5. Eosin negative stain : as with all of the capsule staining methods which involved a drying stage, the eosin negative staining method suffered from the effect of cell shrinkage away from the serum carrier matrix. This not only made difficult authentic capsules

being visualised but also, as in Hiss's method, made the non-capsulate Staph. aureus appear with a large clear 'capsule-like' halo around the cell.

3.1.6. Nigrosin negative stain : as with the other methods involving drying, this procedure caused shrinkage of both capsule and bacterial cell with the same unfortunate results.

3.1.7. Wet India ink method : was attempted with five brands of India ink. Four of the inks gave poor results in that they either stained the capsule and cell and therefore made differentiation difficult, were too granular and did not allow close association of capsule boundary to ink matrix thereby preventing a sharp image of the capsule, or caused clumping of the bacterial cells when mixed with them.

One brand, Higgins India ink (4415, Faber-Castell Corporation, Newark, New Jersey) proved to be a homogenous dense suspension of small particles which allowed excellent contrast of the unstained capsule against the black background of vibrating ink particles. The bacterial cell, being more refractile than the capsule, formed the boundary of the capsule inner edge.

The wet India ink stain using Higgins India ink was considered not only the most reliable method, in that capsules on capsulate organisms were seen clearly and non-capsulate organisms were shown to be non-capsulate, but also it was the easiest method to perform. The only difficulty encountered with this method was the expertise required to produce a film just thin enough to trap

the capsulate organisms between the glass slide and the coverslip, but not too thin as to squash the organisms and cause an apparent increase in capsule size, or so thick as to allow the organisms to move around in the ink matrix making observation difficult.

The expertise required was easily and quickly obtained with practice and it was therefore decided to use the wet India ink method as the definitive capsule staining technique in this study.

3.2. Presence of capsules on *Neisseria gonorrhoeae* on primary isolation

Forty isolates of *N. gonorrhoeae* from the urogenital tract and ten from the upper respiratory tract were examined for capsulation using the wet India ink stain. The results are presented in Table 1.

There is a higher incidence of capsulate organisms in those isolates from the upper respiratory tract than those from the urogenital tract. All the upper respiratory tract isolates were from patients who also provided urogenital isolates and it was assumed that the same strain was isolated from both sites.

There was a wide variation in the incidence of capsulation among the 40 different strains, the incidence varying in the urogenital site isolations from 1:112 to 1:1106. There was no sex difference in the incidence of capsulate gonococci; indeed the lowest and highest indices were from female patients and both male and female derived strains showed a wide spread of incidence ratios.

All 50 isolates had the capacity to form capsules on primary isolation, and Plate 1 shows a packed group of capsulate gonococci from a primary isolation plate.

3.3. Presence of capsules on Neisseria gonorrhoeae in vivo

Exudates from 40 patients (15 male, 25 female) attending the Special Treatment Clinic were emulsified in 0.5 ml Ringer solution and examined for capsulate organisms. Capsulate diplococci were seen in 25 exudates (10 male, 15 female) and these 25 exudates subsequently yielded N. gonorrhoeae on culture. The remaining 15 exudates failed to exhibit capsulate diplococci with India ink staining and also subsequently failed to yield N. gonorrhoeae on culture.

It can be assumed that the capsulate diplococci seen in the exudates were most likely N. gonorrhoeae.

3.4. Assessment of capsule-promoting media

A Gram's stain of a culture grown on each medium indicated that they all supported the growth of N. gonorrhoeae; however, three of the media were found unsatisfactory either with regard to performance of the India ink stain, or harvest of capsulate organisms.

3.4.1. Capsule promoting medium of Hendley et al.

The medium contained a very fine granular deposit, possibly originating from the haemoglobin component. This deposit tended to

'dilute' the smaller granules of the India ink and make recognition of capsulate organisms difficult. The medium did however support rapid, luxuriant growth of N. gonorrhoeae, although no assessment could be made of the presence of capsulate organisms.

3.4.2. Capsule promoting medium of Richardson and Sadoff

Growth of all 10 strains was good, both on the agar slope and in the broth phase. Harvesting of the organisms was made difficult however owing to the production of an 'agar membrane' on the surface of the agar slope which dissociated itself from the slope on shaking and became suspended in the broth. Approximately 50% of the gonococci were assessed to be capsulate, though most capsules were moderate in size and increased the apparent cell diameter by 50%, in some cases they were small and increased the apparent cell diameter by 10 - 25%. Very few cells exhibited large capsules which increased the apparent cell diameter by 100 - 150%.

3.4.3. Capsule promoting medium of Jephcott and Reyn

As with the medium of Richardson above, growth of all 10 strains of gonococci was good. Again, however, an 'agar membrane' was formed on the slope which broke away from the slope and became suspended in the broth phase. Not only did this membrane make harvesting difficult but broke into such small pieces that the reading of the India ink stain was also made difficult. The capsules formed were small to moderate in size and found on approximately 54% of organisms.

3.4.4. Capsule promoting media of Hafiz, McEntegart and Jephcott

The two modified media of Hafiz proved to be the most rewarding with regard to the numbers of capsulate in relation to non-capsulate cells, luxuriance of growth and ease of harvesting for both the India ink stain and production of pure antigen material for the rabbit inoculation to follow. Both modifications gave similar results in their capacity to promote capsule formation on the gonococci and in the size of the capsules so produced. The ease of production of the second modification, with the omission of a step which might lead to contamination, viz. the aseptic addition of the supplement, decided the use of the second modification (2.4.1.5.) as the medium to be used for the promotion of capsules on gonococci.

3.5. Effect on gonococcal capsule size and incidence of an alteration of the carbohydrate : nitrogen ratio of the growth media

In both sets of media, those containing ratios of between 1:1.8 to 1:1.2 of carbohydrate : nitrogen displayed very poor growth or no growth at all, of the gonococcus. The media with carbohydrate : nitrogen ratio of between 1:2 to 1:2.4 in both sets showed good growth of the gonococcus with a ratio of capsulate : total cells of approximately 68%.

The capsule medium was therefore altered to provide a glucose : peptone ratio of 1:2 by reducing the proteose peptone content by 33%.

3.6.1. Detection of anticapsular antibodies

The slide preparation of capsulate gonococci and rabbit pre-immune sera was examined under the microscope first. The capsules on the organisms were very difficult to differentiate even with the lighting adjustment but were just discernible against the clear serum-saline background.

The slide preparation of capsulate gonococci and rabbit immune sera exhibited a marked delineation of capsule against the carrier background with a better-defined capsule boundary, indicating attachment of anticapsular antibody to the capsular material of the gonococci.

3.6.2. Titre of anticapsular antibodies

The results of the titration are shown in Table 3.

The rabbit immune serum was shown to improve visibility of the capsule boundary in dilutions down to 1:31. The 1:63 dilution gave equivocal results and those dilutions below 1:63 gave similar results to the reaction where rabbit pre-immune serum was substituted for the immune serum as a reaction control.

3.6.3. Reactivity of rabbit immune serum towards capsulate *Neisseria gonorrhoeae* strain G.C.A. in a counterimmunoelectrophoresis system

A photograph of the resultant slide is shown in Plate 3.

It can be seen that there is a strong precipitation line between the wells containing the rabbit immune serum and the wells

containing the capsulate gonococcal suspension, indicating a precipitating antibody/antigen reaction in the agar. There is no line of precipitation in the agar between the well containing the capsulate gonococcal suspension and the rabbit pre-immune serum.

3.6.4. Titration of rabbit immune serum against capsulate Neisseria gonorrhoeae strain G.C.A. in the CIE system

A photograph of the resultant slides is shown in Plate 4.

It can be seen that the immune serum reacted strongly in the CIE system against the capsulate gonococcal suspension down to a dilution of 1:64; this compares with an equivocal capsule definition result at this titre.

3.6.5. Reactivity of rabbit immune serum towards 10 strains of capsulate Neisseria gonorrhoeae

A photograph of the two slides containing the rabbit immune serum and the 11 gonococcal strains is shown in Plate 5.

It can be seen that the rabbit immune serum has formed precipitation reaction lines in the agar when reacted against all 11 strains of capsulate gonococci. The rabbit pre-immune serum failed to produce any precipitation reaction against any of the strains. Although the number of strains is small, it seems likely that the immune serum would react similarly against any capsulate strain of N. gonorrhoeae in the CIE system.

3.6.6. Reactivity of rabbit immune serum towards strains of *Neisseria meningitidis* groups B and C, *Branhamella catarrhalis*, *Neisseria subflava*, *Neisseria sicca* and *Neisseria gonorrhoeae* strain G.C.A. in CIE

A photograph of the slide containing the rabbit immune serum and the 6 bacterial strains is shown in Plate 6.

It can be seen that the rabbit immune serum has formed lines of precipitation reaction against all 6 bacterial species although the lines formed between the serum and *B. catarrhalis*, *N. sicca* and *N. subflava* are a little weaker in intensity than those formed against the other 3 strains. None of the strains gave lines of precipitation against the rabbit pre-immune serum.

There would, therefore, seem to be strong cross-reaction between the immune serum raised against *N. gonorrhoeae* strain G.C.A. and the two *N. meningitidis* serogroups and a slightly lesser cross-reactivity against the other 3 species. For the immune serum to be reactive only against strains of *N. gonorrhoeae* all the cross reactions had to be removed.

3.7.1. Reactivity of rabbit immune serum, absorbed with bovine bone marrow towards N. gonorrhoeae strain G.C.A., N. meningitidis serogroups B and C, B. catarrhalis, N. subflava and N. sicca

A photograph of the resultant slide is shown in Plate 7.

It can be seen that a similar result to that using the unabsorbed serum has been obtained, with strong lines of precipitation between the serum and N. gonorrhoeae strain G.C.A., and N. meningitidis serogroups B and C, and slightly weaker reaction between the serum and the other 3 species. The absorption with bovine bone marrow has clearly failed to remove the major portion of the cross-reacting components.

3.7.2. Reactivity of rabbit immune serum, absorbed with bovine bone marrow, B. catarrhalis, N. subflava and N. sicca in a CIE system

A photograph of the resultant slide is shown in Plate 8.

It can be seen that strong lines of precipitation reaction are visible between the wells containing the absorbed immune serum and the wells containing the N. gonorrhoeae, N. meningitidis group B and N. meningitidis group C suspensions. There are however no lines of precipitation between the wells containing the absorbed serum and those containing the B. catarrhalis, N. subflava and N. sicca suspensions indicating that, as would be expected, all the reaction against the 3 absorbing species has been removed leaving only those

reactions against antigens held commonly by the gonococcus and the two meningococcal strains. The serum had therefore to be further absorbed with the meningococci to leave only those antibody molecules which would react with antigens held exclusively by the capsulate gonococci.

