SUMMARY

The aim of this study was to carry out a serological analysis of the human Neisseria with a view to identifying specific gonococcal antigens of potential value in serodiagnosis. For a rational approach to this highly desirable goal the basic serological information is essential, yet few systematic comprehensive investigations have been undertaken. The Neisseria have generally been approached from a narrow view, the subjects for investigations being compartmentalised into either the meningococci or the gonococci or certain gonococcal cell components.

Important features of the present investigation were aspects of planning and technique for selection of cultures for preparing not only reagent antigens but also antisera. It was attempted to achieve (1) species representation by taking type strains of the human Neisseria species (2) gonococcal representation by selecting different antibiotic resistant types (3) antigenically "complete" cells by culture of the gonococcus in the virulent form. In the absence of infrastructure, appropriate methods had to be adapted or developed for our conditions starting from sampling of the patients up to running gradient pore electrophoresis. As a result, useful
Methodologies have been evolved and new observations made in several areas. The work is described in four sections:

(Sec. 2) Investigations related to characterization of culture, (Sec. 3) Optimisation and stabilization of virulent gonococci, (Sec. 4A) analytical serology of Neisseriaceae (whole cell antigen) and (Sec. 4B) analytical serology of gonococcal outer membrane proteins.

Investigations related to standardisation of culture media, preservation of strains, bacteriological diagnosis of patients and characterization of strains, were useful and informative. 1) Rabbit blood supplemented media were found satisfactory for routine culture, antibiotic sensitivity assay and surface preservation of Neisseria cultures. In paraffin layered chocolate slant cultures of gonococci, viability averaged 3 months and increased to 5 months at a lower storage temperature (30°C). The method of "chocolating" was found to affect viability, prolonged heating being advantageous. This may be due to the denaturation of heat-sensitive inhibitors known to occur in whole blood. For preservation in the long term lyophilisation in horse serum containing 5% inositol was found most satisfactory for all Neisseria including virulence type lines of gonococci.
Biochemically the Neisseria were easily distinguished and clear-cut results were obtained by the rapid carbohydrate utilisation test of Young et al. (1976) which assays preformed enzyme. Good growth of gonococci both from stock cultures and from clinical specimens was obtained on the transparent media of Kellogg et al. (1963, 1968). It was used routinely for culture from patients. In laboratory formulated semidefined transparent Oxoid and Difco GC agar media with the Kellogg supplement, we got good growth of gonococci and were able to distinguish four different morphological colony type, a technique which is basic for much of the today's research.

The methods used for bacteriological diagnosis of 170 patients from a local STD clinic were satisfactory. Over 95% male cases and an estimated 80% of female cases of gonorrhoeal infection could be detected in a single culture (Gupta et al., 1982). My results therefore compared favourably with published data.

In the absence of practicable typing techniques for characterization of gonococcal isolates, I developed a modified diffusion assay for multiple antibiotic sensitivity screening (Saikh & Bhattacharyya 1980). It was simple, rapid
and self-compensatory for plating variables it being internally controlled. The method was validated clinically by comparison of results of sensitivities with patients responses to therapy (Gupta et al., 1982). Apart from obtaining a typing method which was reproducible, I also obtained new information on the extent and kind of resistance seen in this region. The incidence of resistance amongst the gonococci isolated was found to be much the highest reported in the country. Two distinct populations of gonococci (non β-lactamase producing) were evident which appeared to be endemic in Calcutta (Gupta et al., 1982). One was characterized by "erythromycin" resistance (33%), the other by "ampicillin" (25%) in addition to their associated multiple resistances. The 'erythromycin' type of strain resembled the New World isolate of Cannon et al. (1980) and appeared therefore to be widespread. The "ampicillin" type of isolate has not so far been described. I found antibiotic resistance markers phenotypically stable and a reliable means of distinguishing gonococcal strains. Clinical evidence of the epidemiological validity of antibiotyping was apparent. The soundness of this approach from a serological point of view has also very recently been established (Bygdeman 1981).
The gonococcus is highly prone to cellular changes in culture which are manifest in colony appearance. It is only since a clear association with loss of infectivity was demonstrated (Kellogg et al., 1963; 1968) that the significance of the phenomenon to our understanding of gonococcal immunobiology was realised. To obtain meaningful information in this investigation, it was considered necessary to use antigenically "complete" colony type-selected cultures. Using the typing media of Kellogg et al. (1963; 1968) and the international typing strain F62, the colony typing technique of Juni and Heym (1977) could be successfully implemented from the published description. The validity of the technique itself has been established by genetic transformation experiments (Juni & Heym 1980) and I found it to be simple and accurate and applicable to two typing (Oxoid & Difco) by cross-plating and statistical assessment. The segregation pattern of type lines of 7 gonococci were followed on successive transfers from the primary isolation. Segregation from the virulent types could not be eliminated; the rate was seen to reduce on repeated selective transfer, but was media and strain dependent. Preservation of established type lines was achieved by lyophilisation but not in paraffin slants. Initially good type stabilisation was obtained on
the Oxoid media (OKS) which contrasted greatly with the high segregation seen on the Difco media (DKS). However, the position was reversed on changing to second lots of the two bases.

