STUDIES ON VIRULENCE PROTEINS OF *STREPTOCOCCUS PNEUMONIAE*

A thesis submitted by
ROBERT ARTHUR LOCK, M.Sc.
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SUMMARY

This work has been concerned with the investigation of some of those proteins produced by *Streptococcus pneumoniae* which may contribute directly to the virulence of the organism, and whose actions may be inhibited within the host by appropriate circulating antibodies. The aim of the work has been to provide evidence for the relative importance of the various proteins to pneumococcal virulence, and to indicate which proteins (either native or as toxoid derivatives) might be most effective as components of an antipneumococcal vaccine.

Those pneumococcal proteins selected for particular attention were pneumolysin, neuraminidase and autolysin. In the first part of this project, all three proteins were purified in quantities large enough for immunization/challenge studies. The purification procedure for pneumolysin was improved substantially from those of previous workers. Neuraminidase had not been purified in its undegraded form prior to the work presented in this thesis; the present work also resolves some previous confusion over the cellular location of the enzyme and its apparant multiplicity of forms. Autolysin was finally purified from a recombinant strain of *Escherichia coli* expressing the pneumococcal *lytA* gene. The use of a recombinant *E. coli* strain as source for the enzyme avoided the complications caused by association of autolysin with pneumococcal cell wall fragments to form heterogeneous complexes. Also purified from recombinant *E. coli* during the course of the present work was GPL, a specifically-inactivated toxoid version of pneumolysin.

In the latter part of this project, chemical means of inactivating the purified toxic proteins pneumolysin and neuraminidase were investigated, with the aim of generating non-toxic derivatives still having substantial immunological cross-reactivity with the native proteins. Mice which had been immunized with proteins or their toxoid derivatives were then challenged intranasally with virulent *S. pneumoniae*.

The results indicate that circulating antibody capable of inhibiting the activity of either pneumolysin, neuraminidase or autolysin will provide significant partial protection against subsequent pneumococcal challenge. This provides the first direct evidence that each of these proteins is important to the virulence of the organism. The results also indicate that

GPL, and, by extension, other genetically-tailored toxoid derivatives of pneumolysin, would be particularly promising protein candidates for inclusion in future antipneumococcal vaccines for humans.

STATEMENT

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University. The work was done entirely by myself except where otherwise acknowledged. To the best of my belief, this thesis contains no material that has been previously published or written by another person, except where due reference is made in the text.

Robert A. Lock

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ABBREVIATIONS

All abbreviations are defined in the text on their first use.

AL	autolysin
AL$^+$	[bacterial strain] expressing autolysin activity
AL$^-$	[bacterial strain] failing to express autolysin activity
Amp	ampicillin
bp	base pairs
CCIE	counter-current immunoelectrophoresis
C-form	"choline"-type (active) form [of autolysin]
CM-	carboxymethyl-
CRP	C-reactive protein
CSF	cerebrospinal fluid
DEAE-	diethylaminoethyl-
EDTA	ethylenediaminotetraacetic acid
E-form	"ethanolamine"-type (inactive) form [of autolysin]
F-Neu	neuraminidase partially inactivated by treatment with 3.4% formaldehyde, as described in Chapter 5.
GPL	"glycine"-pneumolysin (in which the cysteine residue of the native protein has been replaced by a glycine residue; see Chapter 2
HTP-A	neuraminidase peak "A" ex-hydroxylapatite; see Chapter 3
HTP-B	neuraminidase peak "B" ex-hydroxylapatite; equivalent to 86K form of the enzyme. See Chapter 3
HU	haemolytic units; defined in Chapter 2
kb	kilobases
KHU	thousands of haemolytic units
LB	Luria-Bertani (plates/broth); see Chapter 2
LTA	lipoteichoic acid
lytA	structural gene coding for pneumococcal autolysin
MHU	millions of haemolytic units
MU	methylumbelliferone
MUAN	2′-(4-methyl-umbelliferyl)-α-D-N-acetylneuraminic acid
NANA	N-acetylneuraminic acid
ND	non-denaturing
Neu	neuraminidase

PAGE	polyacrylamide gel electrophoresis
PBP	penicillin-binding protein
PBS	phosphate-buffered saline; see Chapter 2
PL	pneumolysin
PMSF	phenylmethylsulphonyl fluoride
PspA	pneumococcal surface protein A
RIA	radioimmunoassay
SDS	sodium dodecyl sulphate
TBS	Tris-buffered saline; see Chapter 2
TTBS	(Tween)- Tris-buffered saline; see Chapter 2
TSB	Trypticase soy broth; see Chapter2
TSB^{+}	Trypticase soy broth plus additives; see Chapter 2
TSB^{++}	Trypticase soy broth plus additives plus additional phosphate buffering; see Chapter 2

Other abbreviations are as listed in Biochem. J. (1976) *153* : 1-21.

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INTRODUCTION

A] GENERAL

Streptococcus pneumoniae, the pneumococcus, is an important human pathogen responsible for life-threatening infections such as pneumonia, bacteraemia and meningitis as well as for less severe but highly prevalent diseases such as otitis media and sinusitis.

In the early years of this century the pneumococcus was subjected to intensive study by microbiologists and members of the medical profession, and, despite the technical limitations of the times, a vast storehouse of knowledge concerning the biology of the organism was accumulated.

However, following the advent first of the sulphonamides and then of penicillin and other drugs which seemed to promise safe and highly effective means of treatment for pneumococcal infections, interest in the pneumococcus declined precipitously and has only recovered in the last few years. One cause of this renewed interest has been the emergence of multiply drug-resistant strains of the organism. Another has been the realization that even today mortality from pneumococcal disease remains significant in the first few days after antimicrobial therapy has been administered. It is becoming increasingly clear that modern antibiotic therapy has not, after all, eliminated the threats posed by the pneumococcus.

There remains much scope for further research for, despite more than a century of investigation into the nature of pneumococcal disease, there is

still no firm indication of the exact means by which the bacterium can bring about the death of its host.

As the treatment of pneumococcal infections has become more difficult, greater emphasis has fallen upon strategies for their prevention. Individuals considered to be at particular risk of infection have been immunized with vaccines composed of pneumococcal polysaccharides. It is now evident that such vaccines have serious shortcomings.

The present work is concerned with the identification and characterization of protein determinants which may contribute to the virulence of *S. pneumoniae*, and with the application of this knowledge to the development of vaccines more effective than those currently available for the prevention of pneumococcal disease.

In this Chapter I will first indicate, briefly, the importance of further research on the pneumococcus by presenting data on the current prevalence and severity of pneumococcal diseases world-wide.

There follows a historical review of the course of pneumococcal research, setting out the major discoveries which have been made so far concerning the biology of *Streptococcus pneumoniae*.

The last parts of this Chapter are concerned with immunological aspects of the organism.

I will present a summary of present knowledge concerning the surface structures of the pneumococcus - that is, those structures which form the interface between the intact, invading organism and the defensive immune system of its host. I will then consider protein factors produced by the pneumococcus which may play important roles in establishing and maintaining its virulence in the face of resistance by the host immune systems.

Finally, I will consider the modern polysaccharide vaccines which have been developed to aid immunological defences against pneumococcal infections, discuss their strengths and weaknesses and indicate ways in which current research might lead to the development of more effective vaccine formulations.

B] INCIDENCE OF PNEUMOCOCCAL INFECTIONS

Our understanding of the epidemiology of the pneumococcus remains fragmentary.

Central to the problem is the asymptomatic carriage of the organism in the upper respiratory tract of significant numbers of healthy individuals (White, 1938; Riley and Douglas, 1981).

The integuement of the body, if maintained intact, presents a formidable combination of obstacles to the entry of the bacteria into deeper tissues. As well as immune functions, there are mechanical factors, including the flow of secretions, ciliary movements and peristalsis. The development of pneumococcal pneumonia in humans is sometimes a sequel to contusion of the respiratory mucosal tissue, e.g by inhalation of a foreign body. But, more importantly, it may follow infection of the upper respiratory tract by other organisms - often viruses - which damage the lung surface (Austrian, 1983). Hence, factors which favour the spread of these organisms will also serve to spread pneumococcal disease.

It is well-known that pneumococcal disease is a serious problem in developing countries. But there is also accumulating evidence to suggest that pneumococcal infection is still a major cause of serious illness in nearly all societies; although the prognosis for such infections has

improved significantly since the introduction of modern antibiotics, there is little evidence that attack rates have declined (Austrian, 1983).

In the U.S.A., *Streptococcus pneumoniae* is the most frequent cause of pneumonia and bacteraemia (Klein, 1981), and a major cause of infections of the middle ear: there are probably more than 1.2 million cases of pneumococcal otitis media in the U.S.A. each year (Austrian, 1983); one study in the early 1970s suggested a figure greater than 10 million cases each year (Fraser *et al.*, 1973). More recently an epidemiologic study of children born in Birmingham, U.S.A. showed that more than 95% acquired nasopharyngeal carriage of one or more serotypes of pneumococci during the first two years of life, and that 15% of carrier acquisitions resulted in disease such as otitis, bacteraemia or meningitis (Gray *et al.*, 1980).

The pneumococcus remains a serious threat to life not only in developing countries such as Papua New Guinea, where it is the principal cause of admission to hospital and death in hospital (Riley and Douglas, 1981), but also in countries where health services are relatively sophisticated and accessible. At present pneumonia ranks as the fifth most common cause of death in the U.S.A. (where it is the only infectious disease amongst the top ten causes of death). In 1973 the pneumococcus was the second most common cause of purulent meningitis in America with an annual incidence of 1.5 - 2.5 cases per 100,000 population. One half of these cases occurred in children aged one month to four years with a case fatality rate of 40%, and half the survivors were left with permanent neurological damage (Fraser *et al.*, 1973).

In 1977, a five year survey of infections in Sydney, Australia, indicated mortality rates for pneumococcal pneumonia, meningitis and bacteraemia of 8.9%, 7.5% and 20% respectively (Hansman, 1977). Two recent reviews (Burman *et al.*, 1985 and Grandsen *et al.*, 1985), from Stockholm and

London, reported an overall mortality for bacteraemic pneumococcal infections of 20% and 28% respectively.

Groups particularly at risk from the pneumococcus are the young and the elderly as well as splenectomised patients and those with underlying diseases including alcoholism, malignancy, sickle-cell anaemia and severe hepatic or renal disease.

Beyond the observations that pneumococcal carriage and strike rates remain high in most human populations, and that appropriate antibiotic therapy is not always effective in reversing the disease process in affected individuals, particular impetus has been added to pneumococcal research by the recent appearance of wild strains of the organism which are resistant to common antibiotic drugs.

The widespread use of penicillin as a cheap means to counter pneumococcal infection has inevitably led to the emergence in many parts of the world of pneumococci with increased resistance to this drug. Pneumococci resistant to tetracycline, sulphonamides, erythromycin, lincomycin, chloramphenicol, clindamycin, streptomycin and rifampin have also been reported recently and, particularly ominously, South African strains resistant to all the above agents as well as to various β-lactam antimicrobial agents (Ward, 1981). A Swedish study in 1984 showed that, of 191 pneumococcal clinical isolates tested, 13% had reduced susceptibility to one or more of eleven antibiotics (Kalin *et al.*, 1984).

In Australia a study of pneumococci collected by nasal swab from 282 aborigines in 1981 indicated high carriage rates of the organism (89% in children, 39% in adolescents and 34% in adults) and showed also that about 25% of children and 5% of adults carried drug-insensitive pneumococci:

resistance to benzylpenicillin, tetracycline and co-trimoxazole was identified (Hansman *et al.*, 1985).

C] HISTORICAL

In order to understand something of the present state of knowledge concerning the biology of *Streptococcus pneumoniae* it is useful to review briefly the history of research on the organism.

An invaluable source of information on pneumococcal research prior to the Second World War is Benjamin White's classic monograph "The Biology of Pneumococcus" (1938). Unreferenced material in this section was abstracted from that work.

The first isolation of the pneumococcus through animal passage took place in the latter part of 1880. Sternberg, in the U.S.A. and Pasteur in France independently recovered the organism from rabbits which had been inoculated with human saliva. The "micrococcus" associated with the infection appeared in "immense number... usually joined in pairs and having a diameter of 0.5u" (Sternberg). Each particle was "surrounded at a certain focus by a sort of aureole which corresponds perhaps to a material substance" (Pasteur); this was the bacterial capsule which was to perplex many early investigators because of the inconstancy of its appearance under conditions of artificial cultivation and confuse the precise identification of the organism to such an extent that White can list eighteen different names which were assigned to it before the turn of the century. The belief even arose, briefly, that the capsule was not a diagnostic feature of the organism, but that it was perhaps no more than a "degenerative phenomenon."

Another complication to early studies was the observation that fatal septicemia associated with the pneumococcus could be induced in rabbits by the administration of material from some humans who were not apparantly suffering from any disease. Nevertheless, the pneumococcus was soon established as a major threat to human health. Within the first ten years of its discovery it had been generally accepted as being the causative agent for a number of serious disease states in man, including endocarditis, meningitis, otitis media and certain forms of arthritis as well as infections of the lungs and blood.

Research during those years was intense.

In 1886 Fraenkel reported that rabbits recovering from subcutaneous infection of the ear by the pneumococcus resisted a subsequent inoculation with the same organism. This was probably the first controlled observation that pneumococcal infection may induce immunity. In the following year it was discovered that rabbits recovering from inoculation with weakened cultures became immune - the possibility of a vaccine against pneumococcal infection was already emerging.

In 1891 the Klemperers noted that the young of immune mother rabbits were usually passively protected. This led them to try injecting into non-immune rabbits (intravenously - for the first time) serum taken from either actively immunized rabbits or human patients convalescing from pneumonia. They found that these sera did indeed appear to confer upon the recipients immunity against subsequent challenge. So, having first proved the harmlessness of 4 - 6 ml aliquots of immune rabbit sera on themselves, they then tried using it to treat pneumonia patients, and received encouraging results.

Later workers were to try the curative action on human patients of immune sera from cows, asses and ponies, sometimes with apparent success.

The Klemperers detected in the blood of their immune animals a curative subtance, identified as a protein, which had not been there before. They observed that, while it had no direct killing effect on pneumococcal cells, it inhibited their action. Here was an alluring prospect: a biological cure for pneumonia. The Klemperers were the forefathers of antipneumococcal serum therapy which was to be the great hope of antipneumococcal medicine for many years to come (q.v. Siber *et al.*, 1984).

Its rational application, however, had to await the recognition of capsular polysaccharide serotypes. Benzancon and Griffin, in 1897, reported that the agglutination reaction whereby immune serum could cause pneumococcal cells to precipitate in clumps, could be used to distinguish between several "races" of pneumococci, but this observation was not immediately taken up.

Meanwhile, information on the "curative principle" continued to accumulate. In 1897 it was discovered that immune serum, while not in itself bactericidal, stimulated white cells to phagocytosis. Neufeld and Rimpau, seven years later, showed that the target of the curative principle was the pneumococcus rather than the white cell. This sensitization of the bacterium to phagocytosis was later called *piantication* for "preparation for slaughter", but the term which eventually became accepted was *opsonization* meaning "prepared for the feast".

It had long been known that the organism was fragile in culture, that is, prone, under certain conditions, to spontaneous lysis. In 1900 Neufeld discovered another most significant property of the pneumococcus: fresh bile, diluted even 200 - 300 fold, could induce the bacterium's dissolution

in a few minutes at room temperature. It was later determined that detergents such as sodium deoxycholate had the same effect as bile, in both cases by stimulating the activity of a lytic enzyme produced by the bacterium itself. Little more than this was known of the matter for some time.

Undoubtedly the most significant advance made in the early years of this century towards an understanding of pneumococcal disease was the discovery that the organism could be divided into a number of "serotypes" distinguishable by the immune reactions of the invaded hosts. The classification of serotypes was pioneered by Neufeld and Haendel in 1910. They divided pneumococcal strains into two immunologically distinct groups. At last it was becoming recognized that immunity to one strain of the organism did not necessarily confer any protection against another.

As methods for serotyping improved, the number of distinct types increased; so, by 1913, 5 types had been recognized, and by 1916 more than 15; by 1938 White allows 32 types, and today more than 80 have been identified.

At first the rapid multiplication of serotypes was complicated by a wide variation in the methods used to define them. There was also confusion about the chemical nature of the material which defined the serotype. Prior to 1917 none of the constituents of the pneumococcus had been isolated or subjected to chemical analysis. In 1917 Dochez and Avery discovered that the urine and sera of individuals ill with pneumonia contained a soluble pneumococcal material with serotype-specific antigenicity, i.e. a cell-free substance carrying the antigenic determinants of the intact cell. So firmly entrenched was the dogma of the time that immunologic activity was associated only with protein that, despite the fact that their "soluble specific substance" was resistant both to boiling and to treatment with

trypsin, the authors identified it as a protein "or a substance associated with protein."

In fact, the type-specific material was pneumococcal capsular polysaccharide, though early attempts to isolate it directly from the bacterium usually produced fractions more or less contaminated by protein. Nevertheless, by the mid twenties its essentially polysaccharide nature had been generally acknowledged. Pneumococcal capsular polysaccharides were the first non-protein substances shown to be antigenic in humans.

An understanding of the nature of pneumococcal antigenicity was crucial to the formulation of effective vaccines against the organism, and the need for such vaccines had long been recognised.

The earliest large-scale vaccine trials were conducted amongst black workers in the South African diamond fields.

The likelihood of transmission of pneumococci from a carrier to another individual is thought to be directly proportional to the frequency and intimacy of their contact. Hence carriage rates amongst adults are highest in crowded barracks and open dormitories (Finland, 1942). Not only were the South African miners quartered in just such conditions, they were subjected to a constant influx of uninfected, non-immune recruits. Conditions were ideal for the spread of the organism, and the resulting high rate of carriage led to a similarly high incidence of pneumococcal disease - in particular, pneumonia - which exacted an enormous toll of lives.

In 1913 and 1914 vaccines consisting of small doses of live pneumococcus or heat-killed broth cultures of the organism were administered to thousands of workers, and the incidence of pneumonia amongst the vaccinated men diminished significantly. Similar studies were carried out at the end of World War I in American army camps where crowded confinement also

favoured the spread of pneumococcal disease, and the administration of heat-killed cultures was found to reduce mortality, although the protection was only transient.

For the next twenty years a variety of vaccines was to be tested. There were three general types:

1. Suspensions of whole cells, killed by heat or chemical treatment
2. Whole cell lysates
3. Extracts from lysates.

Increasingly, vaccines tended towards the third type. They came to consist generally of combinations of purified capsular polysaccharides, but other formulations were tried.

One experimental vaccine which should be mentioned here because of its relevance to the original research reported in the later Chapters of the present work, was based on cell extracts containing the pneumococcal haemolysin.

It was Libman who first reported, in 1905, that colonies of the pneumococcus, when cultured on blood plates, generated zones of haemolysis. By the early twenties the ability to induce red cell lysis had been shown to be common to cultures of all pneumococcal types, and identified as probably due to an intracellular product - a haemolysin - liberated from the cells by autolysis. The haemolysin was immunogenic, and its activity was inhibited by antiserum raised against whole-cell extracts. In 1927 Neill *et al.*, (1927a,b) described the results of studies on the antigenic or immunizing action of various preparations of the haemolysin, but not until 1941 was there any claim that the substance had been purified even

partially (in fact to less than 1% of total material) and only then was it recognised as probably a protein. Five years later Halbert *et al.*, (1946) immunized mice with "partially purified" pneumococcal haemolysin and showed a protective effect against challenge by the organism in rabbits, but it was not possible to tell whether this was due to the induction of anti-haemolysin or to other factors. The matter was taken no further for many years.

The late twenties and early thirties saw several significant advances in understanding the biology of the pneumococcus.

It had first been noted thirty years before that pneumonia was sometimes accompanied by purpura - i.e. bleeding from superficial blood vessels into skin or mucous membranes. In 1928, Moir suggested that the purpura producing substance might be a cleavage product of pneumococcal protein arising during autolysis, its release being accelerated by the action of bile salts. In the following year Goebel and Avery showed that the autolytic enzyme released soluble protein and lipid into cultures of the organism.

Further work was also undertaken on the pneumococcal capsule. Beginning in 1929, Heidelberger and Kendall published a series of communications on the quantitation of the precipitin reaction, paving the way for rigorous, reproducible methods of serotyping. The importance of the capsule *in vivo* was suggested shortly afterwards when acapsular ("rough") pneumococci were shown to be highly vulnerable to phagocytosis, and Ward and Enders pioneered studies on the action of serum complement in opsonizing the bacterial cell.

In 1944, as a side-product of efforts in Avery's laboratory to find the molecular basis for the invasiveness of encapsulated strains of

pneumococci, DNA was identified as the genetic material of bacteria (Avery *et al.*, 1944). This was the first report of a biological activity for nucleic acid and was to form the cornerstone of the discipline of molecular genetics.

But, with this formidable achievement, the great surge of pneumococcal research which had begun more than sixty years before subsided quite abruptly. Its driving force had always been the urgent need to develop treatments effective against pneumococcal disease. While the pneumococcus *in vitro* was not only prone to autolysis but highly vulnerable to common antiseptics and germicides such as phenol, mercuric chloride and formalin, the organism within its host remained generally secure.

In that portion of his review dealing with drugs effective against pneumococcal infection, White notes that "the great majority of chemical agents which are noxious to the pneumococcal cell are similarly injurious to the cells of animal tissues." Agents which had commonly been used before 1938 included arsenical preparations, modifications of alkaloids (particularly cinchona), coal tar dyes, such metals as gold and silver, iodine, and metallic salts. Therapy had also been attempted with intravenous soaps (e.g. sodium oleate), formaldehyde sulphoxylate and even enemas of potassium permanganate which were tried as treatment for lobar pneumonia "with more or less favourable results."

Another approach, already noted, was "vaccine therapy", and another the injection of pneumococcal antigens, although, as White remarks, the introduction of such antigens into a body infected with millions of pneumococci and so having its tissues already "permeated by the antigenic constituents of the bacteria" seemed paradoxical.

In summary, there was no safe, effective means of treating pneumococcal disease.

Then, in the early 1940's, the antimicrobial effects of certain aniline dyes became apparant.

Many such dyes with related structures were synthesized before one (Prontosil, produced at I.G. Farbenindustrien) was produced that proved to be active against the pneumococcus *in vivo*; its activity was shown to reside in the *p*-aminobenzene sulphonamide (sulphanilamide) which it liberated in the body. Within two or three years of this revelation, several thousand active compounds had been synthesized and tested. Some were therapeutically useful, but all had side effects and sometimes, as for the older treatments, these were severe enough to be life-threatening in themselves (Finland, 1982).

However, the advent of the sulphonamides was followed closely by that of penicillin, and to this generally harmless drug the pneumococcus proved exquisitely sensitive. To most researchers it appeared that the need for a greater understanding of the biology of *Streptococcus pneumoniae* had largely passed, and this view became firmly established despite the fact that, as long ago as 1917, the pneumococcus had been shown to have the capacity to develop mutant strains resistant to an antibiotic drug (in this case optochin = ethyl hydrocuprein), that sulphapyridine-resistant bacteria had been isolated in 1939, and that pneumococcal mutants resistant to both drugs both *in vitro* and *in vivo* had been selected by Eriksen (1945).

During the decades immediately following the Second World War, the history of pneumococcal research was largely one of the consolidation of previous discoveries within the framework of modern molecular biology. More detailed information accumulated concerning the surface components of the

organism, the chemical structures of many capsular polysaccharides were elucidated, and multivalent vaccines consisting of common polysaccharide types were developed for immunization of particularly high-risk populations.

Recently, the pneumococcal autolysin has been characterized, and some attention has been given to the identification of the pneumococcal purpura-producing principle as well as to investigations into the haemolytic protein pneumolysin and the enzymes neuraminidase and IgA1-protease which have been implicated in pathogenesis by more or less circumstantial evidence. However, there has been little advance towards identifying the exact importance of these factors in establishing and maintaining pneumococcal disease and those by which the organism is able to cause the death of its host.

Fresh impetus towards the research which will be needed to gather this information has been supplied in the last few years by the emergence of pneumococcal strains exhibiting resistance to multiple antimicrobial drugs and by the realization that in certain high-risk groups the fatality rate for bacteraemic pneumococcal infections still exceeds 25% even when patients receive prompt and appropriate therapy (Austrian, 1984).

D] THE CELL SURFACE

Much of the more recent research into the biology of *Streptococcus pneumoniae* has been concerned with the nature of the cell surface - the interface between the bacterium and the immune system of its host. Such research has direct bearing on the formulation of vaccines effective against pneumococcal infection.

In this section I will summarize current knowledge of the cell surface by reference to a model based upon that of Tomasz (1981): see Figure 1.1.

The surface of the pneumococcus may be considered as three layers: the capsule, the cell wall and the plasma membrane.

1. The Capsule.

The outermost layer of the bacterium is a capsule of repeating oligosaccharide units, sometimes cross-linked by phosphodiester bonds (Lee *et al.*, 1981). Monomeric sugars commonly present include galactose, mannose, rhamnose and their derivatives.

Although it is possible to generate and maintain in the laboratory strains of the organism which lack a capsule, the vast majority of pneumococci isolated from natural habitats (e.g. the nasopharynx) are encapsulated. Even though it may act to decrease the efficiency of bacterial attachment to mucosal surfaces (Andersson *et al.*, 1982) and so inhibit the initial colonization step of infection, the capsule appears to be essential for virulence, rendering the pneumococcus highly resistant to phagocytosis unless sensitized by serum opsonins (Winkelstein, 1973). Such opsonization is largely dependent upon the levels of anticapsular antibody present in the serum (Chudwin *et al.*, 1983).

Clinical isolates of *S. pneumoniae* have been grouped by capsular polysaccharide antigenicity into eighty three serologically-defined types. Such a diversity of serotypes would have been a serious impediment to the development of effective polysaccharide-based vaccines against the pneumococcus if certain serotypes had not proved to be much more invasive

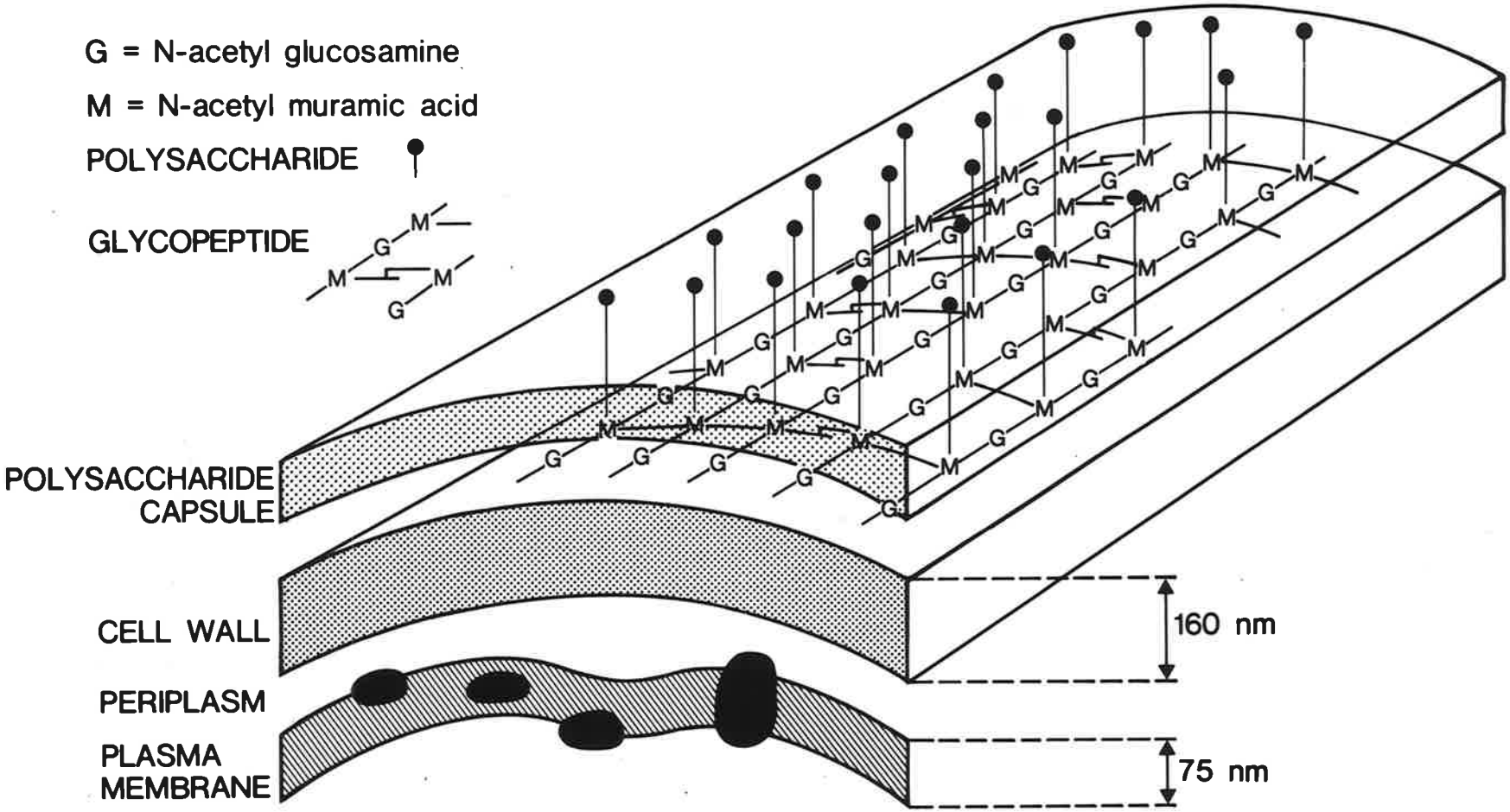
FIGURE 1.1

Diagrammatic sketch of the surface of *Streptococcus pneumoniae*.

(Adapted from Tomasz, 1981).

The thickness of polysaccharide capsule shown is arbitrary.

For the sake of clarity, C-polysaccharide moieties (which are attached to some of the N-acetyl muramyl residues of the cell wall) are not shown; q.v. Figure 1.2.


G = N-acetyl glucosamine
M = N-acetyl muramic acid
POLYSACCHARIDE
GLYCOPEPTIDE
POLYSACCHARIDE CAPSULE
CELL WALL
PERIPLASM
PLASMA MEMBRANE
160 nm
75 nm

to humans than others and hence more likely to cause disease. Both the composition and quantity of capsular polysaccharide appear to play a role in virulence. Amongst strains of a particular capsular type, the degree of virulence may show some positive correlation with the amount of capsular polysaccharide produced (MacLeod and Krauss, 1950). Current knowledge concerning the biochemistry of bacterial capsular polysaccharides and the responses of the immune system to them has recently been reviewed by Lee (1987).

2. The Cell Wall.

Beneath the capsule of the pneumococcus lies a cell wall typical of gram positive bacteria. It is composed largely of glycopeptide and teichoic acid (Figure 1.2).

The glycan chains consist of repeating units of N-acetyl glucosamine and N-acetyl muramic acid, which is an amino sugar derived from glucose. Glycan chains are probably cross-linked through the muramic acid units to form sheets (Fischer and Tomasz, 1985). Carboxyl groups of muramic acid form bonds with the amino groups of N-terminal alanines on peptide side-chains. It is this amide bond linking glycan to peptide that is susceptible to hydrolysis by pneumococcal autolysin. Peptidoglycan solubilized by the action of autolysin is probably the agent known as "purpura-producing principle" responsible for causing tissue damage, oedema and lesions during pneumococcal infection (Chetty and Kreger, 1980, 1981; Tuomanen *et al.*, 1987a,b).

The peptide side-chains form cross-links between glycan layers and are themselves cross-linked via transpeptide bonds, the thickness of the cell wall probably accomodating six to eight layers of glycopeptide. The

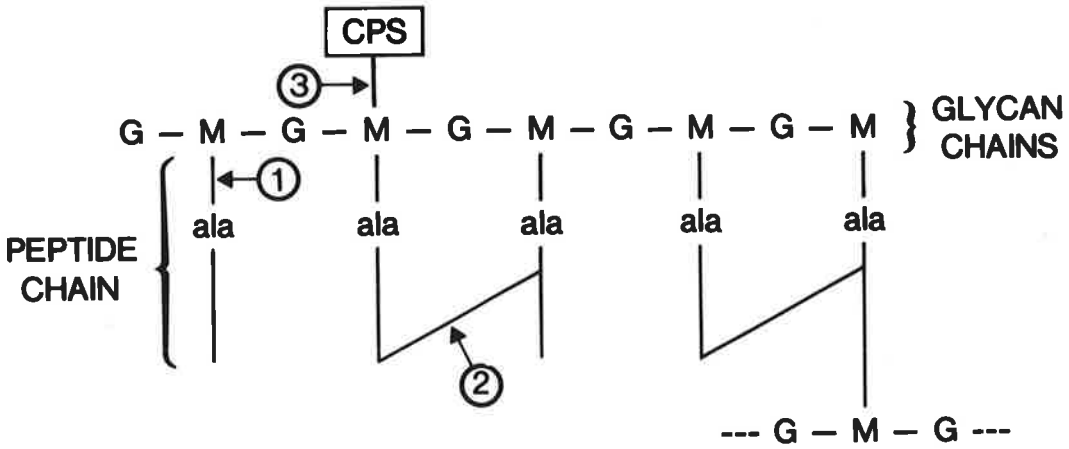
FIGURE 1.2

Diagrammatic model of the structure of the cell wall of *Streptococcus pneumoniae*.

(Adapted from Tomasz, 1981).

The model is partly hypothetical; e.g. the nature of the teichoic acid attachment to glycan, and the frequency of free muramic acid residues are not known. The structures of the peptide network of the cell wall have recently been elucidated by Garcia-Bustos *et al.* (1987). Portions of only two layers of peptidoglycan are shown. The whole cell wall is relatively thick and constructed from several (probably 6 - 8) layers of cross-linked peptidoglycan chains with teichoic acids covalently attached.

CPS	"C-Polysaccharide"; the choline-containing cell-wall teichoic acid. Recent evidence suggests that C-polysaccharide is uniformly distributed on both the inside and outside of the cell walls (Sorensen et al., 1988), but its distribution within the "depth" of the cell wall is not known.
1	The N-acetylmuramyl-L-alanyl bond which is cleaved by autolysin.
2	Transpeptide cross-link.
3	Muramyl- C-polysaccharide cross-link.


CPS
3
G — M — G — M — G — M — G — M — G — M
GLYCAN CHAINS
1
PEPTIDE CHAIN
ala
ala
ala
ala
ala
2
--- G — M — G ---

detailed structure of the peptide network of pneumococcal peptidoglycan has been analysed recently by Garcia-Bustos *et al.* (1987).

While some of the muramic acid units of the glycan chains connect with peptide side-chains, some appear to be linked to the teichoic acid moieties of the cell wall.

Teichoic acid has been defined as a "polymeric ester of phosphoric acid" (Tomasz, 1968; Cleveland *et al.*, 1975), a chemical description broad enough to cover a wide range of molecular species even including, for example, certain of the pneumococcal capsular polysaccharides (Robbins *et al.*, 1983). In the pneumococcus, the teichoic acid of the cell wall is present as "C-polysaccharide." Historically, "C-polysaccharide" has long been used as an imprecise immunological term rather than as a name for a precise chemical formulation; the exact composition of the moiety, and hence its properties, has varied widely according to the method of preparation (Tomasz, 1981). However, it is now possible to define the major C-polysaccharide principle more exactly: it consists of phosphodiester-linked chains of glucose, 2-acetamido -2-, 4-, 6- trideoxygalactose, galactosamine and ribitol phosphate (Jennings *et al.*, 1980).

(It is interesting to note, in passing, that each of these components has been identified as a constituent of one or another pneumococcal capsular polysaccharide, and this, along with other evidence (Austrian, 1983) has suggested the possibility that pneumococcal polysaccharides may have had their evolutionary origins in the cell-wall polysaccharide).

Most importantly, C-polysaccharide is associated with phosphoryl choline, whose presence as a structural component of the cell wall is a feature unique to the pneumococcus. Choline is an essential nutritional

requirement for the growth of pneumococci (Rane and Subbarow, 1940) though it may be replaced by certain other acceptable amino alcohol substitutes, e.g. ethanolamine, which are then utilized at positions which are normally occupied by choline (Mosser and Tomasz, 1970). The presence of choline in the cell wall is important for the ability of the organism to undergo genetic transformation as well as to resist infection by several bacteriophages (Tomasz, 1968; McDonnell *et al.*, 1975). However, its primary effect is probably as a regulatory ligand controlling the activity of autolysin. Choline is essential for the activity of autolysin.

The distribution of C-polysaccharide through the "depth" of the cell wall is not precisely known, although recent electron microscopic evidence (Sorensen *et al.*, 1988) has suggested that it is uniformly distributed on both the inside and outside surfaces. Perhaps surprisingly, those C-polysaccharides on the outside surface of the cell wall are not necessarily masked against host antibodies despite the interpolation of a coat of capsular polysaccharide (Yother *et al.*, 1982). Antibodies directed against C-polysaccharide phosphoryl choline may account for some of the nonspecific immunity of normal mice to pneumococci and may delay the infection until protective levels of anticapsular antibodies are generated (Briles *et al.*, 1981). In mice, both passive and naturally-occuring antiphosphoryl choline antibodies promote clearance of pneumococci from the blood, probably by promoting phagocytosis (McDaniel *et al.*, 1984a,b), but this may not be the case in humans; in elderly human adults with chronic lung disease elevated serum levels of antibody to phosphyoryl choline do not appear to correlate with enhanced ability to opsonize pneumococci (Musher *et al.*, 1986).

Although phosphoryl choline is a component of mammalian membranes and some serum proteins, "natural" anti-phosphoryl choline antibodies do not seem to be disease-causing molecules (Szu *et al.*, 1983).

There is at least one protein inclusion of the cell wall, the polymorphic "pneumococcal surface protein A" (PspA), which appears to be important to the pathogenicity of the organism. McDaniel *et al.*, (1986), have shown that passive immunization with monoclonal antibodies recognizing particular PspA epitopes can protect mice against fatal challenge by some virulent strains of *S. pneumoniae*. Strains of pneumococci which fail to express the intact protein ($PspA^-$) have been produced by specific insertional inactivation of the relevant gene, and shown to have virulence in mice which is significantly reduced from that of an otherwise isogenic $PspA^+$ strain (McDaniel *et al.*, 1987a). In another experiment, mice were immunized intravenously with otherwise isogenic $PspA^+$ and $PspA^-$ strains (which were non-encapsulated, and hence avirulent). When challenged with an encapsulated, virulent strain of pneumococcus expressing the antigen, those mice which had received $PspA^+$ survived significantly longer (McDaniel *et al.*, 1987a). Although PspA is clearly involved in the pathogenicity of the pneumococcus, its exact function has not yet been elucidated.

3. The Plasma Membrane.

The large majority of lipid extractable from pneumococci comes from the plasma membrane (Tomasz, 1981). The lipid bilayer there may form complex foldings giving the appearance of membranous organelles, but the significance of these structures *in vivo* has been the subject of some controversy (Greenawalt and Whiteside, 1975).

Little systematic work appears to have been undertaken on the protein inclusions of the plasma membrane although the association of penicillin resistance with alterations in several penicillin-binding proteins (PBPs) of the plasma membrane has been described (Hakenbeck *et al.*, 1980; Tomasz, 1986; Handwerger and Tomasz, 1986). While these proteins, which catalyse the terminal stages of cell-wall assembly, are acylated and inactivated by penicillin (Handwerger and Tomasz, 1985;), some themselves have penicillin-degrading activity (Ellerbrok and Hakenbeck, 1988).

As well as protein, the plasma membrane contains "Forssman antigen" now identified as lipoteichoic acid (LTA).

In most gram-positive bacteria, the teichoic acid portion of LTA consists of polyglycerophosphate (Cleveland *et al.*, 1976). In pneumococci, however, the composition of this material appears to be similar, if not identical, to the teichoic acid of the cell wall (Fujiwara, 1967). LTA (which appears to be uniformly distributed on the surface of the pneumococcal plasma membrane: Sorensen *et al.*, 1988) is a powerful and highly-specific inhibitor of homologous autolysin, and this may be a most important physiological function for it *in vivo* (Holtje and Tomasz, 1975a,b,c). The lysis induced in pneumococci by low concentrations of detergents such as sodium deoxycholate may proceed by the action of the detergent in abolishing the inhibitory effect of free LTA upon autolysin (Briese and Hakenbeck, 1985).

LTA released from the bacterial cell during pneumococcal infection appears to have a directly toxic effect on the host (Hummell *et al.*, 1985; Hummell and Winkelstein, 1986). Indeed, the ability to bind to host cells and make them susceptible to complement-mediated immunopathogenic damage appears to be a general property of LTA (Weinreb *et al.*, 1986).

E] TOXINS AND VIRULENCE FACTORS

Having considered the surface structures of the pneumococcus, and the ways in which they may interact with the host during infection, we now turn to some of the soluble proteins produced by the bacterium, and the roles which they may have to play in pathogenesis.

In his review, in 1938, White states:

"The idea persists, in spite of many disappointing failures, that some of the symptoms incident to pneumococcal infection, especially those of lobar pneumonia, are caused by a soluble toxin. The idea is a reasonable one, but convincing evidence to support it has never been presented." (White, 1938).

Fifty years later we are still uncertain of the extent to which soluble factors contribute to the pathogenicity of the pneumococcus and the damage to (up to and including the death of) its host.

However, there is now strong evidence that several soluble proteins (some directly toxic and some not) which are released from the pneumococcal cell during infection contribute significantly to pathogenicity and tissue damage. Four such proteins will be considered here. They are autolysin, pneumolysin, neuraminidase, and IgA1-protease.

1. Autolysin.

Pneumococcal autolysin (N-acetylmuramyl-L-alanine amidase) was first purified to homogeneity by Holtje and Tomasz (1976).

Autolysin acts specifically to cleave the covalent bond between the glycan chain and the peptide side-chain of the bacterial cell wall (Howard

and Gooder, 1974; also, see Figure 1.2). Its activity is modulated by choline which binds to an effector site separate from the active site of the enzyme (Giudicelli and Tomasz, 1984). The C-terminus appears to be responsible for the recognition of the choline-containing moiety, while the N-terminus contains the active centre (Garcia *et al.*, 1988). (The binding site may only be necessary for positioning the enzyme correctly for catalysis on an insoluble substrate, since it does not appear to be needed if the substrate is supplied artificially in small, soluble form). Once bound, choline (e.g. that attached covalently to the pneumococcal cell wall teichoic acids) converts the enzyme, by allosteric effect, from its inactive "E" form to its active "C" form. Possibly the hydrophobic C-terminus masks the active site until binding to the choline-containing moieties modifies its folding to expose the active centre (Sanchez-Puelles, 1987). When LTA binds to autolysin, an event which is, again, probably mediated by the choline moieties of the LTA molecules (Horne and Tomasz, 1985), the activity of the enzyme is inhibited.

It is possible that LTA monomers may not in themselves be inhibitory. However, *in vivo*, LTA is probably presented to autolysin in micellar form. After binding to autolysin, these micelles probably cause inhibition by physically blocking access to the enzyme's active site. The reversal of inactivation which is brought about by bile or detergents such as sodium deoxycholate may be due to their disruption of micelles abolishing the steric hindrance (Horne and Tomasz, 1985; Briese and Hakenbeck, 1985).

It is likely that, *in vivo*, autolysin normally remains associated with the bacterial membrane via its attachment to membrane-bound LTA. This LTA inhibits the activity of autolysin until, as a consequence of some trigger event such as treatment with penicillin or infection by phage, the enzyme

is freed into the medium to begin degrading the cell wall (Lopez *et al.*, 1981; Briese and Hakenbeck, 1985).

Autolysin-deficient pneumococci show greatly increased resistance to at least one bacteriophage, Dp-1, a virus which appears to require host autolysin for the liberation of its progeny from the cell at the end of the phage cycle (Lopez *et al.*, 1981). Autolysin-deficient strains also exhibit tolerance to β-lactam drugs (Tomasz *et al.*, 1977; Handwerger and Tomasz, 1985; Sanchez-Puelles *et al.*, 1986).

The bactericidal effect of penicillin appears to be mediated by autolysin. The antibiotic, by inactivating one or more PBPs of the bacterial plasma membrane, may generate some regulatory signal which triggers uncontrolled activity of autolysin, resulting in the death of the cell (Handwerger and Tomasz, 1985). Antibiotic-induced lysis of the pneumococci may have serious consequences for the host, since the cell-wall components generated in the process have been shown to have highly-inflammatory effects (Tuomanen *et al.*, 1987a,b; Ripley-Petzoldt *et al.*, 1988).

The *lytA* gene coding for pneumococcal autolysin has been cloned into *E. coli* and shown to express the authentic enzyme in that organism (Garcia *et al.*, 1985). More recently, the gene has been sequenced and shown to code for a hydrophobic protein with a MW of 36,532 (Garcia *et al.*, 1986b). Mutant strains of pneumococcus in which all (Sanchez-Puelles *et al.*, 1986) or a small part (Lopez *et al.*, 1986) of the *lytA* gene are deleted have also been isolated, as well as mutants producing thermolabile versions of the enzyme (Garcia *et al.*, 1986b).

A recent study with a *lytA*-deficient mutant has confirmed only one of various suggestions concerning physiological roles for the pneumococcal

amidase: that the enzyme catalyses the separation of daughter cells at the end of cell division (Ronda *et al.*, 1987). However, even in mutants entirely deficient in autolysin no functions essential for growth *in vitro* appear to be impaired (Sanchez-Puelles *et al.*, 1986), and, accordingly, there is still some uncertainty as to why an autolytic system of such suicidal efficiency appears to be maintained by strains of pneumococci in the wild.

A hypothesis advanced in the course of the present work is that the selective advantage conferred upon the pneumococcus by autolysin stems from an increase in virulence: the action of autolysin upon the bacterial cell releases substances which enhance the ability of the organism to survive and reproduce during infection against opposition from the host immune system.

Autolysis is a highly effective means of releasing bacterial cell-associated products into the host, and, even although it involves the sacrifice of a proportion of the bacterial cells, its use may be highly advantageous to the invading pneumococcal *genotype* if it helps protect the surviving bacteria against host defences.

During pneumococcal infection the tissue lesions of purpura are probably a consequence of the host immune response to toxic pneumococcal wall components solubilized by autolysin (Chetty and Kreger, 1980, 1981). Both the major cell wall components, teichoic acid and peptidoglycan, can induce pulmonary and meningeal inflammation in rabbits (Tuomanen *et al.*, 1985a,b; 1987b). The minimal biologically active subunit of bacterial peptidoglycan that elicits acute inflammation *in vivo* has been identified as muramyl dipeptide: N-acetylmuramyl-L-alanine-D-isoglutamine (Harrison and Fox, 1985). Once released from the bacterium, cell wall components may adsorb to epithelial surfaces of the host and cause the misdirected activation of

complement there. Such effects have also been observed for LTA, a component of the plasma membrane which is also released from the pneumococcus by autolysis (Weinreb *et al.*, 1986).

In addition to depleting host complement in the immediate vicinity of the invading bacteria, autolysin may have other significant roles to play during infection. There is much evidence to suggest that some pneumococcal proteins liberated during autolysis (in particular, pneumolysin and neuraminidase, which will be considered below) may interefere significantly with the host immune system.

(One approach to determining the exact nature of the selective advantage conferred upon the pneumococcus by the possession of an autolytic system would be to study the virulence of mutants in which the autolysin gene is entirely inactive. However, it should be noted that all work so far published on autolysin-deficient pneumococci (e.g Garcia *et al.*, 1986a,c; Sanchez-Puelles *et al.*, 1986; Lopez *et al.*, 1986) has involved strains which were created by non-specific mutagenesis. It would be difficult to tell whether any reduced virulence in such mutants were due to autolysin-deficiency alone, or to unidentified genetic damage affecting other systems important to pathogenesis).

2. Pneumolysin.

Pneumolysin, first purified from *Streptococcus pneumoniae* by Shumway and Klebanoff (1971), is a potent, lethal toxin belonging to a group of fifteen bacterial sulphydryl-activated cytolysins which share a number of biological properties including immunological cross-reactivity (Bernheimer, 1976; Geoffroy and Alouf, 1984 : reviews). Indeed, DNA sequence data is now

beginning to indicate some amino acid sequence homologies in the structures of these proteins (Mengaud *et al.*, 1987a; Kehoe *et al.*, 1987).