3.7.3. Reactivity of rabbit immune serum absorbed with bovine bone marrow, B. catarrhalis, N. subflava, N. sicca and N. meningitidis serogroup B in a CIE system

The photograph of the resultant slide is shown in Plate 9. It can be seen that there is no line of precipitation reaction between the wells containing the absorbed serum and the wells containing any of the cell suspensions. The absorption with N. meningitidis serogroup B has removed all of the remaining reactivity of the immune serum.

3.8.1. Bulk culture of Neisseria gonorrhoeae strain G.C.B. (I)

After 24 hr. growth, the first bulk culture attempt appeared to show good growth of the gonococcus, when the culture was viewed by transmitted light. A Gram's stain of the culture showed large numbers of gram-negative diplococci in apparently pure culture although when submitted to India ink stain few of the organisms appeared to be capsulate. The culture was therefore incubated, under the same conditions, for a further 24 hr.

After 48 hr. incubation, the culture appeared very turbid

when viewed by transmitted light. A repeat Gram's stain revealed heavy contamination with a large gram-positive bacillus, subsequently shown to be a Bacillus sp.

3.8.2. Bulk culture of Neisseria gonorrhoeae strain G.C.B. (II)

The Gram's stain made after 48 hr. growth of the second attempt at bulk culture again revealed heavy contamination with a large gram-positive bacillus, which, on subculture, proved to be similar to the organism contaminating the first bulk culture. The Gram's stain also revealed large numbers of gram-negative diplococci and on India ink stain these exhibited a high incidence of capsulate organisms, the Bacillus sp. also being shown to possess a capsule.

3.8.3. Bulk culture of Neisseria gonorrhoeae strain G.C.B. (III)

A Gram's stain of the 100 ml inoculum showed large numbers of gram-negative diplococci in pure culture. An India ink stain revealed large numbers of capsulate organisms. The antibiotics had therefore neither prevented growth of the gonococcus in the capsule-promoting broth nor affected the organism's ability to form capsules.

A Gram's stain and India ink stain of the 500 ml inoculum also showed a pure culture of gonococci and a high proportion of capsulate organisms.

The Gram's stain of the bulk culture showed large numbers of gram-negative diplococci after 24 hr. incubation. There was however little capsule formation after this incubation period and the

culture was re-incubated for a further 24 hr. A Gram's stain of the culture after 48 hr. revealed large numbers of gram-negative diplococci, which on subsequent culture and biochemical tests proved to be N. gonorrhoeae, in pure culture. An India ink stain revealed that 66% of the organisms had large capsules, with the remaining 34% showing small to moderate size capsules.

3.9. Reactivity of rabbit immune sera towards N. gonorrhoeae strain G.C.B. and N. meningitidis serogroup B in a capsule definition reaction

The sera taken from the rabbit at 8 and 12 weeks after inoculation failed to show any improvement in the definition of the capsule boundary of either organism. The serum taken at 16 weeks after inoculation indicated that anticapsular antibodies were present in the serum by exhibiting an improvement in the definition of the capsule boundary when compared with a control using pre-immune serum and the gonococcus. There was also visible improvement of boundary definition using the immune serum and the meningococcus, though not as marked as in the gonococcal test.

3.10. Reactivity of rabbit immune sera towards N. gonorrhoeae strain G.C.B. and N. meningitidis serogroup B in a CIE system

The 8 and 12 week sera failed to show any reaction with the bacterial suspensions both over the usual 45 min. run period and also when subjected to a prolonged run of 8 hr. with frequent 'topping up' of bacterial suspension and sera wells.

The 16 week serum also failed to show any reaction with the bacterial suspensions over the usual 45 min. run period but did show lines of precipitation against both strain suspensions when the run was extended to 6 hr. with frequent 'topping up' of both sets of wells. This would indicate very weak antibody activity of the serum and also cross-reactivity of those antibodies against a non-gonococcal organism. The cross-reactions would therefore have to be absorbed from the antiserum using the cross-reacting strain.

3.11. Reactivity of rabbit immune serum absorbed with N. meningitidis serogroup B towards N. gonorrhoeae strain G.C.B. and N. meningitidis serogroup B in a CIE system

The serum failed to react in the system with either the gonococcus or the meningococcus, both over the usual 45 min. run period and an extended run of 8 hr. with frequent 'topping up' of both sets of wells.

A repeat of the above with serum taken from the rabbit 20 weeks after inoculation also failed to react in the system after absorption.

DISCUSSION AND CONCLUSIONS

4.1. Capsule stains

The definitive work on capsule and loose-slime staining was performed by Duguid in 1951 (153). Unquestionable reliance could have been placed on Duguid's work and the method he described as being the most reliable method for capsule staining could have been adopted for use in this work. It was, however, decided that, owing to the great importance a reliable capsule stain had in the project, a thorough investigation of capsule staining techniques, regarding ease of technique, but more importantly reliability of result, would be advisable.

Seven capsule staining methods were assessed, a representative cross-section of methods available, and, indeed, just as Duguid had found in his work, the wet India ink stain did prove to be the most reliable method. There were, however, two minor difficulties associated with the technique. The brand of India ink is of prime importance; the size of the ink particles must be small enough to form an almost continuous outline of the capsule boundary yet not so small as to become integrated into the cellular or capsular material and make visualization of the capsule difficult. Only one of the brands tested performed satisfactorily in this respect. The second difficulty was the expertise required to make the film of ink-culture under the coverslip just the correct thickness to trap the capsulate organisms between the two glass surfaces without causing distortion of the capsule or allowing the organisms to move around the film field. The expertise was gained with practice (3.1.7.).

4.2. Capsule incidence on routine media

In the work which prompted this present investigation Richardson and Sadoff (131) and Hendley *et al.* (132) had both used specially prepared capsule-promoting media for the demonstration of capsules on *N. gonorrhoeae*. This might prompt the suggestion that the capsule on the gonococcus is merely a cultural phenomenon, the cultural conditions directly influencing capsule production. Although Caspar (154), who made the suggestion that gonococci could be found to possess capsules, reported that this was only the case for freshly isolated organisms from acute infections, and that the capsules were lost on subculture. Holmes (4) reported that gonococci only produce capsules as a response to cultural conditions, as did Robbins (155), and stressed the need for specialized media and the disappearance of the capsule on subculture on conventional media.

The 50 *Neisseria gonorrhoeae* isolates on routine selective chocolate agar culture all had evidence of capsule production, and so it seems probable that the phenomenon is demonstrable in all gonococci and is not just a characteristic of a few strains.

Table 1 clearly shows a difference in the number of capsulate organisms isolated from throat culture when compared with the number of capsulate organisms isolated from urogenital tract culture. This may be explained by reference to the conditions prevalent at those sites. Duguid and Wilkinson (156) have shown that a high carbohydrate : nitrogen ratio is favourable to capsule production in culture media and it seems reasonable to suppose that this is the case not only in

culture, but also at sites of infection in the host. The ratio of carbohydrate : nitrogen at both sites would be influenced directly by conditions prevailing on the mucous membrane surfaces of those sites. There would be a more favourable carbohydrate : nitrogen ratio in the throat when compared with the ratio in the urogenital tract. Dietary intake of carbohydrates may to some extent increase the carbohydrate availability in the throat, though this may be transient, but perhaps more importantly would be the presence of urine and urine breakdown products in the urogenital tract which would increase the nitrogen availability there.

There is also a difference in the gas tension prevailing at both sites, which may also influence the growth of the organisms. While the carbon dioxide concentration at the mucous surface of the urogenital tract would closely mirror that found in the atmosphere, the carbon dioxide concentration at the mucous surface of the upper respiratory tract, for approximately 50% of the time, is 4.1%, which is the concentration of carbon dioxide in exhaled breath (157), and approaches the concentration of carbon dioxide in air necessary for the culture of gonococci on artificial media. These two differences in conditions at the two sites may explain the difference in the incidence of capsulation in primary isolation on routine media from the two sites.

There is also a difference in the incidence of capsulate organisms among the 40 patients. This difference possibly reflects the differing cultural conditions present on the mucous surfaces of

individual patients rather than any strain variation among the gonococci. There was no correlation between capsule formation on primary isolation and on culture in capsule-promoting media, some gonococcal strains on primary isolation displaying a 'low' incidence of capsulate organisms on primary isolation yet displaying a 'moderate' to 'high' incidence of capsulate organisms when grown in capsule-promoting media.

4.3. Capsule incidence *in vivo*

The above clearly shows that *N. gonorrhoeae* does possess a capsule when isolated freshly from infected sites, and it might be assumed that the capacity of the organism to produce a capsule *in vitro* would in some way be related to its role as a pathogenic organism. The bacterium's capacity to form a capsule would be dependent on it possessing the necessary enzymic machinery for capsule production, which would reflect its need for a capsule *in vivo* rather than *in vitro*.

One of the aims of the present study was to investigate the possibility of a rapid, reliable serological test for the diagnosis of gonococcal infection by means of a precipitating antigen-antibody reaction using the capsule of the gonococcus as the antigenic component. It was, therefore, necessary to show that *N. gonorrhoeae* does produce a capsule *in vivo*. De Hormaeche, Thornley and Glauert (133) had demonstrated the presence of capsules on gonococci grown in subcutaneous chambers in guinea-pigs; however,

despite light and electron microscope studies of the gonococcus, both in small mammals (158) and in humans (159), no evidence of capsulate gonococci in exudates from humans has been presented to date.

The visualization of capsulate diplococci in exudates from patients subsequently proved to have gonococcal infection provided the evidence that the gonococcus is indeed capsulate when growing *in vivo*, although the limits imposed by the India ink stain allowed only those gonococci growing extracellularly to be visualized.

The possession of a capsule by *N. gonorrhoeae* when growing *in vivo* would presumptively be a phenomenon associated with its role as a pathogenic organism, either as an aid in promoting the infectivity of the organism directly or in protecting the organism from host defences. The most likely explanation for the presence of a capsule would be as a protection against phagocytosis. Hendley *et al.* (132) and Richardson and Sadoff (131) suggest this latter role for the capsule and Richardson *et al.* attempted to prove their theory by adapting a phagocyte slide phagocytosis test. In this test bacterial species are allowed to react with human neutrophils and the reaction viewed under the microscope. They reported that with a strain of *N. gonorrhoeae* grown in a medium detrimental to capsule production, 88% of 400 polymorphs counted had one or more organisms attached or ingested, whilst with the same strain grown in a capsule-promoting medium only 19% of 400 polymorphs

could be found with gonococci attached or ingested.

An insight into the importance that a capsule might have on the infectivity of the gonococcus is afforded by the work of Brooks, Benseman and Peak (160) on the capsule-like K1 antigen of Escherichia coli. They found that the Escherichia coli strains isolated from urinary tract infections had a higher titre of K1 antigen than those strains isolated from other sites. They suggested that the K1 antigen conferred greater resistance to phagocytosis, antibody binding and killing by complement, in sites where opsonins are deficient. This situation would hold true for gonococci found in the urogenital and upper respiratory tract.

