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STUDIES ON PNEUMOCOCCI ISOLATED FROM CHILDREN IN THE SHEFFIELD AREA.

BY

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A THESIS SUBMITTED TO CNAA

IN PARTIAL FULFILMENT

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

RESEARCH CONDUCTED AT SHEFFIELD CITY POLYTECHNIC IN COLLABORATION WITH THE DEPARTMENT OF BACTERIOLOGY, SHEFFIELD CHILDREN'S HOSPITAL.

SEPTEMBER 1984

ADVANCED STUDIES AND CONFERENCES UNDERTAKEN AND ATTENDED IN CONNECTION WITH THE PROGRAMME OF RESEARCH IN PARTIAL FULFILMENT OF THE REQUIREMENTS OF THE DEGREE OF Ph.D.

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2. 30th July - 6th August 1977.
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3. 7th November 1977.
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4. May 1978 and June 1979.
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5. 10th December 1979.
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6. 22nd-24th September 1980.
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7. 1981-84.
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8. 6th May 1983.

   Attended a meeting of the Shunt Infection Group, Newcastle Royal Infirmary.

9. 13th-16th July 1983.

   Attended the Conference of the Association for Research into Spina Bifida and Hydrocephalus, Southampton University.

10. 3rd-10th September 1983.

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    Attended the weekly Research Seminars of the Academic Division of Pathology, University of Sheffield.

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    Attended the weekly Clinico-Pathology meetings, Children's Hospital, Sheffield.

ABSTRACT

STUDIES ON PNEUMOCOCCI ISOLATED FROM CHILDREN IN THE SHEFFIELD AREA. By Richard Howden.

The isolation of the pneumococcus (Streptococcus pneumoniae) was improved, and gave large mucoid colonies, when incubated in an anaerobic environment containing extra CO_2. Both reduction of oxygen tension, with a reduction in the formation of peroxides, and a variable requirement for CO_2 were necessary for optimal recovery of strains from children.

During a four year period 1736 pneumococci isolated from samples at the Children's Hospital, Sheffield were serotyped, their requirement for CO_2 and sensitivity to antibiotics were collated with patients details. This information was analysed for patterns both in health and disease as a background to clinical management and the re-introduction of vaccination in high risk patients.

Serotyping of strains by co-agglutination of sensitised protein-A particles, was quicker and as sensitive as crossed immuno-electrophoresis (CIE), without requiring the complex CIE equipment.

The increased and more active growth obtained under anaerobic conditions enhanced detection of pneumococcal enzymes, including sugar fermentation. New specific substrates, e.g. the APIZYM system, and those linked to nitrophenyl compounds were found to be applicable to the pneumococcus. A second new area of investigation, the differentiation of the pneumococcus by its sensitivity to a range of dye substances, was discovered and developed. Further unique differences were seen in the shape and colour of colonies on Mitis-salivarius agar, CO_2 requirement, haemolysins and sensitivity to tetracycline. Another antibiotic, mecillinam, was found for the practical recognition of penicillin-tolerant strains as an alternative to the use of cloxacillin for this purpose.

Much of the work in this study has not previously been reported and should form the basis of a number of new approaches and may lead to a scheme, at present not available, for the differentiation and characterisation of the pneumococcus beyond the capsular serotype.

ACKNOWLEDGEMENTS.

I wish to express my gratitude and appreciation to Professor M.G. McEntegart, Department of Medical Microbiology, University of Sheffield for his encouragement to start this work, and to Professor B.I. Duerden of the above Department for his advice during the latter part of the study and his constructive criticism in the preparation of this manuscript. I would also like to thank my supervisors, Dr. B. Davis and Dr. J. Mills for their efforts and perseverence throughout this lengthy project. I would also like to thank Mrs. I. Cox for her friendship and assistance in the preparation of the information for the computer input documents. My thanks are due also to Mrs. B. Wing and her helpful colleagues of the Medical Records Department, Sheffield Children's Hospital in the extraction of patients details, and to Mrs. A. Szumski and Mrs. C. Lyons of the Hospital Activity Section, Trent Regional Headquarters for the transfer of this information into the computer. My thanks are due to Mrs. P. Bridge, Mrs. M. Bennett and Mr. M. Pedly of the Health Care Team, Trent Regional Computer Centre for the analysis programming for the data. My appreciation goes to Mrs. D. Huntingdon, Medical Photography, Children's Hospital for her skilful illustrations. Thanks are also due to Mr. C. Dawson for his assistance in the preparation for and printing of this manuscript. API Laboratory Products Limited of Basingstoke, Hampshire are thanked for their APIZYM colour charts and the permission to include these in my thesis.

An especial thanks goes to Mr. L.F. Knapton and my past and present colleagues in the Microbiology Department, Children's Hospital for their co-operation with, and tolerance of, both myself and this project.

The Trent Regional Health Authority NHS Locally Organised Research Grant Commitee is gratefully acknowledged for their financial support.

To my wife, Marjorie, and my children, Karen, Lynn, Jill and David, who were with us before the start of this study, and Suzanne and Jonathan, who were born during the period of this study and who have now embarked upon their own studies - thank you for your forebearance and understanding during my work.

CONTENTS

ABBREVIATIONS

$(NH_4)_2SO_4$	Ammonium sulphate
cm	Centimetre
$FeSO_4.7H_2O$	ferrous sulphate
g	Gram
h	Hour
HCl	Hydrochloric acid
L	Litre
$MnSO_4.4H_2O$	manganese sulphate
$MgSO_4.7H_2O$	magnesium sulphate
Hg	Mercury
µg	microgram
min	Minute
ma	Milliamp
ml	Millilitre
mm	Millimetre
mv	Millivolt
M	Molar
nm	nanometre
N	Normal
KH_2PO_4	potassium dihydrogen phosphate
K_2HPO_4	dipotassium hydrogen phosphate
rpm	revolutions per minute
NaH_2PO_4	sodium dihydrogen phosphate
$Na_2HPO_4.2H_2O$	disodium hydrogen phosphate

Streptococcus pneumoniae is the most frequent bacterial cause of pneumonia, otitis media, and bacteraemia and is the third most common cause of meningitis in infants and children (Klein, 1981). It is difficult to obtain accurate figures of the extent of pneumococcal pneumonia because the pneumococcus is a normal part of the naso-pharnygeal flora of at least 30% of children under 10 years old (Loda et al., 1975 and Dowling et al., 1971). Assuming a 5% mortality rate for the cases producing the 1700 deaths in children, under 5 years old, from acute respiratory infections and pneumonia (Registrar General's report for 1978); this would represent approximately 34,000 infected children per year. The pneumococcus was thought by Heffron in 1939 to be the cause of two thirds of respiratory infections in children and forty years later general practioners in the United Kingdom still reported 55,000 pneumococcal pneumonia cases, in patients of all ages (Vogel, 1982). Diagnosis in children is also complicated by the difficulty in obtaining a full clinical history, a suitable bacteriological sample either because children tend to swallow their respiratory secretions or because they have received prophylactic antibiotic therapy. However, with more stringent criteria, Austrian (1975) reported an estimated 420,000 cases of pneumococcal pneumonia in persons over 18 years old. The pneumococcus remains the only infective agent in the 10 leading causes of death (Austrian, 1975) and accounts for 27% of all

deaths occurring after the first month of life in those under 5 years of age (Court, 1968). In the Newcastle Family Survey (Miller et al., 1960), 80% of illness in children under five years of age was infective and of these 60% were respiratory infections. This group of patients has also the highest pneumococcal carriage rate (Hendley et al., 1975), which is known to be a contributary factor in the production of pneumococcal disease (Hodges and MacLeod, 1946). Spread of the organism from the respiratory tract into the eustachian tube is presumed to result in pneumococcal otitis media (Austrian, 1975), which persists as one of the most common infections of children in developed countries. The attack rate of this infection is estimated at between 15 and 20 cases per 100 children in the first two years of life and by three years of age 70% of children have had at least one attack of otitis media (Teele et al., 1980) which, although rarely fatal, is the cause of life long handicap through deafness (Austrian et al., 1977 and Kamme et al., 1970). The incidence of pneumococcal otitis media reaches a peak at between 6 and 12 months, while in pneumonia cases this happens early in the child's second year and has an attack rate of greater than 32 per 100,000 children (Broome and Facklam, 1981). Early childhood is a vulnerable age for pneumococcal infections when loss of initially protective maternal antibodies leads to systemic infections (Klein, 1981). However Bortolussi et al. (1977) reported in four newborn infants that sepsis arose within the first 24 h of birth,

probably acquired during passage along the mothers genital tract. Pneumococcal meningitis has a peak occurence at between three to five months of age and has been estimated to attack 1.4-2.2 people per 100,000 (Fraser, et al 1973 and 1974). Approximately 8 cases of pneumococcal bacteraemia per 100,000 population, based on hospital findings, has been reported but actual numbers are expected to be much higher (Johnstone, 1981). Recognition of bacteraemia depends on a number of factors, including the decision to perform a venepuncture at the right time of the disease and before the start of antibiotic therapy. Some bacteraemic patients do not present as hospital admissions (MacGowan et al., 1973 and Braddon et al., 1977) and it is probable that if blood cultures were more frequently performed in respiratory illnesses the number of isolates would increase. Whether this would affect the treatment of the patient is unknown (Jacobs et al., 1979). Untreated pneumococcal pneumonia and the concomitant bacteraemia are fatal in 40% of cases (Witt and Hamburger, 1963) and in untreated meningitis the chances of survival are virtually nil (Austrian, 1968).

Two methods have been employed to help the patient combat this organism, firstly vaccination or serum therapy to provide antibodies to enhance phagocytosis of the bacterium, and later replaced by the administration of antibiotics to limit the growth of the organism. The history of pneumococcal serology goes back to 1891 when Metchnikoff cited the agglutination of the organism, and in

1897 Bezancon and Griffin suggested the antigenic diversity of the pneumococcus. Neufeld's description of the now classic quellung reaction, the apparent swelling of the organism's capsule when mixed with specific rabbit antisera, in 1902 preceded the differentiation of the pneumococcal serotypes. Its true importance was overlooked until rediscovered by Armstrong and by Logan and Smeall in 1932, and now forms the basis of the serotyping of isolates. Preventative therapy, dates from 1911, when Sir Almroth Wright prevented pneumonia in South African gold miners by injections of a vaccine prepared from whole killed organisms (Wright et al., 1914). In the next 40 years, following the recognition of the majority of the pneumococcal capsular serotypes and their application to immunisation (Francis and Tillett, 1930; Sutliff and Finland, 1931 and Finland and Brown, 1938), nearly half a million people received polyvalent vaccines composed of either whole pneumococcal cells or their capsular polysaccharides without serious untoward reactions, but with uncertain benefit in some cases (Austrian, 1975). Pneumococcal pneumonia had been a major preoccupation of medicine up to the 1940's and it was ironic that the development of safe and effective multi-factorial vaccines (Heidelberger et al., 1948) coincided with the introduction of effective antimicrobial agents. For the next twenty years there was almost no professional interest in prevention of the disease. A licence to supply polyvalent polysaccharide vaccines by E.R. Squibb and Sons in 1950,

was revoked without prejudice shortly afterwards because of lack of its use and at about this time also, the accompanying bacteriological techniques of mouse inoculation and pneumococcal serotyping fell into widespread disuse and with it the impetus for pneumococcal studies. If the amount of interest in the pneumococcus can be measured by the number of papers published on that subject the figure for 1939 was 120 but by 1955 this had fallen to 29 titles at a period when the total number of papers listed in Index Medicus had begun the spiralling increase over pre-war levels. The exquisite sensitivity of this organism to antibiotics seriously raised the question whether or not it mattered if it was isolated at all, particularly from pneumonia patients. The picture was different in meningitis for, although sulphonamides had reduced the death rate to around 60% in the late 1930's (MacLean et al., 1939) and penicillin in the 1940's had reduced this still further, a fatal outcome for both meningitis and bacteraemia, even in England and the USA, remained at 20-30% (Communicable Disease Reports (CDR), 29/1975, 25/1978, 10/1981, 71/1984 and Broome and Facklam, 1979) and many of the survivors, particularly children, showed residual neurological damage. It is worth remembering that the other main causes of meningitis are much less likely to be fatal, Haemophilus influenzae at 2.9% and the meningococcus at 8%, and with less likelihood of severe sequalae (CDR, 25/1978). Pneumococcal infection continued at a much higher level, and was more often fatal

in less favoured areas; in Nigeria, for example, between 1971 and 1976, 51% of patients with pneumococcal systemic infections died (Baird et al., 1976) and still, in 1984, a small survey of 28 patients with pneumococcal meningitis showed that 14 had died, eight were known to have survived and the fate of the other six was unknown (Kumasi, unpublished data). Survival curves of patients with pneumococcal meningitis and bacteraemia showed that most deaths occurred within the first five days of illness (Austrian and Gold, 1964). These findings suggested that for many patients irreversible physiological changes had already taken place by the time of admission to hospital. This continuing high mortality of pneumococcal systemic infection is a salutary example of the failure of antibiotics to cure infectious disease unaided. The outcome of all infection is dependant on the ability of the victim to combat the effects of the invader and this is most clearly seen in pneumococcal disease. Victims of pneumococcal infection fall broadly into two groups. Firstly those in whom trauma has physically damaged protective membranes and allowed penetration of the organism into the body, e.g., meningitis is a common sequelae of fracture to the skull or cribriform plate. The other much larger group consists of those in whom the immune system is either not developed, as in the infant, or is or has become incompetent because of age, predisposing diseases or drug therapy. Children under five years old and especially those under two years appear unable to make

sufficient or effective anti-polysaccharide antibodies (Howie et al., 1976; Borgono et al., 1978 and Riley and Douglas, 1981). Surveys of the results of vaccination in infants, under one year old, have shown low antibody levels after one or two injections and when examined at two years of age the levels were identical to the unvaccinated group (Sell et al., 1978). Patients whose defences have been reduced include those suffering from sickle cell disease (Seeler et al., 1972), malignancy and other disease in which treatment results in immunosuppression (Allen and Weiner, 1981), alcoholism (Brayton et al., 1970), poor nutritional conditions and being one of or descended from the indigenous natives of America (Herbert et al., 1967 and Davidson et al., 1976), Africa (Johnson, 1981), Australia and New Guinea (Douglas and Riley, 1979). Ethnic origin and any of the other factors when combined exaggerate some inherited tendancy for pneumococcal infection in those peoples (Johnson, 1981). That is except for otitis media which is more common in white children, probably because of anatomic differences in the eustachian tube (Doyle, 1977). In sickle cell disease, in which the spleen can be destroyed or need to be surgically removed, the infection results from lack of the spleen which would otherwise selectively clear the blood of pneumococci and generate the early antibody response (Singer, 1973 and Wara, 1981). This loss in combination with sluggish antibody production and a decreased turnover of the alternative complement pathway components, a source of non-specific opsonising

immunoglobulin (Pearson, 1977), is often fatal. Another large group of victims, even in the so-called developed countries, is the elderly patient in whom the immune system is naturally in decline and may not produce sufficient antibody until it is too late. The natural course of pneumococcal disease is a race to produce sufficient antibodies in the host to remove the organism before the effects of the infection exhaust the patient's strength. The above groups of patients all suffer from the combined disadvantages of a lack of prompt immune reponse and a reduced capacity to cope with the stress of infection. If pneumococcal antibody production reaches the crucial level by the third to the fifth day of overt illness the fever is resolved by crisis, the patient's temperature drops dramatically and survivors suffer prolonged weakness. In the 1930's, when both pneumonia and poor nutritional conditions were common, convalescent pneumococcal patients occupied more than two thirds of the beds on medical wards, for a month or more during six months of each year (Finland, 1981). At this time the other major cause of bed occupancy was typhoid fever whose season of high incidence dove-tailed into the rest of the year. Typhoid fever is largely a disease of the past, in developed countries, but pneumonia is still as common a cause of admission as it was then (Finland, 1981). The order of events from onset of infection to crisis is incompletely understood and varies both with the host and the type of pneumococcus involved (Austrian, 1975). The first line of defence

against invading organism is the mucosal surface of the respiratory tract to which the pneumococcus must attach without being opsonised and then avoid or overcome the alveolar macrophages (Tylewska et al., 1980; Andersson et al., 1981 and Rosenberg, 1981). In vaccinated persons sufficient opsonising antibody is present at the lung surface to induce the macrophages to phagocytose the bacteria (Hof, et al 1980 and Coonrod and Yoreda, 1981). If the pneumococci are not consumed invasion can spread into the interstitial spaces of the lung, to the hilar nodes and thence into the blood stream resulting in meningitis, peritonitis , endocarditis or myocarditis by metastatic spread (G'ad'aleanu , 1980 and Johnston, 1981). Pneumococci are able to effect infiltration of the lungs not by overwhelming the victim with toxins but, by stealth, avoiding activation of the opsonising system. The pneumococcal capsule protects the organism from phagocytosis by wandering macrophages and proteases are released from the organism capable of destroying secretory IgA (Kilian et al., 1979 and Mulks et al., 1980), a phagocytosis-enhancing immunoglobulin present in the mucus. Certain strains reduce the probability of opsonisation further by not activating the alternative complement system (Guckian et al., 1975 and Edwards and Stark, 1977). Once a focus of infection has been established in the lung tissue the combination of any of the potentially reactive constituents of the pneumococcus and the immune system, e.g. capsular polysaccharide with antibody, C substance

with C-reactive protein (CRP) or bacterial cell wall with the alternative complement pathway, leads to the release of potent mediators of inflammation (Tomasz, 1981). The more virulent organisms are less readily phagocytosed, require a greater degree of complement binding for opsonisation and would therefore be expected to have increased persistence in the lung, which would cause excessive release of the acute inflammatory mediators. Interaction of C-reactive protein and pneumococcal carbohydrate can start the complement fixation series by initiating release of the C1 component (Mortensen et al., 1976). The release of the C5 factor of complement and its derivatives enhances chemotaxis of neutrophils, monocytes and macrophages and stimulates the release from the neutrophils of granule enzymes and toxic oxygen metabolites into the lung (Webster et al., 1980). Anaphylatoxins (Dias da Silva and Lepow, 1967) and C567 complexes (Ward et al., 1966) also formed by activation of the complement system cause permeability changes and cell wall damage of the host's own tissue. The degree and extent of the changes seen in the pneumonic lung may be produced by the body's over-reaction to invasion as much as by direct aggressive action of the organism. None of a number of pneumococcal products e.g. haemolysin, purpura-producing principle, virulin, leukocidin and neuraminidase has been proven to be the pathogenic principle in pneumonitis (Johnston, 1981 and Tomasz, 1981). At the time of death the extent of the pneumonitis is insufficient to cause anoxia and the mass of bacterial

particles is not significant in relation to the size of the host (Perry and Cluff, 1963). Injection of large numbers of killed pneumococci fails to cause death therefore the mediators of imflammation probably contribute largely to the fatal outcome and may explain the waves of spreading oedema surrounded by a fringe of polymorphonuclear white blood cells, a characteristic feature of the histology of pneumococcal pneumonia. Factors that weaken the natural resistance of the respiratory system to infection, such as temperature variations and viral infections, assist in the establishment of pneumococcal disease. The question whether chilling facilitates invasion of the lower respiratory tract has been disputed (Douglas and Riley, 1979) but the frequency of pneumonia is clearly related to the effect of temperature changes on certain types of occupation. Exposure to inclement weather and subjection to extremes of heat gave attack rates of 13 and 12.6 cases per 1000 men per year respectively; in factory workers, with only small temperature changes, this rate was 3.9 per 1000 per year (Brundage and Bloomfeld, 1932). Experimental evidence (Green and Kass, 1965) indicates that cold immersion lowers pulmonary antibacterial activity presumably by its effect on the alveolar macrophage, but this could also be by a reduction in pulmonary blood flow (Gwaltney et al., 1975). A strong correlation was shown between weekly mortality from bronchopneumonia in infants and low temperatures at the time of onset of the illness (Payling-Wright and Payling-Wright, 1945). These mortality

rates were related to poverty and poor housing and rose sharply when the temperature fell below 4°C. Lunn et al., in Sheffield (1967), and Douglas and Blomfield (1958) found a significant excess of respiratory infections in children related to social class, pollution and living in a family with an adult with chronic respiratory infection. Seasonal variation in the carriage of pneumococci and transmission on the incidence of pneumonia is also associated with colds and overcrowding (Brimblecombe et al., 1958); conversely lowest carrier rates and infections are both seen in the summer months (Loga et al., 1975). The seasonal incidence of pneumonia parallels the seasonal variation of viral infections of the respiratory tract, largest numbers of isolations being in the winter and spring months. Onset of pneumococcal pneumonia in >90% of cases is preceded, a week or two before, by "cold-like" symptoms (Finland, 1981). The interval, between start of the viral illness and emergence of the symptoms of pneumococcal infection, gives time for pneumococcal antibody production to have begun, and to be present in therapeutic levels only 3-5 days after severe illness has been recognised. A gradually accelerating cyclic process can be envisaged, following in the wake of viral supression of the bodies defences (Hodges and MacLeod, 1946; Harford et al., 1949; Fiala, 1969 and Ferdandez-Peuntes, and Carrasco 1980) when persistence of pneumococci in the lung causes increasing production of inflammatory mediators. This increases the inflammatory response and physiological damage which finally gives rise to clinical

disease, 7-12 days later, at which time the process may have advanced too far for effective treatment. Although doubt has been expressed as to which viruses are involved, rhinovirus, parainfluenza and particularly influenza virus are the prime suspects (Lepow et al., 1968). The increased incidence of pneumococcal bacteraemia following severe pneumonia is a further indicator of influenza virus in the community. The 1957 A_2 influenza virus outbreak increased the incidence of pneumococcal bacteraemia by 300% in the peak month of the epidemic (Austrian, 1968). These gloomy portents receeded as the actual number of deaths from infectious diseases from all causes fell by 60% from the late 1950's to the mid 1970's, although deaths from respiratory diseases stayed the same (Cockburn, 1979). In children however, within five years of the introduction of sulphonamides (Kanof et al., 1943), pneumococcal bacteraemia no longer was the common companion of childhood illnesses. Other problems appeared more pressing, notably outbreaks of hospital acquired sepsis produced by entero-pathogenic Escherichia coli (Kauffman, 1969) and multi-resistant staphylococci (Williams, 1959). It was a case of "out of sight, out of mind" and, although Dowling and Lepper in 1951 and Austrian and Gold in 1964 demonstrated that pneumococcal bacteraemia still carried a significant mortality rate regardless of antibiotics, it was not until the late 1960's and early 1970's that the pneumococcus was brought back into the mainstream of microbiological and clinical attention. The spectre of

clinical pneumococcal isolates resistant to antibiotics first appeared as sporadic reports of strains tolerant to penicillin from Australia, New Guinea and South Africa (Hansman and Bullen, 1967; Hansman, 1972 and Gratten et al., 1980) and progressed to a world wide phenomenon (Hansman, 1977; Watanakukorn et al., 1980; Hansman, 1980) with examples emerging in America (Heiber and Nelson, 1977 and Cooksey et al., 1978), England (Howie and Mitchell, 1976), Canada (Dixon, 1974), Finland (Zacchrisson and Brorsen, 1981), South Africa (Ward et al., 1979), Italy (De Bac et al., 1981) and Japan (Oguri and Kosakai, 1981), showing resistance to an increasing array of antibacterial agents (Jacobs et al., 1978 and Landesman et al., 1979). When the first penicillin tolerant strains were recognised the resistance of S. pneumoniae to tetracycline was widespread (Percival et al., 1969 and Howard et al., 1978) and had reached 13% of isolated strains in Evans and Hansman's paper (1963). Occasional strains resistant to erythromycin (Dixon, 1967) and chloramphenicol (Applebaum et al., 1977 and Garau et al., 1981) had also been seen. The danger of the situation was made apparent when Jacobs et al. (1978) reported strains of pneumococci from hospitals at two South African centres, Johannesburg and Durban, resistant to all of a range of antibiotics, including the penicillins, cephalosporins, erythromycin, tetracycline, clindamycin, sulphonamides rifampicin, streptomycin and chloramphenicol. Because of the exquisite sensitivity of the pneumococcus to penicillin, testing of

isolates for antibiotic susceptibility was only rarely performed until the pneumococcus unexpectedly showed, after thirty years exposure to antibiotics, a capability for developing multiple-resistance and thereby posing a major threat as a hospital and possibly, a community, pathogen (Ward et al., 1979 and Mylotte and Bear, 1981). The pneumococcus has revealed many similarly unusual and fascinating features over the last hundred years, not least of which is the ability, after languishing on the mucus membranes in a commensal role for various lengths of time (Straker et al., 1939; Clifton et al., 1955 and Hendley et al., 1975), before suddenly becoming pathogenic and often rapidly fatal, without the reason for this change or the cause of death being known, despite extensive and intense study. This paradox was evident from the first record of pneumococcal isolations in 1881 when the injection of human saliva into rabbits, independently, by Sternberg and by Pasteur produced septicaemia and death of the animals. The isolation by Fraenkel in 1886 of the coccus, originally named Diplococcus pneumoniae, from the sputa of patients with pneumonia, indicated its role in human disease. However controversy raged over whether the diplococci, reported by Fraenkel, or the bacilli, stained in sections of lung by Gram, from Freidlander's fatal cases of pneumonia were the actual cause of pneumonia (Austrian, 1960). Weichselbaum's extensive study, in 1886, of the bacteriology of pneumonia resolved this heated issue but differences between the presence of organisms in

Gram-stained films and their growth in vitro has continued at the root of more recent problems with the pneumococcus (Barrett-Connor, 1970 and Merrill et al., 1973). The laudable, but often indiscriminant, use of antibiotics before bacteriological specimens have been obtained has clouded the issue, since even a single dose of penicillin can reduce the vitality of the infecting strain (Crofton, 1970; Murray and Hampton, 1980 and Adenyi-Jones et al., 1980). Such interference with growth can produce negative culture reports, in vitro, despite positive Gram film results (Lepow et al., 1968; Spencer and Philips, 1973; Rein et al., 1978 and Sarachik, 1979). Some authors have shown only 40-60% of the isolates expected from the results of Gram-stained smears, even in samples from patients without prior antibiotic therapy (Williams and Kauffman, 1978; Fiala, 1969; Barrett-Connor, 1971 and Boerner and Zwadyk, 1982). Animal inoculation for the recovery of the pneumococcus by its differential pathogenicity was, and by some authors still is, considered to be the better isolation method, particularly when the specimen may contain other bacteria (Austrian, 1959; Rathbun and Govani, 1967 and Converse and Dillon, 1977). However the cost and logistic problems of maintaining a large population of animals has contributed to the decline of this approach. The method should also be abandoned because of the bacteriological problems associated with extrapolating results from animal experiments to human disease. MacLeod (1952) and others have shown that certain serotypes of the

pneumococcus, including type 14, an invasive type of human infection (Heffron, 1939) are non-pathogenic for mice, and in general the higher the serotype number the less likely are they to be demonstrated in the animal (Morch, 1943). Unfortunately the alternative procedure of culture on artificial media, as routinely employed in many laboratories, and quoted in the papers regarding Gram films and poor performance culture results, suffers from a number of weaknesses which may explain the differences in results.

Conventional culture, by aerobic incubation on enriched blood-containing agar plates, produces a small indented (so-called "draughtsman") colony showing a degree of greening or change in the surrounding medium. This appearance can easily be overlooked especially when the sample includes other greening streptococci and if the plate is the subject of an inexperienced or hurried examination. To avoid these pitfalls it is recommended that careful scrutiny of the plate, by a microscope at low power or a hand lens (Austrian, 1971), followed by the testing of at least three colonies from each culture is necessary to maximise the recognition and identification of pneumococci. Recovery rates are increased (Auger, 1939, Kempner and Schlayer, 1942) without markedly enhancing colony size, if the plates are incubated in an atmosphere containing extra CO_2; for example in a candle jar (at 2%) or CO_2 incubator (at 5-10%). Carbon dioxide appears to have two advantages for the artificial culture of pneumococci, firstly in reducing the lag period of growth

following transfer of the organism from the host to a new environment (Valley and Rettger, 1927; Walker, 1932; Broquest and Snell, 1951 and Repaske et al., 1974), and also because growth of certain strains is dependent upon levels of CO_2 above the normal atmospheric value of 0.03% v/v, i.e. these cultures are carboxyphilic (Austrian and Collins, 1966, Sebestény, 1978). Sir Alexander Fleming, in 1941, recommended the advantage of incubating all cultures from the respiratory tract with added CO_2 after repeated attempts to grow a coccus seen in Gram-stained films failed until CO_2 was added to the culture environment from a Kipps apparatus. A further handicap to successful recovery, evident as intense greening or bleaching around colonies on blood containing agars, especially when the catalase content of the blood has been reduced by heating, is produced by the toxic accumulation of hydrogen peroxide (MacLeod and Gordon 1922 and Holt, 1962). The formation of hydrogen peroxide and therefore the need for a peroxide handling enzyme system by the organism would be minimised if cultures were performed in an anaerobic environment. It is often overlooked that the natural habitat of the pneumococcus, the respiratory tract, besides being an area with a high CO_2 level is in fact a reduced environment since the oxygen content of the mucus is removed by the metabolism of the bacteria harboured within the secretions.

The adaption of the pneumococcus to a reduced environment with an increased CO_2 concentration is further seen when the focus of infection in tuberculosis of the lung and

pneumococcal pneumonia is contrasted. Tuberculosis, produced by the aerobic mycobacteria, starts in the well oxygenated upper lobes of the lung and the pneumococcus, a facultative anaerobe, invades the lower lobes, an area of high CO_2 and relatively low O_2 tension. Early papers by Bolognesi (1907) and Smith (1936) reported anaerobic pneumococcal strains but the advantage of using both anaerobiosis and increased CO_2 as the atmosphere for their clinical isolation was not appreciated.

A preliminary report (Howden, 1976) confirmed the enhanced effect of the combined atmosphere. Culture in an anaerobic atmosphere, containing extra CO_2, significantly increased the isolation rate, size of the pneumococcal colony and minimised peroxide production, indicated by a lack of greening when grown anaerobically on heated blood agar.

Both animal inoculations and artificial culture methods suffer from the practical disadvantage of reliance on suitable samples being obtained before antibiotic therapy is started, followed by speedy transfer to and processing in the laboratory. A number of techniques for the detection of bacterial antigens, directly from a patient's sample, avoiding the uncertainties of culture and prior antibiotics (Tugwell and Greenwood, 1975) began to be applied to pneumococcal disease (Dorff et al., 1971; Coonrod and Rytel, 1972 and Whittle et al., 1974). The first to come into vogue was crossed-immunoelectrophoresis (CIE), in which pneumococcal antigen, in the patient's sample, was made to migrate in a buffer system across an agar gel

towards pneumococcal capsular antibodies migrating in the opposite direction and observed for precipitation lines (Anhalt and Yu, 1975; El-Rafaie and Dulake, 1975 and Silverman et al., 1977). In comparisons of media, direct Gram-stained smears and rapid antigen methods, the latter appeared the most sensitive indicator of pneumococcal infection (Olcen, 1978; Fossiek and Fedorko, 1979 and Edwards et al., 1980). But the bacteriological isolation procedures used were aerobic culture only, either with or without extra CO_2. The co-agglutination technique, in which the pneumococcal antigen is agglutinated with specific antibody, previously attached to staphylococcal cells, was next applied to pneumococcal work both for serotyping isolates (Kronvall, 1972) and later for the detection of antigen in patient's samples (Edwards et al., 1980). Other rapid diagnostic methods, enzyme-linked immunosorbent assay (ELISA) (Barrett et al., 1980 and Drow and Manning, 1980), fluorescent antibody technique (FAT) (Clausen, 1981), pneumococcal antibody labelled latex particles (Coonrod and Bauer, 1976) radio-immuno assay (Leinonen, 1980) were introduced during the course of my study, each appear to have merit for the study of pneumococcal disease but were not investigated due to lack of time and the necessary equipment. Antibody coated latex particles, a similar principle to co-agglutination, has been reported to be more sensitive than CIE (Kumar et al., 1980).

The antigen detection methods were developed for rapid

diagnosis of infection but can also be used to serotype the increased number of isolates produced by anaerobic incubation with extra CO_2. An extended survey of the strains obtained by this new culture method to determine the serotypes involved was indicated, as the strains may include previously unknown types whose distribution may differ from other reports. The pattern of serotypes recovered may have relevance to the effective use of pneumococcal vaccines, especially in this hospital which includes a Regional Centre for children with malignant disease, a high risk group for pneumococcal infections. Many surveys of the frequency of serotype distribution both in time and place have shown significant differences (Finland and Barnes, 1977 and Broome and Facklam, 1981) including the types associated with childhood and adult illness (Austrian, 1959; Gray et al., 1979; Riley and Douglas, 1981 and Klein, 1981). Morch in a 1944 Danish study, found types 06, 14, 19 and 23, in 59.9 per cent of isolates from children aged 2 years or less and in only 12.1 per cent of strains from those aged 12 years and more (Morch, 1944). Serotypes responsible for bacteraemia in adults and children differed from those isolated from focal infections such as otitis media (Kamme et al., 1970; Loda et al., 1975 and Gray et al., 1979). Other reports confirmed serotypes 06, 14, 19 and 23 as responsible for most cases of bacteremia or meningitis in children and also included serotype 18 in that group of strains (Seigel et al., 1978). Serotype 03 was given as the principal cause

of invasive disease in general and serotype 19 as the leading cause of otitis media (Kamme et al., 1970; Austrian et al., 1977 and Gray et al., 1979). Although serotype 03 was often seen in otitis media it was an infrequent cause of sepsis or meningitis in children. Frequency of particular serotypes, notably serotype 02, changed from the dominant isolate, up to the late 1930's (Griffiths, 1928 and Heffron, 1939), to being a rare finding by 1945 (Austrian, 1968); most other serotypes have not shown such dramatic change (Finland and Barnes, 1977). There has been concern that the introduction of pneumococcal vaccination into an area would affect the distribution of strains but MacLeod, (1949) and Austrian et al. (1976) showed that the carriage of non-vaccine serotypes was not altered in populations after vaccination. Variation has also been seen with serotypes responsible for the bulk of infections differing from one part of the world to another (Lund, 1959; Hansman 1977a; De Bac et al., 1979; Gaustad, 1979; Jacobs et al., 1979 and Kalin et al., 1980). Serotypes 02 and 25 seldom cause bacteraemia or pneumonia in the United States but are common causes of disease in Papua New Guinea (Riley and Douglas, 1981) and in South Africa (Austrian, 1981), and a survey of pneumonia and meningitis in Nigeria and Senegal indicated that serotypes 01 and 05 were the most frequent types (Greenwood et al., 1980). Frequency of occurence is not always a direct indication of the incidence of disease, pneumococcal serotypes have widely different invasive potential in and between man and animals

and there is an inverse correllation between case rates and carriage (Riley and Douglas, 1981). For example, 60% of carriers of serotypes 01 or 05 developed pneumococcal pneumonia when they developed an upper respiratory infection, whereas only 5% of carriers of serotype 20 did so (Hodges and MacLeod, 1946). Although the proportion of cases caused by each serotype has changed over the years the same twelve types are reponsible for the majority of bacteraemic infections (Tilghan and Finland, 1937 and Austrian and Gold, 1964) and eight serotypes account for 70% of ear infections (Kamme et al., 1970 and Douglas and Riley, 1979). Severity of the disease can also be a product of the serotype, bacteraemic infections caused by type 01 have a fatality rate of 6% when treated with penicillin, similarly treated infections caused by pneumococcus type 03 are accompanied by a mortality of 48% (Austrian, 1968). This difference in the prognosis caused by different serotypes provides an additional reason for identifying the capsular type of the organisms causing infection. Even within a serotype virulence is greater for certain strains (Griffith , 1981) than others and satisfactory rapid differential markers beyond the organisms capsular polysaccharide are required. Recognition of these aggressive variants is essential to our understanding and possible treatment at an early stage of this life-threatening infection. Differentiation of pneumococci at present is limited to identifying the serotype of the strain's capsular polysaccharrides by its

reaction with the 46 type or group antisera covering the 83 known serotypes. Sera are available only in the Danish Serum Institute to recognise the antigens of which each type is composed because of stability problems of the highly absorbed antisera and the complexity of the number of antigens represented in each type. Antisera for serotype 06, for example, is a group reagent which contains antibody to detect sub-types 06A and 06B, which have the antigenic formulae 06a, 06b and 06a,06c respectively; some other groups have much more complicated antigen compositions. Therefore, under most situations, relatively crude strain to strain differences only can be detected. Essentially a differential scheme should preferably be simple to perform, reproducible and perhaps relate to the infective process under review. All typing systems depend on the genetic stability of the markers chosen and it is theoretically impossible to devise a perfect system for epidemiology (Elek and Higney, 1970). Study of the pneumococcus urgently needs a simple distinguishing test or group of tests to provide suitable strain markers. Much of the work on the pneumococcus was performed before modern characterisation techniques were available and a re-examination of some of these, for example enzyme detection, derived from the old carbohydrate fermentation tests, may have merit (Morch, 1943). Unique pneumococcal enzymes have been recognised (H'Oltje and Tomasz, 1975; Glasgow et al., 1977 and Kobata and Takasaki, 1978) which could form the starting point for differentiation by

characteristic enzyme patterns. The enzyme profile tests, such as the APIZYM strip, a commercial source of prepared substrates have been applied to detecting enzymes in cells and bacteria (Humble et al., 1977 and Tharragonnett et al., 1977) including the non-haemolytic streptococci (Waitkins, 1977), and may be applicable to the pneumococcus. Alternatively indicator labelled substrates, e.g. nitrophenyl or methylumberiferryl derivatives, provide an inexpensive, rapid and sensitive indicator of enzyme activity. Production of haemolysins has also been used as a factor to recognise pathogenicity in the streptococci and the clostridia and if more reliably produced in the new anaerobic culture method could find application in the pneumococci. Other observations, made early in the study, on growth inhibition by the pH indicator, bromothymol blue, and the Eh indicator, methylene blue, were expanded into investigations of these and other dyes as differential indicators of individual strains. These new aspects confirm that, nearly 100 years after its first recgnition, there are still many fascinating and potentially rewarding facets to the association of man and the pneumococcus.

The aims and objectives of this thesis therefore will be:-

i) to serotype, over a four year period, the pneumococci isolated by the combined atmosphere culture method from routine hospital laboratory specimens;

ii) to examine methods for this serotyping and the detection of pneumococcal antigen in the patient;

iii) to examine factors affecting the recovery of

pneumococci under different gaseous and cultural conditions;

iv) to attempt to recognise differential properties of the strains by their enzyme patterns and by their production of haemolysins, and to assess the suitability of dye inhibition in distinguishing among the pneumococci; a number of different pneumococcal strains would be examined during the study in order that recent clinical isolates be tested in preference to laboratory maintained cultures which may have lost their viruleunce during storage;

v) to test for antibiotic resistance;

vi) to collect and process epidemiological information on the patients and strains seen during the study.