(As part of a project including the work which is presented in this thesis, the pneumococcal gene for pneumolysin was recently cloned into *E. coli*; Paton *et al.*, 1986. Subsequently, other workers determined its complete nucleotide sequence; Walker *et al.*, 1987).

Of the sulphydryl-activated cytolysins, perhaps the most closely studied has been streptolysin-O, with which pneumolysin shares 42% amino acid homology (Kehoe *et al.*, 1987). Streptolysin-O attaches to the cholesterol present in mammalian cell membrane, inserts itself into the membrane, where it aggregates to form hydrophilic channels, destroying the integrity of the membrane. A single pore is probably enough to induce lysis (Bhakdi and Tranum-Jensen, 1986). Pneumolysin also has a high affinity for cholesterol, and can induce the lysis of mammalian cells, probably in the same manner.

Uniquely amongst sulphydryl-activated cytolysins, pneumolysin is a cytoplasmic protein which appears not to be actively secreted (Johnson, 1977). This conclusion has been confirmed by an examination of the amino acid sequence predicted for pneumolysin from the nucleotide sequence of its cloned gene, which indicates no secretory signal sequence at the N-terminus of the protein (Walker *et al.*, 1987). Nevertheless, substantial titres of antibody directed against pneumolysin may be detected in the sera of patients with pneumococcal pneumonia (Kanclerski *et al.*, 1988) indicating that the protein is released from the cell into its host during infection. Presumably this release is due to lysis of the bacteria, and may be mediated by the action of pneumococcal autolysin.

When injected intravenously into rabbits, pneumolysin has been shown to cause spherocytosis and increased osmotic fragility of erythrocytes

(Shumway and Klebanoff, 1971), effects which could also be observed during the course of a pneumococcal infection (Shumway, 1958). The injection of purified pneumolysin into rabbit eyes resulted in an acute inflammatory response (Johnson and Allen, 1971, 1975) possibly because of the interaction of the toxin with polymorphonuclear leukocytes (Johnson *et al.*, 1981). Pneumolysin appears to be produced by virtually all clinically-isolated strains of pneumococci (Lorian *et al.*, 1973; Paton *et al.*, 1983b; Kanclerski and Molby, 1987).

Initially, some doubt was cast upon the pathogenic importance of the toxin because of the fact that substantial amounts of it can be inactivated *in vitro* by concentrations of cholesterol similar to those found in human serum (Johnson *et al.*, 1980). But the cholesterol in plasma is not found in its free form. Rather, it is combined with other lipids and with protein to form hydrophilic lipoprotein complexes (Mayes, 1981) whose affinity for pneumolysin is not known. Recent research (arising from the work presented in this thesis) has shown that purified pneumolysin, in nanogram amounts, can significantly inhibit the antimicrobial properties of human neutrophils (Paton *et al.*, 1983a) and monocytes (Nandoskar *et al.*, 1986) *in vitro*. Furthermore, the purified toxin is capable of activating human serum complement and depleting serum opsonic activity, and that these latter effects can actually be enhanced by pre-treatment of the toxin with free cholesterol (Paton *et al.*, 1984). Thus it seems likely that, despite the presence of cholesterol in human serum, pneumolysin can inhibit bacterial clearance, even in patients with high levels of antibody to capsular polysaccharide.

Nanogram doses of pneumolysin inhibit the blastogenic response of human lymphocytes to mitogens and block the subsequent production of lymphokine and immunoglobulins *in vitro* (Ferrante *et al.*, 1984). If this occured *in*

vivo then the ability of a host to mount an antibody response during pneumococcal infection would be severely impaired.

3. Neuraminidase.

Neuraminidases (mucopolysaccharide N-acetyl-neuraminhydrolases, E.C. 3.2.1.18) have been reported in a wide variety of bacteria (Drezniek, 1972). These enzymes are capable of cleaving terminal sialic acids, such as N-acetyl-neuraminic acid (NANA) from many biological substrates such as glycolipids, glycoproteins and oligosaccharides (Rafelson, 1963).

There is considerable indirect evidence to indicate that the neuraminidase of *Streptococcus pneumoniae* contributes to the virulence of the organism and causes significant damage to the bacterium's host.

The enzyme appears to be produced by all pneumococci freshly isolated from infected tissue (Kelly *et al.*, 1967; O'Toole *et al.*, 1971) (although the ability to elaborate it may be lost by strains maintained in laboratory culture: Kelly *et al.*, 1966).

While there has been some uncertainty as to whether pneumococcal neuraminidase is a cell-associated or a secreted protein, evidence arising during the course of the present work has suggested that, at least in some strains, the release of neuraminidase from the pneumococci *in vitro* parallels the release of pneumolysin, a protein which is not actively secreted and remains in the cytoplasm until release by cellular lysis (Johnson, 1977). In addition, recent work carried out in the author's laboratory has indicated that *lytA*$^-$ mutants (which were constructed by insertion-duplication mutagenesis directed at the *lytA* gene, and are hence defective in autolysin expression alone) fail to release any neuraminidase

into the culture medium during growth. Back-transformant mutants into which the *lytA* gene specifically has been re-introduced regain the ability to release neuraminidase (A. Berry, personal communication). This suggests that the release of neuraminidase into the host during infection could be mediated by autolysin.

The neuraminidases of several species of bacteria other than *S. pneumoniae* which are able to survive on mucosal surfaces have been shown to remove sialic acid residues from salivary glycoproteins, so hindering the protective role of these secretions against invading micro-organisms (Gottschalk, 1960; Pardoe, 1974).

Microgram quantities of partially-purified pneumococcal neuraminidase administered intracerebrally or intraperitoneally are lethal to mice (Lorian *et al.*, 1973). Histochemical studies of organs taken from mice dying after inoculation with partially-purified pneumococcal neuraminidase have demonstrated marked decreases in the sialic acid content of liver and kidney when compared to controls (Kelly and Greiff, 1970).

Elevated concentrations of free NANA detected in the cerebrospinal fluid (CSF) of some human patients with pneumococcal meningitis correlate significantly with the development of coma and bacteraemia in such patients (O'Toole *et al.*, 1971). As well as serum glycoproteins present in CSF, neurons (which are rich in NANA-bearing glycoproteins and gangliosides) contiguous with the meninges and bathed in infected CSF, may be important substrates for the enzyme (O'Toole *et al.*, 1971).

It has also been suggested that pneumococcal neuraminidase cleaves neuraminic acid from IgG, converting this antibody into an antigenic moiety (Ward and Kunkel, 1983; Ginsberg, 1985 : reviews). One consequence of this may be the production of autologous immune complexes: cryoprecipitable

("cold-insoluble") proteins which are a feature of sera collected from patients with acute post-streptococcal glomerulonephritis (McIntosh *et al.*, 1971a,b). Cryoproteins appear to deposit as nodules on the glomerular basement membrane, causing inflammation there (McIntosh *et al.*, 1971a). When neuraminidase-containing extracts (derived from *Streptococcus pyogenes*) or neuraminidase-treated rat IgG were administered to rats, a significant proportion of the animals showed signs of immune deposit renal disease. Similar results have also been demonstrated using rabbits treated with neuraminidase derived from *Clostridium perfrigens* (Griswold *et al.*, 1975).

It is interesting to note, in passing, the results of recent studies (Svanborg Eden *et al.*, 1987; Beuth *et al.*, 1987; Andersson and Svanborg Eden, 1988; Andersson *et al.*, 1988) on the pneumococcal adhesin by which the bacterium anchors itself to human pharangeal epithelial cells. The specific surface molecules which the adhesin appears to recognise on the surface of the target cells are glycolipid sugar moieties such as NANA, and should be highly susceptible to degradation by neuraminidase. Presumably, the enzyme is not released from the pneumococcus at this earliest stage of infection.

Pneumococcal neuraminidase was partially purified by Tanenbaum and Sun (1971) and by Stahl and O'Toole (1972). The enzyme showed an apparant multiplicity of molecular forms whose relationship to eachother had not been determined prior to the work presented in this thesis.

4. IgA1-protease.

IgA1-proteases are a family of bacterial enzymes that specifically cleave and inactivate human IgA1, a major immunoglobulin secreted by

mucosal surfaces (Mulks *et al.*, 1980a,b). IgA1 is an antibody species which is highly resistant to most common proteolytic enzymes. The ability of the pneumococcus, like some other bacterial pathogens that colonize mucosal surfaces, to specifically inactivate IgA1, may be an important factor in establishing infection in humans.

Kilian and Reinholdt (1987) have suggested that the Fabα fragments of IgA1 generated by the action of the pneumococcal IgA1-protease and retaining full antigen-binding capacity, may bind to epitopes on the bacterial cell surface. Because, being monovalent, they would be unable to induce bacterial agglutination, these fragments would then protect the bacterium from the immune system by blocking the access of intact antibody molecules.

Pneumococcal IgA1-protease is remarkably specific for human IgA1; human immunoglobulins of other classes, including IgA2 and IgA proteins from other animal species, are not susceptible (Kilian *et al.*, 1980). Such precise specificity of the bacterial enzyme for a particular element of the human immune system suggests strongly that IgA1-protease plays an important role in the disease process in humans.

Research on the nature and action of the IgA1-proteases of pathogenic bacteria has been reviewed by Plaut (1983).

Apart from pneumolysin, neuraminidase and IgA1-protease, there may well be other soluble factors important to pneumococcal virulence. There is, for example, a human-elastase inhibitor, possibly an RNA, produced by the organism which may play a role in inhibiting the intracellular proteolysis and killing of phagocytosed pneumococci by human neutrophils (Vered *et al.*,1984, 1985a,b, 1988).

The existence of a pneumococcal extracellular DNase with a significant role in pathogenesis has been suggested (Firshein *et al.*, 1982).

Also, there is evidence (Wikstrom *et al.*, 1984) that many strains of the pneumococcus have the ability to degrade classes of human immunoglobulins other than IgA1, suggesting the action of specific anti-Ig proteases other than IgA1-protease.

No comprehensive survey of pneumococcal toxins has been attempted. However, the importance of pneumolysin, neuraminidase and IgA1-protease to pathogenicity may be indicated by their prevalence in clinical isolates of the organism; indeed, these three proteins appear to be produced by all clinical isolates of *S. pneumoniae* examined so far (Kelly *et al.*, 1967; Mulks *et al.*, 1980a; Paton *et al.*, 1983b). Immunity to one or more of these putative virulence determinants could confer substantial protection on the host. It would be appropriate, therefore, to investigate their protective efficacy as components of pneumococcal vaccines.

The nature and efficacy of pneumococcal vaccines as presently formulated will be summarized in the next section.

F] VACCINES

In 1938 Benjamin White wrote:

"The very nature of pneumococcal disease would seem to preclude the possibility of engendering by artificial means any secure or lasting protection against invasion of the body by pneumococci... it is unreasonable to expect that the administration of pneumococci or of pneumococcal derivatives, no matter how artfully modified so as to be no longer capable of harm, would confer upon the recipient more than a short

period of freedom from subsequent invasion by pneumococcus. The attempts of immunologists to devise a suitable immunizing agent have been more notable for their ingenuity than for their success in establishing an immune state of high degree or of long duration".

Fortunately, his pessimism was not justified. By 1950 it had been demonstrated unequivocally that type-specific pneumococcal infection in man could be prevented by a tetravalent vaccine of capsular polysaccharides; the groundwork for modern polyvalent vaccines had been laid (Heidelberger *et al.*, 1950).

In 1948-50 rigorous trials of polyvalent polysaccharide vaccines containing six capsular serotypes in a single formulation demonstrated that antibodies persisted at between one half and one third of peak levels for five to eight years after a single immunizing dose of vaccine (Austrian and Gold, 1964). Hexavalent polysaccharide vaccines protective against the pneumococcus were marketed in the late 1940's, but they were soon withdrawn, because it was thought that the emergence of modern antibiotics had made them obsolete. In fact, this was not so.

Most of those people who die as a result of pneumococcal disease do so because, early in the course of infection, often before the invading organism has been tested for antibiotic sensitivity, they sustain some (unidentified) physiological injury which cannot be reversed by the later intervention of antibiotic therapy (Austrian and Gold, 1964). Death from pneumococcal pneumonia can occur days after antibiotic therapy has been administered, when pneumococci can no longer be isolated from blood or tissues, and when the extent of pneumonia appears insufficient to cause death by anoxia (Johnston, 1981).

At least for high-risk populations, then, there is a clear case for applying measures designed to prevent the onset of the disease rather than merely relying on antibiotics to eliminate the infection once it has been contracted. It was this conclusion, followed more recently by the emergence of antibiotic-resistant strains of the pneumococcus, which gave impetus to the redevelopment and world-wide marketing of polyvalent polysaccharide pneumococcal vaccines.

The first of these modern vaccines contained capsular polysaccharides from 14 pneumococcal serotypes. The most recent has been a 23-valent version designed with reference to newly-acquired data on the epidemiology of pneumococcal types, the degree of cross-reactivity of human antibodies to serologically-related types, and the stability of polysaccharides in solution (Cadoz *et al.*, 1985); it has been claimed (Robbins *et al.*, 1983) that this latter vaccine is potentially capable of preventing or attenuating 85-95% of bacteraemic pneumococcal infections in immunocompetent persons.

Nevertheless, there remains controversy and confusion over the effectiveness of polysaccharide vaccines. One important reason for this is that many of those persons at particular risk from the pneumococcus are not fully immunocompetent. For some high-risk groups it has been suggested (Austrian *et al.*, 1981) that these vaccines may be of less than ideal efficacy, and indeed there have been several reports of serious and even fatal infections occurring in vaccinated, high-risk individuals (Giebink *et al.*, 1979; Patel, 1981; Sumaya *et al.*, 1981; Nielsen *et al.*, 1982). Broome (1981) has claimed that while trials of the 14-valent polysaccharide vaccine have conclusively demonstrated its efficacy for populations of young adults acutely exposed to a high risk of pneumococcal disease, the efficacy of the vaccine has not been well documented for other, and

particularly high-risk groups, notably the elderly (in whom the humoral antibody response is sometimes rather transient; Riley and Douglas, 1981), those persons with sickle-cell anaemia, asplenia, multiple myeloma, nephrotic syndrome, cirrhosis or alcoholism. (A defective response to thymus-independent antigens, such as pneumococcal polysaccharide, is also a distinctive consequence of HIV infection; Ballet *et al.*, 1987). There is evidence to suggest that people affected by such conditions have a poorer antibody response and are afforded less protection. For healthy adults the initial response to pneumococcal polysaccharide antigens may last at least five years (Mufson *et al.*, 1983), but for immunocompromised patients the duration of protection may well be shorter than in normal persons (Landesman and Schiffman, 1981). Indeed, some high-risk groups may not be protected at all (Forrester *et al.*, 1987).

A recent review of clinical evidence concerning the efficacy of the current pneumococcal polysaccharide vaccine has concluded that while it appears to be effective in the healthy elderly, there are serious concerns about its efficacy in high-risk elderly patients (La Force and Eickhoff, 1988).

Although vaccine-induced levels of antibody of 250-300 ng antibody nitrogen per ml serum have generally been considered protective for bacteraemic infections, the vaccine may not necessarily be effective even when such "protective antibody levels" have been induced because host factors other than antibody (e.g. complement) are also important for optimal protection against pneumococcal disease.

A recent study (Musher *et al.*, 1986) has shown that level of antibody against pneumococcal polysaccharide does not correlate with capacity to opsonize the organism: although pre- and post-vaccination levels of polysaccharide antibody in elderly adult humans with chronic lung disease

were similar to those of younger vaccinated adults, opsonizing activity for six of the nine serotypes investigated actually dropped after vaccination.

The levels of antibody which are protective against non-bacteraemic pneumococcal infections are not known. Young children form a high-risk group of particular concern. Several studies have shown that vaccination with pneumococcal polysaccharides has little or no demonstrable clinical benefit in children under the age of five years (Makela *et al.*, 1981; Riley *et al.*, 1981; Wright *et al.*, 1981; Douglas and Miles, 1984). The apparent failure of the vaccine here is probably due to the poor immunogenicity of bacterial polysaccharides in children (Barrett, 1985: review). An effective immune response to polysaccharides depends on the development of humoral antibodies. Because of this, the administration of polysaccharide vaccines to children is of little benefit until they have achieved immunologic maturity at approximately six to eight years of age (Austrian, 1984a).

G] DIRECTIONS FOR VACCINE RESEARCH

Current pneumococcal vaccines have two critical shortcomings.

The first is that the protection they impart is type-specific; that is, immunization with one polysaccharide type confers no protection against infection by the majority of the other distinct serotypes of *S. pneumoniae:* current vaccines are mixtures of polysaccharides derived from only the most commonly invasive types. Because of this, a formulation of serotypes which is effective for one population may have much reduced efficacy for another: e.g. studies on the type distribution of pneumococcal isolates from Asian populations show only 63 - 73% of serotypes included in the 23-valent vaccine, as against 88 - 93% for the U.S. population (Lee, 1987).

The second shortcoming of present vaccines is that even the protection they provide against those serotypes whose polysaccharides they contain is by no means complete, particularly for people in some groups which are at high risk from pneumococcal infection. For at least one such group - young children - pneumococcal polysaccharides are such poor immunogens that the current vaccine is of little or no benefit.

Protein-based vaccines would have important advantages over the current polysaccharide formulations.

Proteins are more immunogenic than polysaccharides, at least in young children who, as a group, respond well to protein-based vaccines (Chu *et al.*, 1983). Also, since it seems likely that proteins of critical importance to virulence would be produced by all invasive strains of the pneumococcus, a vaccine supplemented with such proteins should provide protection against pneumococci of all serotypes in contrast to the strictly type-specific protection conferred by the present formulation.

Four proteins were initially considered for particular attention in the present study, because of strong evidence suggesting their involvement in pathogenicity.

Pneumolysin is known to be toxic. It has a high specificity for cholesterol, a molecule exclusive to higher organisms. Pneumolysin has well-documented detrimental effects on animal tissues (e.g. ocular tissue) and on components of the human immune system *in vitro*, and it can cause lysis of animal cells. It appears to be produced by all pathogenic strains of *S. pneumoniae*.

Neuraminidase is also toxic, and is capable of cleaving a wide range of biological substrates within animal hosts, including cell-surface receptors and immunoglobulins. Clinical data in humans has implied its involvement in

pneumococcal meningitis, and in post-streptococcal glomerulonephritis. Like pneumolysin, neuraminidase appears to be produced by all clinically-isolated strains of pneumococcus.

Autolysin releases highly-inflammatory LTA and cell-wall fragments from the pneumococcus, and may be the prime mediator for the release into the host of a number of other soluble factors (such as, perhaps, pneumolysin and neuraminidase) which are important to pathogenesis. Autolysin also appears to be produced by all clinically-isolated pneumococcal strains.

IgA1-protease, an enzyme capable of inactivating human IgA1, was also considered for investigation in the current work, but its narrow substrate specificity precluded this. Since animal immunoglobulins are not susceptible to the pneumococcal protease, animal models would be useless for assessing the protective effect of the enzyme as an immunogen. IgA1-protease was therefore excluded from the present study.

The principle behind using bacterial proteins or their toxoid derivatives as immunogens is that the antibodies whose production they direct can be expected to inhibit the activity of the native bacterial proteins to which they bind *in vivo* during infection. Pneumolysin and neuraminidase are free in the medium when they have their effects, and would be expected to be accessible to neutralizing antibody. The exact location of autolysin *in vivo* is not certain, and it may be immobilized on the plasma membrane or cell wall, but its accessibility to neutralizing antibody (at least for a non-encapsulated strain) has been established by Garcia *et al.*, (1982), who have shown that rabbit anti-autolysin serum, when added to cultures of the organism, is capable of suppressing the functions of the enzyme, including autolysis.

It is suggested here that pneumolysin, neuraminidase, autolysin and IgA1-protease may be considered as virulence factors for the pneumococcus. A definition of the term needs to be stated. In a trivial sense, any vital component of the bacterial cell may be said to be a "virulence factor" in that its absence or the inhibition of its function reduces the viability, and hence the virulence, of the cell. In the present work, the term "virulence factor" is used with a more specific meaning. It is applied to bacterial products which:

1) are not necessarily required by the organism for normal growth *in vitro*; and

2) facilitate the infective process either by interacting specifically with host molecules or structures, participating in the release of substances which do so, or otherwise protecting the bacterial cell from host defences.

Such substances would be expected to operate outside the cytoplasm of the living bacterial cell, and hence be potentially accessible to immunological inactivation by the host. When administered to the host as immunogens, protein virulence factors (or toxoid versions of them) should therefore offer some protection against subsequent infection by the organism.

H] AIMS OF THE PRESENT WORK

The present project was conceived with the following design.

Selected pneumococcal proteins for which there was strong evidence suggesting involvement in pathogenesis (i.e. pneumolysin, neuraminidase and autolysin) were to be purified, in toxoid form if necessary, and used to

immunize mice. These mice were subsequently to be challenged with virulent pneumococci, and the degree of protection afforded by each immunogen was then to be assessed.

This research plan addressed two inter-related aims.

Firstly, direct evidence was to be obtained concerning the relative importance of the various proteins to the pathogenic process, making a valuable contribution towards answering the "simple but profound question (which) remains unanswered... why do people die from pneumococcal infection?" (Johnston, 1981).

The second aim of this work was to assess, via an animal model, the potential of the various proteins (or their toxoid derivatives) as supplements to, or replacements for, the polysaccharides presently used in human pneumococcal vaccines.

CHAPTER TWO

MATERIALS AND GENERAL METHODS

A] MATERIALS

1. Biological Materials.

a. Bacterial strains.

i. Streptococcus pneumoniae

Wild-type. Pneumococcal strain numbers in the main text refer to clinical isolates collected by D. Hansman, Microbiology Department, Adelaide Children's Hospital, South Australia, except for D39 (Avery *et al.*, 1944) which was obtained from the National Collection of Type Cultures, London, U.K. (strain number NCTC7466), and Rx-1, an unencapsulated, highly transformable strain (originally described by Guild and Shoemaker, 1974), which was supplied by Dr. J. Yother, University of Birmingham, Alabama, U.S.A.

Autolysin mutants. Autolysin mutants were prepared by A. Berry, Microbiology Department, Adelaide Children's Hospital, South Australia (Berry *et al.*, 1989a) and kindly supplied by her.

Autolysin-negative mutants **AL-2** and **AL-6** were constructed in independent cloning experiments by insertion-duplication mutagenesis using the vector pVA891 (Macrina *et al.*, 1983). This is a deletion derivative of the *Escherichia-Streptococcus* shuttle plasmid pVA838 (Macrina *et al.*, 1982)

which has lost the capacity to replicate autonomously in *Streptococci*. However, pVA891 retains a streptococcal gene encoding erythromycin resistance. In the first stage of the mutagenesis procedure, the 1213 base-pair (bp) pneumococcal DNA insert containing the complete autolysin gene was excised from pGL80 (see below) by digestion with *Hin*dIII and purified by agarose gel electrophoresis. This was then digested with *Taq*I, and a 375 bp fragment from the middle of the coding sequence was isolated after gel electrophoresis. This was ligated into pVA891 which had been linearized with *Cla*I, and transformed into *E. coli* DH1. The recombinant plasmid was purified from its *E. coli* host and used to transform *S. pneumoniae*. Homologous recombination between the 375 bp of pneumococcal DNA in the plasmid and the *S. pneumoniae* chromosomal autolysin sequence would be expected to result in simultaneous incorporation of the plasmid (encoding erythromycin resistance) into the chromosome and interruption of the autolysin coding sequence. The efficiency of direct transformation of the encapsulated type 2 strain D39 to erythromycin resistance, using recombinant pVA891 derivatives, appears to be very low, even in the presence of exogenous competence factor (Berry *et al.*, 1988). Therefore a two-step approach was adopted. Firstly, the non-encapsulated strain Rx-1 was transformed with the recombinant plasmid, yielding a single mutant which was both erythromycin-resistant and autolysin-negative (as judged by resistance to deoxycholate-induced lysis). Secondly, D39 was transformed with DNA extracted from this mutant. Two independent transformation experiments were carried out to minimize the possibility of co-transformation of spurious *S. pneumoniae* sequences along with the interrupted-autolysin/erythromycin-resistance locus. Each experiment yielded several erythromycin resistant D39 transformants, all of which produced a type 2 capsule (confirmed by Quellung reaction), but failed to produce autolysin. One transformant from each of the experiments

(designated AL-2 and AL-6) was selected for use in the current study. Confirmation that the autolysin gene in each transformant had been inactivated by insertion of the plasmid was obtained by Southern blot hybridization analysis using ^{32}P-labelled pVA891 or the *Hin*dIII fragment excised from pGL80 as probe. The hybridization patterns obtained were consistent with those expected to arise from insertion duplication inactivation of the autolysin gene.

AL-6R, an autolysin-positive erythromycin-sensitive revertant of AL-6 was also constructed by transforming AL-6 with the purified 1.2 kb *Hin*dIII pneumococcal DNA insert from pGL80. Homologous double recombination between this fragment and the AL-6 chromosome would be expected to reconstitute a complete copy of the autolysin gene, with the concomitant elimination of the pVA891-derived sequences, including the erythromycin-resistance determinant. Southern blot analysis confirmed that the autolysin gene had been reconstructed.

ii. Escherichia coli

E. coli JM109: *recA*1, *endA*1, *gyrA*96, *thi*, *hsdR*17, *supE*44, *relA*1, λ^-, Δ(*lac-proAB*), [F′, *traD*36, *proAB*, *lacI*^{q}Z, ΔM15]; see Yanisch-Perron *et al.*, (1985).

E. coli K-12 strain DH1 (CGSC6040) (F$^-$ *gyrA*96, *recA*1, *relA*1, *endA*1, *thi*-1, *hsdR*17, *supE*44, λ^-); see Hanahan (1983).

b. Plasmids.

pGLY008: A subclone of the pneumolysin gene inserted into pUC18, and subjected to oligonucleotide-directed mutagenesis which introduced a single DNA base mutation into the cysteine codon at amino acid number 428 (DNA +strand sequence 5′-TGT-3′) such that this codon now specifies a glycine residue (DNA sequence 5′-CGT-3′); (unpublished). Supplied by Dr. G. Boulnois, University of Leicester, U.K.

pGL80: A recombinant plasmid carrying the complete structural gene (*lyt*A) for the pneumococcal autolysin (Garcia *et al.*, 1986b). It was supplied by Dr. R. Lopez, Centro de Investigaciones Biologicas, Madrid, Spain.

pJCP401: A recombinant plasmid containing the coding sequence for the pneumococcal autolysin gene, *lytA*. It was constructed by inserting a 1.2 kb *Hin*dIII fragment of *S. pneumoniae* strain 3551 chromosomal DNA into pUC19, and was isolated from the chromosomal library by use of a 23-mer oligonucleotide probe which had been synthesised, in accordance with the sequence data of Garcia *et al.*, (1986b), to hybridize to a region within the autolysin gene. This plasmid was constructed and supplied by A. Berry.

c. Mice and rabbit.

Mouse strains used were **Prince Henry** (initial source Prince Henry Hospital, Sydney, NSW, Australia) and **BALB/c** (initial source University of Adelaide Central Animal House, South Australia). The mice used in experiments came from breeding colonies maintained within the Adelaide Children's Hospital. The rabbit, a **Lop-Eared Albino**, was supplied by the University of Adelaide Central Animal House.

2. Fine Chemicals.

Sources of various chemicals were as follows:

Acrylamide: Bio-Rad Laboratories, Richmond CA, U.S.A.

Ampicillin, sodium, (Amp): Commonwealth Serum Laboratories, Melbourne, Vic. Australia.

Antifoam A Emulsion: Sigma Chemical Co., St. Louis, MO, U.S.A.

Carbodiimide hydrochloride: Bio-Rad.

[methyl-^{3}H] choline chloride, s.a. 2.96 TBq/mmol: Amersham Laboratories, Bucks, U.K.

Cyanogen bromide: Ajax Chemicals, Sydney, N.S.W. Australia.

(±) - 1,2:3,4 - diepoxybutane: Sigma.

1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide: Sigma.

Fentanyl citrate: David Bull Laboratories, Melbourne, Australia.

Freund's adjuvant (complete and incomplete): Commonwealth Serum Laboratories.

Goat anti-mouse (or anti-rabbit) IgG (H+L) horseradish peroxidase conjugate: Bio-Rad.

HRP Western blot colour development reagent, containing 4-chloro-1-naphthol: Bio-Rad.

Metomidate: "Hypnodil", Janssen Pharmaceutica, Belgium.

Molecular weight marker proteins for SDS polyacrylamide gel electrophoresis; normal: Bio-Rad; or **Dye-conjugated "Rainbow Markers":** Amersham.

Moniarix heptadecavalent pneumococcal polysaccharide vaccine: Smith Kline and French Laboratories, Frenchs Forest, N.S.W. Australia.

N,N'-methylene-bis-acrylamide (Bis): Bio-Rad.

2'-(4-methyl-umbelliferyl)-α-D-N-acetylneuraminic acid, (MUAN): Sigma.

Mucin, bovine submaxillary: Boehringer Mannheim Australia, North Ryde, N.S.W. Australia.

N-acetyl-neuraminic acid: Boehringer Mannheim.

Pentobarbitone: Adelaide Children's Hospital Pharmacy.

Phenylmethylsulphonylfluoride, (PMSF): Sigma.

Pneumococcal polysaccharide, Danish Type 19F: American Type Culture Collection, Rockville, MD, U.S.A.

Ready Value liquid scintillation cocktail: Beckman Instruments Inc., Fullarton, CA, U.S.A.

Restriction enzymes: Boehringer Mannheim.

Other chemicals used were of at least AR grade.

3. Media, Solutions.

Blood-Agar plates: prepared according to "The Oxoid Manual" 5th Edition. Oxoid Ltd., Basingstoke, Hants. U.K.

Cofactor solution (prepared as 100X stock): containing metal ions, biotin, nicotinic acid, pyridoxine, thiamine, riboflavin, calcium pantothenate, glucose and tryptophan: prepared as described by Lacks and Hotchkiss (1960).

Defined Medium: Also see Lacks and Hotchkiss (1960). In addition to cofactors, the medium contains casamino acids, yeast extract and cystine. Bovine serum albumin was omitted, and, when appropriate, unlabelled choline chloride was replaced by the tritiated form. The medium was buffered with sodium acetate and potassium phosphate.

Luria-Bertani (LB) broth and plates: Prepared as described by Maniatis *et al.* (1982).

Phosphate buffered saline, (PBS): 7 mM sodium phosphate, 150 mM sodium chloride, pH 7.2.

Serum broth: 10% (by volume) defibrinated horse serum (Commonwealth Serum Laboratories) and 90% (by volume) Meat Extract Broth, which was prepared as described by Cruickshank *et al.*, (1968).

Todd-Hewitt broth: Prepared from powder, Oxoid CM189.

Tris-Buffered Saline, (TBS): 150 mM NaCl, 20 mM Tris-HCl, pH 7.4.

Trypticase soy broth, (TSB): Prepared from powder, BBL Microbiology Systems, Cockeysville, MD, U.S.A; BBL11768.

TSB^{+}: TSB supplemented with 0.4 g $MgSO_4.7H_2O$, 0.15 g cysteine-HCl and 13 mg $CaCl_2.2H_2O$ per litre.

TSB^{++}: TSB^{+} supplemented with 2.63 g Na_2HPO_4 and 1.15 g $NaH_2PO_4.2H_2O$ (pH 7.4) per litre.

Tween/Tris-Buffered Saline (TTBS): 150 mM NaCl, 0.05% (vol/vol) Tween-20, 20 mM Tris-HCl, pH 7.4.

4. Chromatography Materials.

Sources of chromatography materials were as follows:

Affi-Gel 10: Bio-Rad Laboratories, Richmond, CA, U.S.A.

Carboxymethyl (CM) Sepharose CL-6B,: Pharmacia Fine Chemicals AB, Uppsala, Sweden.

Diethylaminoethyl (DEAE) cellulose): DE52. Whatman, Kent, U.K.

Dyematrex gels: Amicon Corp., Lexington, MA, U.S.A.

Hydroxylapatite, Bio-Gel HTP (DNA Grade): Bio-Rad.

PBE94 Polybuffer exchanger for chromatofocusing: Pharmacia.

Phenyl-Sepharose 4B: Pharmacia.

Sephacryl S-200 Superfine: Pharmacia.

Sepharose CL-4B: Pharmacia.

Sepharose CL-6B (epoxy-activated): Pharmacia.

Thiol-Sepharose 4B (activated): Pharmacia.

B] METHODS

1. Bacterial Culture.

a. *Streptococcus pneumoniae.*

Stock cultures were grown at 37°C in serum broth to A_{600} about 0.4, then snap-frozen in a dry-ice/isopropanol bath and stored at -80°C. When required, pneumococci were subcultured from stocks onto blood-agar plates and incubated aerobically at 37°C for 16 h. Typically, pneumococcal plates were prepared in batches of six. The bacteria from each plate were used to seed 1 litre of TSB^{++} which was incubated in a capped 1-litre bottle without shaking at 37°C for 6 - 8 h. Each starter culture was then added to a further 8 litres of TSB^{++} in a 10-litre glass bottle, and these larger bottles were incubated at 37°C for 16 h.

b. *Escherichia coli.*

Stock cultures were grown in LB broth containing 50 ug/ml ampicillin (for the maintenance of plasmids carrying the resistance marker for this antibiotic) at 37°C for 16 h in a shaking incubator. Glycerol was added to a final 15% (vol/vol), then the cultures were snap-frozen in a dry-ice/isopropanol bath and stored at -80°C. When required, *E. coli* were subcultured from stocks onto plates of LB plus ampicillin (50 ug/ml), and incubated aerobically at 37°C for 16 h. Typically, inocula were then used to seed two 1-litre flasks each containing 250 ml of TSB^{++} plus ampicillin. These starter cultures were incubated at 37°C for 6 - 8 h in a shaking incubator, then each was added to 8.75 litres of TSB^{++} containing 200 ul

Antifoam A Emulsion, in a 10-litre bottle. The large bottles were then incubated at 37°C with aeration for 16 h.

2. Fractionation and Analysis.

a. Column chromatography.

Specific conditions are described in the relevant Chapters of the text. Generally, during fractionation of proteins by column chromatography, the A_{280} of the eluant was monitored continuously using an Isco model 226 absorbance monitor and plotted by a Houston Instruments Omniscribe chart recorder. Fractions were collected by an Isco model 328 fraction collector whose event marker, operating through the optical monitor, permitted correlation of fractions with the absorbance trace.

b. Analytical polyacrylamide gel electrophoresis.

Discontinuous polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulphate (SDS) was carried out as described by Laemmli (1970), except that slab gels were used instead of tube gels. PAGE in the absence of SDS was carried out in identical manner except that SDS was omitted from all buffers and protein samples were not boiled before being loaded. A Protean-II gel electrophoresis apparatus (Bio-Rad) was used. Analytical slab gels consisted of a separating gel, 15 cm wide and 11 cm long, of either 10% (wt/vol) or 12.5% (wt/vol) acrylamide, topped by a stacking gel, 15 cm wide and 2.5 cm long, of 3% (wt/vol) acrylamide, and were 1.5 mm thick. After electrophoresis, gels were stained with Coomassie brilliant blue R250.

c. Preparative polyacrylamide gel electrophoresis.

Preparative slab polyacrylamide gel electrophoresis was carried out using the Protean-II apparatus equipped with an electroelution attachment. Preparative gels were 3 mm thick. The top layer was a stacking gel, of 3% (wt/vol) acrylamide, 15 cm wide and 2.5 cm long. Beneath this was a separating gel of 10% (wt/vol) acrylamide, 15 cm wide and 8 cm long. Beneath the separating gel was a horizontal channel 2 mm deep. The bottom 3 cm of the gel was sealed with a plug gel of 20% (wt/vol) acrylamide. Glycerol (10% vol/vol) and bromophenol blue (0.005% wt/vol) were added to the sample, up to 4 ml of which could be loaded onto each gel. The sample was subjected to electrophoresis at 100 V for 20 h at 4°C while elution buffer (375 mM Tris-HCl, pH 8.8) was pumped through the horizontal channel at a rate of 100 ml/h. Sample elution was monitored and fractions collected as described for column chromatography (see (a) above).

d. Western blot analysis.

Proteins were electrophoretically transferred from SDS-polyacrylamide gels onto nitrocellulose filters as described by Towbin *et al.* (1979), at 250 mA for 2 h using a BioRad Transblot apparatus fitted with a water-cooled coil. The transblot buffer was 25 mM Tris-HCl, 200 mM glycine, 5% (vol/vol) methanol, pH 8.3. A "Rainbow Marker" mix was used to provide molecular weight standards. This is a mix of known proteins each of which had been distinctively coloured by conjugation to a dye; after transfer to nitrocellulose the standard proteins are visible without the necessity for staining. Buffers used in subsequent procedures were: 20 mM Tris-HCl, 150 mM NaCl, pH 7.4 (TBS); and TBS containing 0.05% (vol/vol) Tween-20 (TTBS). After the transfer of proteins, each filter was agitated gently for 20 min at room temperature in 200 ml TTBS containing 5% (wt/vol) instant skim milk

powder. After a brief rinse with TBS, each filter was incubated overnight with gentle shaking at room temperature in 50 ml TTBS containing 0.02% (wt/vol) skim milk, 50 ul antiserum or control serum (as appropriate) from either rabbit or mouse, and 1 ml concentrated *E. coli* lysate. The next morning the solution was discarded and each filter washed for 30 min at room temperature through 3 changes of 100 ml TTBS. Goat anti-rabbit or anti-mouse IgG (as appropriate) conjugated to horseradish peroxidase was added (50 ul conjugate in 100 ml TTBS, 0.02% (wt/vol) skim milk) and the filters were agitated gently at room temperature for 60 min. The filters were then each washed through 4 changes of 100 ml TTBS, then 2 changes of 100 ml TBS at room temperature over 30 min. Enzyme-labelled bands were visualized by the addition of HRP Colour Development Reagent (9.9 mg first dissolved in 3.3 ml cold (-15^{o}C) methanol and then taken up in 20 ml TBS) and 50 ul hydrogen peroxide (30% wt/vol).

e. Counter-current immunoelectrophoresis.

Counter-current immunoelectrophoresis for the detection of capsular polysaccharide was carried out as described by El-Refaie and Dulake (1975) using gels consisting of 1% (wt/vol) agarose in 5 mM diethylbarbituric acid, 50 mM sodium diethylbarbiturate, 80 mM sodium acetate, pH 8.6.

f. Immunodiffusion gel analysis.

Immunodiffusion gel analysis was carried out essentially as described by Ouchterlony (1949) using gels having the composition given in (e) above.

g. Sample concentration and storage.

Protein fractions pooled after column chromatography or preparative polyacrylamide gel electrophoresis were neutralized (if appropriate), then concentrated on ice under N_2 pressure in an Amicon stirred cell apparatus (200 ml capacity) fitted with a YM10 membrane (10,000 MW retention).

Protein solutions which were not to be further fractionated immediately were stored in 50% (vol/vol) glycerol at -15°C until just prior to use when the glycerol was removed and the buffer adjusted by diafiltration through the Amicon stirred-cell concentrator.

h. Estimation of protein.

Protein concentrations were estimated by the method of Bradford (1976), which is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when the dye binds to protein. Bio-Rad Protein Assay Solution was used, and the protein standard was bovine serum albumin.

i. Estimation of polysaccharide.

The anthrone hexose assay method for estimation of polysaccharide was essentially that of Trevelyan and Harrison (1952). Anthrone reagent (0.2% (wt/vol) anthrone, 1.4 M NaCl, 72% (vol/vol) conc. sulphuric acid) was prepared immediately before use. One ml of anthrone reagent was added to 200 ul of each fraction to be assayed, and the mixture was heated to 100°C for 10 min, then cooled on ice. The A_{620} of samples was then determined using a Pye Unicam Spectrophotometer, and polysaccharide concentrations

estimated by reference to A_{620} readings which had been obtained from standard samples containing known amounts of the appropriate polysaccharide.

3. Activity Assays.

a. Pneumolysin.

i. Plate assay

This method was used for the semiquantitative determination of pneumolysin activity in fractions collected from chromatography columns and preparative polyacrylamide gels. Samples for assay were first activated for 5 minutes at room temperature in the presence of 30 mM 2-mercaptoethanol. A 50 ul portion of each sample was then diluted serially in PBS along one row of 12 wells in a 96-well microtitre plate. A 50 ul amount of a 1% (vol/vol) suspension of packed, washed human group O erythrocytes in PBS was added to each well, and the plates were incubated at 37°C for 30 min and then centrifuged. The highest dilution of each sample resulting in at least 50% haemolysis was then estimated visually.

ii. Tube assay

Samples were activated by the addition of 2-mercaptoethanol, then serial twofold dilutions were prepared with PBS. A 1 ml amount of each dilution was mixed with an equal volume of a 1% (vol/vol) suspension of human erythrocytes in PBS. Tubes were incubated at 37°C for 30 min and centrifuged at 3,000 x *g* for 5 min, and the absorbance at 540 nm was measured. The percentage of erythrocytes lysed was plotted against dilution for each sample, and the pneumolysin activity (expressed as haemolytic

units (HU) per ml) of the sample was defined as the reciprocal of the estimated dilution at which 50% of the erythrocytes would have lysed.

b. Neuraminidase: fluorescence assay.

Neuraminidase activity was measured using a fluorimetric assay adapted from that of Hoogeveen *et al.* (1980). The fluorigenic substrate was 2'-(4-methyl-umbelliferyl)-α-D-N-acetylneuraminic acid (MUAN) which was prepared as a 0.03% (wt/vol) stock solution and stored in small aliquots at -15°C. 10 ul of substrate solution was added to 10 ul of enzyme sample (appropriately diluted in PBS) and incubated for 5 min at 37°C. The reaction was stopped by the addition of 2 ml of 0.5 M sodium carbonate buffer (pH 10.5). Neuraminidase activity was determined by measuring the amount of 4-methylumbelliferone (MU) which had been released. MU was quantitated by use of a Perkin Elmer spectrofluorimeter (excitation wavelength 366nm, emission wavelength 446nm) which had been calibrated with standards of known MU concentration. A unit of enzyme activity was defined as that amount which released one micromole of MU from MUAN in one minute at 37°C. Previous workers (e.g. Tanenbaum *et al.*, 1970; Stahl and O'Toole, 1972) have usually expressed the activity of neuraminidase samples in terms of the amount of N-acetyl-neuraminic acid (NANA) liberated by the enzyme from a sialomucoid substrate, with free NANA quantitated by a colorimetric method such as that of Warren (1959). Under the assay conditions given above, an amount of pneumococcal neuraminidase which liberates one micromole of NANA from an excess of bovine submaxillary mucin in one minute will release about 20 micromoles of MU per minute from an excess of MUAN.

The fluorimetric assay of neuraminidase was not affected by the presence of 1mM PMSF and 10mM Na_2EDTA.

c. Autolysin.

i. Preparation of autolysin substrate

The preparation of pneumococcal cell wall containing tritiated choline was based upon the method of Holtje and Tomasz (1976). Pneumococcal strain 3551 was grown in 100 ml defined medium (see A(3) above) in which unlabelled choline had been replaced by 18.5 MBq (6.25 nmoles) of the labelled compound. At an A_{600} of about 0.4, cells were pelleted by centrifugation at 20,000 x *g* at 4°C for 15 min, washed in PBS, resuspended in 10 ml PBS, and incubated at 75°C for 20 min. After thorough sonication, SDS was added to a final concentration of 2% (wt/vol), and the material was heated to 100°C for 10 min. The material was then centrifuged at 20,000 x *g* for 20 min at room temperature. The pellet (approximately 200 ug) was washed twice with water, and taken up in 2 ml water. In different preparations, between 32,000 and 60,000 dpm were incorporated into each microlitre of this substrate, representing up to about 10% incorporation of label. The substrate was stored at -15°C in small aliquots.

ii. Preparation of unlabelled carrier cell wall

Unlabelled pneumococcal cell wall is a byproduct of the procedure for harvesting pneumococcal cell-associated proteins, which is described in the General Methods section of Chapter 3. Following deoxycholate-induced lysis of the pneumococci concentrated from 54 litres of culture, the cell debris was removed by centrifugation at 20,000 x *g* for 20 min at 4°C. When this debris was to be used as unlabelled carrier, it was resuspended in 250 ml PBS, 2% (wt/vol) SDS, and boiled with stirring for 30 min. After cooling, this material was dialysed for 16 h at 4°C against 5 litres of water, then centrifuged at 30,000 x *g* for 20 min at 4°C. The pellet was resuspended in

20 ml of water and dialysed as before. The dialysate was stored at -15°C in 5 ml aliquots.

iii. Assay

To each 200 ul fraction of material to be assayed (diluted, if appropriate, in PBS) was added 1 ul tritiated substrate (i.e. 30 - 60,000 dpm) and 4 ul 10% (wt/vol) sodium deoxycholate. The assay mix was incubated at 37°C for 15 min and the reaction was stopped by the addition of 50 ul unlabelled carrier cell wall and 30 ul 37% (wt/wt) formaldehyde. Each assay mix was then centrifuged at 35,000 x *g* for 20 min at 4°C. Two hundred and fifty microlitres of the supernatant was carefully removed from each sample into a plastic vial containing 3 ml "Ready Value" scintillation cocktail, and radioactivity was determined in a liquid scintillation counter.

5. Immunological Methods.

a. Production of antisera.

i. Mice

Purified antigen (generally 200 - 250 ug/ml in PBS) was emulsified with an equal volume of Freund's Complete Adjuvant. Mice, each weighing about 30 g, were injected intraperitoneally with 0.2 ml volumes containing 20 - 25 ug antigen. At 10 - 14 day intervals, mice were given two further injections of antigen in PBS emulsified with Freund's Incomplete Adjuvant. Control mice were subjected to a similar course of injections, but with the antigen omitted. Seven days after the final injection, blood samples were collected by suborbital bleeding.

ii. Rabbit

A Lop-Eared Albino rabbit, three months old, was given a course of injections similar to that for the mice, except that the volume of each injection was 1.5 ml (containing about 500 ug protein) which was administered subcutaneously at 3 sites about the neck or flank. The delay between administration of doses was 4 weeks. Prior to immunization, and again one week after the final injection, the rabbit was bled via the marginal ear vein.

b. Determination of inhibitory-antibody titres.

i. Antipneumolysin

Sera were serially diluted with PBS in microtitre plates. Each well also contained 4 HU of pneumolysin in a final volume of 50 ul. Plates were incubated at 37°C for 15 min to allow serum antibody to bind to the pneumolysin; 50 ul of a 1% erythrocyte suspension was added, and the plates were incubated at 37°C for a further 30 min. Plates were then centrifuged and, for each serum, the highest dilution that inactivated the pneumolysin was determined visually. Antihaemolytic titre was expressed as anti-HU per ml.

ii. Antineuraminidase

For each serum to be tested 50ul of PBS was added to the 8 wells in one column of a 96-well microtiter plate. Fifty microlitres of serum was added to the first well and diluted serially (2-fold) down the column. The plate was pre-warmed at 37°C and then 10 ul of enzyme (0.02 units) was added to each well and incubated at 37°C for 20 min. Ten microlitres of stock MUAN was added to each well and the plate was incubated at 37°C for a further 5

min. The enzymic reaction was stopped by the addition of 200 ul of 0.5 M sodium carbonate (pH 10.5), and the contents of each well removed into 2 ml of sodium carbonate for measurement of its fluorescence as described above. The amount of MU released was plotted against the serum dilution and the anti-neuraminidase titre of each serum was expressed as the reciprocal of the dilution causing 50% inhibition of the enzyme activity.

iii. Antiautolysin

Before the availability of pure autolysin, the inhibitory activity of antisera was assessed against freshly-prepared crude enzyme. An inoculum of pneumococcal strain D39 from a fresh overnight blood-agar plate was used to seed TSB^{++}, and the culture grown to an A_{600} of about 0.55. The cells were pelleted by centrifugation at 20,000 x *g* at 4°C for 15 min, and washed twice into the original volume of PBS. Sodium deoxycholate was added to a final concentration of 0.2% (wt/vol), the cells left to lyse at 37°C for 10 min, then centrifuged at 35,000 x *g* for 15 min at 4°C to pellet the cell debris. The autolytically-active supernatant was dispensed in 200 ul aliquots. Serial twofold dilutions (volume 50 ul) of serum in PBS were added, the samples were incubated at 37°C for 10 min, and then autolysin assays were carried out as previously described.