Kellogg and Thayer (161) and Jephcott and Reyn (149) have reviewed the virulence and infectivity of the gonococcus and attempted to correlate virulence with colonial morphology. They suggested that gonococci found in urethral exudates may possess a 'virulence factor', which is lost on subculture, and which prevents the bactericidal action of complement and/or natural or immune antibodies. Kellogg and Thayer described four characteristic colony types and showed that types 1 and 2 occurred in primary culture from acute infections, whilst types 3 and 4 were obtained on subculture. The phenomenon of colonial morphology alteration in subculture allied with loss of 'virulence factor' may be associated with capsule possession by freshly isolated strains and their subsequent loss on subculture. Richardson and Sadoff (131), however, cultured both 'virulent' and 'less virulent' colony types of strains of

N. gonorrhoeae and both types formed capsules. When both capsule-forming types were subcultured, they reverted to their original colonial form. This would suggest that there is both a 'virulence factor' closely associated with colonial morphology and in addition a 'virulence factor' associated with capsule formation, both factors being independent of each other.

4.4. Media for capsule production

Neisseria gonorrhoeae is a fastidious organism and requires for growth a medium rich in organic and inorganic supplements. Indeed one of the problems associated with the diagnosis of gonococcal infection has been the difficulty associated with culture of the causative organisms both on laboratory media for primary isolation and also on media for biochemical identification. In order to raise antibodies in rabbits to the gonococcal capsule, capsulate gonococci had to be cultured in large amounts, either in liquid or on solid media, and the media had not only to provide ideal conditions for rapid growth but also to promote capsule production by a large percentage of the organisms in culture. Kellogg and Thayer (161) provided evidence that some factor present in vivo, at infection sites, manifested itself both in the altered infectivity of the gonococcus and also in the colonial morphology exhibited by it on primary isolation. Richardson and Sadoff (131) had shown that there was no relation between the 'virulence factor' associated with colonial morphology and the 'virulence factor' associated with

capsule formation. The possibility still existed however that gonococci which had been dissociated from growing in vivo for a number of subculture steps, or held in culture for long periods would revert permanently to their non-capsulate form, which would hinder attempts to produce capsulate organisms in sufficiently large amounts.

Each individual strain of Neisseria gonorrhoeae requires particular growth requirements with regard to amino acids, purines, pyrimidines or vitamins, (162, 163, 164, 165). Carifo and Catlin (162) have shown that most strains of N. gonorrhoeae are auxotrophic, requiring one or more growth factors that may or may not be necessary for the growth of other strains. The only common factor that strains require would appear to be an energy source of glucose, pyruvate or lactate. All the media in this present study contained glucose as the energy source, although a side study was undertaken to establish the effect of substitution for the glucose, either in part or whole, of glycogen. The glycogen did not effect the capacity of the broth to support growth of the gonococcus or the capacity of the gonococcus to form capsules, when compared with media containing glucose alone.

Five media were tested for the production of capsulate N. gonorrhoeae. Three of the media were single liquid phase media and two were biphasic liquid and solid media. All the media allowed good growth of the gonococci and capsule production; however three of the media proved unsatisfactory for the present study.

The two media containing haemoglobin (2.4.1.1., 2.4.1.2.) made visualization of capsulate organisms, by means of the India ink stain, difficult owing to the presence in the media of large amounts of granular material. The granular material could not be removed by centrifugation and contaminated the harvest of gonococci from the culture. The two bi-phasic media also yielded gonococci contaminated with culture-derived debris. An agar 'skin' formed on the surface of the solid phase and this was harvested with the organisms. The contamination by large amounts of organic debris would prevent any subsequent attempt to inoculate a pure culture of gonococci into the animal model and the presence of this organic debris might alter the immune response of the animal. The haemoglobin-containing media were also rejected because some strains of gonococci had been found to be inhibited by the presence of this compound in culture media (166, 167).

As with the two classes of media above, it was decided to use a medium lacking in serum or other body fluids, which would be difficult to remove from the harvested organisms and, if carried over to the inoculation step, in the procedure, would complicate the production of anticapsulate-gonococci antibodies by the rabbit. A synthetic medium was therefore thought to be the medium lacking in problems associated with media-derived contamination.

The medium of Hafiz et al. (150) satisfied all these requirements. The medium was however modified in several ways. Originally Hafiz et al. had included a supplement in the medium which

had to be sterilized by filtration and then added aseptically to the other ingredients after autoclaving. On several occasions this step resulted in contamination of the medium and so it was decided to assess the medium without added supplement. The modified medium was found to be as satisfactory as the original with regard to capsulate organisms but did not provide as luxuriant a growth. The latter problem was overcome by the addition to the medium of yeast extract, which could be added to the other ingredients before autoclaving thereby minimising post production contamination of the medium.

The pH of culture media is of prime importance and Morse and Hebeler (168) have reported that the pH of the medium can alter cellular metabolic processes. They determined that at pH 6.0 the cells utilized glucose by means of the pentose phosphate pathway and the tricarboxylic acid cycle, whereas at pH values between 7.2 to 8.0 the tricarboxylic acid cycle was not active and 80% of the glucose was metabolized by means of the Entner-Doudoroff pathway and the remaining 20% by means of the pentose phosphate pathway. At pH 6.0 the cells incorporate approximately twice as much glucose carbon as cells growing at pH 8.0. Richardson and Sadoff (131) had noticed enhanced production of capsules by gonococci in one of their cultures which had been contaminated with a 'viridans streptococcus'. This may have been caused by the accumulation in the medium of lactic and acetic acids formed by the metabolic processes of the streptococcus, causing a lowering of the pH of the medium and consequently an alteration in the metabolism of the gonococcus favouring the greater

incorporation of hexose sugars into the cells.

The pH of the medium chosen for the study was originally adjusted to pH 6.6; however, it was noticed that the growth of the organisms in the medium, although rapid for the first 24 hr. slowed down and there did not appear to be any increase in opacity of the culture after approximately 36 hr. growth. The pH of the medium was measured after 36 hr. incubation and found to be approximately 5.8. A pH of 5.8 is below that which is favourable for the growth of the gonococcus (168) and this would explain the apparent cessation of growth after the initial 24 hr. incubation period. The pH of the medium would not only be affected by the metabolic by-products of the growing organisms but also by the dissolving, in the liquid medium, of the carbon dioxide in the atmosphere of the incubator. The initial pH of the medium had to be adjusted to a value which would drop to pH 6.3 - 6.5. after 24 hr. incubation. After trial and error it was found that an initial pH of 7.2 - 7.4 was suitable and this gave a pH of between 6.8 - 7.0 for the greater part of the first 18 hr. incubation period, this pH being optimum for the most rapid generation time (168), followed by a drop in pH over the next 12 hr. period to 6.5 - 6.6 for maximum hexose sugar incorporation.

Duguid (153) disagrees that acid pH is beneficial to production of capsules on organisms and suggests that the most important parameter in the production of capsules is the ratio between polysaccharide and nitrogen in the culture medium (156).

Duguid and Wilkinson (156) showed that, in a medium containing excess sugar, growth was complete after 24 hr. and polysaccharide production complete after 96 hr. They also found that the polysaccharide content of cells was 10 to 20 -fold greater in cultures where excess sugar was present than in cultures where excess nitrogen was present, the increase in polysaccharide content being estimated by the increase in capsule diameter of the cells. An assessment of the effect of an alteration in carbohydrate : nitrogen ratio in the capsule medium chosen on capsule production by the gonococcus was therefore made.

The ratio of carbohydrate : peptone was altered by keeping the glucose content constant and reducing the peptone content to give ratios of carbohydrate : peptone from 1:3 to 1:1.2, and these same ratios were obtained by keeping the peptone content constant and altering the glucose content (Table 2). The performance of each medium was assessed by estimation of the ratio of capsulate to non-capsulate cells and also by the size of the capsules produced. Those media with carbohydrate : peptone ratios of 1:1.8 and above proved to be unsuitable for the growth of the gonococcus. In the media where the peptone content had been reduced to increase the carbohydrate : peptone ratio the cessation of growth was probably a result of a decrease in nitrogen availability below that which the gonococcus required for growth. In those media where the glucose content had been increased to increase the carbohydrate : peptone ratio, the cessation of growth was unexpected but may have been caused

by the rapid lowering of the pH of the media to a value below which the gonococcus was able to survive. This medium was subjected to an increased incubation time of 4 days because of the lack of organisms with large capsules found after the normal incubation period, owing to the time necessary for the glucose to reach 'excess'. This extra incubation time was almost certainly responsible for the unfavourable lowering of the pH to an unacceptable level and hence cessation of growth.

In both series, the media containing the ratio of carbohydrate : peptone of 1:2 gave the maximum number of capsulate organisms for a given bacterial count, though a difference in capsule size between each of the media which supported good growth was not apparent.

The addition of casein hydrolysate to the modified medium of Hafiz et al. was prompted by the work of Hendley et al. (132), who found that the incorporation of this compound into their medium improved the production of capsules during the log phase of growth. Although the casein hydrolysate was incorporated in the final medium no significant increase in capsule size or in capsule incidence was apparent in media containing casein hydrolysate when compared with media lacking in this compound.

For the preliminary challenge of the rabbits with whole capsulate gonococci, small scale production of capsulate organisms had been attempted using 100 ml of capsule medium in Roux flasks. This however proved a long and laborious process and 1500 ml of

culture media incubated in this way yielded approximately 5 g wet weight of organisms with 60 - 70% of the organisms being capsulate. It was therefore thought that bulk culture in a large fermentation vessel would yield more organisms ml^{-1} of culture media than culture in Roux flasks. The fermenter allowed constant agitation of the culture, rather than intermittent agitation as in the Roux flask cultures, which would allow more efficient diffusion of the carbon dioxide/air mixture into the medium, as well as the disruption of clumps of organisms into smaller units and hence more efficient use of the culture medium. The Roux flask cultures always resulted in the formation of a pellicle on the surface of the medium where the gas concentration in the medium would be greatest, and it was hoped that the constant agitation of the medium in the fermenter would facilitate better availability of gas to the gonococci, better availability of fresh culture medium to the gonococci and hence better capsule production.

The first attempts at bulk culture of N. gonorrhoeae resulted in contamination of the culture medium with a gram-positive bacillus, which proved to be a Bacillus sp.. This contaminating organism may have been introduced into the medium during the inoculation of the starter culture, or by means of a contaminated seal on the agitation apparatus of the fermenter, or may have survived the autoclaving process. The problem of contamination was solved by the incorporation of antibiotics into the culture medium after autoclaving and by immersing the agitator seals in disinfectant.

Antibiotics are commonly incorporated into media used for primary isolation of the gonococcus from sites of infection, in order to minimise contamination by other organisms which form the normal flora of these sites. Hafiz and McEntegart (169) used vancomycin, trimethoprim and colistin, Thayer and Martin (67) preferred the use of nystatin instead of trimethoprim, as did Philips, Humphrey and Middleton (69). The initial reluctance to use antibiotics in the medium for capsule production was primarily because the bulk culture of organisms was being carried out in Roux flasks using small volumes of culture medium and the contamination of a few cultures did not present a great problem with regard to wasted time and materials.