2.1 PATIENTS

The patients were children aged between 1 day and 16 years who attended the Children's Hospital, Western Bank, Sheffield, for a variety of reasons not always directly related to pneumococcal infections. Children were either referred to the Hospital's Out-patient departments initially by their General Practitioner, admitted for a pre-arranged operation, admitted as medical or surgical emergency admissions to the wards at Western Bank or the Thornbury Annexe (approximately 1.5 miles away) or came to the Accident and Emergency Department. Several children were referred from other Sheffield hospitals, e.g. Jessop Hospital for Women (Maternity) and a further group of children were transferred from other hospitals in the Trent Regional Health Authority, i.e. Rotherham, Doncaster, Chesterfield, Scunthorpe, Grimsby and Lincoln, most commonly because of complications following meningitis and spina bifida.

2.1.1 Samples

Specimens were obtained from the patients by medical or nursing staff by routine procedures.

i) Swabs. A sterilised cotton wool swab on a wooden stick (Hospiswabs MW130, Medical Wire) was used to obtain material from the throat, nose, mouth, eyes, ears, burns, operative and accidental wounds, vaginal secretions, skin (finger, face and other sites), parotid duct or peritoneum

of the patient as appropriate. Swabs were also used to sample the lung surface, oesophagus, middle ear and bronchial airways at autopsy. After sampling the swab was returned to its plastic container for transfer to the laboratory.

ii) Sputum was obtained with the help of a physiotherapist or by suction after passing a catheter into the trachea of an intubated child and collected into a sterile disposable plastic bottle.

iii) Cerebrospinal fluid (CSF) was aspirated from the lumbar spinal region by the attending doctor. Occasionally the fluid was taken from the ventricular space in the brain or from the valve chamber or reservoir of a CSF shunt system inserted in patients with hydrocephalus to reduce the intercranial pressure.

(iv) Blood culture was performed by the medical staff after skin disinfection of the venepuncture site with Chlorhexidine (Hibitane, ICI). Approximately 2 ml of blood was divided between two bottles of commercially prepared "Bloodgrow" culture broth (MW 900, Medical Wire). The bottles were incubated at 37°C for one week with daily observation for growth. Gram-stained films were made from bottles thought to show growth and if positive subcultures made on two blood agar plates, one each for aerobic and anaerobic culture and on MacConkey agar for aerobic culture; all were incubated at 37°C for up to 48 h. After 7 days, apparently negative bottles were subcultured on to a heated blood agar plate which was incubated at 37°C for

18 h aerobically with 10% CO_2.

v) Sinus-antral washouts. Sterile normal saline was instilled via a catheter to free the mucus and pus obstructing the nasal sinuses. A sample of the washings was removed by suction and placed in a sterile plastic bottle.

vi) Urine. A midstream specimen of urine was collected, after cleansing the external genitalia, and transferred into a screw capped glass 25 ml bottle.

vii) Pleural Effusion Fluid from an empyema or pleural effusion was obtained by the medical staff from the pleural cavity with a syringe and needle and transferred to a 25 ml glass bottle.

viii) Bladder Discharge. The contents of the bladder in spina bifida patients with a non-functional bladder were obtained by washing out the bladder with sterile saline into a 25 ml glass container.

2.1.2 Transport of specimens to the Laboratory

During normal laboratory hours, specimens were sent routinely from the ward with minimum delay to reduce loss of bacterial viability. Outside these hours, swabs were placed into Amies transport medium (Oxoid), held at room temperature and cultured the next day.

2.2 ISOLATION MEDIA AND CULTURAL CONDITIONS

All the pneumococcal strains were routine isolates using the procedure developed in this laboratory (Howden,

1976). The media used were Blood agar (CBA) and heated blood agar (HBA). Blood agar was Columbia agar base (Oxoid) autoclaved at 15 lbs/sq in for 15 min and allowed to cool to 55°C before the addition of 7% (v/v) defibrinated horse blood (LabM). Heated blood agar was prepared by similar methods except that the complete medium was heated to 90°C until its colour changed to dark brown. The agar media were dispensed in 15 ml volumes in petri dishes, allowed to set and the surfaces were dried for 10 min at 37°C. If the plates were not required on the day of preparation they were stored at 4°C for up to 2 days. Specimens were spread evenly over approximately 20% of the area of a CBA or an HBA plate and spread with a sterile nichrome wire loop in three stages to obtain discrete colonies. Plates were incubated at 37°C for 18 h, the HBA plate aerobically in an atmosphere enriched with 5-10% CO_2 (Distillers Co.) and the CBA plates anaerobically as described in the gaseous environment, Section 2.4.1.

2.3 IDENTIFICATION OF ISOLATES

Gram-stained films and a catalase slide test were performed on suspected pneumococcal colonies. Gram-positive diplococci that were catalase negative were identified by tests for bile solubility and optochin sensitivity.

i) The rapid bile solubility test (Howden, 1979) was carried out by placing a loopful of a 10% (w/v) aqueous sodium deoxycholate solution, pH 7.0 on the suspected

colony. After incubation at 37°C for 10 min pneumococcal colonies had dissolved and non-pneumococcal colonies remained entire. If a doubtful result occurred, the rapid test was confirmed by overnight bile solubility and optochin sensitivity tests.

ii) Overnight bile solubility. To 1 ml of an overnight culture was added 1 ml of 10% (w/v) sodium deoxycholate solution. After incubation at 37°C for 10 min, the fluid was examined; clearing of the culture was a positive result and a negative result was when the culture remained turbid.

iii) Optochin test (Lund, 1959). The test culture was spread on to half of a blood agar plate and an optochin paper disk (Mast Laboratories) added; the plate was incubated in air plus 10% CO_2 at 37°C for 18 h. A zone of inhibition >18 mm in diameter was a positive result.

2.4 EFFECTS OF GASEOUS ENVIRONMENT ON GROWTH

2.4.1 Incubation conditions for the clinical specimens

The cultures were incubated under the standard anaerobic conditions used in the laboratory. The inoculated plates were placed, with the lid uppermost, in an anaerobic jar (Baird and Tatlock) equipped with a palladium catalyst (Oxoid). The air was evacuated to a vacuum of 660 mm Hg and replaced with a gas mixture of 90% H_2 and 10% CO_2. Reaction of the oxygen remaining in the jar and the added hydrogen under the influence of an active catalyst produced a secondary vacuum of 20 mm Hg within 10

min which was equalised with more of the gas mixture. The jars were incubated at 37°C for 18 h. A nutrient agar plate streaked with a culture of Pseudomonas aeruginosa was included in each jar as a control. The Pseudomonas, an obligate aerobe, would not grow if all the oxygen had been removed and the vessel remained airtight.

2.4.2 Culture of pneumococci to determine the optimum conditions for growth

Pneumococcal cultures were incubated in seven distinct gaseous atmospheres given below:

a) 90% hydrogen (H_2) and 10% carbon dioxide (CO_2);

b) 100% H_2;

c) 100% nitrogen (N_2);

d) 5% H_2, 85% N_2 and 10% CO_2;

e) 5% CO_2 in a CO_2 incubator;

f) aerobic without extra CO_2;

g) candle jar container;

For suppliers of the gases see Appendix A.

The routine laboratory procedure as described in 2.4.1 was followed for all gases with the exception of nitrogen which required an extra stage. After evacuating the jar to 660 mm of Hg nitrogen was added, followed by a further evacuation to 660 mm Hg and a second introduction of the gas. A secondary vacuum was not produced by the nitrogen gas and the jar was immediately placed in the incubator. The candle jar conditions were obtained by

placing a lighted candle in the jar which burns until approximately 3% of the O_2 has been consumed and approximately 2.5% CO_2 has been produced.
All cultures were incubated at 37°C unless otherwise specified.

2.4.3 Comparison of aerobic and anaerobic incubation for the isolation of pneumococci from routine nasal swabs

During a three month period routine nasal swabs were cultured on to two CBA plates, one incubated anaerobically with CO_2 and the other aerobically with CO_2. The swabs were then broken off into 7 ml of Todd-Hewitt broth (T-H), containing 1% (w/v) liver digest (T-HL), in a 150 X 12 mm loose capped glass test tube. After 18 h at 37°C the plates were examined for pneumococcal colonies and the broths subcultured on two CBA plates, incubated and examined as the original plates. A total of 89 specimens were tested.

2.4.4 The growth of pneumococci under different gaseous conditions.

To further compare the effect of gaseous conditions on pneumococcal growth eight recently isolated strains were cultured in an extended range of gas mixtures. The culture method was in two stages, firstly the test strain was grown anaerobically with CO_2 on CBA for 18 h at 37°C, a single colony transferred to 5 ml of T-H broth, incubated for 4 h at 37°C, centrifuged, washed in sterile distilled water and

finally resuspended in 1 ml of sterile distilled water. Each suspension was seeded on seven freshly prepared CBA plates using the standard loop method (McGeachie and Kennedy, 1963). Since it is known that small numbers of bacteria are more exacting in their *in vitro* growth requirements the suspension was diluted 1 in 100 in sterile distilled water and subcultured using the standard loop method. All plates were incubated at 37°C for 18 h in the atmospheres given in Section 2.4.2.

The loss of viability after diluting the supension 1 in 100 was investigated using a strain each of serotypes 6 and 18 grown and washed in sterile water as in the above experiment, diluted 1 in 10, 1 in 50 and 1 in 100 in sterile distilled water, PBS and T-H broth; each dilution was subcultured on to CBA and incubated anaerobically with CO_2 and aerobically at 37°C for 18 h.

Four cultures were tested by a modified procedure for gaseous requirement, the second stage dilution was 1 in 100 in PBS. After 18 h at 37°C the viable count was recorded.

2.4.5 Direct isolation and gaseous environment

To study the need for different atmospheres, on primary isolation, nasal swabs were obtained from four patients suspected of carrying pneumococci. The organisms were washed from the swabs into 0.5 ml of PBS and the standard loop method used to inoculate seven CBA plates; 5 ml of T-H was added to the remaining suspension. All cultures were incubated at 37°C for 18 h with one of each

of the seven seeded plates incubated under the seven conditions given in Section 2.4.2.

2.4.6 The requirement for CO_2

It is generally accepted that certain pneumococci require extra CO_2 (Austrian, 1965) for artificial culture, nd comparisons were made of growth on CBA under the following conditions:-

Anaerobically in H_2,	CO_2 level approximately nil
Aerobically,	CO_2 level approximately 0.03%
Candle jar,	CO_2 level approximately 2.50%
Carbon dioxide incubator,	CO_2 level approximately 5.0%

Four strains, one each of serotypes 03, 09, 19 and 33, recently isolated from clinical samples were cultured under each of the above conditions at 37°C for 18 h.

Repeat nasal samples were obtained from two patients previously shown to carry pneumococci. The organisms were washed from the swabs into 0.5 ml of sterile distilled water and a loopful of the suspension plated on to 4 CBA plates and 4 heated CBA plates (HBA). One plate of each medium was incubated aerobically, aerobically plus 5% CO_2, anaerobically and anaerobically plus 10% CO_2.

A simple method was developed to assess the requirement for CO_2 by recently isolated cultures. A light suspension of the strain was made in PBS, subcultured on to two CBA plates by the standard loop procedure; one plate was incubated in the aerobic incubator and the second in the CO_2 incubator, both at 37°C for 18 h.

Absence of growth on the aerobic and growth on the CO_2 plate was taken to indicate CO_2 dependence. The test was repeated on all dependent strains to confirm the need for CO_2.

2.5 IDENTIFICATION OF CAPSULAR SEROTYPES

Four methods were used for capsular typing of isolates.

2.5.1 Capsule swelling test

The test strain was mixed with a loopful of each of the Pooled antisera (Statens Seruminstitute, Copenhagen) on a glass slide and observed for apparent increase in capsular size. The pooled antisera consisted of an Omniserum, containing antibodies raised against all pneumococcal antigen types, and nine sera that contained antibodies to a smaller group of serotypes; by these sera the unknown strain could more readily be assigned to a single serotype group.

2.5.2 Immunodiffusion

Prior to obtaining electrophoresis equipment capsular antigen was detected in pneumococcal broth cultures by immunodiffusion.

A glass microscope slide (6.5 x 2.5 cm) was placed on a level platform and 2 ml of molten Nobel agar in half strength Veronal buffer (Table 2.1) pH 8.6, poured on to it. When the agar was set, wells, 1.5 mm diameter with

inter-well gaps of 3 mm, were cut in the agar. This pattern of holes allowed four antigens to be tested against the 10 pooled antisera. The respective solutions were added to the wells using glass capillary tubes and the slide placed in a moist chamber for 18 h at room temperature. If a precipitation line, observed against a dark background, formed between an antigen and antiserum well, the antigen was retested with the individual antisera of that pool. A permanent preparation was made by staining the precipitation lines after the soluble proteins remaining in the gel had been removed by immersing the slide in physiological saline for 24 h at 37°C followed by 4 h in tap water. The gel was stained with the Amido Black solution (Table 2.2) for 30 min and the excess colour removed by decolourisation in 1% (v/v) glacial acetic acid containing 1% (v/v) glycerol. The agar was finally dried at 37°C and examined for black stained precipitation lines. Four methods of pneumococcal antigen extraction were compared :-

i) T-H broth culture supernate, after 18 h at 37°C;

ii) Alcohol extraction of T-H culture broth. Two volumes of 74 OP ethyl alcohol mixed with 1 volume of culture at 4°C for 18 h; supernate removed and the deposit made up to 0.1 volume;

iii) T-H culture, lysed by 1% of a 10% (w/v) solution of sodium deoxycholate at 37°C for 2 h, followed by alcohol precipitation as for method ii);

iv) Protein reduced lysed T-H culture by boiling with 1% of

50% (v/v) acetic acid, cooling, neutralising with sodium hydroxide before proceeding as for method iii).

2.5.3 Immuno-electrophoresis

Pneumococcal antigen was obtained from the supernate of an overnight culture in Todd-Hewitt liver digest broth (T-HL). On a glass slide on a level surface, 2 ml of 1% (w/v) molten agar in either Veronal buffer, pH 8.6, or Tris buffer, pH 8.1 (Table 2.1), were poured and allowed to set. Parallel rows of wells, 2 mm in diameter, were cut 5 mm apart. The holes in one row were filled with the test antigen and the opposite row with pneumococcal antisera. The slide was placed on the central platform in the electrophoresis chamber and paper wicks were led from the edge of the agar into the buffer chamber containing the same buffer. Tris buffer was diluted 1 in 9 in distilled water for use. A constant current or constant voltage was applied from the the power pack to the electrophoresis tank for a time specified in the experiments described below. The heat produced by current passing across the slide was absorbed by placing a plastic platten containing ice beneath the slide before each electrophoretic run. Lines of precipitation were first observed against a dark background. The gel, washed overnight in physiological saline, was transferred to tap water for 4 h, and stained in coomassie blue (Table 2.2). The dye solution, diluted 1 in 20 in 12.5% (w/v) trichloroacetic acid (TCA), was left on the slide for 15 min, the excess stain removed in TCA

TABLE 2.1

Buffer and staining solutions used in electrophoresis

Veronal buffer (pH 8.6)

Sodium barbitone	10.00 g
Sodium acetate	6.50 g
Distilled water	1.00 l
Thymol (5% (w/v) aqueous)	5.0 ml

The buffer was brought to pH 8.6 with HCl

Tris buffer (pH 8.1)

Tris (hydroxymethyl) aminomethane	0.60 g
Glycine	2.90 g
HCl (1 N)	0.5 ml
Distilled water	100 ml

TABLE 2.2

Staining solutions used in electrophoresis

Amido Black Stain

Amido Black	1 g
Acetic acid (glacial)	7 ml
Distilled water to	100 ml

Decolourising Solution I

Ethyl Alcohol (66 0P)	90 ml
Acetic acid (glacial)	20 ml
Distilled water	900 ml

Decolourising Solution II

10% (w/v) aqueous Trichloracetic acid

Coomassie Blue Stain

Brilliant Blue R	1 g
Distilled water	100 ml

before the slide was dried in air.

Crossed immunoelectrophoresis or electroendosmosis, a rapid but more complicated technique than immunodiffusion, required a series of experiments to select the most suitable method. The supernate from 100 ml of an 18 h Tryptone soya broth (TS) culture, at 37°C, of a pnuemococcal strain found to react with pool H antiserum by the immunodiffusion method was used throughout the first series of tests:-

i) Agar/agar and indubriose in Veronal buffer were compared at a constant current of 10, 20 or 30 ma for 30 min and at 250 mv constant voltage.

ii) A repeat of series i) with Tris buffer replacing the Veronal buffer.

iii) Repeat testing in agar/agar gel in Tris and Veronal buffers at a 100 mv.

iv) Agar/agar and indubriose gels were repeated in Tris buffer at 50 ma constant current.

v) Indubriose gel in Tris buffer at 100 mv for 30 min; the reaction temperature was reduced by a frozen platten beneath the slide during electrophoresis.

vi) Fresh group H antigen prepared in Todd-Hewitt broth was tested under the conditions used in v).

vii) Source of the precipitation artefact was investigated by obtaining antigen as supernate from both TS and T-H broths with and without 1% (w/v) liver digest and/or 1% (w/v) haemoglobin solution and passing a constant current

of 100 mv for 1 h in indubriose agar and Tris buffer.

viii) New antigen was prepared from whole culture, whole culture lysed with sodium deoxycholate and the culture supernate, both with and without alcohol precipitation, and compared under the conditions used in vii).

ix) The Veronal buffer system was retested with antigens from cultures of serotypes 06, 23 and group E identified by slide agglutination. A constant current of 20 ma was applied for 30 min on the antigens against Omni, pool B, pool H, pool E, serotype 06 and serotype 23 antisera in indubriose and agar/agar gels.

x) Sensitivity of each system was assessed with neat, 1 in 3, 1 in 6, 1 in 12 and 1 in 24 dilutions of serotypes 06 and 23 antigens in both gels under the conditions used in vii).

Cultures of pneumococci in T-H, previously tested by immunodiffusion and stored at 4°C, were tested by electrophoresis at 20 ma per slide for 30 min in Veronal buffer against their respective pool and component sera; strains non-reactive by immunodiffusion were tested with the sera against the pool and serotype 06, 19, 23 and 18 antigens.

The interwell gap in the initial tests, 5 mm, was compared with gaps of 2 and 3 mm using 10 strains of groups A, D and F in agar/agar, Veronal buffer at 100 mv for 30 min.

Agar/agar and indubriose gel were both tested at pH 8.6 and pH 6.6, using antigens from ix), at 30 ma constant current for 30 min.

Sensitivity of the pH 6.6 gel for pool antisera was tested using antigens of groups A, B, C, F, G and H, neat and diluted 1 in 12 phosphate buffered saline (PBS).

xi) Detection of pneumococcal antigen in the following body fluids was attempted in agar/agar with Veronal buffer at 30 ma for 30 min:-

a) CSF was centrifuged at 2000 rpm for 10 min and the supernate used as the antigen;

b) Sputum was liquified with an equal volume of 2% (w/v) N-acetyl cysteine (Airbron), centrifuged at 2000 rpm for 15 min and the supernate used as the antigen;

c) Whole blood was allowed to clot in a sterile glass bottle and the serum obtained after centrifugation at 2000 rpm for 10 min used as the antigen.

2.5.4 Co-agglutination

The method used was essentially that of Kronval (1973). A freeze dried ampoule of Staphylococcus aureus Cowan Type 1 (NCTC 8530) was obtained from the National Collection for Type Cultures, Colindale, London.

i) Preparation of Protein-A suspension. The organism was grown for 18 h at 37°C either:-

a) in a cotton wool plugged flask (17cm base diameter) containing 150 ml of Casein-Cysteine-Yeast broth (CCY, Table 2.3) (Ardvison et al., 1971) from which the contents were harvested by centrifugation at 2000 rpm for 20 min in universal containers, or

b) as lawns on the surface of eight petri dishes containing

TABLE 2.3

Casein-Cystine-Yeast medium for the culture of Staphylococcus aureus

Basal medium

Casein	8 g
Yeast extract	2 g
Sodium lactate	1.5 ml
$Na_2HPO_4.2H_2O$	0.2 g
KH_2PO_4	0.08 g
$(NH_4)_2SO_4$	0.2 g
Tryptophane	0.016 g
L-cystine	0.02 g
Distilled water	200 ml

To the medium, autoclaved at 10 lbs/sq in for 15 min, when cool were added 2 ml of Supplements A and B (given below)

Supplement A Vitamin solution

Thiamine	0.02 g
Nicotinic acid	0.04 g
Distilled water	100 ml

Supplement B Mineral solution

$MgSO_4.7H_2O$	0.2 g
$MnSO_4.4H_2O$	0.1 g
$FeSO_4.7H_2O$	0.06 g
Citric acid	0.06 g
Distilled water	100 ml

15 ml of Columbia base agar (LabM). The growth from each plate was removed with the edge of a sterile microscope slide into 10-15 ml of PBS and the suspension centrifuged at 2000 rpm for 20 min in universal containers.

The deposit from either method was formalised by suspending in 25 ml of PBS containing 0.5% (v/v) formaldehyde solution BP 36% (v/v) (Evans Medical) for 3 h at room temperature. After washing four times in PBS, the concentration of treated cells was adjusted to 10% (v/v) and placed in a 100 ml beaker. A 2 metre length of sterile silicone tubing (internal diameter 0.5 cm) and peristaltic pump were arranged to pass the suspension continously through an 80°C water bath for 3 h.

ii) Modification to the CCY broth method was sought to enhance growth and speed harvesting of the staphylococci:-

a) CCY agar plates were prepared, covered with a sterile semi-permeable membrane (cellophane jam pot cover) seeded with the staphylococcus, incubated for 48 h at 37°C.

b) Alternatively the agar was seeded without membranes and the growth removed by scraping into PBS;

iii) Preparation of protein-A co-agglutination reagent (PCR). To 0.035 ml of the formalised heat treated protein-A preparation was added 0.007 ml of pneumococcal capsular antiserum. After 30 min at room temperature this was diluted 1 in 4 with PBS containing 0.1% (w/v) sodium azide before use.

iv) Testing of an unknown pneumococcal isolate.

Pneumococcal antigen was obtained by either emulsifying an anaerobically cultured colony in a loopful (0.005 ml) of saline or as the supernate from a T-HL culture. A loopful of each of the PCR pools in turn was mixed with the antigen on a glass slide and observed for aggregation of the suspension (co-agglutination) for up to 1 min. If co-agglutination was obtained with a pool PCR, the PCR of each of the individual serotypes of that pool were tested.

To investigate certain pneumococcal strains which, when grown in T-H broth, gave no or slow agglutination with pool PCR reagents two strains showing this effect were grown in TS, RCM, CCY and T-H broths with and without 10% (v/v) serum at 37°C for 18 h.

Extraction and concentration of the antigen released into T-H broth was attempted by acetone and alcohol precipitation both with and without prior boiling to remove protein.

v) Modification in the preparation of PCR reagents was necessary to cause stronger reactions with certain strains. At the dilution previously used for all reagents, 1 in 28, although more concentrated than Kronvall's recommended 1 in 100, Omni, pool D and serotype 03 PCR reagents gave weak or negative pool reactions with some of their specific antigens. The volume of antiserum added to the prepared protein-A and the amount of PBS for the in-use dilution was varied for each of the screening reagents. Sensitising dilutions (SD) were made at 1 in 2 (SD1), 1 in 7 (SD2) and 1 in 14 (SD3) for each of the antiserum pools and serotype

03 in 10% (v/v) treated protein-A for 30 min and further diluted for use (UD) to 1 in 2 (UD2), 1 in 3 (UD3) and 1 in 4 (UD4) and examined for co-agglutination of the test strains indicated below:-

a) Omni serum was tested with strains of serotypes 03, 04, 06, 09, 11, 14, 15, 19, 21 and 23 at SD1, neat and UD2, SD2 and SD3;

b) Pool B: strains of serotypes 03, 06, 08 and 19 were tested at SD1 and SD2 and UD4;

c) Pool H: strains were tested of serotypes 13, 14, 15, 23 and 28 at SD1, SD2, SD3 and UD4;

d) Pool E: with serotypes 10, 21 (two strains) and 33 at SD1, SD2, SD3, UD2, UD3, UD4;

e) Pool D: serotypes 09 (two strains), 11 (two strains), 16 and 37 at SD1, SD2, SD3 and UD2;

f) Pool F: serotypes 17 (three strains) at SD1, SD2, SD3 and UD2;

g) Pool G: serotypes 34 and 35 (four strains) at SD1, SD2 and SD3;

h) Pool A: serotypes 01 (two strains), 04 and 18 at SD1, SD2 and UD3;

i) Pool C: serotypes 07, 27 and 31 at SD1, SD2 and OD2;

j) Pool I: two strains "x" and "y" (not individually serotyped) at SD1, SD2 and UD2;

k) Serotype 03: three strains termed "M", "N" and "R" at SD1, SD2 and SD3, the antigen from strain R was diluted 1 in 25, 1 in 100, 1 in 150, 1 in 250 and 1 in 625.

vi) Alternative methods of antigen extraction of a serotype

03 culture, incubated on 10 CBA plates anaerobically with CO_2 at 37°C for 18 h were performed as follows:-

a) growth from three plates was scraped into sterile distilled water, lysed with 1% (w/v) sodium deoxycholate and boiled to remove protein; when cool the polysaccharide was precipitated with 2 volumes of cold 74 OP ethyl alcohol;

b) as for a) except that cold acetone was the precipitant;

c) colonies from 2 plates were lysed in situ with the deoxycholate solution and washed off and treated as a);

d) as for c) except that acetone was used to extract the polysaccharide.

Growth in RCM broth at 37°C for 18 h was similarly treated as follows:-

e) 10 ml of culture as in a) precipitated with alcohol;

f) 10 ml of culture as in b) with acetone;

g) 10 ml of culture boiled and precipitated with alcohol but without prior lysis;

h) as in g) except that acetone was used.

After 18 h at 4°C the extracts were centrifuged, the supernate removed, the precipitate reconstituted in 2 ml of distilled water and titred against serotype 03 PCR reagent.

vii) Loss of antigenicity was noted for certain strains after storage in T-H broth when compared to growth of the same scraped from blood agar culture. This effect was examined by growing a strain each of serotypes 01, 03, 06, 17, 19, 20, 22, 23, 33, 35 and group I at 37°C for 18 h in T-H broth and on CBA plates. Half of each broth was stored

at 4°C and RT; a suspension from each plate was also made in PBS and stored at 4°C. The reaction of each strain was confirmed against its pool and component reagents at that time and again after 7 days.

2.6 THE EFFECTS OF MEDIA ON PNEUMOCOCCAL GROWTH

The ability of certain media to actively support pneumococcal growth for isolation and antigen and enzyme testing was investigated. Media were prepared from Todd-Hewitt broth, formulated for the production of streptococcal toxins, Re-inforced clostridial medium broth, an anaerobic culture medium, and Tryptone soya broth, a blood culture medium.

2.6.1 Reinforced Clostridial broth

The commercial Reinforced clostridial medium (RCM) was prepared as three variants, in 5 ml volumes:-

i) BRCM, a 1% (w/v) solution of methylene blue was added at 1% (v/v) to RCM from which the agar had been removed by filtration.

ii) BRC, RCM prepared by removal of the agar (without the addition of methylene blue);

iii) RCM, prepared with agar but without the dye.

A series of broths was seeded with 0.2 ml of a faintly turbid suspension in PBS of each of 5 strains (one culture of serotypes 06, 08 and 14, and two of serotype 03), incubated at 37°C for 18 h.

2.6.2 Liver digest

Liver digest powder (LD, Oxoid), a source of growth factors, was added to three commercial media (LabM), tryptone soya broth, RCM and Todd-Hewitt broth at a concentration of 1% (w/v). Doubling dilutions of these media in their respective broths was made down to 0.03%. To each dilution and a control tube, without LD, was added 0.1 ml of a light suspension of a serotype 03 culture in PBS; incubation was at 37°C for 18 h.

2.6.3 Growth at different pH values

Three cultures (serotypes 01, 03 and 33) were examined in the following eight media:-

a) THA78 : prepared by solidifying T-H broth with 1% (w/v) Agar No.3 (Oxoid), pH 7.8;

b) THA73: prepared from THA78, after adjusting the pH of the medium to 7.3 with N HCl, with the addition of 1% (v/v) of a 0.2% (w/v) bromocresol purple solution (BCP);

c) LTGA68: prepared from 1 g, Lab Lemco , 2 g tryptose, 0.2 g glucose and 1g of Agar No.3 in 100 ml of distilled water, final pH 6.8;

d) THTA78: prepared by adding 1% (v/v) of a 0.2% (w/v) bromothymol blue solution (BTB), 0.2% (w/v) sodium bicarbonate and 0.04% (w/v) disodium phosphate to THTA, pH 7.8,;

e) RCMA68: prepared by adding 0.8% (w/v) Agar No. 3 to RCM broth;

f) RCMA73: by adding 0.2% (w/v) sodium carbonate to RCMA,

adjusting the pH of the medium to 7.3, and 1% (v/v) BCP;
g) RCMA78: with sodium bicarbonate at 0.2% (w/v), disodium phosphate at 0.04% (w/v) and BTB at 1% (v/v), final pH 7.8;
h) CBA, pH 7.3;

Each of the media, after being autoclaved at 15 lbs/sq in, were spread with the three strains, serotypes 01, 03 and 31, and incubated, anaerobically with CO_2, for 18 h at 37°C.

2.6.4 Viability and turbidity

Three pneumococcal strains, serotypes 06, 19 and 23 were cultured in 5 ml of T-H broth, aerobically and on a CBA plate incubated, anaerobically with CO_2. After 18 h at 37°C the broths were centrifuged and the deposits made up to 5 ml in PBS; the growth from the plate was scraped with a sterile glass slide into 5 ml PBS. Viable counts were performed on each sample by a standard loop method. The turbidity of each suspension was measured in a spectrophotometer at 660 nm, and at 260 nm, after the suspension had been lysed with bile for 1 h at 37°C.

2.6.5 The effects of cysteine and sodium bicarbonate on growth in Todd-Hewitt broth

T-H broth, suitable for the production of capsular material for serotyping, had the disadvantage that viability of the culture was rapidly lost and the formulae could not be used for other studies. The action of cysteine and/or sodium bicarbonate to improve growth in

this medium was investigated in the following manner:-

i) Cysteine hydrochloride. A 0.05% (w/v) aqueous sterile solution was diluted 1 in 10 in T-H and then double diluted in 5 ml of T-H to give 2 rows of five tubes at concentrations from 0.05 to 0.003% (w/v). A control tube, 5 ml of T-H without cysteine, was included in each row. Into each tube was mixed 0.2 ml of a light suspension of a serotype 03 strain, prior to incubation at 37°C.

ii) Sodium bicarbonate was added, after 5 h incubation, as 0.2 ml of a 0.2% (w/v) sterile aqueous solution to each of one row of the above, including a control tube, before incubation for a futher 16 h.

iii) Adjustment of the cysteine solution to pH 7.0 was made, with 0.1N NaOH, and four media produced:-

a) T-H only;

b) T-H plus 0.025% (w/v) neutralised cysteine (T-HC);

c) T-H plus 0.025% (w/v) neutralised cysteine plus 0.1% (w/v) sodium bicarbonate (T-HCB);

d) T-H plus 0.1% (w/v) sodium bicarbonate (T-HB).

Three further cultures, serotypes 08, 19 and 23 strain, were cultured in the four media at 37°C. After 24 h the turbidity was noted and viable counts were made by the standard loop method.

A further six cultures, of serotypes 03, 06, 11, 14, 15 and a non-capsulate strain, were cultured in the cysteine broth (T-HC); the cysteine and bicarbonate broth (T-HCB), and T-H with liver digest and haemoglobin solutions at 1% (w/v) (T-HLH) for 18 h at 37°C.

2.6.6 Selective agents for pneumococcal isolation

The practical advantages of selective culture methods for the recovery of pneumococci from clinical samples was examined in three ways:-

i) Broth cultures. Six mixed cultures were prepared with the Oxford strain of Staph. aureus (SOX) and six recent pneumococcal isolates to examine the selective effect of sodium azide or gentamicin in T-H broth. The strains, serotypes 03 (three cultures), 04 (one culture) and 14 (two cultures), cultured on CBA anaerobically with CO_2 at 37°C for 18 h, were suspended in 5 ml of PBS, and to each was added 0.2 ml of a suspension of a fresh culture of SOX, suspended in PBS. Three media were prepared in 5 ml volumes: Todd-Hewitt broth containing 1% (w/v) liver digest (T-HL), T-HL containing 0.01% (w/v) sodium azide (AT-HL) and T-HL containing a 2 µg gentamicin disc (0.4 µg/ml) (GT-HL). A 0.1 ml volume of the pneumococcal/SOX suspension was added to each of the three media, incubated at 37°C for 18 h and subcultured on CBA agar for 18 h at 37°C. Control tubes of each of the pneumococci and the SOX were made in T-HL and similarly incubated.

ii) Antibiotic discs placed on plate cultures were assessed for their inhibition of respiratory isolates. Nine pneumococcal and four non-haemolytic streptococcal (NHS) isolates from respiratory specimens were examined for inhibition by commercial discs of 2 µg gentamicin, 10 µg fucidin, 30 µg kanamycin and 200 µg nitrofurantoin.

A light suspension of the test organism, in PBS, was spread on to lysed blood sensitivity agar plates by the comparative zone method (Stokes, 1968); SOX acted as the control organism. After 18 h aerobic incubation without extra CO_2 at 37°C the zone diameters produced by the test and control cultures were measured.

iii) Gentamicin containing blood agar.

a) The amount of gentamicin necessary for inclusion in agar plates for selective culture was assayed by measuring the minimal inhibition concentration (MIC) of gentamicin for pneumococci. Plates of heated blood sensitivity agar were prepared containing:- gentamicin sulphate at 0.12, 0.25, 0.5, 1, 2, 4 and 8 µg/ml. A loopful of the nine pneumococcal suspensions and the SOX control were seeded on to each of the dilutions, incubated at 37°C for 18 h and the presence or absence of growth recorded. The plate containing the first concentration not permitting growth was taken as the MIC.

b) Throat and nasal swabs from a total of 59 patients were cultured on to three plates, two containing 5 µg/ml gentamicin in CBA (GCBA) and the third a CBA plate without gentamicin. One GCBA was incubated aerobically, the other in the CO_2 incubator and the CBA plate anaerobically with CO_2; all were incubated at 37°C for 18 h.

c) A short series of 12 routine swabs was cultured on CBA aerobically with and without CO_2, anaerobically with and without CO_2 and on GCBA anaerobically with CO_2.

d) With eight further swabs a light suspension was made of

the growth obtained anaerobically with CO_2 on CBA in 1 ml of PBS cultured aerobically and anaerobically both with CO_2 onGCBA plates for 18 h at 37°C.

2.7 Enzyme Studies

A number of approaches were made to demonstrate pneumococcal enzymes to investigate potential differential characters.

2.7.1 Carbohydrate fermentation

Enriched serum-free media were examined for suitability and speed of reaction in the detection of carbohydrate fermentation as an alternative to the serum water sugars usually used with the pneumococcus:

i) CTA medium (BBL), recommended for fermentation studies of fastidious organisms, was prepared in 7 ml volumes in sealed bijoux bottles, with and without 1% (w/v) liver digest. A bottle of each medium was seeded with seven pneumococcal cultures, serotypes 13 (T52), 03 (T69), 23 (T109), 06 (four strains, T122 and W154, from different patients, and T123 and T124, from the same patient) and a strain of Streptococcus faecalis. Repeat cultures of the same strains were tested in the media supplemented with 20% (v/v) egg yolk emulsion and CTA with yeast extract, 0.3% (w/v). Each series was incubated at 37°C for 48 h.

ii) Defined Gonnococcal broth (Difco), described for the cultivation of Neisseria gonorrohoea without serum, was prepared in three forms i.e. as the base only, as the base

containing supplements and as the base containing 0.5% (w/v) mannitol. The above eight strains were cultured for 48 h at 37°C in each of the three formulae and the growth and approximate final pH noted.

iii) Modified Todd-Hewitt broth was prepared, without glucose, to contain Lab Lemco 10g; tryptone 20g; sodium bicarbonate 2g; sodium chloride 2g; disodium phosphate 0.4g in a litre of distilled water. Four media were made from this base by the addition of 0.5% (w/v) of either glucose, mannitol, salicin or sucrose and tested with four pneumococcal strains and a Staph. aureus (SOX) culture incubated at 37°C; the broths were examined after 18 and 42 h incubation.

iv) Columbia agar base was prepared with 0.75% (w/v) sucrose and 1% (v/v) bromocresol purple indicator solution and with 10% (v/v) horse serum or with 10% (w/v) finely powdered chalk. Cultures of seven pneumococci and a S. faecalis were spread on the three media and growth and acid production recorded after 18 h incubation at 37°C.

2.7.2 Basal sugar testing medium

Alternative serum-free media for carbohydrate studies were assessed for use with the pneumococci.

i) Trypticase-yeast-liver-succinate broth (TYLS). The suitability of TYLS for carbohydrate fermentation studies was tested with pneumococci grown in the new medium with and without haemoglobin and cystein, and in Columbia base broth (CB) and RCM broth. TYLS was prepared from:-

Trypticase	1.00 g
Liver digest	0.50 g
NaCl	0.50 g
Yeast extract	0.30 g
Sodium succinate	0.25 g
Distilled water	100 ml

(final pH 7.2).

Columbia broth (CB) was obtained by suspending 3.9 g of Columbia agar base in 100 ml of distilled water, allowing the agar to settle for 30 min and decanting the clear fluid above the agar into a flask for sterilisation. Supplemented formulae of TYLS and CB were made by adding to each 10 ml of the basic medium 0.1 ml of separately sterilised haemoglobin (500 mg/ml) and cystein HCl (3.7%) solutions prior to use. Six cultures, serotypes 03 (T1279), 06 (T1093), 09 (T1512), 17 (W801), 19 (T1453) and 33 (W787), each suspended in 0.5 ml of PBS, were added in 0.1 ml volumes to 3 ml of each of the five broths and a loopful spread on to two CBA plates. One plate was incubated aerobically without extra CO_2 and the other aerobically with CO_2; cultures T1093, T1512 and W801 had been shown to be carboxyphilic (Section 2.4.6).

ii) Streptococcal basal medium for carbohydrate fermentation

a) Streptococcal basal broth (SB), an alternative new medium for carbohydrate testing was made from:-

Tryptose	1.00 g
Proteose peptone	0.50 g

Yeast extract	0.30 g
Liver digest	1.00 g
NaCl	0.50 g
Cystein HCl	0.04 g
Distilled water	100 ml
(final pH 7.2)	

Suspensions from eight isolates, serotypes 06 (T2907 and T2908, from the throat and nasal swabs of the same patient), 09 (T2860), 16 (T2744), 19 (T2645 and W1799), 23 (T2894 and T2901), were each made in 1 ml of PBS and a 0.2 ml volume transferred to 5 ml of SB, RCM and T-H broths, incubated at 37°C for 18 h.

b) Streptococcal basal Agar (SBA) was SB solidified with 1% (w/v) agar No.1. Plates of SBA and CBA were used for the subculture of the above strains grown in SB under aerobic, aerobic with CO_2 and anaerobic with extra CO_2 conditions for 18 h at 37°C.

c) Attempted enhancement of growth in SB: the value of ,dditives to SB was assessed by preparing the basal broth formula to contain one of the following:-

sodium acetate	0.30% (w/v)
sodium bicarbonate	0.20% (w/v)
ornithine	0.50% (w/v)
arginine	0.50% (w/v)
choline	0.50% (w/v)
glucose	0.50% (w/v)
sodium succinate	0.25% (w/v)
haemoglobin solution	1.00% (w/v)

agar No.1 0.05% (w/v)

phosphate buffer (K_2HPO_4 1.21 g/L and KH_2PO_4 0.34 g/L).