When pure autolysin became available, the anti-autolytic assay was modified to the following. Autolysin (20 ug/ml in PBS) was dispensed in 10 ul aliquots to each of which was added a known dilution of serum in PBS. After 10 min incubation at 37°C, 185 ul of assay mix (180 ul PBS, 4 ul 10% (wt/vol) sodium deoxycholate, 1 ul tritiated substrate) was added to each sample, and autolysin assays were carried out as previously described. In the absence of antiautolytic activity, the amount of enzyme present in each assay was just sufficient to cause complete solubilization of label. After anti-autolysin assays, radioactivity released from each sample was plotted

against serum dilutions, and anti-autolysin titre expressed as the reciprocal of that dilution estimated to inhibit the release of 50% of the available label.

CHAPTER THREE

PURIFICATION OF PROTEINS FROM STREPTOCOCCUS PNEUMONIAE

A] INTRODUCTION

Pneumolysin, neuraminidase and autolysin are three pneumococcal proteins which may have important roles to play in the processes by which the pneumococcus invades and damages its host. The first concern of the present study was to devise ways by which each of these proteins might be isolated in pure form in milligram quantities, so that they could then be assessed as protective antigens.

This Chapter describes the development of large-scale procedures for the preparation of pure pneumolysin and neuraminidase from cell lysates of *Streptococcus pneumoniae*, and preliminary work on the preparation of autolysin from the same source. The general method for harvesting cell-associated protein from pneumococcal broth cultures is described in Section B of this Chapter (General Methods).

Part 1 of Section C then details the method for the purification of pneumolysin, Part 2 describes the purification of neuraminidase, and Part 3 deals with autolysin.

In the case of autolysin, the preparative method which was finally adopted did not use *S. pneumoniae* as its starting material, but instead a culture of *Escherichia coli* (strain DH1) carrying a recombinant plasmid, pGL80, including the DNA coding sequence for pneumococcal autolysin. The

procedure for preparing autolysin from recombinant *E. coli* is given in Chapter 4, along with details for the preparation of a mutant (inactivated) version of pneumolysin.

In the present Chapter there is a brief assessment of fractionation steps which were tested as possible components of a preparative purification procedure for each protein but were then rejected.

Prior to the present study pneumolysin had been purified by other workers, but this Chapter describes modifications to their published method which both simplify it and increase the yield and the specific activity of the final product.

Pneumococcal neuraminidase had not been purified in undegraded form prior to the work presented here.

B] GENERAL METHODS

1. Harvesting of Pneumococcal Cell-associated Protein.

Stock cultures of *Streptococcus pneumoniae* were maintained in serum broth at -70°C (see General Methods, Chapter 2). An inoculum from this stock was grown on a blood-agar plate for 16 h at 37°C, then subcultured onto six others which were incubated 24 h at 37°C. The bacteria from these plates were used to inoculate six bottles each containing 1 litre of TSB^{++}, and these "starter cultures" were incubated for 8 h at 37°C. Each "starter" was then seeded into 8 litres of TSB^{++} in a 9-litre glass bottle and incubated for 16 h at 37°C. The final A_{600} of the pneumococcal culture was about 1.1.

The 54 litres of pneumococcal culture was concentrated to about 1.5 litres using an Amicon DC10LA ultrafiltration apparatus fitted with an H5MP01-43 hollow-fibre cartridge (0.1 um exclusion) and a water-cooled jacket. The concentrate was collected and the ultrafiltration reservoir washed with 500 ml of 200 mM sodium phosphate, pH 7.0 which was then combined with the concentrate. Sodium deoxycholate (10% wt/vol) was added to a final concentration of 0.2% (wt/vol), and the culture was stirred for 1 h at room temperature. Lysis of the bacterial cells was confirmed by microscopy. The lysate was then centrifuged at 27,000 x *g* for 15 min at 4°C.

The supernatant was stirred, vigorously, at room temperature, and solid ammonium sulphate was added gradually to a final concentration of 60% (wt/vol). The precipitate was collected by centrifugation at 27,000 x *g* for 15 min at 4°C and redissolved in 100 ml 10 mM sodium phosphate, pH 7.0. It

was dialysed at 4°C against three changes of 5 litres of the same buffer over 20 h, and then clarified by centrifugation at 27,000 x *g* at 4°C for 20 min.

Subsequent procedures are described in the relevant Sections below.

2. Note on the Use of the French Pressure Cell.

Following the successful use of the French pressure cell to lyse recombinant *E. coli* (yields of protein were improved substantially over those obtained after other means of lysis: see Chapter 4) the same method was tried for pneumococci. The potential advantage of the French pressure cell lay in its shortening of the time required for the preparative procedure. Following lysis (directly into a low volume of DEAE-cellulose starting buffer) and the removal of cell debris by centrifugation, the ammomium sulphate precipitation step for concentration of the lysate supernatant is not required, nor the subsequent overnight dialysis to remove the ammonium sulphate from the resuspended protein pellet. Overall, there is a saving of about 24 hours.

S. pneumoniae cells were resuspended in 60 ml 50 mM sodium phosphate, pH 7.0, and passed twice through an Aminco French pressure cell at a pressure of about 50 MPa. Microscopic examination then indicated that about 90% of the cells had lysed. The lysate was centrifuged at 27,000 x *g* for 20 min at 4°C, then the supernatant was loaded directly onto a DEAE-cellulose chromatography column. The procedure was not a success. A large amount of a milky suspension, presumably small fragments of cell wall, remained in the preparation even after centrifugation, obscuring the absorbance trace and clogging the ultrafiltration membrane used in the concentration of column

fractions. The French pressure cell was therefore not used further for the routine lysis of pneumococci.

C] METHODS AND RESULTS

1. Pneumolysin.

a. Background.

Pneumolysin was first purified by Shumway and Klebanoff (1971).

Their final pneumolysin preparation had a specific activity of about 550 KHU/mg with a yield of 10% of the activity of the initial crude lysate. The purity of the final product under denaturing conditions was not demonstrated.

In 1983 the present author published an adaptation of Shumway and Klebanoff's method (Paton, Lock and Hansman, 1983). The new method was simpler, eliminating several steps and modifying others. The specific activity of the final product was about the same as that obtained by Shumway and Klebanoff, but the yield was increased to about 15% of the original activity. Also, the final product was shown to be substantially homogeneous on both denaturing and non-denaturing gel systems. The yield of protein, however, was still low. Only about 500 ug of highly purified pneumolysin could be prepared from 18 litres of cell culture.

This Section details the development of further modifications to the method for preparing pneumolysin. The final method described here is simpler again, the purity of the end product is maintained, about 25% of the initial activity is recovered, and the specific activity of the final product is approximately doubled to 1 - 1.2 MHU/mg protein.

b. Assays.

The procedures used for assaying pneumolysin activity are described in full in Chapter 2.

Briefly, activity was determined from the dilution of activated pneumolysin required to lyse a 1 ml amount of a 1% (vol/vol) suspension of washed, packed human erythrocytes after incubation for 30 min in PBS at 37°C.

c. Screening pneumococcal strains for production of pneumolysin.

Pneumolysin is located in the cytoplasm of the pneumococcus and is released only by cell lysis (Johnson, 1977). In order to choose an appropriate strain for large-scale production of pneumolysin, cell lysates from 150 clinical isolates of *S. pneumoniae* grown in TSB^+ were checked for pneumolysin activity by plate assay (see General Methods, Chapter 2). All strains showed some haemolytic activity, but only five yielded activities as high as 256 HU/ml (Figure 3.1). After tube assay (see General Methods, Chapter 2) of these five, a type 1 strain, designated 3551, which produced the greatest amount of the toxin, was selected for further study. This strain had the added advantage that it appeared to be less prone to spontaneous autolysis late in the log phase of growth than the other high producing strains, retaining 85 - 98% of total pneumolysin within the cell.

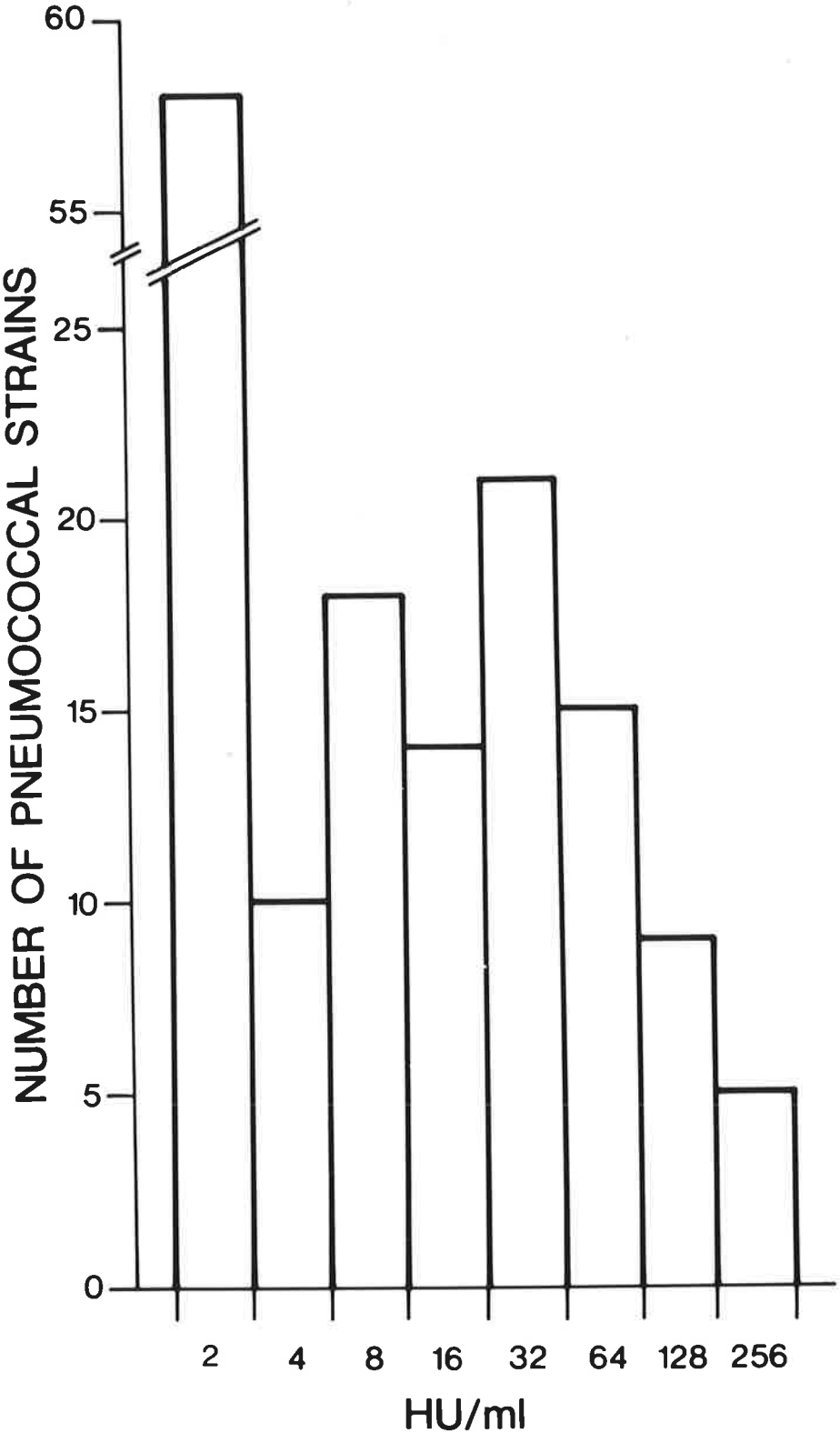
d. Culture media.

Unlike the Brain Heart Infusion Broth used by Shumway and Klebanoff, TSB is a relatively cheap medium containing little high molecular weight

FIGURE 3.1

Pneumolysin production by various pneumococcal strains.

150 clinical isolates of *S. pneumoniae* were grown at 37°C in TSB^+ to an approximate A_{600} of 0.6. Cells were pelleted by centrifugation and resuspended in PBS, and autolysis was induced by the addition of 0.2% (wt/vol) sodium deoxycholate. Lysates were then assayed for haemolytic activity by the plate method (see Chapter 2, General Methods). The horizontal axis of the histogram indicates approximate haemolytic activity per ml cell culture. The vertical axis indicates the number of pneumococcal strains in each class.


60
55
25
20
15
10
5
0
NUMBER OF PNEUMOCOCCAL STRAINS
2
4
8
16
32
64
128
256
HU/ml

protein which might contaminate the cell culture concentrate from which the pneumococcal proteins are purified. Previous experience in this laboratory had shown that, when supplemented with cysteine, Ca^{++} and Mg^{++}, TSB could support substantial growth of the pneumococcus (to $A_{600} \geq 0.9$). In early experiments in the present study, TSB^{+} was the usual culture medium for growing the pneumococci.

Todd-Hewitt Broth was also considered for this purpose.

Total pneumolysin production by strain 3551 was slightly reduced in Todd-Hewitt Broth compared with TSB^{+} (about 205 HU/ml and 250 HU/ml total pneumolysin were produced in the two broths, respectively, after overnight culture to plateau density) but the proportion of pneumolysin which remained associated with the cells was much reduced: only 69% of pneumolysin remained cell-associated in Todd-Hewitt Broth compared with 98% in TSB^{+}.

Despite being substantially the more expensive medium, Todd-Hewitt Broth was judged to have no significant advantage over TSB^{+} for the culture of pneumococci for the preparation of pure pneumolysin; TSB^{+} was therefore retained as the standard medium.

e. Preparation of pneumolysin: initial method.

The initial method used in the present study for the purification of pneumolysin was adapted directly from that of Shumway and Klebanoff (1971).

Briefly, their method was:

1. Pneumococci grown in Brain Heart Infusion Broth supplemented with glucose;

2. Cells pelleted by centrifugation;
3. Cells resuspended, disrupted by sonication;
4. Sonicate centrifuged to pellet cell debris;
5. Protein precipitated from the supernatant by the addition of acetic acid;
6. Pellet washed, recentrifuged;
7. Protein precipitated from the supernatant by the addition of ammonium sulphate;
8. Pellet redissolved, dialysed;
9. Protein fractionated by DEAE-cellulose chromatography;
10. Lytic fractions concentrated by ultrafiltration;
11. Protein fractionated by Sephadex G-100 chromatography;
12. Lytic fractions concentrated by ultrafiltration;
13. Protein fractionated by preparative discontinuous tube gel PAGE;
14. Pneumolysin concentrated by ultrafiltration, stored.

Modifications to this method were made with the following considerations in mind:

Firstly, the disruption of pneumococcal cells may be achieved simply by the addition of a small amount of a detergent such as sodium deoxycholate at room temperature, without the need for sonication; secondly, the acetic acid precipitation step in the Shumway and Klebanoff method does not appear to be necessary; and, thirdly, preparative PAGE of milligram quantities of protein may be carried out more conveniently using a slab gel rather than by tube gels.

These considerations led to the development of a modified method for the purification of pneumolysin. The details of this method have previously

been published by the present author, in collaboration (Paton *et al.*, 1983).

In summary, the method is this:

1. Pneumococcal strain 3551 grown in 18 litre batches in TSB^+;
2. Cells harvested by centrifugation;
3. Cells lysed by the addition of 0.2% (wt/vol) sodium deoxycholate;
4. Cell debris removed by centrifugation;
5. Protein precipitated from lysate supernatant by the addition of ammonium sulphate;
6. Pellet redissolved, dialysed;
7. Protein fractionated by DEAE-cellulose chromatography (1.6 x 20 cm column);
8. Fractions with haemolytic activities greater than 1 KHU/ml pooled, concentrated by ultrafiltration;
9. The concentrate fractionated by Sephacryl S-200 chromatography (2.6 x 100 cm column);
10. Fractions with haemolytic activities greater than 1 KHU/ml pooled, concentrated by ultrafiltration;
11. Protein fractionated by preparative discontinuous polyacrylamide slab gel electrophoresis;
12. Pneumolysin concentrated, washed, stored.

The purity of the material at various stages in the procedure was monitored by PAGE.

As previously mentioned, the final product appeared to be essentially homogeneous when analysed by PAGE either in the presence or absence of SDS,

and the specific activity of the final product was comparable to that obtained by Shumway and Klebanoff. However, although the yield was higher than that from their method (15% of original activity compared with 10%), still only about 500 ug of highly purified pneumolysin was obtained from 18 litres of cell culture.

The main problem seemed to be the final purification step - preparative PAGE. While being highly effective at removing trace protein contaminants, preparative PAGE often brought about a drop in the specific activity of the pneumolysin recovered, probably because of the effects of trace contamination of the sample by unpolymerized acrylamide from the gel. More importantly, however, about two thirds of the pneumolysin loaded onto each preparative gel was not recovered. During electrophoretic concentration in the stacking gel much pneumolysin formed aggregates too large to penetrate the separating gel. After electrophoresis, this material could be visualised by Coomassie staining as an intense band at the interface between the two gel phases.

Clearly, the method devised in 1983 needed re-examination.

f. Investigation of various purification procedures.

During the development of the final method (which is described in (g) below) for purifying pneumolysin, a number of procedures, which fractionate proteins according to a variety of their physical and chemical properties, were investigated. The results are summarized below. (No Figures are presented).

i. Chromatofocusing

Chromatofocusing is a purification technique whereby a pH gradient is applied to a column of ion exchange resin causing proteins on the column to elute in order of their isoelectric points (pI). Focusing effects which take place during elution result in sample concentration and band sharpening, leading to high resolution.

The pI of pneumolysin had previously been estimated at pH 4.9 (Kreger and Bernheimer, 1969).

Pneumolysin, which had been partially purified by DEAE-cellulose chromatography, was subjected to chromatofocusing on a 1.3 x 40 cm column of Pharmacia PBE94 gel using Polybuffer 74 - HCl to generate a linear 400 ml gradient across the pH range 7.4 - 4.0. After neutralization, fractions were assayed for haemolytic activity which was detected as a peak eluting at pH 4.9.

Some purification was achieved, but such large proportions of the haemolytic activity and the pneumolysin protein were lost that chromatofocusing could not be considered appropriate as part of a preparative method.

ii. Dye-ligand chromatography

Dye-ligand chromatography is a variant of affinity chromatography in which the ligands immobilized on the inert gel support are synthetic dyes rather than naturally occuring molecules with a known affinity for the material which is to be purified. Various dyes able to serve as high capacity, group-selective ligands have been synthesized, but theoretical understanding of their affinities has not yet advanced sufficiently to

allow the prediction of which ligands, if any, will best serve a particular purpose.

Pneumolysin which had been partially purified by passage through DEAE-cellulose was applied to six small test columns each containing a small amount of Amicon Matrex gel. One gel was a "control" consisting of the cross-linked agarose support matrix only. The other five were agarose gels each carrying a covalently-bound Matrex dye, either Blue-A, Blue-B, Red-A, Orange-A or Green-A. The conditions for loading and elution of protein were those suggested by the manufacturers.

Pneumolysin in 80 mM sodium phosphate buffer, pH 7.0, failed to bind to Blue-A, Blue-B, Red-A, Orange-A or Green-A Dyematrex gels, and no significant purification was achieved by passage through these gels.

iii. Hydrophobic interaction chromatography

In hydrophobic interaction chromatography using Pharmacia phenyl-Sepharose Cl-4B the affinity ligands are phenyl groups immobilized on a cross-linked agarose support. The immobilized hydrophobic groups have affinity for the hydrophobic regions of proteins, so that proteins passing through a column of phenyl-Sepharose are fractionated on the basis of differences in their hydrophobicity.

The initial binding of the protein to the gel takes place in a buffer containing a chaotropic salt (normally ammonium sulphate) which is present in high enough concentration to sequester a large proportion of the water molecules from the region surrounding the immobilized ligands and so minimize hydrophilic disruption of the hydrophobic bonding between protein

and the gel. The elution of bound proteins is then achieved by lowering the salt concentration.

In the present case, firstly, it was shown that pneumolysin was soluble at 2 M ammonium sulphate, and that its activity could be recovered by dialysis back into PBS.

At an ammonium sulphate concentration of 1.5 M, pneumolysin bound readily to the column, but then proved difficult to dislodge. Even 50 mM Tris-HCl, pH 7.4 in the complete absence of ammonium sulphate failed to elute it. A wipe with 1 M Tris base was necessary; after neutralization, 27% of the activity loaded was recovered.

When pneumolysin was loaded at 150 mM ammonium sulphate, 50 mM Tris-HCl, pH 8.2, it bound and could be eluted with 50 mM Tris-HCl, pH 9.2.

Pneumolysin which had been partially purified by passage through DEAE-cellulose was loaded onto the column in the presence of 150 mM ammonium sulphate, 50 mM Tris-HCl, pH 8.2 and eluted with a pH gradient, pH 8.2 - 9.2, of 50 mM Tris-HCl.

About 70% of activity was recovered, and some degree of purification was achieved, but not enough to justify the use of hydrophobic interaction chromatography for the purification of pneumolysin on a routine basis.

iv. Affinity chromatography through activated thiol gel

Affinity chromatography of protein on thiol-gel involves the selective coupling of protein thiol groups to thiol groups immobilized on the support matrix, with the consequent formation of mixed disulphide bonds. The coupling reaction is reversible, and the thiol-containing protein can be

eluted (after the washing away of unbound material) by reduction of the disulphide bond.

Prior to the present study, thiol-gel affinity chromatography had been used effectively for the purification of streptolysin-O, a thiol-activated toxin related to pneumolysin (Prigent *et al.*, 1978).

In the present study, some purification of pneumolysin (post-DEAE) was achieved by the elution of the toxin from a column of activated thiol-Sepharose 4B by the application of a linear 10 - 500 mM gradient of buffered 2-mercaptoethanol. However, the degree of purification was no better than that achieved by other techniques, and the unpleasantness of working with buffers containing high concentrations of reducing agent did not recommend thiol-gel affinity chromatography as a method for routine use.

g. Preparation of pneumolysin: final method.

i. Growth medium

In parallel with unsuccessful attempts to employ various alternative protein fractionation techniques in the purification of pneumolysin (see above), investigations were made into the possibility that pneumococcal growth (and hence production of pneumolysin, which is expressed constitutively) in TSB^+ might be enhanced by further supplementation of that medium.

The buffering capacity of the medium was increased by the inclusion of either sodium carbonate or sodium phosphate. In case the availability of a carbon source was limiting to growth, glucose was added. Also tested was the effect of adding a mixture of seven common growth-medium cofactors.

The results, summarized in Table 3.1, suggested that pneumolysin production could be improved significantly by the addition of 50 mM sodium phosphate, pH 7.4 to the medium in the absence of extra glucose or cofactors. Phosphate-supplemented medium (TSB^{++}) was used in the final method for pneumolysin preparations and for all subsequent pneumococcal protein preparations.

ii. Protein purification

Details of the purification of pneumolysin are shown in Figure 3.2.

Crude pneumococcal cell protein from 54 litres of culture (see Section B: General Methods) was applied to a column (4.4 x 60 cm) of DEAE-cellulose (Whatman DE-52) which had been pre-equilibrated with 10 mM sodium phosphate, pH 7.0. The column was eluted with a linear gradient of 10 - 250 mM sodium phosphate, pH 7.0 at 4°C, and fractions were assayed for haemolytic activity (Figure 3.2a). Fractions with activities greater than 10 KHU/ml were pooled and concentrated to about 10 ml by ultrafiltration. The concentrate was then applied to a column (2.6 x 100 cm) of Sephacryl S-200 which was then eluted with 50 mM sodium phosphate, pH 7.0 at 4°C (Figure 3.2b). Fractions with activities greater than 10 KHU/ml were again pooled and concentrated by ultrafiltration. Active fractions were pooled as before and reapplied to the Sephacryl S-200 column (Figure 3.2c). Fractions from this column with activities greater than 10 KHU/ml were then again pooled and concentrated. Pneumolysin prepared in this way could be stored in 50% (vol/vol) glycerol at -15°C for at least 12 months without significant loss of activity.

TABLE 3.1

Effect of additives to TSB+ on pneumolysin production by pneumococcal strain 3551.

		HU per ml		
Additives	Final pH	S/N	Pellet	Total
None	4.80	6	254	260
Phosphate	5.15	17	417	434
Carbonate	4.90	6	242	248
Glucose	4.88	6	213	219
Cofactors	4.85	6	255	261
Phosphate + glucose	5.08	7	400	407
Carbonate + glucose	4.87	6	206	212
Phosphate + cofactors	5.15	12	375	387

Strain 3551 was grown in TSB+ plus additives to approximately maximum culture density.

Phosphate = 50 mM sodium phosphate, pH 7.4;

Carbonate = 50 mM sodium carbonate, pH 7.4;

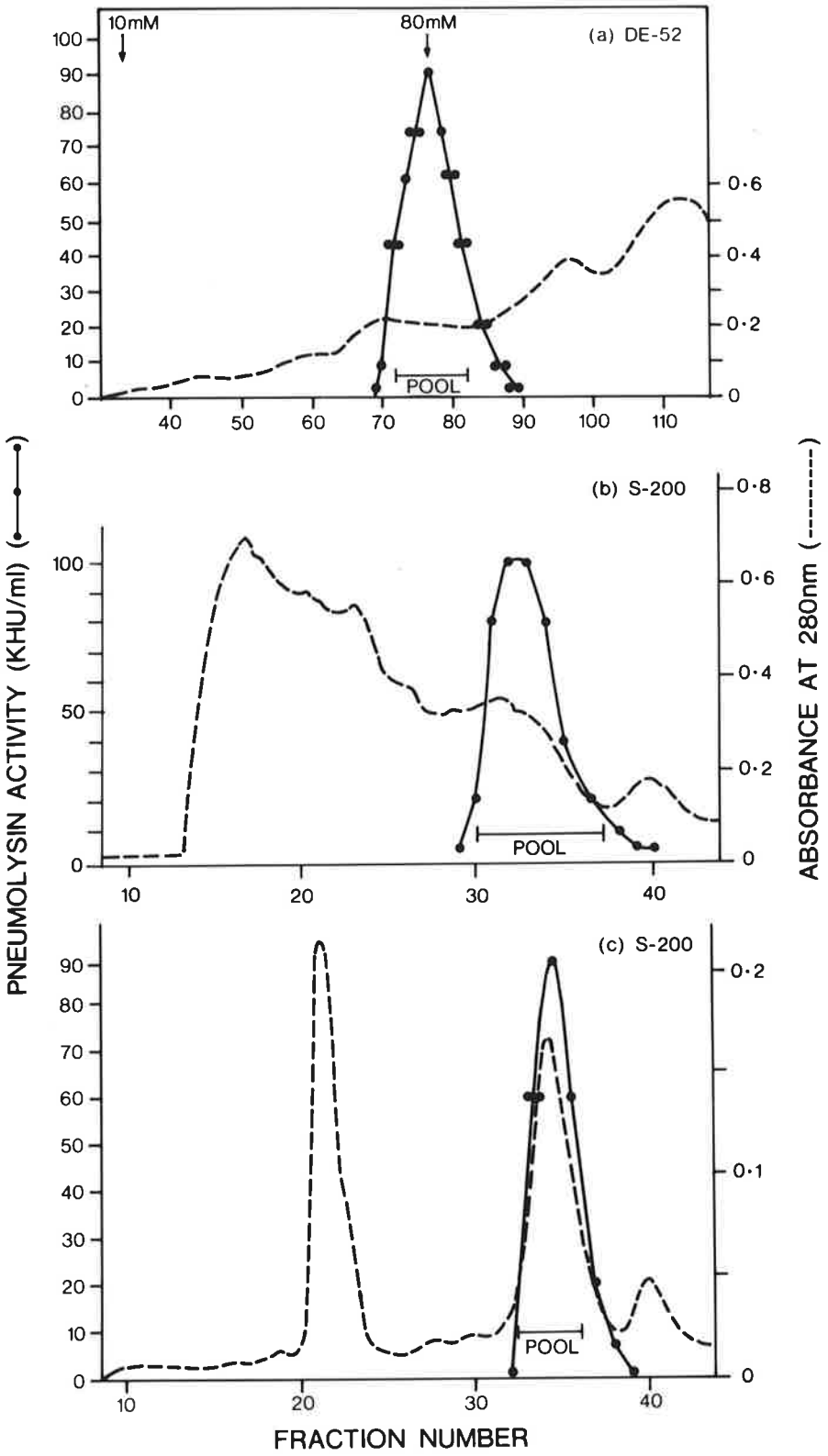
Glucose was added to 1 g/l;

Cofactors = 1/100 vol of 100 X Cofactor Stock, as defined in Chapter 2, General Methods.

Figure 3.2

Chromatographic purification of pneumolysin (final method).

(a) DEAE-cellulose; (b) Sephacryl S-200 (first run); (c) Sephacryl S-200 (second run). Vertical arrows indicate relevant concentrations of sodium phosphate, pH 7.0. Horizontal bars indicate those fractions which were pooled prior to the next step. Haemolytic activity of fractions was determined by the plate assay method (see Chapter 2, General Methods).


10mM
80mM
(a) DE-52
POOL
(b) S-200
POOL
(c) S-200
POOL
PNEUMOLYSIN ACTIVITY (KHU/ml) (——●——)
ABSORBANCE AT 280nm (--------)
FRACTION NUMBER

h. Purity and yield.

Yields and specific activities of pneumolysin samples from various stages of the purification procedure are given in Table 3.2.

When samples were analysed by SDS-PAGE (Figure 3.3), the final preparation migrated as a single major protein species accounting for greater than 97% of the total protein (as determined from densitometer scans of stained gels). Comparison of the mobility of this major species with that of molecular weight markers on SDS gels indicated that it had an apparent molecular weight of 52,000. No capsular polysaccharide material could be detected in the final preparation by counter-current immunoelectrophoresis (see Chapter 2, General Methods). The final pneumolysin preparation had a specific activity of 1 - 1.2 MHU/mg of protein which represented a net purification of at least 500 fold compared with crude cell lysate. Approximately 25% of the original activity was generally recovered with a final yield of about 4 - 5 mg of highly purified pneumolysin per 54 litres of cell culture.

i. Discussion.

Whereas, in the present author's previously published procedure for the purification of pneumolysin, 18 litres of cell culture could be made to yield about 500 ug of highly purified pneumolysin having a specific activity of about 500 KHU/mg protein, the procedure described in this Chapter yielded, per 54 litres of cell culture, 4 - 5 mg pneumolysin of comparable purity having a specific activity of at least 1 MHU/mg protein.

The improvement in yield in the later procedure is probably due in part to the supplementation of the growth medium with sodium phosphate allowing

TABLE 3.2

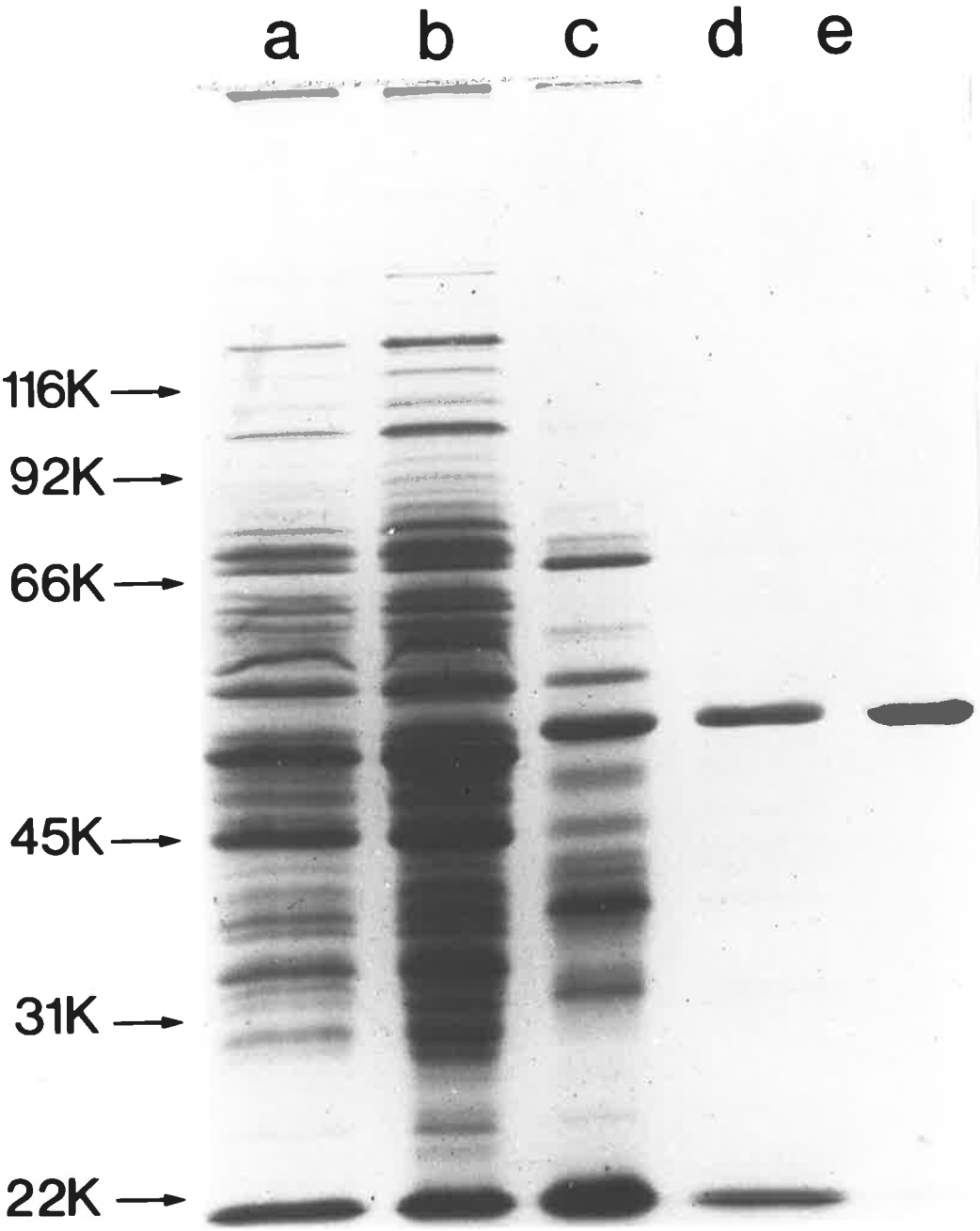
Yields and specific activities of haemolytic fractions during the preparation of pneumolysin by the final method.

	Total activity (KHU)	Specific activity (KHU/mg protein)
crude cell lysate	21,950	2.2
post - ammonium sulphate	16,200	2.6
post - DEAE	10,900	190
post - Sephacryl S-200 (1)	7,200	690
post - Sephacryl S-200 (2)	5,290	1150

Figure 3.3

SDS-PAGE (10% gel) of samples from various stages in the final method for the purification of pneumolysin.

(a) crude cell lysate (40 ug); (b) post - ammonium sulphate precipitation (40 ug); (c) post - DEAE-cellulose chromatography (25 ug); (d) post - first step Sephacryl S-200 chromatography (5 ug); (e) post - second step Sephacryl S-200 chromatography (5 ug). Protein bands were visualised by staining with Coomassie brilliant blue R250. The mobilities of various molecular weight markers are indicated.


a
b
c
d
e
116K
92K
66K
45K
31K
22K

growth of the culture to higher cell density. Also, in the later procedure, cells were harvested by ultrafiltration rather than centrifugation. Since ultrafiltration was by far the faster procedure for concentrating large volumes of the cells, it might be expected to reduce the loss of cell contents into the supernatant which occurs due to autolysis during concentration. However, the most important factor was probably the omission of the final (preparative PAGE) step from the earlier method. Preparative PAGE, while highly effective at removing trace contaminants, also entailed the loss of much protein because, during electrophoretic concentration in the stacking gel, some pneumolysin formed aggregates too large to penetrate the separating gel.

In the final method, the purity of the end product was maintained despite the omission of preparative PAGE. This was achieved partly by using a DEAE column with a much higher capacity resulting in superior fractionation at the critical first chromatographic step, and partly by including an additional column chromatography step using Sephacryl S-200. In addition, fractions generated by column chromatography were chosen with greater selectivity: the haemolytic activity required for inclusion was 10 KHU/ml rather than 1 KHU/ml.

The higher specific activity of the pneumolysin after the final method described in this Chapter was a result of the omission of the preparative PAGE step. Preparative PAGE caused a substantial drop in the specific activity of pneumolysin, possibly because of the effect of contamination of the final material by residual, unpolymerised acrylamide from the gel.

Shumway and Klebanoff (1971) demonstrated the electrophoretic homogeneity of their final product only under non-denaturing conditions. In the present study, the final product was shown to migrate on polyacrylamide gels essentially as a single band under both non-denaturing and denaturing

(i.e. in the presence of SDS) conditions. SDS-PAGE analysis also permitted the first accurate determination of pneumolysin's molecular weight at 52,000. Previous estimates had been 63,000 (Shumway and Klebanoff, 1971) and 66,000 (Kreger and Bernheimer, 1969).

(Recently the cloning and sequencing of the pneumolysin gene has allowed the precise determination of its molecular weight at 52,800: Walker *et al.*, 1987).

The isoelectric point of the final product was 4.9, as previously reported for pneumolysin (Kreger and Bernheimer, 1969).

The yield of pneumolysin was 25% of the original activity (compared with 10% by the method of Shumway and Klebanoff) and the final specific activity about 1 MHU/mg protein (compared with 550 KHU/mg).

Using the method detailed here, a single batch of pneumococcal cells could be made to yield 4 - 5 mg pure pneumolysin, an amount permitting detailed investigation of the effectiveness of the protein as a protective immunogen in mice (see Chapter 5).

Since completion of the work presented here, Kanclerski and Mollby (1987) have reported a method for the purification of pneumolysin involving sequential chromatography of pneumococcal extract through DEAE-Sepharose, thiopropyl-Sepharose and Sephacryl S-200 - i.e. their method is similar to that described here except for the inclusion of a thiol-gel step (using thiopropyl-Sepharose 6B rather than thiol-Sepharose 4B, which was tested and rejected as part of the present study).

Since the publication of Kanclerski and Mollby's method, there has been some difficulty in reproducing its results (R. Mollby, personal communication).

2. Neuraminidase.

a. Background.

Previous workers, finding pneumococcal neuraminidase activity in their preparations apparently associated with a number of molecular species, suggested that neuraminidase exists as a group of isoenzymes with molecular weights of approximately 70,000 (Tanenbaum *et al.*, 1970; Tanenbaum and Sun, 1971; Stahl and O'Toole, 1972). These proteins proved difficult to separate, and since, prior to the present study, no subfraction of pneumococcal neuraminidase had been shown to be pure as judged by SDS-PAGE, the relationship between the different forms or components of the enzyme was difficult to determine.

There was also some uncertainty as to whether pneumococcal neuraminidase is a cell-associated or a secreted protein, with some workers choosing to study enzyme from the pneumococcal culture supernatant (Hughes and Jeanloz, 1964; Lee and Howe, 1966; Tanenbaum *et al.*, 1970; Tanenbaum and Sun, 1971; Stahl and O'Toole, 1972; Glasgow *et al.*, 1977) and some choosing to study enzyme that remained cell-associated (Kelly *et al.*, 1967; Kelly and Greiff, 1970). In the present study, neuraminidase was eventually purified from strain 3551 in which about 87% of the enzyme activity remains associated with the cell pellet under standard culture conditions.

b. Assays.

i. Neuraminidase assay

In previous studies on neuraminidase the activity of the enzyme has usually been determined by the use of colorimetric assays to measure the

release of N-acetyl neuraminic acid (NANA) from a sialomucoid substrate such as salivary, submaxillary or colostral mucins from any of a number of species.

Aminoff (1961) investigated two such assays: the so-called "thiobarbituric acid" method originated by Warren (1959), and the "alkali-Ehrlich" method. Both were capable of quantitating free NANA to the exclusion of NANA which was still substrate-bound, and both assays, or variants based upon them, have been used frequently by subsequent workers for the determination of neuraminidase activity.

However, all these colorimetric assays for neuraminidase activity share two major disadvantages. Firstly, they are relatively insensitive, and, secondly, they are inconveniently complicated for the routine, simultaneous assay of large numbers of samples (e.g column chromatography fractions).

The assay used in the present study was adapted from that of Hoogeveen *et al.*(1980). It is rapid and convenient and has proved over a thousand times more sensitive than either the thiobarbituric acid or alkali-Ehrlich methods. The basis of the assay is the fluorimetric quantitation of the methylumbelliferone (MU) which is released by neuraminidase from the non-fluorescent substrate 2′-(4-methylumbelliferyl)-α-*D*-N-acetylneuraminic acid (MUAN).

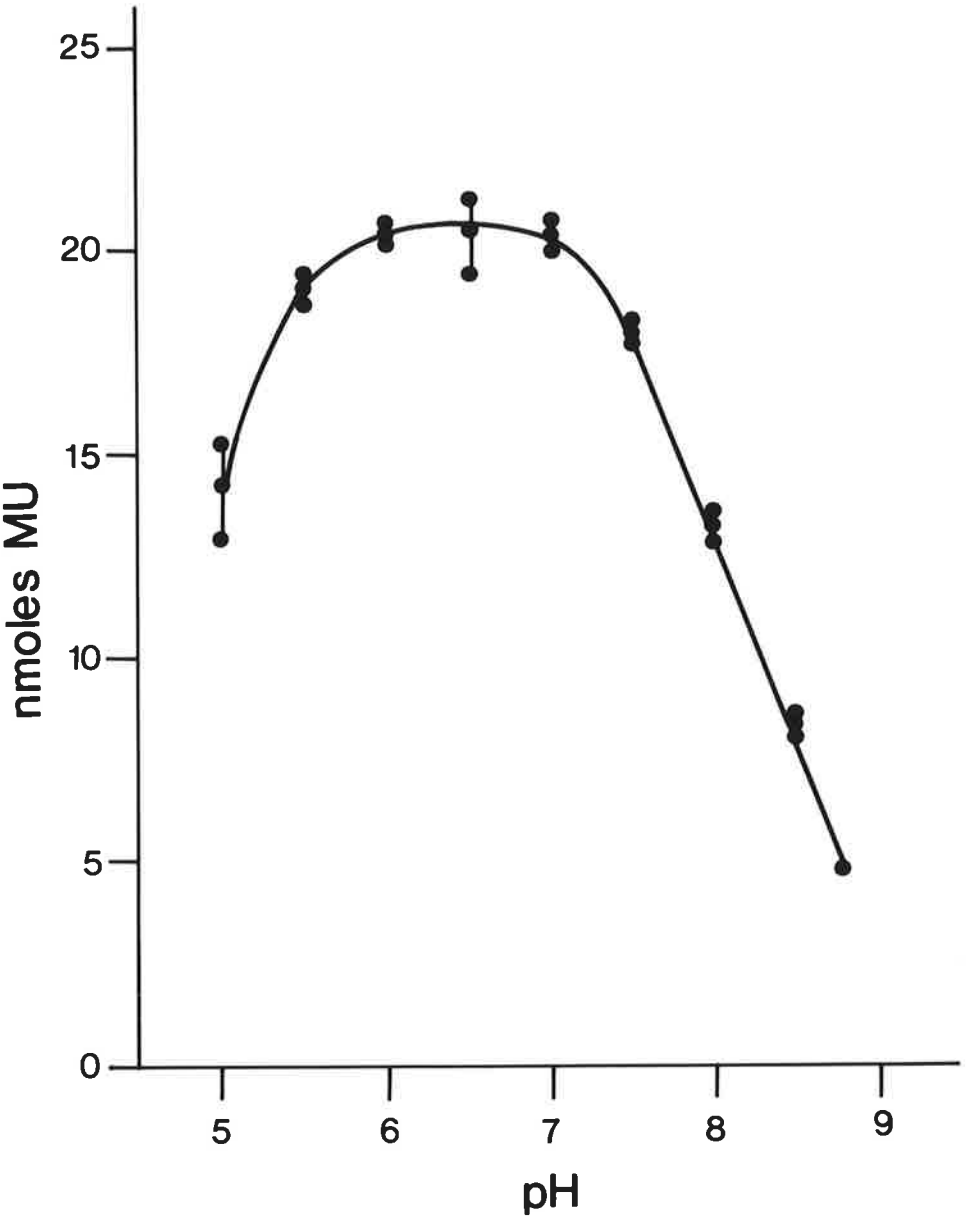
Hoogeveen *et al.* presented their method in outline only. In the present study the assay system was examined for its response to varying pH and ionic strength, as well as to the presence of cations and non-ionic detergent, using crude fractions of neuraminidase containing a mixture of active species with molecular weights between 86,000 and 107,000.

In a system buffered with either Tris-HCl or sodium phosphate the optimal pH for the enzyme was found to be 6.0 - 7.0 (Figure 3.4). This is

Figure 3.4

Effect of pH on the activity of neuraminidase.

Conditions for the fluorimetric assay of neuraminidase are given in Chapter 2, General Methods. The vertical axis indicates nmoles MU liberated from MUAN during the course of each assay (triplicate points). The enzyme used was a crude (post-DEAE) fraction prepared in the absence of protease inhibitors and containing a mixture of active species with molecular weights between 86,000 and 107,000.


25
20
15
10
5
0
nmoles MU
5
6
7
8
9
pH

consistent with previously published data for neuraminidase acting upon more complex biological substrates (Hughes and Jeanloz, 1964; Stahl and O'Toole, 1972).

Cations (Ca^{++} as $CaCl_2$, in concentrations up to 30 mM, and Mg^{++}, as $MgCl_2$, up to 3 mM) caused only marginal improvement in enzyme activity (results not shown). This is also consistent with earlier reports (Tanenbaum *et al.*, 1970).

The enzyme was tolerant to concentrations of NaCl up to at least 1M (Figure 3.5). Triton X-100, at a concentration of 0.1% (vol/vol), increased enzyme activity significantly (Figure 3.6), and this increased activity was maintained at concentrations of Triton up to 1% (vol/vol); (not shown). Tolerance to high ionic strength and activation by Triton X-100 have not previously been reported for pneumococcal neuraminidase.

The standard assay system developed from these results is detailed in Chapter 2, General Methods. Because of the very high sensitivity of the fluorimetric method (which necessitated 10^2 - 10^3 fold or greater dilution of most enzyme fractions in order to obtain results lying within the linear range of the assay) there was no necessity to optimize substrate cleavage, so, for convenience, cations and Triton were generally omitted.

The activity of a neuraminidase sample was estimated from the amount of MU it released after incubation with an excess of substrate for five minutes using a dilution of enzyme such that the rate of MU release was essentially linear over this period of time.

Values of enzyme activity obtained by the fluorimetric method correlated well with values obtained by the alkali-Ehrlich method of Aminoff (1961). One colorimetric unit of enzyme activity (i.e. that required to release one micromole of NANA from an excess of mouse submaxillary mucin in a one min

Figure 3.5

Effect of ionic strength on the activity of neuraminidase.

The vertical axis indicates nmoles MU liberated from MUAN during the course of each assay (triplicate points). The horizontal axis expresses ionic strength in terms of molar concentration of NaCl. Similar results were obtained when the ionic strength was supplied by sodium phosphate, pH 7.0. The enzyme was crude (post- DEAE) neuraminidase prepared in the absence of protease inhibitors.