The preparation of a large volume of medium in the fermentation vessel did, however, require a significant amount of preparation and monitoring of the autoclaving process to prevent caramelisation of the glucose and the contamination of the bulk medium provided the impetus to assess the value that antibiotics would have in the production of an uncontaminated culture of capsulate gonococci.

Antibiotic control of contamination is not without its complications and another reason for their being omitted from the first culture medium was the possibility that their use would adversely affect not only capsule production but also the growth of the gonococci in the medium during the initial assessment trial of capsule production by many gonococcal strains. It was known

that certain strains of gonococci are sensitive to antibiotics used routinely in primary isolation media (70, 170, 171) and that solving the problem of contamination may have resulted in the gonococci either not growing in the medium or not producing capsules of the size and number required. A small trial involving the Neisseria gonorrhoeae strain to be used in the bulk culture and the antibiotics to be incorporated into the medium indicated that the use of antibiotics to control contamination would not be counter-productive.

4.5. Production of anticapsular antibodies

The first attempt at producing hyperimmune serum to gonococcal capsular material in the rabbit was made using capsulate whole organisms rather than purified capsular antigen. Gotschlich, Goldschneider and Artenstein (172) and Artenstein et al. (173) working with the Neisseria meningitidis serogroup C polysaccharide found the polysaccharide to be a highly immunogenic protective antigen in human volunteers as was also the case with N. meningitidis group A polysaccharide. In a later investigation, however, Gotschlich, Lui and Artenstein (174) suggested that, although these purified polysaccharides were immunogenic in the human, only weak immunity was produced in laboratory animals with N. meningitidis group B polysaccharide, whereas polysaccharide from groups A and C produced a good antigenic response. Limjuco et al. (175) suggested that no common pattern of lipopolysaccharide exists among the Neisseriaceae,

the lipopolysaccharide of N. meningitidis group B being markedly different from that of N. meningitidis group C and the lipopolysaccharide of N. perflava, N. sicca and N. catarrhalis. This may also be the case for capsular polysaccharide which would explain the differing immune responses in animals to different capsular types.

Molecular size alone is not sufficient to confer antigenicity on a particular substance; however the polysaccharide dextran of molecular weight 600,000 is a good antigen whilst dextran of molecular weight 100,000 is not (175a). Although no obvious reason for this exists, it may be that the larger molecule possesses sufficiently more repeating sub-units for the immune system to recognise non-self and produce an immune response.

Gotschlich et al. (174) suggested that the polysaccharides they isolated were of molecular weights exceeding 100,000, whereas Mergenhagen, Martin and Schiffman (176) suggested that the molecular weights were much smaller. This difference in molecular weight might have been due to the possible enzymic or chemical depolymerization of the larger molecules into smaller sub-units during the different isolation procedures. Gotschlich agrees that the appearance of smaller sub-units of polysaccharide was due to the action of enzymes on the larger molecules, and found, with N. meningitidis group B polysaccharide, that cultures incubated for 16 hr. or longer showed a considerable increase in the production of low molecular weight polysaccharide over those incubated for 7 hr.

where smaller sub-units were not apparent. He suggested that this was due to the production of an enzyme by the meningococcus, which broke down the capsular material.

The problem, therefore, was that the gonococcus exhibited maximum capsule production after the first 24 hr. of incubation when the nitrogen-containing compound became limited, very little being produced during the log-phase and immediate post log-phase of growth. This would mean that there would be a likelihood of obtaining small molecular weight polysaccharide as found by Mergenhagen *et al.* (176) even without the use of a 'harsh' extraction procedure, and this polysaccharide would not necessarily have proven to be antigenic. The use of an extraction procedure would increase the likelihood of the larger polysaccharide molecules being broken into smaller sub-units and because of this it was decided to use capsulate whole organisms for the first attempt at antiserum production. It was hoped that any cross-reactions due to antibody response to cell wall, somatic or nuclear-derived antigens could be absorbed by conventional absorption techniques.

The culture, harvest and purification of capsulate organisms was performed using the minimum of mechanical techniques likely to cause dissociation of capsule from the cell. Richardson and Sadoff (131) found that the capsules produced on their strain of gonococcus were easily removed by forcing the capsulate organisms through an 18 gauge needle, and although no such phenomenon was observed with the isolates used in this study it was thought prudent

to keep mechanical agitation to a minimum and to monitor the integrity of the capsules on the cells at each step of the procedure.

1500 ml of capsule medium in 100 ml aliquots was required to provide 15 1ml aliquots of capsulate gonococci in suspensions containing 10^9 cells ml^{-1} for the preliminary challenge of the rabbits using capsulate whole organisms. All aliquots were stored at -20° until all of the material for injection was ready. The freezing and thawing of the material had no visible adverse effect on the integrity of the capsulate cells in the saline carrier, either in the numbers of intact cells or incidence of capsulate organisms. The heating of the culture media to 56° to kill the organisms prior to harvesting the cells was thought necessary, not only to make the manipulation of the cultures easier but also to ensure that the rabbit was not being subjected to challenge using live organisms, although it was suspected that the freezing process would kill the gonococci during their storage prior to inoculation.

The use of killed organisms for injection was thought not to affect the production of antibody, either by prolonging the timing of antibody appearance in the rabbit or the titre of antibody produced, and would prevent the rabbit succumbing to an injection process elicited by live organisms. A number of workers had used killed organisms previously for the production of antibodies in rabbits. The methods of killing the cells usually involved heat, as with Mannel and Mayer (177) with enterobacterial polysaccharide,

Danielsson et al. (178) with N. gonorrhoeae and Esquenazi and Streitfeld (179) with N. gonorrhoeae and N. catarrhalis, or formalin as Danielsson et al. (178), Peacock (95) and Brodeur (128), all working with N. gonorrhoeae. Danielsson and Sandstrom (180) working with N. gonorrhoeae had shown that the use of heat or formalin-killed organisms did not significantly affect antibody production when compared with the use of live gonococci. The use of heat- rather than formalin-killed organisms was in order to keep chemical contamination of the antigen as low as possible and also because of the effect any chemical substance might have on the integrity of the capsule-cell unit and subsequent antibody production.

Adjuvants, as a means of promoting the antigenic effect of administered compounds are widely used, both for the study of the immunogenicity of whole cells and cellular components. The most commonly used adjuvant is 'Freund's adjuvant', composed of a water in oil emulsion, either with or without living or dead mycobacteria in suspension. The majority of workers involved in the serology of N. gonorrhoeae infections have used this procedure (121, 126, 180, 181, 182). Vijay et al. (183) used aluminium hydroxide as an adjuvant and McCabe and King (184) described the use of hog gastric mucin as an adjuvant with N. intracellularis, this method also being used by Weiss and Long (185) to assist carbohydrate antigens from N. meningitidis group A and C. The presence of an adjuvant is believed to promote the enhanced

production of antibody by direct effect on the membrane of the phagocyte, leading to more effective transformation of the cell for antibody formation, or by making the soluble antigen more readily phagocytosed, or by providing a storage facility for antigen and allowing slower release of antigen to the lymphocytes, or by combination of all three processes. These phenomena may be true for soluble antigens such as the purified capsular material used in the second attempt at antibody production where a modified Freund's adjuvant (152) was used, but adjuvant probably has no effect on the antigenicity of particulate antigens, as in the case of whole organisms, and many workers have administered adjuvant-free material with satisfactory results (93, 128, 132, 177, 178, 179, 186). Danielsson and Sandstrom (180) found no difference in the antibody-promoting capacity of antigens prepared from N. gonorrhoeae administered with adjuvant and administered alone. It was thought therefore that the use of adjuvant in the preliminary antibody production using whole capsulate organisms was not necessary and this would simplify the production of injection material and also minimise the use of mechanical manipulation which might adversely effect the integrity of the capsulate organisms.

The schedule of antigen administration was decided upon after careful consideration of the procedures used by previous workers in the field of antibody production using rabbits, both with capsulate organisms other than N. gonorrhoeae and also with whole gonococci. There is a wide variety of administration schedules ranging from the

two injections with a one month interval between them, used by Buchanan et al. (126) working with gonococcal pili, to the more complicated schedule of Angelsen and Maeland (93) working with various antigens derived from N. gonorrhoeae, using varying quantities of antigenic material administered at varying time intervals over a four week period. Danielsson and Sandstrom (180) used a combination of the two techniques in a procedure lasting approximately eight weeks.

It was decided to use a schedule of administration, using whole capsulate organisms, with a regular inter-injection period of three days over a six week period, similar to Edmunds (186) and Peacock (95), using a constant antigen volume of 1ml containing 10^9 cells. The decision to use 10^9 cells in each ml of antigen material was influenced by previous work involving whole organisms, where Hendley et al. (132), Esquenazi and Streitfeld (179), Brodeur, Ashton and Diena (128), Danielsson and Sandstrom (180), and Edmunds (186) all used antigenic material containing approximately 10^9 organisms ml^{-1} in obtaining a good antibody response in the rabbit.

The decision not to use adjuvant with the whole capsulate gonococci allowed the more direct route of intravenous, as opposed to subcutaneous, injection. The administration of relatively large volumes of antigenic material over an extended period, which would effect a continuing challenge of the animal model's immune system, as opposed to the administration of a large volume of antigenic

material, with or without adjuvant, which would provide a store of material to be leached out over a long period, seemed better suited to the use of whole organisms in this study. Any attempt to reduce the volume of material from 15 ml to a volume capable of being administered by subcutaneous route without the need to use a large number of injection sites in the animal model resulted in an extremely viscous solution which required extreme pressure to pass through the injection needle. The subcutaneous route of administration is best suited to a small number of injections of material combined with adjuvant, as was the case in the second attempt at antibody production using purified capsular material, the intravenous route of administration of both living and killed whole organisms being the most popular method among the previous workers in the field.

Ten days after the last injection of whole organisms intravenously, and 8, 12 and 16 weeks after the subcutaneous administration of purified capsular material with adjuvant, the rabbits were bled using an ear vein slit and the sera taken for assessment of anticapsular antibody in a 'capsule swelling' or Quellung reaction (187) and in a counterimmunoelectrophoresis system.

4.6. Assessment of anticapsular antibodies

4.6.1. 'Capsule swelling' or Quellung reaction

Neufeld (187) described the 'capsule swelling' reaction in 1902 using the pneumococcal capsule as an example. This phenomenon of apparent 'capsule swelling' by means of specific capsular antisera raised against capsulate organisms has been shown not to rely on a swelling of the capsule. Indeed there is no evidence to suggest any increase in capsule size, as has been shown by Mudd, Heinmets and Anderson (188) with electron microscope studies of the pneumococcal capsule, nor indeed is there an increase in total cell volume after 'capsule swelling' when compared with untreated cells, as has been shown by Johnson and Dennison (189). The apparent 'swelling' as viewed under the light microscope, is caused by a change in the refractive index of the capsule-carrier interface owing to the adherence of antibody to the outer wall of the capsule, thereby making it more easily visible and hence apparently larger.