A 0.2 ml volume of suspensions of four cultures, serotypes 14 (T2989), 15 (T2860 and W2077) and 19 (T2645) in 2.5 ml of PBS were added to each of the 10 broths, plus a control without additives and into T-H and RCM broths, and incubated at 37°C for 18 h.

Growth was recorded and the broths subcultured on to CBA incubated anaerobically with added CO_2 for 18 h. Tests for residual glucose in the broth after incubation was made with glucose indicator strips (Clinistix, Ames).

d) Assimilation studies using SB agar were prepared by spreading a light suspension of the organism, in PBS, over the surface of the agar, cutting six wells, 7 mm in diameter, and adding a sterile 0.1% (w/v) solution of one of the following:-

glutamic acid

pyruvic acid

thiamine

L-histidine

tryptophan

aneurin

Four pneumococci of serotypes 15 (T2860 and W2077), 14 (T2989) and 19 (T2645), were tested by this method, incubated at 37°C for 24 h aerobically and for a further 24 h anaerobically with added CO_2.

2.7.3 Microtitre plate method for carbohydrate fermentation testing

The rapid demonstration of carbohydrate fermentation of high concentration substrates in microtitre plates has been successfully applied to organisms difficult to test by conventional methods, e.g. the Bacteroides group.

i) Preliminary studies of this method was performed on eight pneumococcal suspensions, serotypes 03 (T1279), 06 (W765), 09 (T1512), 16 (T1093), 17 (W801), 19 (T1453), 23 (W372) and 33 (W787), obtained by scraping the growth resulting from anaerobic culture on a CBA plate into 1 ml of bacteroides basal medium (BM) broth. The carbohydrate substrates were 20% (w/v) solutions of fructose, inulin, maltose, raffinose, rhamnose, sucrose, trehalose and xylose. Inulin and trehalose gave saturated solutions at this level and were prepared at 6% (w/v). Two drops of each suspension, two drops of each test solution and two drops of a 2% (v/v) Andrade's solution, in BM, were added to a well in a microtitre plate. Aesculin hyrolysis was similarly tested for each strain. The plate, sealed in a plastic bag, was incubated at 37°C for 4 h and examined for colour changes in the pH of the medium.

ii) The effect of suspending broth on microtitre carbohydrate reactions of three strains, serotypes 06 (T4269), 09 (T4257) and 17 (T4273), was examined by culturing the strains on three CBA plates anaerobically with CO_2, for 18 h at 37°C. The growth from the three plates was scraped into 4 ml of PBS and 0.1 ml of the

suspension added to each of 30 microtitre wells in three rows of 10 wells. To the first row 0.1 ml of double strength Columbia broth was added; to the second 0.1 ml of double strength SB broth and to the third 0.1 ml of double strength BM broth; each medium contained 2 ml of Andrades solution/100 ml. Across each row 0.1 ml of "sugar" solution was added prior to sealing the plates and incubating at 37°C for 4 h; the final well of each row contained the media and organism only as a negative control. The nine solutions tested were fructose, inulin, melibiose, raffinose, rhamnose, sucrose, trehalose, xylose and aesculin.

iii) Further tests, based on the results obtained from ii) above, were performed on five cultures, serotypes 06 (T4269), 14 (T4334), 17 (T4273), 19 (W2856), and 23 (T4329) suspended in SB broth, in the substrates and method used in ii).

iv) An extended series of fermentable substances, at 20% (w/v) concentrations, was tested with a further eight isolates, serotypes 03 (T1453), 04 (W787), 09 (T1093), 14 (T1512 and W801), 19 (T1279), and 23 (T795 and W372), in SB by the microtitre plate method. The substrates were arabinose, dulcitol, fructose, galactose, inulin, mannose, maltose, melibiose, raffinose, salicin, sorbitol and trehalose. It was only possible to obtain solutions at 5% (w/v) for dulcitol and 6% (w/v) for mannose; salicin crystallised out of solution on storage at 4°C. The growth from a CBA plate, cultured anaerobically for 18 h, was

scraped into 1.5 ml of SB broth and 0.1 ml of the suspension added to 0.1 ml of substrate in a microtitre plate. The microtitre plate was incubated in the CO_2 incubator for 4 h before the colour changes were recorded.

2.7.4 The APIZYM system

The commercially available APIZYM freeze dried enzyme substrates (API Products, Table 2.4) were used to detect pneumococcal enzyme profiles.

The basic method consisted of adding a suspension of the test organism to each of the 19 enzyme substrate and the negative control cupules of the APIZYM strip, followed by incubation at 37°C for 4 h before the development of enzyme products with two indicator reagents.

It was therefore necessary to standardise the preparation and concentration of pneumococcal suspension, the length of and gaseous conditions incubation prior to applying the method for pneumococci.

i) Growth standardisation.

a) bacterial mass was measured by centrifuging the growth from 5 ml of an 18 h T-H culture at 37°C, removing 4.5 ml of the supernate, resuspending the deposit in the remainder, heating to 60°C for 30 min and centrifuging in an Haematocrit centrifuge at 10,000 rpm for 5 min.

b) Photometric estimation of the growth of two strains was made both in T-H broth, after 18 h culture at 37°C, divided and examined as whole broth and as centrifuged supernate and from the colonies, from the well of a CBA plate scraped

TABLE 2.4

The APIZYM substrates

2-naphthyl phosphate;

2-naphthyl butyrate;

2-naphthyl caprylate;

2-naphthyl myristate;

L-leucyl-2-naphthylamide;

L-valyl-2-naphthylamide;

L-cystyl-2-naphthylamide;

N-benzoyl-DL-arginine-2-naphthylamine;

N-glutaryl-phenylalanine-2-naphthylamine;

2-naphthyl phosphate;

Naphthol-AS-BI-phosphodiamide;

6-Br-2-naphthyl-alpha D-galactopyranoside;

2-naphthyl-beta D-galactopyranoside;

Naphthol-AS-BI-beta D-glucuronic acid;

2-naphthyl-alpha D-glucopyranoside;

6-Br-2-naphthyl-beta D-glucopyranoside;

1-naphthyl-N-acetyl-beta D-glucosaminide;

6-Br-2-naphthyl-alpha D-mannopyranoside;

2-naphthyl-alpha L-fucopyranoside.

into 2 ml of PBS. Absorbance of all samples was measured on the Cecil spectrophotometer from 380-700 nm.

An area, 3 cm square, from a lawn of growth on a CBA plate, incubated anaerobically with CO_2, was scraped into 2 ml of PBS, lysed by 0.2 ml of 1% (w/v) sodium deoxycholate solution. The lysate, was scanned in the visible and ultra-violet wavelengths from 200-700 nm against a blank of 2 ml PBS plus 0.2 ml of deoxycholate.

d) Repeat spectrophotometric assays of lysed and whole suspensions were made at 350 and 660 nm respectively. The suspension was also cultured on CBA anaerobically with CO_2 for 18 h at 37°C for viable count neat and when diluted 1 in 25 and 1 in 625 in PBS.

e) Further estimations were performed to compare readings at 660 and 260 nm for suspensions prepared from plates and for T-H and RCM broths. Growth in 5 ml of each broth was washed three times in PBS before resuspending in 2 ml prior to lysis and measurement.

f) Standardisation of bacterial dose of strain 55, grown on CBA anaerobically, scraped into PBS, and read at 660 nm was adjusted to 1.0 absorbance unit. This suspension, neat and at 1 in 2 and 1 in 4 dilutions was added in 0.1 ml portions to each cupule of three APIZYM strips and incubated at 37°C in a moist chamber for 4 h. The colour of the enzyme products in each cupule, developed by a drop of reagents A and B (API Products) was scored from 0 to 5 against the colour chart for that enzyme (Appendix B). The turbidity of the same strain, grown under the same conditions, was

adjusted to 1.0 absorbance unit, and part of this suspension concentrated by centrifugation to a turbidity equivalent to 2.0 and 2.5 absorbance units. The three suspensions were added to three API strips and treated as for the dilute suspensions.

ii) Gaseous environment. The effect of the gaseous environment on the APIZYM results was studied with suspensions of four pneumococcal strains, obtained as in f), and adjusted to 2.0 absorbance units. Three strips were filled for each strain and incubated under aerobic conditions with and without added CO_2, and anaerobically with CO_2 for 4 h at 37°C.

iii) Length of incubation. The length of time for incubation of the substrate strips before development of the reaction colours was examined in the same four strains similarly prepared and added to three strips. One strip of each strain was developed after 4, 5 and 6 h incubation at 37°C.

iv) Testing of pneumococcal isolates by a standardised APIZYM method. From the results (Section 4.2.5) obtained from the above studies (Section 2.7.4, i, ii and iii) a standard method was adopted for the testing of pneumococci in the APIZYM substrate strip as follows:- The bacterial dosage for the strips was prepared from a lawn of growth produced by subculture of three colonies of the test strain on a CBA plate, incubated anaerobically with 10% CO_2 for 18 h at 37°C. The growth removed from the plate with a wire

loop, was suspended in 2.2 ml of PBS and a 0.2 ml volume diluted in 0.8 ml of PBS. The turbidity of the diluted sample was measured in a spectrophotometer (Cecil Instruments, model CE 393) at 660 nm. The concentration of the remaining suspension was adjusted by dilution with PBS or concentration after centrifugation and removal of part of the supernate, to give a turbidity equivalent to 2.5 absorbance units. For the test, a 0.1 ml volume of the adjusted suspension was placed into each of the 19 test and the single control cupules of the APIZYM strip and incubated in a moist chamber tray at 37 °C for 4 h. The degree of enzyme activity was read 5 min after adding one drop of reagent ZYM A (API Products, tris aminomethane 250g, 37% hydrochloric acid 110 ml, laurylsulphate 100g and distilled water up to 1 L, pH 7.6-7.8) and one drop of reagent ZYM B (API Products, fast blue BB, Sigma No. F 0250, 3.5 g and 2-methoxyethanol up to 1 L) to each cupule. A photographic spotlight was held 10 cm above the tray for 10 sec to eliminate any yellow colour produced by an excess of unreacted Fast blue.

The colour of each cupule was compared with a standard colour chart supplied with the kit and graded from 0 to 5 - the higher the score the more intense was the colour. This method was used to test 101 routine clinical pneumococcal isolates.

2.7.5 Nitrophenyl enzyme substrates

Nitrophenyl substrates when cleaved by enzyme release

the yellow coloured nitrophenyl group and can be used as a simple rapid alternative to the conventional sugar media and the APIZYM system to detect enzyme activity. The presence of enzyme activity was investigated using four nitrophenyl enzyme substrates (Koch-Light Laboratories) at their optimum reaction pH 5.4:

p-nitrophenyl-alpha-D-glucosaminide;

p-nitrophenyl-alpha-D-galactosaminde;

p-nitrophenyl-N-acetyl-beta-D-glucosaminide;

o-nitrophenyl beta D-galactosaminide;

To obtain the optimum reaction pH the substrates were made up in citrate phosphate buffer, pH 5.4, prepared from:- 27.8 ml of Solution B (0.2 M disodium phosphate 71.7 g/L) and 22.2 ml of Solution C (0.1 M citric acid 18.2 g/L) mixed with 50 ml of distilled water. Four methods were used to examine the suitability of this approach:-

i) Glass capillary tubes, 3 cm in length, were half filled with the test substrate and an equal volume of the pneumococcal suspension, in PBS, incubated at 37°C for up to 2 h and examined for colour change.

ii) Paper discs, impregnated with the substrate, were placed on the pneumococcal colonies, produced on a CBA plate by anaerobic culture for 18 h, incubated for up to 2 h at 37°C and examined for colour change.

iii) Spectrophotometric assay was made, from 300-600 nm, of the enzyme products from beta-galactosaminide substrate reacted with a pneumococcal culture. The sensitivity of the method was assessed by adding one volume of

pneumoccocal suspension (containing approximately 10^4 cfu) to 3 volumes of ortho nitro-phenyl galactopyranoside solution, incubated at 37 °C for 1 h. The substrates were diluted to 1 in 2, 1 in 4 and 1 in 8 and tested against a suspension of the same strain (containing approximately 10^8 cfu) under the same conditions.

iv) Alpha-galactosidase, alpha-glucosidase and N-acetyl glucosaminidase activity was tested in precipitation tubes in 0.2 ml volumes against four strains at 37°C; colour changes were noted at 2 h and 18 h.

2.7.6 Neuraminidase

The ability of pneumococci to produce neuraminidase (Receptor Destroying Enzyme; RDE), was demonstrated by two methods:-

i) Pan-agglutinability of human blood group O red cells with blood group AB antiserum can occur after exposure to neuraminidase. This effect was tested for by adding 2 ml of either an overnight T-H broth culture or 2 ml of the broth culture, previously lysed with 0.4% (w/v) sodium deoxycholate, to 2 ml of a 1% (w/v) suspension of group O Rh negative red cells in a 0.85% (w/v) sodium chloride solution containing 0.1% (w/v) calcium chloride. A control tube of 2 ml T-H broth and 2 ml of 1% (v/v) red cells was included and all tubes incubated at 37°C for 30 min. After centrifugation at 2000 rpm for 2 min, the supernate was discarded and a volume of the deposit mixed with a volume of AB serum on a microscope slide and observed for

agglutination for 2 min.

ii) The World Health Organization (WHO) method for influenza A virus neuraminidase serum inhibitors was applied to Todd Hewitt broth cultures of three pneumococcal cultures (W, T and C) incubated for 18 h at 37°C. The neuraminidase substrate was fetuin, an ammonium sulphate precipitate of foetal calf serum; the full list of reagents is given in Table 2.5. For the test 0.05 ml volumes of broth culture, phosphate buffer and fetuin were mixed with 0.5 ml of saline and incubated at 37°C for 18 h. When cooled to 20°C 0.1 ml of periodate reagent was added and incubation continued at 20°C for exactly 20 min before 1 ml of arsenite reagent and 2.5 ml of thiobarbituric reagent were added. After thorough mixing, the tubes were placed in a boiling water bath for 15 min, allowed to cool and 4 ml of Warrenhoff's reagent added. The colour of the upper layer produced by vigorous shaking on a mechanical mixer for 2 min and centrifugaton at 1000 rpm for 5 min was removed and read at 549 nm in the spectrophotometer. The value obtained was compared with the turbidity of the original culture measured at 660 nm in the same instrument.

2.7.7 Proteinase

Production of proteinase, after stimulation in a milk-containing broth, was recognised as zones of clearing produced on milk agar.

i) Proteinase stimulation in each of four broths prepared as follows:-

TABLE 2.5

Reagents for the neuraminidase assay

1. Periodate reagent

Sodium meta-periodate	4.28 g
Distilled water	38 ml
Syrupy o-phosphoric acid	62 ml

Stored in a glass stoppered bottle.

2. Arsenite reagent

Sodium meta-arsenite	10 g
Anhydrous sodium sulphate	7.10 g
Distilled water	100 ml
Concentrated sulphuric acid	0.3 ml

3. Thiobarbituric acid

Thiobarbituric acid	1.2 g
Anhydrous sodium sulphate	14.2 g

The above reagents were each made up, once a week, and dissolved in a boiling water bath.

4. Warrenoff reagent

N-butanol	100 ml
Concentrated HCl	5 ml

This reagent was made up fresh as required.

5. Phosphate buffer

A. Sodium dihydrogen orthophosphate (Na_2PO_4)	0.4 M
B. Disodium hydrogen orthophosphate (Na_2HPO_4)	0.4 M

For use 19 ml of solution B was mixed with 81 ml of solution A (final pH 5.9).

a) Tryptose liver digest broth (TL): 1% (w/v) tryptose, 1% (w/v) liver digest and 0.5% (w/v) sodium chloride in distilled water;

b) PTL: the TL formulae without salt and dissolved in PBS;

c) Todd-Hewitt broth (T-H);

d) RCM.

The broths, dispensed in 10 ml volumes, were autoclaved, at 15 lbs/sq in for 15 min, and allowed to cool before 1 ml of separately autoclaved 10% (w/v) skimmed milk in distilled water was added when required. Three strains of pneumococci, serotypes 03, 06 and 23, and a clinically isolated culture of Serratia marsescens were incubated in each of the broths and a control broth, TL without milk, at 37°C. After 2 days incubation, 0.05 ml of each broth was added to a 5 mm well cut in a 2% (v/v) sterile skimmed milk Columbia agar plate and incubated aerobically at 37°C for a further 18 h. All the broths were subcultured on CBA aerobically and anaerobically with CO_2 at 37°C for 18 h.

ii) Milk containing agar plates, prepared from the TL and PTL broths, by the addition of 1% (w/v) agar No.1, were spread with the previous cultures, together with a second control culture, Clostridium perfringens; all plates were incubated anaerobically with CO_2 for 2 days at 37°C. After growth in TL and PTL broths tests for proteinase production were repeated on 2% (v/v) milk TL and PTL agars, under the same conditions as in i).

2.7.8 Haemolysis

Preliminary culture of pneumococci on azide blood agar showed that immediately after anaerobic incubation in CO_2 at 37°C colonies were not haemolytic but if these plates were left at 4°C for 3 h, 10 of the 14 strains tested showed haemolysis. The other four strains were also haemolytic after a further 3 h at room temperature. Haemolysis was not seen with the same strains if cultured on CBA and experiments were undertaken to examine the cause of this effect.

i) Four horse blood containing media, Columbia agar base, with and without 0.02% (w/v) sodium azide and tryptose blood agar base (the base for azide blood agar) with and without 0.02% (w/v) sodium azide, were seeded with eight pneumococcal strains and incubated anaerobically with CO_2 at 37°C for 18 h. The plates were removed from the anaerobic culture jar, placed at 4°C for 3 h and examined for zones of haemolysis against an illuminated background.

ii) Reductions in the azide content of azide blood agar (Difco) were sought to increase the size of colonies produced on the medium without loss of the haemolysis zones. The eight test strains were cultured as in experiment i) but on three formulations of azide blood agar, containing either 0.02%, 0.01% or 0.005% (w/v) sodium azide, and 5% (v/v) horse blood.

iii) Todd-Hewitt medium, recommended for the production of haemolysins by the beta-haemolytic streptococci, was prepared as two forms of Todd-Hewitt agar with 1% (w/v)

agar No.1, 5% (v/v) horse blood and with (AzT-H) and without (T-H) 0.005% (w/v) sodium azide. The above eight strains were cultured on the T-H, AzT-H media, azide blood agar and CBA under anaerobic conditions with CO_2 at 37°C for 18 h.

iv) Haemolysis of sheep and horse red cells: Tryptose base agar plates, with and without 0.005% (w/v) sodium azide, and containing 5% (v/v) of sheep or horse blood and CBA with 5% (v/v) horse blood were seeded with 12 pneumococcal strains and incubated anaerobically with CO_2 at 37°C for 18 h. Cultures were made of six further strains on the five media under aerobic conditions with and without CO_2 and anaerobically with CO_2 for 18 h at 37°C. The strains were of serotypes 03, 06, 09, 14, 23 and a non-capsulate isolate.

v) Haemolysis of human and horse red cells: human blood group O plates were prepared, with and without 0.005% (w/v) sodium azide and also plates of the same basal medium but containg 5% (v/v) horse blood, to examine the type of haemolysis formed by three strains, serotypes 19, 23 and on-capsulate isolate (strains T705, W338 and W317 respectively), incubated under the standard double zone haemolysis conditions.

vi) Camp reaction. Four strains of beta-haemolytic streptococci (Lancefield group B) obtained from the Jessop Hospital (strains RS11, Q90, G64 and I49) were used in the Camp reaction test on Tryptose blood agar prepared with 5% (v/v) group O negative human blood and the same medium

previously spread with 1 ml of 0.05% (w/v) sterile sodium azide solution. Three strains of pneumococci, serotypes 04 (W155), 06 (W148) and 19 (T328) and a control strain of Cl. perfringens were streaked on to two plates of each medium and two of the Group B isolates streaked, 5 cm apart, across the plates at right angles to the pneumococcal inocula. Three further strains, serotypes 18 (W261), 19 (T345) and 23 (T587) with the control were cultured on to two similar sets of plates with the other two Group B isolates spread at right angle. All plates were incubated at 37°C, one set anaerobically with added CO_2 and the other aerobically with CO_2.

2.8 DYE INHIBITION STUDIES

Some pneumococci were found to be inhibited by methylene blue (Section 3.3) and bromothymol blue (Section 3.5.2), the effects of these and other "dyestuffs" were studied in an attempt to demonstrate differential sensitivity patterns to growth inhibitors and therefore recognise variations between strains.

2.8.1 Inhibition of broth cultures by bromocresol purple, toluidine blue and methylene blue

The ability of serotype 33 pneumococcal strain to grow in T-H broths containing bromothymol blue (0.2% (w/v)), bromocresol purple (0.2% (w/v)), toluidine blue (0.1% (w/v)) and methylene blue (0.1% (w/v)) each diluted to 1 in 100, 1 in 1000 and 1 in 10000 in the medium was

tested as a preliminary study. The twelve dye broths and a control T-H broth were lightly seeded with the pneumococcus and incubated for 18 h at 37°C.

2.8.2 Inhibition of growth on dye-containing agar slopes

The method was used to provide a concentration gradient of potential inhibitory agents to simplify selection of suitable dyes and concentrations.

i) Sixteen dyes tested against four strains. Stock solutions were prepared, in distilled water, from bromothymol blue (BTB), bromocresol purple (BP), congo red (CR), methyl red (MR), and phenol red (PR) at concentrations of 0.2 % (w/v); cresol red (RR), neutral red (NR), janus green (JG), nile blue (NB), crystal violet (CV), methylene blue (MB), toluidine blue (TB) and phenolphthalein (PP) at 0.1 % (w/v); saffronin (SF) and coomassie blue (CB) at 0.5 % (w/v) and from malachite green (MG) at 0.02 % (w/v). For use the dye solutions were diluted 1 in 10 in sterile water and 1 ml was mixed with 9 ml of molten T-H agar. Plates were poured and allowed to set at an angle to form a slope whose thinnest edge just reached the raised edge of the base. When the dye agar had set the plate was returned to the level and 9 ml of T-H agar, without dye, was poured on top. Four test strains, serotypes 09 (T891), 16 (T895) and 34 (T904 and T918), were streaked across the agar, from the highest to the lowest dye concentration area. The seeded plates were incubated at 37°C for 18 h anaerobically with CO_2. The presence of

growth and its length across the plate was noted.

ii) Testing of nine of the above sixteen dyes and a further nine dyes against six pneumococcal strains by the agar slope method.: solutions of the dyes MB, TB, NR, NB, CB, CV, orange G (OG), luxol fast blue (LB), Best's carmine (BC), celestin blue (CEB), brilliant blue (BB) and rhodamine blue (RB) were made at 0.1% (w/v) concentrations in distilled water. Acridine orange (AO), alcian blue (AB), MG and JG were prepared at 0.2% (w/v) and BTB and eosin-yellowish (EY) as 0.05% (w/v) solutions. A 1 in 10 dilution of these solutions, in 10 ml of sterile molten agar, was sloped in a petri dish and 10 ml of plain agar poured as the reverse slope. The six test strains, four from the previous experiment and strains T966 (serotype 33) and strain W984 (serotype 14), were streaked along the gradient, incubated anaerobically for 18 h at 37°C and the length of growth produced measured.

iii) Inhibition studies by the agar slope method of ten dyes selected from those previously tested. Dye solutions were prepared at 0.1% (w/v) for NB, TB, BTB, JG and EY; at 0.00002% (w/v) for MG; at 0.05% (w/v) for MB; at 0.2% (w/v) for AB; 0.05% (w/v) for AO and at 0.002% (w/v) for CV. These dye solutions were diluted 1 in 10 in agar and sloped plates prepared. The six strains, previously used, were seeded on to the plates, incubated at 37°C for 18 h, and the extent of growth inhibition along the line of the streak measured.

2.8.3 The effects of aerobic and anaerobic culture on dye inhibition of growth

Pairs of CB plates were seeded with the four test strains, serotypes 09 (T891), 16 (T895) and 34 (T904 and T918), and nine wells, 5 mm in diameter,were cut in the agar and carefully filled with solutions of the dyes at the concentrations used in Section 2.8.2, ii. The dyes tested were BTB, MB, TB, NR, JG, NB, MG, CB and CV. All plates were incubated at 37°C for 18 h, one from each pair under aerobic conditions and the other anaerobically, both with added CO_2. Zones of growth inhibition around the wells were measured.

2.8.4 Paper strip method for dye inhibition studies

Dye impregnated paper strips were prepared by adding 0.3 ml of a 0.1% (w/v) dye solution to a 3 x 1 cm square of Whatman's No.1 filter paper. The strips, when dry, were placed at right angles to each of the six pneumococcal test strains streaked across CBA plates, before incubation under anaerobic conditions at 37°C for 18 h. The cultures and the dyes tested were those used in Section 2.8.2 ii together with luxol fast blue, nigrosin, naphthol yellow, orange G, rhodamine B, saffron, Best's carmine, celestin blue, brilliant blue, acridine orange, alcian blue and eosin yellowish.

2.8.5 Inhibition studies by the agar plate containing method

A progressive series of 19 biological titrations was performed in stages starting with the ten dyes selected following the agar slope results. The testing was performed on batches of eight to ten dyes but the concentration used and the number of each stage are presented in Tables 2.6 to 2.9 for clarity, and the pneumococcal strains tested in Table 2.10. Two of the ten dyes, eosin-yellowish and alcian blue, not included in the tables only featured in the early tests and were then discarded. Eosin yellowish was tested in Stage 1 at 1.0 and 0.5 ml of a 0.4% (w/v) solution and in Stage 2 at the same volumes from a 0.033% (w/v) solution; alcian blue was tested in Stage 1 at 1.5 and 1.0 ml of a 0.4% (w/v) solution.

The test procedure for all experiments was to include the dye in 15 ml of T-H agar, to spread the organism over approximately 1 cm^2 of the plate and then to incubate aerobically with added CO_2 for 18 h at 37°C. Growth was recorded as presence, absence or as reduced colony size.

ii) Batch testing for reproducibility of inhibition results (Stage 17): using the dye concentrations given in Tables 2.8 and 2.9 four plates were poured for each dye solution by adding 12 ml of the diluted dye to 48 ml of T-H agar. A series of four experiments were performed on consecutive days.

Day 1: baseline testing of the dyes by the standard method

TABLE 2.6

Dye concentrations and volumes added to Todd-Hewitt agar plates in the dye inhibition Stages 1-8. A

Stage No.	Dye, concentration (%) and volume (ml) added			
	Bromothymol blue	Nile blue	Acridine orange	Crystal violet
1	0.02 (1.0, 0.5)	0.10 (1.0, 0.5)	0.10 (1.5, 1.0)	0.01 (1.0, 0.5)
2	0.02	0.20	0.033	0.033
	(1.0 and 0.5 ml was taken from each dye)			
3	0.02 (0.8, 0.6)	0.10 (0.3, 0.1)	0.033 (0.8, 0.6)	0.01 (0.8, 0.5)
4	0.01	0.025	0.04	0.002
	(1.0 ml was taken from each dye)			
5	0.01 (0.8)	0.10 (0.3)	0.04 (1.04)	0.002 (0.8)
6	0.01 (0.9)	0.025 (0.4)	0.04 (0.4)	0.002 (1.2)
7	0.0033	0.0035	0.008	0.001
	(3.0 ml was taken from each dye)			
8	0.0033 (3.4)	0.0035 (3.6)	0.008 (3.4)	0.001 (3.4)

In stages 1-6 the volume of dye solution was added to 15 ml of Todd-Hewitt agar. In stages 7 and 8 the volume of dye solution was added to 12 ml of the agar.

TABLE 2.7

Dye concentrations and volume added to Todd-Hewitt agar plates in the dye inhibition Stages 1-8. B

Stage No.	Dye, concentration (%) and volume (ml) added			
	Janus green	Methylene blue	Malachite green	Toluidine blue
1	0.01 (1.0, 0.5)	0.10 (0.5, 0.2)	0.002 (1.5, 1.0)	0.10 (0.5, 0.2)
2	0.20	0.01	0.002	0.01
	(1.0 and 0.5 ml was taken from each dye)			
3	0.02 (0.8, 0.6)	0.01 (1.5, 1.2)	0.002 (0.7, 0.5)	0.01 (0.8, 0.6)
4	0.02 (0.8)	0.01 (0.7)	0.00002 (1.0)	0.01 (0.8)
5	0.01 (1.0)	0.01 (0.9)	0.00002 (0.7)	0.01 (1.0)
6	0.02 (0.4)	0.01 (0.4)	0.00002 (0.8)	0.01 (0.4)
7	0.0028	0.0014	0.000005	0.0020
	(3.0 ml was taken from each dye)			
8	0.0028 (3.4)	0.0014 (3.4)	0.000005 (2.6)	0.0020 (3.4)

In Stages 1-6 the volume of dye solution was added to 15 ml of Todd-Hewitt agar. In Stages 7 and 8 the volume of dye solution was added to 12 ml of the agar.

TABLE 2.8

Dye concentrations and volume added to Todd-Hewitt agar plates in each dye inhibition Stages 9-19. A

Stage No.	Dye and concentration (%) added			
	Bromothymol blue	Nile blue	Acridine orange	Crystal violet
9	0.003	0.003	0.013	0.0008
10	0.00375	0.001	0.000325	0.0008
11	0.005	0.0015	0.0044	0.00026
12	0.005	0.0015	0.0046	0.0004
13a	0.005	0.0015	0.0043	0.0006
b	-	-	0.0046	0.0004
14a	0.005	0.0015	0.0043	0.00044
b	-	0.0010	0.00325	0.00026
15	0.0043	0.00107	0.00406	0.00296
16	0.0043	0.00107	0.00406	0.00296
17 18 19	0.0043	0.00107	0.00406	0.00245

In all of the above stages 3 ml of the dye solution was added to 12 ml of Todd-Hewitt agar.

TABLE 2.9

Dye concentrations and volume added to Todd-Hewitt agar plates in the dye inhibition Stages 9-19. B

Stage No.	Dye and concentration (%) added			
	Janus green	Methylene blue	Malachite green	Toluidine blue
9	0.0027	0.0013	0.000005	0.0013
10	0.0038	0.00043	0.000005	0.000325
11	0.00348	0.00052	0.000010	0.00026
12	0.0036	0.00059	0.000025	0.00028
13a	0.0038	0.00059	0.000025	0.000325
b	0.0036	0.00052	0.000050	0.00028
c	0.00348	-	-	0.00026
14a	0.00348	0.00059	0.000035	0.000236
b	0.0027	-	0.000050	0.000216
15	0.00225	0.00060	0.000040	0.000163
16	0.00225	0.00060	0.000040	0.000163
17				
18	0.00290	0.00060	0.000040	0.000163
19				

In all of the above stages 3 ml of the dye solution was added to 12 ml of Todd-Hewitt agar.

TABLE 2.10

Pneumococci tested in the agar plate dye inhibition stages.

Stage No.	Serotype (number of strains)
1	
2	04, 06, 09, 14, 16, 33 and 34 (2)
3	
4	01, 03, (3), 06 (6), 07, 09 (5), 14 (2), 15 (2), 16 (3), 19 (4), 22, 23 (3), 31 (2), 33 (2), 34 (3), a pool I a non-capsulate variant
5	03 (3), 14 (2), 16, 19(2), 23 and pool I
6	
7	01, 03, 06, 09, 14 (2), 16, 19, 23, pool I and a non-capsulate variant
8	03, 14 (2), 16, 19, 23 and pool I
9, 10	
11, 12	
13, 14	03, 04, 09, 14 (2), 19 and 23 (2)
15, 16	
17	
18	03 (3), 06 (4), 09 (2), 11, 14 (4), 19 (9) and 23 (4)
19	03 (5), 06 (4), 08, 09 (2), 11, 14 (8), 15 (2), 19 (5) and 23 (8)

with the eight test strains;

Day 2: two series of plates were produced: a) the diluted dye solutions were autoclaved in the T-H agar: b) the dye solutions were added after sterilisation of the agar;

Day 3: repeat testing of the standard strains as on Day 1.

iii) Testing of pneumococcal strains for sensitivity to the dyes and potassium tellurite (Stages 16 and 18). Potassium tellurite agar was prepared for both experiments by adding 1.2 ml of a 0.1% (w/v) solution of potassium tellurite and 1.8 ml of sterile distilled water to 12 ml of T-H agar.

iv) Dye inhibition testing and colonial appearance on Mitis-salivarius agar of 36 strains (Stage 19). These strains were cultured on the dyes given in Tables 2.9 and 2.10. They were also tested for colonial effects when grown on Mitis-salivarius agar (Section 2 8.9) and for CO_2 requirement by the routine method (Section 2.4.6).

2.8.9 Colonial appearance on Mitis-salivarius agar.

Originally described as a selective medium for S. mitis and S. salivarius and the differentiation of dextran producing strains preliminary studies with this medium in the selective isolation of pneumococci gave novel colonial forms.

i) Mitis-salivarius agar (M-S) (Difco) and TYC agar (LabM), a similar formula for the isolation of dextran producing organisms, were seeded with 10 isolates, serotypes 01, 14, 17, 19 (three strains), 23 (three strains) and 34, cultured aerobically and anaerobically at 37 °C for 18 h and examined

for colonial size and appearance. A MOC (made in the U.S.S.R) plate microscope was used to examine the culture plate by oblique trans illumination at 12.5 x 0.6 and 12.5 x 2 magnifications.

ii) The effect of sucrose on the production of the colonial forms of six strains, serotypes 09, 14, 19 (two isolates) and 23 (two isolates), was examined on M-S and M-S prepared without sucrose (S-M-S), cultured anaerobically at 37°C for 18 h.

iii) The effects of base medium and trypan blue on the colony types were investigated in twenty four strains grown on M-S, S-M-S, T-H containing 1% of 0.75% (w/v) trypan blue, RCM with 1% of 0.75% (w/v) trypan blue and CB agars, anaerobically at 37°C for 18 h. The pneumococci were of serotypes 01, 06 (three strains), 08, 09, 14 (four strains), 17, 19 (five strains), 23 (six strains), 34 and serogroup I.

iv) The next 70 routinely isolated pneumococcal strains were tested for colonial appearance on M-S, at 37°C for 18 h.

v) To examine the basis for the irridescence effect two positive and a negative strain were grown in M-S broths and on M-S agar at 37°C for 18 h. The growth in the broth was centrifuged and a drop of the deposit observed under the plate microscope on a glass slide. Colonies, scraped directly from the M-S agar and as a heavy suspension in PBS were similarly observed under the microscope.

vi) A further 60 isolates were tested on M-S agar under

the same conditions, two years after the original irridescence experiments.

vii) M-S agar, containing potassium tellurite is the recommended formula for selective streptococcal culture. Plates of M-S were prepared to include 1 ml of 1% potassium tellurite/litre of medium of both the Oxoid and Difco formulae, and tested for growth inhibition of the eight cultures used in the dye inhibition studies (Section 2.8.5) incubated anaerobically at 37°C.

2.9 SENSITIVITY TESTS

The increased reports of antibiotic resistance in the pneumococcus warranted a reappraisal of laboratory sensitivity testing procedures for this organism. Tetracycline and co-trimoxazole resistance has become common but strains tolerant to chloramphenicol and the penicillins are still rare in Britain. To facilitate the early recognition of antibiotic tolerant strains it was necessary to investigate aspects of the in-vitro tests for pneumococcal susceptibility.

i) Media and atmosphere effects on the interpretation of sensitivity results were investigated for two strains, by the disc diffusion method, against penicillin, 2 units; tetracycline, 10 µg; trimethoprim, 2.4 µg, and co-trimoxazole, 25 µg. These were tested on Isosensitivity agar (IS), IS plus blood heated as for HBA (CIS) and on IS plus 7% horse blood (BIS) under anaerobic and aerobic conditions with added CO_2 at 37°C for 18 h.

ii) Sensitiviy testing of 22 pneumococcal isolates by the antibiotic disc and agar containing methods using lysed blood sensitivity agar (LIS) was as follows:

a) Discs of tetracycline (5 µg) and trimethoprim (2.5 µg) were placed on LIS previously seeded with the test strain.

b) Antibiotic plates containing 1 µg/ml trimethoprim and a second series containing 1 µg/ml of tetracycline were prepared by adding the drug when the agar had cooled to approximately 55°C. A loopful of a dilute suspension of the test culture ,in PBS, was spread over 1 sq cm of these plates and incubated at 37°C for 18 h. The performance of the antibiotics and the agar was controlled by Staph. aureus (NCTC 6571, SOX).

c) Plates containing trimethoprim at 2.5, 1.0 and 0.75 µg/ml were poured and a loopful of a PBS suspension of 20 of the above pneumococcal isolates spread over 1 sq cm of the agar and incubated at 37°C for 18 h.

Repeat tests were performed by the above method on the resistant cultures after incubation in RCM broth for 4 h.

iii) Extended series of antibiotic disc susceptibility tests. A known penicillin tolerant culture obtained from the Microbiology department at the Royal Hallamshire Hospital, Sheffield and 12 test strains from this department were allowed to grow in T-H broth for 4 h at 37°C in the CO_2 incubator. Each strain was spread on to BIS and LIS agars with a rotary plating machine; SOX served as the control organism. Commercial antibiotic discs were placed at the junction of the two seeded areas; the

antibiotics and concentrations used are given in Table 2.11. Zone diameters were measured after incubation at 37°C for 18 h.

The tests for mecillinam and cloxacillin were repeated for six further recent clinical isolates, the previous resistant strain and two resistant cultures supplied by the Streptococcal Reference Laboratory, Colindale.

iv) Sensitivity to Tetracycline and Trimethoprim by the disc diffusion method of 50 pneumococcal strains was performed on heated blood sensitivity agar against tetracycline, 5 µg, and trimethoprim, 2.5 µg. The test strain was applied to the central 6.5 cm diameter area of the plate on a rotary plater, the control organism SOX was spread on the remainder of the plate and the discs placed on the junction of the two seeded areas. After incubation at 37°C for 18 h the inhibition zones of the test strains were measured.

TABLE 2.11

The antibiotics tested

Paper discs impregnated with antibiotics in the following concentrations were supplied by Mast (except where indicated).

Penicillin	1.5	units
Tetracycline	10	µg
Trimethoprim	2.5	µg
Co-trimoxazole	25	µg
Sulphadimidine	200	µg
Mezlocillin	30	µg
Azlocillin	75	µg
Chloramphenicol	2	µg
Erythomycin	5	µg
Cefotaxime	30	µg
Ticarcillin	75	µg
Cefuroxime	30	µg
Mecillinam	10	µg
Ceftazidime	10	µg
Cloxacillin	5	µg
Cefamandole	75	µg (Oxoid)

2.10 COLLECTION OF EPIDEMIOLOGICAL DATA

A daybook register of the samples positive for pneumococci was generated in the laboratory and contained the following information:-

a) Patient's name, sex, registration number, date of birth, ward and consultant;

b) The type of sample, and its laboratory number together with clinical details of the patient were taken from the laboratory request form;

c) To these were added the sensitivity pattern, capsular type and requirement for CO_2.