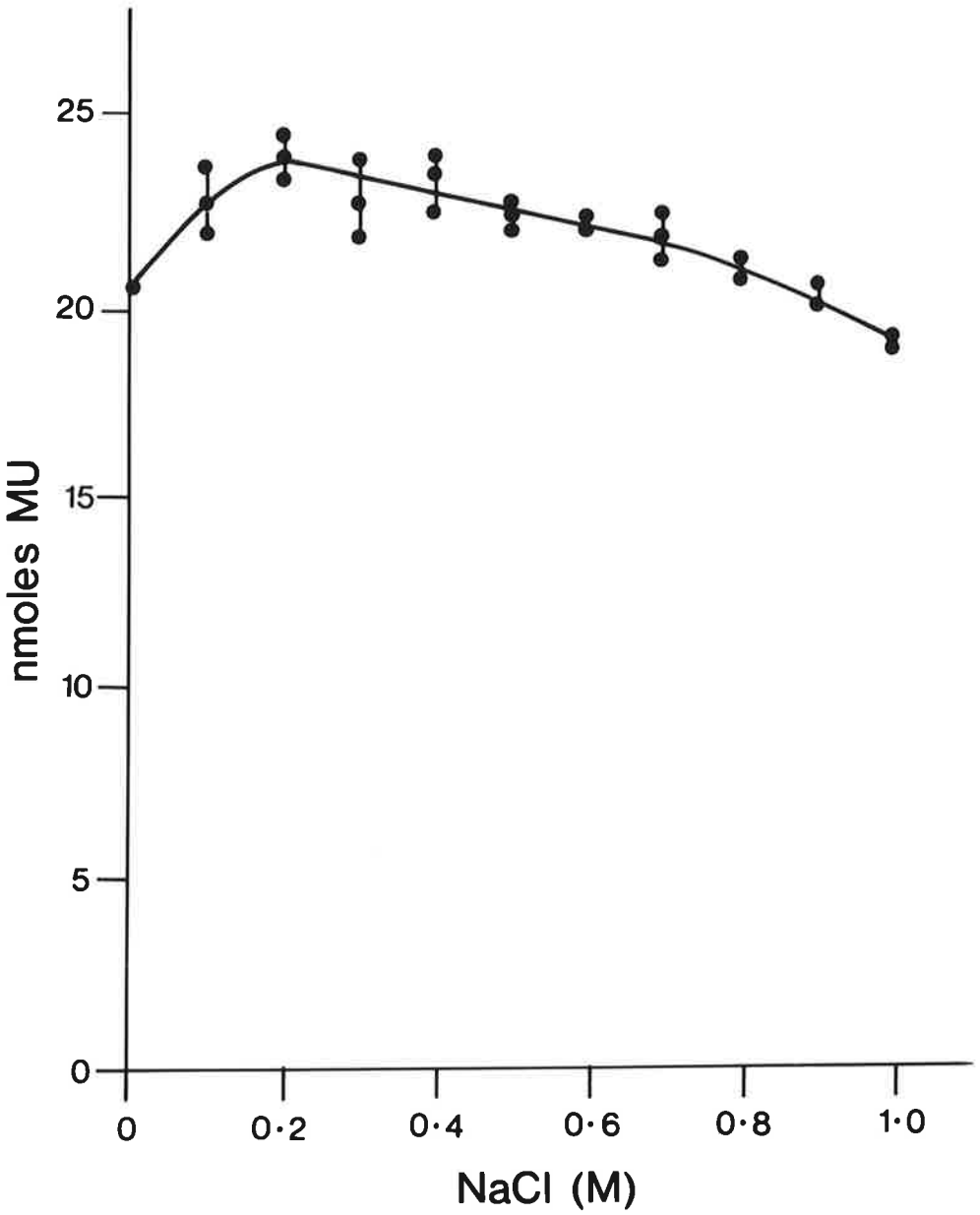

25
20
15
10
5
0
nmoles MU
0
0·2
0·4
0·6
0·8
1·0
NaCl (M)

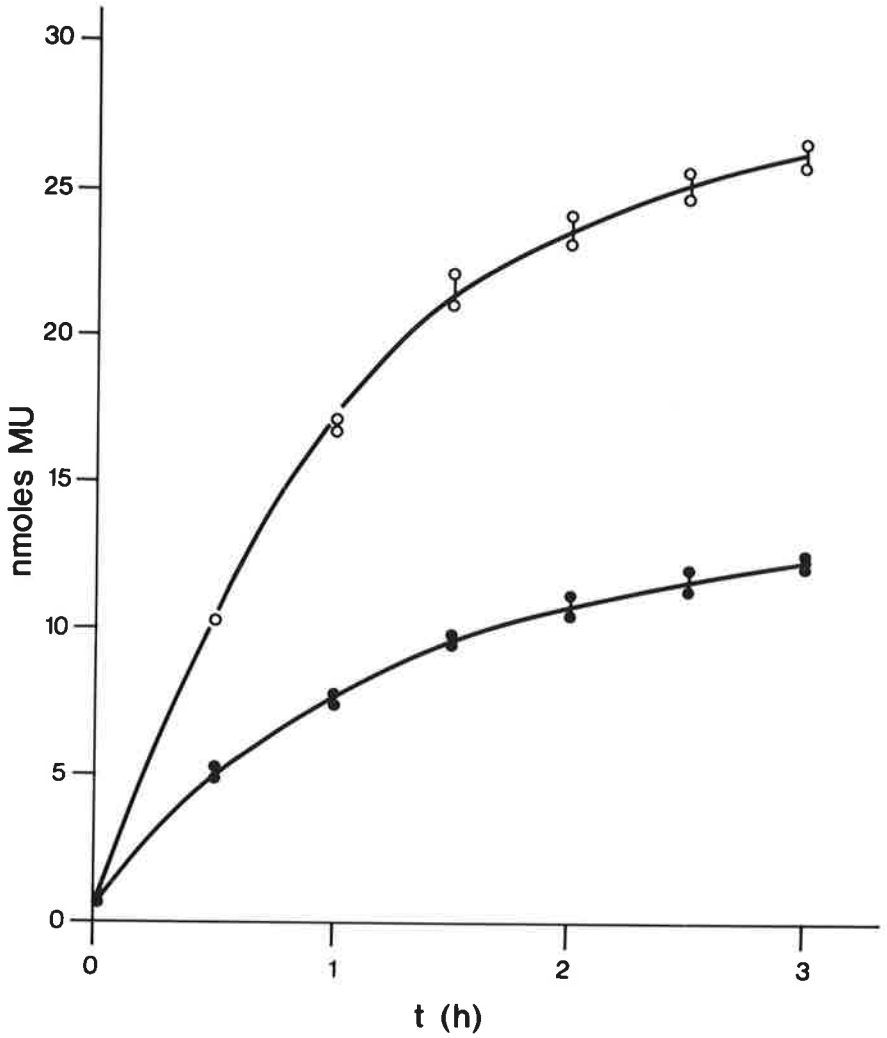
Figure 3.6

Effect of Triton X-100 on the activity of neuraminidase.

The vertical axis indicates nmoles MU liberated from MUAN during the course of each assay (duplicate points). Crude (post- DEAE) neuraminidase was diluted 1/1000 and incubation time was extended up to 3 h.

● : minus Triton X-100

○ : plus Triton X-100, 0.1% (vol/vol)


30
25
20
15
10
5
0
nmoles MU
0
1
2
3
t (h)

incubation) is equivalent to about 20 fluorimetric units (i.e. one micromole of MU released from an excess of MUAN per min) under equivalent assay conditions (salt concentrations, pH and temperature as given for fluorimetric assay in Chapter 2, General Methods).

The fluorimetric assay of neuraminidase was not affected by the presence of 1 mM PMSF and 10 mM Na_2EDTA.

ii. Determination of serum antineuraminidase titres

The antineuraminidase titres of sera were determined as described in Chapter 2, General Methods.

c. Culture medium / Screening of strains for neuraminidase activity.

Culture supernatants and cell lysates were prepared from thirty clinical isolates of *S. pneumoniae* grown in Todd-Hewitt Broth, a medium which had been used in several early studies on pneumococal neuraminidase (Tanenbaum et al., 1970; Tanenbaum and Sun, 1971; Stahl and O'Toole, 1972). Strains tested included 3551, 32973 and 3520, all of which had been shown earlier to produce large amounts of pneumolysin.

Culture supernatants and cell lysate supernatants were assayed by the fluorimetric method (see Chapter 2, General Methods) for neuraminidase activity. The results are presented in Table 3.3a,b.

Cell-pellet lysate is a convenient starting material for the preparation of protein because it can be produced easily in concentrated form. The preparation of protein from large volumes of culture supernatant is generally inconvenient. It was therefore of some importance to select as a

TABLE 3.3a

Neuraminidase activities of various strains of *S. pneumoniae*.

	TODD - HEWITT				TSB +			
Strain	S/N	Pell	Tot	%P	S/N	Pell	Tot	%P
D39	0.09	0.22	0.31	71	0.10	0.18	0.28	64
3551	1.13	1.98	3.11	64	0.18	1.23	1.41	87
6266	0.33	0.25	0.58	43	0.03	0.12	0.15	79
6979	1.33	0.46	1.79	26	0.07	0.52	0.59	89
5493	2.15	0.78	2.93	27	0.10	0.27	0.37	73
6276	0.94	0.23	1.17	19	0.10	0.11	0.21	53
6687	0.82	0.47	1.29	36	0.07	0.32	0.39	83
7136	2.84	1.49	4.33	34	0.08	0.39	0.47	83
3799	1.31	1.49	2.80	53	0.05	0.35	0.40	86
3798	0.68	0.27	0.95	28	0.08	0.36	0.44	82
7781	1.62	0.97	2.59	37	0.08	0.47	0.55	86
7945	0.67	0.59	1.26	47	0.04	0.46	0.50	91
8050	0.58	0.34	0.92	37	0.07	0.40	0.47	86
8812	0.71	0.26	0.97	26	0.04	0.46	0.50	91
9044	0.41	0.67	1.08	62	0.09	0.43	0.52	83

Strains were cultured to A_{600} of about 0.5. Figures given are units of neuraminidase activity per ml cell culture. S/N = culture supernatant; Pell = cell pellet; Tot = total neuraminidase produced; %P = percentage of total neuraminidase activity found in cell pellet. Strain numbers are Adelaide Children's Hospital pneumococcal strain library code numbers, except for D39 (see Chapter 2, Materials).

TABLE 3.3b

Neuraminidase activities of various strains of *S. pneumoniae*.

	TODD - HEWITT			
Strain	S/N	Pell	Tot	%P
8956	1.87	0.45	2.32	23
3800	1.14	0.27	1.41	19
3801	0.84	0.93	1.77	53
3802	1.08	0.21	1.29	16
3803	0.84	0.33	1.17	28
3804	1.23	0.21	1.44	15
3805	1.74	0.48	2.22	22
3806	0.99	0.54	1.53	35
3807	1.77	0.24	2.01	12
9208	1.74	0.18	1.92	9
9578	1.62	0.21	1.83	14
9811	1.32	0.36	1.68	21
9899	0.96	1.02	1.98	52
10138	1.74	0.75	2.49	30
10159	1.56	0.27	1.83	15

Strains were cultured to A_{600} of about 0.5. Figures given are units of neuraminidase activity per ml cell culture. S/N = culture supernatant; Pell = cell pellet; Tot = total neuraminidase produced (supernatant plus pellet); %P = percentage of total neuraminidase activity found in cell pellet. Strain numbers are Adelaide Children's Hospital pneumococcal strain library code numbers.

source for neuraminidase a pneumococcal strain in which the bulk of the enzyme remained cell-associated during the time in which the culture grew to maximum density.

Strain 3551 proved to be an excellent producer of neuraminidase; it was surpassed only by one other strain, 7136. In addition, when the cells were grown in Todd-Hewitt Broth, 64% of the neuraminidase activity detected in 3551 was associated with the cell pellet, a percentage which was also surpassed by only one other strain, D39.

Table 3.3a also compares neuraminidase production in Todd-Hewitt Broth with that in TSB^+ for fifteen pneumococcal strains.

Despite generally poorer bacterial growth in Todd-Hewitt Broth, total neuraminidase production (i.e. in cell pellet plus culture supernatant) was higher than in TSB^+ for every one of the fifteen strains for which the comparison was made. On average, production in Todd-Hewitt Broth was 3.6 fold higher than in TSB^+. However, the proportion of neuraminidase found in the cell pellet was far lower: on average (for the 15 strains compared: see Table 3.3a) only 41% for Todd-Hewitt Broth, while it was 81% for TSB^+. In TSB^+ the amount of cell-associated neuraminidase was greater than that found in the supernatant for every strain tested.

For strain 3551 specifically: total production of neuraminidase in Todd-Hewitt Broth was 2.2 times higher than in TSB^+, but only 64% of this was cell-associated as compared with 87% in TSB^+.

Unlike Todd-Hewitt Broth, TSB lacks higher molecular weight polypeptides which may complicate the purification of bacterial proteins. When this was taken into account, along with the difference in cost and neuraminidase yields between the two media, TSB was judged more suitable for culturing pneumococci from which to prepare cell-associated neuraminidase.

d. Investigation of various purification procedures.

During the development of a method for the purification of neuraminidase, a number of procedures were tried, more or less unsuccessfully. These are summarized briefly below. (No Figures are presented).

i. Chromatofocusing

The neuraminidase peak recovered after DEAE-fractionation of pneumococcal cell lysate supernatant was fractionated further on a 1.3 x 40 cm column of Pharmacia PBE94 chromatofocusing gel using polybuffer 74 - HCl to generate a linear 360 ml gradient across the range pH 7.4 - 4.0. After neutralization, the fractions were assayed for neuraminidase activity which was detected (on both occasions when this procedure was tried) as a broad peak eluting between pH 5.3 and 4.3. (The pI of pneumococcal neuraminidase has been estimated previously as 4.90: Stahl and O'Toole, 1972). When peak fractions were pooled and analyzed by SDS-PAGE the material recovered was extremely heterogeneous. The degree of purification achieved was judged insufficient to warrant the routine use of chromatofocusing in the purification of neuraminidase.

ii. Affinity chromatography using mucin-Affigel

Pneumococcal neuraminidase is a mucopolysaccharide N-acetyl-neuraminhydrolase whose substrates include glycoproteins such as mucin. Mucin, immobilized on a suitable support matrix, might therefore be an effective medium for the purification of neuraminidase by affinity column chromatography provided that enzymic digestion did not proceed

rapidly enough to strip the column of its immobilized ligands during the course of fractionation.

Bio-Rad Affigel 10 is a cross-linked agarose support bearing active N-hydrosuccinimide ester functional groups which will react with all materials containing free amino groups and bind them to the support. Affigel is designed for the creation of specific gels for affinity chromatography.

On two occasions mouse submaxillary mucin was coupled to Affigel-10 using the procedure recommended by the manufacturer in attempts to construct a chromatography gel with specific affinity for neuraminidase.

Neuraminidase, which had been partially purified through DEAE-cellulose then Sephacryl S-200 chromatography, was applied to the mucin-Affigel in 50 mM sodium phosphate, pH 7.0. About one third of the activity bound to the column and could be eluted with buffered 1 M NaCl. The resulting material, however, when analysed by SDS-PAGE, was disappointingly heterogeneous.

iii. Preparative polyacrylamide gel electrophoresis

While preparative PAGE using the BioRad Protean gel apparatus had proven a very effective technique in the purification of pneumolysin, it was much less successful with neuraminidase.

In preliminary studies to determine the behaviour of neuraminidase on non-denaturing polyacrylamide gels, identical paired samples of the partially-purified enzyme were loaded onto adjacent tracks of analytical ND gels of varying compositions and electrophoresed. One track of each pair was then stained with Coomassie blue and the other sliced into 5 mm

sections each of which was soaked overnight at 4°C in PBS. The buffer was then withdrawn and assayed for neuraminidase activity. The profile of such activity could then be matched against the appearance of stained bands of protein on the twin track.

At acceptable voltages ($\leq$ 200 V) with a polyacrylamide concentration in the separating gel of 10% (wt/vol), neuraminidase barely penetrated the gel. In 7.5% gels the protein showed a marked tendency to smear as it moved into the gel, and activity was recovered as a broad peak co-migrating with the major concentration of protein. When detergent (Triton X-100 at concentrations of 0.1 - 1%, vol/vol) was included in the gel and the electrode buffer, neuraminidase activity moved more readily into the gel, but even at very low loadings of protein smearing was not resolved, nor was the peak of enzyme activity sharpened.

A preparative gel run was attempted using only a very short (2 cm) separating gel, but even under optimal conditions for fractionation neuraminidase activity migrated so slowly and was so diffuse upon elution that it was clear that preparative PAGE was unlikely to be a useful technique for the routine preparation of the enzyme, and it was abandoned.

iv. Hydrophobic interaction chromatography

In preliminary experiments it was established that pneumococcal neuraminidase remains soluble in ammonium sulphate concentrations of at least 2.0 M. Enzyme which had been partially purified by DEAE-cellulose chromatography followed by passage through Sephacryl S-200, remained unbound to phenyl-Sepharose CL-4B in 1.0 M ammonium sulphate, 50 mM sodium phosphate, pH 7.0, but bound at 1.5 M ammonium sulphate, 50 mM sodium phosphate, pH 7.0. Once bound, however, neuraminidase activity failed to

elute even when the ammonium sulphate and buffer concentrations were reduced to zero. Enzyme activity could be recovered if the column were wiped with 250 mM Tris base and the pH of the eluate adjusted immediately to 7.0, but this activity remained asociated with a complex mixture of proteins (as determined by analytical SDS-PAGE).

v. Dye-ligand chromatography on gels other than Red-A

Column chromatography using a Red-A dye-ligand gel was later found to be useful as part of an effective method for the purification of neuraminidase (see (e) below). However, other dye-ligand gels were also tested, less successfully.

Neuraminidase which had been partially purified by passage through DEAE-cellulose was applied to five small test columns each containing a small amount of Amicon Matrex gel. One gel was a "control" consisting of the cross-linked agarose support matrix only. The other four were agarose gels each carrying a covalently-bound Matrex dye, either Blue-A, Blue-B, Orange-A or Green-A. The conditions for loading and elution of protein were those suggested by the manufacturers.

Enzyme activity was essentially unbound to the control gel, Blue-A, Blue-B and Orange-A, and partially bound to Green-A. Both unbound material (that which could be eluted from the column with low-salt buffer at pH 7.0) and bound material (that which was eluted with buffered 1 M NaCl) was assayed for neuraminidase activity and analysed by SDS-PAGE.

No useful degree of purification was observed in material which had passed through any column.

e. 86K form purification procedure: final method.

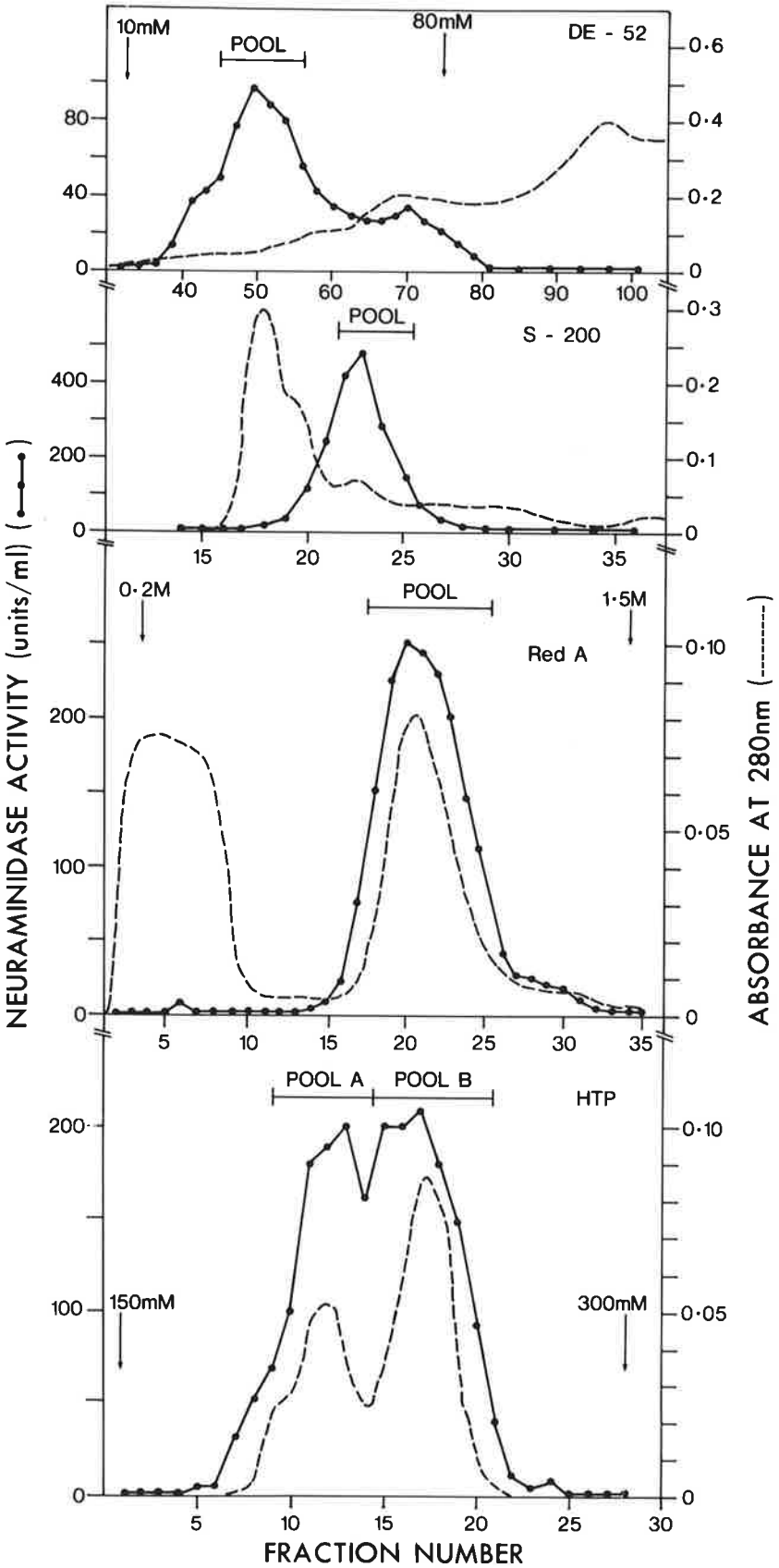
Details of the chromatographic purification of the molecular weight 86,000 (86K) form of neuraminidase are shown in Figure 3.7.

Pneumococcal cell lysate supernatant was applied to a column (4.4 x 60 cm) of DEAE-cellulose (Whatman DE-52) which had been pre-equilibrated with 10 mM sodium phosphate, pH 7.0. The column was eluted with a 2 litre linear gradient of 10 - 250 mM sodium phosphate, pH 7.0, at 4°C, and fractions were assayed for neuraminidase activity (Figure 3.7a). Fractions with high activity were pooled, concentrated to about 10 ml in a 200 ml capacity Amicon stirred cell ultrafiltration apparatus fitted with a YM10 membrane (10,000 molecular weight retention), and the concentrate was then applied to a column (2.6 x 100 cm) of Sephacryl S-200 and eluted with 50 mM sodium phosphate, pH 7.0, at 4°C (Figure 3.7b). Fractions with high activity were again pooled and concentrated by ultrafiltration to about 10 ml. The composition of the buffer was adjusted by the addition of NaCl to a final concentration of 0.2 M and the concentrate was applied to a column (1.6 x 20 cm) of Red-A dye-ligand chromatography gel pre-equilibrated with 0.2 M NaCl, 50 mM sodium phosphate, pH 7.0, and eluted with a 400 ml linear gradient of 0.2 - 1.5 M NaCl in 50 mM sodium phosphate, pH 7.0, at 4°C (Figure 3.7c). Fractions with high neuraminidase activity were again pooled, then washed and concentrated by ultrafiltration into 10 ml of 0.15 M sodium phosphate, pH 7.0. This concentrate was loaded onto a column (1.6 x 20 cm) of DNA-grade hydroxylapatite which had been pre-equilibrated with the same buffer and was eluted with a 200 ml linear gradient of 0.15 - 0.35 M sodium phosphate, pH 7.0, at room temperature (Figure 3.7d). Fractions within each of two major peaks of activity recovered from this column were pooled separately and this final material was stored in 50% (vol/vol)

Figure 3.7

Chromatographic purification of the 86K form of neuraminidase.

Column chromatography profiles for each step of the purification method are shown. (a) DEAE-celluolose; (b) Sephacryl S-200; (c) Red-A Dyematrex gel; (d) Hydroxylapatite-HTP. Vertical arrows indicate relevant salt concentrations. Horizontal bars indicate those fractions pooled prior to the next step.


10mM
POOL
80mM
DE - 52
POOL
S - 200
0·2M
POOL
1·5M
Red A
POOL A
POOL B
HTP
150mM
300mM
NEURAMINIDASE ACTIVITY (units/ml) (——)
ABSORBANCE AT 280nm (- - - - -)
FRACTION NUMBER

glycerol at -15°C. Neuraminidase could be stored in this manner for at least 6 months without detectable loss of activity.

f. 86K form: purity and yield.

Specific activity data for each stage of the purification procedure are given in Table 3.4, and sodium dodecyl sulphate discontinuous polyacrylamide slab gel electrophoresis (SDS-PAGE) analysis is shown in Figure 3.8. The final chromatographic step (Bio-Gel hydroxylapatite HTP) resolved the neuraminidase into two peaks of activity, HTP-A and HTP-B. Fraction HTP-A, which had a specific activity of 4,300 U/mg protein, comprised three protein species with apparent molecular weights (as determined after SDS-PAGE by comparison with molecular weight markers) of 88,000, 91,000 and 95,000. Fraction HTP-B had a specific activity of 3,700 U/mg and, during SDS-PAGE, migrated as a single band with an apparent molecular weight of 86,000.

When HTP-B (i.e. the 86K form of the enzyme) was electrophoresed under non-denaturing conditions it migrated slowly as a diffuse band. Material which was eluted from gel slices taken across all portions of this band had neuraminidase activity (result not shown), a finding which is consistent with those reported by Milligan *et al.* (1980) for neuraminidase isolated from a group B streptococcus. Bands of contaminating protein species were not detected in HTP-B by analytical non-denaturing PAGE.

g. Identification of the 107K form.

Neuraminidase from various stages of the above purification procedure was subjected to Western blot analysis with mouse anti-HTP-B serum as the

TABLE 3.4

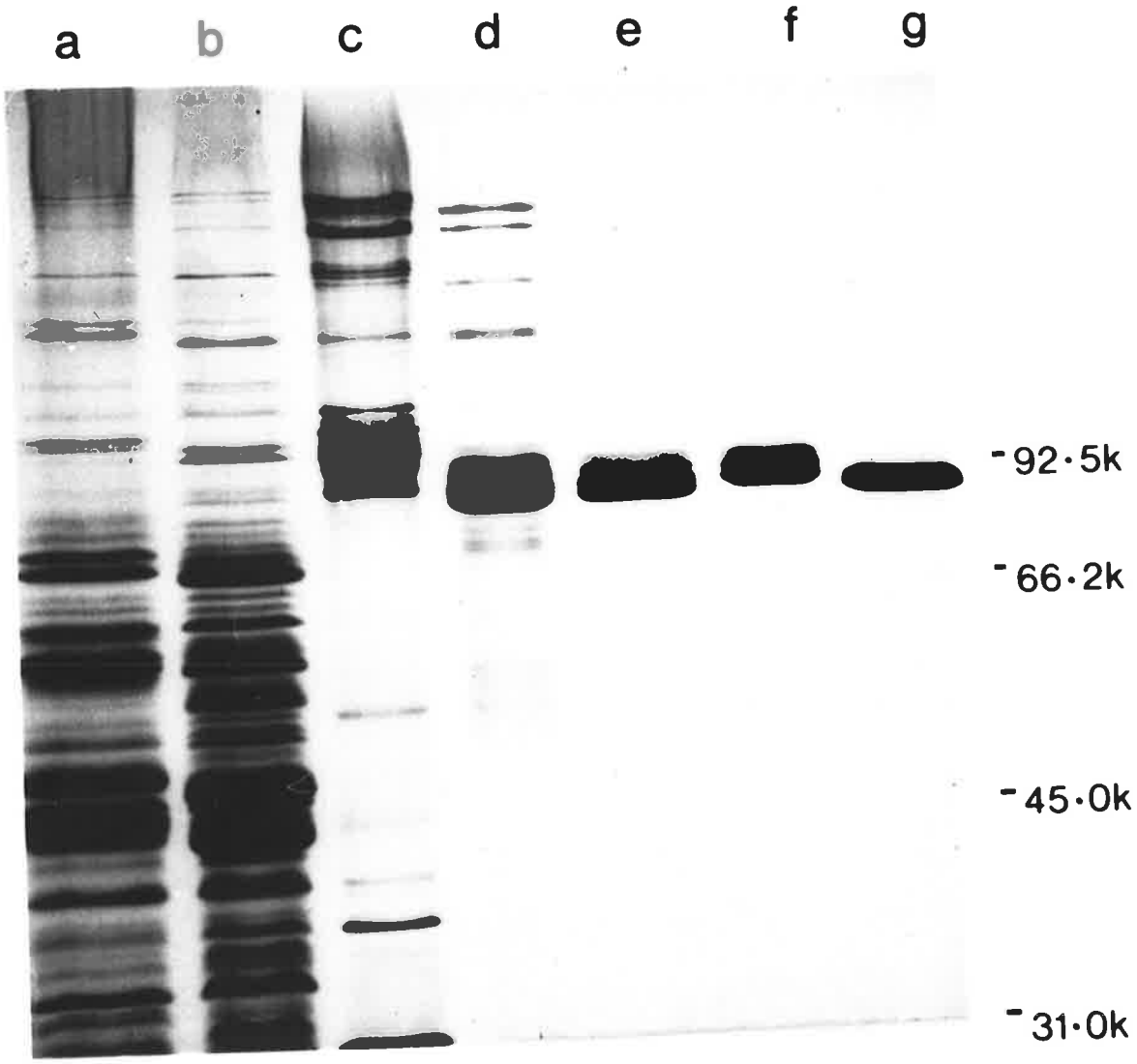
Yield and specific activity of neuraminidase at each step in the procedure for the purification of the 86K form of the enzyme.

	Total activity (units)	Specific activity (units/mg protein)
crude cell lysate	61,600	10.0
post- ammonium sulphate	39,200	10.8
post- DEAE	14,800	1,230
post- Sephacryl S-200	14,000	3,300
post- Red-A	9,000	3,460
post- hydroxylapatite A	2,230	4,300
B	3,870	3,700

Figure 3.8

SDS-PAGE (10% gel) of samples from various stages in the purification of the 86K form of neuraminidase.

(a) Crude cell lysate supernatant (40 ug); (b) ammonium sulphate pellet dialysate (40 ug); (c) post-DEAE (20 ug); (d) post- Sephacryl S-200 (10 ug); (e) post- Red-A (5 ug); (f) post- hydroxylapatite, peak HTP-A (5 ug); (g) post- hydroxylapatite, peak HTP-B (5 ug). The mobilities of molecular weight marker proteins are indicated. Proteins were stained with Coomassie brilliant blue R250.


a
b
c
d
e
f
g
92·5k
66·2k
45·0k
31·0k

antibody probe. While the neuraminidase in fresh pneumococcal cell lysate was visualised as a single band with a MW of about 107,000 (107K), neuraminidase from the cell culture supernatant was seen to be already partially degraded. The continuing degradation of the cell-associated enzyme during the purification of the 86K species was also clearly evident (Figure 3.9). These results indicate that the 86K form of the enzyme is an enzymically active breakdown product of the 107K form.

Pneumococcal cell lysate does not appear to exhibit generalized proteolytic activity. If post-DEAE lysate is incubated at 37°C for several hours in PBS and then subjected to SDS-PAGE, the general pattern of protein bands visualized after Coomassie staining is the same as that of non-incubated lysate (not shown). In addition, crude pneumococcal lysate fails to liberate dye from Remazo brilliant blue hide powder, an insoluble, non-specific protease substate (Rinderknecht *et al.*, 1968).

h. Effect of protease inhibitors.

In order to establish whether the presence of known inhibitors of proteolysis could protect high-MW neuraminidase against breakdown, crude neuraminidase (recovered after the DEAE fractionation step of the purification procedure) was incubated for several hours at 37°C either in the absence of inhibitors or in the presence of either N-ethylmaleimide, phenylmethylsulphonylfluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), benzamidine or ϵ-aminocaproic acid. Western blot analysis of the protein samples using mouse anti - HTP-B serum as the antibody probe, then established that both PMSF (1 mM) and Na_2-EDTA (10 mM) significantly inhibited the degradation of the enzyme (Figure 3.10).

Figure 3.9

Western blot analysis of neuraminidase from various stages in the purification of the 86K form of the enzyme.

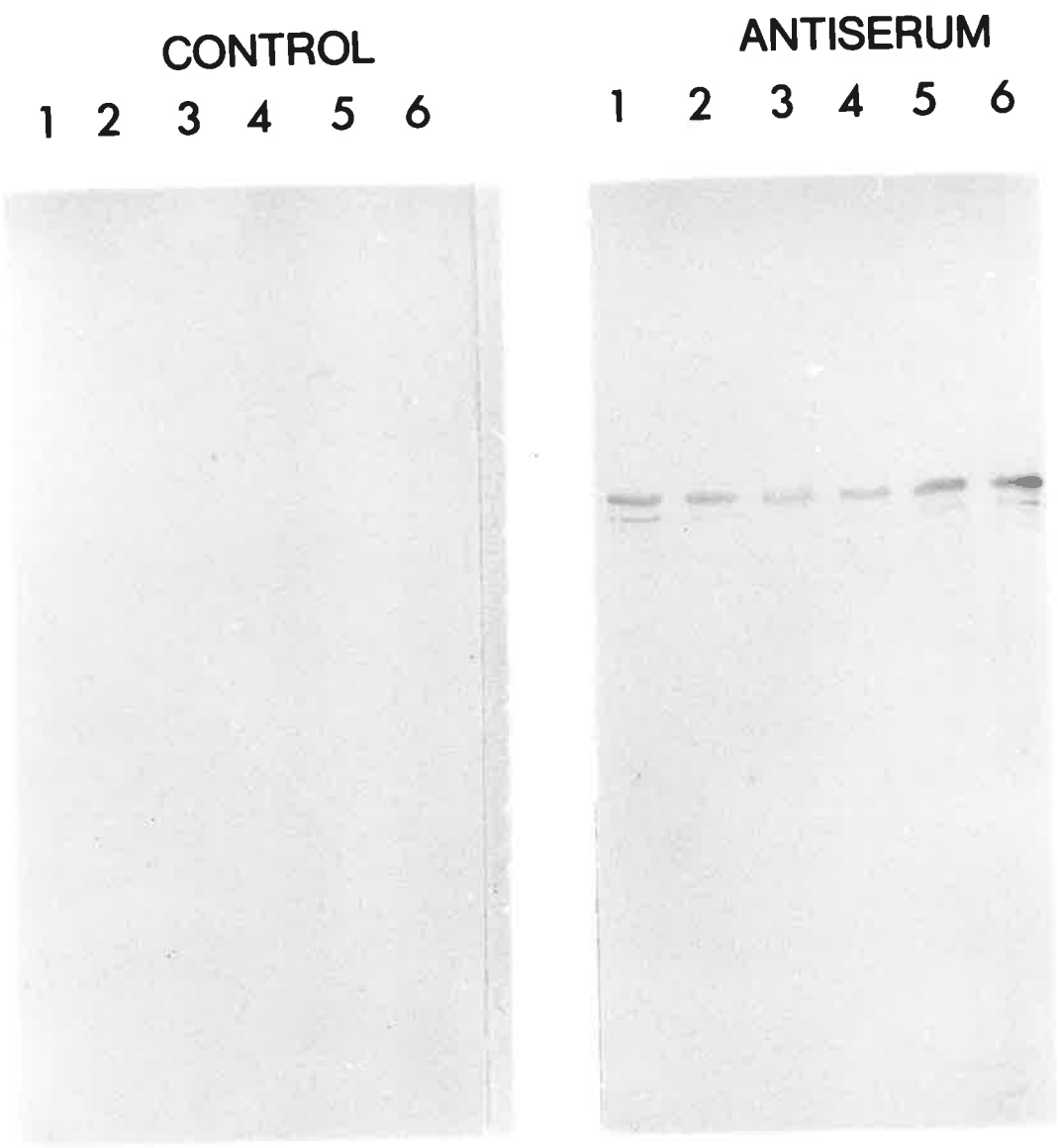
(a) crude pneumococcal cell lysate; (b) 38,000 x *g* supernatant of pneumococcal culture; (c) 38,000 x *g* supernatant of crude cell lysate; (d) post- ammonium sulphate precipitation; (e) post- DEAE-cellulose chromatography; (f) post- Sephacryl S-200 chromatography; (g) post- Red-A chromatography; (h) post- hydroxylapatite chromatography: HTP-A; (i) post-hydroxylapatite chromatography: HTP-B. The antibody probe was mouse serum raised against HTP-B. The mobilities of molecular weight marker proteins are indicated.


a
b
c
d
e
f
g
h
i
—116·3K
—92·5K
—66·2K

Figure 3.10

The effect of protease inhibitors on the degradation of neuraminidase.

Western blot analysis of crude (post- DEAE) neuraminidase which had been incubated for several hours, either (1) in the absence of protease inhibitors; or in the presence of: (2) N-ethylmaleimide (1 mM); (3) phenylmethylsulphonylfluoride (10 mM); (4) ethylenediaminetetraacetic acid (100 mM); (5) benzamidine (100 mM); (6) ϵ-aminocaproic acid (100 mM). The antibody probe was mouse antiserum raised against HTP-B, and the control was non-immune mouse serum.


CONTROL
1 2 3 4 5 6
ANTISERUM
1 2 3 4 5 6

i. 107K form purification procedure: final method.

Details of the purification procedure for the 107K form of neuraminidase are shown in Figure 3.11.

Except for the inclusion of 1 mM PMSF and 10 mM Na_2-EDTA in the dialysis buffer after the ammonium sulphate precipitation step and in all subsequent buffers, the purification procedure for the 107K form of neuraminidase was exactly as for the 86K form until after Sephacryl S-200 chromatography.

After Sephacryl S-200 chromatography, pooled fractions were washed and concentrated by ultrafiltration into 10 ml of 1 mM PMSF, 10 mM Na_2-EDTA, 10 mM sodium phosphate, pH 6.0, and loaded onto a column (1.6 x 20 cm) of CM-Sepharose CL-6B which had been pre-equilibrated with the same buffer. Neuraminidase was eluted with a 400 ml linear gradient of 10 - 200 mM sodium phosphate, pH 6.0, containing 1 mM PMSF and 10 mM Na_2-EDTA, at 4°C (Figure 3.11). Fractions with high neuraminidase activity were pooled, and the material was concentrated and stored in 50% (vol/vol) glycerol/50 mM sodium phosphate, pH 7.0, at -15°C. Under these conditions its activity was stable for at least six months.

j. 107K form: purity and yield.

SDS-PAGE analysis (Figure 3.12) indicated that PMSF and EDTA were effective in protecting the neuraminidase against breakdown and that after the CM-Sepharose step the enzyme could be visualized essentially as a single band co-migrating with its parent (cell lysate) form. It had a MW of 107,000 and a specific activity of 3,5000 U/mg. Detailed data on specific activities and yields are given in Table 3.5.

Figure 3.11

Chromatographic purification of the 107K form of neuraminidase.

Column chromatographic profiles for each step in the purification are shown. Vertical arrows indicate relevant salt concentrations. Horizontal bars indicate those fractions which were pooled prior to the next step. Top: DEAE-cellulose. Middle: Sephacryl S-200. Bottom: CM-Sepharose.

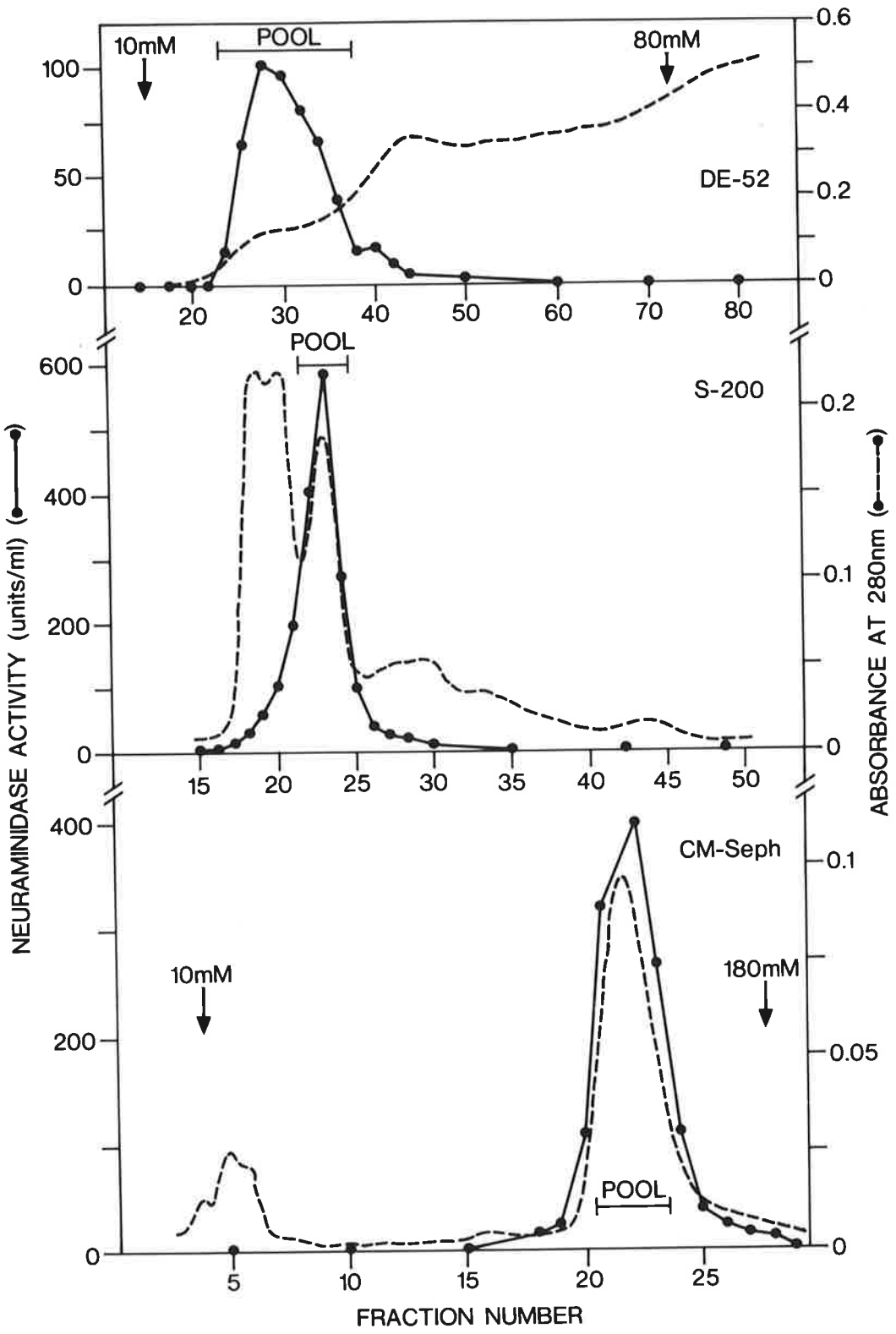

10mM
POOL
80mM
DE-52
POOL
S-200
CM-Seph
10mM
180mM
POOL
NEURAMINIDASE ACTIVITY (units/ml)
ABSORBANCE AT 280nm
FRACTION NUMBER

Figure 3.12

SDS-PAGE (10% gel) of samples from various stages in the purification of the 107K form of neuraminidase.

(a) crude pneumococcal cell lysate (25 ug); (b) post- ammonium sulphate precipitation (25 ug); (c) post- DEAE-cellulose chromatography (10 ug); (d) post- Sephacryl S-200 chromatography (5 ug); (e) post- CM-Sepharose chromatography (5 ug). The mobilities of molecular weight markers are indicated. Proteins were stained with Coomassie brilliant blue R250.

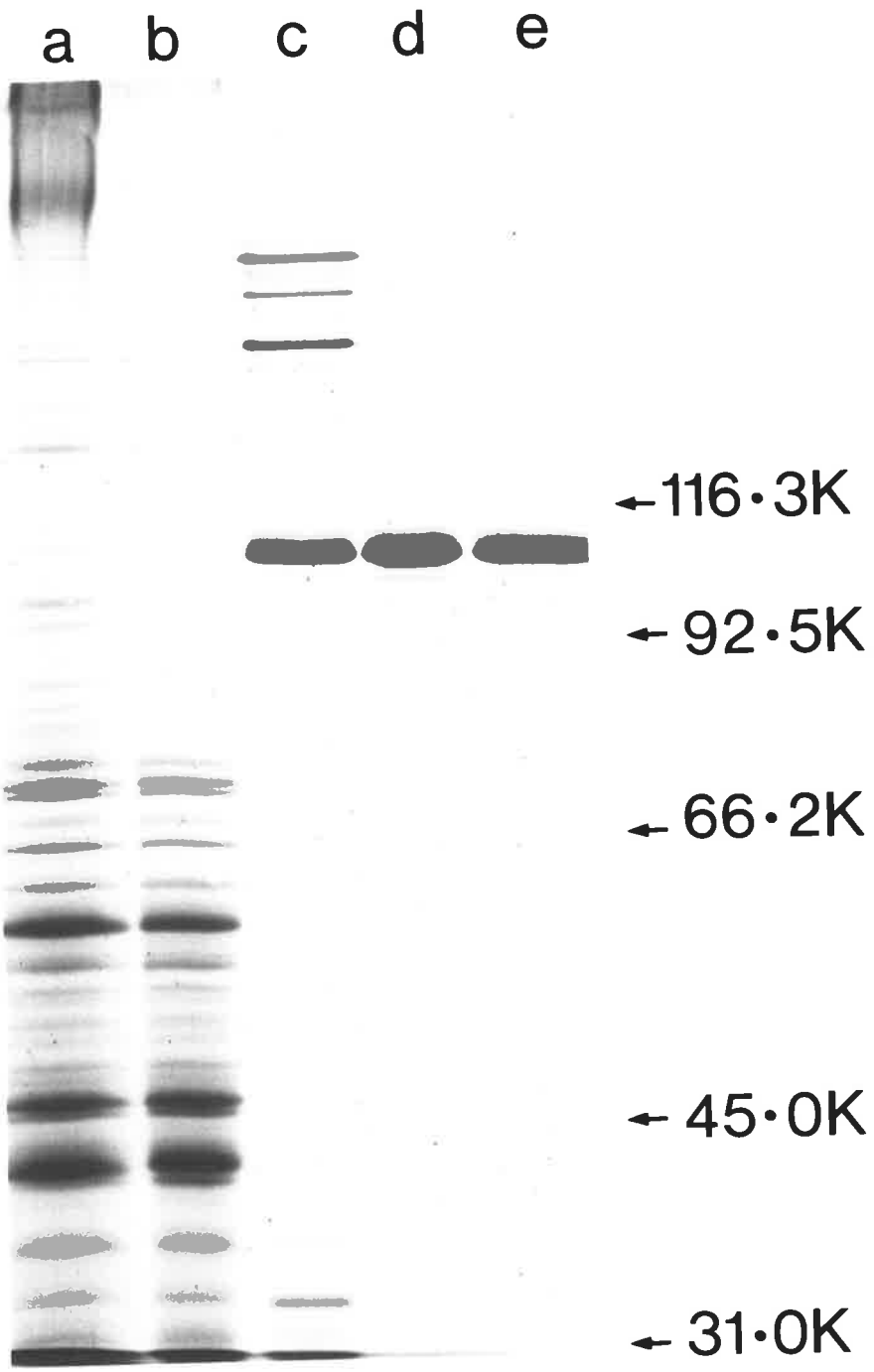

a
b
c
d
e
116·3K
92·5K
66·2K
45·0K
31·0K

TABLE 3.5

Yield and specific activity of neuraminidase at each step in the procedure for the purification of the 107K form of the enzyme.

	Total activity (units)	Specific activity (units/mg protein)
crude cell lysate	52,900	11.5
post- ammonium sulphate	31,500	14.0
post- DEAE	22,000	1,100
post- Sephacryl S-200	14,080	3,200
post- CM-Sepharose	4,900	3,500

k. Relationship between the 86K and 107K forms.

Sera were collected from mice which had been immunized either with HTP-A or with the 86K form or the 107K form of neuraminidase. Immunodiffusion analysis indicated that each antiserum reacted strongly against all three antigens (Figure 3.13). A strong antigenic relationship between the various forms of the enzyme was confirmed by the finding that each antiserum was capable of neutralizing the neuraminidase activity in each of the protein preparations. When tested against each of the three neuraminidase preparations, the three antisera all exhibited neutralization titres in excess of 100. Control sera had antineuraminidase titres of less than 10.

l. Discussion.

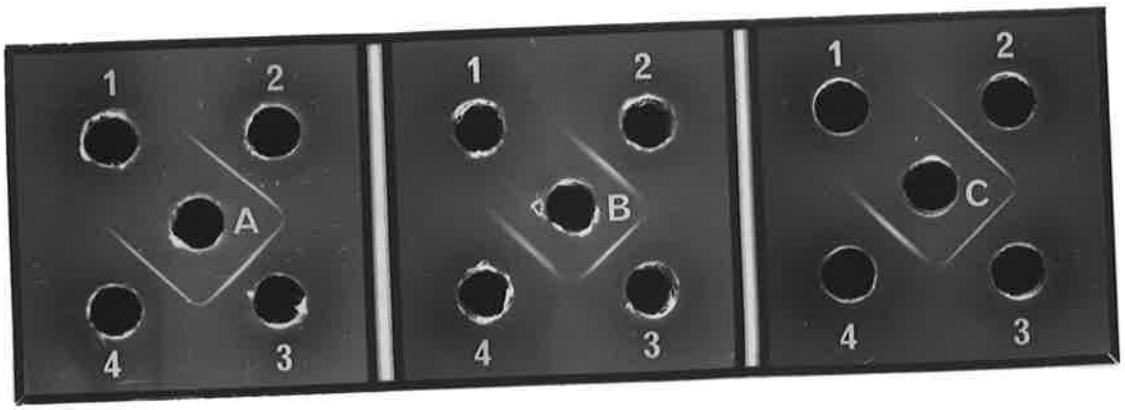
There has previously been some confusion concerning some of the properties of pneumococcal neuraminidase.