The Quellung or capsule boundary definition reaction was used to detect the presence or absence of anticapsular antibodies in the rabbit immune serum. The phenomenon has been shown to occur with capsulate organisms other than pneumococci and it seemed reasonable to suppose that capsulate gonococci would behave similarly.

The serum taken from the rabbit 10 days after the last intravenous injection did react well in the Quellung reaction either

when undiluted or when diluted to a titre of 1:32 with rabbit pre-immune serum. The Quellung reaction was found to be equivocal at dilutions of antiserum below 1:32 and it was found that an increase in reaction time tended to increase the positivity of the reaction. It was therefore decided to read all Quellung reactions after a standard reaction time of 30 min. The sera taken from the rabbit 8 and 12 weeks after the second series of subcutaneous injections of purified capsular material failed to react in a Quellung reaction indicating little or no antibody response in the rabbit detectable at that time. The serum taken from the rabbit 16 weeks after the last subcutaneous injection did show reaction in the Quellung reaction both against the strain of N. gonorrhoeae and also the N. meningitidis serogroup B, although the reaction against the meningococcus was not as definite as in the case of the gonococcus. Serum taken from the rabbit 20 weeks after the last subcutaneous injection gave similar results to the serum taken 16 weeks after injection.

Anticapsular antibodies were therefore shown to be present in the sera obtained from both inoculation schedules and an assessment of their capacity to react in a precipitating reaction with capsulate whole organisms in a CIE system was therefore initiated.

4.6.2. Counterimmunoelectrophoresis

Although the results of the Quellung reaction would indicate that there are anti-capsular antibodies present in the antiserum

produced in the rabbit, it should be acknowledged that the antigen-antiserum interaction demonstrated in the CIE procedures described provide insufficient evidence to demonstrate a specific capsule-anticapsular reaction. The interactions detected with CIE may well be directed against any or all of the components of the antigenic mosaic that makes up the outer membrane envelope of the gonococcus and not just against any capsular material that may be present. Such interactions could involve any of the organelles and components situated on the outer membrane which have been described previously and have been used by other workers to investigate gonococcal serology; outer membrane complex, pili, cell wall, lipopolysaccharide, etc.

The essentially negative results experienced with the extraction of capsular material, tend to suggest lability of the capsule-like material and this contrasts with the distinct and easily extractable capsule of sero-groupable meningococci.

It would therefore seem prudent to regard the material associated with the interactions in the CIE as being capsule-like material rather than capsular material per se. This of course would not invalidate the use of the procedure in the diagnosis of gonococcal infection if the material is found to be specific for the gonococcus. The presence of several cellular components in the material, however, would clearly increase the likelihood of cross-reaction taking place.

The rationale behind the use of CIE in this work and the protocol of the experimental procedure involved in the use of CIE

has been described above. In summary, the system of CIE was chosen as the means of detecting capsulate gonococci in exudates because of its reliability, as shown by previous workers with several bacterial species implicated in disease, its simplicity of use and the rapidity of the test procedure, important parameters to be considered in the development of a screening test for bacterial infection. The reliability of the method is not determined by the internal mechanics of the system but is more closely associated with the antiserum used, only those antisera possessing antibodies in sufficiently high titre to the antigens in question giving positive reaction in the system.

The production of a positive result in the CIE depends wholly on the combination of antigen and antibody in a visible precipitating reaction in the agar carrier, and the success of this method in the current work would depend on the production of specific precipitating antibodies by the rabbit directed against the capsular material of the gonococcus. There are however other interactions in a system of precipitating antigen-antibody complexes other than the specific interaction of antigen and antibody, as described by Marrack's 'Lattice Theory' (190), particularly the physical conditions imposed by these detection systems whether it be liquid or semi-solid agar. The precipitation reaction involves two stages in a liquid-based carrier: the first stage is the formation of soluble antigen-antibody complexes, as described in the 'Lattice Theory', while the second stage is the slow conversion

of these complexes into larger visible precipitates. The close binding of these aggregates to each other depends on the formation of ionic bonds between oppositely charged groups, a result of which is an increase in the hydrophobic nature of the complex and hence an increase in their insolubility. The ionic strength of the carrier medium in the CIE system therefore bears directly on the possibility of an antigen-antibody reaction resulting in the formation of a visible precipitate, the lower the salt concentration the less the likelihood of a visible precipitate being formed.

Another factor influencing the likelihood of a precipitating antigen-antibody reaction is the presence of non-precipitating antibodies in the serum. It seems likely that the antibodies produced in the rabbit would be those principally of the IgG class, most rabbits when challenged with most antigens producing IgG antibodies (190). It is known that certain types of IgG antibody are non-precipitating (190) either by reason of their low affinity for their antigenic ligands or else by reason of their physical make-up, perhaps by the possession of large numbers of closely arranged binding sites making them incapable of binding with large antigen molecules. The presence of non-precipitating antibodies is greatest in serum obtained soon after immunization has begun (190); similarly, antisera obtained at increasingly later dates after immunization seem to possess antibody capable of forming increasingly stable antigen-antibody complexes.

There is, however, a major drawback to the use of antisera

obtained at times greatly removed from the start of the immunization programme. The serum obtained after a lengthy challenge procedure tends to be less specific and exhibits more cross-reactions with heterologous antigenic ligands. The reasons for this change in antibody property may be that certain antigenic groupings need longer exposure times to elicit an immune response or that certain antigenic groupings are in such low concentration that a longer challenge period, such as that provided by the use of adjuvant, is necessary to elicit any detectable response using a particular detection system to detect that response (190).

It can be seen from the results, that the rabbit immune serum did indeed produce a line of precipitation in the CIE system when reacted against capsulate gonococci of the strain used to immunise the rabbit. A titration of the immune serum gave a titre of 1:64 in the CIE system, compared with a titre of 1:32 in the Quellung reaction. This difference is possibly of little significance and may simply be within the limits of experimental error; however several factors may explain a difference between titres of antibody-antigen reactions using different surveillance techniques.

It may be thought that the use of whole cells to produce antibody would result in a more reactive antiserum by reason of the potentially large number of antigenic determinants both on the surface of and internal to the cell wall, thereby increasing the likelihood of a precipitating reaction between antigen and antibody.

Although this might result in a 'stronger' precipitation line in CIE owing to the formation of multiple complexes, essentially the titre of the reaction would not be affected because all the antibodies would have been diluted out to the same extent. Although those antigens which caused the formation of anticapsular antibodies may have elicited a weaker immune response than those antigens which caused the formation of anti-somatic or anti-cell wall antibodies, the former antibodies being detected in the Quellung reaction whilst all antibodies directed against bacterially-derived antigens are potentially detectable in the CIE. One would therefore have expected a higher antibody titre in the CIE than in the Quellung reaction. There is, however, evidence that antigen-antibody reactions observed by means of different reaction phenomena, that is, precipitation, agglutination, etc., do differ in their detectability or observability. Precipitation reactions in liquid or gel carriers are generally not visible when dilutions exceeding 50-fold are used, whereas agglutinating reactions involving bacteria may be observed in dilutions approaching 1000-fold and beyond (190). The sensitivity of the Quellung reaction is nearer that of agglutinating rather than precipitating reactions and it would therefore seem more likely that the titre of antiserum in the Quellung would be greater than that in the CIE system, but this was not the case, and the reasons for this are discussed below.

When the rabbit immune serum was reacted against several different strains of capsulate N. gonorrhoeae in the CIE system, a

positive result was obtained. A similar result, however, was observed when the immune serum was reacted against N. meningitidis, serogroups B and C and several non-pathogenic members of the Neisseriaceae. The cross-reactions were almost certainly caused by the use of capsulate whole gonococci possessing antigens derived from cellular components closely related in their antigenicity to components possessed by other related species. Surface antigens are much more likely to elicit an immune response when administered as part of an intact cell, rather than when they are administered in a purified form (190), possibly because, as described above, they tend to persist in the animal and hence are more immunogenic. It can therefore be seen that the very reason for using intact cells, in order to improve the immunogenicity of the capsule by carrying it on a protein-rich intact cell, has resulted in an increase in the immunogenicity of other cross-reacting cellular components.

Cross-reactions in precipitating antigen-antibody systems are almost always caused by the imperfect specificity of the antibodies produced, rather than the presence of identical antigenic determinants possessed by closely related bacterial species (190). The initiation of an antigen-antibody reaction does not require that the antibody possesses a ligand which is a precise fit with the antigenic determinant that initiated its production, but merely requires that the antigenic determinant and its corresponding ligand be capable of binding in fairly close proximity. Cross-reactions are caused therefore by structurally similar though not necessarily

identical determinants binding with an insufficiently specific antibody site. It is indeed found that all antisera produce some cross-reactions even with immunogens whose determinant groups are not precisely duplicated in any other antigen.

It is true that antibodies react more strongly with their initiating immunogens than with cross-reacting antigens, principally because any ligand will only react with a part of the total immunogen present and also the antibody will bind preferentially with the specific initiating ligand rather than with any cross-reacting ligands which may be present. Similarly antigen-antibody complexes formed between dissimilar groupings will tend to be less stable than those between related complexes and would tend to dissociate more easily.

Fewer cross-reactions are exhibited by low-affinity antibodies than by high-affinity antibodies (190). Polysaccharide antigens and their respective antibodies are known to be low-affinity systems and assuming that the gonococcal capsule is polysaccharide in nature, then one would expect that the system used in this work would show a similar lack of cross-reactions. The presence of proteinacous cellular components owing to the use of whole cells, however, has almost certainly resulted in the production of relatively non-specific anti-protein antibodies. If anti-protein antibodies were as specific as anti-polysaccharide antibodies then the cross-reactions would almost certainly have been restricted to the closely related meningococcal species.

Protein antibodies, however, are relatively unspecific owing to the three-dimentional confirmation of the molecule and it is here that cross-reactions owing to similarity rather than identity are important. We would therefore expect antibodies to protein components of the cell to cross-react with similar though not identical protein components from other closely related species.

The result of the Quellung reaction with N. gonorrhoeae strain G.C.B. and N. meningitidis serogroup B using the rabbit immune serum raised to the purified gonococcal capsular material, although displaying some cross-reactivity indicated some specific activity against the gonococcal capsule by reason of the more definite improvement of capsule boundary definition. The CIE reaction between the immune serum and the two species however was disappointing in that the strength of reaction appeared to be similar for both species and also the reaction time had to be increased considerably to elicit any visible reaction at all.