To complete the patients' details omitted from the laboratory request form, patients' medical records were obtained from the Medical Records Department, Accident and Emergency Department and the post mortem files from the Histology Department, Children's Hospital, Sheffield (with the permission of the Consultants responsible for the patient). The patients' home address and the reason for their admission or visit to the hospital were taken from these notes.

The details of those patients who had been admitted to the Children's Hospital or the Thornbury Annexe were processed by the Medical Records Department for analysis of bed occupancy and usage, using the Trent Regional Computer. A print out of each admission was produced and the appropriate episode matched with the laboratory details. A special computer file for this project and the material to be entered was coded on to specially produced computer

input documents; Document A for In-patients and Document B for Out-patients, Accident and Emergency patients and post mortem cases (Appendix C). Document B had an extended patient details section for the information comparable to that on files for the In-patients (Document A).

The two groups of information were merged into one file for subsequent analysis of laboratory and patient data.

3.1 GASEOUS ENVIRONMENT AND GROWTH

The clinical recovery of the pneumococcus can be affected by the gaseous atmosphere used as much as by the growth media. A number of investigations were undertaken to examine the factors involved.

3.1.1 Comparison of anaerobic and aerobic culture for the isolation of pneumococci from nasal swabs

Anaerobic culture with added CO_2 when compared to aerobic culture without CO_2 gave a 100% increase in pneumococcal isolations (Howden, 1976). It was, therefore, necessary to examine the differences between aerobic and anaerobic culture when both atmospheres contained added CO_2. Of the 89 specimens tested in the present study, pneumococci were isolated from both anaerobic and aerobic culture of 14, and in eight of these 14 samples the broth cultures also yielded pneumococci. Two further broths were positive for pneumococci but the parallel direct plate cultures were negative. The number of colonies seen under both conditions were similar however, those produced anaerobically with CO_2 were much larger than those produced aerobically. The numbers and colony size of the majority of the other bacteria present on aerobic cultures were reduced or absent when cultured anaerobically. Anaerobic incubation with added CO_2 did not show a numerical advantage over aerobic incubation with CO_2 but proved to be a simple and effective selective procedure that enhanced

recognition both by the production of large mucoid pneumococcal colonies and by the suppression of many of the mixed flora commonly seen in cultures of respiratory specimens.

3.1.2 The isolation of pneumococci under different gaseous conditions

The atmosphere employed and the eight capsular serotypes tested are given in Table 3.1. None of the strains grew after diluting 1 in 100 with water. The Pseudomonas culture, the negative control organism for anaerobiosis, grew in both the N_2 and the $H_2/CO_2/N_2$ atmospheres indicating that some O_2 remained in these jars. The N_2 atmosphere showed no advantage over aerobic or candle jar. The $H_2/CO_2/N_2$ mixture alone gave similar yields to H_2.

The results (Table 3.2) of dilution in PBS and T-H broth showed that the serotype 06 strain gave similar results in PBS and T-H under both incubation conditions. After dilution in water however anaerobic culture, with CO_2, gave higher yields than the aerobic atmosphere. The serotype 18 culture gave scant growth anaerobically with CO_2 and failed to grow under aerobic conditions regardless of the diluent. The results of the repeat gaseous test of serotypes 18, 19, 23 and a further strain serotype 19 are given in Table 3.3. All cultures grown anaerobically with CO_2 gave larger colonies and comparable or higher viable counts than in the other environments, except for

TABLE 3.1

Pneumococcal growth on Columbia blood agar plates under different gaseous atmospheres. A

Pneumo. Type	Colony count and size after incubation in					
	H_2/CO_2	H_2	N_2	$H_2/CO_2/N_2$	O_2	CJ
19	2	2	-	-	-	-
06	8 L	20 M	15 S	8 S	8 S	15 S
04	10^7 L	10^7 M	10^7 S	10^7 S	10^7 S	10^7 S
18	10^7 L	10^7 M	10^5 S	10^7 M	10^1 S	10^7 S
03	10^7 L	10^7 L	10^7 L	10^7 L	10^5 L	10^3 L
23	10^7 L	10^7 M	10^7 S	10^7 M	10^7 S	10^7 S
19	10^7 L	10^7 M	10^5 S	10^5 M	10^7 S	10^7 S

H_2 = hydrogen

N_2 = nitrogen

CO_2 = carbon dioxide

O_2 = oxygen

L = large colonies

M = medium colonies

S = small colonies

CJ = candle jar

TABLE 3.2

Anaerobic and aerobic culture after dilution in water, phosphate buffered saline or Todd-Hewitt broth of two pneumococcal cultures

Sero type	Atmos	Density of growth after dilution in								
		Water			PBS			Todd-Hewitt broth		
		10	50	100	10	50	100	10	50	100
06	AnO_2+CO_2	++	-	-	++	+	80	++	+	30
	O_2	+	-	-	++	+	80	++	+	30
18	AnO_2+CO_2	10	-	-	9	4	1	12	4	2
	O_2	-	-	-	-	-	-	-	-	-

Atmos = atmosphere used for culture

AnO_2+CO_2 = anaerobic atmosphere with 10% CO_2

O_2 = aerobic atmosphere

++ = moderate semi-confluent growth

\+ = less confluent growth

\- = no growth

The figures in the AnO_2+CO_2 and the O_2 rows indicate the number of colonies recorded.

TABLE 3.3

Pneumococcal growth on Columbia blood agar plates under different gaseous atmospheres. B

Pneumo. Type	Colony count and size after incubation in					
	H_2/CO_2	H_2	N_2	$H_2/CO_2/N_2$	O_2	CJ
19	10^7	10^5	10^5	10^5	10^7	10^7
18	10^5	10^3	10^3	10^5	0	10^7
23	10^7	10^5	10^7	10^7	10^5	10^7
19	10^7	10^5	10^5	10^7	10^6	10^6

H_2 = hydrogen
N_2 = nitrogen
CO_2 = carbon dioxide
O_2 = oxygen
L = large colonies
M = medium colonies
S = small colonies
CJ = candle jar

the serotype 18 strain. This strain was of further interest with its need for CO_2, it failed to grow or gave reduced viable counts in those atmospheres without CO_2 i.e. aerobic, hydrogen or nitrogen.

3.1.3 Direct isolation and gaseous environment

The results from the specimens of patients suspected of carrying pneumococci (Section 2.4.5), incubated under different atmospheres, gave moderate or heavy mixed growths of other respiratory flora as well as pneumococci. None of the pneumococcal isolates (Table 3.4) grew aerobically without extra CO_2. The pneumococcal colonies produced by culture in a H_2 atmosphere, from serotypes 18 and 06 gave the same viable count as under anaerobic conditions with CO_2 although the colony size was greatly reduced. Two serotype 09 isolates required increased CO_2 levels for growth even when the O_2 tension was reduced since they failed to grow in either a H_2 or a N_2 atmophere. The extent of the CO_2 requirement merited further study.

3.2. THE REQUIREMENT FOR CARBON DIOXIDE

The essential requirement for CO_2 shown by certain pneumococci was investigated to establish its importance in the isolation of clinical strains.

3.2.1 Carbon dioxide requirement of individual strains grown on Columbia blood agar

None of the four strains, serotypes 03, 09, 19 and

TABLE 3.4.

The effect of different culture atmospheres on the isolation of pneumococci from clinical specimens

Pneumo. Type	Viable count (cfu/ml) after incubation in						
	H_2/CO_2	H_2	N_2	$H_2/CO_2/N_2$	CO_2	O_2	CJ
06	10^7	10^7	4	10^7	10^7	0	10^7
18	10^7	10^7	10^3	10^7	10^7	0	10^7
09	10^7	0	0	10^5	10^7	0	10^7
09	10^5	0	0	10^5	10^7	0	10^7

H_2 = hydrogen
N_2 = nitrogen
CO_2 = carbon dioxide
O_2 = oxygen
L = large colonies
M = medium colonies
S = small colonies
CJ = candle jar

33, grew aerobically without CO_2, but all strains grew in the other three environments (Section 2.4.6). The pneumococcal colonies grown in the CO_2 incubator were larger and more convex than the smaller more collapsed colonies seen in the candle jar cultures. The improved colony appearance after candle jar culture over aerobic culture could have been produced by the reduction in oxygen within the jar as the candle burnt rather than the increased CO_2 level produced by the combustion. Therefore the tests were repeated but with a further series of plates for a second candle jar which included a bottle of 40% KOH solution to absorb the CO_2 produced. The absorbed candle jar plates gave only small numbers of minute colonies (less than 0.5 mm in diameter) with serotypes 09, 19 and 33; the serotype 03 strain failed to grow. When the absorbed candle jar plates were reincubated anaerobically with CO_2 for a further 18 h at 37°C the serotype 03 strain still did not grow; the other three strains gave a heavy growth of large mucoid colonies, larger than those seen after 18 h incubation anaerobically with CO_2. The requirement for CO_2 could be even more apparent on primary isolation rather than after subculture on laboratory media and the repeat nasal swabs obtained from the two patients previously shown to carry pneumococci were obtained to examine this aspect of pneumococcal isolation. The pneumococcus isolated from one of the patients (Patient A) was present on CBA in moderate numbers after anaerobic incubation with CO_2, in

small numbers anaerobically without CO_2 and failed to grow aerobically either with or without CO_2. A similar pattern was observed on HBA except that smaller numbers of colonies were seen anaerobically with CO_2 and only scant numbers when cultured anaerobically without CO_2.

In the second patient (patient B) pneumococci were present in larger numbers anaerobically with CO_2 and smaller numbers and reducing colony sizes were observed in a progression down to aerobic culture without CO_2. A similar pattern was found on HBA but with lower numbers anaerobically progressing down to no growth aerobically without CO_2. Pneumococcal colonies on HBA that had been incubated anaerobically did not show the characteristic greening and bleaching observed on that medium when incubated aerobically.

Growth on CBA and Sheep blood Todd-Hewitt/liver digest agar (ST-HL) of these four strains, under the same atmospheres, showed that one of the sertype 09 strains could grow aerobically without extra CO_2 on ST-HL but not CBA. This strain also failed to grow on CBA plates when sheep blood replaced the horse blood.

3.2.2 The requirement for CO_2 related to pneumococcal capsular serotype

The four strains used in Section 3.2.1, belonging to serotypes 06, 09 (two isolates) and 18 all failed to grow aerobically without extra CO_2 even after 10 subcultures on artificial media. The simple method adopted for assessing

the CO_2 dependance of recently isolated cultures (Section 2.4.6) was used from April to July 1979 on 103 strains of which 14 (13.6%) were shown to need extra CO_2 for growth *in vivo*. Of these 14 strains, 11 were either serotype 09 or 16. When the survey was extended (Table 3.5) from October 1979 to November 1980, a further 27 CO_2 requiring cultures were found in the 298 strains tested, 11 of which were of these two serotypes. Further details of CO_2 dependance and serotype are included in Section 5.

3.3 THE EFFECTS OF MEDIA ON GROWTH

Growth in a number of media and their modifications was undertaken to improve recovery of the pneumococcocci from clinical samples and viability of the organisms for identification and characterisation studies.

3.3.1 Growth in Reinforced Clostridial broth

The strains of serotypes 06, 08, 14 and the two cultures of serotype 03 all grew in the two RCM formulae without dye but not in the medium with methylene blue. The dye containing broths were blue in the upper 5-10 mm (oxidised) and colourless (reduced) below this level. The broths without agar gave a diffuse light turbidity and the medium with agar a 3-5 mm wide band of heavy growth approximately 7-12 mm below the surface of the fluid. The depth of this band corresponded to the depth at which the methylene blue was reduced in the dye containing medium.

TABLE 3.5

Distribution of CO_2 requirement related to pneumococcal serotype

Serotype	Total number of strains tested	CO_2 dependant strains
09	24	18
01	4	1
18	17	2
42	1	1
19	76	7
16	6	4
23	64	2
03	20	1
28	1	1
I	4	1
15	14	1
06	85	1
04	15	1
Others	69	0
Totals	401	41

Methylene blue appeared to be inhibitory to the pneumococci tested; this phenomenon was further studied in Section 4.7.

3.3.2 Growth enhancement using liver digest

Heavier growth of the serotype 03 strain occurred in each of the media with LD than in the corresponding medium without this supplement. The amount of LD necessary for optimal enhancement, examined by double dilution, showed that turbidity of the broths increased progressively from the control tube without LD to the tube containing 1% LD. Thus 1% was taken as the routine amount of supplement for broth cultures.

3.3.3 The effect of pH change on pneumococcal growth

i) Todd-Hewitt and Reinforced Clostridial media: the initial pH values of T-HL and RCML were 7.8 and 6.8 respectively. After 24 h incubation with a serotype 03 strain, isolated from a patient with an ear infection, these values had fallen to 6.6 and 5.4 and after 72 h to 6.4 and 5.1 respectively. The T-HL had become almost clear within 72 h and the RCML remained very turbid. The supernates titred against serotype 03 protein-A reagent gave values of 3000 after both 24 and 72 h in the T-HL culture and 2000 and then 6000 from RCML. The deposits of both broths gave titres of 100 after 72 h.

A further four cultures, serotypes 03, 04 and 14 (two strains), obtained from respiratory sites, and the above

serotype 03 strain, were incubated for 48 h in RCML and the appearance of growth, the pH and glucose remaining recorded. The following results were obtained in three strains (serotypes 03, 04 and 14) diffuse turbidity, a final pH of 6.6 and absence of glucose, when tested with Dextrostix, were produced; in the other serotype 14 culture the growth was granular and settled out with a final pH of 7.0, a large amount of glucose remained. The initial serotype 03 strain produced a diffuse turbidity as did the respiratory serotype 03 isolate, but the former gave a final pH of 5.8 with some glucose remaining and the latter a pH of 6.6 with all the glucose exhausted.

ii) The effect of the pH of T-H, RCM and Columbia agars on the growth of the three cultures, serotypes 01, 03 and 31 on the eight media (Section 2.6.3) was to give reduced colony size (<0.5 mm) on T-H and RCM at pH 6.8. At pH 7.3, on T-H and CA media, moderate sized colonies, approximately 1.5 mm were observed; large colonies, approximately 2-3 mm formed on RCMA with serotypes 01 and 31 and the serotype 03 strain gave moderate sized colonies. The T-H and RCM media at pH 7.8 gave small colonies < 1 mm. No growth was found on either T-H or RCM agars at pH 7.8 containing bromothymol blue which had been included to demonstrate acid production from the glucose in the formulae. This finding was further investigated and is reported in the dye inhibition section (Section 4.7). The colonies of the serotype 03 strains were generally reduced anaerobically when compared to aerobic culture, in

contrast to the other cultures in which colonies were much larger anaerobically.

3.3.4 Yield and viable count on broth and plate culture

The viable counts and optical density tests (Section 2.6.4) showed that although the turbidity of T-H was only 0.03 to 1.0 unit less than that of the plate culture the viable count was only a quarter to a half of the plate viable count. The values found at 260 nm were too small for this absorbance to be used for further strains. The growth after plate culture was adopted for study following 18 h incubation at 37°C since the viable count was significantly higher.

3.3.5 The effects of cysteine and sodium bicarbonate on growth in Todd-Hewitt broth

Turbidity was evident in all tubes (Section 2.6.5) at 5 h. At 18 h the row of broths containing both cysteine and bicarbonate were more turbid than their respective tubes in the cysteine row. With cysteine only the 0.25% broth was the most turbid, this density gradually decreased to the 0.03% tube; this last tube was still more turbid than the control tube.

The control tube and those with cysteine only were strongly positive and the bicarbonate tubes very weakly positive for glucose. Viable counts and final pH of the control tube, 0.025% cysteine and the 0.025% cysteine with bicarbonate broths were 4×10^6 cfu/ml, pH 6.5; 2.5×10^8, pH 7.2; 2 x

10^8, pH 8.3 respectively.

3.3.6 Neutralised cysteine and sodium bicarbonate.

The serotype 23 strain performed equally well in all 4 T-H media; the growth of the serotype 08 culture was inhibited by bicarbonate (T-HB) and enhanced in T-HC (neutralised cysteine); the serotype 19 strain was inhibited by cysteine and bicarbonate in combination, T-HCB.

No advantage was found for these strains except that the medium plus bicarbonate had a pH of 7.4 - 8.0 and was consistently depleted of glucose; in T-HLH the pH was less than 6.8 and only 2 of the 6 organisms removed all the glucose; in T-HC this only occurred with the serotype 11 strain.

3.4 SELECTIVE CULTURE METHODS

i) Sodium azide (0.01%) and gentamicin (0.4 µg/ml) included in T-H broths for the isolation of six pneumococcal strains when mixed with a suspension of Staph. aureus (Oxford strain, SOX) gave the results in Table 3.6. Gentamicin inhibited the SOX but was also inhibitory to four of the pneumococcal strains tested, at the concentrations used. Sodium azide did not inhibit the SOX but restricted the growth of four of the pneumococcal strains. The serotype 04 pneumococcal culture was inhibited by the SOX in the T-HL broths and one of the serotype 14 strains showed a reduction in growth. Reduced

TABLE 3.6

Pneumococcal recovery from mixed culture with Staph. aureus using sodium azide and gentamicin selective broths.

Sero type	Density of growth on medium						
	T-HL		AT-HL		GT-HL		Contr
	P	S	P	S	P	S	P
03	++	++	++	++	+	-	+++
03	++	++	++	++	++	-	+++
14	+++	++	-	++	-	-	+++
14	++	+++	+	+++	+++	-	+++
03	+++	+++	+++	+++	+++	-	+++
04	-	+++	-	+++	-	-	+++

T-HL = Todd-Hewitt broth containing 1% liver digest;

AT-HL = T-HL containing 0.01% sodium azide;

GT-HL = T-HL containing gentamicin 0.04 µg/ml.

Contr = positive control of growth;

P = pneumococcal growth;

S = SOX growth.

Growth was scored from - = no growth

to +++ = heavy growth.

growth by these strains and the other serotype 14 strain was more pronounced in the azide medium whilst serotypes 03, the second serotype 14 and the serotype 04 gave reduced growth in the gentamicin medium and contrasted with the heavy growth of the pneumococci in pure culture.

ii) The inhibition zones produced with gentamicin, fucidin, kanamycin and nitrofurantoin discs against the nine pneumococci and four non-haemolytic streptococci (NHS) showed that all strains were sensitive to nitrofurantoin with zones exceeding 35 mm, except for one strain from each group that gave a zone of 10 mm. Inhibition zones of all isolates were <10 mm with kanamycin and gentamicin discs and 10-12 mm with fucidin. None of the discs were suitable for selective purposes with the organisms tested.

iii) Gentamicin blood agar:

a) The gentamicin minimal inhibitory concentration (MIC) of the nine pneumococci, taken as the plate containing the lowest concentration of gentamicin not permitting growth, showed that one of the pneumococci was inhibited by 4 µg, the other 8 grew in the presence of 8 µg/ml; the control SOX had an MIC of 0.25 µg/ml.

b) Gentamicin blood agar (GCBA) seeded with 59 consecutive throat and nasal swabs gave eight samples positive for pneumococci under anaerobic conditions, five when incubated aerobically with CO_2 and four on the aerobic (without CO_2) GCBA plates. Growth was present on 17 of the aerobic GCBA, 24 of the CO_2 GCBA and 44 of the anaerobic CBA plates.

The short series of 12 routine swabs cultured on CBA aerobically with and without CO_2, anaerobically with and without CO_2 and on GCBA anaerobically with CO_2 gave 2 samples positive for pneumococci on CBA and GCBA incubated anaerobically. One of the negative swabs yielded a beta-haemolytic streptococcus on the anaerobic CBA but not on the GCBA or the other CBA plates.

Eight further swabs cultured on GCBA under aerobic and anaerobic conditions yielded three specimens positive for pneumococci after aerobic culture; four of the remaining five samples gave no growth. Anaerobic culture on GCBA showed five positives (equivalent to number found on the original CBA plates). All of the remaining plates showed some growth.

3.5 DISCUSSION

Pneumococci can be difficult to isolate from serious infection sites for a number of often interrelated reasons and it is essential that the artificial environment employed for their recovery is optimal for their exacting requirements. The culture method commonly used is rapid transfer of the sample, obtained before the commencement of antibiotic therapy, on to enriched agar with aerobic cultivation in an atmosphere containing 10% CO_2. This has been shown to be unsatisfactory in many surveys of respiratory isolation in which the pneumococcus was recognised in Gram stained smears of the specimen, but grown in only 50 to 70% of cases (Lepow et al., 1968; Spencer and Phillips, 1973; Rein et al., 1978 and Sarchik, 1979). Following the introduction of an improved anaerobic technique (Collee et al., 1971; 1972), pneumococcal isolations in this laboratory appeared to have increased. A study by Howden (1976) showed that more than twice the number of pneumococci were isolated by the new anaerobic procedure which included extra CO_2 than by aerobic incubation. The advantage of anaerobiosis with CO_2 over aerobic incubation without CO_2 was quite clear but the effect of permutations of O_2 and CO_2 concentrations on the recovery of pneumococci was not examined. Qualitative and quantitative experiments of a range of gas conditions on colony size and viable count confirmed that growth of almost all strains was enhanced by anaerobiosis with added

CO_2. The main exceptions were colonies of serotype 03 strains, which although large after anaerobic incubation, remained smaller than the huge mucoid colonies seen after aerobic culture. Other gas mixtures were less than optimal for some of the strains tested. Certain isolates grown anaerobically without added CO_2 gave results comparable to aerobic incubation with added CO_2. In others growth in nitrogen was similar to aerobic culture without added CO_2. The two major factors which combined to give increased pneumococcal isolation from clinical material were reduced O_2 and increased CO_2 levels. In a more easily defined group of strains no growth occurred in air unless extra CO_2 was available. These CO_2 dependent cultures predominantly belonged to serotype 09. Following an extended survey of the proportion of dependant strains related to serotype it was seen that 10% of all isolates required extra CO_2 (Table 3.5). Although these strains were most prevalent in serotypes 09, 16 and 19 other serotypes showed one or more examples and in two serotypes i.e. serotypes 28 and 42, these were the only isolates seen of that serotype. This degree of CO_2 dependence would have meant that more than 40 samples each year could have been incorrectly reported as negative for pneumococci. At least some of these false negatives could have been from patients with septicaemia or meningitis, confirming the essential nature of CO_2 in the isolation of pneumococci.

The marked differences in pneumococcal colony size after aerobic (Fig 3.1) and anaerobic incubation (Fig 3.2) may be

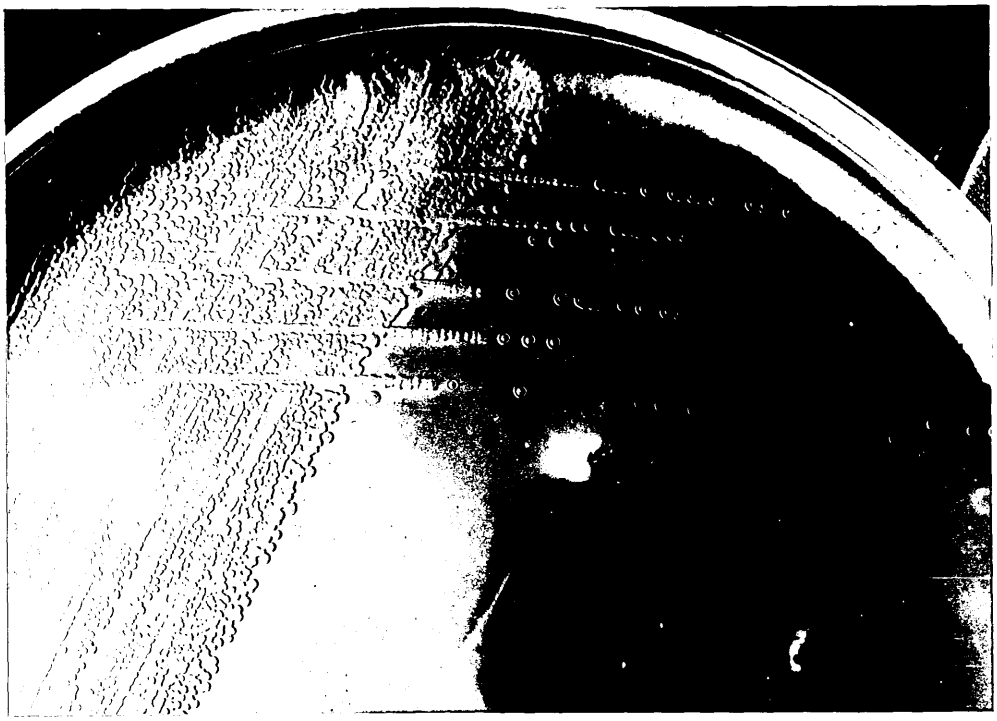

FIGURE 3.1 Colonies of S. pneumoniae after aerobic culture (without added CO_2) on Columbia blood agar.

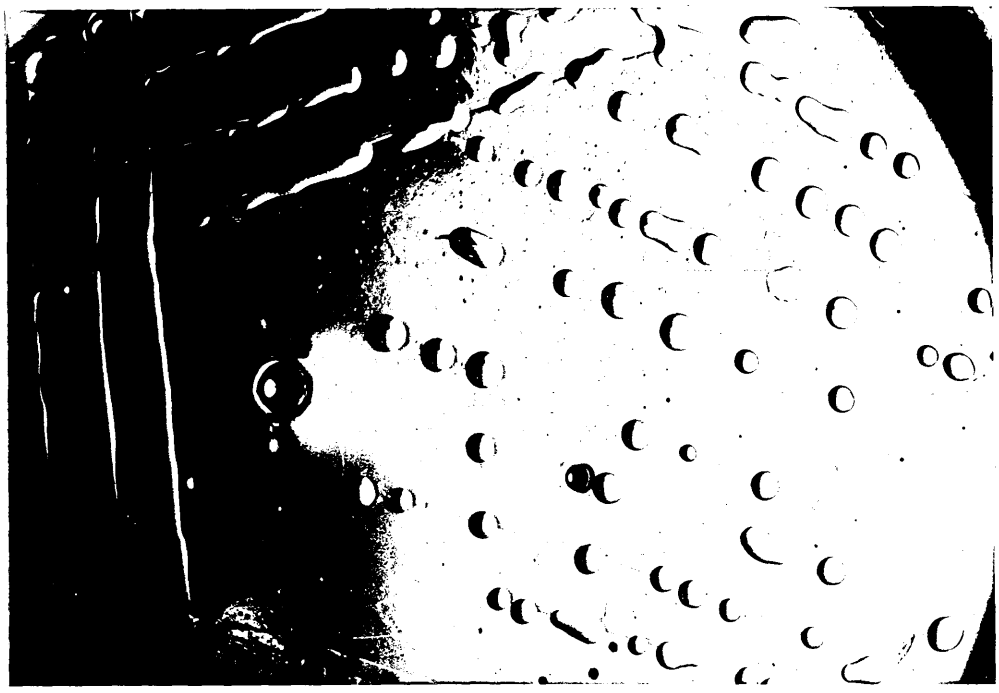

FIGURE 3.2 Colonies of S. pneumoniae after anaerobic culture with added CO_2 on Columbia blood agar.

a combination of a number of effects. The pneumococcus, unique amongst aerobes, is unable to synthesise either catalase or peroxidases (MacLeod, 1921) such that when grown in air accumulates hydrogen peroxide. This is probably a product of oxidative reactions by the organisms flavoprotein enzymes and leads to the characteristic greening or bleaching around colonies formed on blood agar rendered catalase deficient by heating. Under anaerobic conditions hydrogen peroxide should not accumulate and an enzyme deficiency would not, in vivo, be a problem. This lack of peroxide was confirmed in vitro by the absence of changes around colonies grown anaerobically on heated blood agar.

Anaerobic culture has a further advantage for the isolation of pneumococci by suppressing other commonly encountered bacteria, for example the neisseriae, diphtheroids and staphylococci. The Gram negative bacilli, particularly the pseudomonads, are also adversly affected by anaerobiosis since they either fail to grow or the colonies produced are reduced in size. The majority of the organisms listed can be associated with the pneumococcus in infection but most commonly are commensals that contaminate samples during collection. Suppression of aerobes gives the pneumococcus the opportunity to form colonies which are also enhanced in size and appearance (Fig 3.2) facilitating their easy recognition in mixed culture. This is in sharp contrast to the small easily overlooked "draughtsman" colonies (Fig 3.1), amongst the many colonies produced by aerobic

culture.

The role of CO_2 in the increased colony size is uncertain; it has been shown that other streptococci in continuous culture have a reduced lag phase if a particular CO_2 level is provided (Valley 1927; Walker 1932 and Kempner 1942). Carbon dioxide depletion experiments suggested that a similar mechanism exists in the pneumococcus since strains incubated initially without CO_2 gave only minute colonies but when CO_2 was introduced and incubation continued characteristic large mucoid colonies resulted. This supports the belief that lack of CO_2 extends the lag phase of pneumococcal growth. Some carboxyphilic pneumococci were able to grow on Todd-Hewitt (T-H) agar but not on Columbia agar without extra CO_2 being present. The bicarbonate ions in the T-H medium were probably the factor that allowed these strains to overcome their dependance on gaseous CO_2. This independance did not apply to all the CO_2-requiring isolates but whether this was a qualiatative or quantitative effect would require further study. The increased colony size after anaerobic incubation with extra CO_2 is probably a combination of increased viablity of the organism, in part by a reduction in peroxide formation and inhibition of autolytic enzyme release and greater production of capsular material.

Carboxyphilic streptococci are most frequently recognised as initially anaerobic strains which on subculture become aerotolerant if extra CO_2 is provided. In a similar fashion some pneumococci were seen to be initially

intolerant of an aerobic atmosphere unless an excess of CO_2 was present. In other isolates the lack of CO_2 could be overcome if cultures were incubated anaerobically in an H_2 only atmosphere.

The narrow band of pneumococcal growth, 10 mm below the surface of a broth, containing a small amount of agar to reduce convection currents, was similar to the growth of microaerophilic organisms in this kind of medium. Failure of artificial culture conditions to provide optimal concentrations of pneumococcal growth factors would appear to be depend nt upon both gaseous and medium constituents. Enriched agar bases with horse or sheep blood are used for pneumococcal isolation but these have largely been developed with stock laboratory strains and compared with now acknowledged inferior media. The commonly used medium, Columbia blood agar base, considered superior to previously used blood agar formulae (Elner, 1966) may only represent an improved medium and not the ultimate pneumococcal culture base. Todd-Hewitt agar for example proved superior to Columbia agar in the recovery of CO_2-dependent strains and RCM agar gave larger colonies with non-fastidious cultures. Liver digest, a source of essential amino acids and vitamins, further enhanced pneumococcal growth. Although T-H broth culture is less practical for a routine clinical laboratory than Columbia blood agar, it was sucessful in the recovery of the organism from respiratory samples when the plate culture was negative. Whether this was through osmotic support or supply of nutrients is

unknown but it was not possible to extrapolate the findings from T-H broth to growth on agar. Simple alteration of the pH in T-H, RCM or Columbia agars significantly affected pneumococcal viability, autolysis and the amount of capsular material formed. The optimal pneumococcal culture pH on agar was 7.3, with marked reductions in growth either at 6.8 or 7.8, and was pH 7.8 in T-H broth. Growth in T-H broth was enhanced both by cysteine, an antagonist of oxidation and hydrogen peroxide; and by sodium bicarbonate, a pH buffer or possible source of bicarbonate ions. The extended metabolism produced in the broths containing both cysteine and bicarbonate had the disadvantage that the growth curve reached a plateau more quickly and the organisms were therefore in decline after 20 h incubation. However, extended glucose metabolism consequently gave higher capsular polysaccharide titres and was independant of the serotype of the isolate, fall in initial pH of the medium and viability of the culture.

Inhibition of pneumococcal growth by methylene blue and bromothymol blue was an unexpected finding, which was further expanded in Section 4.J.

Recommended selective agents were also unreliable when pneumococci with an MIC of greater than 4 µg/ml to gentamicin were inhibited by 0.4 µg/ml of gentamicin included in a broth medium. Similarly the sodium azide at the concentration used should not have reduced the growth of many of the pneumococcal strains tested but should have inhibited the staphylococci. These practical difficulties

in applying theoretically suitable selective agents to the isolation of small numbers of bacteria confirms the view that anaerobic culture with added CO_2 is the method of choice for the recovery of pneumococci from clinical samples.

4. IDENTIFICATION AND CHARACTERISATION

4.1 CAPSULE SWELLING

The capsule swelling test, performed on 10 pneumococcal strains, was difficult to interpret because the increase in capsule size was not easily recognised with the microscope available; the method was therefore discontinued.

4.2 IMMUNODIFFUSION

All the four methods of antigen extraction (Section 2.5.2) gave similar specific precipitation lines; T-H broth culture supernate (method a) was adopted for routine use. Culture supernates from 151 consecutive clinical isolates tested, initially against the pooled sera, by immunodiffusion showed specific reactions with 91 strains, but 60 cultures did not react. This gave the following distribution:-

Pool A	9 cultures
Pool B	18 cultures
Pool C	2 cultures
Pool D	10 cultures
Pool E	1 cultures
Pool F	7 culture
Pool G	6 cultures
Pool H	37 cultures
Pool I	1 culture
Unidentified	60 cultures

Immunodiffusion was not suitable for routine antigen

detection.

4.3 IMMUNO-ELECTROPHORESIS

4.3.1 Studies to establish an optimal method for electrophoresis

Precipitation lines were not seen under any of the conditions (Section 2.5.3.i) when the serogroup H strain antigen was tested in agar/agar or indubriose in Veronal buffer. When the parallel series (Section 2.5.3.ii), with the substitution of Tris buffer was performed, the results were also negative and the gel distorted after the current had been passed for 15 min. At a constant current of 100 ma in agar/agar (Section 2.5.3.iii) with Veronal or Tris buffers the former was negative and the latter gave blurred lines; the gel again becoming distorted after 15 min. Faint non-specific lines were seen with 50 ma constant current in agar/agar with Tris buffer and faint specific precipitation was observed using indubriose as the support medium (Section 2.5.3.iv). Agar distortion caused by overheating was reduced by placing a frozen platten under the slides before starting each electrophoresis test. A constant voltage of 100mv for 30 min across cooled indubriose in Tris buffer (Section 2.5.3.v) showed strong precipitation lines with Omni-serum and weak lines with pool H serum and pool B.

Fresh group H antigen prepared in T-H broth tested at 100mv for 30 min in Tris buffer and indubriose gave specific reactions with Omni and pool H sera (Section 2.5.3.vi).

The source of this artefact, investigated by electrophoresis of pneumococcal cultures in tryptone soya broth (TSB) and T-H with and without 1% liver digest and/or 1% haemoglobin (Section 2.5.3.vii) showed faint specific lines with all T-H based media and blurred non-specific lines with the TSB media. Todd-Hewitt medium was therefore used for all subsequent broth culture tests.

Sources of antigen (Section 2.5.3.viii), in whole culture, whole culture lysed with sodium deoxycholate and culture supernate both with and without alcohol precipitation, under the conditions used for detection of the artefact showed similar antigen titres for all extracts; the precipitin lines formed however were still weak.

The Veronal buffer system when retested (Section 2.5.3.ix) with antigens from cultures of serotypes 06, 23 and group E identified by slide agglutination at a constant current of 20 ma for 30 min against Omni, pool B, pool H, pool E, serotype 06 and serotype 23 antisera in indubriose and also agar/agar gave strong precipitin lines with serotypes 06 and 23. The group E strain gave a weak line with Omni-serum only.

Sensitivity of the system (Section 2.5.3.x) assessed with neat, 1 in 3, 1 in 6, 1 in 12 and 1 in 24 dilutions of serotypes 06 and 23 antigens in both gels under the same conditions showed equivalent titres for both agars. The precipitin lines in indubriose, in contrast to agar/agar were initially thicker and closer to the well and became more central after dilution.

The reactions given by pneumococcal cultures in T-H, previously tested by immunodiffusion and stored at 4 ºC, when tested by CIE at 20 ma per slide for 30 min in Veronal buffer are shown in Table 4.1. The pool sera were negative by CIE except for the strains of groups A and D, and one of the three group C cultures.

The interwell gap, previously 5 mm, when tested at 2 and 3 mm with 10 strains of groups A, D and F showed specific positive reactions with the A and F sera. Three of the four group D strains, negative immediately after electrophoresis showed precipitation lines after cooling and staining. The 3 mm distance was chosen for further study because the lines formed were more central between the wells; both gaps showed strong precipitation. Agar/agar and indubriose gel tested at pH 8.6 and pH 6.6, using the 10 serotypes A, D and F antigens, at 30 ma constant current for 30 min, gave central clear and stronger lines at pH 6.6 on the indubriose slides.

Sensitivity of the acidic gel for pool antisera tested against neat and 1 in 12 diluted antigens of groups A, B, C, F, G and H showed that Groups A, C, F and G cultures were positive at both concentrations. Group B cultures showed two strains positive at both levels, 18 at 1 in 12 and six negative. Group H c showed 16 strains at both levels and the remaining 13 at 1 in 12 only.

TABLE 4.1

Crossed-immuno-electrophoresis of pneumococcal cultures, previously tested by immunodiffusion

Group /Pool	CIE Results	Not Tested	Not typable	Untyped by ID Typed by CIE
A	9	0	0	0
B	38*	1	0	20
C	2	2	0	0
D	8	1	1	0
E	1	0	0	0
F	7	0	0	0
G	2	0	4	0
H	30*	4	9	7
I	1	0	0	0
Negatives	41**	0	27	27
Totals	139	12	14	27

* = includes cultures positive by ID that were positive by CIE and cultures non-reacting by ID that were positive after CIE.

** = includes cultures negative both by ID and CIE and strains previously positive by ID that were negative by CIE.

4.3.2 Detection of antigen in body fluids

Patient A : the acute CSF sample, which yielded a serotype 06 pneumococcus, and a second CSF, taken two days later, tested against Omni, serotypes 06 and 35 antisera in indubriose (pH 8.6) gave a strong line from the first sample and a faint line from the second, only with serotype 06 serum; the other sera were negative on both samples.

Patient B : sputum, from which a pneumococcus serotype 19 had been isolated, tested against Omni, serotypes 19 and 06 showed precipitin lines with Omni and serotype 19 but not serotype 06.

Patient C : blood and CSF obtained at post-mortem reacted with serotype 03 sera but not Omni or pool B; a serotype 03 pneumococcus was grown from the middle ear swab from this patient.

Patient D : serum from a patient suspected of having pneumococcal septicaemia was negative by electrophoresis and also blood culture.

4.4 CO-AGGLUTINATION

4.4.1 Production of protein-A suspension

Comparisons of bacterial yields on CCY, and tryptone soya (TS) and Columbia (CB) agars with and without CCY supplements showed that CCY agar, TS with supplement and CB with or without additions gave equivalent growth, heavier than TS without supplement. Production of protein-A for co-agglutination was modified to growth on CB agar followed

by the scrape/wash method.