To the extent observed this result was surprising because variability in these typing media has not been reported. It was also a problem in view of our media procurement difficulties and because reproducibility of antigen preparations was compromised. At the same time the phenomenon was of fundamental interest because it implied a role for nutrition in the expression of virulence phenotype which may also operate in vivo e.g. in the suppression of fimbriation. Thus, an investigation of the factors involved was undertaken. Most striking was the variability seen in type stability in 9 batches of both OKS and DKS media which was independent of plating efficiency. Of the four main components of the media, the make of starch or the composition of supplement did not have any effect on the colony type character. Quality of agar and batch of peptone were the main determinants of media performance. The agars differed in content of calcium and magnesium. In OKS for example, calcium and magnesium contents were 2 mM and 0.44 mM respectively which contrast with the low calcium high magnesium concentrations recommended for defined media.
formula for the gonococcus (Lascolea & Young 1974; Manchee et al., 1980). Thus it is likely that disproportionate of limiting conditions for divalent cations provided largely by the agar, influence phenotype through cell wall modification. Supplementation of media, particularly OKS with Mg$^{2+}$ increased viable counts about 30% and also colony size, but had no effect on colony type stability.

Divalent cations provided by the agar were therefore not the sole contributory factors. The peptone itself had an important role. The variability of peptone batch has been demonstrated in both bases (Table 38.1 and 38.2). Peptones are the sources of amino acids for several of which gonococcal auxotypes are known to have absolute requirements for growth. The influence of 14 amino acids on colony type were investigated in two strains by supplementation of the two media. They were chosen on the basis of known importance in defined formula and significant differences in the levels of the two media. Their effects were complex and could be broadly classified in this way:

1) No effect (Leucine & Glutamine)
2) Growth promoting (tyrosine & isoleucine)
3) Lethal (-SH containing amino acids)
4) Type segregation (arginine, lysine, serine)
5) Type segregation at low concentration and lethal at high concentration (glycine, alanine, valine, aspartic acid).

6) Growth promotion and virulence type reversion (proline)

Their effects were strain as well as base dependent. Base differences in response to amino acids (e.g. glycine, arginine, proline) depended on their initial content in the two peptones, thus supporting the view that it is the amino acids themselves rather than polypeptides which influence colony phenotype. SH-containing amino acids were highly lethal, probably because of the generation of hydrogen peroxide on interaction with other amino acids as has recently been shown by Narrod and Morse (1982).

Three other amino acids had pronounced effects and were studied in greater detail. Glycine at low levels had the effect of increasing segregation to the avirulent colony type. Beyond a total molarity of about 20 mM it was toxic and gave rise to aberrant cryptic colonies similar to those seen in one batch of OKS. Virulent colony types appeared to be significantly more sensitive than avirulent types to glycine toxicity. The remarkable sensitivity of the gonococcus to glycine is possibly due to interference in cell wall synthesis and may be a major reason for general media toxicity. Gonococcal peptidoglycan fractions with a low degree of cross linking had a high glycine content (Rosenthal et al., 1980).
Arginine caused loss of the virulent phenotype in direct proportion to concentration in all 3 gonococci investigated. The second amino acid seen to affect colony type specifically was proline. It caused a pronounced type reversion peak in strain F62 but not in N133 when added particularly to DKS. This strain-dependant response was probably due to auxotrophy for this amino acid in strain F62, the genes for which are linked to those for membrane synthesis (Cannon et al., 1981).

These in vitro observations of a definite role of amino acid nutrition in virulence phenotype may be relevant to events in vivo. As in pathogenic Escherichia coli (reviewed by Beachey 1980; Watt 1980), the infective gonococcus is invariably fimbriate (Kellogg et al., 1963, 1968; Walsh et al., 1963; Sparling & Vobs 1967; Lucas et al., 1971), yet once the infection is established it becomes non-fimbriate (Novotny & Cowley 1978; Novotny et al., 1975). This conversion may be brought about nutritionally by progressive changes in the micro-environment of the tissues. It is known for instance that the urethral epithelial cells sit on a basement through which the gonococci penetrate into the submucosa. This membrane is a glycoprotein containing substantial amounts of hydroxy-proline, hydroxy-lysine and
glycine (Watt & Ward 1977). It follows from my results that high local availability of these amino acids at this stage would influence cell phenotype. Support for this view comes from the recent demonstration that in *E. coli* fimbriation is alanine dependent (Girardeau *et al.*, 1982). My findings in this respect add another dimension for further investigation of the gonococcus and indicate the way for improved media design.

The fourth section of this thesis describes serological investigations by micro double diffusion analysis. Firstly a systematic survey of sonicate antigens of all *Neisseria* was undertaken. Sonicates as antigens comprise mainly soluble cytoplasmic components. This was followed by analysis of cross-reactivity in purified membrane antigens of gonococci.