Cross-reactions between the neisseriae have been noticed by previous workers in the field of gonococcal and meningococcal serology and account for the greater part of the failure of serological techniques in the diagnosis of neisserial and particularly gonococcal infection. Most of the studies of cross-reactions between the neisseriae have centred around the close relationship between gonococci and meningococci. Tramont, Sadoff and Artenstein (191) found cross-reactivity between meningococcal serogroups A, B and C and four strains of gonococci, whilst Gotschlich et al. (174) found

that rabbits immunized with meningococci serogroup B produced antisera which cross-reacted with meningococci groups A and C. Danielsson (192) reported cross-reactions between gonococci and 'apathogenic' neisseriae, as well as with meningococci, and Danielsson et al. (178) using purified gonococcal, meningococcal, N. flava and N. sicca 'protoplasm' antigens prepared by gel filtration and ion-exchange chromatography found that they cross-reacted with hyperimmune rabbit serum prepared against gonococci. Esquenazi and Streitfeld (179) using an agar-gel diffusion system with antisera produced in rabbits against a strain of N. gonorrhoeae found cross-reactivity against N. catarrhalis. Danielsson (192), using immunofluorescent and Ouchterlony gel diffusion techniques, indicated that most of the cross-reacting antigens were of intracellular origin and this would correlate with the findings of Maeland (193) who found that a protein determinant obtained from three strains of gonococci did not differ serologically between each strain and he thought this to be a group-reactive antigen common to both N. gonorrhoeae and N. meningitidis.

More disturbing than cross-reactions between closely related bacterial species, several workers have reported cross-reactions between neisseriae and dissimilar species of bacteria. Robbins et al. (194) have reported cross-reactions between meningococcal group C polysaccharide and an Escherichia coli capsular polysaccharide, between another E. coli- derived antigen and a meningococcal group B antigen, and also between antigens

derived from meningococci group A and a Bacillus pumilus. Cross-reactions between dissimilar species, particularly species likely to be found in or near the genital tract as normal flora would clearly pose problems for the reliable screening of gonococcal infections by serological techniques.

Cross-reacting antibodies to other bacterial species are normally removed from antisera by either adsorption or absorption techniques. Absorption involves allowing the cross-reacting antiserum to react with the appropriate cross-reacting species and then removing the bacterial-antibody complexes by centrifugation. Adsorption involves the introduction to the antiserum of soluble cross-reacting ligands to such an excess that the cross-reacting antibodies are saturated and although still present in the system take no further part in the formation of complexes. Because of its simplicity it was decided to use an absorption technique and basically the methods of Peacock (95) were followed.

The removal of cross-reacting antibodies using bovine bone marrow, Branhamella catarrhalis, Neisseria subflava and Neisseria sicca as absorption vehicles was reasonably successful in that the resulting absorbed serum failed to react with these three bacterial species whilst retaining its activity against the gonococcus. The absorbed serum, however, also continued to react in the CIE with the two meningococcal strains and this again illustrates the close relationship between the gonococcus and meningococcus. This is more vividly illustrated by the failure of

the serum to react with either the gonococcus or either of the two meningococcal strains after its absorption with the meningococcus group B. Several factors may explain this finding:

i. The strain of N. meningitidis group B used for the absorption may possess identical antigenic determinants to those determinants possessed by the N. gonorrhoeae strain used to elicit the immune response in the rabbit

ii. The antigenic determinants possessed by the gonococcus may have been absorbed out of the antiserum piecemeal by the previous absorption procedures leaving only those determinants common to the gonococcus and the meningococcus group B which were subsequently removed by absorption with the meningococcus

iii. The reacting antibodies in the system may have been directed against cell wall and internal antigens of the cell, which, having been absorbed from the system, left only anticapsular antibodies in the serum which may have combined with antigen in a non-precipitating reaction.

iv. The antiserum may have been so weak in antibodies reactive against components possessed exclusively by the gonococcus that absorption of all the commonly reactive antibodies left an antiserum incapable of forming a visible precipitate in the CIE system.

The 'strength' of the antiserum may have been affected by several factors:

i. The capsule on the gonococcus may not be a very good immunogen, and although a positive Quellung reaction would suggest that anticapsular antibodies were present in the serum, it may be, as has been discussed previously, that the Quellung is so much more sensitive than the CIE that the anticapsular antibodies were capable of giving a positive Quellung reaction but not a positive precipitation reaction in the CIE

ii. The inoculation schedule may have been inappropriate for the capsular antigens to produce an immune response

iii. In the case of the purified capsular material, it may be that very little capsular material was present in the material used for injection. Richardson and Sadoff (131) have shown how mechanically unstable the capsules are and it may be that the mechanical agitation and chemical extraction procedure destroyed a significant proportion of the capsular material leaving a minor portion which gave the very weak immune response seen in the CIE.

Peacock (95), in his work on preparing a N. gonorrhoeae fluorescent antibody conjugate, suggested that the most critical step in its preparation was the absorption of the antiserum with the

meningococci. He noticed that any increase in either the amount of meningococci used for absorption or the time the reaction was allowed to proceed resulted in a significant loss of reactivity from the system; subsequently he used only small amounts of absorbing antigens. His rationale behind using a technique which did not remove all cross-reacting antibodies was that, from a practical viewpoint, this was not necessary and that the fluorescent antibody technique served only as a screening procedure. Indeed he even suggested that the absorption with the meningococcus could be omitted altogether. The aim of the present study, however, was to provide a simple, rapid yet reliable system for the diagnosis of gonorrhoea and clearly the presence of cross-reacting antibodies in the serum would be counter-productive to this aim.

The aim of this present study was to investigate the production of a capsule by N. gonorrhoeae and to assess the possibility of using capsulate gonococci or purified capsular material to raise an anticapsular antiserum in rabbits and the use of that antiserum to detect capsulate gonococci in exudates, by means of a counterimmunoelectrophoresis system. The results would appear, at first sight, to be discouraging because of the pronounced cross-reactivity of the antiserum produced, both using whole organisms and purified capsular material.

The gonococcal capsular material will, however, have to be investigated more fully and its nature identified by biochemical analysis and compared with the capsular material from other species. The close relationship between the capsular antigens of the neisseriae might be investigated by raising antisera in rabbits to purified capsular material from each of several strains of each species and then by comparing the antigens in a system of intermediate gel electrophoresis whereby lines of identity between each capsular extract can be identified when each extract is reacted against each antiserum.

The components of each extract could then be separated using column chromatography and those components exclusive to the gonococcal capsule used to raise immune serum in a rabbit for use in a more specific CIE technique.

The isolation of a purified gonococcal capsular extract and subsequent raising of antiserum to the capsule has implications not

only for use as a diagnostic procedure. Interest in the use of vaccines to combat bacterial disease, particularly those infections caused by capsulate bacteria, has increased over the last decade, although appreciation of their efficacy has been apparent for many years. McLeod et al. (195) and Anderson et al. (196) showed that injection of capsular polysaccharide prevented pneumococcal pneumonia whilst Schneerson et al. (197) showed that immunization of humans with the capsular antigen of Haemophilus influenzae type b provided immunity to disease caused by that organism. Although most of the recent work on vaccine production has centred around the use of meningococcal and pneumococcal vaccines, investigation into the possible use of gonococcal vaccines has been in progress.

Greenberg (198), using autolysed, unpurified preparations containing three Kellogg type 1 strains, has reported that over 90% of the subjects to which it was administered developed an increased titre of antibody to the gonococcus; unfortunately he did not specify whether the increase in antibody titre was mirrored by a significant decrease in the incidence of gonorrhoea among those vaccinated. Indeed Greenberg (199), in a previous paper, noticed a similar increase in antibody titre which failed to protect the recipients against subsequent challenge with gonococci. Caspar (154) suggests that the use of whole or autolysed gonococci in vaccines is merely an attempt to elicit a response to non-specific proteins of the cell and that a vaccine prepared from some specific cell

component, if possible a purified carbohydrate component, would give greater protection, as has been found with purified meningococcal capsule vaccines.

Buchanan et al. (200) prepared vaccines using the outer membrane complex (OMC) and also purified pili from gonococci. They found that chimpanzees vaccinated with the OMC were better protected than when vaccinated with the purified pili, suggesting that the antigens which induced protection against infection were located in the outer membrane. Subsequent work, however, has shown that the protective effect of OMC is strain-specific and its use as a vaccine would rely on the capacity to produce a vaccine containing antigens from the most commonly occurring strains. The use of a vaccine conferring only partial immunity, by preventing overt infection, while allowing the carriage of viable organisms would be extremely unwise. As with the OMC vaccine studies, the feasibility of using capsular vaccines would depend on the number of strain-specific capsular antigens found and further work on the isolation, identification and characterization of gonococcal capsules and capsular types will have to be carried out.

The use of a vaccine in the prophylaxis of gonococcal infection is not without its problems, one of the major drawbacks being that if a successful vaccine is prepared then who should receive it. The disease itself does not confer long-lasting immunity (201) possibly because of the nature of the infection process, being one principally involved with the mucous membrane

surfaces and it may be that vaccination gives better protection than previous infection, as is the case with tetanus, pertussis and smallpox, where infection does not necessarily confer immunity. Although it may be that the gonococcus does not have the capacity to elicit a long-standing immune response and the production of a useful vaccine may be thwarted, it seems, from the experience of workers in the field of vaccine productions, that a vaccine prepared from the capsule may be the most profitable line of investigation.

There are therefore two main lines of investigation to follow. The first involves the full characterization of the gonococcal capsule and its use as a diagnostic tool and the second involves the assessment of the capsule to provide a prophylactic vaccine in the prevention of gonococcal disease.