4.4.2 Non reaction of pool reagent

Two strains, serotypes 04 and 09, which had shown weak agglutination when tested against pool co-agglutination reagent (PCR) but specific reaction with their individual PCR had been cultured in T-H broth. When the reactions of these strains were tested after growth in TS, RCM, CCY and T-H broths with and without 10% serum (Section 2.5.4), the serotype 04 isolate did not agglutinate satisfactorily with pool A PCR, in any of the media. The serotype 09 isolate gave a positive result against pool D PCR, with all the formulae tested, except CCY medium (Table 4.2).

Extraction and concentration treatments were negative for serotype 04 strain and pool A reagent, and positive for serotype 09 strain and pool D reagent.

4.4.3 Modification of sensitisation of protein-A reagent

Weak or negative pool reactions observed with Omni, pool D and serotype 03 strains, using co-agglutination reagent at a final dilution of 1 in 28 and examined by the modifications described in Section 2.5.4 gave the following results at sensitising dilutions (SD 1 to 3) and use dilutions (UD 1 to 4):-

a) Omni serum when tested as reagent SD1 gave positive results with serotypes 04, 11, 14, 15 and 21; doubtful

TABLE 4.2

Co-agglutination reactions, against pool antisera by two pneumococcal strains grown in eight broths.

Serotype	Media tested							
	TS	TSS	RCM	RCMS	CCY	CCYS	THL	THLS
04								
growth	-	+++	++	+++	+	+	+++	+++
Pool A	.	W	O	O	O	O	O	O
09								
growth	-	+++	+++	+++	+	+	+++	+++
Pool D	.	P	P	P	W	P	P	P

TS = tryptone soya broth;
CCY = CCY broth;
THL = Todd-Hewitt broth plus 1% liver digest;
RCM = reinforced clostridial medium;
Suffix "S" = the medium with 10% horse serum added.
P = positive; agglutination
W = slow/weak; agglutination.
O = no agglutination.
Growth was scored from - = no growth to +++ = heavy growth.

agglutination with serotype 19 and was negative with serotypes 03, 06, 09 and 23. Reagent preparations SD2, SD3 and UD2/SD1 were negative with all strains tested.

a) Pool A:

	SD1	UD3	SD2	UD3
Serotype 01	+/-	+	+	+
Serotype 01	+/-	+	+/-	+
Serotype 04	+	+	+	+
Serotype 18	+	+	+	+

b) Pool B: strains of serotypes 03, 06, 08 and 19 tested at SD1 and SD2 and UD4 showed SD1 to be slightly quicker for all strains and stronger for one of the serotype 03 cultures.

c) Pool C:

	SD1	UD2	SD2	UD2
Serotype 07	+	+	+	+
Serotype 27	+	+	+	-
Serotype 31	+	+	+	+

d) Pool D:

	SD1	UD2	SD3	UD2	SD2	UD2
Serotype 09	+	-/+	+	-	-/+	-
Serotype 09	-/+	-	-	-	-	-
Serotype 11	+	-	+	-	-	-
Serotype 11	-	-	-/+	-	-	-
Serotype 11	+	-	+	-	-/+	-
Serotype 16	+	-/+	-/+	-	-/+	-
Serotype 37	+	+	+	+	+	+

e) Pool E:

	SD1	SD3	UD2	UD3	SD2	UD4
Serotype 10	-	-	-	-	+	+
Serotype 21	-	-	-/+	-	+	+
Serotype 21	+	+	+	-/+	+	++
Serotype 33	-	-	-	-	+	+/-

f) Pool F:

	SD1	UD2	SD3	UD2	SD2	UD2
Serotype 17	+	+	+	+	+	+/-
Serotype 17	+/-	-/+	+	+	+	+/-
Serotype 17	+/-	-/+	+	+	+/-	-/+
Serotype 22	+	-/+	+	+	+	+/-

g) Pool G:

	SD1	UD3	SD2	UD3
Serotype 34	+	+	+	+
Serotype 35	+/-	-	+	-/+
Serotype 35	+	+	+	+
Serotype 35	-/+	-	+	+/-
Serotype 35	+	+	+	+

h) Pool H: strains tested of serotypes 13, 14, 15, 23 and 28 gave equivalent reactions at SD1, SD2, SD3. The UD4 dilutions of SD2 gave much stronger reaction than the other UD4 reagents.

i) Pool I:

	SD1	UD2	SD2	UD2
"Type x"	+	-	+	+
"Type y"	+/-	-	+	+

j) Serotype 03:

		SD1	SD2	SD3
Strain M		+	-/+	-
Strain N		+	+	+
Strain R		+	+	+
Titre				
Strain R	1:25	+	+	+
	1:100	+	+	+
	1:150	+	+/-	-
	1:200	+	+/-	-
	1:250	+	-/+	-
	1:625	+	-	-

The results from the above tests were used to modify the reciprocal dilution of each stage of the preparation of the particular pool antisera PCR:-

	Sensitising Dilution	Use Dilution
Omni	2	0
Pool A	2	2
Pool B	2	3
Pool C	2	2
Pool D	2	0
Pool E	14	4
Pool F	7	2
Pool G	14	3
Pool H	14	4
Pool I	14	1
Serotype 03	2	0

4.4.4 Detection of antigen by co-agglutination and immunoelectrophoresis

Overnight broth cultures of serotypes 01, 04 and 18 (group A), and a serotype 19 strain (group B) were double diluted in PBS from 1 in 10 to 1 in 320. Testing of the group A strains against pool A antisera and their individual antisera by co-agglutination and immuno-electrophoresis in veronal buffer at pH 6.6 and 8.6, 30 ma constant voltage for 30 min gave the results in Table 4.3. The serotype 19 culture was a negative control of the pool A antiserum. The PA reagent was approximately one dilution less than immuno-electrophoresis at pH 6.6 but roughly equivalent at pH 8.6.

4.4.5 Extraction of serotype 03 antigen

The antigen preparations obtained by the extraction procedures detailed in Section 2.5.4 were titred against serotype 03 PA reagent and the results expressed as per plate or per 10 ml broth:-

a) lysed with 1% sodium deoxycholate, boiled and precipitated with alcohol - 1300

b) as for a) except acetone was used to precipitate the antigen - 1300

c) plates lysed in situ with the deoxycholate treated as a) - 500

d) as for c) except that acetone was the precipitant - 500

e) RCM treated as in a) - 1000

TABLE 4.3

Co-agglutination and immuno-electrophoresis titres of three pool A antigens and a negative control, serotype 19.

Antigen	Antisera	Titres of the antigens tested		
		Electrophoresis*		Co-agglutination
		pH 6.6	pH 8.6	
01	01	160	80	80
	pool A	320	160	160
04	04	160	160	160
	pool A	320	80	160
18	18	160	80	160/320
	pool A	160	80	80
19	pool A	0	0	0

* the results seen after cooling and staining

f)RCM as in b) - 2000

g)RCM boiled, alcohol precipitated without lysis - 2000

h) as in g) except acetone was used - 2000

A serotype 06 culture, used as a negative control of serotype 03 reagent, was negative with all preparations.

4.4.6 Survival of antigen

Strains grown on T-H broth and on CBA plates to test loss of antigenicity were serotypes 01, 03, 06, 17, 19, 20, 22, 23, 33, 35, and group I. The reaction of each strain grown in T-H broth and grown on CB agar when tested against their respective pool and individual PCR gave specific co-agglutination on the day of incubation. After 7 days storage at RT or 4°C the broth and the growth from the CBA culture plate, suspended in PBS at 4°C, gave the following results:-

Serotypes 06 and 17 positive reaction with type but not pool reagents however stored.

Serotypes 22 and 23 positive reaction with type but not pool reactions stored in broth, but with both reagents if stored in PBS.

Serotypes 01, 30, 35 and group I reacted with both pool and type reagents however stored.

Serotype 20 reacted with both reagents and antigens if stored at 4°C but not at RT.

Serotypes 03 and 19 were positive with type reagents however the antigen was stored but doubtful results with the pool reagents were seen when stored in broth.

The loss of pool reaction resulted in subsequent serotyping of strains being performed from plate cultures.

4.5 ENZYME STUDIES

4.5.1 Carbohydrate fermentation

i) Pneumococci tested for carbohydrate fermentation in enriched serum-free CTA medium, without 1% liver digest did not support the growth of the seven pneumococcal cultures; the strain of S. faecalis did grow. When 20% egg yolk emulsion was added to the CTA formula three of the pneumococcal cultures grew but detection of fermentation was masked by the yellow colour of the medium. Yeast extract and liver digest when included permitted slow growth and four of the seven strains showed a weakly acid pH change in the depth of the mannitol medium, after 2 days incubation.

ii) Defined Gonnococcal broth examined for pneumococcal fermentation studies as the base alone, as the base plus supplements and as the base plus 0.5% mannitol gave the growth and approximate final pH results noted in Table 4.4, with the strains used in Section 4.5.1,i). Heavier growth was more generally produced by the supplemented base, which included glucose, than in the base alone, but was only slightly heavier than in the medium with mannitol. Acid production in the medium with mannitol and with supplement but not in the base alone was seen with strain T122 only; T52 and T69 failed to produce acidity.

TABLE 4.4

Growth and fermentation in Gonnococcal culture broth and CTA medium

Sero type	GC base		GC base/ mannitol		GC base/ supplements		CTA/ mannitol
	growth	pH	growth	pH	growth	pH	pH
13	++	K	++	K	++	N	A
23	++	A	+++	A	+++	A	A
06	++	K	+++	A	+++	A	N
06	++	A	+++	A	+++	A	A
06	+	N	+	N	++	A	N
06	++	A	++	A	+++	A	A
03	+	K	+	K	++	N	N
S.f	+++	A	+++	A	+++	A	A

GC base = defined gonnococcal culture basal medium;
S.f = S. faecalis
A = acid;
K = alkaline;
N = neutral.
Growth was scored from + = light turbidity to +++ = heavy turbidity.

iii) Four media modified from Todd-Hewitt broth containing either glucose, mannitol, salicin or sucrose tested with four pneumococcal cultures and the Staph. aureus (SOX) strain gave the results shown in Table 4.5 after 18 h and 42 h incubation. Growth of the pneumococcus was poor except in the glucose medium. The SOX culture produced heavy growth in all the media tested.

iv) Growth and acid production in Columbia agar by the seven pneumococcal strains and the culture of S.faecalis in that medium alone, and with horse serum or finely powdered chalk are recorded in Table 4.6. The formula containing chalk did not show any advantage over the basic medium in growth or acid production and gave reduced colony size. Strain T 408 produced clearing around the colonies on chalk medium and opacity on serum agar. Serum added to the basic formulae yielded the heaviest growth and largest colonies but failed to support the growth of strain T 291.

4.5.2 Basal sugar testing medium.

i) Trypticase-yeast-liver-succinate broth (TYLS): Growth in TYLS and CB broths with and without haemoglobin and cystein showed light growth in TYLS both with and without the additives for five of the six strains, T1512 failed to grow in TYLS with additives. Turbidity in CB without the additions was equivalent to that in TYLS, but growth was enhanced to moderate turbidity in the medium with additives for three of the six strains.

ii) Streptococcal basal medium for carbohydrate tests.

TABLE 4.5

Modified Todd-Hewitt broth as a base for carbohydrate fermentation studies

Sero type	Growth after 1 and 2 days in									
	Basal		Man'tol		Glucose		Salicin		Sucrose	
	1	2	1	2	1	2	1	2	1	2
22	-/+	-	-	-	+++	+++	-	-	-	-
03	-/+	-	-	-	+++	+++	+	+	-	-
19	+	+	+	-/+	+++	+++	-	-	-	-
23	-	-	-	-	+++	+++	+++	+	-	-
Staph	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

Man'tol = mannitol
- = no growth;
-/+ = scant growth;
+ = poor growth;
+++ = moderate to heavy growth.
Staph = Staph. aureus

TABLE 4.6

Columbia agar with and without serum and chalk as a base for fermentation studies

Sero type	Basal		Serum		Chalk	
	growth	pH	growth	pH	growth	pH
22	+/-	K	-	K	-	K
03	+	A	++	A	+/-	A
19	++	A	++	A	+	A
23	++	A	++	A	++	A
01	+	A	++	A	+	A
16	++	A	++*	A	++**	A
03	++	A	++	A	++	A
S.f	++	A	++	A	++	A

A = acid;

K = alkaline

* = marked opacity of the medium around the colonies

** = clearing of the medium around the colonies

S.f = S. faecalis

Growth was scored from - = no growth to ++ = moderate growth.

a) Growth of the eight strains in streptococcal broth (SB), RCM and T-H was heaviest in RCM, less in T-H and least in SB. Strain T2860 failed to grow in SB and T-H but grew in the RCM broth.

b) Streptococcal basal Agar (SBA) used to culture the eight strains used in a) above gave similar numbers of colonies on both media under aerobic, aerobic with CO_2 and anaerobic with CO_2 atmospheres. Strains T2645, T2744 and T2860 (serotypes 09, 16 and 19 respectively) failed to grow in aerobic culture without CO_2 on either medium i.e. those strains were carboxyphilic. The major difference between the media was that smaller colonies were produced on SBA under all three conditions; colonies on SBA after anaerobic incubation were approximately the size of colonies on CBA incubated aerobically without added CO_2.

c) The addition of agents to the Streptococcal basal medium was carried out in an attempt to improve the growth of the four pneumococcal strains. Serotypes 14, 15 (two isolates) and 19, gave the results shown in Table 4.7. Glucose stimulated growth for all four strains equivalent to the turbidity seen in T-H; glucose, detected by the Clinistix test, was still present in the broth after 18 h incubation. Choline and agar marginally improved the growth of two of the strains compared to the blank medium. None of the other agents noticeably improved growth, bicarbonate and arginine inhibited all four strains, haemoglobin inhibited three and ornithine two strains. Moderate to heavy growth was seen on subculture independently of the turbidity of

Table 4.7

The effect of additives on the growth of pneumococci in Streptococcal basal medium.

	Serotype			
Additives	15	14	19	15
acetate	+/-	+	+/-	+
succinate	-/+	+/-	+	+
bicarbonate	-	-	-	-
phosphates	-/+	+/-	+	+
ornithine	-	+/-	-	+
arginine	-	-	-	-
choline	+G	+G	+G	+G
glucose	++G	++S	++S	++S
haemoglobin	-	-	-	-
agar	+	+	+	+
control	+/-	+/-	+	+
T-H broth	++	++	++	++
RCM	+	+	+	+

Growth was graded as turbidity from:
- = no growth, to
++ = moderate turbidy.
G = granular appearance of turbidity;
S = smooth even turbidity.
T-H = Todd-Hewitt medium.
RCM = Reinforced clostridial medium.

of the initial broth for all media except that containing glucose. One of the serotype 15 strains did not grow when subcultured from the glucose containing broth and the other three cultures only yielded 15-20 colonies.

d) Assimilation studies of the four strains, serotypes 14 (T2989), 15 (T2860 and W2077) and 19 (T2645) in Streptococcal basal agar plates containing glutamic acid, pyruvic acid, thiamine, L-histidine, tryptophan and aneurin showed no visible growth with any of the strains after 24 h. After 48 h only the serotype 19 culture showed a halo of growth around the haemoglobin and aneurin wells.

4.5.3 Fermentation of high concentration substrates in microtitre plates.

i) High concentrations of fructose, inulin, maltose, raffinose, rhamnose, sucrose, trehalose, xylose and aesculin in microtitre plates gave a yellow to red colour change (positive) result with fructose, maltose and sucrose with seven of the eight strains tested (serotypes 03, 06, 09, 16, 17, 23 and 33). The other isolate (serotype 19) gave negative results with all substrates. All strains gave negative results with inulin, raffinose, rhamnose, xylose and aesculin and only the serotype 23 strain was positive with trehalose.

ii) The effect of suspending medium on microtitre fermentation tests gave the results presented in Table 4.8. Positive results (acid reaction) were seen with all media containing fructose, melibiose and sucrose. Columbia broth

TABLE 4.8

Fermentation reactions of pneumococci suspended in Columbia base, Streptococcal basal and Bacteroides basal broths using the microtitre plate method.

"Sugars" tested	Broths used								
	Columbia			Strep/base			Bacte/base		
	Serotype								
	9	6	17	9	6	17	9	6	17
Fructose	+	+	+	+	+	+	+	+	+
Inulin	+	-	-	-	-	-	-	-	-
Melibiose	+	+	+	+	+	+	+	+	+
Raffinose	-	+	+	D	+	D	-	+	D
Rhamnose	-	-	-	-	-	-	-	-	-
Sucrose	+	+	+	+	+	+	+	+	+
Trehalose	-	+	+	-	+	-	-	+	+
Xylose	-	-	-	-	-	-	-	-	-
Aesculin	-	-	-	-	-	-	-	-	-
Control	-	-	-	-	-	-	-	-	-

Strep/base = Streptococcal basal medium
Bacte/base = Bacteroides basal medium
+ = red colour change (positive);
- = yellow colour (negative);
D = orange colour change (doubtful);
control = basal medium only.

gave a posiive reaction with inulin for the serotype 09 strain which was negative in the other two broths; CB and BM were positive for the serotype 17 strain that was negative when tested in SB. Raffinose results were inconsistent in SB and BM. However the colour changes from yellow (negative) to orange (doubtful) to pink/red (positive) with the other compounds tested appeared clearer and brighter on SB than on CB or BM.

iii) The repeat microtitre fermentation results in Streptococcal basal broth of serotypes 06, 14, 17, 19 and 23 showed that all strains examined fermented fructose, melibiose, sucrose and lactose, and none fermented inulin, rhamnose, xylose or mannitol. Three cultures, serotypes 06, 17 and 19, gave positive reactions with trehalose; the serotype 19 culture was also positive with raffinose.

iv) The extended series to examine the fermentation of arabinose, dulcitol, fructose, galactose, inulin, mannose, maltose, melibiose, raffinose, salicin, sorbitol and trehalose in microtitre plates gave the following results with the eight isolates tested, serotypes 03, 04, 09, 14 (two strains), 19 and 23 (two strains). All strains gave positive reactions with fructose, galactose, maltose and mannose. Negative results were seen for all strains with arabinose, dulcitol, inulin, raffinose and sorbitol. The serotype 19 culture was the only strain that did not ferment melibiose; the serotype 09 isolate was the only strain that did not ferment trehalose. Positive results were only seen with one of the serotype 23 strains and the

serotype 03 strain and doubtful results were given with one of the serotype 14 strains and the serotype 19 isolate in the salicin containing wells.

4.5.4 The APIZYM system

The standardisation experiments for the three variables, bacterial dose, duration of and suitable gaseous conditions for incubation were as follows:-

i) Growth standardisation

a) bacterial mass measured by centrifugation of a broth culture in a haematocrit centrifuge at 10,000 rpm for 5 min gave a deposit of <1% and was therefore unsuitable for routine estimations.

b) Photometric estimation, in the range 380-700 nm, of the growth from two strains examined as whole culture in broth, as the centrifuged supernate and as the suspension obtained from the colonies on a CBA plate gave the results in Table 4.9. Growth on agar gave a straight line graph of absorbance against wavelength and in broth gave a sigmoid curve with a peak absorbance at 540 nm.

When scanned in the spectrophotometer the lawn of growth from a CBA plate, suspended in PBS and lysed by sodium deoxycholate gave absorbance peaks at 260 and 350 nm against a blank of PBS containing an equivalent amount of deoxycholate.

A repeat of equal areas of growth prepared as lysed and whole suspensions measured for absorbance at 350 and 660 nm, and viable count at the latter absorbance are given in

TABLE 4.9

Photometric estimation of growth on Columbia agar and in Todd-Hewitt broth

Strain	Wavelength (nm)								
	380	420	460	500	540	580	620	660	700
	Plate								
x	1.24	1.10	1.02	1.08	0.92	0.87	0.77	0.80	0.69
y	1.06	0.94	0.86	0.83	0.75	0.71	0.64	0.60	0.52
	Broth (whole)								
x	0.35	0.32	0.45	0.47	0.44	0.37	0.43	0.40	0.40
y	0.25	0.30	0.39	0.47	0.44	0.35	0.37	0.37	0.37
	Broth (supernate)								
x	0.04	0.03	0.03	0.05	0.04	0.05	0.05	0.04	0.04
y	0.04	0.04	0.06	0.07	0.07	0.08	0.07	0.05	0.04

TABLE 4.10

Pneumococcal growth measured as turbidity, viable count and absorbance.

Strain	Wavelength		Culture			Viable count
	660	350	neat	1:25	1:625	
	Experiment A.					
59	0.61	1.80	++++	+++	400 c	1.25×10^6
83	0.36	1.40	++++	+++	100 c	0.31×10^6
49	0.48	1.20	++++	+++	200 c	0.63×10^6
15	0.48	1.52	++++	+++	100 c	0.31×10^6
	Experiment B.					
55	0.61	1.53			80 c	0.30×10^6
49	0.50	1.30			80 c	0.30×10^6
15	0.71	1.60			80 c	0.30×10^6
	Experiment C.					
49 TH	1.02	1.94	++++	+++	++	7.00×10^7
RCM	0.18	1.31	-	-	-	0
15 TH	0.73	1.59	+++	++	200 c	6.30×10^5
RCM	0.20	0.99	20 c	2 c	1 c	1.00×10^2

TH = Todd-Hewitt medium;
RCM = reinforced clostridial medium;
c = colonies;
Growth was graded from
- = no growth, to
++++ = confluent growth.

Table 4.10, experiment A. Further repeat measurements performed at 660 and 260 nm for plate culture, experiment B, and in T-H and RCM broths, experiment C, gave the results shown in Table 4.10. The RCM broth did not support the growth of strain number 55 nor active growth of the other strains unless 1% liver digest had been added. The results of RCM, T-H broth and CBA assayed for viable count and absorbance at 660 and 260 nm, after lysis, are given in Table 4.11, experiment D. Measurement of turbidity at 660 nm appeared the more practical method for standardising inoculum density related to viable count.

The higher viable count after 18 h incubation on CBA, experiment D, led to the adoption of a plate method in the preparation of organisms for APIZYM studies in preference to broth culture.

c) The suspension, at 1.0 absorbance unit, of strain 55, neat and diluted 1 in 2 and 1 in 4 in three APIZYM strips gave the results, recorded in Table 4.12 (experiment A), after 4 h incubation at 37 °C. The intensity of the colours, produced after the addition of APIZYM reagents A and B, were scored from 0 to 5, using the colour chart supplied with the strips (Appendix B).

The APIZYM strip results of the same strain, at 1.0 absorbance unit and concentrated to 2.0 and 2.5 units, when tested under the same conditions are given in Table 4.12, experiment B.

ii) <u>The effects of gaseous environment</u> on the APIZYM reactions of four strains (under the standard test

TABLE 4.11

Pneumococcal growth measured as absorbance and viable count, Experiment D.

Strain	Wavelength (nm) 660			260			Viable Count ($x10^5$)	
	CBA	T-H	RCM	CBA	T-H	RCM	CBA	T-H
49	0.46	0.56	0.35	0.13	0.16	0.07	12.5	6.3
15	0.73	0.64	0.29	0.27	0.18	0.03	19.0	4.7
55	0.66	0.63	0.36	0.19	0.25	0.12	12.5	4.7

CBA = Columbia base agar;
T-H = Todd-Hewitt medium;
RCM = reinforced clostridial medium;

TABLE 4.12

The effect of bacterial dosage on APIZYM results.

Dose	Cupule Number*										
	Experiment A.										
	2	3	4	6	7	8	10	13	14	16	18
Neat	0	0	4	5	4	0	4	4	5	5	5
1:2	0	0	2	4	2	0	0	2	2	4	4
1:4	0	0	0	3	0	0	0	0	0	3	3
	Experiment B.										
Neat	0	1	4	5	5	1	5	5	5	5	5
x2.0	0	1	4	5	4	0	5	5	5	5	5
x2.5	1	1	4	5	5	0	5	5	5	5	5

* cupules 5, 9, 11, 12, 15, 17, 19 and 20, the control cupule was negative in each test.

Figures given under the cupule number are the colour, produced by APIZYM reagents A and B, scored by the comparative chart (Appendix B) from:-
0 = no colour change to 5 = maximum colour change.

conditions, both aerobically, with and without extra CO_2 and anaerobically with 10% CO_2) gave comparable enzyme results; aerobic incubation in the CO_2 incubator was therefore adopted as the standard method.

iii) Incubation of the strips for 4, 5 and 6 h before colour development with the same four strains showed no advantage for continued incubation; the standard length of incubation for the strips was taken to be 4 h.

iv) The results of 101 strains tested by the standard APIZYM method (Section 2.7.4) are summarised as cultures showing the same enzyme profile patterns (Tables 4.13 and 4.14) and analysed by serotype (Tables 4.15) and by source of isolate and serotype (Table 4.16).

4.5.5 Nitrophenyl enzyme substrates

Nitrophenyl substrates when attacked by specific enzyme released the yellow coloured nitrophenyl group in the test methods except when used impregnated into paper discs.

The following results were obtained:-

i) Glass capillary tubes containing the organism and test substrate gave positive (yellow colour) release with alpha-galactosidase, alpha-glucosidase and N-acetyl glucosaminidase substrates with the four strains tested.

ii) Paper discs impregnated with the same nitrophenyl substrates placed directly on to pneumococcal colonies grown anaerobically on CBA plate did not produce a colour change after 2 h further incubation.

TABLE 4.13

The first 20 APIZYM profile codes

obtained from 101 pneumococcal strains.

Number of Strains	Cupule number of Substrates attacked										Profile Code
15	3	4	6	7	10	13	14	16		18	01
11			6	7	10	13	14	16		18	02
10	3	4	6	7	10		14	16		18	03
9			6	7	10		14	16		18	04
7			6	7	10			16		18	05
6	3	4	6					16		18	06
5	3	4	6	7		13	14	16		18	07
3			6	7	10			16			08
3	3	4	6	7	10		14	16			09
3	3	4	6	7			14	16		18	10
4		4	6	7	10	13	14	16		18	11
1			6	7				16		18	12
1			6					16		18	13
1			6	7		13	14	16		18	14
3	3		6	7	10		14	16		18	15
2		4	6		10	13	14	16		18	16
1			6	7	10	13	14	16		18	17
2		4	6	7	10	13	14	16		18	18
1			6		10	13	14	16		18	19
1	3	4	6	7	10	13	14	16	17	18	20

TABLE 4.14

The remaining 12 APIZYM profiles

obtained from 101 pneumococcal strains.

Number of Strains	Cupule number of Substrates attacked										Profile Code
1		4	6	7		13	14	16		18	21
1	3	4	6	7	10		14	16	17	18	22
1		4	6	7	10		14	16		18	23
1	3	4	6		10	13		16		18	24
1	3	4	6			13	14	16		18	25
1	3	4	6	7			14	16		18	26
1	3	4	6		10		14	16		18	27
1			6	7	10			16		18	28
1	3		6	7	10		14	16	17		29
1			6	7	10	13	14	16			30
1			6	7	10		14	16			31
1	3	4	6			13	14	16			32

TABLE 4.15

Relationship of Pneumococcal serotype to APIZYM enzyme profile code

Serotype	Number of strains tested	Number of codes seen	Enzyme profile codes
06	25	14	1, 2, 3, 4, 5, 6, 7, 10, 11, 12, 20, 23, 28, 30, 32.
19	19	9	1, 2, 7, 8, 14, 15, 16, 18, 19.
18	8	8	3, 7, 8, 11, 13, 18, 22, 25.
23	7	4	1, 3, 6, 19.
03	7	5	1, 2, 5, 21, 27.
09	6	3	3, 4, 9.
31	6	3	2, 11, 24.
11	5	3	3, 4, 15.
Others	21	13	2, 3, 4, 5, 6, 7, 8, 9, 10 11, 15, 29, 31

TABLE 4.16

Relationship of the source of sample to serotype and APIZYM profiles.

Sample		Organisms	
Source	Number	Serotype	Enzyme code
Sputum	14	3, 6, 8, 11, 13, 19, 23, 33	1, 2, 3, 4, 10 15, 26, 27
Ear	9	1, 3, 6, 9, 18, 19, 33	1, 2, 3, 8, 11 15, 25
Eye	8	NC, 6, 11, 15, 18	1, 3, 4, 7, 9 20, 29.
Nasal*	6	6, 11, 18, 31, 37	3, 4, 7, 8, 24 30
Wounds	4	1, 11, 17, 31	3, 10, 11, 18

* Nasal swabs, not associated with discharge, provided a further 51 samples; throat swabs were the source of 14 isolates.

NC = non-capsulate strain.

iii) Spectrophotometric assay of the products from the pneumococcus grown in the beta-galactosaminide substrate showed a peak absorbance at 410 nm. The sensitivity of the method assessed by ortho nitrophenyl galactosaminide solution incubated with approximately 10^4 cfu of organism, had an absorbance of 0.96. Dilutions of the substrate at 1 in 2, 1 in 4 and 1 in 8 tested against a suspension of the same strain at a concentration of approximately 10^8 cfu showed absorbances of 0.72, 0.54 and 0.35 respectively.

iv) Alpha-galactosidase, alpha-glucosidase and N-acetyl glucosaminidase solutions tested in 0.2 ml volumes against four pneumococcal strains showed yellow colour changes in alpha-glucosidase and N-acetyl glucosaminidase and no change in alpha-galactosidase within 2 h. When incubation was continued for 18 h the negative tubes became positive.

4.5.6 Neuraminidase

Neuraminidase was demonstrated by:-

i) Pan-agglutinability of human blood group O red cells with AB antiserum was not possible since the red cells lysed during exposure to either an overnight T-H broth culture or a deoxycholate lysed broth. Sodium deoxycholate, which had been used to lyse the pneumococci in the culture broths, when diluted from 0.4% to 0.02% in saline, lysed untreated red cells within 3 min at each concentration. The control was not lysed and did not show agglutination. If the deoxycholate was diluted in 5% saline, lysis did not occur at levels below 0.02% during

the 30 min incubation period. Pan-agglutination was not demonstrated in this modified method.

ii) Pneumococcal neuraminindase content, measured by the WHO method (Section 2.7.2), for three T-H broth cultures (strains W, T and C) and the turbidity of the broths at 660 nm were recorded and expressed as a ratio:-

Strain Code	Turbidity (T) at 660 nm	Neuraminidase (N) activity	N/T ratio
W	0.59	0.78	1.32
T	0.33	0.68	2.06
C	0.40	0.54	1.35

4.5.7 Proteinase activity

Proteinase production by the three strains of pneumococci, serotypes 03, 06 and 23, and the clinically isolated culture of Serratia marsecens, was indicated if zones of clearing on Columbia milk agar (CBM) were produced. Prior stimulation in three of the four milk containing broths, Tryptose liver digest broth (MTL), TL without salt but in PBS (MPTL) and Reinforced Clostridial broth (MRCM), showed clotting and partial clearing of the milk with all three pneumococci. No changes were seen in the fourth medium, Todd-Hewitt broth (MT-H) or in any of the broths with the Serratia culture. Pneumococci were recovered from each of the subcultures of the control broths, without milk, but not from the test broths with milk. The Serratia strain gave moderate turbidity in TL, no apparent growth in PTL, T-H or RCM but was recovered on

subculture from all of the broths. A zone 5 mm greater than the well size was produced on the CBM agar by the Serratia strain after culture in MTL only. None of the pneumococcal culture broths produced zones of clearing in the milk containing agar after prior culture in any of the four broths.

ii) The Serratia strain when grown on the milk agar plates prepared from the MPTL and MTL formulae produced clearing on aerobically cultured plates but not on anaerobic plates. On both agars after anaerobic but not aerobic culture the pneumococci and the Cl. perfringens gave minimal clearing of the medium in the initial well but not around individual colonies. Colonies on MPTL were relatively smaller than on MTL and this was reflected by slightly smaller zones of clearing.

Repeat tests on 2% milk TL agar gave a similar effect of clearing and sparse growth as seen in i). The pneumococcal cultures in TL and MTL broths, added to wells in MTL and MPTL agar, gave no change in the medium and a 5 mm zone of clearing around the well was seen with the Serratia strain.

4.5.8 Haemolysis

i) Sodium azide induced haemolysis:

a) Four horse blood containing media, Columbia agar base, with and without 0.02% sodium azide and tryptose blood agar base, with and without 0.02% sodium azide, seeded with pneumococcal strains were incubated anaerobically with CO_2 for 18 h. The plate was removed from the anaerobic culture

jar and stored at 4°C for 3 h and observed for zones of haemolysis against an illuminated background. Although colony size and viable count was greatest on CBA only tryptose blood agar with azide showed haemolysis around colonies. The results can be summarised as:-

	Columbia	Columbia azide	Tryptose	Tryptose azide
Haemolysis	-	-	-	+
Growth	+++	+	++	+

Both tryptose and azide were therefore essential for production and/or release of the haemolysins.

ii) Reductions in the azide content of the medium to increase colony size but still promote haemolysis showed that culture of the eight test strains on tryptose agar with 0.005% azide gave larger heamolytic colonies than at 0.02 and 0.01% levels. This concentration reduced the tendancy of red cells to autolyse in azide medium during storage.

iii) Culture on Todd-Hewitt agar prepared both with 0.005% azide (T-HA) and without (T-H), azide blood agar (TAz) and CBA for the eight strains gave the results in Table 4.17. Although the haemolysis produced on T-HA was equivalent to that on TAz the double haemolytic zone was only seen on the latter medium. Collapsed "draughtsman" colonies were commonly seen on all media except TAz.

iv) Haemolysis of sheep and horse red cells by the 12 pneumococcal strains cultured anaerobically with CO_2 on Tryptose base agar plates, with and without 0.005% sodium

TABLE 4.17

Haemolysis on Todd-Hewitt and Tryptose blood agars,

containing sodium azide

Sero-type	Media tested							
	Columba		Todd-H		Azide Todd-H		Tryptose Azide	
	Shape	Media Effect	Shape	Media Effect	Shape	Media Effect	Shape	Media Effect
16	D	a	D	2H	D	4H	F	4H Dz
09	F	a	F	2H	F	4H	F	4H Dz
01	C	a	F	2H	F	4H	F	4H Dz
NC	C	ap	C	2H	C	4H	F	4H Dz
I	C	ap	F	2H	F	4H	F	4H Dz
23	D	a	F	2H	F	4H	F	4H Dz
14	C	a	C	2H	F	4H	F	4H Dz
19	C	a	D	2H	D	2H	F	4H Dz

Todd-H = Todd-Hewitt agar;
C = convex colony;
a = alpha haemolysis;
D = draughtsmen colony;
ap = alpha prime haemolysis;
F = flat topped colony;
2H = twice the haemolysis zone produced on CBA;
4H = four times the zone of haemolysis;
Dz = double zone of haemolysis;
(absence of Dz in the media effect column indicates that Dz was not produced).

azide, and 5% of sheep or horse red cells and on CBA with 5% horse blood showed zones only on the azide containing media. The zones on horse blood were slightly smaller and not as sharply defined as those on sheep blood which also differed in producing a greenish central area around the colonies.

Cultures of the six strains on the above five media incubated aerobically, aerobically with CO_2 and anaerobically with CO_2 showed that the T-H agar with azide produced haemolysis of both sheep and horse red cells. The haemolytic activity in azide medium was not influenced by incubation in CO_2; anaerobiosis tended to reduce the degree of haemolysis.

v) Haemolysis of human and horse red cells. The cultures on human blood tryptose agar base without azide gave moderate-sized colonies without haemolysis; with azide smaller colonies having a double zone of haemolysis were produced. Horse blood plates without azide showed similar colony sizes and a single haemolysis zone and with azide a double zone. A faint extra zone of haemolysis was shown by all strains on the human blood plates with azide, but these plates appeared to contain less haemoglobin than the horse blood media.

vi) The Camp reaction. was positive only with the C. perfringens control culture after anaerobic incubation, the pneumococci were negative after both aerobic and anaerobic incubation. The plates spread with azide did not show the

double zone of haemolysis produced on horse blood containing agar.

4.6 DYE INHIBITION STUDIES

4.6.1 Inhibition of cultures in Todd-Hewitt broth.

The results of the first tests for dye inhibition of the serotype 33 strain in broth culture (Section 2.8.1) showed complete inhibition of growth in the bromothymol blue solution (BTB) 1% but not at 0.1 or 0.01%. Inhibition was produced by methylene blue (MB) and toluidine blue but only in the aerobic zone, a 10 mm blue region at the top of the broth tube. Bromocresol purple did not affect growth at the three concentrations tested.

4.6.2 Inhibition studies by the agar slope method.

i) Initial testing of sixteen dyes: the results obtained from the four strains, serotypes 09 (T891), 16 (T895) and 34 (T904 and T918) in the dye inhibition tests by the agar gradient method (Section 2.8.2, i) are given in Table 4.18.

Bromothymol blue (BTB), methylene blue (MB), toluidine blue (TB), neutral red (NR), janus green (JG), nile blue (NB), malachite green (MG), coomassie blue (CB) and crystal violet had shown some degree of inhibition and were selected for further study.

ii) Inhibition tests of nine from the initial sixteen and a further nine dyes.: the six test strains, tested against

TABLE 4.18

Growth inhibition studies of 16 dyes

by the agar slope method.

Dyes	Results obtained with serotypes				
	34	34	09	16	SOX
BB	+	+	+	+	+
BP	+	+	+	+	+
CR	+	+	+	+	+
CO	+	+	+	+	+
NR	+	+	+	+	+
MR	+	+	+	+	+
SF	+	+	+	+	+
CB	+	+	+	+	+
PR	+	+	+	+	+
PP	+	+	+	+	+
NB	+	15	15	15	+
JG	+	+	10	10	+
CV	15	15	15	15	I
MB	40	40	70	40	I
TB	5	I	5	I	I
MG	I	I	I	I	I

+ = growth along the length of the streak
I = no growth
SOX = Staph. aureus (Oxford strain).

The figures represent the length of growth of the test strains along the streak, in mm.

The dyes were included at the following concentrations: BB, CR, MR, PR at 0.002%; RR, NR, JG, NB, CV, MB, TB and PP at 0.001%; SF and CB at 0.005% and 0.0002% for MG.

BB = bromothymol blue;
BP = bromocresol purple;
RR = cresol red;
CR = congo red;
NR = neutral red;
MR = methyl red;
SF = saffronin;
CB = coomassie blue;
PR = phenol red;
PP = phenolphthalein;
NB = nile blue;
JG = janus green;
CV = crystal violet;
MB = methylene blue;
TB = toluidine blue;
MG = malachite green;

the eighteen dye concentrations given in Section 2.8.2, ii, gave the inhibition patterns shown in Table 4.19. Potentially useful differential effects were seen with BTB, TB, JG and EY.

iii) Inhibition studies of ten dyes, selected from the twenty-five previously tested, on the six test strains, at the dye concentrations given in Section 2.8.2, iii, are recorded in Table 4.20. These findings suggested possible dye concentrations for agar plates containing a single dye level.

iv) Agar slope inhibition studies of nine dyes against four strains under aerobic and anaerobic conditions on the four pneumococcal cultures, at the concentrations given in Section 2.8.3 are recorded in Table 4.21. In general the organisms were more dye sensitive anaerobically than aerobically, e.g. the inhibition of strains T918 and T895 by BB, JG, NB and MG. It was not possible to compare the effect of dyes on T891, under the two conditions, as this culture failed to grow aerobically.