Firstly, it has not been clear whether the enzyme is intracellular (and released from the cell only by lysis) or actively secreted. Work recently undertaken in the author's laboratory has shown that autolysin-negative pneumococci created by insertion-duplication mutagenesis specifically directed at the *lytA* gene (i.e. pneumococci deficient in autolysin function alone) do not release neuraminidase into the culture medium during growth. When such mutants are provided with a functional copy of the *lytA* gene, their ability to release neuraminidase is restored (A. Berry, personal communication). These results imply that neuraminidase is normally a cell-associated protein which is released only by cell lysis. This conclusion was confirmed in the present study when amounts of the enzyme present in the culture supernatant and the cell pellet were determined for

Figure 3.13

Immunodifussion gel analysis of neuraminidase preparations.

Wells contained the following: (A) HTP-A; (B) 86K neuraminidase (HTP-B); (C) 107K neuraminidase; (1) control mouse serum; (2) mouse antiserum raised against HTP-A; (3) mouse antiserum raised against 86K neuraminidase; (4) mouse antiserum raised against 107K neuraminidase. Each well contained either 10 ug antigen or 25 ul serum.


1
2
A
4
3
1
2
B
4
3
1
2
C
4
3

thirty strains of *S. pneumoniae*. In all cases the proportion of neuraminidase in each fraction roughly paralleled that of pneumolysin, which has been previously shown to be cytoplasmically located and released only after cell lysis (Johnson, 1977). When 3551, the strain used in the present study, was cultured in TSB^+, 87% of the total neuraminidase activity remained cell-associated at the end of logarithmic phase growth. Neuraminidase was prepared from this cell pellet.

Secondly, greater confusion concerning the nature of neuraminidase has been occasioned by the apparent existence of multiple molecular forms of the enzyme (Tanenbaum *et al.*, 1970; Tanebaum and Sun, 1971; Stahl and O'Toole, 1972). In the present study, using sequential column chromatography through DEAE-cellulose, Sephacryl S-200, Red-A Matrex gel and hydroxylapatite-HTP, a single pneumococcal polypeptide species having neuraminidase activity was purified to electrophoretic homogeneity on denaturing (SDS) polyacrylamide gels. Antiserum raised against this 86K species was then used as a probe to determine the relationship between the various forms of the enzyme.

Western blot analysis indicated that the neuraminidase present in fresh pneumococcal cell lysate exists as a single high molecular weight (107K) form, and that this is progressively degraded during the purification procedure into a number of lower molecular weight species, at least some of which retain enzyme activity. Neuraminidase in the culture supernatant was detected primarily as the 107K form, but a significant amount of breakdown was already apparant, indicating that the process is not an artifact of the purification procedure. These observations account for reports of multiple neuraminidase isoenzymes in *S. pneumoniae*.

SDS-PAGE and Western blot analyses of partially purified (post-DEAE) extracts of neuraminidase which had been incubated to allow breakdown of

the enzyme showed that this breakdown is not due to generalized proteolysis, but appears to be preferential for neuraminidase. Cleavage of neuraminidase takes place in a series of discrete steps generating a "ladder" of related products as visualised by SDS-PAGE. It should be noted that the 86K species which was isolated during this study appears to be only one of the more stable intermediates in the degradation process, not the end-product.

These results suggest the existence of a previously undescribed pneumococcal protease with apparant specificity for neuraminidase. This protease, which appears to co-purify with its substrate during the early steps of the purification procedure described in this Chapter, seems to cleave neuraminidase in a stepwise fashion generating a family of related proteins at least some of which retain neuraminidase activity.

While it is possible to speculate on functions that the neuraminidase protease may have in *vivo*, an investigation of the role of the putative pneumococcal neuraminidase protease lies outside the scope of the present study.

PMSF and EDTA were found to inhibit the degradation of neuraminidase, and the use of these compounds in all buffers during the purification procedure allowed the isolation of the 107K species largely undegraded.

When PMSF and EDTA were included in the column buffers, the peaks of neuraminidase activity were tightened. This was particularly the case during fractionation on DEAE-cellulose. Previously the enzyme had exhibited a broad elution profile at this step, consistent with the occurrence of proteolysis (c.f. Figures 3.7a and 3.11a).

Mouse antiserum was raised against the pure 107K species as well as against the pure 86K species (HTP-B) and an intermediate group of related

neuraminidase species (HTP-A). Immunodiffusion and enzyme neutralization analysis showed that each serum reacted strongly with homologous and heterologous antigens, confirming the relationship between the various forms of the enzyme.

Both the undegraded 107K form of the enzyme and its breakdown product, the 86K form, were later used in immunization/challenge experiments in mice (see Chapter Five).

m. Note: On the preparation of pneumolysin and neuraminidase from the same batch of pneumococcal cells.

The early stages (i.e. until after DEAE-cellulose chromatography) in the methods for preparing pneumolysin and the 86K form of neuraminidase from pneumococci are identical, and the preparation of both both proteins from the same batch of cells was found to be possible. However, pneumolysin could not be prepared succesfully from cell lysate which had been treated with PMSF and EDTA to preserve the 107K form of neuraminidase. Whenever the attempt was made, the resolution of the pneumolysin after DEAE-cellulose chromatography suffered badly, and this was also the case after subsequent fractionation of the material by Sephacryl S-200 chromatography even in the absence of the protease inhibitors, to the extent that little useful purification was achieved by this step (results not shown). The reason for the loss of resolution was not investigated.

3. Autolysin.

a. Background.

Pneumococcal N-acetylmuramyl-L-alanine amidase (autolysin) was first purified to biochemical homogeneity by Holtje and Tomasz (1976). Because of problems with the stability of the native, C-form of the enzyme, they purified the E-form, i.e. that form of autolysin which is recoverable from pneumococci cultured in medium in which the essential growth component choline has been replaced by its analogue, ethanolamine. The E-form of autolysin is enzymically inactive, but is converted to the active C-form by a brief incubation with pneumococcal cell-wall.

In outline, Holtje and Tomasz' method for the purification of autolysin from *S. pneumoniae* was as follows:

1. Cells were grown in 90-litre batches of ethanolamine-containing growth medium;
2. Cells were disrupted by French press;
3. Cell debris was removed by centrifugation;
4. Protein was precipitated from the supernatant by the addition of ammonium sulphate;
5. Protein was redissolved, dialysed;
6. The dialysate was applied to a column of hydroxylapatite (Bio-Gel HT);
7. Fractions with autolysin activity were pooled and concentrated by ultrafiltration;
8. The concentrate was subjected to gel filtration through a column of Bio-Gel A-5m;
9. Active fractions were again pooled and concentrated;
10. The concentrate was dialysed against electrophoresis

buffer;

11. The dialysate was subjected to PAGE on tube gels;
12. The gels were sliced, and each slice eluted with phosphate buffer;
13. The eluate was again subjected to hydroxylapatite column chromatography;
14. Active fractions were pooled and concentrated.

The final material was shown to migrate as a single band after PAGE in the presence of SDS. The yield of pure autolysin from 90 litres of culture was only 1.7 mg.

A second method for purifying the enzyme directly from *S. pneumoniae* has also been published. Briese and Hakenbeck (1985) showed that the E-form of pneumococcal autolysin could be purified by sequential column chromatography through hydroxylapatite, and an affinity column consisting of lipoteichoic acid (LTA) bound to Sepharose. Autolysin, which bound specifically to the LTA-Sepharose, could be eluted from it by the application of choline. Only very small amounts of autolysin (0.04 mg per preparation) were obtained by Briese and Hakenbeck, and, when their final preparation was analysed by SDS-PAGE, a number of bands of contaminating protein were detected.

Holtje and Tomasz' method was chosen as the starting point for the preparation of autolysin to be used in this study.

b. Assay.

The assay for autolysin activity was adapted from that of Holtje and Tomasz (1976). It is described in full in Chapter 2, General Methods.

Briefly: extracts containing autolysin were incubated at 37°C in the presence of pneumococcal cell walls labelled with [^{3}H-choline]. The reaction was stopped by the addition of formaldehyde and a large excess of unlabelled cell wall. Undigested substrate was then pelleted, along with unlabelled cell wall carrier, by centrifugation. Supernatants containing tritiated cell wall fragments which had been solubilized by the action of autolysin were then removed into scintillation fluid, and radioactivity was quantitated.

c. Culture medium.

The ethanolamine-containing medium used by both Holtje and Tomasz (1976) and Briese and Hakenbeck (1985) is a chemically-defined growth medium consisting of many components. It is tedious to make and supports pneumococcal growth only to relatively low culture densities. Holtje and Tomasz do not cite the density of the cultures from which they prepared autolysin, but Briese and Hakenbeck state that they harvested their cultures at an absorbance of only 0.15 - 0.20, which is consistent with the maximum cell densities which could be obtained in the present study when their medium was used. The poor growth of pneumococci in the ethanolamine-containing defined medium explains, in part, the poor yield of autolysin which was obtained by these workers.

For the purposes of the present study it was not important that the autolysin which was obtained at the end of the purification procedure should have enzymic activity, as long as it could be used to raise antiserum capable of inhibiting the autolytic activity of the native enzyme. Accordingly, there was no need to prepare autolysin in its stable, E-form, from cells grown in ethanolamine-containing medium. The

pneumococcal cells used as a source for autolysin for the work described in this Chapter were grown in TSB^{++}, the standard medium for pneumococcal culture in this study.

d. Screening of pneumococcal strains for autolysin activity.

Twelve clinical isolates of *S. pneumoniae* were cultured at 37°C overnight on blood-agar plates. Pneumococci from each plate were suspended in 5 ml TSB^{++}, then the absorbance of each culture was adjusted, by the addition of fresh TSB^{++}, to 0.15. The cultures were incubated for 2h, during which their absorbances increased, generally, to between 0.3 and 0.4. At 0 h, 1 h and 2 h, 1 ml samples were removed. Twenty microlitres of sodium deoxycholate (10% wt/vol) and 100 ul 0.5 M sodium phosphate, pH 7.0 were added to each sample, and the cells incubated at 37°C for a further 20 min to lyse. The cell debris was removed by centrifugation (Eppendorf microfuge, 10 min at 4°C) and the supernatants were assayed for autolysin activity.

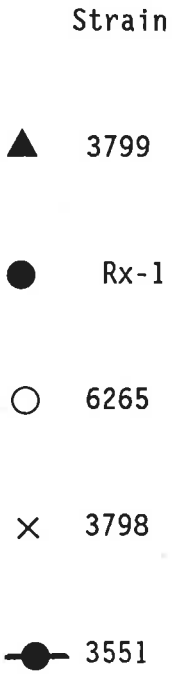
The results for five representative strains are shown graphically in Figure 3.14, and autolysin activities for all 12 strains at the 2 h point are given in Table 3.6.

Clearly, 3551, the pneumococcal strain which had proved highly suitable for the production of both pneumolysin and neuraminidase, produces very little autolysin activity. Nevertheless, most of the preliminary work (described below) which was undertaken towards the development of a method for the purification of autolysin used 3551 as starting material. The reasons for this are given in the Discussion (see (f) below).

Figure 3.14

Autolysin activity in cultures of five representative strains of pneumococci.

Broth cultures of five clinical isolates of *S. pneumoniae*, all at initial A_{600} of 0.15, were incubated for 2 h at 37°C. Autolysin activities (duplicate points) were determined as described in Chapter 2, General Methods. ΔDPM = crude readings minus a background of 995 d.p.m.



These strain numbers are Adelaide Children's Hospital pneumococcal strain library code numbers, except for Rx-1 (see Chapter 2, Materials).

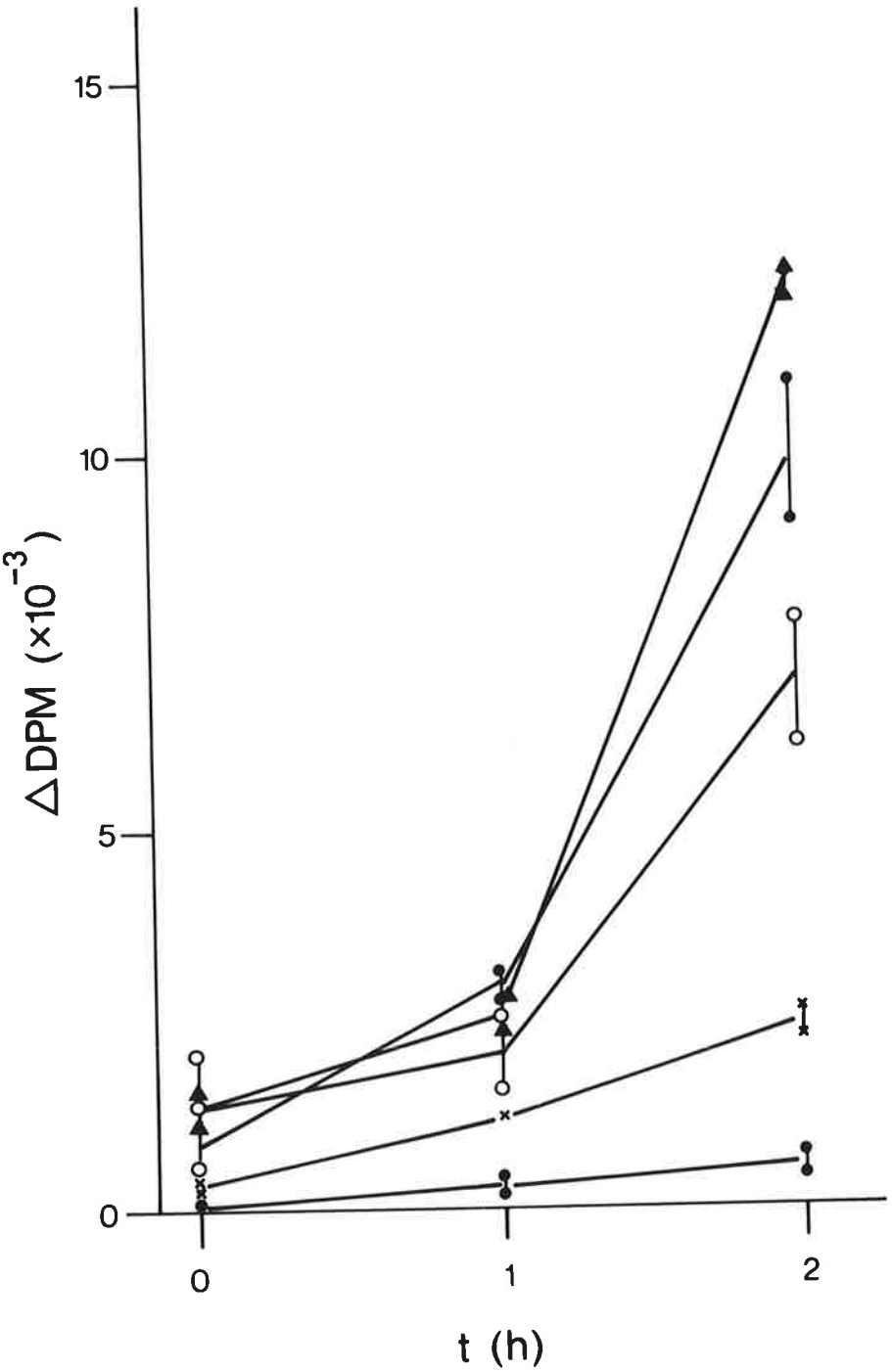

15
10
5
0
ΔDPM (x10^-3)
0
1
2
t (h)

TABLE 3.6

Autolysin activities of various strains of S. pneumoniae.

Strain	ΔDPM x 10^{-3}
3551	0.81
6266	8.41
6979	6.02
5493	8.28
6276	4.71
6265	6.95
RX-1	10.01
3799	12.19
3798	2.07
7781	6.85
8050	2.07
3802	4.21

Strains were grown for 2 h in TSB^{++} from an initial A_{600} of 0.15 to a final 0.3 - 0.4, then autolytic activities of washed cell pellets were determined. Strain numbers are Adelaide Children's Hospital pneumococcal library strain numbers except for Rx-1 (see Chapter 2, Materials). ΔDPM = crude readings minus a background of 995 dpm.

e. Investigation of various purification procedures.

The results from this Section are summarized in Figure 3.15.

Initially, pneumococcal cell-associated protein (see Section B of this Chapter: General Methods) was subjected to column chromatography through DEAE cellulose under the same conditions as those which had previously been used for the DEAE-cellulose fractionation of both pneumolysin and the 86K form of neuraminidase (see 1(g) and 2(e) above). While autolysin activity was separated from that of pneumolysin and neuraminidase, it was recovered from the column as a very broad, heterogeneous peak co-incident with a large amount of material having absorbance at 280 nm (Figure 3.16). Even after subsequent chromatography through Sephacryl S-200 (under conditions which were again the same as those used for the fractionation of pneumolysin and the 86K form of neuraminidase: see 1(g) and 2(e) above) the autolysin-containing material remained very heterogeneous (Figure 3.15a).

In the next attempt at purification, the autolysin-containing fractions from DEAE-cellulose were applied to a column (2.6 x 40 cm) of hydroxylapatite (BioGel HT) which had been equilibrated with 10 mM sodium phosphate, pH 7.0, and autolysin was eluted by the application of an 800 ml linear gradient of 10 - 400 mM sodium phosphate, pH 7.0, at room temperature. Subsequently, autolysin-containing material was applied to Sephacryl-S200 (under conditions previously given), then subjected to preparative PAGE (see Chapter 2, General Methods). An analytical SDS-polyacrylamide gel of the final product is shown in Figure 3.15b. The putative autolysin band (MW 36,500) comprises about 15 - 20 % of the total material.

In later attempts to purify autolysin, pneumococcal cell-associated protein was fractionated initially through hydroxylapatite (Figure 3.17)

Figure 3.15

Summary of trial autolysin (C-form) preparations from pneumococcal cell-associated protein.

Details are given in the text, C3(e). Shown here are analytical SDS-PAGE (12.5%) gels of material which was recovered after each of the final preparative steps. The mobilities of molecular weight marker proteins are indicated.

The near-comigration of proteins in preparation (b) and the molecular weight marker standards is co-incidental.

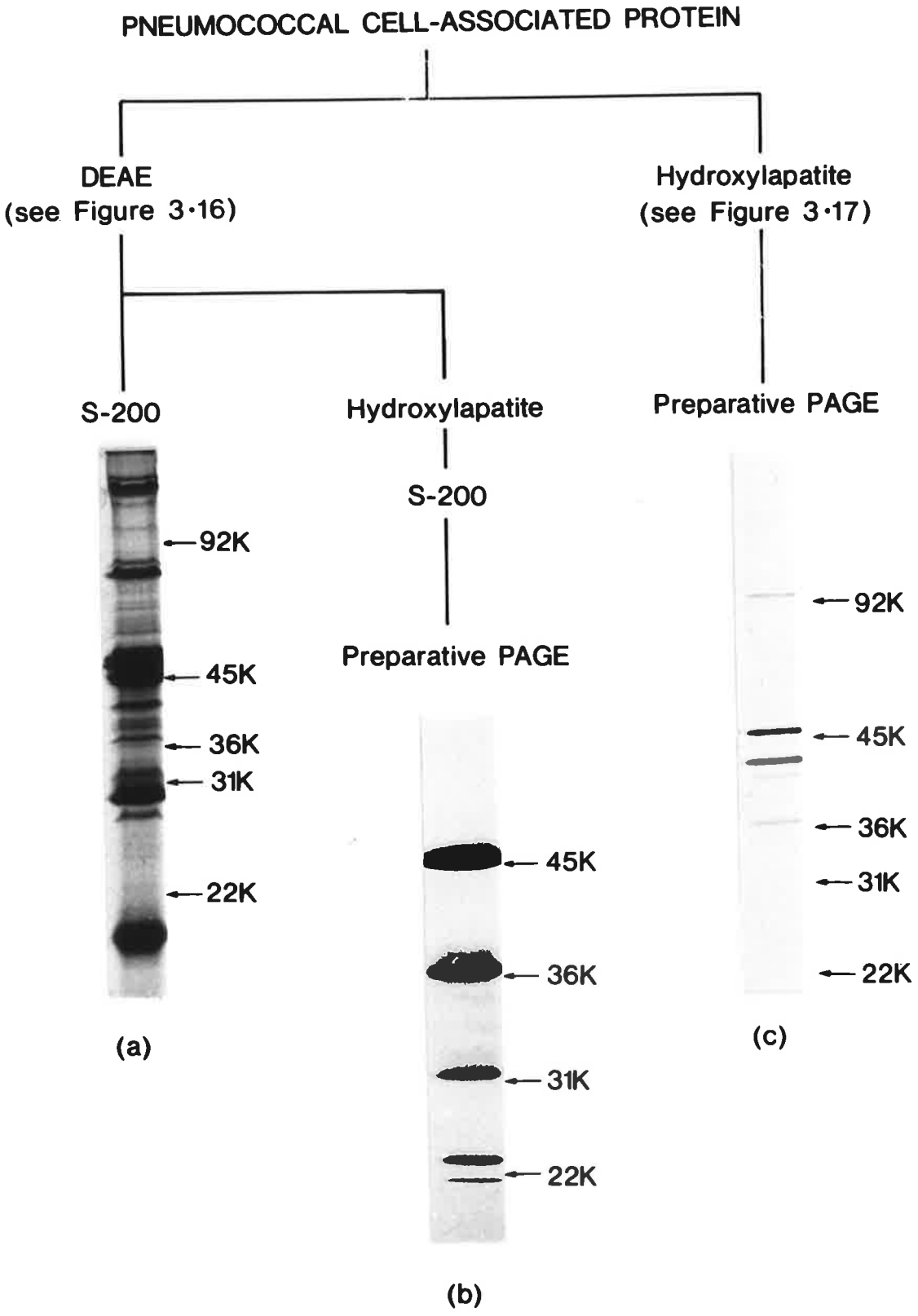

PNEUMOCOCCAL CELL-ASSOCIATED PROTEIN
DEAE
(see Figure 3·16)
Hydroxylapatite
(see Figure 3·17)
S-200
Hydroxylapatite
Preparative PAGE
S-200
Preparative PAGE
92K
45K
36K
31K
22K
(a)
45K
36K
31K
22K
(b)
92K
45K
36K
31K
22K
(c)

Figure 3.16

Trial purification of autolysin (C-form): DEAE-cellulose column chromatography of pneumococcal cell-associated protein.

q.v. Figure 3.15.

Vertical arrows indicate relevant salt concentrations. Horizontal bar indicates those fractions which were pooled prior to the next step.

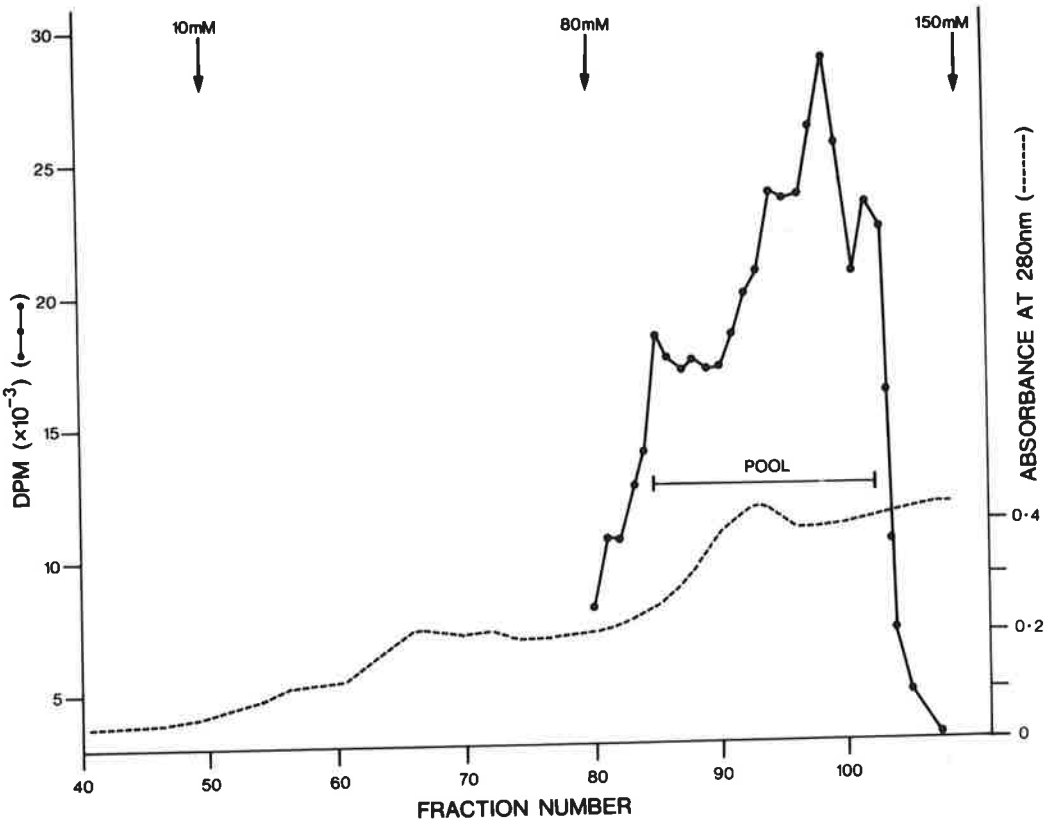
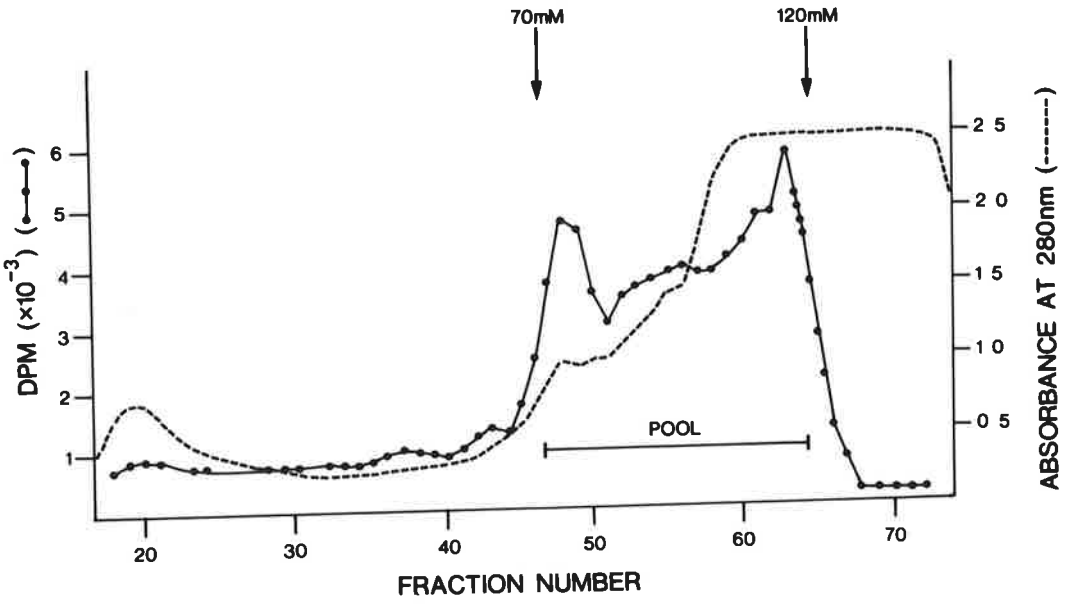

10mM
80mM
150mM
30
25
20
15
10
5
DPM (×10⁻³) (●—●)
POOL
ABSORBANCE AT 280nm (------)
0·4
0·2
0
40
50
60
70
80
90
100
FRACTION NUMBER

Figure 3.17

Trial purification of autolysin (C-form): hydroxylapatite column chromatography of pneumococcal cell-associated protein.

q.v. Figure 3.15.

Vertical arrows indicate relevant salt concentrations. Horizontal bar indicates those fractions which were pooled prior to the next step.


70mM
120mM
DPM (×10⁻³) (——)
ABSORBANCE AT 280nm (-------)
POOL
FRACTION NUMBER
1
2
3
4
5
6
0 5
1 0
1 5
2 0
2 5
20
30
40
50
60
70

and this step was followed by preparative PAGE. Once again, in the final product, material migrating on analytical SDS polyacrylamide gels with mobility corresponding to the molecular weight which had been reported for autolysin comprised only a minor proportion of the total material (Figure 3.15c). On the S-200 column, the elution volume of autolysin activity suggested a MW substantially higher than the expected value of 36,500, possibly because the protein was forming non-covalent aggregates. However, the inclusion of a mild, non-ionic detergent, Triton X-100, at concentrations up to 1% (vol/vol) in the sample and column buffer did not improve resolution.

Holtje and Tomasz (1976) have reported that the C-form of autolysin is highly labile, and this appeared to be confirmed during the present work. Over 90% of the autolytic activity which was detectable in the crude preparation of cell-associated protein was lost from each preparation during the first column chromatographic step, and over 90% of the surviving activity was lost during the next step. After the third step of the multistep purification procedure shown in Figure 3.15b, autolysin-containing fractions could be only tentatively identified on the basis of MW as judged by SDS-PAGE.

f. Discussion.

Critical to the following discussion is the difference between the C-form and the E-form of the pneumococcal autolysin, a difference which is related to the role of choline in the structure of the pneumococcal cell wall.

S. pneumoniae requires exogenous choline for incorporation into its cell wall. Autolysin probably attaches to the pneumococcal cell wall

specifically via choline-containing sites (Giudicelli and Tomasz, 1984; Garcia *et al.*, 1985).

If cells which have been grown in the presence of choline are disrupted, the autolysin in the resulting crude mixture is present in its C-form. Although the structure of the C-form has not been completely established, it is likely that it consists of pieces of pneumococcal cell-wall teichoic acid - glycan polymers associated with multimers of the autolysin protein (Holtje and Tomasz, 1976).

Pneumococci can grow in medium which lacks choline if that compound is replaced by certain close analogues, such as ethanolamine. Since pneumococci in which ethanolamine has been substituted for choline have cell walls lacking binding sites for autolysin, the autolysin which is produced from such cells remains as free, monomeric protein, i.e. the E-form.

The E-form of the enzyme is stable, but not active until it has been incubated with cell walls containing choline, which convert it to the active, but labile C-form (Holtje and Tomasz, 1976). Previously-published methods for the purification of autolysin have dealt only with the E-form, but the difficulties associated with growing pneumococci in large volumes of chemically-defined medium, and the poor yield of autolysin which other workers had obtained from such cultures, suggested that, in the present case, it might be preferable to purify autolysin from pneumococci which had been grown in a standard broth culture medium such as TSB^{++}. The autolysin protein produced under such conditions would be the C-form, and would have to be purified free from other components of the complex before it could be used in immunization/challenge experiments (whose results would otherwise be confused by the effects of antibodies directed against these other antigens).

Pneumococci were grown in TSB^{++}. As well as being easy to prepare and capable of supporting the growth of cells to relatively high culture densities, this medium had another advantage: it had already been proven effective for the growth of cells from which pneumolysin and neuraminidase could be prepared.

Since the early stages (i.e. until immediately after the DEAE step) of the preparations which had already established for both pneumolysin and for the 86K form of neuraminidase were identical (see note B 2(m) above), both proteins had, on some occasions, been purified from a single batch of cells, with considerable saving both of time and materials. Strain 3551 produced large amounts of pneumolysin and neuraminidase. If it could be have been used successfully as a source for autolysin also, then all three proteins of interest might have been purified from each batch of cell lysate. Indeed, if DEAE-cellulose had proven suitable as a first column chromatography step for the purification of autolysin, the initial column purification step for all three proteins might have been accomplished simultaneously.

Given the fact that 3551 was selected as a source for pneumolysin and neuraminidase partly on the basis that large proportions of both proteins remained cell-associated (i.e. were not released by cell lysis) during culture, it is not surprising that 3551 is poorly autolytic. However, this might not have been a disadvantage. The paucity of autolysin activity in strain 3551 does not necessarily reflect a low production of autolysin protein by that strain. Instead, it might be due to low specific activity, which would be of little consequence to the present study, so long as antisera raised against the autolysin from 3551 could be shown to be effective at inhibiting the activity of the enzyme from more highly autolytic strains. In the course of the work described in this Chapter, no

attempt was made to compare the amounts of autolysin protein produced by different strains of pneumococcus, but this would have been necessary if the preparation of autolysin from pneumococci had proven to be the most suitable way of obtaining the enzyme.

One problem with attempting to isolate C-form autolysin from a poorly-autolytic strain was that the lability of the C-form ensured that what little autolytic activity there was to monitor rapidly disappeared during the course of purification. After two chromatographic steps, or a chromatographic step followed by preparative PAGE, virtually no activity remained, and after any subsequent steps, autolysin-containing fractions could only be identified by the appearance of a strong 36.5K band on SDS polyacrylamide gels. If 36.5K material had been isolated in pure form, its identity would then have had to be confirmed by immunological means (see next Chapter).

A second, more serious problem related to the purification of autolysin from its C-form became apparant during the course of the work described in this Section. After early column chromatographic steps, autolytic activity could only be recovered as broad peaks, a result which probably reflects a substantial degree of heterogeneity in the composition of C-form complexes. The addition of the non-ionic detergent Triton X-100 in concentrations up to 1% (vol/vol) failed to sharpen the activity profiles. (In view of the fact that the enzyme is activated by the presence of detergent, the failure of detergent to dissociate it from its natural substrate is, in fact, not unexpected).

Another problem, less easy to assess quantitatively, arose from the use of pneumococcal cell lysate supernatant as the source for the C-form material. Only autolysin associated with cell wall fragments which had solubilized during the deoxycholate-induced lysis step would be expected to

partition into the supernatant. Some autolysin, and perhaps a high proportion of it, presumably remained associated with larger, insoluble fragments and was lost when these were pelleted and discarded along with the rest of the cell debris.

The work described in this Section was not exhaustive, and despite the various difficulties described here, the preparation of relatively large amounts of pure autolysin protein from its C-form from pneumococcal cells might have been achieved if other combinations of purification procedures had been carefully tested. However, the availability of recombinant *E. coli* carrying the gene for pneumococcal autolysin and capable of synthesizing very large amounts of highly active enzyme in the absence of interfering cell wall suggested another approach to the problem.

The development of a method for the preparative purification of autolysin from recombinant *E. coli* is described in Chapter Four.

D] SUMMARY

This Chapter describes the development of methods by which pneumolysin and undegraded (107K form) neuraminidase may be purified in milligram quantities from cultures of *Streptococcus pneumoniae*. Preliminary attempts to find a similar method for the preparation of pneumococcal autolysin were not successful.

In the course of the work described here, many preparative techniques were applied more or less unsuccessfully to the purification of the proteins of interest. Within each of these techniques, there was scope for the variation of a number of parameters, such as pH, salt and detergent concentrations, temperature, column size and the exact characteristics of the fractionating medium. Not all potentially useful combinations of conditions could be tested, and neither could all potentially useful combinations of techniques. The important point is that effective methods were devised, empirically, for the preparation of both pneumolysin and neuraminidase from *S. pneumoniae* cultures, and eventually of autolysin also, although from a different source.

Methods for the preparative purification of autolysin, and of a specifically-inactivated mutant form of pneumolysin, both from recombinant strains of *E. coli*, are described in the next Chapter.

CHAPTER FOUR

PURIFICATION OF PNEUMOCOCCAL PROTEINS FROM RECOMBINANT ESCHERICHIA COLI

A] INTRODUCTION

The previous Chapter describes methods for purifying pneumolysin and neuraminidase from cultures of *S. pneumoniae* for the purpose of testing their efficacy as protective immunogens. Since both proteins are toxic (Shumway and Klebanoff, 1971; Lorian *et al.*, 1973) ways of inactivating them prior to their administration to experimental animals were investigated. The details of this work are given in the next Chapter, but a summary of the results is relevant here: physical and chemical means of detoxifying the proteins were either ineffective, or substantially altered their immunological characteristics.

Recombinant DNA technology suggested a way of addressing this problem. If the pneumococcal gene for a protein can be inserted into in an appropriate *E. coli* host, it becomes amenable to genetic manipulation. The base triplet coding for an amino acid essential for the maintenance of toxicity may then be specifically mutated, resulting in a gene product which has little or no activity but retains the immunological epitopes of the native protein.

With this possibility in mind, the present author, in conjunction with co-workers, cloned the gene for pneumolysin into *E. coli* (Paton *et al.*, 1986, see Publications Appendix; also, see Chapter 5). Subsequently, other researchers, who had also cloned this gene, determined its base sequence

(Walker *et al.*, 1987) and then, by site-directed mutagenesis, produced a clone expressing a mutant form of pneumolysin having a much reduced specific haemolytic activity (G. Boulnois, personal communication). The clone producing this mutant pneumolysin was kindly made available to the present author, and the first part of this Chapter describes a method for purifying its product.

The pneumococcal gene for neuraminidase has also been cloned into *E. coli* in the author's laboratory (Berry *et al.*, 1988), but it has proved rather more difficult to handle. Fragments of pneumococcal DNA containing the neuraminidase gene appear to be highly unstable in *E. coli*, and the clone which has been successfully maintained, although expressing neuraminidase activity, does not carry sufficient pneumococcal DNA to code for the entire, parent (107K) form of the enzyme. The fact that a partial (in this case 98K) product of the neuraminidase gene has enzymic activity is not surprising in the light of the evidence, presented in Chapter Three, that at least some of the natural degradation products of the parent form retain activity also. At the time of writing, the successful manipulation of the cloned neuraminidase gene to produce a specifically-inactivated protein has not been accomplished, and the purification of neuraminidase from recombinant *E. coli* is not considered in this Chapter.

In contrast to pneumolysin and neuraminidase, autolysin, an enzyme whose substrate is exclusively bacterial in origin, is unlikely to be directly toxic to animals. However, it presents a different problem. Autolysin is difficult to purify in substantial amounts from its parent organism because of its tendency to associate with cell-wall fragments to form heterogeneous (and highly labile) complexes (see Chapters 1 and 3). Two recombinant strains of *E. coli* harbouring the pneumococcal autolysin (*lytA*) structural gene were available to the present author. One of these strains

(JM109[pJCP401]) had been constructed in the author's laboratory, while the other (DH1[pGL80]) (Garcia *et al.*, 1985) was kindly supplied by R. Lopez. Since *E. coli* does not produce cell wall to which autolysin can bind, both clones express the enzyme in its pure (and stable) E-form. It was expected, therefore, that autolysin prepared from a recombinant source would retain its activity during the purification procedure, thereby much simplifying its identification. The latter part of this Chapter deals with the purification of pneumococcal autolysin from recombinant *E. coli*.

B] GENERAL METHODS

1. Culture Conditions.

Cultures of recombinant *E. coli* which had been maintained at -80°C (see Chapter 2, General Methods) were used to inoculate LB plates containing ampicillin (50 ug/ml) which were incubated at 37°C overnight. Cells from each plate were then transferred into 200 ml TSB^{++} containing ampicillin (50 ug/ml) and shaken for 8 h at 37°C. Ampicillin was present as a selective agent in both the agar plates and the starter broth culture because the cloned pneumococcal genes used in the work described in this Chapter were all carried on recombinant plasmids which conferred ampicillin resistance. The starter culture was used to inoculate two 8-litre bottles of TSB^{++} which were incubated overnight at 37°C with aeration. Final A_{600} was about 1.0.

2. Harvesting of Cell-associated Protein.

The culture was concentrated to 1.5 litres using an Amicon DC-10 ultrafiltration apparatus fitted with an H5-MP01 hollow-fibre cartridge (0.1 um pore size) and a water-cooled jacket. Cells were then pelleted by centrifugation at 27,000 x *g* for 20 min at 4°C, and lysis induced by one of three methods:

a. Cells were resuspended in 60 ml PBS, 5 mg/ml lysozyme, 50 mM EDTA, pH 7.2. Cells were incubated for 1 - 2 h at 37°C, then placed on ice and sonicated for 3 x 4 min at 150 watts, 80% duty pulse, using a Sonifier Cell

Disruptor model B-30 (Branson Sonic Power Co., Danbury, Conn., U.S.A.) fitted with a microtip probe. Microscopic examination of the cells at this stage indicated 70 - 80% lysis.

b. Cells were resuspended in 60 ml 25% (wt/vol) sucrose, 100 mM EDTA, 10 mM Tris-HCl, pH 8.0, and lysozyme was added to a final concentration of 1.3 mg/ml. The suspension was incubated for 30 min at 37°C, 160 ml 1% (vol/vol) Triton X-100, 63 mM EDTA, 50 mM Tris-HCl, pH 6.0 was added, the suspension was placed on ice for 15 min, and then sonicated as for (a). Microscopic examination indicated greater than 90% lysis of cells.

Following methods (a) or (b), the lysate was centrifuged at 27,000 x *g* for 20 min at 4°C, and the supernatant dialysed at 4°C overnight against 2 changes of at least 25 volumes of appropriate column loading buffer, then clarified by further centrifugation at 27,000 x *g* for 20 min at 4°C. The supernatant was then applied to a chromatography column as described in Methods and Results.

c. Cells were resuspended in 60 ml of appropriate chromatography column buffer, then passed twice through an Aminco French Pressure Cell at a pressure of 50 - 60 MPa, after which microscopic examination indicated greater than 95% lysis of cells. The lysate was centrifuged at 27,000 x *g* for 20 min at 4°C, then the supernatant was loaded directly onto a chromatography column as described in Methods and Results below.

C] METHODS AND RESULTS

1. Inactivated Pneumolysin (GPL).

a. Background

The pneumococcal gene encoding pneumolysin has been cloned into *E. coli* (Paton *et al.*, 1986; Walker *et al.*, 1987) and its nucleotide sequence completely determined (Walker *et al.*, 1987). The nucleotide sequences for the genes encoding the related thiol-activated toxins streptolysin-O and listeriolysin have also been determined (Kehoe *et al.*, 1987; Mengaud *et al.*, 1987a). The predicted amino acid sequences for all three proteins contains a single cysteine residue. Although significant homologies between the three proteins were not detectable at the DNA level, there were striking homologies at the amino acid sequence level. In particular, there was absolute conservation of a sequence of 12 amino acid residues which, in each protein, contained the unique cysteine residue (Mengaud *et al.*, 1987a,b). These results strongly suggested that the region around the cysteine was an active site in all three proteins. In addition, the conservation of the cys residue itself and the absolute requirement for reducing conditions for the haemolytic activity of all the toxins suggested that the cys residue was particularly critical.

Oligonucleotide-directed mutagenesis was used by workers in the Department of Microbiology at the University of Leicester, U.K., (G. Boulnois and T. Mitchell, personal communication) to introduce a single DNA base mutation into the cysteine codon (corresponding to amino acid position number 428) of the cloned pneumolysin gene, converting the DNA +strand sequence from 5′-TGT-3′ to 5′-CGT-3′, a triplet which codes for a glycine

residue. Data supplied by Drs Boulnois and Mitchell suggested that the mutant protein retained less than 2% of the haemolytic activity of native pneumolysin. The mutant gene was supplied to the present author as an insert within the *Kpn*I - *Hinc*II sites of pUC18. The resulting plasmid, designated pGLY008, introduced into the *E. coli* host strain JM109, was the source of the glycine-substituted pneumolysin (GPL) whose purification is described below.

b. Assay.

Despite the fact that GPL has a much reduced specific haemolytic activity (in fact, about 0.5% - 0.6% that of the native toxin; see (d) below), it was active enough to be traced, during purification, by the standard haemolytic plate assay which is described in Chapter 2, General Methods. The only modification which was made to this method was the omission of the 2-mercaptoethanol activation step.

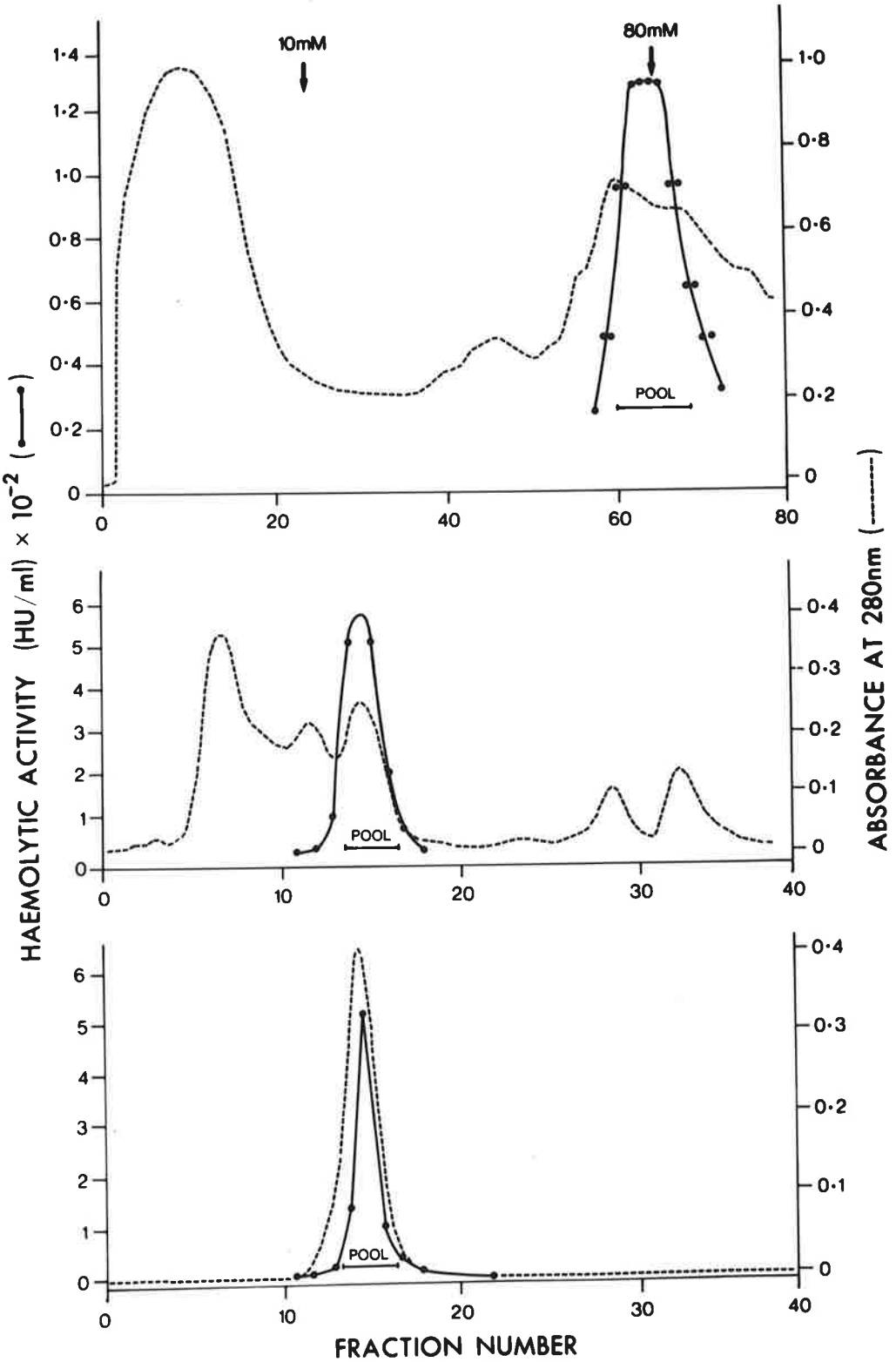
c. Preparative method.

The final method previously devised for the purification of native pneumolysin (see Chapter 3) was taken as a starting point for the purification of the mutant toxin, and, in the event, this proved entirely adequate without modification. Briefly, *E. coli* JM109[pGLY008] lysate (see B 2 above) was loaded onto a column of DEAE-cellulose and eluted by the application of a linear 10 mM - 250 mM gradient of sodium phosphate, pH 7.0. The elution profile is shown in Figure 4.1a. Fractions having haemolytic activity $\geq$ 100 HU/ml were pooled, concentrated, and applied to a column of Sephacryl S-200 (Figure 4.1b). Active fractions were pooled as

Figure 4.1

Chromatographic purification of GPL.