PLATES

Plate 1

Capsulate gonococci from a primary isolation culture shown under India ink stain

Microscope Magnification x 1000

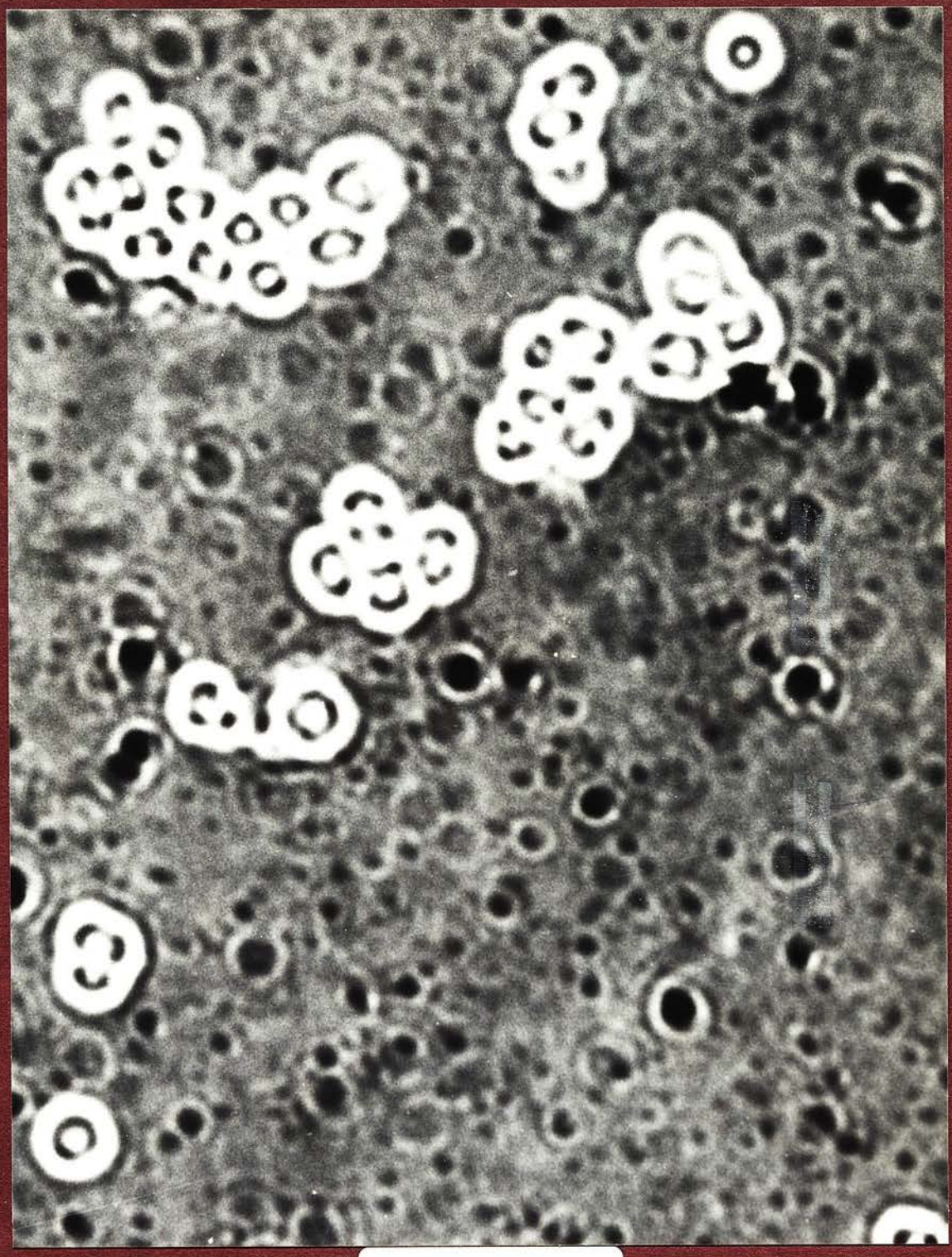


PLATE ONE

Plate 2

A single capsulate gonococcus in an exudate shown by India ink stain

Microscope Magnification x 1000

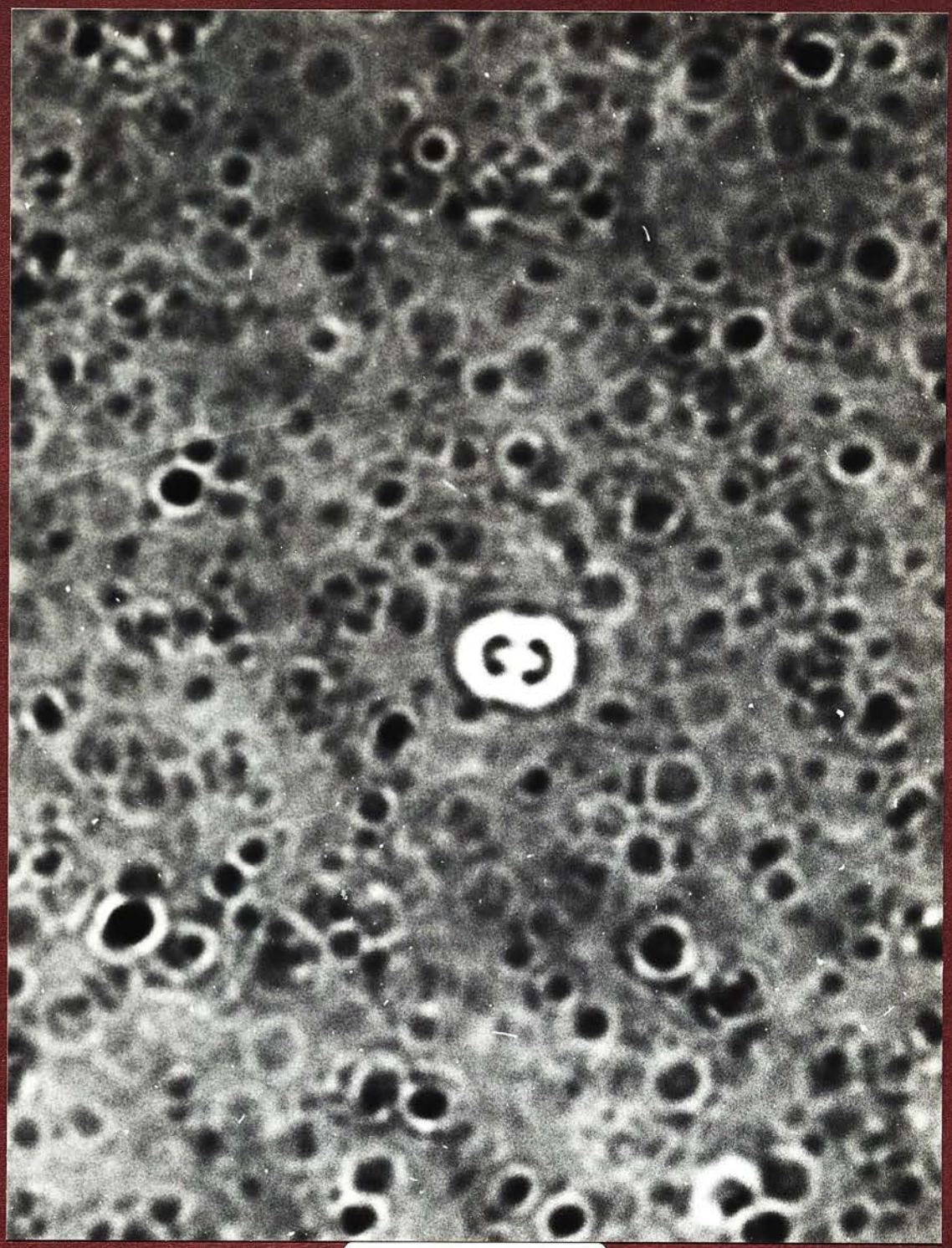


PLATE TWO

Plate 3

Top row:- (left to right)

1 - 3 Rabbit immune serum

4 Rabbit pre-immune serum

Bottom row:- (left to right)

1 - 4 Capsulate gonococci suspension

Plate 4

Top row:- (left to right)

1 Diluted rabbit immune serum titre 1 : 32

2 Diluted rabbit immune serum titre 1 : 64

3 Diluted rabbit immune serum titre 1 : 128

4 Diluted rabbit immune serum titre 1 : 256

5 Rabbit pre-immune serum

Bottom row:- (left to right)

1 - 5 Capsulate gonococci suspension

PLATE THREE

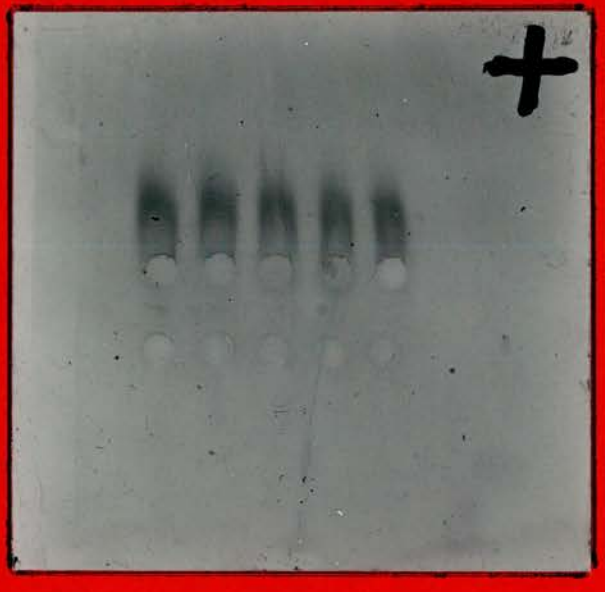


PLATE FOUR

Plate 5 (top)

Top row:- (left to right)

1 - 6 Rabbit immune serum

Bottom row:- (left to right)

1 - 6 6 strains of capsulate gonococci suspension

Plate 5 (Bottom)

Top row:- (left to right)

1 - 5 Rabbit immune serum

6 Rabbit pre-immune serum

Bottom row:- (Left to right)

1 - 5 5 strains of capsulate gonococci suspension

6 Capsulate N. gonorrhoeae strain G.C.A. suspension

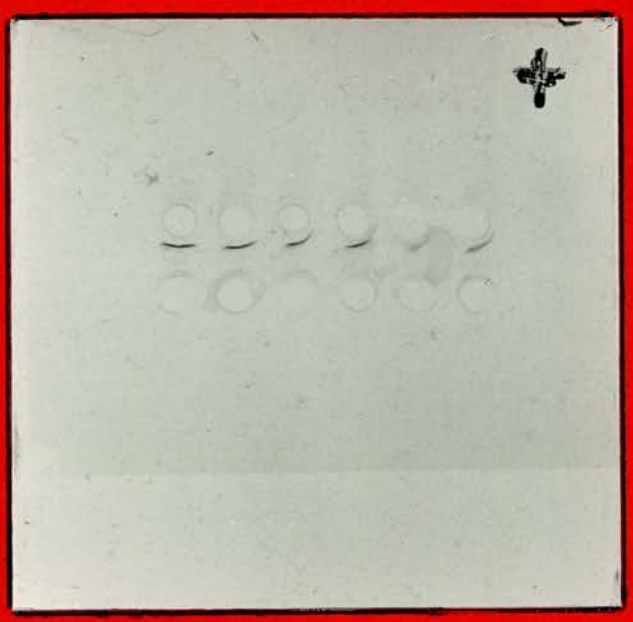


PLATE FIVE
(top)

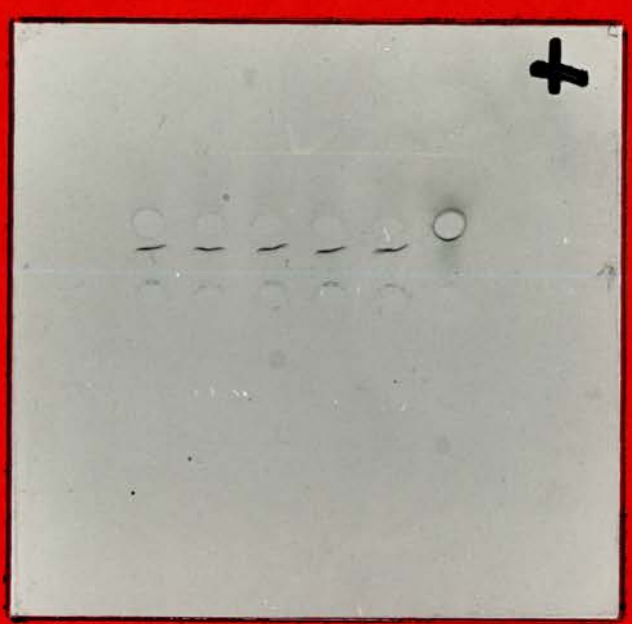


PLATE FIVE
(bottom)