4.6.3 Paper strip method.

Of the 21 dyes tested by the impregnated paper strip method (Section 2.8.4) luxol fast blue, nigrosin, naphthol yellow, orange G, rhodamine B, saffron, Best's carmine, celestin blue, and brilliant blue showed no inhibitory effect. Acridine orange gave slight inhibition of strains if the bacterial dose was light; alcian blue inhibited one strain and eosin yellowish inhibited all but one of the six

TABLE 4.19

Repeat agar slope inhibition tests of eighteen dyes.

Dye	Test strain					
	T891	T895	T904	W894	T918	T966
AO	n	d	d	n	d	d
AB	+	+	+	+	+	+
EY	+	I	I	+	I	n
BB	I	+	I	I	I	n
MB	+	+	+	+	+	+
TB	I	+	I	I	I	+
NR	+	+	+	+	+	n
JG	+	+	I	I	I	n
NB	+	+	+	+	+	+
MG	+	+	+	+	+	+
COB	+	+	+	+	+	+
CV	I	I	I	I	I	I
OG	+	+	+	+	+	+
LB	+	+	+	+	+	+
BC	+	+	+	+	+	+
CEB	+	+	+	+	+	+
BRB	+	+	+	+	+	+
RB	+	+	+	+	+	+

I = growth inhibited;
d = doubtful result - reduced colony size produced;
+ = no inhibition of growth;
n = no growth on the culture control plate.

AO = acridine orange; AB = alcian blue;
BC = Best's carmine; BRB = brilliant blue
CEB = cestin blue; EY = eosin yellowish;
LB = luxol blue; OG = orange G;
RB = rhodamine blue.

The dyes were included at the following concentratios:-
0.0010% - MB, TB, NR, NB, COB, CV, OG, LB, BC, CEB, BB AND RB.
0.0020% - AO, AB, MG, AND JG.
0.0005% - BTB AND EY.

TABLE 4.20

Further inhibition studies of ten dyes by the agar slope method.

Dye	Test strains					
	T891	T895	T904	W894	T918	T966
NB	I	12	10	15	6	10
CV	I	35	40	35	40	35
TB	I	I	I	I	I	I
BTB	I	2	10	10	10	I
JG	I	10	15	15	15	2
MG	I	5	30	30	35	30
MB	I	I	I	I	I	I
EY	30	+	d	d	d	+
AB	+	+	+	+	+	+
AO	I	30	30	25	30	30

I = inhibition of all growth along the streak;
+ = no inhibition of growth;
d = doubtful result - reduced colony size produced;

Figures used are the length of growth along the streak, in mm.

The dye agar base contained dyes at the following concentratios:-

0.0010% - BTB, TB, NB, JG and EY.
0.0020% - AB.
0.0005% - MB and AO.
0.000002% - MG.

TABLE 4.21

Inhibition studies of nine dyes

on strains grown on Columbia agar

under aerobic and anaerobic cultural conditions

Dye	Atmos	Strains tested			
		T904	T918	T891	T895
BTB	A	I	I	I	I
	O	I	+	+	+
MB	A	I	I	I	I
	O	I	I	n	I
TB	A	I	I	I	I
	O	I	I	n	I
NR	A	+	+	d	+
	O	+	+	n	+
JG	A	I	I	I	I
	O	I	+	n	+
NB	A	I	I	I	I
	O	I	I	n	+
MG	A	I	I	+	I
	O	+	+	n	I
CB	A	+	d	+	d
	O	+	+	n	+
CV	A	I	I	I	I
	O	I	I	n	I

A = anaerobic incubation with added CO_2;
O = aerobic incubation without CO_2;
I = inhibition zone >5 mm;
d = doubtful result- inhibition zone <5 mm;
+ = no inhibition of growth;
n = no growth on the control plate;
Atmos = cultural atmosphere.

The dyes were included at the following concentrations: BB, at 0.02%; NR, JG, NB, CV, MB, TB and at 0.01%; CB at 0.05% and 0.002% for MG.

cultures used. Only bromothymol blue, malachite green and janus green, from the nine dyes of the initial sixteen tested, gave satisfactory results.

4.6.4 Inhibition studies by the dye containing agar plate method

The dye concentrations and the pneumococcal strains used in these experiments are given in Tables 2.6-10. The investigation of dye inhibition was a series of progressive biological titrations of initially ten and subsequently eight dyes. The results of each experiment were used to determine the conditions for the next, which in turn modified the next stage. The modifications were chosen specifically for each dye at every stage and are therefore given collectively in Tables 2.6-7 for Stages 1-8, and in Tables 2.8-9 for Stages 9-19. The many stages of the modifications to dye concentrations produced, however, a large number of results; these are given in Tables 4.22-43 and summarised below.

Stage 1 : The results of the eight cultures tested against the ten dyes prepared at two concentrations are given in Table 4.22. Comparable results at both dye levels were seen with NB, BTB, JG and TB. The higher dye levels of CV, MG, AO, MB and EY inhibited all cultures and the results given in the table are those seen with the lower concentrations. The majority of dyes inhibited or greatly reduced pneumococcal growth at the concentration used.

Stage 2 : The results for the eight strains used in Stage

TABLE 4.22

Inhibition studies by the agar plate containing method. Stage 1.

Dye	Test strains							
	T891	T895	T904	W894	T918	T966	T1214	T1217
NB	I	I	I	I	I	I	I	I
BTB	I	I	I	I	I	I	I	I
JG	I	I	I	I	I	I	I	I
CV*	d	I	d	I	d	d	I	I
MG*	I	I	I	I	d	I	I	I
AO*	d	d	d	I	d	d	I	I
TB	I	I	I	I	I	I	I	I
MB*	I	I	I	I	I	+	I	I
EY*	+	d	d	d	d	d	d	+
AC*	+	+	+	+	+	+	+	+

I = inhibition of growth;
d = reduced colony size;
+ = no inhibition of growth;
* = the results given are for the more dilute plate for these dyes; the higher concentration gave total inhibition. Those dyes without the * gave the same results at both dilutions.

Dye, concentration (%) and volume (ml) added

Bromothymol blue	Nile blue	Acridine orange	Crystal violet
0.02	0.10	0.10	0.01
(1.0, 0.5)	(1.0, 0.5)	(1.5, 1.0)	(1.0, 0.5)
Janus green	**Methylene blue**	**Malachite green**	**Toluidine blue**
0.01	0.10	0.002	0.10
(1.0, 0.5)	(0.5, 0.2)	(1.5, 1.0)	(0.5, 0.2)

1, when tested on the modified dye concentration plates, containing 1.0 and 0.5 ml of each dye, gave the results shown in Table 4.23. If comparable results were obtained at both the levels tested only one result is listed. The levels were not optimal but promising inter strain differences were indicated.

Stage 3 : The results of testing the eight test at the new dye levels are given in Table 4.24. Dye concentrations were chosen from these results for the testing of 40 pneumococcal strains for inhibition patterns (Stage 4).

Stage 4 : The results of the 40 strains tested against the eight dyes are given in Tables 4.25-26. Patterns of inhibition were seen with a number of strains and the two pairs of isolates of the same serotypes from the same patients gave reproducible results. The reactions of MG and JG were not sufficiently discriminatory and variations to these and the other dyes were sought to further refine the method.

Stage 5 : The results of the revised dye concentration plates against the ten test strains are given in Table 4.27. The concentrations were in general too inhibitory.

Stage 6 : The results of testing levels modified after the results of stage 5 are recorded in Table 4.28. These values were generally not sufficiently inhibitory and again modifications were sought.

Stage 7 : The results of the inhibition tests made in parallel for the five cultures and the single tests for the six other strains are given in Table 4.29. Comparable

TABLE 4.23

Inhibition studies by the agar plate containing method. Stage 2.

Dye agar plates		Test strain							
		T891	T895	T904	W984	T918	T966	T1214	T1217
BTB	i	I	I	I	I	I	I	I	I
"	ii	+	+	+	+	+	+	+	I
JG	i	+	+	I	I	I	+	I	I
"	ii	+	+	+	+	+	+	+	+
NB		+	+	+	+	+	+	+	+
CV		+	+	+	+	+	+	+	+
MG		+	+	+	+	+	+	+	+
TB		+	+	I	I	+	+	I	I
AO	i	+	+	+	I	+	+	I	I
"	ii	+	+	+	I	+	+	+	+
EY	i	d	d	d	d	d	d	d	d
"	ii	+	d	d	d	d	d	d	d
MB	i	+	d	d	I	+	+	I	I
"	ii	+	+	d	I	+	d	d	I

I = inhibition of growth;
d = reduction in colony size;
+ = no reduction in growth.

Dye, concentration (%) and volume (ml) added

Bromothymol blue	Nile blue	Acridine orange	Crystal violet
0.02	0.20	0.033	0.033
Janus green	**Methylene blue**	**Malachite green**	**Toluidine blue**
0.20	0.01	0.002	0.01

i = 1.0 and ii = 0.5 ml of dye/15ml agar plate)
Where i and ii are not specified in the table the same results were obtained at both concentrations.

TABLE 4.24

Inhibition studies by the agar plate containing method. Stage 3.

Dye		Test strain							
		T891	T895	T904	W984	T918	T966	T1214	T1217
NB	i	+	+	+	I	+	+	I	I
"	ii	+	+	+	+	+	+	+	+
CV	i	+	+	+	d	+	+	I	I
"	ii	+	+	+	+	+	+	d	d
MG	i	+	I	+	I	+	I	I	I
"	ii	+	I	+	d	+	d	d	d
BTB	i	I	I	+	I	+	+	I	I
"	ii	I	+	+	I	+	+	d	I
MB	i	d	d	I	I	d	d	I	I
"	ii	+	d	d	I	d	I	I	I
TB	i	d	d	I	I	I	I	I	I
"	ii	+	+	d	I	+	+	I	I
JG		+	+	+	+	+	+	d	d
AO		+	+	+	d	+	+	I	I

I = inhibition of growth;
d = reduced colony size;
+ = no inhibition of growth;

Dye, concentration (%) and volume (ml) added

Bromothymol blue	Nile blue	Acridine orange	Crystal violet
0.02	0.10	0.033	0.01
(0.8, 0.6)	(0.3, 0.1)	(0.8, 0.6)	(0.8, 0.5)
Janus green	**Methylene blue**	**Malachite green**	**Toluidine blue**
0.02	0.01	0.002	0.01
(0.8, 0.6)	(1.5, 1.2)	(0.7, 0.5)	(0.8, 0.6)

(i = first volume, ii = second volume ml of dye/15ml agar plate)

TABLE 4.25

Inhibition studies of 20 of the 40 pneumococcal strains by the agar plate method. Stage 4.

Test Strain Serotype	Dye agar							
	BTB	AO	TB	MG	JG	CV	NB	MB
01	I	I	I	I	I	d	I	+
03	I	I	I	I	I	+	I	+
03*	I	I	I	I	I	I	I	I
03*	I	I	I	I	I	I	I	I
06	I	I	I	+	I	+	I	I
06*	d	I	I	+	I	d	I	I
06*	d	I	I	+	I	d	I	I
06	I	I	d	+	I	I	I	I
06	I	I	I	+	I	+	I	I
06	d	I	I	+	I	+	I	I
07	+	I	I	+	I	+	I	d
09	+	+	+	+	I	+	d	+
09	I	+	I	+	I	+	d	+
09	+	I	+	+	I	+	d	d
09	d	+	d	+	I	+	I	d
09	d	+	d	+	I	+	I	d
14	+	I	I	+	I	+	I	+
14	+	I	I	+	I	+	I	I
15	d	I	I	+	I	d	I	I
15	d	I	I	+	I	+	I	d

I = inhibition of growth; * = paired isolates.
d = reduction in colony size; NC = non-capsulate strain;
+ = no reduction in growth; PI = serogroup I strain.

Dye, concentration (%) and volume (ml) added

Bromothymol blue	Nile blue	Acridine orange	Crystal violet
0.01	0.025	0.04	0.002
Janus green	Methylene blue	Malachite green	Toluidine blue
0.02	0.01	0.002	0.01
(0.80)	(0.70)	(1.0)	(0.80)

(1.0 ml was taken from each dye)

TABLE 4.26

Inhibition studies of the remaining 20 of the 40 pneumococcal strains by the agar plate method. Stage 4.

Test Strain Serotype	Dye agar							
	BTB	AO	TB	MG	JG	CV	NB	MB
16	+	I	I	+	I	+	I	+
16	I	+	d	I	I	+	I	+
16	+	+	+	+	I	+	d	I
19	+	I	I	+	I	+	I	I
19	I	I	I	+	I	+	I	I
19	+	I	I	+	I	+	I	I
19	+	I	I	+	I	+	I	I
22	d	I	d	+	I	I	I	I
23	+	+	I	+	+	+	I	+
23	+	+	+	+	I	+	I	I
23	I	+	I	+	I	+	I	I
31	d	I	I	+	I	+	I	I
31	d	I	I	+	I	d	I	I
33	I	+	+	I	I	+	d	+
33	d	I	d	+	I	d	I	I
34	I	+	I	+	I	+	I	+
34	d	+	d	+	I	+	I	d
34	I	+	I	+	I	+	I	I
NC	I	d	I	d	I	d	I	I
PI	+	I	I	+	I	+	I	+

I = inhibition of growth;
d = reduction in colony size; NC = non-capsulate strain;
+ = no reduction in growth; PI = serogroup I strain.

Dye, concentration (%) and volume (ml) added

Bromothymol blue	Nile blue	Acridine orange	Crystal violet
0.01	0.025	0.04	0.002
Janus green	**Methylene blue**	**Malachite green**	**Toluidine blue**
0.02	0.01	0.002	0.01
(0.80)	(0.70)	(1.0)	(0.80)

(1.0 ml was taken from each dye)

TABLE 4.27

Inhibition studies by the agar plate method. Stage 5.

Test strain serotype	Dye							
	BTB	AO	TB	MG	JG	CV	NB	MB
03	I	I	I	I	d	+	I	I
19	I	d	I	I	d	+	I	I
03*	I	I	I	I	I	I	I	I
03*	I	I	I	I	I	I	I	I
16	d	d	I	I	d	+	I	I
14	d	I	I	I	I	d	I	I
PI	I	I	I	I	I	d	I	I
23	d	d	I	I	d	+	I	I
14	I	I	I	I	I	I	I	I
19	I	I	I	I	I	+	I	I

* = isolated from two sites on the same patient.
I = inhibition of growth;
d = reduced colony size;
\+ = no inhibition of growth;

Dye, concentration (%) and volume (ml) added

Bromothymol blue	Nile blue	Acridine orange	Crystal violet
0.01	0.10	0.04	0.002
(0.80)	(0.30)	(1.04)	(0.80)
Janus green	Methylene blue	Malachite green	Toluidine blue
0.01	0.10	0.002	0.10
(1.0)	(0.90)	(0.70)	(1.0)

TABLE 4.28

Inhibition studies by the agar plate method. Stage 6.

Test strain serotype	Dye							
	BTB	AO	TB	MG	JG	CV	NB	MB
19	+	+	I	I	+	+	+	+
16	+	+	+	I	+	+	+	+
14	+	+	+	I	+	+	+	+
PI	+	+	+	I	+	+	+	+
23	+	+	+	I	+	+	+	+
14	+	I	I	I	I	I	I	I
19	+	I	I	I	+	d	+	+

The three strains of serotype 03 failed to grow on the control plate.

I = inhibition of growth;
d = reduced colony size;
+ = no inhibition of growth;

Dye, concentration (%) and volume (ml) added

Bromothymol blue	Nile blue	Acridine orange	Crystal violet
0.01	0.09	0.04	0.002
(0.90)	(0.40)	(0.40)	(1.2)
Janus green	**Methylene blue**	**Malachite green**	**Toluidine blue**
0.02	0.01	0.00002	0.01
(0.40)	(0.40)	(0.80)	(0.40)

TABLE 4.29

Inhibition studies by the agar plate method. Stage 7.

Test strain serotype	Dye							
	BTB	AO	TB	MG	JG	CV	NB	MB
PI	I	+	+	I	+	d	+	+
PI*	I	d	+	I	+	d	+	+
23	+	+	d	I	+	d	+	d
23*	I	+	+	I	+	d	+	+
14	+	+	d	I	+	d	+	d
14*	I	+	+	I	+	d	+	+
19	I	+	I	I	+	d	+	I
19*	I	d	I	I	d	d	+	I
16	+	+	+	I	+	+	+	+
16*	+	+	+	I	+	+	+	+
09	+	+	d	I	+	+	+	d
01	+	+	+	I	+	+	+	+
NC	I	+	d	I	+	I	+	d
14	+	I	+	I	d	d	+	+
03	+	d	+	I	+	d	+	+
06	I	I	I	I	+	d	+	I

I = inhibition of growth;
d = reduction in colony size;
+ = no reduction in growth;
* = repeat cultures of the above strain.

Dye, concentration (%) and volume (ml) added

Bromothymol blue	Nile blue	Acridine orange	Crystal violet
0.0033	0.0035	0.008	0.001
Janus green	Methylene blue	Malachite green	Toluidine blue
0.0028	0.0014	0.000005	0.002

(3.0 ml was taken from each dye)

patterns were seen with the tests for the five pairs. Inter-strain differences were more apparent with plates prepared by adding 3 ml volumes of dye instead of fractions of 1 ml; further modifications were considered necessary.

Stage 8 : The results of the dye inhibition studies of slightly different amounts of dye than those tested in stage 7 are given in Table 4.30. The results were not suitable for routine testing of strains.

Stage 9 : The effect of the two levels bacterial dosage (approximately 10^8 and 10^3cfu/ml) on pneumococcal susceptibility to dyes are given in Table 4.31. The lower bacterial dosage increased the senstivity of strain 6 (serotype 03) on BTB, strains 7 (type 14) and 8 (type 19) on JG and on TB, and strains 3 (type 23) and 5 (type 04) on AO and strain 7 on MB. The inhibition results shown by the majority of dyes were unaffected by the concentration of pneumococci present. The dosage used was standardised after the results of Stage 10.

Stage 10 : The eight strains, grown into the log phase and diluted for use, gave viable counts of approximately 10^5cfu/ml and the patterns of inhibition in Table 4.32.

Stage 11 : The tolerance of the eight strains to the adjusted dye levels following standardisation of the bacterial dose are given in Table 4.33.

Stage 12 : The results of growth on the further adjusted dye agar plates are given in Table 4.34.

Stage 13 : The results of the parallel testing of the eight strains on agar plates containing narrow ranges of the dye

TABLE 4.30

Inhibition studies by the agar plate method. Stage 8.

Test strain serotype	Dye							
	BTB	AO	TB	MG	JG	CV	NB	MB
14	d	d	I	I	d	d	d	I
PI	d	+	I	d	+	d	+	I
23	d	+	I	d	+	d	+	I
14	d	+	I	I	+	d	I	I
19	d	+	I	d	+	d	+	I
03	d	+	d	I	+	I	+	+
16	d	+	d	I	+	d	+	+
NC	d	d	I	I	d	I	+	I

I = inhibition of growth;
d = reduction in colony size;
+ = no reduction in growth;

Dye, concentration (%) and volume (ml) added

Bromothymol blue	Nile blue	Acridine orange	Crystal violet
0.0033	0.0035	0.008	0.001
(3.40)	(3.60)	(3.40)	(3.4)
Janus green	**Methylene blue**	**Malachite green**	**Toluidine blue**
0.0028	0.0014	0.000005	0.002
(3.40)	(3.40)	(2.60)	(3.40)

TABLE 4.31

Inhibition studies at two bacterial concentrations by the agar plate containing method. Stage 9.

Sero Type	Dye							
	TB		BB		AO		MB	
	L	H	L	H	L	H	L	H
14	d	d	I	I	I	I	d	d
09	d	d	I	I	I	I	d	d
23	I	I	+	+	d	I	I	I
23	d	d	I	I	d	d	I	I
04	d	d	+	+	d	I	I	I
03	I	I	I	+	+	+	I	I
14	I	d	+	+	+	+	I	d
19	I	d	+	+	+	+	I	I
	JG		NB		CV		MG	
	L	H	L	H	L	H	L	H
14	+	+	+	+	+	+	+	+
09	+	+	+	+	+	+	+	+
23	d	+	+	+	+	+	+	+
23	+	+	+	+	+	+	+	+
04	+	+	+	+	+	+	+	+
03	d	+	+	+	+	+	+	+
14	I	+	+	+	+	+	+	+
19	I	+	+	+	+	+	+	+

I = inhibition of growth;
d = reduction in colony size;
+ = unrestricted growth;
L = low pneumococcal dose (10^3 cfu/ml);
H = high pneumococcal dose (10^8 cfu/ml).

Dye, concentration (%) and volume (ml) added

Bromothymol blue	Nile blue	Acridine orange	Crystal violet
0.003	0.003	0.013	0.001
Janus green	Methylene blue	Malachite green	Toluidine blue
0.0027	0.0013	0.000005	0.0013

(3.0 ml of each dye was added to 12 ml of agar)

TABLE 4.32

Inhibition studies by the agar plate containing method.

Stage 10.

Sero type	Dye							
	TB	BB	AO	MB	JG	NB	CV	MG
14	I	+	I	I	d	+	+	I
09	I	+	I	d	d	+	+	+
23	I	+	I	I	I	+	+	+
23	I	+	d	+	+	+	+	+
04	I	+	I	d	+	+	+	+
03	I	+	I	d	I	I	+	+
14	I	+	I	I	d	+	+	+
19	I	+	+	I	+	+	+	+

I = inhibition of growth;
d = reduction in colony size;
+ = unrestricted growth;

Dye, concentration (%) and volume (ml) added

Bromothymol blue	Nile blue	Acridine orange	Crystal violet
0.00375	0.001	0.00325	0.0008

Janus green	Methylene blue	Malachite green	Toluidine blue
0.0038	0.00043	0.000005	0.00325

(3.0 ml of each dye was added to 12 ml of agar)

TABLE 4.33

Inhibition studies by the agar plate containing method.

Stage 11.

Sero type	Dye							
	TB	BB	AO	MB	JG	NB	CV	MG
14	I	+	+	+	I	+	I	+
09	I	+	+	+	d	+	I	+
23	I	+	+	+	I	+	I	+
23	d	+	+	+	+	+	I	+
04	I	+	+	+	I	+	I	+
03	I	I	+	+	I	I	I	+
14	I	+	+	+	I	I	I	+
19	I	+	+	+	I	d	I	I

I = inhibition of growth;
d = reduction in colony size;
+ = unrestricted growth;

Dye, concentration (%) and volume (ml) added

Bromothymol blue	Nile blue	Acridine orange	Crystal violet
0.005	0.0015	0.0044	0.00026

Janus green	Methylene blue	Malachite green	Toluidine blue
0.00348	0.00052	0.000010	0.00026

(3.0 ml of each dye was added to 12 ml of agar)

TABLE 4.34

Inhibition studies by the agar plate containing method.

Stage 12.

Sero type	Dye							
	TB	BB	AO	MB	JG	NB	CV	MG
14	d	I	+	+	+	I	+	+
09	+	+	I	+	+	+	+	+
23	d	+	+	+	+	I	+	+
23	+	I	+	+	+	+	+	+
04	+	+	+	+	+	+	+	+
03	I	I	+	I	+	I	+	+
14	d	+	+	d	+	I	+	+
19	d	I	+	d	+	I	+	+

I = inhibition of growth;
d = reduction in colony size;
+ = unrestricted growth;

Dye, concentration (%) and volume (ml) added

Bromothymol blue	Nile blue	Acridine orange	Crystal violet
0.005	0.0015	0.0046	0.00040
Janus green	Methylene blue	Malachite green	Toluidine blue
0.0036	0.00059	0.000025	0.00028

(3.0 ml of each dye was added to 12 ml of agar)

solutions are given in Table 4.35. The BB results were comparable with those seen in Table 4.34 but the repeat NB concentration inhibited all of the test strains. Suitable inhibitory levels of MG and MB appeared to be between the concentrations tested. The CV, TB, AO and JG patterns were either not reproducible from the previous stage or the inhibition seen was not suitable for differentiation.

Stage 14 : Further testing of dye inhibition at narrow concentration ranges gave the results in Table 4.36. More suitable patterns appeared to be produced at these levels for NB, GV, AO and JG. Less satisfactory results were shown by MB, BB and TB in which non-reproducible or difficult discriminatory inhibition was seen.

Stage 15 : Adjusted dye concentrations following stage 14 gave the discriminant patterns of inhibitions, given in Table 4.37, except for the CV concentration which was not sufficiently inhibitory.

Stage 16 : Modified dye studies and testing of potassium tellurite inhibition gave the results in Table 4.38. Discriminant results were suggested by most of the dyes tested except for CV, which was at a too inhibitory level.

Stage 17 : Testing of inhibition on the same batch of agar plates on three consecutive days and plates prepared from dye solutions autoclaved in the agar, gave the results in Tables 4.39 and 4.40. Variation in inhibition patterns were seen over the three days with all dyes and with a range of strains, and little evidence of deterioration of dye activity. Autoclaving the dye in the agar reduced the

TABLE 4.35

Inhibition studies by the agar plate containing method.

Stage 13.

Dye and dilution tested	Strain used							
	1	2	3	4	5	6	7	8
MB a	I	+	+	+	+	I	I	I
b	+	+	+	+	+	+	+	d
AO a	I	I	I	I	I	I	d	d
b	I	I	I	I	I	I	d	d
CV a	I	I	I	I	I	I	I	I
b	I	I	I	I	I	I	I	I
c	I	d	+	I	I	I	I	I
TB a	I	d	d	d	I	I	I	I
b	I	d	I	d	d	I	I	I
c	I	d	d	d	d	I	I	I
MG a	+	+	+	+	+	+	+	+
b	I	+	+	I	I	I	I	I
JG a	I	d	I	d	I	I	I	I
b	I	d	I	d	I	I	I	I
c	I	d	I	d	I	I	I	I
BB	I	+	+	I	+	I	+	I
NB	I	I	I	I	I	I	I	I

I = inhibition of growth;
d = reduction in colony size;
+ = unrestricted growth;
a, b, c = progressively more dilute solutions as below.

Dye, concentration (%) and volume (ml) added

Bromothymol blue	Nile blue	Acridine orange	Crystal violet
0.005	0.0015	0.0043, 0.0046	0.0004 0.0006

Janus green	Methylene blue	Malachite green	Toluidine blue
0.0038, 0.0036 0.00348	0.00059 -	0.000025 0.000050	0.000325, 0.00028 0.00026

(3.0 ml of each dye was added to 12 ml of agar)

TABLE 4.36

Inhibition studies by the agar plate containing method.

Stage 14.

Dye and dilution tested		Strain used							
		1	2	3	4	5	6	7	8
AO	a	I	I	I	d	I	I	I	+
	b	d	d	+	+	+	+	+	+
CV	a	I	I	I	I	I	I	I	I
	b	+	+	+	I	+	d	+	I
TB	a	d	d	I	+	d	I	d	I
	b	d	d	I	+	+	I	d	I
NB	a	I	I	I	I	I	I	I	I
	b	+	+	+	+	+	I	I	+
MG	a	+	+	+	I	d	I	+	+
	b	I	I	+	I	I	I	I	I
JG	a	I	d	I	+	d	I	d	I
	b	I	+	I	+	+	I	d	I
BB		I	I	I	I	I	I	I	I
MB		+	+	+	+	+	d	I	d

I = inhibition of growth;
d = reduction in colony size;
+ = unrestricted growth;
a, b = progressively more dilute solutions as below.

Dye, concentration (%) and volume (ml) added

Bromothymol blue	Nile blue	Acridine orange	Crystal violet
0.005	0.0015	0.0043	0.00044
-	0.0010	0.00325	0.00026

Janus green	Methylene blue	Malachite green	Toluidine blue
0.00348	0.00059	0.000035	0.000236
0.0027	-	0.000050	0.000216

(3.0 ml of each dye was added to 12 ml of agar)

TABLE 4.37

Inhibition studies by the agar plate containing method.

Stage 15.

Sero type	Dye							
	TB	BB	AO	MB	JG	NB	CV	MG
14	+	+	+	d	d	+	+	+
09	+	+	I	I	d	I	+	+
23	+	+	+	d	d	+	+	+
23	+	I	+	+	+	+	+	+
04	+	+	+	+	d	+	+	+
03	I	I	+	I	I	I	+	I
14	I	+	I	I	I	I	+	I
19	+	I	+	I	I	I	+	+

I = inhibition of growth;
d = reduction in colony size;
+ = unrestricted growth;

Dye, concentration (%) and volume (ml) added

Bromothymol blue	Nile blue	Acridine orange	Crystal violet
0.0043	0.00107	0.00406	0.000296

Janus green	Methylene blue	Malachite green	Toluidine blue
0.00225	0.00060	0.000040	0.000163

(3.0 ml of each dye was added to 12 ml of agar)

TABLE 4.38

Inhibition studies of dyes and potassium tellurite by the agar plate containing method. Stage 16.

Sero type	Dye								
	TB	BB	AO	MB	JG	NB	CV	MG	PT
14	+	I	I	I	I	I	I	I	I
09	+	+	I	I	d	I	I	+	+
23	+	+	+	I	I	+	I	+	+
23	+	I	+	+	+	+	I	+	+
04	+	+	d	I	d	I	I	+	+
03	I	I	d	I	I	I	I	I	I
14	d	+	d	I	I	I	I	+	+
19	+	I	+	I	I	I	I	+	+

I = inhibition of growth;
d = reduction in colony size;
+ = unrestricted growth;

Dye, concentration (%) and volume (ml) added

Bromothymol blue	Nile blue	Acridine orange	Crystal violet
0.0043	0.00107	0.00406	0.000296

Janus green	Methylene blue	Malachite green	Toluidine blue
0.00225	0.00060	0.000040	0.000163

(3.0 ml of each dye was added to 12 ml of agar)

TABLE 4.39

Inhibition studies of a batch of dye containing agars and agars prepared by autoclaving with the dye. Stage 17 A.

Dye and Day tested	Strain used							
	14	09	23	23	04	03	14	19
TB 1	I	I	I	+	+	I	I	I
2	I	+	I	+	+	I	d	I
A	I	d	d	+	+	I	I	I
3	+	d	d	+	+	I	I	I
BB 1	I	+	I	I	+	I	+	I
2	I	+	+	I	+	I	+	I
A	I	+	+	I	+	I	+	I
3	I	+	+	I	+	I	+	I
AO 1	+	+	+	+	+	I	+	+
2	I	I	+	+	+	d	I	+
A	I	+	+	+	+	I	d	+
3	+	d	+	+	d	d	I	+
MB 1	I	I	I	I	+	I	I	I
2	I	I	I	I	I	I	I	I
A	I	I	I	+	I	I	I	I
3	d	d	d	d	d	I	I	I

A = dye solution autoclaved, Day 2.
I = inhibition of growth;
d = reduction in colony size;
+ = unrestricted growth;

Dye, concentration (%) and volume (ml) added

Bromothymol blue	Nile blue	Acridine orange	Crystal violet
0.0043	0.00107	0.00406	0.000245

Janus green	Methylene blue	Malachite green	Toluidine blue
0.00225	0.00060	0.000040	0.000163

(3.0 ml of each dye was added to 12 ml of agar)

TABLE 4.40

Inhibition studies of a batch of dye containing agars and agars prepared by autoclaving with the dye. Stage 17 B.

Dye and Day tested	Strain used 14	09	23	23	04	03	14	19
JG 1	d	d	d	+	+	I	d	+
2	I	d	+	+	d	I	I	I
A	+	d	+	+	I	I	+	+
3	d	d	d	+	d	d	I	I
NB 1	+	I	+	+	+	I	I	I
2	I	I	+	+	d	I	I	I
A	+	+	+	+	+	I	I	+
3	+	I	+	+	d	I	I	I
CV 1	I	+	+	I	+	I	I	I
2	I	+	+	I	+	I	I	I
A	I	+	+	+	+	I	+	I
3	d	d	d	d	+	+	I	I
MG 1	+	+	+	+	+	I	+	+
2	+	+	+	+	+	+	+	+
A	+	+	+	+	+	d	+	+
3	+	+	+	+	+	+	+	+

A = dye solution autoclaved, Day 2.
I = inhibition of growth;
d = reduction in colony size;
+ = unrestricted growth;

Dye, concentration (%) and volume (ml) added

Bromothymol blue	Nile blue	Acridine orange	Crystal violet
0.0043	0.00107	0.00406	0.000245
Janus green	**Methylene blue**	**Malachite green**	**Toluidine blue**
0.00290	0.00060	0.000040	0.000163

(3.0 ml of each dye was added to 12 ml of agar)

inhibitory effects of JG, NB and CV for a significant number of strains, AO and MB each with a single culture, whilst the TB and BB results were unaffected.

Stage 18 : The results of testing 18 recent isolates and the standard eight strains against the dye levels modified from the previous stage's results and the tellurite levels used in stage 16 are given in Table 4.41. Inter-strain differences in dye susceptibility were seen for all serotypes to some degree. All the serotype 19 strains except one gave the same inhibition pattern.

Stage 19 : The results of testing 36 strains for dye susceptibility, CO_2 requirement and colonial appearance on Mitis-salivarius agar (M-S) are given in Tables 4.42-43. Differences were seen amongst serotypes 03 and 06 both with dye inhibition and colonial appearance on M-S agar. Only three of the 36 strains were CO_2 dependant. The two serotype 09 cultures differed in that the CO_2 dependant strain was not inhibited by, and the non-dependant strain was inhibited by CV. All of the five cultures of serotype 19 gave similar dye inhibition patterns including the two CO_2 requiring isolates; differences within this serotype were only seen on M-S agar. Little difference was seen in the serotype 14 cultures except that two strains were not inhibited on TB agar, one of these failed to grow on M-S agar. The serotype 23 strains gave similar patterns on dye and M-S agars except that two of the eight strains tested were tolerant of BB.

TABLE 4.41

Inhibition studies of dyes and potassium tellurite on 26 pneumococcal strains by the agar plate containing method. Stage 18.

Sero type	Dye								
	TB	BB	AO	MB	JG	NB	CV	MG	PT
23 a	+	+	d	d	+	+	+	+	+
" b	I	I	I	I	I	I	I	+	+
" c	+	I	I	+	+	+	I	+	+
" d	+	I	d	+	+	+	I	+	+
14 a	d	I	I	I	I	d	+	+	+
" b	I	I	d	I	I	I	I	+	+
" c	+	I	d	d	+	+	+	+	+
" d	I	I	I	I	I	I	I	+	+
19 a	I	I	I	I	I	I	d	I	I
" b	I	I	d	I	I	I	d	+	+
" c	I	I	I	I	I	I	I	+	+
" d	d	I	d	I	I	d	I	+	+
" e	I	I	d	I	I	I	I	+	+
" f	I	I	d	I	d	I	I	+	+
" g	I	I	d	I	d	I	I	+	+
" h	I	I	d	I	d	I	I	+	+
" i	I	I	d	I	d	I	I	+	+
03 a	I	I	+	I	I	I	I	+	+
" b	I	I	d	I	d	d	+	+	+
" c	I	I	d	I	d	d	+	+	+
09 a	I	+	I	I	d	I	I	+	+
" b	I	I	I	I	d	I	I	+	+
11	I	I	d	I	I	d	d	d	+
06 a	I	I	I	I	I	I	I	+	+
" b	I	I	I	I	I	I	d	+	+
" c	I	I	d	I	I	I	I	d	+
" d	+	I	+	d	d	+	d	+	+

I = inhibition of growth;
d = reduction in colony size;
+ = unrestricted growth;
Lower case letters denote examples of that serotype.
The dye levels were the same as in Table 4.40.

TABLE 4.42

Inhibition studies, CO_2 requirement and colonial appearance on Mitis-salvarius agar of 36 pneumococcal strains. Stage 19.

Sero type	M-S agar	Dye								
		BT	AO	TB	MG	JG	CV	NB	MB	PT
03	G	I	I	I	+	I	I	I	I	+
03	G	I	I	d	+	+	+	I	I	+
03	BR	+	+	+	+	d	+	+	d	+
03	n	I	I	d	+	d	I	I	I	+
03	G	d	+	d	+	d	d	I	I	+
06	BR	+	I	I	+	I	I	I	I	+
06	R	I	I	I	+	d	+	I	I	+
06	R	I	d	d	+	d	+	I	d	+
06	R	I	d	d	+	d	+	I	I	+
15	R	+	I	d	+	d	+	I	d	+
15	R	d	I	d	+	d	d	I	I	+
11	n	I	I	d	+	d	d	I	I	+
08	n	I	I	I	+	I	I	I	I	+
09	R	+	I	d	+	d	I	I	d	+
09□	R	+	I	d	+	d	+	I	d	+
19□	sc	I	I	I	+	I	I	I	I	+
19□	R	I	I	I	+	I	I	I	I	+
19	R	I	I	I	+	I	I	I	I	+
19	R	d	I	I	+	I	I	I	I	+
19	R	I	I	I	+	I	I	I	I	+

I = inhibition of growth; d = reduction in colony size;
+ = unrestricted growth; n = failure to grow;
□ = CO_2 requiring strain. sc = small negative colony
BR = bluish irridescence colony;
DB = dark blue coloured colony;
G = greyish colony.
GR = greenish irridescent colony;
R = irridescence colony;
The dye levels used were the same as in Table 4.40.

TABLE 4.43

Inhibition studies, CO_2 requirement and colonial appearance on Mitis-salvarius agar of 36 pneumococcal strains. Stage 19 B.

Sero type	M-S agar	Dye								
		BT	AO	TB	MG	JG	CV	NB	MB	PT
23	R	I	I	I	+	I	I	I	I	+
23	R	I	I	d	+	d	I	I	d	+
23	R	+	I	d	+	d	I	I	d	+
23	R	I	I	d	+	d	I	I	d	+
23	R	I	I	d	+	d	I	I	d	+
23	R	+	I	d	+	d	d	I	d	+
23	R	I	I	I	+	I	I	I	I	+
23	R	I	I	I	+	I	I	I	I	+
14	DB	+	I	d	+	I	I	I	I	+
14	n	+	d	n	+	I	I	I	I	+
14	DB	+	d	n	+	I	I	I	I	+
14	DB	+	I	I	+	d	I	I	I	+
14	DB	+	I	I	+	+	I	I	I	+
14	DB	+	I	I	+	I	I	I	I	+
14	DB	+	d	d	+	d	+	I	I	+
14	DB	+	I	I	+	d	I	I	I	+

I = inhibition of growth;
+ = unrestricted growth;
d = reduction in colony size;
n = failure to grow;

BR = bluish irridescence colony;
DB = dark blue coloured colony;
G = greyish colony.
GR = greenish irridescent colony;
R = irridescence colony;
sc = small negative colony.

The dye levels used were the same as in Table 4.40.