(a) DEAE-cellulose; (b) Sephacryl S-200 (first run); (c) Sephacryl S-200 (second run). Vertical arrows indicate relevant concentrations of sodium phosphate, pH 7.0. Horizontal bars indicate those fractions which were pooled prior to the next step. Haemolytic activity of fractions was determined by the plate assay method (see Chapter 2, General Methods).


10mM
80mM
POOL
POOL
POOL
HAEMOLYTIC ACTIVITY (HU/ml) × 10^{-2} (——)
ABSORBANCE AT 280nm (- - - - -)
FRACTION NUMBER

before and again applied to the Sephacryl S-200 column (Figure 4.1c). Fractions with haemolytic activity $\geq$ 100 HU/ml were then pooled, concentrated, and stored in 50% (vol/vol) glycerol at -15°C.

d. Purity and yield.

Fractions from various stages of the purification method were analysed by SDS-PAGE (Figure 4.2), and the homogeneity of the final material confirmed by PAGE in the absence of SDS (not shown). The final yield was about 30 mg of pure GPL having a specific activity of 6.2 KHU/mg, which represents 0.5% - 0.6% of the specific activity of native pneumolysin.

e. Toxicity.

While 50 ug of native pneumolysin injected intraperitoneally in 200 ul PBS was generally fatal to mice, a similar injection of at least 200 ug of GPL was not. (Higher doses of GPL were not tested). The intravenous toxicity of GPL was also lower. While 5 ug of native pneumolysin injected in 100 ul PBS was almost instantly fatal, mice survived a similar injection of at least 25 ug GPL. (Once again, higher doses of GPL were not tested).

f. Immunological characterization.

Mouse antiserum directed against either PL or GPL was prepared by the procedure described in Chapter 2, General Methods. Gel immunodiffusion analysis then indicated strong immunological cross-reactivity between each antiserum and its homologous and heterologous antigen (Figure 4.3). In addition, mouse antiserum directed against GPL was shown to inhibit the

Figure 4.2

SDS-PAGE (10% gel) of samples from various stages in the purification of GPL.

(a) Ammonium sulphate pellet dialysate (40 ug); (b) post-DEAE (30 ug); (c) post- Sephacryl S-200, first run (10 ug); (d) post- Sephacryl S-200, second run (5 ug). The mobilities of molecular weight marker proteins are indicated. Proteins were stained with Coomassie brilliant blue R250.

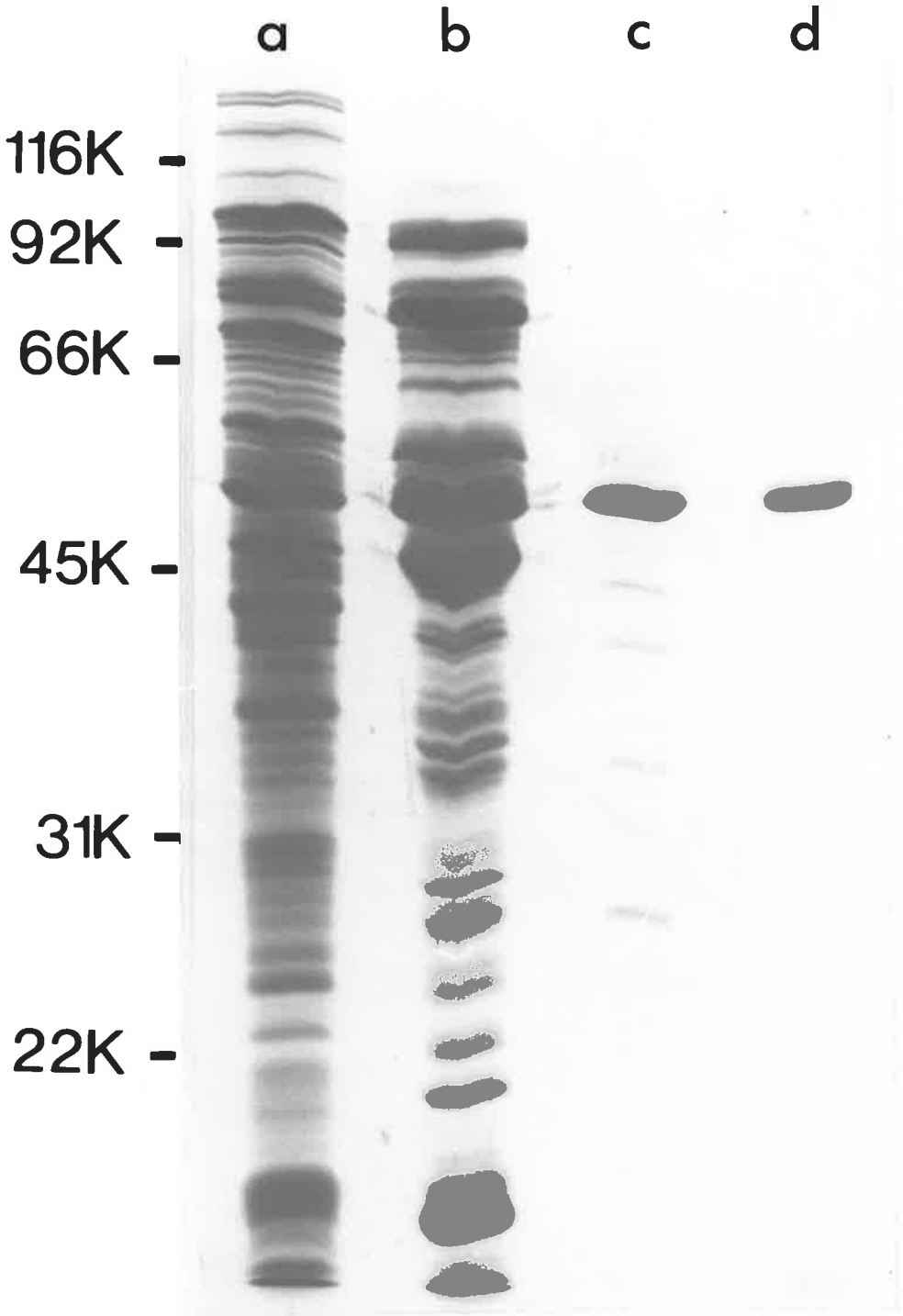
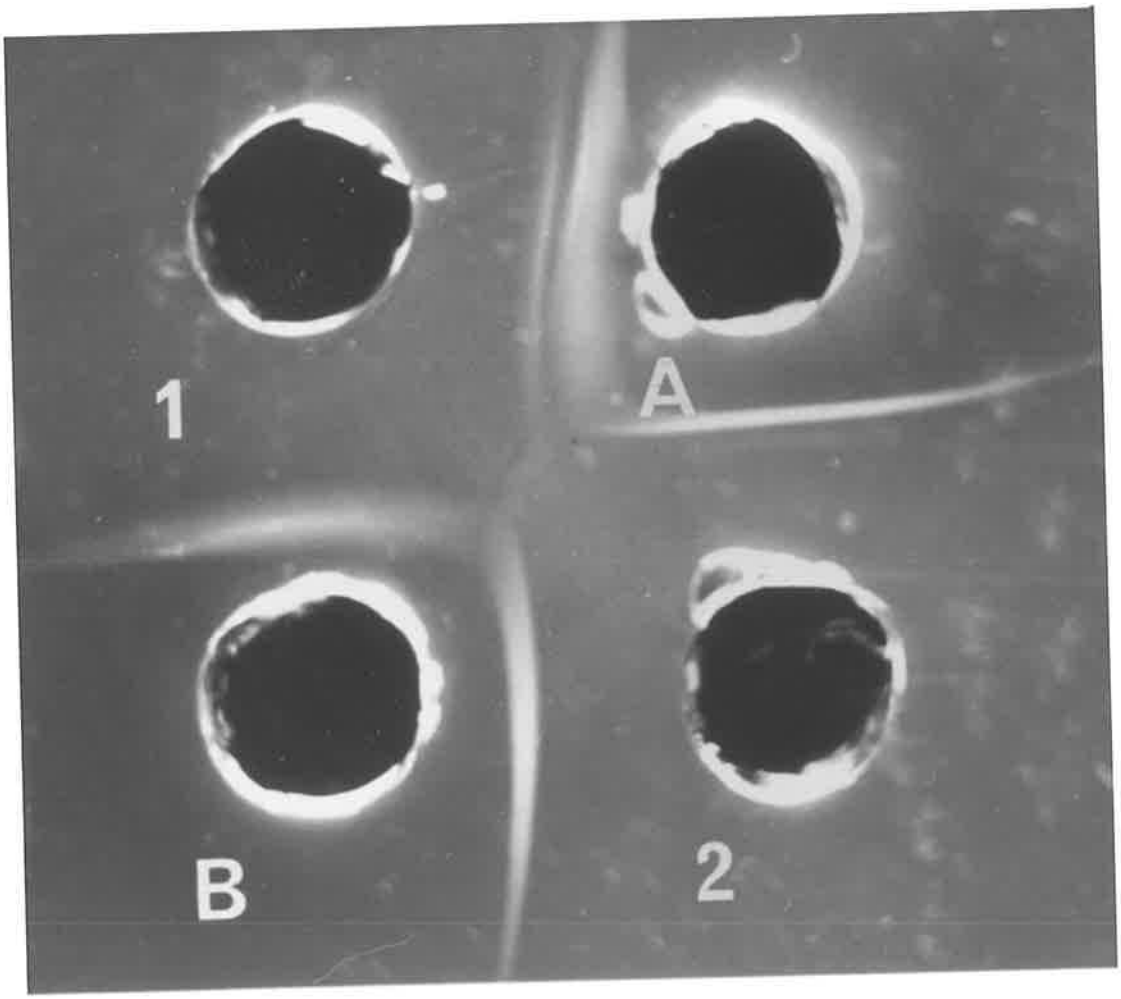

a
b
c
d
116K
92K
66K
45K
31K
22K

Figure 4.3

PL and GPL: Immunological cross-reactivity.

Immunodiffusion gel analysis. (1) GPL; (2) pneumolysin; (A) mouse antiserum raised against GPL; (B) mouse antiserum raised against pneumolysin. Each well contained either 10 ug antigen or 25 ul serum.

(The sera used here were pooled from groups of mice used in immunization/challenge series C: see Chapter 5).


1
A
B
2

haemolytic activity of native PL. In a typical inhibition experiment, the antihaemolytic titre of pooled control (non-immune) mouse serum was about 200, while the titres for pooled anti - PL and anti - GPL sera were about 2,800 and 2,300, respectively.

g. Discussion.

The work presented here shows that GPL, a mutant, inactivated form of pneumolysin, may readily be prepared in pure form from the recombinant *E. coli* JM109[pGLY008] by the application of a method very similar to that previously used for the preparation of the native toxin from cultures of *S. pneumoniae*. About 30 mg of GPL may be prepared routinely from 16 litres of broth culture. The final material, which is homogeneous as judged by PAGE in the presence or absence of SDS, has a haemolytic activity which is no more than 0.6% that of PL. Its intraperitoneal and intravenous toxicities in mice are also much reduced. Mouse antiserum directed against GPL gives a strong immunological reaction with PL, and inhibits the activity of the native toxin almost as effectively as antiserum directed against that protein.

In conclusion, the GPL prepared here was suitable for testing as a protective immunogen in mice.

2. Autolysin.

a. Background

The structural gene for pneumococcal autolysin (*lytA*) has been cloned into *E. coli* by Garcia *et al.* (1985) and its complete nucleotide sequence determined (Garcia et al., 1986b). A recombinant plasmid (designated pGL80) harbouring the *lytA* gene and its accompanying promotor was supplied to the present author by R. Lopez *et al.* for the purposes of the work described in this Section. The host organism into which is was introduced was *E. coli* strain DH1.

The *lytA* gene and its promotor have also been cloned in the author's laboratory (A. Berry, unpublished) as a 1.2 kb chromosomal DNA fragment from pneumococcal strain 3551 inserted into the *Hind*III site of pUC19. The host organism for the plasmid (designated pJCP401) was *E. coli* JM109. The recombinant organism JM109[pJCP401] was made available for the present work. Also provided, for analytical purposes, were AL^- strains of pneumococcus (designated AL-2 and AL-6) which had been prepared by insertion-duplication mutagenesis, and a back-transformant version of AL-6, designated AL-6R, which had been restored to AL^+ phenotype (see Chapter 2, Materials).

b. Assays.

Both the assay for autolysin activity, which is based on the enzymic liberation of [^{3}H-methyl] - choline from insoluble, labelled cell wall, and the anti-autolysin assays which determine the inhibitory activities of sera, are described in Chapter 2, General Methods.

c. Attempts at purifying autolysin from JM109[pJCP401].

In a preliminary experiment, the recombinant organism JM109[pJCP401] was cultured in broth under the conditions described in Chapter 2, General Methods, to an A_{600} of about 0.8, then centrifuged at 27,000 x *g* at 4°C for 15 min and the autolysin activity of the supernatant and cell pellet fractions determined. About 75% of the enzyme activity was associated with the cell pellet. When the cells were lysed by either of the methods using lysozyme (see Sections B 2a and B 2b above), the cell debris removed by centrifugation, the proteins precipitated from the supernatant by the addition of ammonium sulphate and the pellet resuspended in and dialysed into sodium phosphate buffer (see General Methods B 2, above) the dialysate retained a high degree of autolytic activity.

Initially, CM-Sepharose was tried as the first fractionation step, but even at low salt concentration (10 mM sodium phosphate) and reduced pH (pH 6.0), autolysin activity remained largely associated with the bulk of the protein, which failed to bind or bound only very loosely to the column, and little purification was achieved (result not shown).

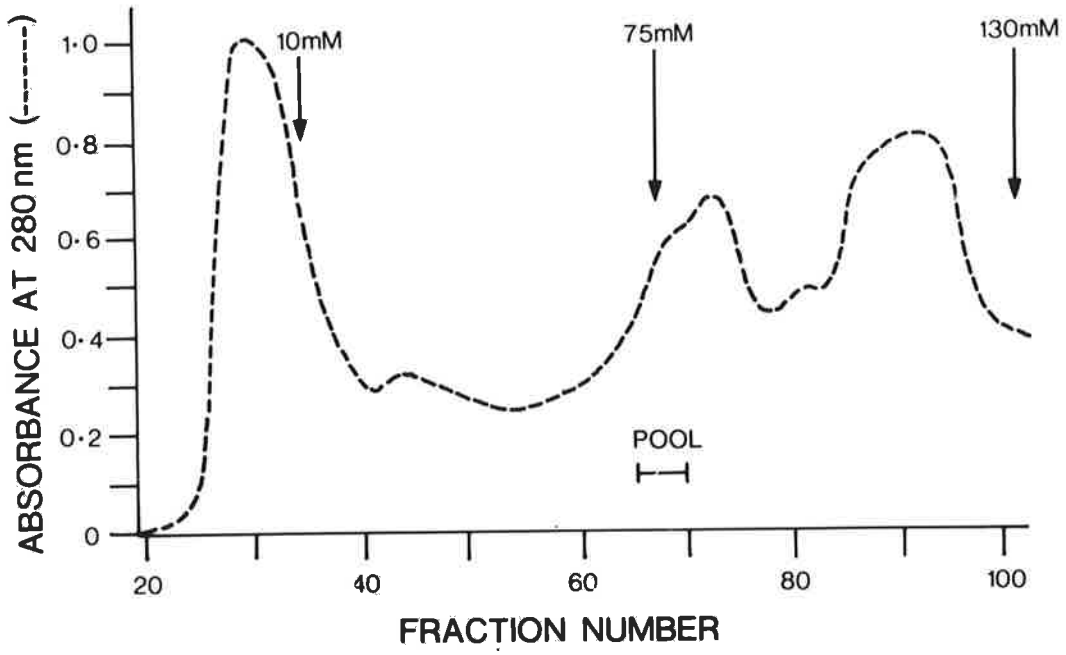
Next, DEAE-cellulose column chromatography was tried under conditions identical to those by which crude protein from *S. pneumoniae* had previously been fractionated: the crude protein mix, in about 400 ml 10 mM sodium phosphate, pH 7.0, was applied to a column (4.4 x 60 cm) of DEAE-cellulose which had been pre-equilibrated with 10 mM sodium phosphate, pH 7.0, and was eluted with a linear 2-litre gradient of 10 - 250 mM sodium phosphate, pH 7.0 at 4°C (Figure 4.4a). No activity was detected in any fractions recovered after DEAE-cellulose chromatography. Because of this, the further fractionation of the enzyme was monitored only by SDS-PAGE of column fractions: the major protein having the appropriate molecular weight (36,500) was assumed to be autolysin. When fractions were analyzed by

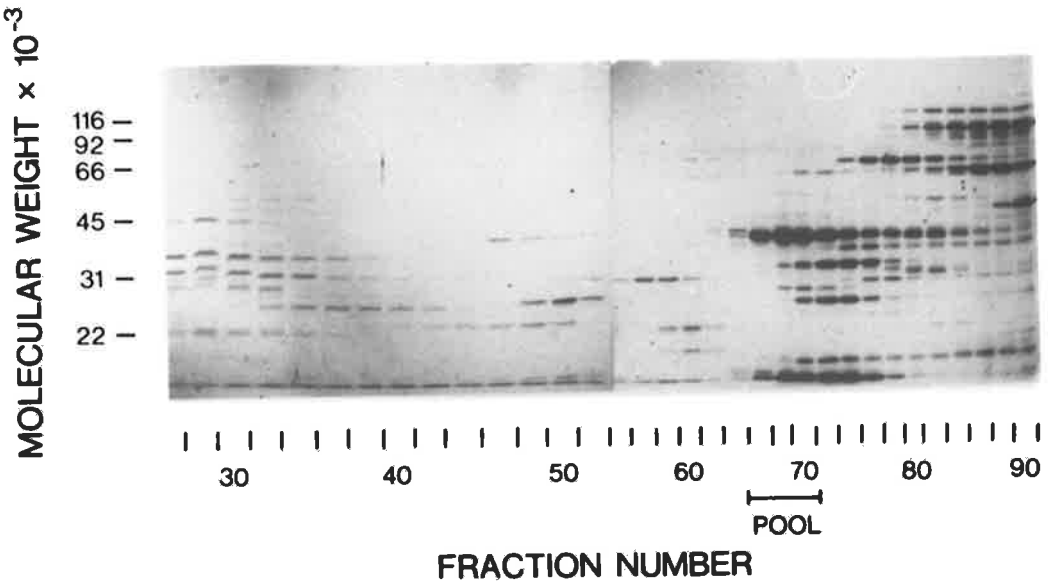
Figure 4.4

Chromatographic purification of 36.5K protein from *E. coli* JM109[pJCP401]: DEAE-cellulose fractionation.

a. (Top): Chromatographic profile. Vertical arrows indicate relevant salt concentrations. The horizontal bar indicates those fractions which were pooled prior to the next step.

b. (Bottom): SDS-PAGE (10% gel) of fractions from the DEAE-cellulose column. The mobilities of MW marker proteins are indicated. The horizontal bar shows pooled fractions.


ABSORBANCE AT 280 nm (------)
1·0
0·8
0·6
0·4
0·2
0
10mM
75mM
130mM
POOL
20
40
60
80
100
FRACTION NUMBER


MOLECULAR WEIGHT x 10^{-3}
116
92
66
45
31
22
30
40
50
60
70
80
90
POOL
FRACTION NUMBER

SDS-PAGE for the presence of the major 36.5K protein species (Figure 4.4b), a substantial purification of this material was observed. Relevant fractions were pooled and concentrated, with diafiltration, into about 10 ml 50 mM sodium phosphate, pH 7.0, then applied to a column (2.6 x 100 cm) of Sephacryl S-200 and eluted with 50 mM sodium phosphate, pH 7.0 at 4°C (Figure 4.5). Once again, fractions were analyzed by SDS-PAGE. Those having the greatest concentration of the 36.5K material were pooled, concentrated and stored in 50% (vol/vol) glycerol at -15°C. The final material was highly pure (>95%) as judged by SDS-PAGE (Figure 4.6[1]), but, at this stage, could not be definitely identified as autolysin because it lacked the appropriate enzymic activity.

d. Comparison of autolysin production between clones.

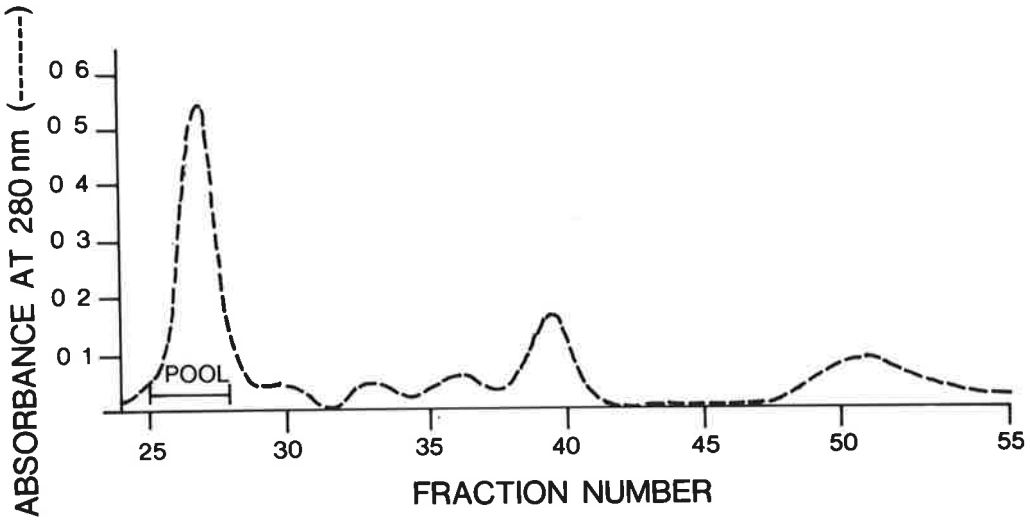
The autolysin production of DH1[pGL80] was then compared with that of JM109[pJCP401]. Both organisms were grown to an A_{600} of 0.72 in LB, lysed by treatment with lysozyme followed by sonication, and assayed for autolytic activity. Strain DH1[pGL80] catalysed the release of 1.00×10^7 DPM per ml cell lysate, while JM109[pJCP401] released 7.76×10^5 DPM per ml, but despite this difference, the intensity of the 36.5K protein band (as visualised after SDS-PAGE) appeared to be approximately the same in lysates from both organisms (Figure 4.7). As DH1[pGL80] expressed more than twelve times the autolysin activity of JM109[pJCP401], it was used in all further experiments on the preparation of the enzyme.

Figure 4.5

Chromatographic purification of 36.5K protein from *E. coli* JM109[pJCP401]: Sephacryl S-200 fractionation.

a. (Top): Chromatographic profile. The horizontal bar indicates those fractions which were pooled prior to the next step.

b. (Bottom): SDS-PAGE (10% gel) of fractions from the Sephacryl column. The mobilities of MW marker proteins are indicated. The horizontal bar shows pooled fractions.


ABSORBANCE AT 280 nm (------)
0 6
0 5
0 4
0 3
0 2
0 1
POOL
25
30
35
40
45
50
55
FRACTION NUMBER

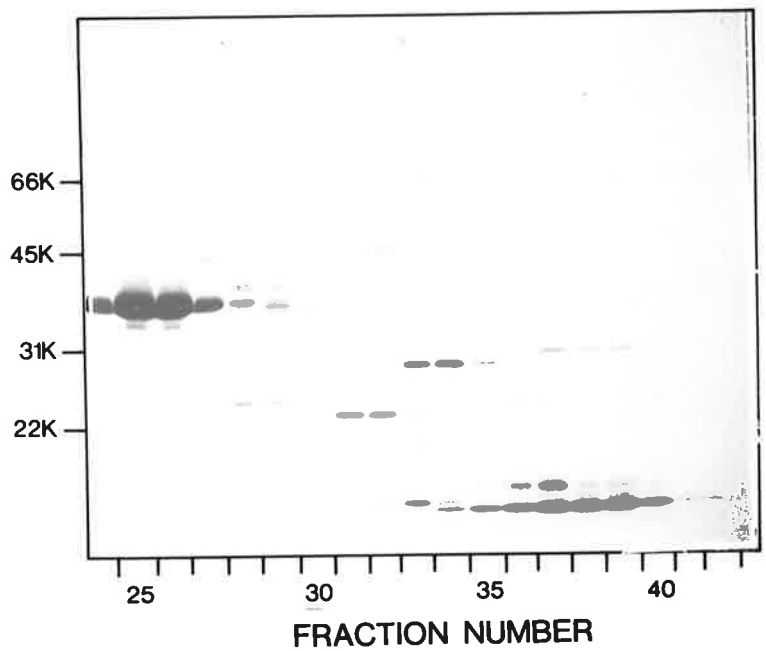

66K
45K
31K
22K
25
30
35
40
FRACTION NUMBER

Figure 4.6

SDS-PAGE (10% gels) of candidate autolysin fractions.

A composite photograph showing SDS-PAGE analysis of various fractions.

1. Purification series for 36.5K protein from JM109[pJCP401] (see Figures 4.4 and 4.5).

(a) Dialysate of the ammonium sulphate precipitate of crude cell protein (30 ug); (b) post- DEAE (15 ug); (c) post- Sephacryl S-200 (10 ug).

2. Final product (post- Sephacryl S-200) 36.5K protein prepared from sonicate of DH1[pGL80] (see Figure 4.9) (15 ug).

The same set of MW markers mobilities (as indicated) applies to both (1) and (2).

3. Final products (post- Sephacryl S-200) from French press lysate of [DH1]pGL80 (see Figure 4.10).

(a) Pool B (8 ug); (b) pool A (8 ug).

Mobilities of MW marker proteins are indicated.

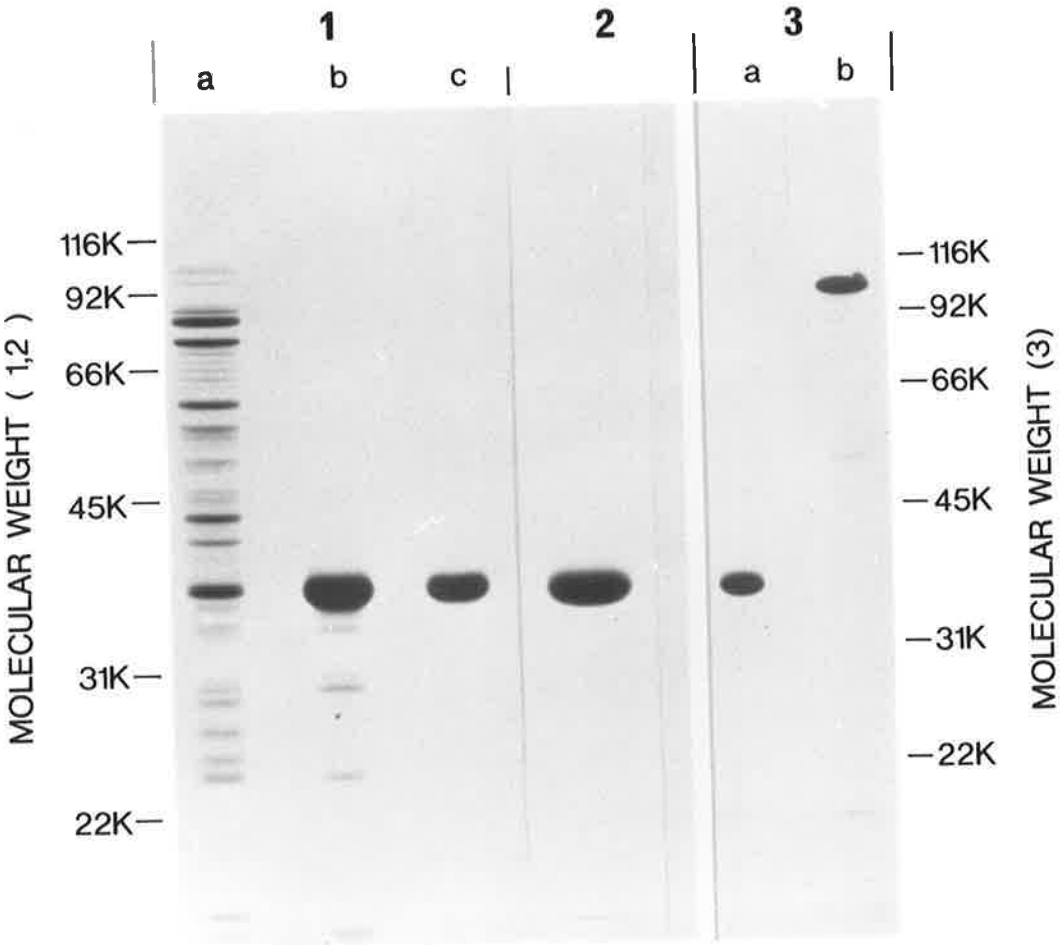
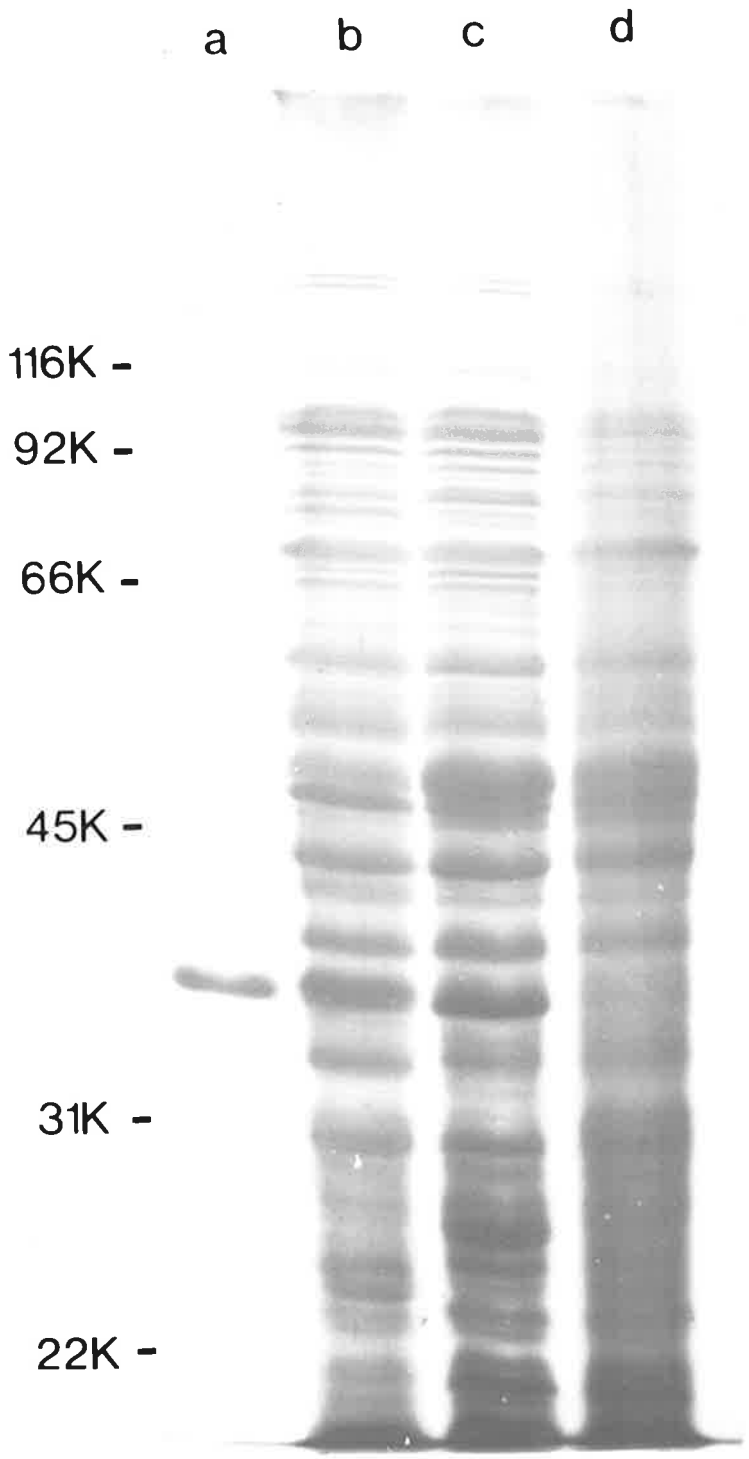

1
2
3
a
b
c
a
b
MOLECULAR WEIGHT (1,2)
116K
92K
66K
45K
31K
22K
116K
92K
66K
45K
31K
22K
MOLECULAR WEIGHT (3)

Figure 4.7

SDS-PAGE (12.5% gel) of *E. coli* lysates.

(a) Marker 36.5K protein prepared from JM109[pJCP401] (5 ug); (b) JM109[pJCP401] crude cell lysate (40 ug); (c) DH1[pGL80] crude cell lysate; (d) DH1 crude cell lysate (40 ug). Mobilities of MW marker proteins are indicated.


a
b
c
d
116K -
92K -
66K -
45K -
31K -
22K -

e. Initial attempts at purifying autolysin from DH1[pGL80].

i. Strain DH1[pGL80] was cultured in broth, and lysed by treatment with EDTA, Triton X-100 and lysozyme followed by sonication, then crude cell protein was prepared as described in General Methods above, and this material was applied to a column of DEAE-cellulose and eluted as described for JM109[pJCP401]. The result is shown in Figure 4.8. Once again, no autolytic activity was recovered, and the putative autolysin-containing fractions had to be identified solely by SDS-PAGE. SDS-PAGE analysis had previously shown (Figure 4.7) that the amount of 36.5K material which could be visualised in lysates of the clone was markedly more than in lysates of the parent organism, and this suggested that DH1[pGL80] expresses the foreign protein with a degree of efficiency high enough to ensure that the major 36.5K protein species in clone lysates was likely to be autolysin. Those fractions which contained the most 36.5K material were pooled, concentrated, and applied to Sephacryl S-200, as before (Figure 4.9), and fractions comprising the major peak were then pooled, concentrated and stored. SDS-PAGE analysis of the final material, which appears to be of >95% purity, is shown in Figure 4.6[2]. The yield was 12 - 17 mg.

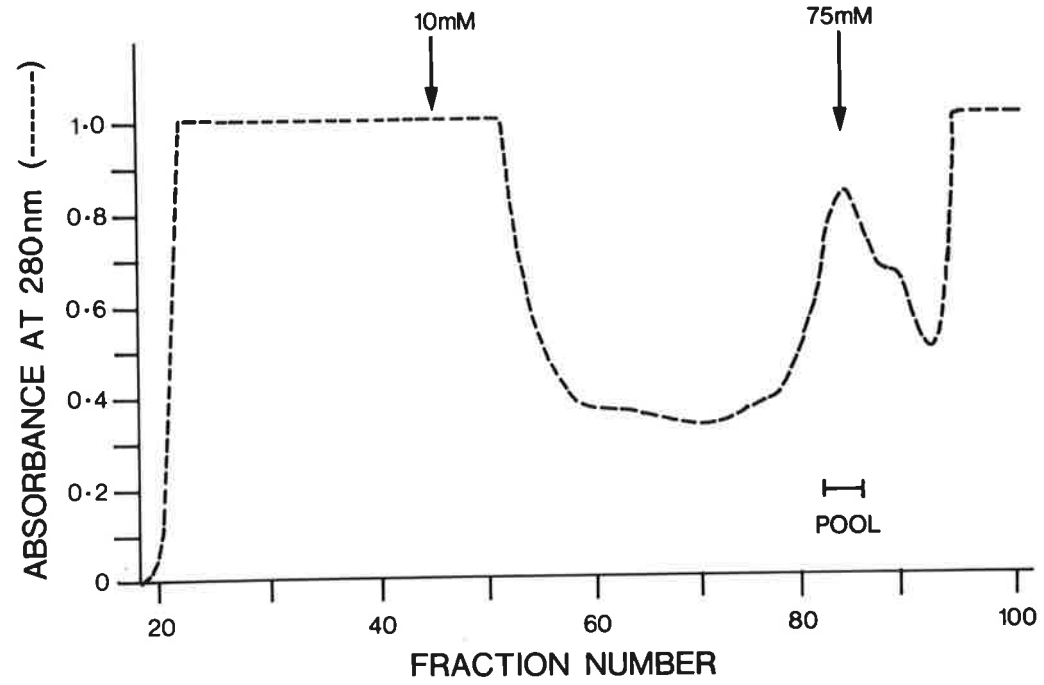
ii. Alternatively, after the DH1[pGL80] cells had been pelleted by centrifugation and resuspended in 50 mM sodium phosphate, pH 7.0, they were passed through a French pressure cell (see Section B 2c above). This method of lysis avoided any of the possible detrimental effects of EDTA, Triton, lysozyme and sonication upon autolysin activity. The lysate was centrifuged to remove cell debris, and the supernatant was loaded directly onto the DEAE column which had been pre-equilibrated with 50 mM sodium phosphate, pH 7.0 (see Section B 2c). This procedure further avoided ammonium sulphate precipitation of the protein and dialysis into low salt (10 mM phosphate)

Figure 4.8

Chromatographic purification of 36.5K protein from sonicate of *E. coli* DH1[pGL80]: DEAE-cellulose fractionation.

a. (Top): Chromatographic profile. Vertical arrows indicate relevant salt concentrations. The horizontal bar indicates those fractions which were pooled prior to the next step.

b. (Bottom): SDS-PAGE (10% gel) of fractions from the DEAE-cellulose column. The mobilities of MW marker proteins are indicated. The horizontal bar shows pooled fractions.


10mM
75mM
ABSORBANCE AT 280nm (-------)
1·0
0·8
0·6
0·4
0·2
0
POOL
20
40
60
80
100
FRACTION NUMBER

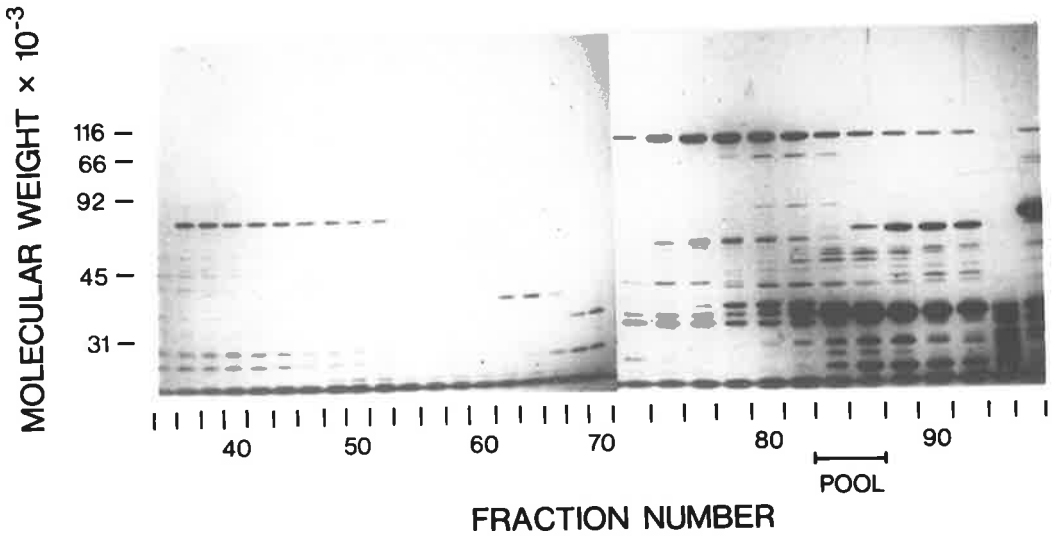
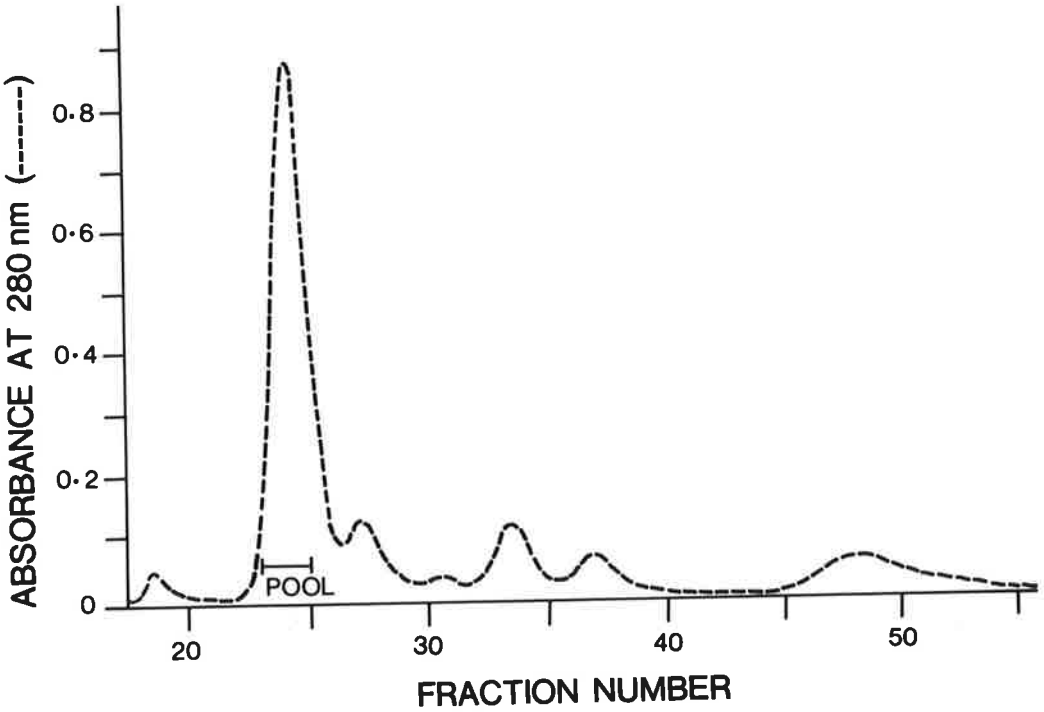

MOLECULAR WEIGHT × 10⁻³
116
66
92
45
31
40
50
60
70
80
90
POOL
FRACTION NUMBER

Figure 4.9

Chromatographic purification of 36.5K protein from sonicate of *E. coli* DH1[pGL80]: Sephacryl S-200 fractionation.

Chromatographic profile. The horizontal bar indicates those fractions which were pooled prior to the next step.


ABSORBANCE AT 280 nm (------)
0·8
0·6
0·4
0·2
0
POOL
20
30
40
50
FRACTION NUMBER

buffer, as well as the 18 - 20 h delay before chromatography during which the crude protein was precipitated, resuspended and dialysed.

Protein was eluted from the DEAE column at 4°C by the application of a linear 2-litre gradient of 50 - 250 mM sodium phosphate, pH 7.0, and this time, enzymic activity was recovered (Figure 4.10a). Active fractions were pooled and concentrated. At this stage, so much protein had been recovered that the sample volume was reduced to 20 ml rather than 10 ml and the material was fractionated through Sephacryl S-200 in two 10 ml batches. Once again, a peak of enzymic activity was recovered (Figure 4.10b). However, SDS-PAGE analysis of the material eluted from the Sephacryl column indicated that the enzymically-active peak (designated "A") consisted very largely of a 102K protein species (i.e. having an apparant MW of 102,000; Figure 4.6 3b). The 36.5K species was found instead in the adjacent Sephacryl peak ("B") which completely lacked autolysin activity (Figure 4.6 3a) . The final yield of this "B" material had been greatly improved by the French press method of lysis: 27 mg of highly pure 36.5K material was recovered from the Sephacryl column. Yet it could still not be identified by activity as autolysin.

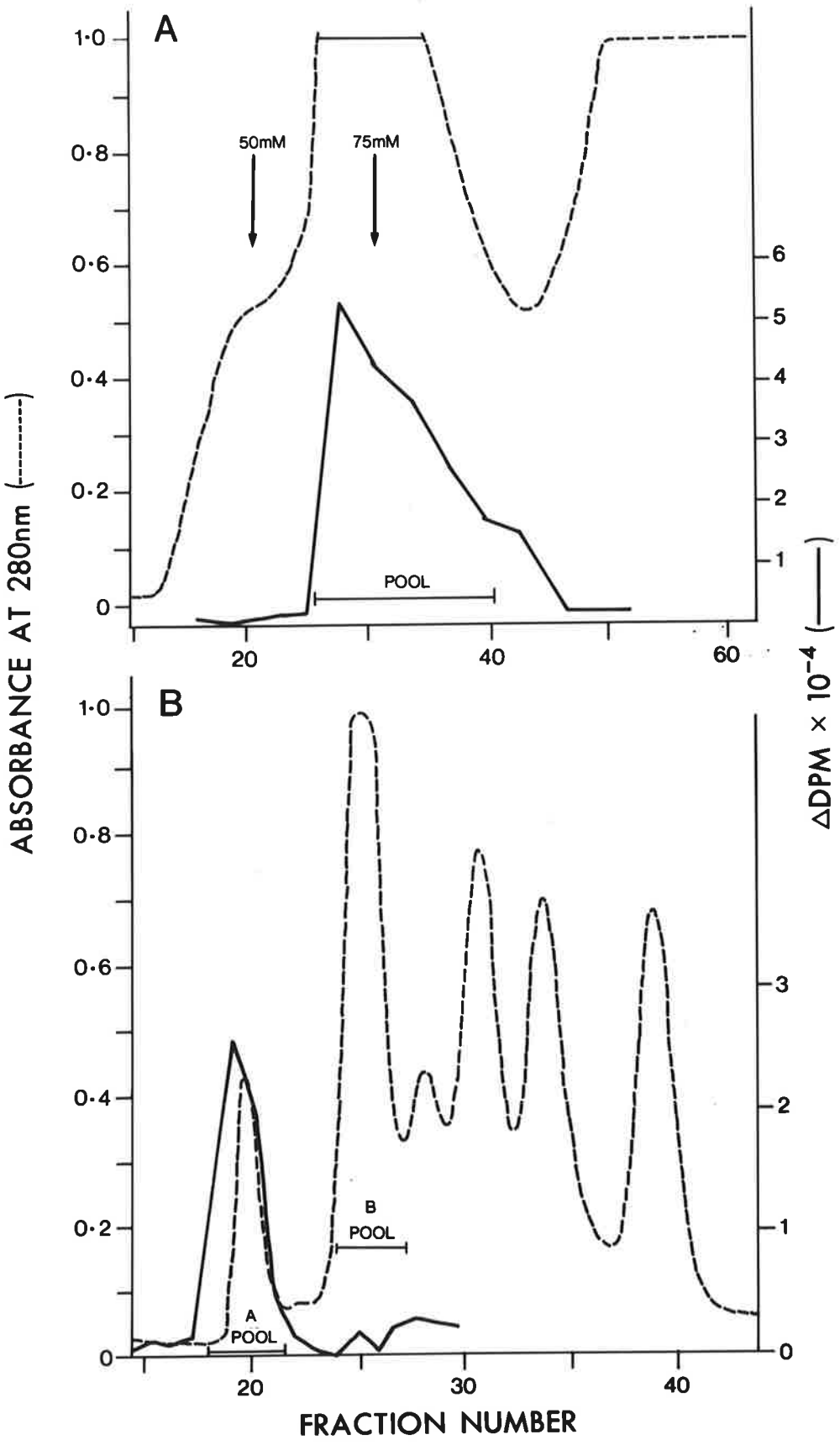
f. Attempts at characterization of candidate "autolysin" fractions.

One way of definitely identifying as autolysin the 36.5K protein species which had been isolated from both recombinant organisms would have been to show that, when administered as an antigen to experimental animals, it directed the production of antibodies which were capable of inhibiting the activity of the native enzyme. However, when administered to Prince Henry strain mice by routine methods (see Chapter 2, General Methods) using dosages of 5, 10, 15, 20, 25, 50 or 100 ug of protein per mouse per

Figure 4.10

Chromatographic purification of 36.5K protein from French-press lysate of *E. coli* DH1[pGL80].

Column chromatographic profiles for each step in the purification are shown. Vertical arrows indicate relevant salt concentrations. Horizontal bars indicate those fractions which were pooled prior to the next step.
a. (Top): DEAE-cellulose. **b.** (Bottom): Sephacryl S-200.


A
1·0
0·8
0·6
0·4
0·2
0
50mM
75mM
POOL
6
5
4
3
2
1
20
40
60
B
1·0
0·8
0·6
0·4
0·2
0
B
POOL
A
POOL
3
2
1
0
20
30
40
ABSORBANCE AT 280nm (--------)
ΔDPM × 10⁻⁴ (——)
FRACTION NUMBER

inoculation, the material was poorly antigenic. Immunodiffusion gel analysis failed to indicate the induction of any antibody to the protein, and sera from inoculated mice, when tested for ability to inhibit autolysin activity, failed to do so.