Plate 6

Top row:- (left to right)

1 - 6	Rabbit immune serum
7	Rabbit pre-immune serum

Bottom row:- (left to right)

1	Capsulate *N. meningitidis* group B suspension
2	Capsulate *N. meningitidis* group C suspension
3	Capsulate *B. catarrhalis* suspension
4	Capsulate *N. subflava* suspension
5	Capsulate *N. sicca* suspension
6,7	Capsulate *N. gonorrhoeae* strain G.C.A. suspension

Plate 7

Top row:- (left to right)

1 - 6	Rabbit immune serum absorbed with bovine bone marrow
7	Rabbit pre-immune serum

Bottom row:- (left to right)

as above

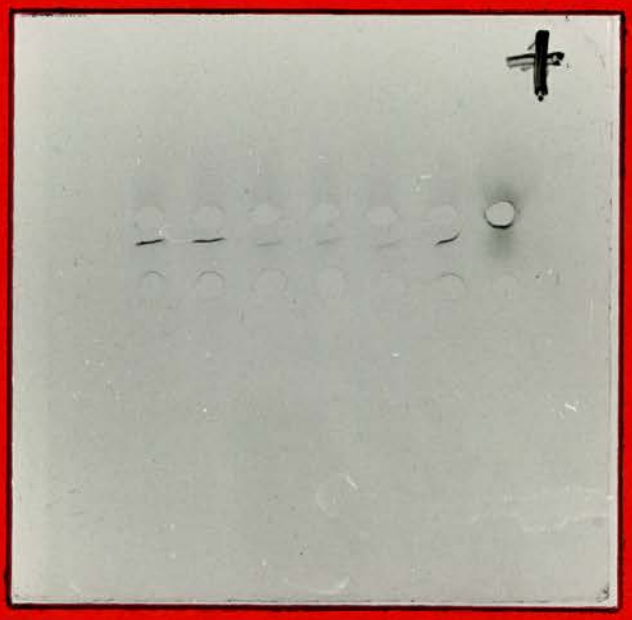


PLATE SIX

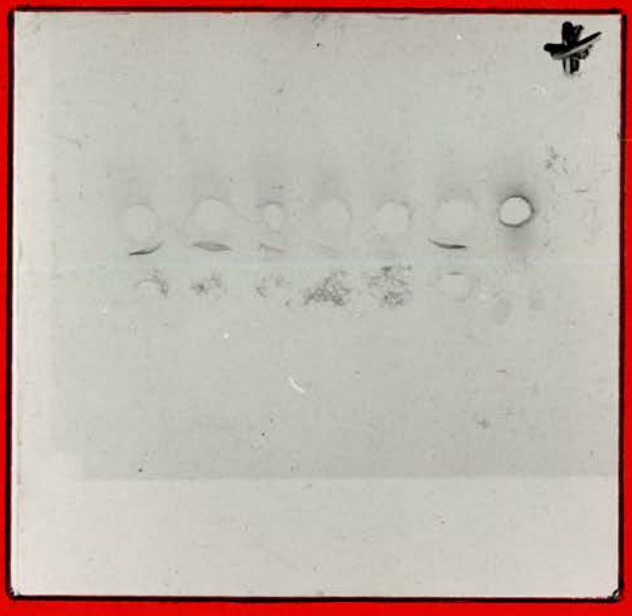


PLATE SEVEN

Plate 8

Top row:- (left to right)

1 - 5	Rabbit immune serum absorbed with bovine bone marrow, *N. sicca*, *N. subflava*, *B. catarrhalis*
6	Unabsorbed rabbit immune serum

Bottom row:- (left to right)

1	Capsulate *N. meningitidis* group B suspension
2	Capsulate *N. meningitidis* group C suspension
3	Capsulate *B. catarrhalis* suspension
4	Capsulate *N. subflava* suspension
5	Capsulate *N. sicca* suspension
6	Capsulate *N. gonorrhoeae* strain G.C.A. suspension

PLATE EIGHT

Plate 9

Top row:- (left to right)

1 - 3	Rabbit immune serum absorbed with bovine bone marrow, *N. sicca*, *N. subflava*, *B. catarrhalis* and *N. meningitidis* group B
4	Unabsorbed rabbit immune serum

Bottom row:- (left to right)

1	Capsulate *N. meningitidis* group B suspension
2	Capsulate *N. meningitidis* group C suspension
3,4	Capsulate *N. gonorrhoeae* strain G.C.A. suspension

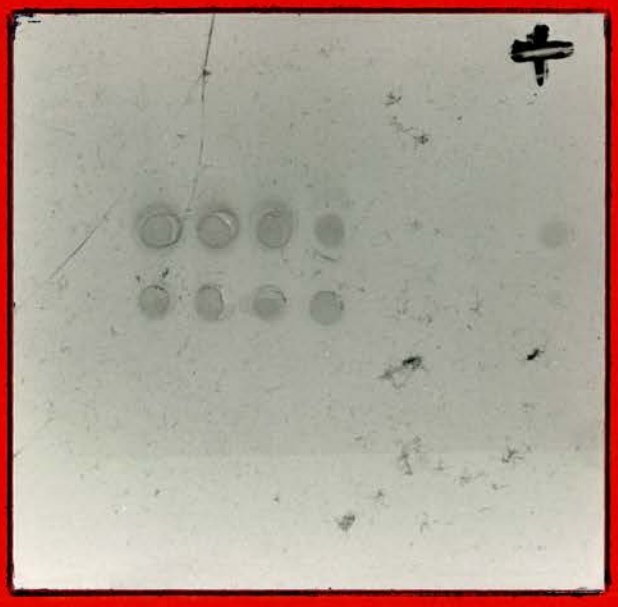
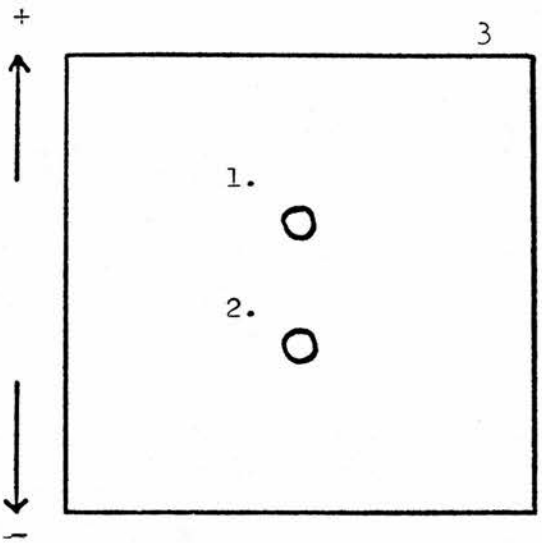
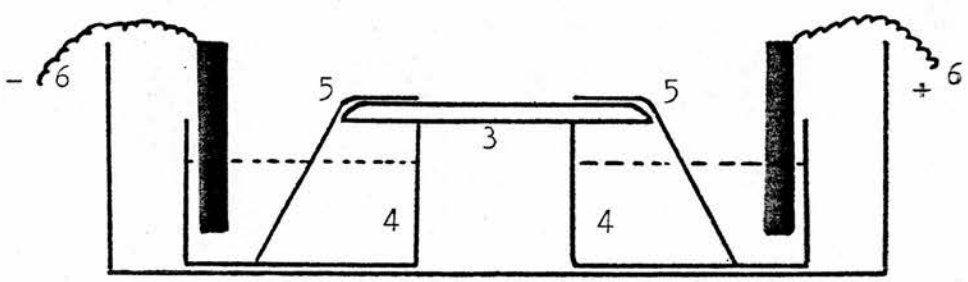


PLATE NINE

FIGURES

FIGURE 1





TABLES

TABLE 1

ISOLATE	RATIO CAPS : NON-CAPS	ISOLATE	RATIO CAPS : NON-CAPS	ISOLATE	RATIO CAPS : NON-CAPS
1. GENITAL	1 : 865	21. GENITAL	1 : 241	41. THROAT	1 : 80
2. "	1 : 903	22. "	1 : 112	42. "	1 : 126
3. "	1 : 245	23. "	1 : 642	43. "	1 : 43
4. "	1 : 567	24. "	1 : 250	44. "	1 : 8
5. "	1 : 417	25. "	1 : 529	45. "	1 : 64
6. "	1 : 935	26. "	1 : 340	46. "	1 : 26
7. "	1 : 819	27. "	1 : 361	47. "	1 : 21
8. "	1 : 312	28. "	1 : 712	48. "	1 : 11
9. "	1 : 133	29. "	1 : 961	49. "	1 : 19
10. "	1 : 530	30. "	1 : 783	50. "	1 : 25
11. "	1 : 713	31. "	1 : 271		
12. "	1 : 601	32. "	1 : 311		
13. "	1 : 473	33. "	1 : 376		
14. "	1 : 182	34. "	1 : 581		
15. "	1 : 419	35. "	1 : 1007		
16. "	1 : 987	36. "	1 : 727		
17. "	1 : 153	37. "	1 : 198		
18. "	1 : 1106	38. "	1 : 658		
19. "	1 : 863	39. "	1 : 531		
20. "	1 : 381	40. "	1 : 842		

TABLE 2

Media No.	Weight in g. in 10 ml.		Ratio
	Glucose	Peptone	Glucose : Peptone
1	0.5	1.5	1 : 3
2	0.54	1.5	1 : 2.8
3	0.58	1.5	1 : 2.6
4	0.63	1.5	1 : 2.4
5	0.68	1.5	1 : 2.2
6	0.75	1.5	1 : 2.0
7	0.83	1.5	1 : 1.8
8	0.94	1.5	1 : 1.6
9	1.07	1.5	1 : 1.4
10	1.25	1.5	1 : 1.2
11	0.5	1.5	1 : 3
12	0.5	1.4	1 : 2.8
13	0.5	1.3	1 : 2.6
14	0.5	1.2	1 : 2.4
15	0.5	1.1	1 : 2.2
16	0.5	1.0	1 : 2.0
17	0.5	0.9	1 : 1.8
18	0.5	0.8	1 : 1.6
19	0.5	0.7	1 : 1.4
20	0.5	0.6	1 : 1.2

TABLE 3.

Volume in ml G.C.A. suspension (10^9 cells ml^{-1})	Rabbit anti-sera dilution	Volume in ml anti-sera dilution	Enhanced capsule definition
0.1	NEAT	0.1	+
"	1 : 1	"	+
"	1 : 3	"	+
"	1 : 7	"	+
"	1 : 15	"	+
"	1 : 31	"	+
"	1 : 63	"	±
"	1 : 127	"	-
"	1 : 255	"	-
"	NEAT	0.1 ml pre-immune	-

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