4.6.5 Colonial forms on Mitis-salivarius media

i) The growth on Mitis-salivarius and TYC agars of 11 pneumococcal strains, serotypes 01, 14, 17, 19 (three cultures), 23 (three cultures) and 34, showed that very small colonies were produced on TYC. These strains however on M-S agar gave larger colonies showing a number of distinctive colours and sizes. The observation of the phenomena depended upon examining the culture under the plate microscope after positioning the substage mirror to direct oblique light through the colonies. Under these conditions the observed characters were divided into three types: large irridescent colonies (I), smaller dark blue colonies (B) and large grey colonies (G). The 11 strains were divided into four groups:-

Colony-type I (Ct I): five cultures, two each of serotypes 23 and 19, and a serotype 34;

Colony type B (Ct B): one culture, serotype 14;

Colony type G (Ct G): one culture, serotype 19;

Colony type O (Ct O): five strains, which failed to grow on M-S, serotypes 06, 17, 19 and 23.

ii) Growth of the six strains on M-S and M-S-s (M-S without sucrose) produced five Ct I and one Ct B on M-S and on M-S-s four Ct I and two cultures unclassified because the colonies produced were too small.

iii) The results of the 24 pneumococcal strains cultured on M-S, M-S-s, T-H agar with trypan blue (T-Htb), RCM agar with trypan blue (RCMtb) and CA agar are given in Table 4.44. Irridescence was shown by 16 strains on M-S, 14

TABLE 4.44

Growth of 24 pneumococcal strains on Mitis-salivarius, with and without sucrose, and on T-H and RCM agar with trypan blue.

Strain Type	Medium				
	M-S	M-S-s	T-Htb	RCMtb	CBA
23	R	R	d	R	M
23	O	O	O	O	O
23	R	O	d	R	M
23	R	R	R	R	M
23	R	R	d	d	M
23	R	R	d	d	S
14	B	B	-	O	S
14	B	B	-	-	S
14	B	B	-	-	S
14	B	B	-	-	M
19	R	R	-	R	M
19	R	R	d	R	M
19	R	R	R	R	M
19	R	R	d	d	M
19	R	R	d	O	M
06	R	R	d	d	M
06	R	R	d	d	M
06	R	d	R	R	M
09	*	d	d	-	M
17	R	R	R	R	M
01	*	R	R	R	M
34	R	d	-	-	M
gI	G	G	d	-	M
08	O	R	d	d	M

R = irridescence, Ct I; d = doubtful Ct I;
B = dark blue colonies; G = greyish colonies;
- = colourless colonies; O = no growth;
* = culture gave blueish irridescent colonies, Ct Ib;
M = medium sized colonies; S = small colonies;
M-S = Mitis-salivarius agar; CBA = Columbia base agar;
M-S-s = Mitis-salivarius agar without sucrose;
T-Htb = Todd-Hewitt agar with toluidine blue;
RCMtb = reinforced clostridial agar with toluidine blue.

strains on S-M-S, six strains on T-Htb and nine strains on RCMtb. Growth on CA, the control plate, produced medium sized colonies with 19 cultures, small colonies with four strains and one culture, serotype 23, failed to grow on all media. Seven strains on M-S did not produce irridescences; four gave Ct B colonies and were all of serotype 14; the other three cultures, serotypes 01 and 09 and the serogroup I strain appeared as large bright blue colonies. Repeat testing of these seven strains confirmed the results for five strains, the four type 14 and the serogroup I cultures. The two other isolates, serotypes 09 and 01 both gave blueish irridescenct colonies and were classified as Ct Ib, variants of Ct I.

iv) The results of growing 70 consecuetive pneumococcal isolates on M-S showed 55 cultures of Ct I which included two isolates, both serotype 08 having a pink blue irridescence. The other 15 non-irridescent strains were divided into 10 Ct B cultures, consisting of eight serotype 14 and two non-capsulate isolates; four greyish colonies, Ct G, belonging to serotypes 03 (two cultures), 37 and 19, and one whiteish colony, Ct W, a serotype 37 strain. These results were summarised as:-

55 strains Ct I: mixed serotypes;
10 strains Ct B: serotype 14 (eight strains),
non-capsulate (two strains);
4 strains Ct G: serotype 03 (two strains),
serotypes 19 and 37;
1 strains Ct W: serotype 37.

v) Examinations for irridescence of pneumococci grown in M-S broth, and colonies transferred from M-S agar to microscope slides were negative. The suspension of colonies from M-S agar in PBS was initially negative but during observation on the microscope slide with the consequent drying a weak irridescence was seen.

vi) Testing of 60 strains, two years after the first experiments, confirmed the colonial effects and showed 45 cultures were of Ct I, two of which were of Ct Ib, both serotypes 19. The other 15 strains were divided into seven of Ct B, six serotype 14 and one non-capsulate variant; five were of Ct G, serotypes 03 (two strains), serogroup I (two strains) and serotype 19, and two of Ct W, serotypes 01 and 03.

A further 40 pneumococci, tested for growth characteristics on M-S as part of a larger investigation of differential factors, also showed the distinct colonial types; the detailed results are included with the Dye inhibition studies, Tables 4.42-3.

vii) The growth of eight strains on the two M-S agar formulae, with and without potassium tellurite showed that four strains were unaffected by the concentration of tellurite, three cultures were inhibited by the tellurite and one strain did not grow on M-S. The inhibition of pneumococci by tellurite was further investigated in the Dye inhibition studies, Tables 4.41 to 4.43.

4.7 ANTIBIOTIC SENSITIVITY TESTS.

4.7.1 Media and atmosphere effects on inhibition zones produced by penicillin, tetracycline, trimethoprim and co-trimoxazole

The results of testings the two pneumococcal strains on the sensitivity test agars (Section 2.9) against the above antibiotics are recorded in Table 4.45. On iso-sensitest (IS) and "chocolate"-IS (CIS) plates zone diameters for penicillin and tetracycline for sensitive strains were larger after aerobic than after anaerobic incubation. On blood-iso-sensitest agar (BIS) little difference was seen. Trimethoprim when tested on IS and BIS incubated anaerobically gave reduced zones but on CIS anaerobic culture gave larger zones. Co-trimoxazole zones were larger on IS and CIS under anaerobic conditions and smaller on BIS. The larger inhibition zones produced by co-trimoxazole in comparison could be the result of the sulphonamide fraction of this combined anti-microbial agent.

4.7.2 Sensitivity testing of 22 pneumococcal strains against tetracycline and trimethoprim by the disc and agar containing methods

The testing of 21 strains resistant to trimethoprim by the plate method and four cultures resistant by the disc technique by both the plate and disc methods gave the following results. Tetracycline gave comparable results by

TABLE 4.45

Media and atmosphere effects on penicillin, tetracycline, trimethoprim and co-trimoxazole inhibition zones on three versions of Isosensitest agar.

Strain	Atmosphere	Pen	Tet	Trim	Co-trim
		Isosensitest agar (IS)			
1	O_2	30	24	16	20
	AO_2	40	34	12	30
2	O_2	30	10	16	20
	AO_2	40	10	12	30
		Heated blood IS (CIS)			
1	O_2	30	14	10	20
	AO_2	40	30	14	24
2	O_2	30	0	10	20
	AO_2	40	10	14	24
		Blood IS (BIS)			
1	O_2	30	24	12	24
	AO_2	30	24	10	20
2	O_2	30	0	12	24
	AO_2	30	0	10	20

O_2 = aerobic incubation with added CO_2;
AO_2 = anaerobic incubation with added CO_2;
Pen = penicillin, 2 units;
Tet = tetracyclin, 5 µg;
Trim = trimethoprim, 2.4 µg;
Co-trim = co-trimoxazole, 25 µg.
The figures under the antibiotic columns are the diameter of the inhibition zones in mm.

either method with the same three cultures showing resistance. The control organisms appeared sensitive on both the plate and disc methods.

Growth occurred on the trimethoprim containing plates at 2.5 µg for two cultures, a further strain at 1.0 µg, 6 more at 0.75 µg and 11 cultures grew only on the control plate without antibiotic.

Repeat testing of the resistant strains on trimethoprim agar after initial culture for 4 h in RCM broth showed that none grew at 2.5 µg but all did so at 1.0 and 0.77 µg. Further tests on four of the sensitive strains from the first experiment showed growth at 1.0 and 0.75 but not at 2.5 µg.

Resistance or drug tolerance to trimethoprim could be a product of both the dose of the agent and the viability of the organism. This problem was not seen with tetracycline in which the repeats of the plate containing method gave comparable findings.

4.7.3 Extended series of antibiotic disc diffusion senstivity tests

All 13 strains tested, except for the known resistant culture, gave zones of inhibition greater than the control organism, i.e. zone diameters 22-35 mm. Chloramphenicol and trimethoprim gave zones smaller than the control (15-18 mm). A comparison of a resistant and a representative sensitive strain are given in Table 4.46. The resistant culture generally gave smaller zones with all antibiotics

TABLE 4.46

Antibiotic inhibition diameters of an antibiotic tolerant and a representative sensitive strain

	Sensitive strain	Resistant strain
Penicillin	35	20
Cloxacillin	30	15
Erythromycin	30	25
Chloramphenicol	15	17
Piperacillin	40	30
Mecillinam	22	8
Trimethoprim	18	17
Cefotaxime	40	30
Mezlocillin	40	32
Azlocillin	40	30
Ceftazidine	28	22
Cefuroxime	40	30
Cefamandole	35	22
Ticaricillin	35	25

Figures given are the inhibition zone diameters in mm. The concentrations in the discs were the recommended blood level for each antibiotic (see Table 2.11).

and markedly reduced zones were seen for cloxacillin and mecillinam. Cloxacillin has been suggested as a suitable test disc for penicillin tolerant strains but the finding with mecillinam was not expected.

The repeat testing for mecillinam and cloxacillin for the routine isolates gave zones >25 mm for Cloxacillin. The three resistant strains (the previously tested strain and the two Colindale cultures) gave zones <18 mm with cloxacillin and <12 mm when tested against mecillinam, compared to >22 mm sized zones with the routine cultures.

4.7.4 Sensitivity testing for tetracycline and trimethoprim by the disc method

The 50 cultures tested by the disc diffusion method against tetracycline and trimethoprim were considered sensitive to the antibiotic if an inhibition zone >18mm was produced.

Four strains, one of the four serotype 06 cultures and three of the eight serotype 14 strains tested, were resistant to tetracyclin. None of the 50 strains tested were resistant to trimethoprim.

4.8 DISCUSSION

The studies included in this chapter form two separate but interrelated parts for the differentiation and characterisation of the pneumococcus. These consist of firstly, serological typing of the organisms' capsule, and secondly, attempts to distinguish within and between the capsular serotypes by other means, such as the presence of enzymes and growth inhibition by dyes.

Identification of pneumococcal capsules is, of necessity, by elimination, since there are 83 known serotypes. Four methods of performing this elimination process are described, capsule swelling, immuno-diffusion (ID), crossed immuno-electrophoresis (CIE) and co-agglutination (CA), each of which were assessed for specificity, speed and simplicity of the technique. The Quellung reaction for capsular typing, the apparent swelling of the pneumococcal capsule when mixed with homologous antisera (Neufeld, 1902), has a long and distinguished history in the identification of pneumococci (Armstrong, 1932 and Logan and Smeall, 1932) and as a prelude to serum-therapy (Austrian, 1968). Attempts to use this rection were not successful because either poor technique or equipment failed to give clear evidence of capsule swelling. Strains tested by this method had been cultured anaerobically and gave large mucoid colonies with presumably a large capsule which may not have been suitable for capsule swelling. Immunodiffusion, the precipitation of antigen and antibody

at optimal concentrations in an agar gel was applied as an interim procedure in this study prior to the arrival of electrophoresis equipment. Although ID has been used to detect microbiological antigens it appeared to be neither sensitive nor rapid enough for typing the pneumococcus in a clinical laboratory. Identification by ID required a minimum of two days; obtaining a result with the pool antisera occupied the first and the individual antisera tests the second day. Only 91 of the 151 culture supernates tested were positive by ID, which may in part be explained by the findings of El-Rafie and Dulake (1975) that ID required 5-10 times more antigen to produce precipitation by this method than by CIE. Although more sensitive and more rapid than ID, difficulties were experienced in applying CIE to pneumococcal typing. The CIE method, derived from procedures for detecting Hepatitis antigen, has been described as either a discontinuous or continuous electrophoresis of the antigen and antibody. This reaction has been performed in barbital based buffers with a variety of agarose gel supports at a particular current or voltage for 30-60 min (Coonrod and Rytel, 1972; Tugwell and Greenwood, 1975 and El-Rafie and Dulake, 1975). The problems experienced with the initial test antigen, eventually found to contain non-specific precipitants, hindered the assessment of the relative merits of buffers, agar gels and electrical system in CIE. The composition of the buffer used is critical because the mobility of pneumococcal capsular polysaccharides in an electrical

field is affected by pH and ions present. An alkaline pH value of 8.2, or more commonly 8.6, should detect the negatively charged pneumococcal polysaccharides (Michaels and Poziviak, 1976). However two of the serotypes, frequently isolated from serious disease, serotypes 07 and 14, are said to have either neutral (Anhalt and Yu, 1975) or positively charged capsules (Michaels and Poziviak, 1976) and in any event are not detectable in the usual technique according to most authorities (Kenny et al., 1972; El-Rafie and Dulake, 1975 and Michaels and Poziviak, 1976). Tugwell and Greenwood (1975), in their Nigerian studies, did however detect these serotypes by CIE. In contrast El-Rafie and Dulake (1975) showed that an acidic buffer system (pH 6.6) was a more sensitive method for all serotypes, except serotype 14, and that any precipitate formed centrally between the wells was therefore more easily recognised. Once a fresh antigen preparation had been obtained more specific and reproducible lines were obtained which confirmed the advantages of the acidic buffer over the alkaline system. Strong precipitation lines were formed centrally between wells, in an indubriose support gel, at pH 6.6 but not in the same gel at pH 8.6, when the gap between the antigen and antibody wells had been reduced from 5 mm to 2 mm. Application of CIE to serotyping, at pH 8.6, was unable to serotype 41 cultures, which was little better than the 60 strains untyped by ID. All the typeable strains by ID were serogroup positive, only strains of serogroups A and D reacted with their pool

antisera by CIE. When CIE was performed in a pH 6.6 buffer (Section 4.3.1), with antigens neat and at a 1 in 12 dilution, pool reactions, at both concentrations, were seen with all of the serotypes tested, with the exception of some of the pool B and H strains previously untyped by ID. This was in general agreement with El-Rafie and Dulake (1975) who showed prozone to be more pronounced in alkaline than in acidic buffers systems. Other difficulties experienced with CIE concerned speed and simplicity of the method, for as other workers have shown (Coonrod and Rytel, 1973; Tugwell and Greenwood, 1975 and El-rafie and Dulake, 1975) precipitation lines were not always visible immediately after electrophoresis, and usually required leaving for four hours or overnight at 4°C and/or staining with Coomassie blue before reliable results were seen. Immuno-electrophoresis when applied to the detection of antigens in samples from patients suspected of meningitis, septicaemia or pneumonia, presented problems, especially as it was performed on an irregular basis requiring reagents such as buffers and agar to be prepared immediately before each test. The positive results that were seen by CIE in the clinical situation were obtained retrospectively because the samples arose during the evening and the serotype of the isolate was known before the patients's sample came to be tested. No extra clinical information was offered by CIE, in this small survey, to warrant persistence with this generally slow and unreliable method and it was subsequently abandoned in favour of

co-agglutination. Co-agglutination had been developed from the observation that staphylococcal protein-A can attach to the Fc structures of antibody molecules (Forsgren and Sjoquist, 1966; Kronvall and Williams, 1969 and Kronvall and Frommel, 1970) to leave the Fab-located antigen combining sites exposed and produce a sensitised particle able to demonstrate the combination of antigen and antibody as a slide test. Preparation of the protein-A carrier particle, as originally described by Kronvall (1972), recommended growth of the staphylococcus on CCY medium (Ardvison et al., 1971) a relatively complicated broth which requires two vitamins and four minerals as supplements. Tryptic soy broth was used by Edwards et al. (1980) to prepare the reagent but growth on Columbia agar base (Section 2.5.4) was found in this study to be superior to the two broths. Harvesting of this growth was found to be easier from a plate culture than from large volumes of broth recommended in the original method. Sensitisation of the stabilised protein-A, by formalisation and heat was achieved using the Danish Serum Institute's pneumococcal antiserum, but Lund (personal communication) underlined that these sera were only for the capsular swelling reaction and not for CIE or agglutination. Anhalt and Yu (1975) and Coonrod and Rytel (1972) advocated the production of a serum especially for CIE when they found weak precipitation and cross reaction with S. viridans by their techniques. Poor results had been found with Omni-serum and serotype 03 sera with CIE (Coonrod and

Rytel, 1972; Kenny et al., 1972 and Anhalt and Yu, 1975) and this also occurred initially with CA reagents. The titres of Omni-serum components are lower than those in the pool antisera, and serotype 03 strains are known to produce an excess of capsular material which may cause prozone effects with antiserum. Experiments were performed (Section 4.4.2) to overcome the slow or weak reactions seen with these and other early CA reagents in order to obtain more rapid results than the recommended 2 min for agglutination to appear after adding the reagent (Kronvall, 1972 and Edwards et al., 1980). After modifications in reagent preparation (Section 4.4.3) CA with Danish serum gave satisfactory co-agglutination results in less than 15 seconds, in contrast to the time taken for ID and CIE. Omni-serum was the least satisfactory by CA, but after identification of the test strain by bile solubility and colonial appearance, testing proceeded to the pool reagents and only rarely was Omni reagent used. Serotype 03 pneumococci were further examined to determine the optimal antigen extraction method (Section 4.4.4) and showed that culture in RCM broth, after boiling and precipitation of the capsular material by either alcohol or acetone, gave the highest titres. The reduced titres found in pneumococci scraped from CB agar plates, when lysed by deoxycholate, before boiling and precipitation, were much higher than the results obtained when the plate growth was lysed in situ, possibly because the polysaccharide released during growth adheres to or diffuses into the agar. The advantage of

broth culture for the release of serotype 03 antigen was lost when capsular polysaccharides from this and ten others strains grown in broths and on blood agar plates were tested after storage for one week. Antigen reactions were more reliably detected from the growth on an agar plate, suspended in PBS and held at 4°C, than the same strain cultured in broth and similarly stored (Section 4.4.5). The antigenicity of the capsule, in broth, had been destroyed or considerably reduced in a short time and may explain why some strains, stored in T-H broth at 4°C for many weeks, between testing by ID, CIE or CA, failed to be serotyped. The CA method proved to be superior for serotyping than capsule swelling, ID or CIE both for accuracy and speed of reaction and CA was adopted for the epidemiological survey of serotypes after identifying 265 (90%) of the 293 strains available between April and October 1976, the majority of which had been stored in T-H broths.

Once the identification of the capsular serotypes was resolved the second stage, pneumococcal characterisation, began with a search for "sugar" fermentation test media. Pneumococcal fermentation studies, previously by serum agar slopes, have largely been abandoned because of a failure to provide rapid or useful information. Modern media can more reliably supply nutrients and the need for serum in carbohydrate test media should be superfluous, particularly since the effect of serum on the pneumococcus is largely as a pH buffer (Holt, 1962). Some of the more recent

fermentation substrates and methods have been tested (Sections 4.2.3-5) for their ability to demonstrate pneumococcal enzymes, in an attempt to provide quicker and more relevant results. Suitable serum-free media were not found, neither CTA medium nor a modified T-H medium (prepared without glucose), were suitable for pneumococcal growth as a basic medium for fermentation. Defined gonococcal medium although slightly better than CTA in promoting growth was no more suitable for fermentation studies since pH changes were seen both with and without fermentable additives (Table 4.4). Growth produced on Columbia agar with sucrose was not very heavy, and when supplemented by chalk, to demonstrate and contain acid production in the streptococci, the colonies formed were much reduced. Only the addition of serum gave moderate sized colonies for seven of the eight pneumococcal strains tested. These media failures were largely overcome when dense suspensions of the test organism were mixed with high concentrations of substrates in microtitre plates. Fermentation reactions were slightly affected by the suspending medium used but the results were generally clearly evident after 4-6 h in contrast to 4-6 days by the serum agar slope method.

An alternative approach to enzyme detection, the commercial version of the Auxotab method, the APIZYM system also detects enzyme reactions within 4 h. The 19 APIZYM cupules contain substrates for carbohydrate splitting enzymes, esterases, phosphatase and protein peptidases, each

selected to recognise particular enzymes rather than the gross effects demonstrated in conventional bacteriological fermentation tests. The APIZYM system, as a controlled set of enzyme substrates, could form the basis of a standardised intra and inter-laboratory scheme to characterise pneumococci by their enzyme profiles. This could be done in a similar manner to the differentiation of Enterobacteriaceae and streptococci by the API20E and APISTREP strips (API Products) respectively, but potentially may give patterns associated with clinical infections. To produce a reproducible enzyme detection scheme it was first necessary to standardise enzyme preparation by first considering the growth conditions. Pneumococci can be difficult to characterise because vigorous growth is difficult to obtain (Section 4.5.1-2). Cultures in broths (Section 4.5.4) did not give the highest yields probably because the growth curve had passed to the decline phase through the action of inhibitory metabolic products, acidic pH shift by glucose fermentation and peroxide accumulation. The excess capsule release in the latter stages of metabolism may trigger pneumococcal autolytic enzyme (Tomasz, 1981) and enhance the decline in the organisms viability. This did not appear to be the case with strains grown anaerobically with CO_2 on CB agar which, when first removed from the anaerobic jar, showed large and convex colonies and only developed the collapsed "draughtsman" colonies, characteristic of aerobic culture, after the plates had remained at room temperature for

number of hours. The growth produced by anaerobic culture with CO_2 was heavy enough to allow a dense suspension to be prepared from one culture plate and sufficiently viable to permit between four and ten strong enzyme reactions in the APIZYM strip, depending upon the strain tested. Pneumococci had not previously been tested in the system, although the development laboratory of the French parent company of API Products stated that the pneumococcus was unreactive in the APIZYM system. The only available reports of enzyme reactions of related organisms in the APIZYM system, were the few Gram-positive cocci tested by Humble et al. (1977) in a general paper on the APIZYM method and the more intensive study of Waitkins et al. (1976) on the alpha and non-haemolytic streptococci. All the pneumococci tested (Section 4.5.4, ii) showed leucine arylamidase and alpha glucosidase activity, the former shown by most of the streptococci and both shown by S. faecalis and some of the other streptococci. Differences in enzymes detected were seen in the production of esterase lipase, chymotrypsin, alpha and beta galactosidases and beta glucosaminidase by the majority of the pneumococci, and their absence in the rest of the streptococci. Conversely many of the other streptococci had been shown to produce acid and/or alkaline phosphatases, cysteine aminopeptidase and phosphoamidase, none of which were detected in the pneumococci tested. The 32 enzyme profile codes, obtained from the 10 possible APIZYM enzyme-positive results found in the pneumococci tested (Tables 4.14-16),

were randomly distributed amongst the serotypes of the strains. Amongst each group of serotypes from three (serotypes 09, 11 and 31) to fifteen profiles (serotype 06) were found, and although 63% of the cultures gave enzyme codes 1-7 there was no pattern related to capsular serotype which indicated a use for this method to differentiate within the pneumococcus. The examination of APIZYM profile, serotype and source of the isolates (Table 4.9) was less useful as a possible indicator of pathogenicity; the four wound site isolates, for example, showed four codes, only one of which was in the seven most common codes. A similar picture was seen with strains from the other sites; perhaps more relevant information would be obtained from a larger survey. The possession of neuraminidase, expressed as a ratio to the density of the test suspension and detected by the WHO method and not by the pan-agglutinability of red cells, also warranted further investigation. An inverse ratio of neuraminidase activity seen in the two isolates from infection samples when compared with the commensal strain (Section 4.2.6, ii) was difficult to explain since the invading strains would have been expected to more actively attack the sialo-mucins, the naturally ocurring substrate for the enzyme in the patient. Proteinases, another group of potentially aggressive enzymes had been demonstrated in pneumococci when tested in the APIZYM system but only very weak activity was seen even after stimulation in milk agar.

Bacterial pathogenicity can also in part result from the

organisms' production of haemolysins as observed in the streptococci and clostridia, but in the pneumococcus these have been difficult to determine. A double zone of haemolysis, best demonstrated on azide containing tryptose blood agar (Section 4.5.8), was a reproducible and reliable characteristic on this medium only. The "hot-cold" nature of the activation of this haemolysin was similar to the phenomenon produced by Cl. perfringens which also shares possession of a neuraminidase. Slight differences were seen on the three red cell species tested with a suggestion of an extra zone of haemolysis on human blood agar plates, again an area demanding further studies. The future examination of the pneumococcus requires a more flexible approach which probably includes using a simpler version of the APIZYM system. Only ten of the 19 APIZYM substrates were positive for any one pneumococcus and enzyme activity could therefore be examined as individual tests, without recourse to the expense of the complete APIZYM strip. A wide range of carbohydrates and other substrates, including many similar to those in the APIZYM series, are available linked to a nitrophenyl group and in the small number of substrates reported in Section 4.5.5 provided a sensitive and rapid enzyme detection method. To complete the evaluation of the biochemical testing of the pneumococcus parallel study of the nitrophenyl method, the APIZYM system and carbohydrate fermentation in microtitre plates with the examination of other substrates appear to be promising areas for further study.

Much of this chapter has been occupied with numerous experiments examining another promising new approach to the differentiation of the pneumococcus that is by its susceptibility paricularly to the blue and green dyes. Patterns of resistance to dyes and chemicals, to give a so-called resistogram have been used in the staphylococci, E. coli, the shigella and in the brucella to differentiate within the species and between strains that would not be possible by biochemical or serological means. The most difficult aspect of inhibition methods are the biological titrations to find the optimal concentrations of suitable inhibitors; pneumococcal inhibition testing was no exception. Initial studies with agar slopes established general concentrations for investigations in agar plates containing each of eight dyes from the 25 screened for suitability. It was soon realised that the amount of dye had to be very carefully controlled, within a very narrow range, to reduce the inherent error of preparing agar plates to contain an exact concentration after a number of dilutions from a stock solution. This was started by adding 3 ml of a weaker solution to 12 ml of agar rather than the previously used volumes of less than 1 ml of a more concentrated solution added to 15 ml. The mathematical exaggerations inherent in diluting dilute solutions, and the time available for this part of the study, prevented the definition of optimal levels of the dyes tested. The levels used (Section 2.8.5, Table 2.6~10) had been gradually finely adjusted but small changes in the

concentration of the stock solutions gave apparently large changes in the plate values and the later stages (Tables 4.27-43) showed how critical were the dye concentrations. Reproducibility of the results obtained from the same batch of dye-containing agar plates between repeat tests of the same isolate or the same serotype from the same patient proved satisfactory (stage 7 and 17). However in the later stages in which some of the dyes in consecutive tests were at the same concentrations some of the expected results differed. A panel of test strains had earlier been introduced to remove one of the test variables. From stage 9 the test pneumococci were used in the log phase of growth since active growth should be the most standard and sensitive stage of the organisms for inhibition. The last four stages (15-19) gave dye inhibition patterns that were close to being optimal as differential markers, with the probability that closer study of the individual dye in fine detail variation tests would give the elusive levels indicated in the attempts to study eight dyes at the same time. To bring this work to completion would need the selection of pure standardised internationally coded dye powders, probably obtained from one supplier and the precise weighing of the light dye powder. This, combined with the selection of stock concentrations for careful in-use dilution on a larger scale than the one or two plates per batch in this study, should average out the described pitfalls of the method. If the stock solutions were able to be preserved, over a few months, testing of a

significant number of strains using one batch of solutions could enable standardisation of the technique. Once this had been achieved a panel of pneumococcal cultures could be chosen from these strains and maintained to control subsequent batches of dyes by biological titrations.

Differentiation of the pneumococcus in Mitis-salivarius agar (M-S) has not previously been reported although the medium was developed for the selective isolation and colonial forms of the oral streptococci, S. mitis and S. salivarius.

The distinctly coloured colonial forms produced on M-S agar by the pneumococci studied (Section 4.6.5) was only partly the product of two of the components of the medium, trypan blue dye and sucrose. The effect seemed to be a combination of these two components and the shape of the colony produced on this particular formula. Simple and reproducible colonial differentiation was available when viewed under the critical microscopic conditions (Section 2.8.6). The majority of strains comprised one group which produced large irridescent colonies unrelated to serotype. Other strains gave colony forms more often associated with serotype, e.g. the dark blue colonies of serotype 14 and the non-capsulate variants, and the greyish colonies produced by a significant number of the serotype 03 isolates. Potassium tellurite, a required additive in the original M-S formula, gave inhibition patterns when included in this medium but was unsatisfactory when included in T-H agar (Tables 4.38 and 4.41-2).

The final section of the chapter was in response to the changes in the clinical and laboratory reports concerning the reduction in the susceptibility of the pneumococcus to antibiotics. The incidence of pneumococci, seen in this laboratory, that were resistant to tetracycline was higher than would be expected since our patients are all under the age of sixteen and one would not expect that they would have been prescribed this drug. The increasing use of co-trimoxazole both for urine and respiratory infections may explain the other increased incidence of apparent antibiotic resistance seen in this laboratory. Disparate results were found in bench tests of 22 pneumococci for resistance to trimethoprim (the antibiotic component of co-trimoxazole) (Section 4.7, ii). The disc diffusion method showed only four resistant cultures but in the agar incorporation plate 21 strains grew in the presence of 1 µg/ml of the drug. Studies of agar incorporation between 0.75 and 2.5 µg/ml were made again using lysed blood containing sensitivity agar without reproducible results unless the organism had been grown in broth for four hours before the plates were seeded with the strain. In general the incorporation tests gave an MIC between 1.0 and 2.5 but the disc diffusion method gave zone diameters of inhibition >15 mm. The discrepancies were not seen when tetracycline was tested in a similar manner and serves to highlight the known difficulties of laboratory testing of trimethoprim (and co-trimoxazole) susceptibility. When a further 50 strains were tested on heated blood agar plates by the disc

diffusion method for both tetracycline and trimethoprim, no cultures were seen which showed resistance to trimethoprim but four were tolerant of tetracycline. The major concern with the pneumococcus is the emergence of penicillin-tolerant strains which are difficult to recognise by routine disc diffusion tests using a 1.0 or 1.5 unit disc since the reduction in inhibition size is so slight as to be easily mixed in a busy department. Cloxacillin (5 μg) discs have been recommended to detect the penicillin-tolerant variants as the fall in zone size around this disc is greatly reduced in an antibiotic tolerant strain. The results produced by the three resistant strains tested (Section 4.7, iii) confirmed this opinion and showed an almost constant reduction in zone diameter in all the sixteen drugs tested. Mecillinam proved to be at least as sensitive an indicator of penicillin-tolerance as cloxacillin in the very limited number of cultures available.

A total of 1736 pneumococcal isolations were made between November 1976 and October 1980 from 1361 patients at the Children's Hospital. The results are presented in Table 5.1. During 1976/7 the monthly isolations were highest during November, February and March and at their lowest in August, September and October. In 1977/8 total isolations were reduced; the highest monthly total was seen in July and the lowest during August and September. The highest return in the four periods was made in 1978/9 in which all months showed a general increase with December and January providing the most and August and September again the least number of isolations. A more evenly distributed year with winter counts similar to the summer returns was seen in 1979/80; March 1980 gave the highest monthly total for the four years.

When the isolation rates were examined against the patient types i.e. In-patient, Out-patient, Accident and Emergency and post-mortem (Table 5.2) the proportion of In-patients yielding pneumococci increased markedly from 220 in 1977/8 to 375 in 1978/9 with a corresponding fall in isolations from 137 down to 106 in the other categories of cases.

Distribution of pneumococci amongst patients by age and sex (Table 5.3) showed an excess number of male patients (56%) but this was in line with the ratio seen for all admissions (59%). Age distibution showed a higher incidence of pneumococcus positive patients in children less than 5 years old and a lower incidence in those greater than 5

TABLE 5.1

Pneumococcal isolations by month and year (1976-80) at the Children's Hospital, Sheffield.

Month	Number of isolations in the given month in 1976/7	1977/8	1978/9	1979/80	Total
November	51	35	40	31	157
December	32	38	55	26	151
January	30	32	56	33	151
February	62	21	38	41	162
March	59	21	50	79	209
April	37	17	42	35	131
May	43	35	49	51	178
June	29	27	38	36	130
July	27	51	41	41	160
August	21	24	17	22	84
September	12	23	23	37	95
October	19	33	31	45	128
Totals	422	357	480	477	1736

TABLE 5.2

Source of pneumococcal positive samples 1976-80

at the Children's Hospital, Sheffield

Source	Number of isolates from the given source in 1976/7	1977/8	1978/9	1979/80	Total
IN/pt.	303	220	374	371	1268
OUT/pt.	59	74	50	44	227
A+E	43	45	42	53	183
P/M	17	18	14	9	58
Total	422	357	480	477	1736

IN/pt. = In-patient;
OUT/pt. = Out-patient;
A+E = Accident and Emergency Department;
P/M = Post-mortem.

TABLE 5.3

Age and sex distributions of pneumococcal patients against all patients admitted to the Children's Hospital, Sheffield (1976-80).

	Number (%) of isolations from patients aged					
	<1	1-5	6-10	11-15	total	%
Pnuemococcal patients						
Females	205	335	113	50	703	44
Males	290	509	167	67	1033	56
Totals	495	844	280	117	1736	
	(28)	(49)	(16)	(7)		
All admissions (aver./yr)						
Females	290	777	847	633	2547	41
Males	383	1020	1339	895	3637	59
	673	1797	2186	1528	6184	
	(11)	(29)	(35)	(25)		

years, when contrasted to the expected value for all admissions.

The number of isolates related to the hospital medical departments and the number of respiratory sample culture requests are given in Table 5.4. The four medical consultants were considered in one group and all other departments as a second group. The increase in medical requests for bacteriological culture of respiratory specimens and the number of In-patient isolations both occurred in 1978-80.

Association of the most frequently isolated serotypes with hospital ward are given in Table 5.5. The medical units (Wards 3, 4, Isolation, and Ground floor, Thornbury Annexe) provided 721 (41.5%), surgical units (Wards 5, 10, 11 and First floor, Thornbury) 324 (18.6%), Plastic surgery (Wards 2, 8 and 9) 95 (5.5%), mixed surgical and medical wards (1, 6, 7, Intensive Care unit, Conservatory, Thornbury and the Ryegate Annexe) 128 (7.4%) and Out-patients, Accident and Emergency, and post-mortem departments 468 isolates (27%).

The distribution of serotypes over the period (Table 5.6) showed that the 1736 strains belonged to 34 serotypes, serogroup I and non-capsulate variants. Serotypes 06, 19 and 23 were represented by 823 cultures (47.4%), and serotypes 03, 09, 11 and 14 provided 321 strains (18.5%). Strains of the serotypes represented in the Pneumovax vaccine (Thomas Morson Pharmaceuticals, U.S.A.) accounted for 1226 (71%) of all the isolates. Of the 24 blood culture or CSF positive patients 16 yielded vaccine

TABLE 5.4

Distribution of pneumococcal isolations by consultant per year and the total number of respiratory samples received

	Number of isolates from the given source				
Consultant	1976/7	1977/8	1978/9	1979/80	Total
Medical*					
A	65	58	107	128	358
B	18	26	29	Ret.	73
C	66	31	91	104	292
D	44	46	90	60	240
Total	193	161	317	292	963
Others**					
	229	196	163	185	773
Totals	422	357	480	477	1736
Respiratory samples					
	3590	3761	5548	5744	18643

Ret. = retired from Hospital practice.

* = The four main medical firms;

** = All the other consultant firms.

TABLE 5.5

Distribution of the frequently isolated pneumococcal serotypes by Ward.

Ward or Unit	Number of the serotypes isolated from a given Ward or Unit								Total
	03	04	06	09	14	19	23	Others	
Medical									
Three	9	5	28	14	11	23	20	44	154
Four	6	4	47	8	10	44	25	52	196
Isol	3	3	35	8	8	26	30	55	168
G/f	5	7	45	8	19	44	27	48	203
Surgical									
Ten	3	2	11	1	1	5	7	22	52
Eleven	2	2	4	2	2	8	4	22	46
F/f	10	2	37	14	12	23	20	48	166
Two	1	0	9	3	4	9	6	28	60
Mixed surgical and medical									
	27	22	31	7	9	31	26	70	223
Totals	66	47	247	65	76	213	165	389	1268

Non-admission patients provided 469 isolates.
F/f = First floor ward, Thornbury Annexe;
G/f = Ground floor ward, Thornbury Annexe;
Isol = Isolation ward, Thornbury Annexe

TABLE 5.6

Distribution of serotype isolated 1976-80.

Serotype	Numbers isolated
06	313
19	294
23	216
14	99
09	86
03	77
11	59
15	56
18	49
04	45
17	40
31	35
35	33
33	29
10	28
01	23
22	29
21	20
08	17
16	14
34	14
20	10
02, 05, 07, 12, 13, 27, 28, 37, 40, 42	37
Serogroup I	15
Non-capsulate	45
Un-typed	53
Total	1736

serotypes were isolated. Vaccine serotypes were recovered from 79/89 (88.8%) ear swabs, 107/153 (69.9%) sputa, 52/68 (76.5%) wound swabs and 75/140 (53.6%) of the eye swabs received.

Culture reports as pneumococci alone or mixed with other organisms are recorded in Table 5.7. Pneumococci only were reported in 1283 (73.9%) specimens and Haemophilus was present in the majority (77.5%) of the 453 mixed culture reports and in half of the nasal swabs. The proportion of haemophili isolated from sputa was significantly higher ($P<0.1\%$) and the seven strains recovered from ear swabs was significantly lower than expected ($P<0.5\%$). Presence of Staph. aureus with pneumococci in sputa, eyes, ears and wound specimens, and beta-haemolytic streptococci with throat swab isolates were both significantly raised ($P<0.5\%$).

Analysis of isolations from particular sites related to capsular serotype (Table 5.8) showed that pneumococci were found in 919 nasal swabs (52.9%) of the positive specimens) without any serotype showing a significant relationship to that site. Serotype 03 with ear swabs and the non-capsulate variants with eye swabs were the only strains significantly associated with site ($P<0.1\%$).

When serotypes of isolates received from different sample sites at the same admission were studied (Table 5.9) the majority (96.3%) yielded the same serotype from each specimen. In contrast when repeat admission episodes were considered mixed patterns of change in serotype between

TABLE 5.7

Occurrence of pneumococci with other organisms at particular sites.

Site	Alone	Numbers of isolates for a given site Mixed with *		
		Haem.	Staph.	BHS
Throat	276	73	3	16[1]
Nasal	732	178	1	13
Sputum	72	42[1]	12[1]	1
Eye	93	35	12[2]	0
Ear	63	7[3]	11[1]	3
Wounds	20	3	20[1]	1
Others	27	0	0	0
Total	1283	353	66	34

* Isolates were mixed with more than one organism.
Pneumo = Pneumococci;
Haem. = Haemophilus species;
Staph. = Staph. aureus;
BHS = beta-haemolytic streptococci.

[1] = significant at P<0.1%
[2] = significant at P<0.5%
[3] = inversely significant at P<0.5%

TABLE 5.8

Distribution of pneumococcal serotypes against sample site.