When the 36.5K material which had been isolated from JM109[pJCP401] was administered to a rabbit (see Chapter 2, General Methods), immunodiffusion analysis (Figure 4.11) indicated that antiserum had been obtained. This antiserum cross-reacted with 36.5K material which had been prepared from sonicated DH1[pGL80], (see (e)*i* above), and with the 36.5K protein of Sephacryl peak "B", which had been prepared from French press lysate of DH1[pGL80] (see (e)*ii* above). However, the antiserum failed to react with the 102K protein of peak "A".

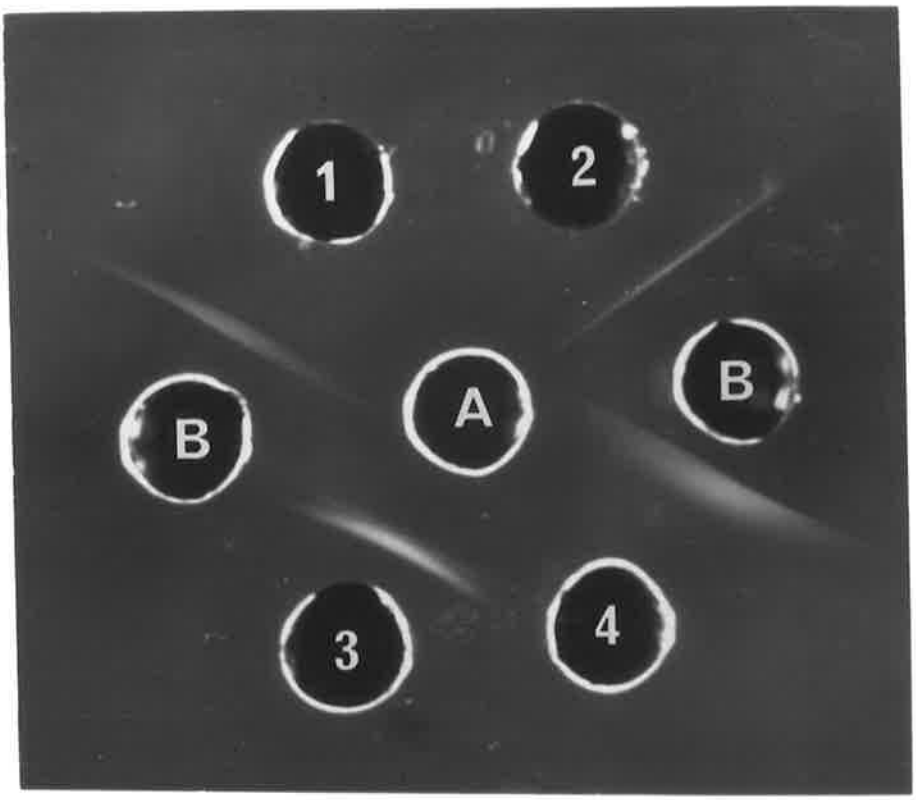
When tested for anti-autolysin activity, the antiserum expressed none.

Next, an attempt was made to identify the 36.5K protein on the basis of binding to phosphoryl choline, a property which is highly specific to autolysin. One milligram of 36.5K protein was concentrated with diafiltration into 2 ml PBS to which was added an equimolar amount of [^{3}H-methyl] - phosphoryl choline (approximately 8 x 10^6 DPM). The mixture was incubated for 1 h at 37^{o}C, then applied to a 50 ml column of Sephadex G-50 pre-equilibrated with PBS, and eluted with the same buffer. The A_{280} of the eluate was monitored in order to identify those fractions containing protein, and an aliquot of each fraction was added to Ready Value scintillation cocktail and its radioactivity determined in a liquid scintillation counter. The results (not shown) indicated a complete separation of protein and phosphoryl choline peaks; the 36.5K protein had entirely failed to bind phosphoryl choline.

Figure 4.11

Immunological cross-reactivity of candidate autolysin proteins.

Immunodiffusion gel analysis. (A) Rabbit antiserum raised against DH1[pGL80] Sephacryl S-200 pool A (see Figure 4.10); (B) mouse antiserum raised against pool B (see Figure 4.10); (1) 36.5K protein from sonicate of DH1[pGL80]; (2) 36.5K protein from sonicate of JM109[pJCP401]; (3) pool A material from French-press lysate of DH1[pGL80]; (4) pool B material from French-press lysate of DH1[pGL80]. Each well contained either 10 ug antigen or 25 ul serum.


1
2
B
A
B
3
4

Finally, Western blot analysis (not shown) indicated that antisera directed against the 36.5K material labelled protein in whole-cell extracts from *E. coli*, but failed to label protein in extracts from *S. pneumoniae*.

Clearly, the 36.5K protein which had been isolated at this stage was not autolysin.

An attempt was now made to characterize the autolytically-active 102K protein which had been purified from French press lysates of DH1[pGL80]. This material was highly antigenic in mice, as indicated by immunodiffusion gel analysis (Figure 4.11). However, anti-102K serum failed to cross-react with the 36.5K protein (Figure 4.11), and did not inhibit the activity of native autolysin.

g. Final procedure for the purification of autolysin from DH1[pGL80].

After the failure of the attempts described above, the following procedure (essentially that of Garcia *et al.*, 1987) was tested and established as effective for the preparation of pneumococcal autolysin from *E. coli* DH1[pGL80]. Purification, which is achieved in a single affinity-column step, depends upon the strong and highly-specific affinity between autolysin and choline.

Firstly, a choline-Sepharose affinity column was prepared as described by Garcia *et al.* (1987). Fifteen grams of epoxy-activated Sepharose-6B (Pharmacia) were swollen in water and washed under suction, with water, on a sintered-glass filter for 1 h. One hundred millilitres of choline-coupling solution (0.1 M NaOH, 1.5 M choline chloride, 0.2 M sodium borohydride, pH 13.0) was added, and the gel was shaken gently for 16 h at

37°C, then packed into a chromatography column (1.6 x 20 cm) and thoroughly pre-equilibrated with 50 mM Tris-acetate, pH 6.9 at 4°C.

E. coli DH1[pGL80] was cultured in 16 litres of medium (as described in Chapter 2, General Methods), and lysed by use of a French pressure cell (also described in Chapter 2, General Methods). The lysate was centrifuged at 27,000 x *g* for 20 min at 4°C, and the supernatant was applied to the choline-Sepharose column, which was then washed, firstly with 4 column volumes of 50 mM Tris-acetate, pH 6.9, 1 M NaCl, and subsequently with 4 column volumes of 50 mM Tris-acetate, pH 6.9 containing 2% (wt/vol) choline chloride. During these washes, A_{280} of the eluate was monitored (traces not shown). The material which was eluted after the first wash retained only about 8% of the autolysin activity present in the crude lysate. The material which was eluted after the second wash retained about 86% of the original enzymic activity, and about 16 mg protein was recovered, in close agreement with the results obtained by Garcia *et al.* (1987). SDS-PAGE analysis of this material indicated that it consisted almost entirely (> 95%) of a single protein species having molecular weight 36,500 (Figure 4.12). Garcia *et al.* did not further purify the material they obtained from their affinity column. In the present study, some attempts were made to remove the minor contaminants which remained in the preparation at this stage. Although they were not removed either by DEAE-cellulose chromatography (eluting with a buffer containing 1.5 M NaCl and 2% (wt/vol) choline, as recommended by Sanz *et al.*, 1988) or by passage through Sephacryl S-200 (not shown), they could be eliminated very effectively by preparative polyacrylamide gel electrophoresis as described in Chapter 2, General Methods (result: see Figure 4.12), However, the losses of material incurred during this step were not considered to be justified, and it was generally omitted from the procedure which was used for the preparation of

Figure 4.12

Purification of autolysin from E. coli DH1[pGL80]: SDS-PAGE analysis (12.5% gel).

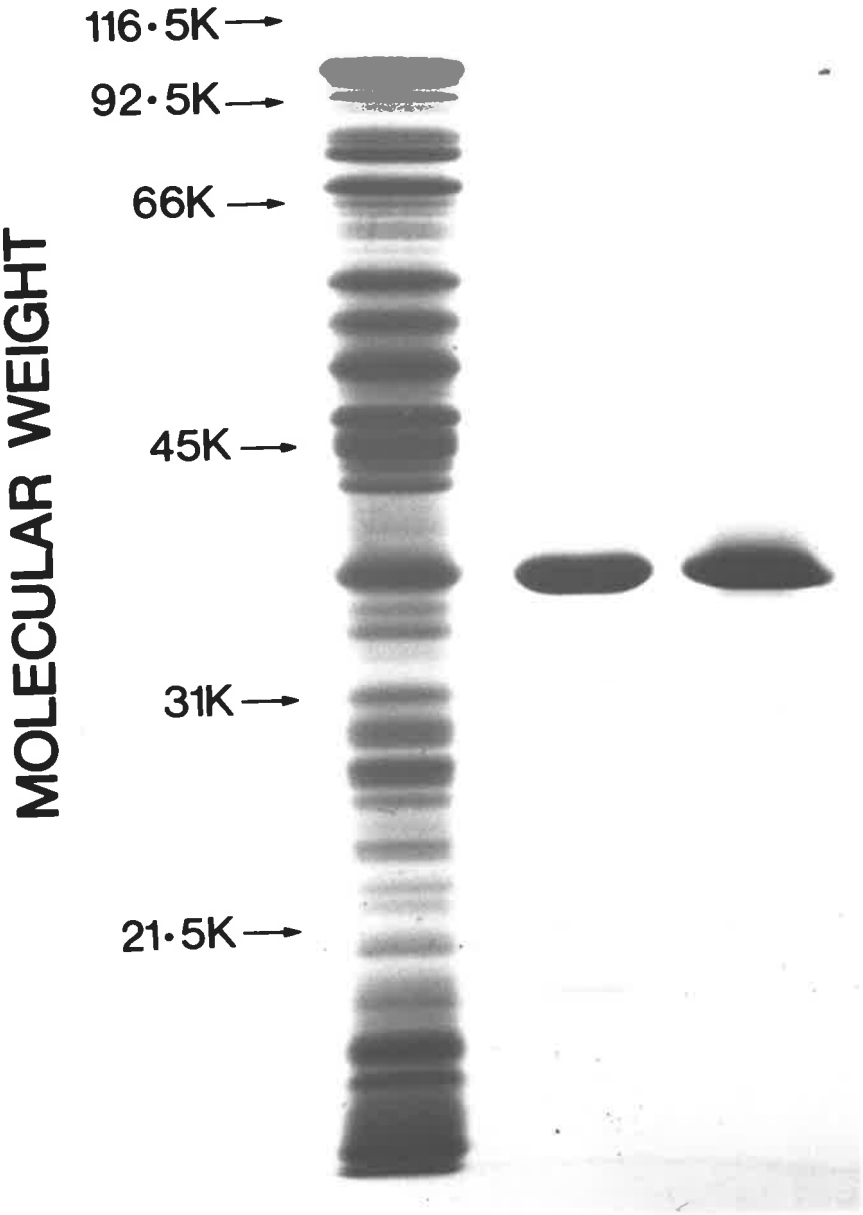
From left to right:

Crude lysate of DH1[pGL80] (40 ug);

Autolysin, post - choline-Sepharose affinity column (8 ug);

Autolysin, post - preparative-PAGE (8 ug).

Mobilities of MW marker proteins are indicated.


MOLECULAR WEIGHT
116·5K
92·5K
66K
45K
31K
21·5K

autolysin for use in the immunization/challenge experiments reported in Chapter 5.

h. Characterization of autolysin.

A 20 ng amount of enzyme was sufficient to solubilize all [^{3}H-methyl] - choline label from 100 ng cell wall substrate in 15 min at 37^{o}C in PBS. The enzyme was also capable of lysing a *lytA*$^-$ mutant of *S. pneumoniae*; when a 100 ml culture of AL-2 (see Chapter 2, Materials) a strain completely lacking autolytic activity, was grown to an A_{600} of 0.7, and the cells were pelleted by centrifugation and resuspended in 5 ml 50 mM sodium citrate, pH 7.0 containing 0.1% (wt/vol) sodium deoxycholate, the addition of 30 ug of purified autolysin caused complete cell lysis after 10 min at 37^{o}C.

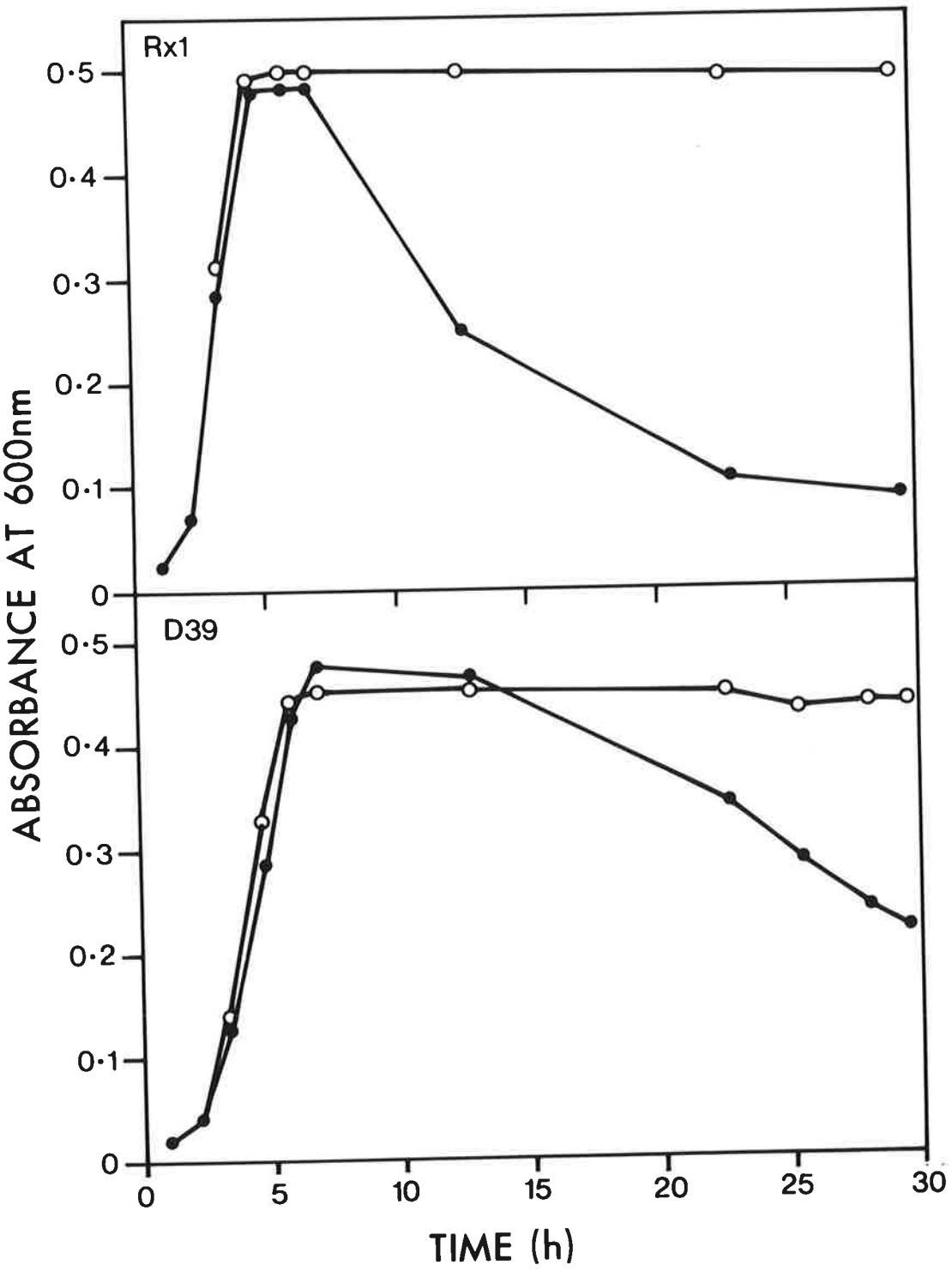
The purified protein was highly antigenic. Mice which had been immnunized with autolysin by the standard method (see Chapter 2, General Methods) produced antiserum which reacted strongly with the protein, as judged by gel immunodiffusion analysis (not shown). The anti-autolysin titres (estimated by the procedure described in Chapter 2, General Methods) obtained for antisera were 320 - 420, while the corresponding titres of control sera were less than 1 (i.e. undiluted control serum did not cause significant inhibition of autolysin activity).

The antiserum was capable of preventing the autolysis of *lytA*$^+$ pneumococcal strains in culture. Either strain D39 or its non-encapsulated derivative Rx-1 was cultured at 37^{o}C in Todd-Hewitt broth in the presence of 5 ul/ml of either anti-autolysin or control serum, and the A_{600} of the cultures was monitored. The results (Figure 4.13) indicate that both strains were effectively protected from autolysis by the antiserum, but not by the control serum, a result which parallels that which was obtained for

Figure 4.13

Effect of anti-autolysin serum on pneumococcal culture density.

A 5 ul/ml amount of mouse serum, either control (●) or anti-autolysin (○) was added at time zero, and the cultures (either Rx-1 or D39, in Todd-Hewitt broth) were incubated at 37°C.


Rx1
D39
ABSORBANCE AT 600nm
0·5
0·4
0·3
0·2
0·1
0
0
5
10
15
20
25
30
TIME (h)

rabbit anti-autolysin serum by Garcia *et al.* (1982), (although these workers tested their sera only on non-encapsulated pneumococci). Microscopic examination of the pneumococcal cultures at maximum cell density also indicated that antiserum had inhibited the separation of daughter cells: in the presence of antiserum, the cultures consisted largely of long chains of cells, while, in the presence of control serum, diplococci predominated.

Anti-autolysin serum was also analysed by the Western blot procedure, in which it was used as a probe against lysates of *E. coli* strains DH1 and DH1[pGL80], and against pneumococcal wild-type strain D39, *lytA*$^-$ (and hence AL$^-$) mutants AL-2 and AL-6 (see Chapter 2, Materials), and the back-transformant version of AL-6, AL-6R, which had been reconverted to AL$^+$ phenotype (also see Chapter 2, Materials). The results (Figure 4.14) indicated that while autolysin serum failed to recognize any protein in DH1 lysate, in lysate from DH1[pGL80] it recognized two proteins, one having MW 36.5K, and one of MW 31.5K. The 36.5K band (but not the 31.5K band) was clearly visualized also in both D39 and in AL-6R, but was absent from the *lytA*$^-$ mutants. Interestingly, all pneumococcal strains probed (but neither *E. coli* strain) expressed an identical pattern of other material which was recognized by anti-autolysin serum. There were two major bands having mobilities corresponding to MW 68K and 92K, and other, poorly-defined material migrating between these.

i. Discussion.

Two recombinant strains of *E. coli*, JM109[pJCP401] and DH1[pGL80], were available as sources of pneumococcal E-form autolysin. Although JM109[pJCP401] was used in some preliminary work, a comparison between this

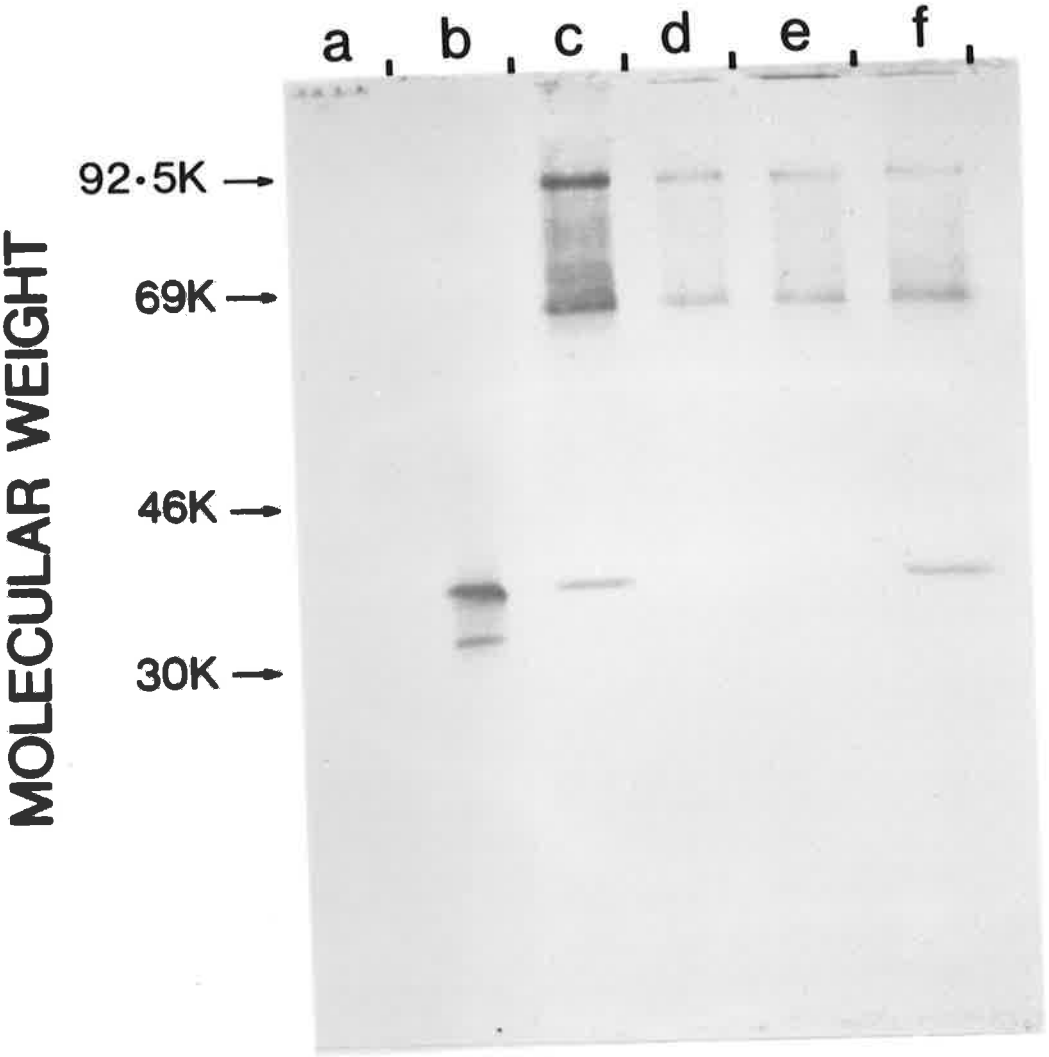
Figure 4.14

Western blot analysis of anti-autolysin serum.

The filter shown was probed with mouse anti-autolysin serum. An equivalent filter probed with control serum was blank. The crude lysates probed were derived from:

(a) *E. coli* DH1; (b) *E. coli* DH1[pGL80]; (c) *S. pneumoniae* D39; (d) *S. pneumoniae* AL-2; (d) *S. pneumoniae* AL-6; (e) *S. pneumoniae* AL-6R.

Equivalent amounts (about 40 ug) of each lysate were loaded. MW mobilities shown were determined by reference to Rainbow MW markers.


a
b
c
d
e
f
92·5K
69K
46K
30K
MOLECULAR WEIGHT

strain and DH1[pGL80] indicated that the latter expressed about twelve times more autolysin activity, and it was used in all subsequent experiments.

During early work, CM-Sepharose chromatography was tried briefly as a first purification step. Autolysin activity survived. Despite this, however, the loss of autolysin activity in subsequent work where DEAE-cellulose chromatography was used as a first purification step was wrongly interpreted as being due to lability of the enzyme, a conclusion which was fostered by the demonstrable lability of C-form autolysin during attempts to purify it from pneumococcal lysates (see Chapter 3). In the absence of autolysin activity, the presence of the enzyme during its purification was monitored by means of MW alone, on the assumption that the major 36.5K species present in the protein mix at any time would be autolysin. (This assumption was based on the observation that SDS-PAGE analysis had indicated 36.5K protein present in much higher amounts in lysates of the recombinant strains than in lysate from the *E. coli* host). Unfortunately, purification procedures guided by molecular weight assays for autolysin lost track of the genuine enzyme completely. The 36.5K material purified after such procedures was eventually shown not to be autolysin, and is, presumably, either a protein native to *E. coli*, or one encoded by the cloning vectors.

(Also identified, along the way, was an *E. coli* protein of MW 102,000 capable of digesting the same assay substrate as autolysin. The existence of this enzyme is not, perhaps, surprising, since pneumococcal autolysin is only one of a "ubiquitous" family of bacterial peptidoglycan hydrolases: see Rogers *et al.*, 1980, for review. Despite the fact that the *E. coli* enzyme can hydrolyse pneumococcal [^{3}H-methyl]-choline cell wall, it is unlikely to be closely related to pneumococcal autolysin because the

antiserum whose production it directs fails to cross-react immunologically with the pneumococcal enzyme).

The impasse over autolysin purification was broken by two recently-published papers detailing single-step purification procedures based on the affinity of different ligands for the autolysin effector site.

The first paper is that of Sanz *et al.*, (1988). These workers showed that the effector site of pneumococcal autolysin has a remarkably high affinity for DEAE-cellulose. Once the enzyme has adsorbed to DEAE, its elution requires the presence not only of a high concentration of salt (1.5M NaCl, a concentration of NaCl higher than that which was used routinely in the present study to remove residual protein from DEAE-cellulose columns prior to their reuse) but also of 2% (wt/vol) choline. As a consequence, it would appear that autolysin which was applied to DEAE-cellulose columns during the course of the unsuccessful attempts at purification described in this Chapter was never eluted from them.

Autolysin molecules in lysates of *S. pneumoniae* which had been grown in the presence of choline would not be expected to display a high affinity for DEAE because they would be in their C-form, with effector sites already blocked by their natural effector, i.e. choline-containing cell-wall fragments. This accounts for the fact that, in work previously reported (see Chapter 3), autolysin activity was recovered after the DEAE-cellulose fractionation of autolysin from *S. pneumoniae* lysates.

The second relevant paper is that of Garcia *et al.* (1987). These authors prepared E-form autolysin from recombinant *E. coli* by adsorbing it to choline which had been immobilized on a Sepharose support, then eluting the pure enzyme from the choline-Sepharose column by the application of high salt containing free choline. This method provided the means by which

autolysin could be prepared reliably as part of the present study in quantities suitable for the immunization/challenge experiments reported in the next Chapter. The pure enzyme was identified not only by its molecular weight and its affinity for choline, but by its activity, against both artificial substrates and the cell walls of *lytA*$^-$ pneumococcal mutants in culture. In addition, it was shown to direct the production of antiserum which, even at a dilution of 1/200, completely prevented at autolysis of *lytA*$^+$ pneumococcal strains, either encapsulated or non-encapsulated, in culture. Western blot analysis showed that this antiserum recognized a 36.5K species present in lysates of *S. pneumoniae* or recombinant *E. coli* having AL$^+$ pheonotype, but not in strains which were AL$^-$.

The Western blot results (Figure 4.14) have two interesting features requiring comment. The first is the existence, in lysate from the AL$^+$ *E. coli* clone DH1[pGL80], not only of a 36.5K protein band recognised by anti-autolysin serum, but also of a band having MW 31.5K. Neither species appears to be an *E. coli* protein, since neither was visualised in the parental *E. coli* strain DH1. The 31.5K species did not appear in pneumococcal lysates. SDS-PAGE analysis (Figure 4.12) of the autolysin preparation used to produce the antiserum indicates no trace of the 31.5K species. Possibly, this species is the *B*-lacatmase which provides the antibiotic resistance marker on pGL80. A strong 31.5K band had previously been visualised (Paton *et al.*, 1986) in analytical gels of protein translated from plasmid pBR322, which also carries the coding sequence for the β-lactamase. This protein may be a very minor but highly antigenic contaminant of autolysin prepared from DH1[pGL80]. Such a possibility could be investigated by repeating the Western blot experiment shown here with lysate of a DH1 strain carrying the parent plasmid to pGL80, pBR325 (Garcia *et al.*, 1986). Another unexpected feature of the Western blot analysis was the appearance, in pneumococcal lysates alone (whether of AL$^+$ or AL$^-$

strains) of an identical pattern of material having MW 68 - 92K. This seems to indicate the presence of heterologous pneumococcal material without autolysin activity but sharing antigenic determinants with the enzyme.

In summary, however, the important conclusion from the Western blot studies is that AL^+ strains, whether *E. coli* or *S. pneumoniae*, express 36.5K material recognised by the antiserum, while AL^- strains, *E. coli* or *S. pneumoniae*, do not. This clearly confirms the identity of the protein which directed the production of the antiserum.

One property of mouse anti-autolysin serum observed in this study was its inhibition of the separation of daughter cells in pneumococcal culture. This parallels effects observed by Garcia *et al.*, (1982), for anti-autolysin sera prepared from rabbits. It is interesting to mention here, in passing, results obtained in the author's laboratory by A. Berry (personal communication) after adding the mouse antiserum prepared here to cultures of the AL^- mutants, AL-2 and AL-6. While small chains of 4 - 6 cells did form in such cultures (a result consistent with those previously obtained by other workers using AL^- mutants: e.g. Sanchez-Puelles *et al.*, 1986) long chains were rare, and their formation was not promoted by the presence of anti-autolysin serum. The formation of long chains appears to require the presence of both autolysin and anti-autolysin. Conceivably, it may be a result of antibody cross-linking of autolysin which is immobilized on the pneumococcal cell wall.

The preparative method described by Sanz *et al.*, (1988) was not used in the present study, except (unsuccessfully) to remove trace contaminants present in material which had already been purified by the method of Garcia *et al.*, (1987). The purity of the autolysin obtained by the latter method alone was judged sufficient for use in further experiments.

D] DISCUSSION

This Chapter details procedures by which GPL, (a toxoid derivative of pneumolysin), and pneumococcal autolysin may be purified from recombinant *E. coli*.

The use of recombinant *E. coli* as sources for pneumococcal proteins provides several benefits.

Firstly, smaller volumes of cell culture are required to obtain useful amounts of pure protein. Pneumolysin and autolysin each appear to constitute only about 0.1% of total pneumococcal cell protein. Such low rates of expression necessitate the handling of large volumes of (virulent) *S. pneumoniae*, if this organism is to be used as the source of the proteins. While, in the present study, autolysin was not successfully prepared from pneumococci, the amount of pure pneumolysin which could be recovered from 54 litres of pneumococcal culture at maximum cell density was only about 4 - 5 mg. The results presented in this Chapter indicate that much greater yields can be obtained from recombinant *E. coli*. Only 16 litres of JM109[pGLY008] yields about 30 mg of GPL, and the same volume of DH1[pGL80] will provide about 15 mg of pure autolysin.

Once the coding sequence for a pneumococcal protein has been introduced into *E. coli*, it becomes amenable to further manipulation resulting in even higher yields: e.g. it may be repositioned downstream from a powerful, inducible promotor which strongly enhances the expression of the product. Indeed, this has already been accomplished in the case of autolysin. The "super-producing" strain of *E. coli* from which Garcia *et al.*, (1987) prepared their autolysin was host to a plasmid which carried the pneumococcal structural gene for autolysin *(lytA)* positioned downstream from both a modified lipoprotein promotor and a lactose promotor. When the

bacterium was grown in the presence of lactose, autolysin accounted for 7% of total cellular protein. The availability of such a strain (and an analogous strain which "superproduces" GPL, if such were constructed) would provide even higher yields of protein from low volumes of cell culture, and would be of considerable benefit to future extensions of the present study, up to and including the commercial production of vaccine antigens.

A second set of advantages stemming from the use of recombinant DNA technology in the present case relates to difficulties arising from specific properties of pneumolysin and autolysin.

In the case of pneumolysin, this property is toxicity. GPL was originally obtained by genetic manipulation of the pneumococcal pneumolysin gene introduced into *E. coli*. Such techniques were necessary to produce a toxoid which differs from native pneumolysin only by one critical amino acid residue, which has only 0.5 - 0.6% of the haemolytic activity of the native protein, and has much-reduced toxicity in mice, while retaining immunological cross-reactivity against pneumolysin.

In the case of autolysin, the problem which is addressed by recombinant DNA technology is the high affinity of the effector site of the enzyme for choline-containing pneumococcal cell wall fragments, a property which causes the enzyme present in pneumococcal lysates to associate as heterogeneous, labile complexes, and renders its purification from such lysates a difficult task (see Chapter 3). Since *E. coli* does not possess a cell wall which binds autolysin, this problem did not arise with the autolysin produced by DH1[pGL80]. Application of the procedure devised by Garcia *et al.*, (1987) (a procedure relying upon the very strong and specific affinity of the enzyme for choline) permitted the rapid isolation of large amounts of pure autolysin.

Another benefit of using pneumococcal protein isolated from recombinant *E. coli* results when only a single pneumococcal protein is present in lysate from each recombinant strain. (This is known to be the case for *E. coli* carrying pGLY008 or pGL80 whose pneumococcal DNA inserts have been precisely characterized, and, indeed, sequenced). The presence of any minor (*E. coli* - derived) contaminants in the final product is then far less critical to the execution of immunization/challenge experiments involving that product, because they would be expected to have no protective effect against challenge by pneumococci.

In summary: the present Chapter details methods for purifying GPL and autolysin from recombinant *E. coli* in amounts large enough to permit an assessment of their efficacy as protective immunogens in mice. The proteins purified from *E. coli* were both shown to be capable of directing the production of antisera which could prevent the functions of the relevant, native pneumococcal proteins *in vitro*: i.e. anti-GPL effectively inhibits the activity of pneumolysin, and anti-autolysin effectively inhibits pneumococcal cell lysis.

CHAPTER FIVE

IMMUNIZATION AND CHALLENGE

A] INTRODUCTION

The work presented in the previous Chapters was concerned with the isolation, in pure form, of (a) the putative pneumococcal virulence proteins pneumolysin (PL), neuraminidase (Neu) and autolysin (AL); and (b) a specifically-modified version of pneumolysin (GPL) having much reduced haemolytic activity.

In the work presented here, the toxicities of these proteins were considered, along with ways by which the toxicities of the more harmful proteins, pneumolysin and neuraminidase, might be reduced by chemical treatment while as much of their antigenicity as possible was maintained. The proteins were then tested for their efficacy as immunogens protecting mice against nasal challenge with *S. pneumoniae*.

B] GENERAL METHODS

1. Immunization of Mice.

The intraperitoneal immunization regime is given in detail in Chapter 2, General Methods. Mice were normally given a course of three intraperitoneal injections of 20 ug antigen, initially presented in Freund's complete adjuvant, then in Freund's incomplete adjuvant. At the end of the course of injections, the mice were bled sub-orbitally and sera prepared for antibody assays.

2. Challenge.

One week after bleeding, intraperitoneally-immunized mice were anaesthetised by one of two methods.

a. Intraperitoneal injection with 1.8 mg of pentobarbitone was used in challenge series A.

b. The method recommended by Green *et al.* (1981) was used in all other challenge experiments: mice were anaesthetised by intraperitoneal injection with 2 mg metomidate and 2 ug fentanyl in 0.2 ml PBS.

After anaesthesis had been induced, a 50 ul amount of a 4 h serum broth culture (i.e. about 5 x 10^6 colony-forming units) of *S. pneumoniae* strain D39 was introduced into the nostrils of each mouse. The mice regained consciousness after about 1 h. The survival time of each mouse was then recorded.

3. Counter Current Immuno-Electrophoresis (CCIE).

CCIE for the detection of pneumococcal capsular polysaccharides was carried out as described by El-Refaie and Dulake (1975). The method is detailed in Chapter 2, General Methods.

4. Radioimmunoassay (RIA) for Antibody to Capsular Polysaccharides.

RIA for the detection of antibody directed against pneumococcal capsular polysaccharide was carried out essentially as described by Schiffman *et al.*, (1980). ^{14}C-polysaccharides, having specific activities 20 - 60 x 10^6 DPM/mg, were supplied by Dr. G. Schiffman, State University of New York, Downstate Medical Centre, Brooklyn, NY, USA. To each 20 ul serum sample was added 0.5 ml (10^4 DPM) of ^{14}C-polysaccharide of an appropriate serotype, and the mixtures were incubated at 37^{o}C for 15 min. A 0.5 ml amount of saturated ammonium sulphate solution was then added and the samples were chilled on ice for 15 min. The samples were centrifuged at 27,000 x *g* for 15 min at 4^{o}C and all traces of the supernatant carefully removed. Pellets were solubilized by the addition of 0.25 ml of 2% (vol/vol) Triton X-100, then 1 ml scintillation fluid (Beckman Ready Value) was added and radioactivity was determined with a liquid scintillation counter. Nanograms of antibody nitrogen per ml of serum were estimated by reference to results obtained from rabbit standard antisera supplied by Dr. K. Amiraian, Health Research, Albany, NY, USA. Specific antibody levels in these sera had been previously determined by quantitative precipitation.

5. Determination of Inhibitory Titres of Antisera.

Inhibitory titres of antisera were determined as described in Chapter 2, General Methods.

6. Statistical Analysis.

The results of nasal challenge experiments were analysed by Mann-Whitney U test (1-tailed).

C] METHODS AND RESULTS

1. Setting Parameters for Immunization.

a. Mice.

Two strains of mice were readily available: Prince Henry and BALB/c.

Initial experiments indicated that both responded well to protein antigens. For example, both strains, receiving pneumolysin as antigen in the standard immunization regime, produced sera having antihaemolytic titres which were 40 - 80 times higher than those of control mice.

However, Prince Henry mice responded much less efficiently than BALB/c mice to pneumococcal capsular polysaccharide antigens. The polysaccharide was presented in the form of the commercial 17-valent polysaccharide vaccine, Moniarix, and was administered sub-cutaneously as a single dose equivalent to 0, 0.1, 0.2, 0.5 or 1 ug of polysaccharide per serotype in 200 ul PBS. Figure 5.1 indicates the antibody titres (determined by RIA and expressed as nanograms of antibody nitrogen per ml serum) produced by mice of both strains for five serotypes: types 2, 3, 8, 18 and 19. Experience in the author's laboratory with clinical samples of sera from children of various ages tested before and after administration of pneumococcal vaccine had suggested that these five serotypes were useful indicators for the response of young humans to the range of pneumococcal capsular polysaccharide types: where vaccinated children were old enough to respond at all to the polysaccharide, their response to serotypes 18 and 19 was generally poor, to types 2 and 8 relatively moderate, and to type 3

Figure 5.1

Antibody titres for BALB/c and Prince Henry mice immunized with Moniarix.

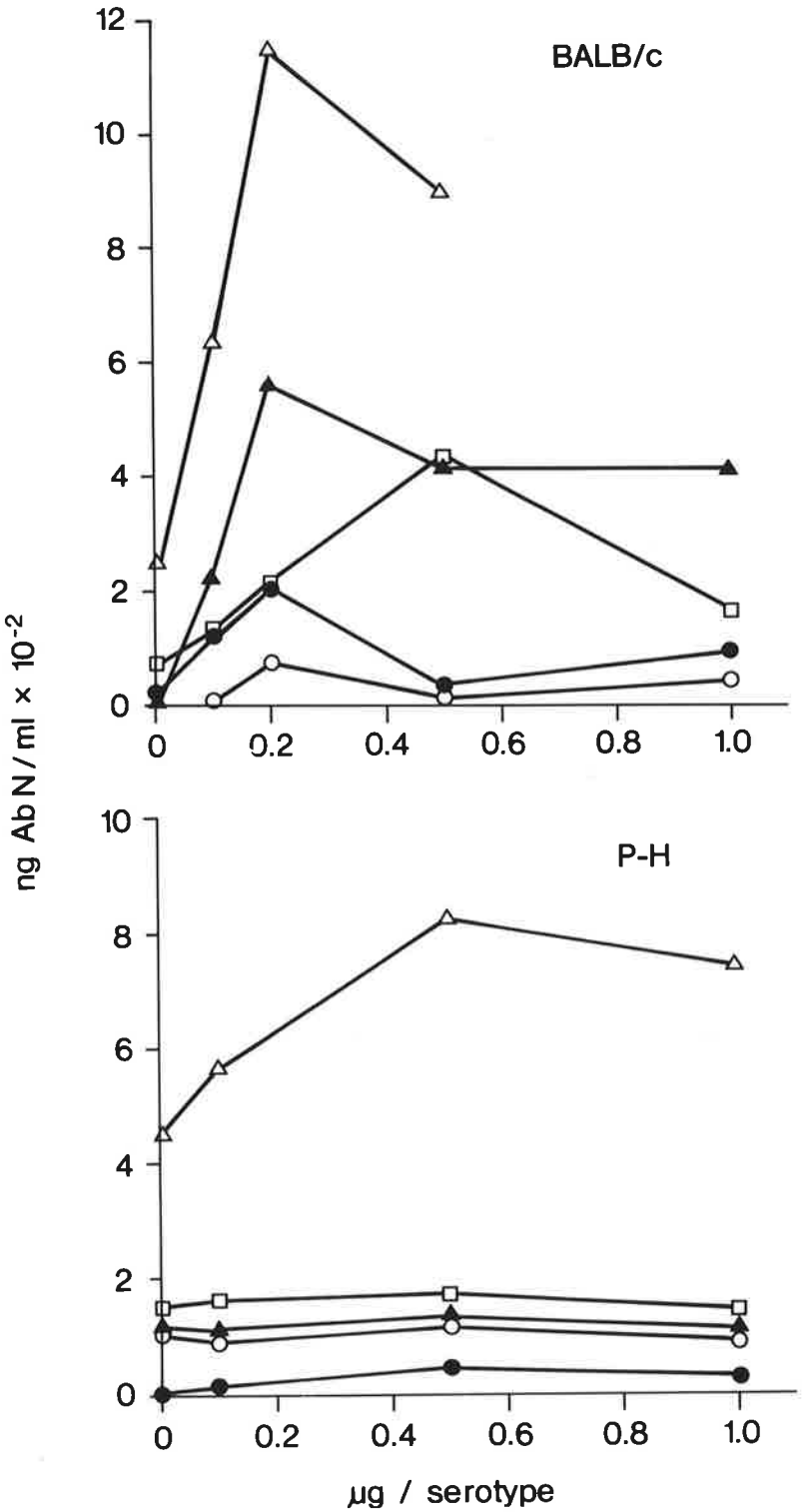
Mice were immunized sub-cutaneously with a single dose of Moniarix 17-valent pneumococcal capsular polysaccharide vaccine in 200 ul PBS, and were bled 2 weeks later.

The vertical axis indicates nanograms of antibody nitrogen per ml of serum ($\times 10^{-2}$).

The horizontal axis indicates polysaccharide dose in nanograms per serotype.

Antibody titres for five representative serotypes are shown:

	Type
▲	2
△	3
●	8
○	18
□	19


BALB/c
P-H
ng Ab N / ml × 10-2
µg / serotype
0
2
4
6
8
10
12
0.2
0.4
0.6
0.8
1.0

relatively good (see also Douglas *et al.*, 1983). While immunized BALB/c mice showed significant increase in antibody nitrogen per ml over non-immunized controls for all five of the representative serotypes, immunized Prince Henry mice showed a significant increase for only one serotype - type 3 - and this increase was, at best, less than 2-fold.

The fact that young Prince Henry mice responded poorly to capsular polysaccharide antigens, but well to proteins, suggested that the strain was useful for modelling the response of young humans to pneumococcal antigens, both polysaccharide and protein. Since this will be of some importance in projected studies with vaccines having both protein and polysaccharide components, the Prince Henry strain was used in all subsequent immunization/challenge experiments in the present study.

b. Modification of the toxicity of antigens.

When a toxic protein is administered in sub-lethal doses to experimental animals while retaining some or all of its activity, the protection it provides as an immunogen may be partially countered or overcome by the detrimental effects due to its toxicity. Ways in which the toxic activity of the protein may be reduced (e.g. by appropriate chemical treatment) while maintaining as much of its antigenicity as possible should therefore be investigated.

i. Pneumolysin

The native protein is toxic; limited data from mice suggested that the LD_{50} dose of active pneumolysin injected intraperitoneally in 200 ul PBS was about 50 ug. When the protein was injected in 100 ul PBS via a tail

vein, a dose of 1 ug was survived, but 5 ug was almost immediately lethal. Attempts were made to reduce this toxicity by chemical means before using the pneumolysin as an immunogen.

Pneumolysin requires reducing conditions for activation. Because of this, in the early stages (i.e. Series A) of the immunization/challenge experiments detailed below, the protein was treated with oxygen before being used as an immunogen. Little inactivation was achieved, however, even after pure oxygen had been bubbled through solutions of pneumolysin for up to 4 h at room temperature.

Since the cysteine residue of pneumolysin evidently has a vital role to play in the haemolytic activity of the toxin, attempts were made to covalently modify the sulphydryl group by carboxymethylation in order to block its participation in the haemolytic process. Pneumolysin was therefore treated at room temperature for 30 min with 1 M iodoacetic acid in 0.75 M Tris-HCl buffer, pH 8.6. This was ineffective: after reactivation with 2-mercaptoethanol, the protein retained full haemolytic activity.

Pneumolysin was also treated with formaldehyde. After overnight incubation at 37^{o}C of a 0.1 mg/ml solution of pneumolysin in PBS in the presence of a 1%, 2% or 3% final concentration (vol/vol) of formaldehyde, the activity of the toxin was reduced by 69%, 96% and >99% respectively. When the concentration of pneumolysin was reduced to only 5 ug/ml, similar degrees of inactivation could be achieved by using only one tenth the concentrations of formaldehyde.

Preliminary experiments (not shown) suggested that pneumolysin which had been 96% inactivated by treatment with formaldehyde retained its antigenicity when administered to mice by the intraperitoneal method, but indicated that there was a fine line between formaldehyde treatment which

accomplished this and treatment which destroyed the antigenicity of the protein completely. No systematic evaluation of the efficacy of formaldehyde-inactivated pneumolysin as a protective immunogen was undertaken because of the subsequent availability of GPL, a stable and highly-antigenic toxoid version of the protein differing from it only by the specific modification of the single cysteine residue at the active site. Formaldehyde-inactivated pneumolysin was tested as an additive to polysaccharide vaccine (administered sub-cutaneeously), and the results of this experiment are presented in c(*ii*) below. All immunization/challenge experiments from Series B onwards, however, used either native pneumolysin, or GPL.

ii. Neuraminidase

Neuraminidase is also toxic to mice. Although a mouse receiving 50 ug of fully-active 107K form neuraminidase intraperitoneally in 200 ul PBS survived, 10 ug administered intravenously in 100 ul PBS was lethal. For this reason, the possibility of inactivating the enzyme by treatment with formaldehyde was investigated. Samples of the 107K form of neuraminidase, at 0.5 mg/ml in PBS, were exposed to concentrations of formaldehyde of between 0% and 7% (vol/vol) at 23^{o}C for 18 h. The effect of this upon the activity of the enzyme is shown in Figure 5.2. Neuraminidase which had been exposed to 3.4% formaldehyde was inactivated by approximately 60% (from about 3,500 units/mg to about 1,400 units/mg) and showed strong reactivity with antisera which had been raised against the native enzyme, as determined by gel immunodiffusion analysis. Higher concentrations of formaldehyde (or more extended exposure to it) reduced enzyme activity further (e.g. 7% formaldehyde for 18 h abolished virtually all activity), but also reduced immunoreactivity (not shown). In all immunization

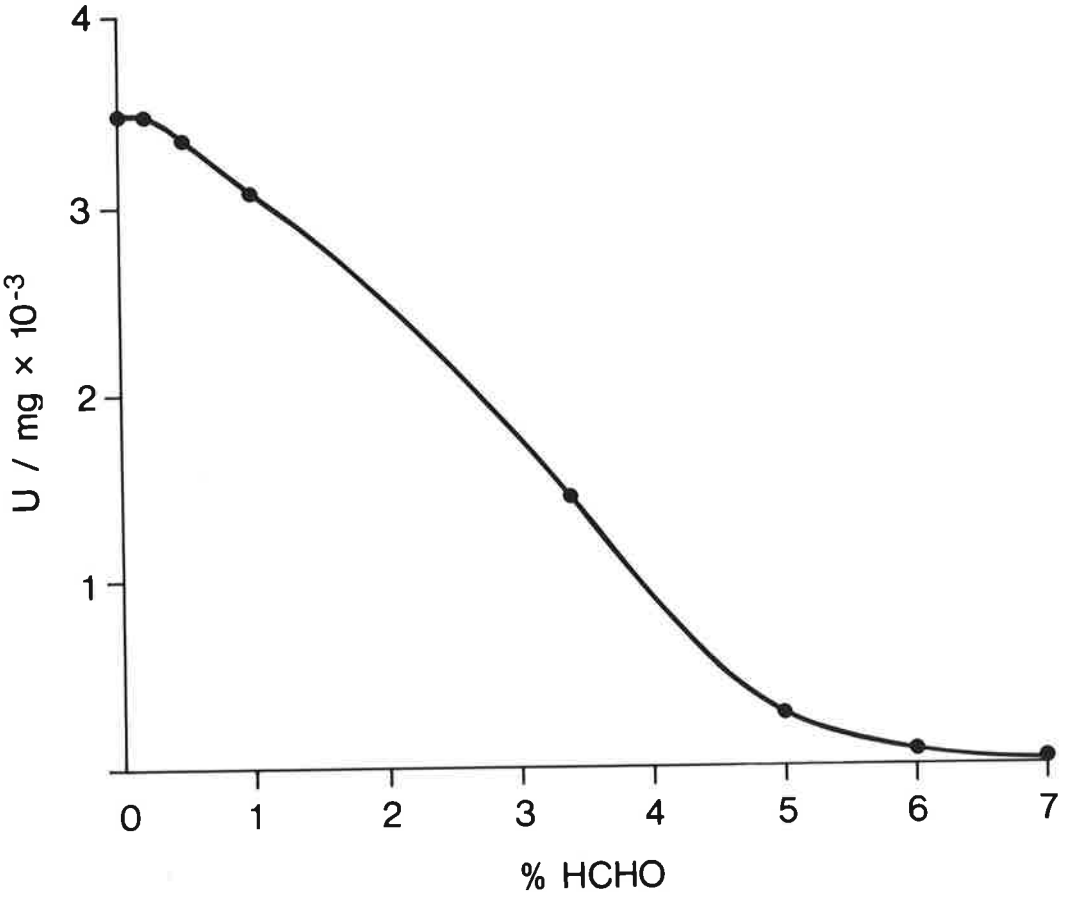
Figure 5.2

Inactivation of neuraminidase by formaldehyde.