The initial problem encountered in sonicate analysis of gonococci was the extreme variability of rabbit immune-response. A gonococcal reference serum to strain N133 was constructed by judicious pooling of rabbit antisera to achieve maximal precipitin pattern complexity. Five precipitin zones were produced by this serum, all of which consisted of multiple components. No individual rabbit responded comprehensively to N133 antigens and 2 of 5 totally failed to produce antibody for three of the five precipitin zones. This variability must inevitably generate anomaly in interpretation.
and reproducibility. It was therefore encouraging to find considerable agreement between the results of this study and published work.

The distribution of components of the 5 reference precipitin zones in eleven representative gonococci, four meningococci and five Neisseria species was examined by both direct comparison and sonicate absorption analysis. Cell location of antigens as surface or internal was determined by absorption with intact gonococci. Of the 5 precipitin zones 1 to 4 were internal cell antigens and corresponded to zones A to C of Danielsson (1965). Slow moving zone 5 found adjacent to the antigen well was surface located and corresponded to Danielsson's band D and also the serotype precipitins of Geizer (1975) and Johnston et al. (1976). A similar precipitin complex exists in the meningococci which has recently been developed for their serotyping (Ashton et al., 1980).

In agreement with Danielsson (1965a) extensive cross-reactivity throughout the Neisseria was found in the internally located precipitins. Unlike him however, I found gonococcal-meninogococcal cross-reactivity in zone 5. Several other important features of Neisseria serology not evident in his very similar study were revealed in this analysis.
This was certainly due to the reactivity of the reference antiserum, the colony type controlled reagent antigens as well as methodological differences such as the absorption technique used. Antigens with specificities at several levels of generic organisation were detected. Precipitins 1 and 3 can be said to be genetic antigens because they were common to all "true" Neisseria. N. catarrhalis, the only non-reactor, is now classified with the Branhamella on the basis of established taxonomic criteria e.g. guanine-cytosine base ratios. Precipitin 4 was present in all the human Neisseria with recognised pathogenic potential i.e. N. gonorrhoeae, N. meningitidis and N. flavescens. Further investigation of this novel component should prove of great fundamental interest. Cross-reactivity in zone 5 was restricted to meningococci and gonococci. Since this activity is surface located, a study of its role in specificity of the host cell interaction in these species should be fruitful.

Specificity at the sub-specific level was found in two precipitin zones. Serotype specific determinants in the cell surface complex in zone 5 was to be expected from detailed investigations of the major OM proteins of gonococci (Geizer 1975; Johnston et al., 1976). A second serotype antigen was identified amongst internal antigens, precipitin 2, which has not so far been described. Preliminary evidence of its distribution in Calcutta and international strains
indicated epidemiological significance which is worthy of further investigation. The obstacle to progress here will be erratic rabbit response to this component which however it may be possible to overcome by using the hen which is known to respond well to other gonococcal antigens (Wallace et al., 1978). This survey has thus opened up several potential areas for further research, of both fundamental and applied interest.

Among the outer membrane (OM) components of *N. gonorrhoeae* LPS has proved to be highly cross-reactive with other *Neisseria* and fimbrial preparations are still undergoing assessment. Therefore, I have concentrated upon OM proteins. The most abundant, protein 1, accounts for about 60% of the total protein mass of the OM. This protein contributes to great serological diversity in gonococci (Johnston et al., 1976) and thus is of little potential value for serodiagnosis or as a vaccine.

Most of the other proteins were present in much smaller amounts and have not been fully investigated. Recently Newhall et al., (1980) biochemically detected a high molecular weight protein in low strength polyacrylamide gel electrophoresis. It was found in all the gonococci they tested irrespective of virulence type, but not in other *Neisseria*. Using the basic methodology of Newhall et al., (1980) gonococcal OM preparations were made from two strains and fractionated on sepharose 4B columns. Of the four elution peaks, fraction B was found by gel
filtration to correspond to the high molecular weight protein of Newhall et al. in elution characteristics and PAGE electrophoresis. Strain N123 produced relatively large amounts of protein B (about 20%) of total OM protein. In agreement with Newhall et al. I found peak B to contain a single protein high molecular weight equivalent to IgM in SDS-PAGE.

All the four fractions peaks were examined in immunodiffusion for reactivity with antisera to gonococci and all other Neisseria species. Protein B was reacted with human sera from a wide spectrum of gonorrhoeal infections control subjects. Fraction B was of interest from the immunological point of view. It was present in all 9 gonococcal cell lysates tested by direct comparison in ID. Moreover hyper-immune rabbit antigonococcal antisera to 5 of 6 representative gonococcal strains contained antibody to this fraction. The exception was reference antiserum of NL33. It was of interest to note that the serum from patient NL33 reacted with fraction B. Eight of 16 sera from proved gonococcal patients but none of six controls reacted with fraction B.

The function of such a large protein in the OM apparently present in only the gonococci, is an intriguing question. Newhall's group recognised that it was a unique
gonococcal species protein. I have demonstrated that it is a gonococcal species antigen and that a human antibody response to it occurs in gonorrhea. This sets the stage for further investigation of this protein and its exploitation in sero-diagnosis.