Sero type	Number of isolates of the given serotype from								
	Thr	Nas	Eye	Ear	W/s	Spt	Ur	Rem	Total
01	2	10	1	7	1			2	23
03	23	25	3	12	4	9	1		77
04	7	25	2	1	2	6		2	45
06	60	184	21	11	7	24		6	313
09	17	50	5	4	3	5	1		86
10	6	16	1		2	1	1		28
11	13	34	2	1	3	5		1	59
14	13	59	10	6	2	6		3	99
15	1	38	1	1		9		2	56
17	5	23		1		4	3	4	40
18	8	27	1	4	4	4		1	49
19	65	139	19	20	17	33		1	294
22	3	16	1	3	1	4		1	29
23	42	128	8	12	6	17		3	216
31	6	16	6	1	2	4			35
33	4	16		1		7		1	29
35	4	23	1	1		4			33
NC	2	3	40						45
Others	32	87	16	1	10	13		26	180
Total	313	919	138	87	64	154	6	54	1736

Thr = throat swabs;
Nas = nasal swabs;
W/s = wound swabs;
Spt = respiratory sample, not throat or nasal swab;
Sin = sinus samples;
Ur = urine.
Rem = remainder of sample sites.
NC = non-capsulate strains.

TABLE 5.9

Pneumococcal isolation from different patient sites during the same admission episode.

Site of sample	Number of samples from the given site		
	Same serotype	Different serotypes	Total
Thr and Nasal	92	2[1]	94
Eye	11	0	11
Ear	1	0	1
Skin	3	0	3
Eye and Nasal	13	2[2]	15
Others	11[3]	1[4]	12
Total	131	5	136

[1] = a throat swab strain type 03 and nasal swab type 06; a throat swab type 19 and nasal strain type 14.

[2] = one eye swab contained a non-capsulate strain and the nasal swab type 23, and the other pair was of a type 16 strain in the eye swab and a type 23 isolate in the nasal swab.

[3] = nasal and ear swabs; blood and CSF (three patients); blood and throat swab; skin and throat swabs; sputum and ear swabs; wound and nasal swabs; ear and throat swabs; vaginal swab and urine; CSF and valve system.

[4] = a non-capsulate eye swab strain and a type 19 throat swab isolate.

samples was seen (Table 5.10). The total number of patients admitted on more than one occasion was 111 but only isolates from 104 patients were serotyped on each occasion. In 80 patients the serotype recovered differed between visits and from 24 patients the same serotype was isolated on more than one occasion. Certain patients had three or four admission episodes such that 134 examples of either persistence or change in pneumococcal serotype isolated was seen. Changes in the serotype isolated often occurred within the first month following the initial sample, however patterns were difficult to follow because of the random nature of the admission intervals. The examples given in Table 5.11 are those patients sampled on more than three separate occasions. Persistence of the same serotype for longer than one or two months was unusual, the exception was example 12 in which serotype 19 persisted over a three year period.

All specimens obtained in the mortuary from 39 cases, listed for cause of death, serotype and site of the isolate (Table 5.12), contained the same serotype from each patients' samples. Pneumococci were found in either blood or CSF from 15 of the children, nine of whom had died of sudden infant death syndrome (cot death).

It was not practical to analyse the small groups formed when the 2545 clinical diagnosis codes (International Classification of Diseases, WHO) for the 1367 patients were referenced against the 205 diagnosis codes obtained from the patients laboratory request form. Similarly when the

TABLE 5.10

Repeat episodes of pneumococcal isolations from the same patient at different admission episodes.

	Number of samples at each interval, in months							
	1	2	3	4	5	6	>6	Total
Change of serotype	18	8	8	12	2	6	43	97
Persistence of serotype	15	10	5	1	4	0	2	37

TABLE 5.11

Examples of serotypes seen in patients providing three or more samples.

Patient	Date	Type	Date	Type	Date	Type	Date	Type
1)	3/78	04;	7/78	23;	6/80	06;	8/80	06
2)	11/76	06;	2/77	23;	1/79	23;	11/79	18
3)	[1]12/77	08;	12/77	19;	6/78	15;	8/78	15
4)	7/77	19;	8/77	19;	[2]11/77	19;	11/77	17;
	1/78	19						
5)	3/78	17;	11/79	19;	4/80	23;	10/80	15
6)	12/77	19;	1/78	06;	4/78	R;		
7)	6/78	14;	7/79	23;	11/79	15;	1/80	15
8)	5/78	15;	9/78	15;	12/78	18;	2/79	18
9)	11/79	06;	3/80	19;	[3]7/80	23;	7/80	23
10)	3/79	04;	4/79	04;	6/79	04;	7/79	15;
	8/79	15						
11)	6/78	31;	9/78	31;	11/78	19, 09[4]		
12)	1/77	19;	7/77	19;	12/77	19;	9/80	19
13)	5/77	23;	7/77	04;	8/77	15;	1/78	15;
	1/79	11;	4/79	09				
14)	11/77	19;	1/78	22;	12/78	06;	1/79	06;
	5/79	23						
15)	3/77	10;	1/79	19;	2/79	09.		

[1] = CSF isolate type 08, throat swab strain type 19;
[2] = nine days interval between admission episodes;
[3] = twelve days interval between admission episodes;
[4] = nasal swab strain type 19, ear swab isolate type 09;

TABLE 5.12

Pneumococcal isolations from post-mortem patients

Patient	Cause of death	Serotype	Site
6497	septicaemia	09	mid ear
6790	respiratory infection	19	mid ear
6791	respiratory infection	19	mid ear, nose
6796	Haemophilus meningitis	03	mid ear
6804	Letterer-Siwe syndrome	11	lung
6827	bronchopneumonia	19	lung
6840	sudden infant death	17	mid ear
6842	repiratory infection	14	lung
6855	myeloid leukaemia	18	blood, nose
6873	sudden infant death, O.M	06	mid ear
6874	respiratory failure	15	lung
6859	sudden infant death	23	mid ear
6965	sudden infant death, O.M	23	C.S.F, mid ear
7007	pneumonia	22	blood, lung
7017	sudden infant death	19	blood, mid ear
7020	sudden infant death	14	nose
7026	respiratory infection, O.M	18	mid ear
7035	bronchitis	03	lung
7037	respiratory failure	11	C.S.F, mid ear
7147	sudden infant death	14	blood
7155	respiratory failure	14	lung
7156	congen abnormal glands	31	lung
7189	chest infection	23	ear
7205	chickenpox	14	throat
7240	respiratory infection	19	mid ear
7266	respiratory failure	19	mid ear
7286	sudden infant death	14	mid ear
7288	bronchopneumonia	33	blood
7312	congen hydrocephalus	06	lung, nose
7352	respiratory failure	22	mid ear
7356	congen hydrocephalus	23	operation wound
7357	bronchopneumonia	23	C.S.F,lung, ear
7369	pelvic neoplasm	I	blood
7410	gastroenteritis	22	mid ear
7419	bronchopneumonia	01	mid ear
7437	sudden infant death	03	lung, mid ear
7493	lung neoplasm	35	lung
7536	sudden infant death	23	mid ear
7445	sudden infant death	06	mid ear

Total: 39 cases

Sudden infant deaths: 9 cases (3 blood or C.S.F positive)

Other patients either blood or C.S.F positive: 6 cases.

O.M. = otitis media; congen = congenital;
mid ear = middle ear.

laboratory details were plotted against the serotypes of isolates the groups formed were too small for analysis. The clinical detail codes were condensed into 11 groups of related conditions and a final group for the remaining miscellaneous diagnoses (Appendix D). Distribution of pneumococcal serotypes amongst these clinical detail groups is given in Table 5.13. The significance of serotype 03 with ear disease and non-capsulate isolates with eye infections were confirmed together with the association of serotype 03 with patients of the "sore throat" group.

The distribution of tetracycline and co-trimoxazole resistant strains amongst pneumococcal serotypes given in Table 5.14 are a continuation of the studies presented in Section 4.7. This showed tetracycline resistance in 3.7% of all isolates, with a high incidence in serotypes 01, 03, 14 and 21.

TABLE 5.13

Distribution of pneumococcal serotypes amongst laboratory diagnosis codes.

Diagnosis groups	Serotype											
	03	04	06	09	11	14	15	19	23	R	X	Total
Nasal	7		21	6	6	6	8	20	14	1	56	145
Eye	2	1	16	6	1			15	3	30	28	102
Lower	2	5	28	4		6	6	14	14	2	51	132
Upper	7	5	76	25	12	19	16	81	73	1	115	430
"Sore throat"	11		14	4	6	8	3	18	18	2	28	112
Ear	10	3	14	8	3	7	2	21	7		20	95
"Serious" infection	2	5	24	4	4	15	1	22	4	3	37	121
Immuno comprsd	1		7	1	2	2	4	2	2		14	35
Plastic op. pts.	1		10	7	1	4	1	16	12		26	78
Heart op. pts.	1		8		2		4	8	13		13	49
CSF-valve pts.			9	1		1	1	5	1		7	24
Others	32	26	86	20	22	31	10	72	55	6	51	412
Totals	76	45	313	86	59	99	56	294	216	45	446	1736

X = the other serotypes;
Immuno comprsd = immunologically compromised patients;
op.pts. = operation patients.

For the composition of the diagnosis groups which were derived from the clinical details on the laboratory request forms, see Appendix D.

TABLE 5.14

Distribution of pneumococcal serotypes related to sensitivity to tetracycline and co-trimoxazole

Sero type	Total Tested	Total Sens	Total Res	% Res	Total Resistant Tet	Co-tr	Tet and Co-trm
06	251	230	21	8.4	9	10	2
14	71	57	14	19.7	10♦	4	
01	20	11	9	45.0	8♦	1	
19	232	224	8	3.4	7		1
23	173	166	7	4.2	4	3	
03	67	60	7	10.2	7		
21	13	8	5	38.5	5♦		
04	36	32	4	12.5	3	1	
18	37	34	3	8.1	3		
31	29	26	3	10.3	2	1	
35	23	21	2	8.7	1	1	
15	44	43	1	2.3	1		
NC	45	44	1	2.3	1		
17	33	32	1	3.0	1		
05	1	0	1	100.0	1		
12	3	2	1	33.3	1		
09	60	59	1	1.7		1	
34	13	12	1	7.7		1	
UT	29	27	2	6.9	1	1	
Rem	185	185	0				
Total	1364	1272	92	6.7	65	24	3

Sens = sensitive;
Res = resistant;
Tet = tetracycline;
Co-tr = co-trimoxazole;
NC = non-capsulate strains;
UT = untyped strains;
♦ = significant at P<0.1%.

It seems that the pneumococcus, like the poor, will always be with us, for even after over a hundred years of study we are still a long way from determining the basis for its life-threatening invasion of the lungs, blood-stream and cerebro-spinal system. The pneumococcus also continues to be an enigma by its ability to change into this systemic invader after a varied period as a benign inhabitant of the naso-pharyngeal tract. A plethora of information, accumulated over many years has provided glimpses of many aspects of this complex organism, without revealing the crucial infective characteristic which could lead to preventive measures against the often fatal outcome. Pneumococcal disease was such a dominant feature of medicine into the late 1930's that the organism received intensive study which eventually led to effective vaccines and serum therapy, and to the administration of one of the first anti-bacterial agents, optochin. The pneumococcus was also however one of the first organisms to show resistance to a chemotherapeutic agent and optochin treatment was soon abandoned. Ultimately successful treatment was achieved with the sulphonamides and, during the Second World War, with penicillin. Although thirty years were to elapse before pneumococcal resistance to penicillin became a clinical problem the only pathological or epidemiological marker to investigate these resistant strains is still serotyping of the organism's capsule, a technique available before the First World War (Neufeld, 1902).

The aims of this thesis were to collect epidemiological information of the pneumococci isolated in the Sheffield Children's Hospital following the introduction of the new anaerobic culture method (Collee et al. , 1971; 1972) and to use the improved growth obtained to seek alternative ways to characterize this complex organism. This latter area eventually formed the largest part of the study with the recognition and development of new approaches to differentiation, independent of capsular serotype (Sections 4.5.3-5 and 4.6-7).

Following Mrs. Beeton's instruction for hare pie - "first catch your hare", this essential requirement was achieved by the introduction of the new anaerobic culture method, with added CO_2, which increased pneumococcal isolation and formed the basis for the other studies. Characteristically large mucoid colonies (Howden, 1976) and heavier bacterial growth, little affected by the lytic action of the organism's autolysin, enhanced detection of enzymes (Section 4.5) and the other differential methods (Section 4.6). The desire to obtain new information from this fascinating organism co-incided with renewed interest in the pneumococcus during the early 1970's, resulting from the emergence of antibiotic tolerant strains. Another development at this time was the detection of bacterial antigens in body fluids, initially by CIE. It was anticipated that this method would greatly reduce the time for and increase laboratory diagnosis of serious pneumococcal infections, without the delays inherent in

artificial culture; particularly when patients had received prior antibiotic therapy. Unfortunately CIE was not found to be suitable in this laboratory (Section 4.3), in part because the method was unreliable and also because too few samples were received to maintain a routine service. A further application of CIE, the serotyping of isolates, was no more successful; neither were the capsule swelling or immunodiffusion methods practical for this purpose (Sections 4.1-3). All three techniques were either too slow or more importantly did not give specific results, and often a combination of both. These difficulties were overcome by co-agglutination (Section 4.4) a quick, specific and simple technique, suitable for use in most laboratories. A practical system was established to serotype all routine isolates (Section 4.4.6), following confirmation of the isolate as a pneumococcus by the rapid bile solubility test (Howden, 1979). Current practice in the majority of clinical laboratories is to confirm suspect "draughtsmen" colonies, produced after aerobic culture, by the bile solubility of a broth culture and the susceptibility of the strain to optochin, both of which require overnight incubation before results can be read (Lund, 1959). The large mucoid colonies, following anaerobic culture with added CO_2, in conjunction with the rapid bile test permitted a positive report to be issued within minutes of the culture plates being examined, without the delays inherent in the overnight tests. Serotyping of pneumococci was also simplified and quickened

as positive results could be obtained, again within minutes of isolation, by the co-agglutination slide test using only one or two of these large colonies. The mucoid colonies formed with the new anaerobic culture method with added CO_2 were similar to those of serotype 03 strains, previously the only characteristically mucoid pneumococcus. Strangely, whilst almost all other strains gave larger mucoid colonies when grown anaerobically with added CO_2 the colonies of the serotype 03 isolates were smaller than when grown aerobically, although they retained their extremely mucoid appearance. Serological typing confirmed that the mucoid isolates were not predominantly serotype 03 and in fact belonged to a wide range of capsular types. Unfortunately serotype distribution patterns found in the survey (Table 5.6) could not be contrasted against earlier distributions because serotyping of isolates had not previously been undertaken in this laboratory. A day-book register of positive samples for earlier years had been collected and it was hoped to compare the isolation rates before and after the introduction of the improved anaerobic culture method, which had occurred in September 1973. However, the only laboratory records book missing from a series spanning the last 24 years was the one for 1971-2. The nearest complete year, prior to commencement of the new method, was 1969-70 which showed an isolation rate of 4.8% (210 isolates from 4380 respiratory samples). In the eight months immediatly before the new method, January to August 1973, 5% of the 5,340 samples tested were positive (267

strains). For the two years following improved anaerobic culture with added CO_2 the isolation rate rose to 8.2% and 10.0%, and the year preceding the survey an isolation rate of 8.4% was recorded from a peak number of 7,238 respiratory specimens. These improved results confirmed the view that introduction of anaerobic culture with added CO_2 had at least increased recognition of pneumococci. The major source of pneumococcal-positive samples were throat and nasal swabs which usually contain a rich microbial flora that tends to obscure or inhibit pneumococcal colonies in artificial culture. The new method contributed to pneumococcal recovery in two ways. Firstly, the production of anaerobic conditions inhibited many of the competing organisms (Section 3.1), and secondly the extra CO_2 encouraged or was essential for the growth of many pneumococci (Section 3.2). These factors in combination produced larger and more readily visible colonies, although the value of the two factors, as would be expected in any biological system, was found to vary from strain to strain. Some strains required anaerobiosis, some were carboxyphilic and in some both conditions were necessary for optimal growth (Sections 3.1-2). The advantage of anaerobiosis may, in this catalase-negative organism, simply be a reduction in its production of hydrogen peroxide, a recognised inhibitor of pneumococci in artificial culture (McLeod, 1922 and Holt, 1960). Reduced oxygen tension and not necessarily exacting anaerobiosis may be sufficient since growth of the pneumococcus in a

semi-solid broth (containing 0.2% agar) was similar to that of micro-aerophilic organisms i.e. a narrow band of turbidity was seen approximately 10 mm below the surface of the medium (Section 3.3). The study of Wu et al. (1980) confirmed the advantage of strict anaerobiosis in successful pneumococcal isolation using a GasPak anaerobic system (BBL Microbiology Systems, Cockeysville, Maryland, America). Baseman and Strand (1984), on the other hand, could not confirm this advantage. However their anaerobic cultures were performed in a Bio-Bag Environmental ChamberA anaerobic system (Marion Laboratories, Kansas City, Mo, America) which they speculated could have been the cause of their failure through an inability to attain suitable anaerobiosis. The role of CO_2 was also not straightforward although it seemed to be essential for a high proportion of strains (Section 3.2). It could be, as in other carboxyphilic bacteria, that CO_2 is incorporated into the carboxylic acid cycle via the phosphoenol-pyruvate pathway (Kapke et al., 1980). The actual mechanism could be confirmed by determining those molecules in the pneumococcus which accumulate radio-active carbon when grown in an atmosphere containing radio-isotope labelled CO_2. A biochemical explanation of the apparent increase in capsular material, indicated by the mucoid colonial appearance, produced by growth in extra $CO_2$is required. It could be that a combination of the gas with pyrimidine precursors in the organism leads to the production of uridine phosphate. The uridine compound, under the control

of pyrophosphorylase, could combine with glucose-1-phosphate, released from the anaerobic metabolism of carbohydrate, resulting in a cyclic process to form and extend polysaccharide chains. The actual process would no doubt be more complicated to account for the different polysaccharides composing the 83 capsular types (Lee et al., 1981). Whether these individually recognised pathways in the above sequence are applicable to polysaccharide formation is another relevant area for biochemical study of the pneumococcus. Whatever pathways are responsible for CO_2 dependance this requirement is strongly linked with certain capsular serotypes. An average 10% of all strains recovered by the new method were shown to require extra CO_2 even after a number of sub-cultures on artificial media (Section 3.2). This requirement increased to 66% of serotype 16 strains and to 75% of the capsular serotype 09 isolates (Table 3.5). The isolation rate following anaerobic culture with added CO_2 had not merely increased by 10%, as would be expected from the general level of CO_2 dependant isolates, but had in fact increased by over 60%. Perhaps pneumococci are even more sensitive to CO_2 tension when first transferred from the host, particularly if this transfer is on relatively inhospitable culture swabs, or perhaps mucoid colonies are more easily seen.

Isolations with the new method were at a high rate (11.7% and 9.5%, Table 5.1) during the first two years of the study although the number of samples examined had slumped from over 7,000 in the year before to less than 3,700

(Table 5.4). When, in the final two years, the number of samples increased to over 5,000 the recovery rates fell back to 8.7 and 8.3% respectively. It is of interest to speculate on the factors involved in the changing patterns of sampling before and during the survey. Why was the number of samples received before the survey so high and why did the total number of requests vary so much? The increased number of samples cultured in the second part of the study coincided with increases seen in requests from particular consultants (Table 5.4). The changes may reflect an increase in the number of patients seen by these consultants and/or the introduction of policies to sample certain patients, for example, all patients on admission. The latter seems most likely as the increase in samples from three of the medical consultants was in contrast to the relatively static number of requests received from the remaining consultants. Without a background of previous assessment of these and other study, results were greatly handicapped. Slowly the value of this type of information is now becoming accepted in many laboratories. The depth of information collected in the survey (Tables 5.1-14) may be impractical for some departments but an on-going record of bacteriological, and hopefully relevant clinical information, would help recognise trends relevant both to the hospital and to the patient. These benefits would include the improved management of resources and the selection, based on the sensitivity records of recently tested strains, of effective antibiotic therapy. Thus both

retrospective and prospective evidence could be an aid in discerning the clinical significance of one of the more difficult aspects of the association of man and the pneumococcus. When present in CSF or blood culture samples the significance of the pneumococcus is rarely in doubt, however, when isolated from respiratory material, for example, its importance can be less clear. The respiratory tract, particularly the anterior nares, is a reservoir for the pneumococcus both as a harmless visitor and as the probable starting point for many of the organism's infectious forays, e.g. sinusitis, otitis media, pneumonia and meningitis. For the clinician the nose provides one of the "routine" bacteriological sampling sites for a large number of patients. Not surprisingly nasal swabs were the source of 919 (55%) of the 1736 pneumococcal isolates (Table 5.8). How many of these isolates were harmless visitors and how many were potential invaders is unknown. When recovered from sputa in patients with suspected chest infection, the role of the pneumococcus can be even more confused. Mere presence in the sample does not confirm the pneumococcus as the cause of infection as it may be a contaminant of the specimen acquired during transit from the lung to the laboratory container. In certain situations clarification can be deduced from the clinical details, provided on the specimen request form, when taken in conjunction with demonstration of the organism in purulent material, by Gram's stain, capsule swelling, CIE or co-agglutination, and its recovery in "significant"

numbers on a culture plate. Clinical details if relevant and if on the request form can be most helpful, however, the reality often falls well below this standard. As was shown in Table 5.13 strains of particular types, such as serotypes 03 and 14, which have been frequently isolated from particular sites in disease states, can also be isolated on occasions when their significance is uncertain. Serotype 03 strains, a common cause of otitis media, were isolated from 10% of the 95 patients said to have ear infections but were also isolated from 10% of those in the "sore throat" group of cases. In another example non-capsulate strains were isolated from 30 patients said to have eye infections. This was an unexpected finding since these variants are considered to be non-pathogenic (Lund, 1971). A total of 40 non-capsulate isolates were made from eye swabs (Table 5.8) most of which were clinically significant as these swabs are generally only taken from patients with infected eyes. Again this underlines that clinical details on laboratory request form can be unreliable. The isolation of these variants from so many patients with clinical conjunctivitis warrants closer investigation as it is possible that they are an overlooked cause of infection. The small colonies produced by the non-capsulate strains can easily be missed as they resemble colonies of capsulate pneumococci and were similar to those produced by those non-haemolytic streptococci generally regarded as clinically insignificant. These non-capsulate strains also differed from the other pneumococci in having

a negative rapid plate bile solubility test. They were however positive in the broth bile solubility test and were sensitive to optochin. Two representative cultures sent to Dr. G. Colman, Streptococcal Reference Laboratory, P.H.L.S., Colindale, were confirmed as non-capsulate pneumococci.

Just as the clinical details supplied to the laboratory were not always useful in assessing significance of an isolate, demonstration of the organism in material received was not always appropriate. This, however, was largely a problem of obtaining satisfactory samples, a common feature of almost all bacteriological investigations, especially those involving children. Sputa, as distinct from saliva, was only infrequently obtained during the survey and although presumptive pneumococci were occasionally seen in direct Gram-stained smears it was not practical to demonstrate antigen in these few samples. Similarly the small number of cases of pneumococcal meningitis seen in this hospital made the use of CIE unsuitable for the detection of antigen in CSF, especially as all such cases had been detected by Gram-stained film and/or anaerobic culture with added CO_2. The epidemiological survey had been based on the need to know the distribution of pneumococcal serotypes in health and particularly in disease. This background information would be essential prior to the introduction of vaccination for high-risk children. Protection of all young children from pneumococcal infections, especially from otitis media, has

been proposed, although those most at risk are under two years old and are also the group least able to produce raised antibody titres (Klein, 1981). The most likely group in this hospital are immuno-compromised children, mainly those with acute lymphoblastic leukaemia, in whom pneumococcal bacteraemia is often the terminal event when their immune capabilities and white cell count are extremely low. Of the 13 pneumococcal blood culture isolates six patients (46%) were infected with strains that were not included in the commercial vaccine, serotypes 10, 15, 22, 33 and group I (two strains). This vaccine contains polysaccharide of serotypes 01, 02, 03, 04, 06, 07, 08, 09, 12, 14, 18, 19, 23 and 25. A slightly different picture was seen in those serotypes isolated from CSF in which five of the 14 isolates (35%) were non-vaccine strains, serotypes 11, 17 (three strains) and a group I strain (which was from the same patient as one of the group I blood culture isolates). The ratio for the 138 eye swab isolates taken from the information presented in Table 5.8 showed that 45% (62 strains) were non-vaccine serotypes, although this number included 40 non-capsulate variants. Non-vaccine isolates only accounted for 10% (nine of the 87 strains) of ear infection cases. When the average ratio of non-vaccine to vaccine strains was extracted from the total isolations, including both commensal and pathogenic cases, listed in Table 5.6, non-vaccine strains composed 22% (394) of the 1,736 isolates. Other authors who have considered the distribution of vaccine strains in serious pneumococcal

infections have shown that less than 20% of clinically significant isolates belonged to the non-vaccine capsular types (Broome and Facklam, 1981; Klein, 1981; Hansman, 1983 and Turk, 1984). Composition of the serotypes included in the vaccine was a compromise arrived at from world-wide distribution pattern and unfortunately its composition could not be tailored to each country's individual serotype patterns. The distribution of strains in ear infections indicated that vaccination would be effective for this group of patients. Vaccination would be of less value in bacteraemia or meningitis if the serotype distribution persisted, nonetheless half of these very serious infections may have been prevented if the patients had been vaccinated. It is an interesting aside to remember that despite at least 100 times more deaths from pneumococcal infection than from tetanus vaccination against the latter is part of normal medical procedure, but protection against the pneumococcus is almost never used (Vogel, 1982). Serotype distributions of the strain in the survey showed that serotypes 06, 19 and 23 represented 46.8% (813) of the isolates and serotypes 03, 09 and 14 accounted for a further 15.1% (262); the remaining 661 strains were divided amongst 27 serotypes or groups (Table 5.6). As others have found, the pattern of serotypes is unique to the survey community, in this case the Sheffield area, but certain similarities were shown with other surveys in children (Dowling et al., 1971; Lcda et al., 1975 and Gray et al., 1979). Serotypes 06, 19 and 23 were also the most common

serotypes encountered in serious childhood infections (Morch, 1944; Douglas and Riley, 1979 and Finland, 1981) which underlines the double edged sword of pneumococcal isolations, the ability for the same serotype to occur both as a commensal or a pathogen. The presence of serotype 03, generally recognised as a severe pathogen, has also been found in up to 33% of healthy children. In this survey <5% of strains were of this type and associated both with ear infection and probable "routine" sample swabs. Serotype 09, 75% of which in the survey were carboxyphilic, was a major cause of systemic disease in an Australian survey (Hansman, 1983) and may have been less recognised in British surveys because of the CO_2 requirement of so many of these strains. Turk (CDR 17/84) was surprised to note a significant number of this serotype amongst children with severe pneumococcal disease seen at the Northern General Hospital, Sheffield when compared to his earlier findings in his Oxford studies (Turk, 1980). This may be another example of variation in serotype distribution from community to community, or different pathogenicity of serotype 09 strains for children. The carboxyphilic and non-carboxyphilic strains of this serotype isolated in this survey were not tested with subtype sera. This could have determined whether this characteristic requirement was related to a particular subgroup and to the subgroup included in the pneumococcal vaccine (subtype 9v), which would of course have been relevant to any protection afforded by the vaccine.

The epidemiological evidence (Section 5) whilst providing necessary background material for subsequent studies could not be expected to provide conclusive evidence for the significance of many of the isolates. The investigations presented, primarily those in Section 4, gave a number of pointers to the development of differential studies. Capsular serotyping has been a necessary obsession of pneumococcal investigations because of its link with pathogenicity. Other methods to recognise invasiveness have faltered through lack of suitable methods or the extended nature of some investigations. Previous carbohydrate fermentation testing of the pneumococcus needed serum-water media and incubation for at least five days (Morch, 1949). The active growth produced by anaerobic culture with added CO_2 reduced this time to less than 5 h, and therefore an extended re-examination of differentiation by carbohydrate fermentation reactions would seem appropriate. Detection of enzyme reactions by labelling substrates with indicators, e.g. nitrophenol or methylumberriferryl (Maddocks and Grenan, 1976), or developing coloured products of the reaction (APIZYM) are other rapid and sensitive methods to finding characteristic pneumococcal or invasive enzymes. Pneumococci in the limited number of substrates included in the APIZYM strip have shown esterase lipase, chymotrypsin, galactosidase and glucoseaminidase activities (Section 4.5.4), which had not been found in other streptococci (Waitkins, 1977). It may therefore be possible to find further differential

substrates amongst the array of biochemicals now available.

Significant antibody production against specific pneumococcal products, e.g. those against phosphocholine (Briles et al., 1981), have been related to infection. Specific enzymes if released into body fluids by the pneumococcus during tissue invasion could allow early diagnosis of infection and also end the speculation of this organisms' isolation from sputum samples for example. Fine-print examinations of pneumococcal enzymes have been made in recent years, largely by biochemists using the organism as a source of novel enzymes (Kobata and Takasaki, 1978 and Yamashata et al., 1981). To fulfill the promise of the survey's characterisation results it is essential that detailed biochemical techniques are applied to the pneumococcus to add the meat to the skeleton of observations reported. As mentioned above the basis for CO_2 dependence should be investigated although the results of this requirement still provide a simple differential test. The production of "hot-cold" haemolysins and neuraminidase with their similarities to those of Cl. perfringens require further study and in the case of the latter test a simplified method. The scientific basis of all investigations must be with the premise that the pneumococus is a clinically important entity and not a biochemical novelty; and tempered by the practical application of these in the hospital laboratory. The API enzyme substrate strips form an execellent starting point for the enzyme studies, with their standardised simple

procedure providing for collation of results between study centres.

Dye inhibition studies may provide an alternative or complementary scheme for characterisation, which require only the minimal amount of equipment. Determination of the critical dye concentrations by biological titrations was, as Elek and Higney (1970) had found with other organisms, a necessary stage if this type of work is to be reproducible.

From the relatively small amount of work presented, on a few of the many hundreds of dyes available for study, a vast area of investigation for the pneumococcus was revealed. An even simpler differential test, the production of coloured colonies on Mitis-salivarius agar (Section 4.6.5), also showed an association of certain colonial types to capsular serotypes. In the light of the many new aspects revealed by this study, a fresh examination of the pneumococcus is indicated so that the science of biochemistry can be applied to the practise of bacteriology.

The results presented here have increased our knowledge of the pneumococcus based upon an improved ability to isolate the organism and the supplementation of capsular serotyping by the introduction of new methods to characterise and differentiate this interesting bacterium.

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TABLE A1

The suppliers and order codes of the commercial media.

Name	Code	Supplier
Amies Transport Medium		Difco
Azide Blood agar base		Difco
Columbia agar base	Lab 1	Lab M
DNase agar	CM 321	Oxoid
Iso-sensitest agar	CM 471	Oxoid
Liver digest powder	L 27	Oxoid
MacConkey agar	CM 7	Oxoid
Mitis-salivarius agar	CM 157	Oxoid
Phosphate buffered saline	BR 14a	Oxoid
Proteose peptone		Difco
Reinforced Clostridial medium	CM 149	Oxoid
Todd Hewitt broth	CM 189	Oxoid
Tryptone	L42	Oxoid
TYC agar	Lab 35	Lab M

TABLE A2

The suppliers of media.

API System, S.A.,
La Baline Les Grottes,
38390 Montalieu Vercieu,
France.

Difco Laboratories,
Detroit,
Michigan 48201
U.S.A.

L'Industrie Biologique Francais, S.A.
9 Genevilliers,
France.

London Analytical and Bacteriological Media Ltd. (Lab M),
Ford Lane,
Pendleton,
Salford, M6 6PB

Mast Laboratories Ltd.
Mast House,
Derby Road,
Bootle, Merseyside.

Medical Wire and Equipment Co. Ltd.,
Potley,
Corsham,
Wiltshire.

Oxoid Ltd,
Wade Road,
Basingstoke,
Hants.

TABLE A3

The suppliers of chemicals.

BDH Chemicals Ltd,
Poole,
Dorset.

Duncan, Flockhart Co. Ltd.,
700 Oldfield LaneNorth,
Greenford,
Middlesex.

Evans Medical,
Speke,
Liverpool.

ICI PLC,
Alderley Park,
Macclesfield,
Cheshire.

Koch-Light Laboratories Ltd.,
Poyle Estate,
Willow Road,
Colnbrook,
Slough, S23 0DZ

Sigma London Chemical Co. Ltd.,
Fancy Road,
Poole,
Dorset.

Statens Serum Institute
Amager Boulevard 80
DK -2300
Copenhagen 5,
Denmark

TABLE A4

The suppliers of equipment and gases.

Equipment:

Cecil Instruments Ltd.,
Green End Road,
Cambridge.

Daly Instruments Ltd.
Bolney Cross,
Bolney,
Sussex.

Gec-Elliott Process Instruments
c/o. Baird and Tatlock,
P.O. Box 1,
Romford,
Essex.

Medcalf Bros. Ltd.
Cranborne Road,
Potters Bar,
Herts.

Shandon Southern Instruments Ltd.,
Frimley Road,
Crawley,
Surrey.

Gases:

BOC Ltd Special Gases,
National Accounting,
Dept. E5,
P.O. Box 12,
Worsley,
Manchester

Distillers - The Distillers Company,
(Carbon Dioxide Ltd),
Cedar House,
39 London Road,
Reigate,
Surrey, RH29QE

FIGURE B1 Colour chart for the developed reactions of the APIZYM enzyme substrates cupules.

Quantity of hydrolysed subtrate Quantité de substrat hydrolisé		0 nanomole	5 nanomoles	10 nanomoles	20 nanomoles	30 nanomoles	⩾ 40 nanomoles
Activity mark Activité chiffrée		0	1	2	3	4	5
Control — Témoin	1						
2 - naphtyl - phosphate	2						
2 - naphtyl - butyrate	3						
2 - naphtyl - caprylate	4						
2 - naphtyl - myristate	5						
L - leucyl - 2 - naphtylamide	6						
L - valyl - 2 - naphtylamide	7						
L - cystyl - 2 - naphtylamide	8						
N-benzoyl-DL-arginine-2-naphtylamide	9						
N-glutaryl-phénilalanine-2-naphtylamide	10						
2 - naphtyl - phosphate	11						
Naphtol-AS-BI-phosphodiamide	12						
6-Br-2-naphtyl-αD-galactopyranoside	13						
2-naphtyl-βD-galactopyranoside	14						
Naphtol-AS-BI-βD-glucuronate	15						
2-naphtyl-αD-glucopyranoside	16						
6-Br-2-naphtyl-βD-glucopyranoside	17						
1-naphtyl-N-acétyl-βD-glucosaminide	18						
6-Br-2-naphtyl-αD-mannopyranoside	19						
2-naphtyl-αL-fucopyranoside	20						

Appendix C

Document A for the collection of laboratory details which were combined with the patient's details held in the Hospital Activity Computer File.

DOCUMENT A

FOR ADDING EXTRA DATA TO INPATIENT RECORDS

Unit Number

Admit Date

Patient Type 1

iagnosis Details

If diagnosis on record correct leave this blank.

Lab. Number

Site of Sample | Type | If mixed | Sens.

Ward | Date Taken

Post Code

Lab Diagnosis | Test

Document B for the collection of both laboratory and patient details, for patients whose details were not on the Hospital Activity Computer file.

FOR ADDING NEW PATIENTS

Unit Number

Admit Date

Patient Type

1. Inpatient
2. Outpatient
3. Accident and Emergency
4. PM

Surname Key

M
F

Date of Birth

Age at Admission

Consultant/Speciality

Diagnosis Details

Lab. Number

Site of Sample

Type

If mixed Sens.

Ward

Date Taken

Post Code

Lab Diagnosis

Test

Appendix D1

Patients diagnosis groups of laboratory obtained clinical detail codes used in Table 5.13.

Nasal:-

- Nasal Discharge/Catarrh
- Rhinitis
- Sinusitis

Eye :-

- Blepharitis
- Brused/Infected eye
- Conjunctivitis
- Tear duct Obstruction

Lower (respiratory) :-

- Bronchitis
- Bronchoscopy
- Bronchopenumonia
- Consolidation, right lower lobe
- Cystic Fibrosis
- Empyema
- Pneumonia, pneumococcal
- Pneumonia (unspecified)
- Pneumonia, aspiration

Pneumonia, left lower lobe

Pneumonia, right lower lobe

Pneumonia, right upper lobe

Pneumonia, staphylococcal

Lung, collapse lobe of

Lung, consolidation of

Thick Secretions unspecified

Tracheostomy

Upper (respiratory):-

Apnoea

Asthma

Bronchial asthma

Bronchiolitis

Bronchspasm

Bronchospasm with bronchiolitis

Cold

Cough

Croup

Epiglottitis

Influenza unspecified/query

Nerve Palsy with cough

Respiratory tract infection, unspecified

Respiratory Tract infection, upper

Stridor

Tachypnoeia

Wheezing

Whooping cough/Pertussis

"Sore throat":-

Cervical adenitis

Cervical Lymphodenopathy

Folliculitis/Acute Tonsillitis

Irritable hip

Joint Pains

Knee, painful right

Laryngitis and Tracheitis

Limp

Pharyngitis

Rheumatic Fever

Sore Mouth/Thrush

Sore Throat (unspecified)

Ear:-

Ear Unspecified/Discharge

Mastoid Cavity Infection

Otitis Media, bilateral

Otitis Media, unspecified

Strabismus

"Serious infections":-

Cerebro-spinal fluid, leaking from head wound

Convulsion/Febrile Episode

Encephalitis

Fever, recurring

Meningitis, haemophilus

Meningitis, E. coli

Meningitis (unspecified)

Meningococcal septicaemia

Pyrexia of Unknown Origin

Septicaemia (non-specified)

Immuno-compromised:-

Anaemia, aplastic

Anaemia, iron deficiency

Angiosarcoma - skull

Burkitt's Lymphoma

Haemophilia

Leukaemia, acute lymphoblastic

Leukaemia, acute myeloid leukaemia

Lymphosarcoma

Medulla blastoma

Neoplasm, malignant

Neoplasm unspecified

Neuronblastoma

Tumour, saccrocoxygeal

Tumour, spinal

Plastic operation cases:-

Burns, hand

Burns, Leg

Burns, face

Choanal atresia

Cleft Palate with Cleft Lip

Cleft Palate

Cleft Lip only

Cleft Lip, repair

Excision, naevus

Hypospadias

Paratoid Duct Blocked

Plastic Surgery (unspecified)

Polydactyly

Scalds, chest

Scalds, feet

Scalds, shoulder

Scald, trunk

Scald, unspecifed

Skin Graft

Tongue Tie

Heart operation patients:-

Atrial Septal Defect

Closed Heart Surgery

Coarction Aorta

Heart Anomaly - unspecified

Open Heart Surgery

Patent Ductus Arteriosus

Transposition of Great Vessels

CSF-valve patients:-

Bulging fontonelle

Blocked shunt

Hydrocephalus

Hydrocephalus unspecified

Hydroceph with valve

Hydroceph, ventriculitis

Myelomeningocoele

Shunt/Valve system, infected

Spina Bifida

Valve insertion

Other diagnoses:- 91 descriptions that did not form groups of conditions