Neuraminidase (107K form, 0.5 mg/ml in PBS) was treated for 18 h at room temperature with various concentrations of formaldehyde. The formaldehyde was removed by diafiltration, and specific activity of the neuraminidase samples determined.

The vertical axis shows specific activity in units (fluorimetric assay) per mg x 10^{-3}.

The horizontal axis shows the final concentration of formaldehyde as a percentage (vol/vol).


4
3
2
1
U / mg × 10-3
0
1
2
3
4
5
6
7
% HCHO

experiments where formaldehyde was used to achieve the partial inactivation of neuraminidase, the formaldehyde was thoroughly removed by diafiltration before the protein was administered to mice. F-Neu (i.e. neuraminidase which had been partially inactivated by treatment with 3.4% formaldehyde) was significantly less toxic than the native enzyme: mice were able to survive at least 40 ug of the treated protein when it was injected intravenously in 100 ul of PBS. (Higher doses were not tested).

iii. Autolysin

Since the natural substrate for autolysin is exclusively bacterial in origin, the enzyme was not expected to be significantly toxic and was not pre-treated before use as an immunogen.

iv. GPL

The haemolytic activity of GPL was only about 0.6% that of native, unmodified pneumolysin (see Chapter 4, C 1d), and the protein appeared to have correspondingly low toxicity. Mice survived the intraperitoneal injection, in 200 ul PBS, of 200 ug of GPL, and of at least 25 ug of the protein injected intravenously in 100 ul PBS. (Higher doses were not tested).

c. Dose optimization.

i. Intraperitoneal

The intraperitoneal immunization regime for mice is described in Chapter 2, General Methods. Briefly, it consists of a course of 3 injections of antigen in 50% Freund's adjuvant administered at two week intervals. Using this procedure, antigen doses were optimized (with respect to appropriate inhibitory activity of sera collected 1 week after the final injection) for PL, GPL, F-Neu and AL. Doses of 5, 10, 20 and 40 ug of each antigen were assessed. The results (presented in Figure 5.3) indicated that about 20 ug of antigen per mouse was generally optimal, a 20 - 25 ug dose was used for all protein antigens in subsequent experiments.

ii. Subcutaneous

The subcutaneous route of immunization was tested only for formaldehyde-inactivated PL.

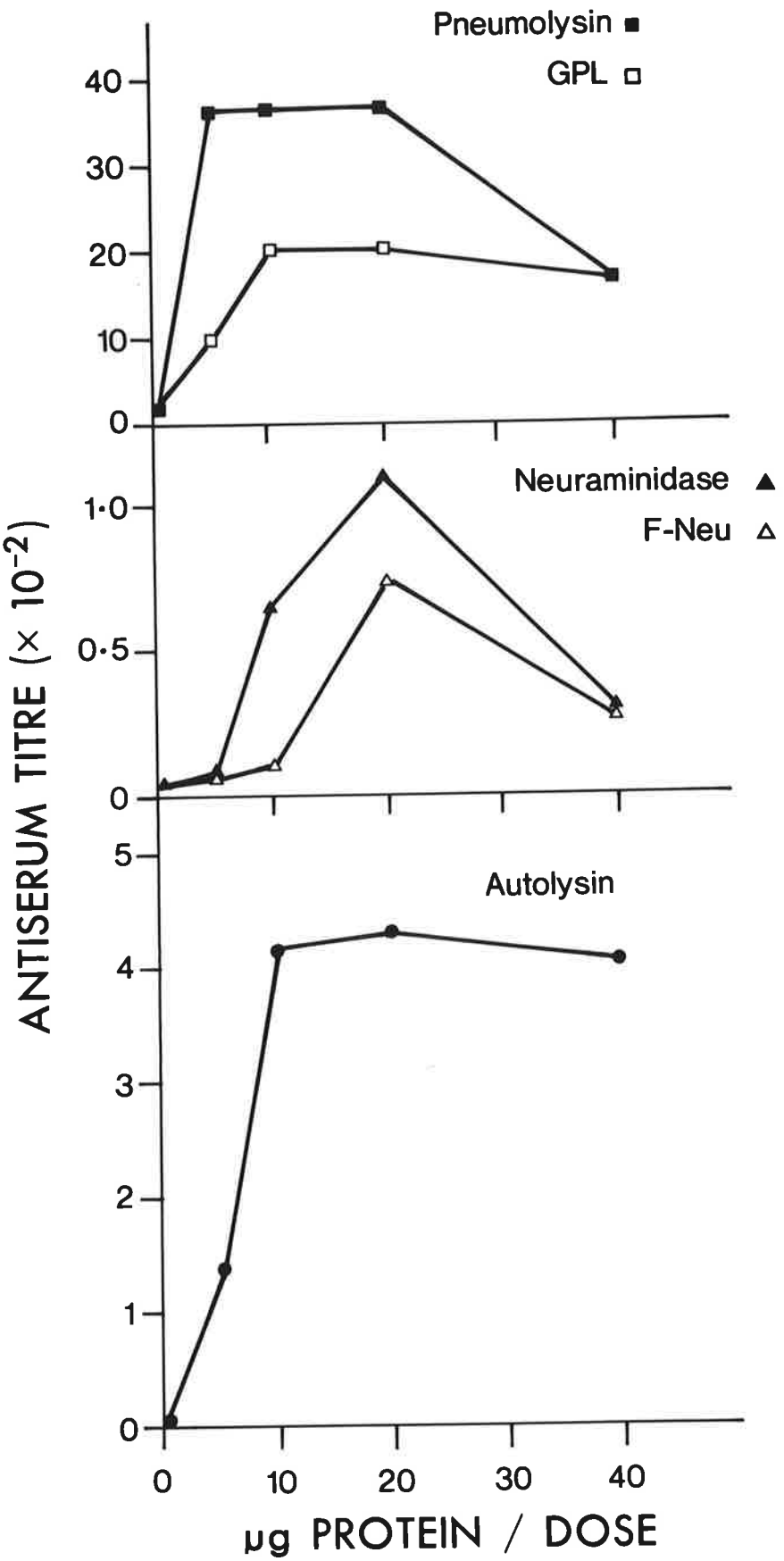
Pneumolysin which had been 99%-inactivated by treatment with formaldehyde was injected subcutaneously into batches of Prince Henry strain mice (five mice per batch). The antigen (0, 5, 10, 15, 50, 100 or 200 ug in 200 ul PBS) was presented in a course of three injections given at two week intervals, and sera were collected one week after the second and third injections. The maximum response was obtained in the mice which had been given two injections of 50 ug PL, but the antihaemolytic titre was only about five times that of control mice. Formaldehyde-inactivated PL (50 ug administered subcutaneously in a single dose to Prince Henry mice) did not affect the response of these mice to the simultaneous injection of

Figure 5.3

Mouse immunization with various antigens: dose response.

Prince Henry strain mice were immunized with varying doses of protein antigen (as indicated) according to the standard method (see Chapter 2, General Methods), and the appropriate antititres of resulting sera were estimated (also by procedures given in Chapter 2, General Methods).

(Note that the apparent large variation in the response of the mice to each of the three groups of antigen stems from the fact that the definition of antititre units in each case is to some degree arbitrary).


Pneumolysin ■
GPL □
40
30
20
10
0
Neuraminidase ▲
F-Neu △
1·0
0·5
0
5
Autolysin
4
3
2
1
0
0
10
20
30
40
ANTISERUM TITRE (× 10⁻²)
µg PROTEIN / DOSE

Moniarix, as assessed by RIA estimation of antibody directed against representative serotypes 3, 7, 8 and 18 (data not shown).

2. Setting Parameters for Challenge.

Mice were challenged with *S. pneumoniae* D39, a type-2 strain. This was chosen because of its high virulence (the mouse intraperitoneal 50% lethal dose was established as being of the order of 10 organisms) and because it was of a different capsular serotype to 3551, the type-1 strain from which pneumococcal pneumolysin and neuraminidase had been isolated. Thus the challenge experiments determined non-type-specific protection.

The intranasal dose of D39 was set at 5×10^6 organisms once it had been determined that this was the minimal dose required to reliably kill > 95% of non-immunized control mice. The intranasal LD_{50} for D39 in non-immunized Prince Henry mice is approximately 5×10^4 organisms (Berry et al., 1989b).

Anaesthetic made the introduction of the pneumococcal culture into the nostrils of the mice easier, suppressed the sneezing reflex, and was expected also to suppress the ciliary activity of the respiratory epithelial cells which acts to clear the pneumococci from the respiratory tract (Svanborg Eden *et al.*, 1987). The dose of anaesthetic was adjusted so that the sneezing reflex was completely suppressed during the challenge. The mice then spent an average of about one hour unconscious.

The pneumococcal culture was introduced into the nostrils in a 50 ul volume of serum broth culture using an Eppendorf automatic micropipette.

Figure 5.4

Effect of immunization with pneumolysin on the survival time of mice challenged with S. pneumoniae.

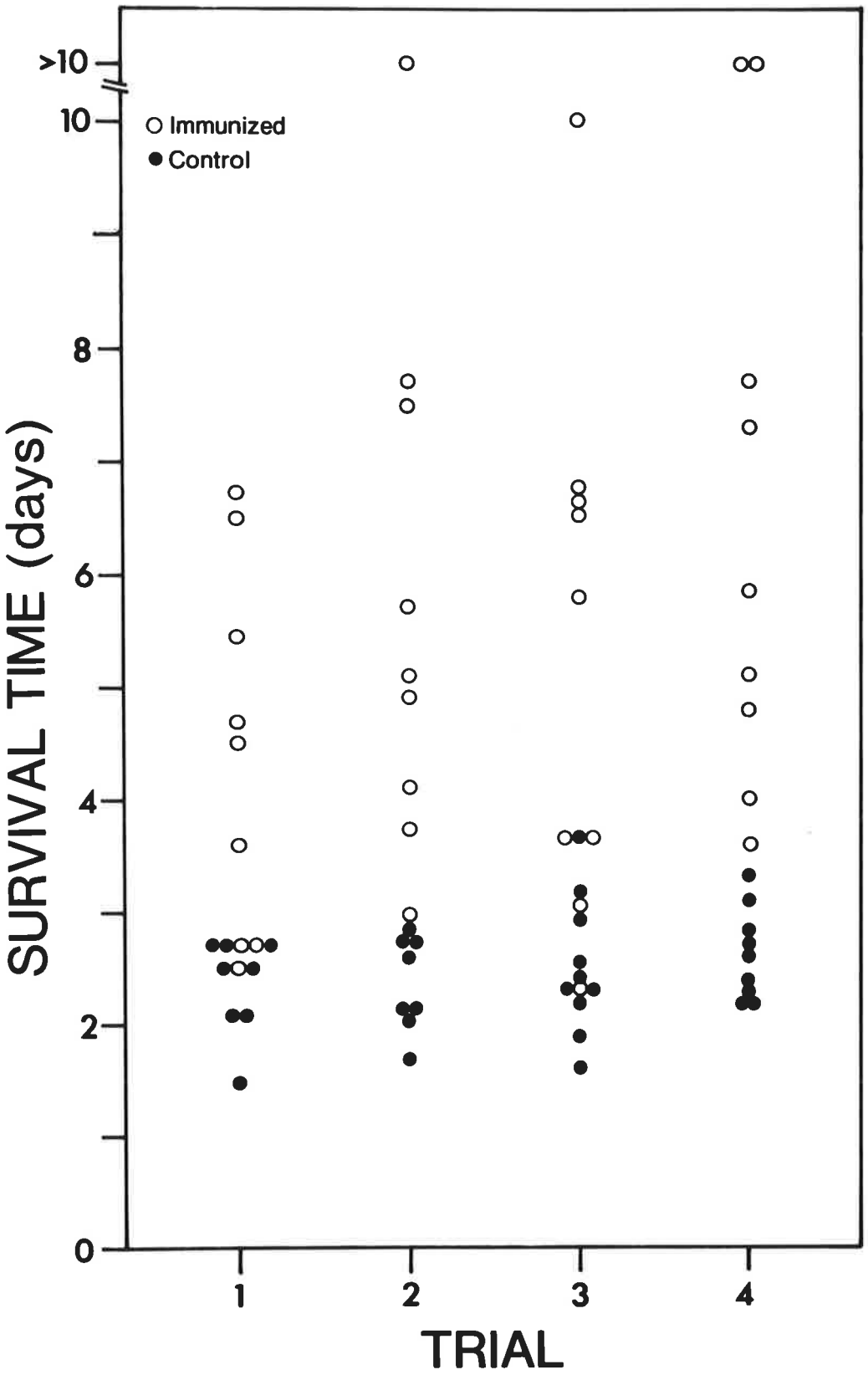
See text, immunization/challenge series A.

The survival time of immunized mice (○) and control mice (●) is indicated for each of four separate trials.

3. Immunization/Challenge Experiments.

Series A: Pneumolysin

Four groups of mice, with 8 to 10 mice in each group, were immunized with pneumolysin (20 ug per injection) which had been prepared by the old method (see Chapter 3, C 1e) and partially inactivated by exposure to oxygen. All immunized mice gave sera which produced a single preciptin line when tested by immunodiffusion gel analysis against either highly purified pneumolysin or a crude (post - ammonium sulphate) pneumococcal extract, but no precipitin lines were visible when sera from control mice were similarly tested (not shown). All sera were tested by radioimmunoassay for the presence of antibody to type 1 and type 2 pneumococcal capsular polysaccharides, and no significant difference in antibody levels between control and immunized mice was detected for either serotype. Sera were also assayed for antihaemolytic activity, and the geometric mean antihaemolytic activity for immunized mice was about 38 times that for control mice. Mice were anaesthetised by intraperitoneal injection with pentabarbitone, and challenged intranasally with about 5×10^6 colony-forming units of D39. All mice died within 10 days, with the exception of three immunized mice which were still alive and well after 20 days. A type 2 *S. pneumoniae* could be isolated from the heart blood of each mouse that died as a result of the challenge, and histological examination revealed evidence of pneumonic consolidation in both control and immunized mice at the time of death. The survival time of each mouse was recorded, and the results for the four separate trials are shown in Figure 5.4. In each trial, the median survival time of immunized mice was greater than that of control mice. The overall median survival time for mice immunized with pneumolysin was 5.50 days, compared with 2.50 days for control mice. When the results were analyzed by


>10
10
8
6
4
2
0
SURVIVAL TIME (days)
○ Immunized
● Control
1
2
3
4
TRIAL

the Mann-Whitney U test (1-tailed) the observed differences were highly significant, with p values less than 0.005, 0.001, 0.002 and 0.001 for trials 1 to 4, respectively.

Series B: Neuraminidase

i. Two groups of ten mice were used, control mice receiving a course of intraperitoneal injections from which protein had been omitted, and immunized mice receiving active 86K form neuraminidase. The antineuraminidase titre achieved by control mice was about 10, while that of immunized mice was about 80. When the mice were subsequently challenged (Figure 5.5), 3 immunized mice and 1 control mouse survived for longer than 14 days, but this difference was not statistically significant. The median survival time for immunized mice (2.40 days) was also not significantly different from that of control mice (2.50 days).

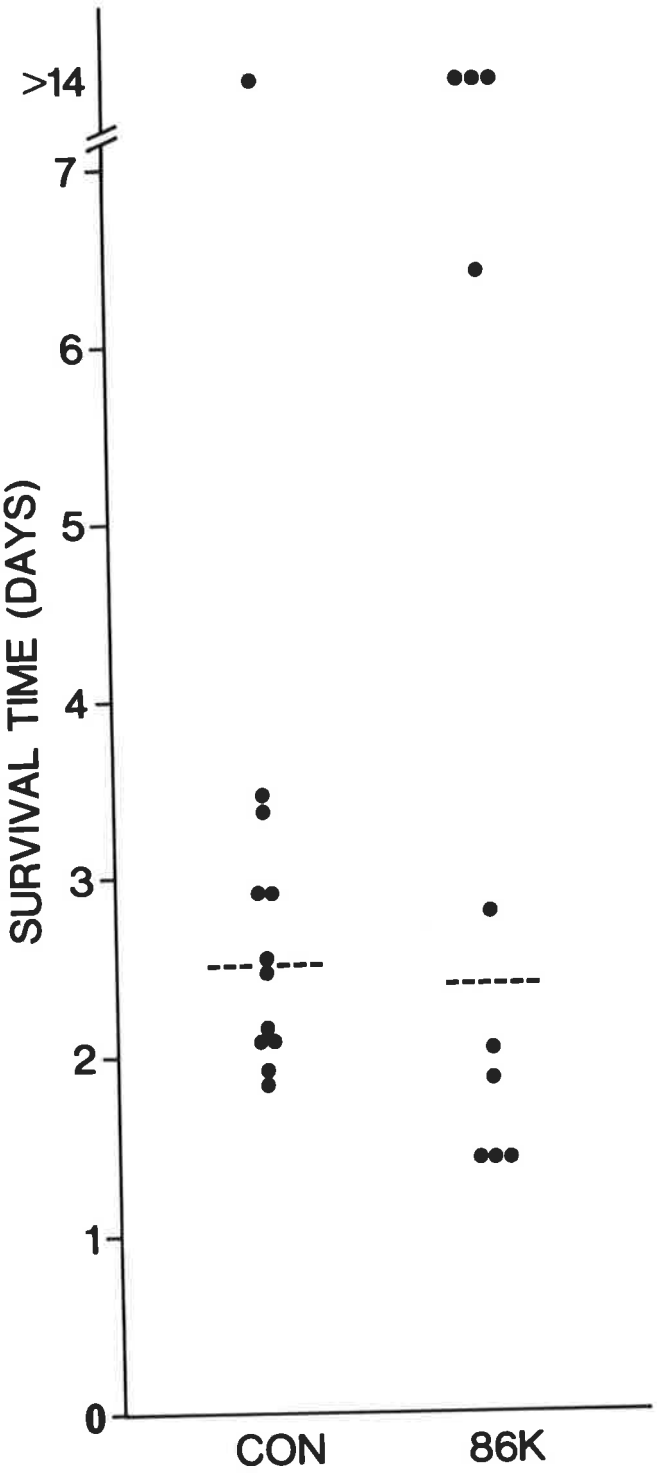
ii. The second set of experiments in this series used mice (10 - 12 per group) which were immunized with either the 107K form of neuraminidase (Neu), neuraminidase which had been pre-treated with 3.4% formaldehyde (F-Neu), pneumolysin (PL prepared by the final method described in Chapter 3) or a combination of F-Neu and PL. The antigen dose was either 25 ug (per injection) of a single protein, or 25 ug of each of two proteins. Serum samples collected from mice seven days after their last injection were examined by gel immunodiffusion, and strong immunoreactivity was observed between all sera and appropriate antigens (not shown). No antibody to pneumococcal capsular polysaccharide was detected by radioimmunoassay.

Figure 5.5

Effect of immunization with 86K - form neuraminidase on the survival time of mice challenged with S. pneumoniae.

See text, immunization/challenge series B (*i*).

The survival times of control mice (CON) and immunized mice (86K) in a single trial are shown. The broken lines indicate the median survival times for each group.


SURVIVAL TIME (DAYS)
>14
7
6
5
4
3
2
1
0
CON
86K

Antineuraminidase and antipneumolysin titres of sera were determined: the results (Table 5.1) indicated that substantial inhibitory activities had been achieved. Mice were anaesthetized by intraperitoneal injection with metomidate and fentanyl before challenge, the results of which are given in Figure 5.6. Median survival times are presented in Table 5.2, and the significance of differences between groups are given in Table 5.3; these data are summarized below, along with those of the next set of experiments in this series.

iii. The third set of mice was the same as the second except that the Neu group was omitted and the number of mice in each of the other groups was increased to 15 - 18. Once again, immunodiffusion analysis indicated strong immunoreactivity between all sera and the appropriate antigens (not shown). The results of challenging the second set of mice are given in Figure 5.7, median survival times are presented in Table 5.2, and the statistical significance of differences between groups are given in Table 5.3.

Taken together, the first three sets of experiments in Series B indicate that, while immunization with Neu (in either its degraded, 86K form, or its intact 107K form) had no protective effect, mice which had received F-Neu gained a modest but reproducibly significant increase in median survival time (3.20 days vs 2.50 days; $p < 0.05$, and 2.75 days vs 2.25 days; $p < 0.01$ in two independent experiments). Mice which had been immunized with PL alone survived substantially longer than control mice (4.50 days vs 2.50 days and 3.90 days vs 2.25 days at $p < 0.01$ and $p < 0.001$, respectively) confirming the results of Series A. These mice also survived significantly

TABLE 5.1

Antineuraminidase and antipneumolysin titres of pooled mouse sera.

Immunogens	Anti-Neu	Anti-PL
Control	< 10	< 200
PL	< 10	2,800
Neu	110	< 200
F-Neu	85	< 200
PL plus F-Neu	70	3,100

Figure 5.6

Effect of immunization with Neu, F-Neu, PL, and [F-Neu + PL] upon the survival time of mice challenged with S. pneumoniae.

See text, immunization/challenge series B (*ii*).

The broken lines indicate median survival times.

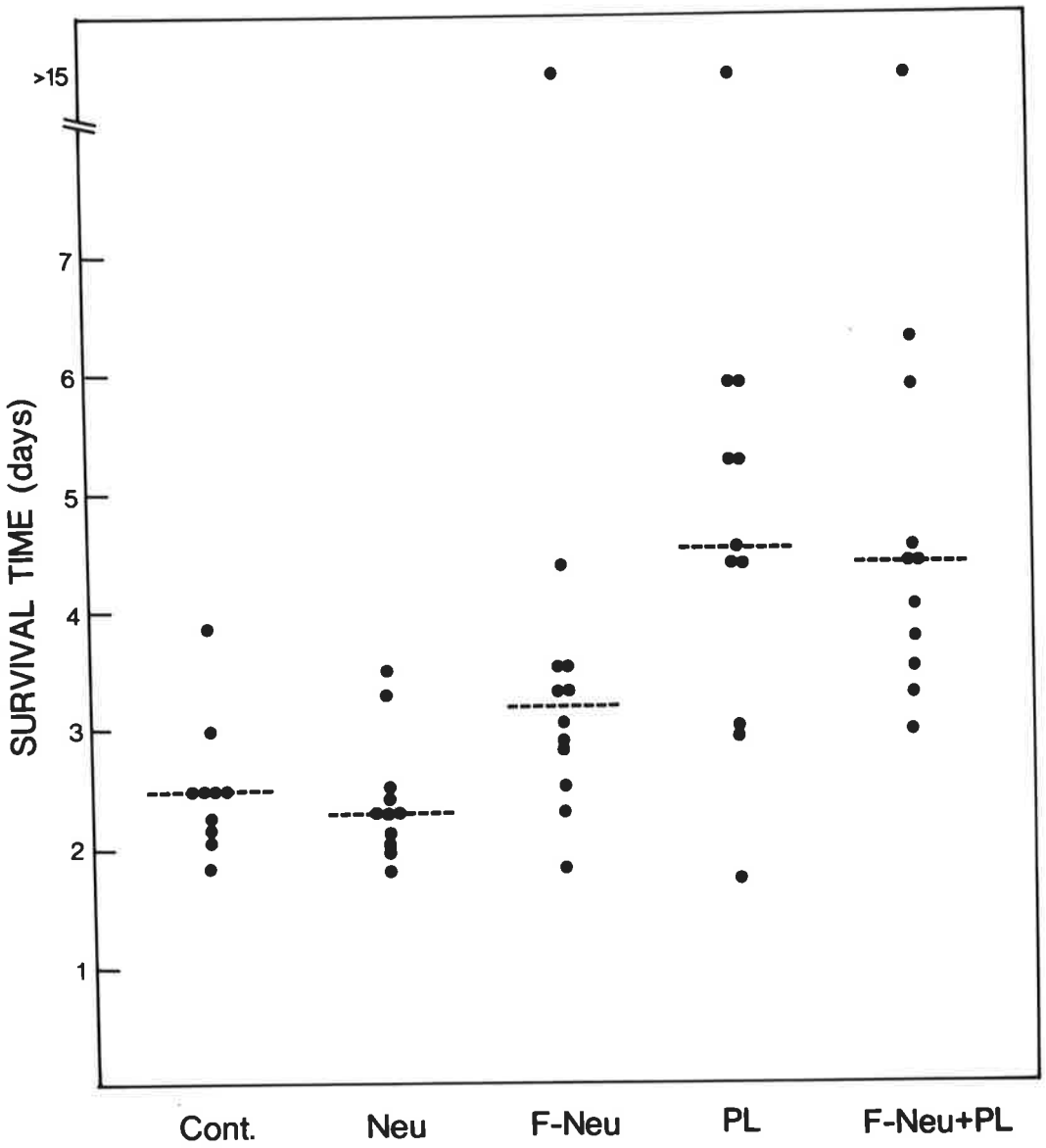

SURVIVAL TIME (days)
>15
7
6
5
4
3
2
1
Cont.
Neu
F-Neu
PL
F-Neu+PL

TABLE 5.2

Median survival times (in days) of immunized and control mice following challenge with S. pneumoniae.

Series	Immunogen	Immunized	Control
A	PL (average of 4 trials)	5.50	2.50
B	i) 86K Neu	2.40	2.50
	ii) 107K Neu	2.30	
	F-Neu	3.20	2.50
	PL	4.50	
	F-Neu + PL	4.40	
	iii) F-Neu	2.75	
	PL	3.90	2.25
	F-Neu + PL	4.70	
	iv) Neu (5%)	2.40	2.40
	Neu (7%)	2.40	
C	PL	3.90	2.20
	GPL ~	4.90	
D	AL	3.30	2.30

TABLE 5.3

Statistical analysis of the results of challenge trials (1-tailed Mann-Whitney U Test).

	Trial 1	Trial 2
Neu vs Control	NS	-
F-Neu vs Control	$p < 0.05$	$p < 0.05$
PL vs Control	$p < 0.01$	$p < 0.001$
F-Neu plus PL vs Control	$p < 0.001$	$p < 0.001$
PL vs F-Neu	$p < 0.05$	$p < 0.01$
F-Neu plus PL vs PL	NS	NS
F-Neu plus PL vs F-Neu	$p < 0.01$	$p < 0.01$
AL vs Control	$p < 0.01$	-

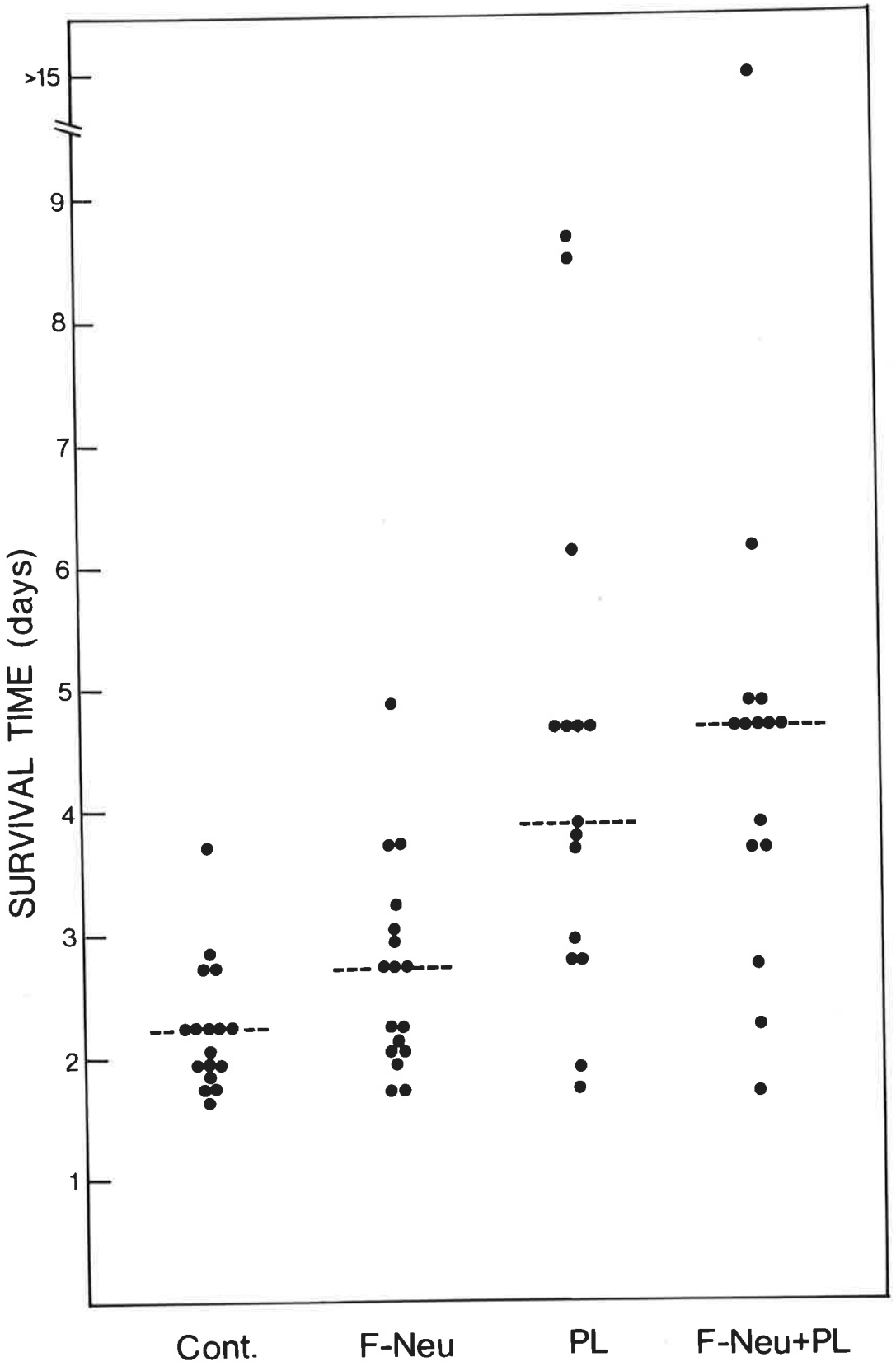
NS = Not Significant.

Figure 5.7

Effect of immunization with F-Neu, PL, and [F-Neu + PL] upon the survival time of mice challenged with S. pneumoniae.

See text, immunization/challenge series B (*iii*).

The broken lines indicate median survival times.


SURVIVAL TIME (days)
>15
9
8
7
6
5
4
3
2
1
Cont.
F-Neu
PL
F-Neu+PL

longer than those immunized with F-Neu (4.5 days vs 3.20 days, and 3.90 days vs 2.75 days at $p< 0.05$ and $p< 0.01$, respectively). The difference in median survival times between groups of mice immunized with PL alone and those receiving both PL and F-Neu was not statistically significant.

iv. The fourth set comprised three groups of 10 - 12 mice. In addition to the control group, there was a group of mice immunized with neuraminidase which had been pre-treated with 5% formaldehyde, and a group immunized with neuraminidase which had been pretreated with 7% formaldehyde. The antigen dose was 25 ug per injection. Immunodiffusion gel analysis indicated that the sera of immunized mice cross-reacted with neuraminidase which had not been pre-treated with formaldehyde, although with slightly reduced efficiency (not shown), but antineuraminidase titres for the pooled sera of the 5% group and the 7% group were only 11 and 6, respectively, compared with 17 for the pooled sera of control mice, and 240 for F-Neu mice. Mice were anaesthetized with metomidate and fentanyl and challenged intranasally with about 5×10^6 c.f.u. of D39 as before. The results (shown in Figure 5.8) indicate no difference in mean survival time between the groups; immunization with neuraminidase pre-treated with either 5% or 7% formaldehyde conferred no protection against challenge.

Series C: GPL

Three groups of 15 - 18 mice were used. One was a control group, one was immunized with native PL, and one received GPL. Immunodiffusion gel analysis indicated that sera collected from both immunized groups reacted strongly with both antigens (see Chapter 4, Figure 4.3). Antihaemolytic titres of control, PL and GPL mice were 200, 2,800 and 2,300, respectively.

Figure 5.8

Effect of immunization with neuraminidase pre-treated with 5% or 7% formaldehyde upon the survival time of mice challenged with S. pneumoniae.

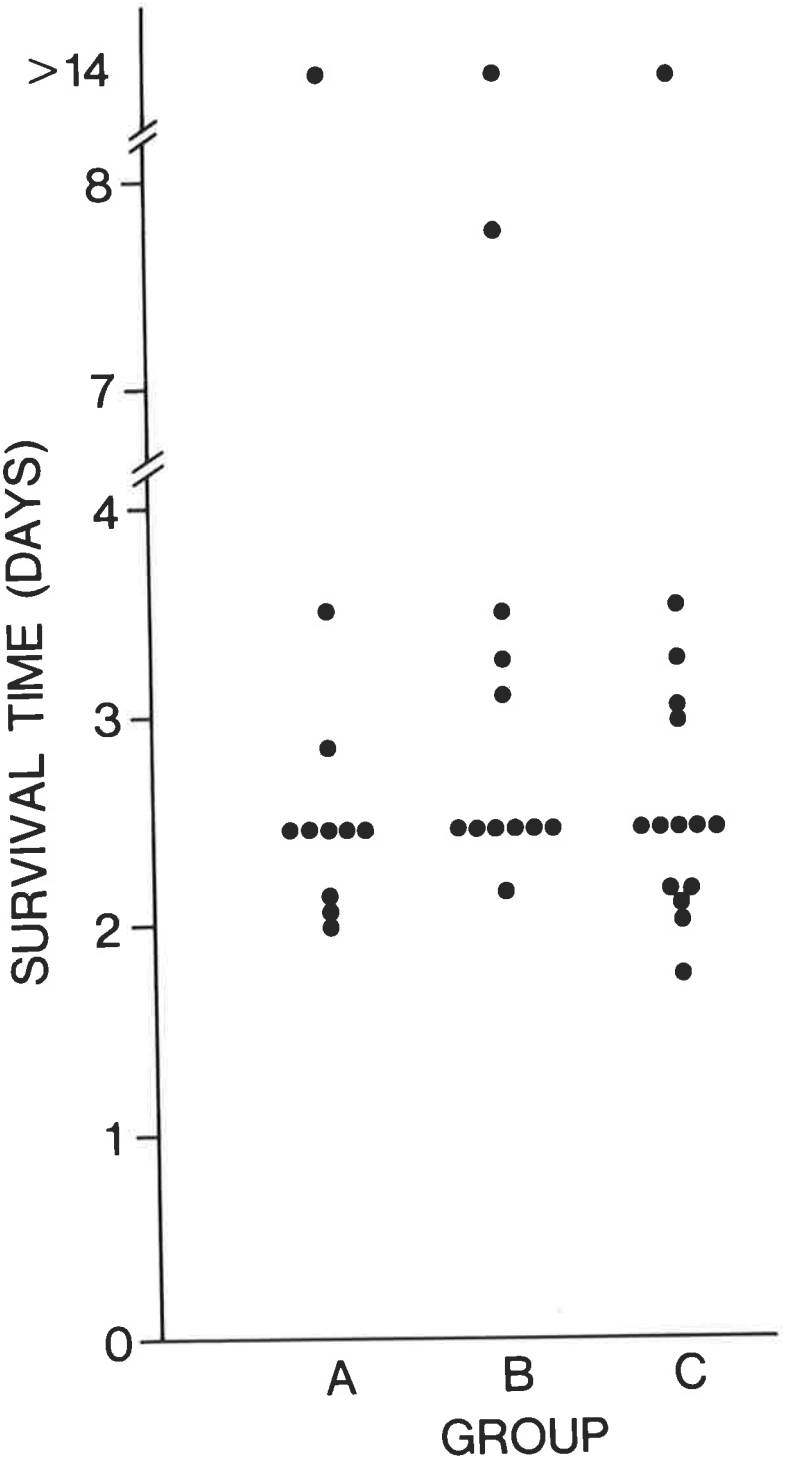
See text, immunization/challenge series B (*iv*).

Group A: Control (non-mmunized) mice;

Group B: Mice immunized with neuraminidase pre-treated with 5% formaldehyde

Group C: Mice immunized with neuraminidase pre-treated with 7% formaldehyde

Details of the formaldehyde-inactivation of Neu are given in the text.


>14
8
7
4
3
2
1
0
SURVIVAL TIME (DAYS)
A
B
C
GROUP

The results of the challenge are shown in Figure 5.9. The median survival times for both the PL group (3.9 days) and the GPL group (4.9 days) were significantly greater ($p < 0.01$) than that of the control group (2.2 days), but the difference between the PL and GPL groups was not statistically significant.

Series D: Autolysin

Two groups of 17 mice each were used, one a control group, and one immunized with autolysin. Immunodiffusion analysis (not shown) indicated that the mice which received autolysin produced antibodies directed against the protein. The anti-autolysin titre of pooled immune sera was 320, while for control sera it was less than 1. The results of the challenge, shown in Figure 5.10, indicate that the median survival time for control mice was 2.3 days, while for immunized mice it was 3.3 days. The difference is significant at $p < 0.01$.

4. Summary of Challenge Results.

Median survival times for all groups of mice are presented in Table 5.2.

- Mice immunized with native PL survived about twice as long as control mice;
- Mice immunized with native neuraminidase, either in its 86K (degraded) or 107K (intact) form were not protected, and neither were mice which had been immunized with 107K neuraminidase which had been pre-treated with formaldehyde at concentrations $\geq$ 5%;

Figure 5.9

Effect of immunization with pneumolysin and GPL upon the survival time of mice challenged with S. pneumoniae.

See text, immunization/challenge series C.

Broken lines indicate median survival times.

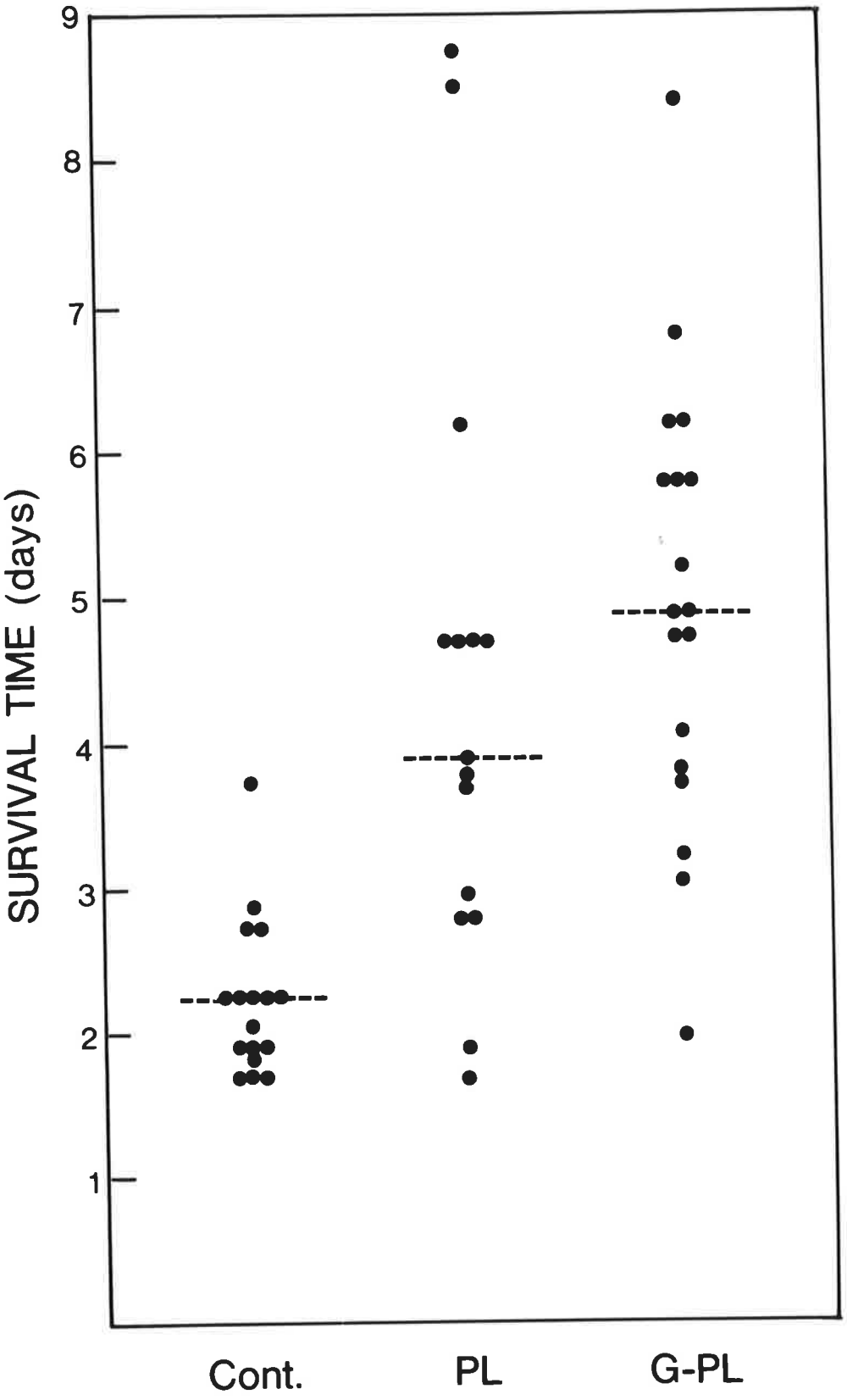
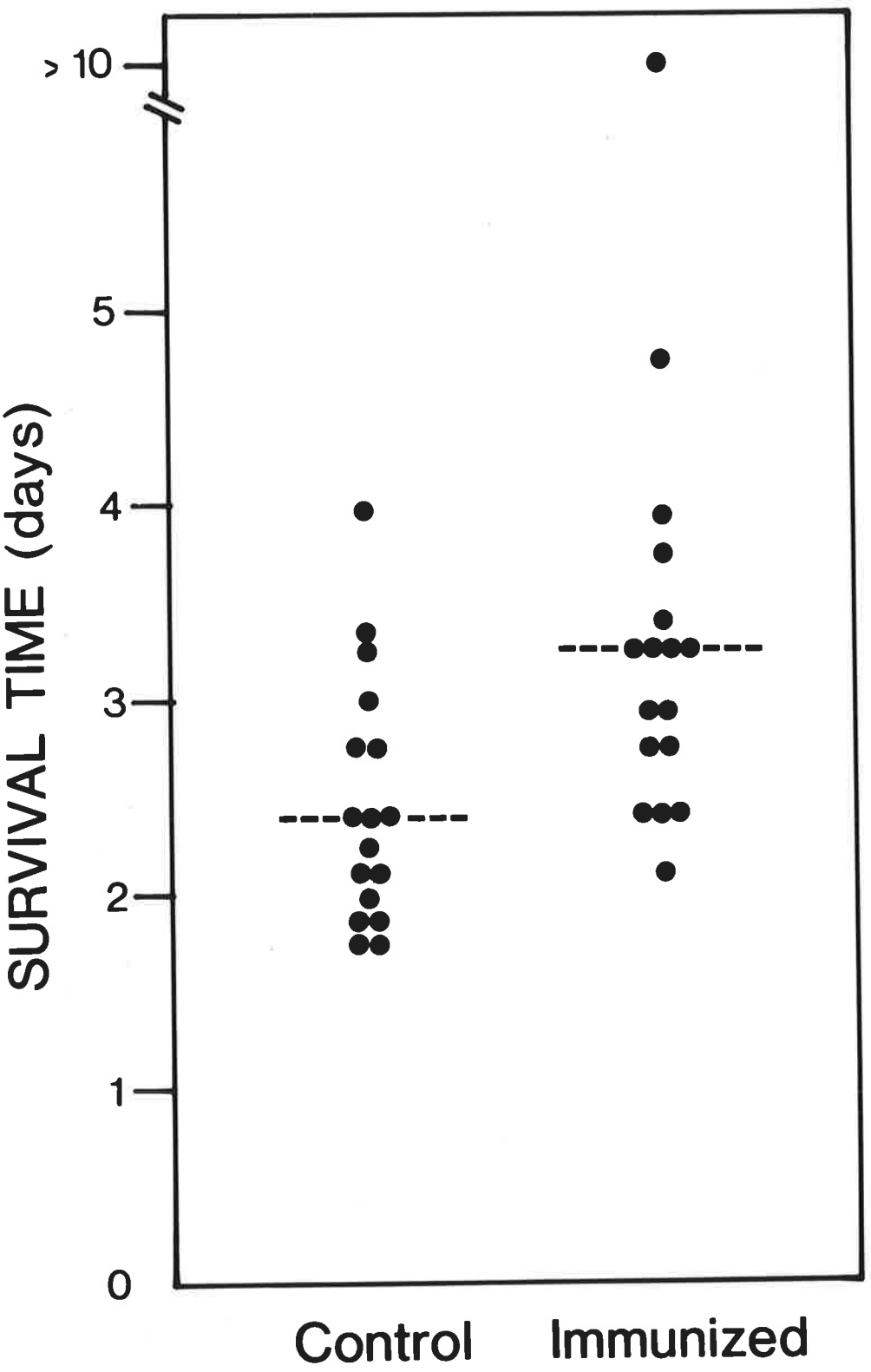

SURVIVAL TIME (days)
9
8
7
6
5
4
3
2
1
Cont.
PL
G-PL

Figure 5.10

Effect of immunization with autolysin upon survival time of mice challenged with S. pneumoniae.

See text, immunization/challenge series D.

Broken lines indicate the median survival time for each group.


SURVIVAL TIME (days)
> 10
5
4
3
2
1
0
Control
Immunized

- Mice immunized with F-Neu (i.e. 107K-form neuraminidase which had been pre-treated with 3.4% formaldehyde) survived significantly longer after challenge than control mice, but the magnitude of protection was less than that provided by pneumolysin;
- When PL and F-Neu were administered together, the protective effect was not significantly greater than that provided by PL alone;
- GPL provided protection which was at least as great as that of the native protein, PL;
- Mice immunized with autolysin survived significantly longer than control mice, but the magnitude of protection was less than that provided by PL or GPL;
- In all experiments, protection, when observed, was only partial, resulting in extended survival time rather than complete survival.

D] DISCUSSION

The work presented in this Chapter indicates that: 1) both native pneumolysin and its toxoid derivative GPL can be used as immunogens to protect mice partially but significantly against intranasal challenge with virulent *S. pneumoniae*; 2) while native pneumococcal neuraminidase is not protective, reducing the activity of the enzyme by appropriate treatment with formaldehyde can convert it to a partially protective immunogen; 3) autolysin is also a partially protective immunogen.

The immunization/challenge experiments which were carried out in the present study left unexamined a number of variables which should be considered here.

1. Antigens.

A particular combination of doses of the immunogens PL and F-Neu was found not to provide a protective effect greater than that of PL alone. Other combinations of immunogens could be tested, to determine if their protective effects might be additive, or even act synergistically.

In the present study, the chemical inactivation of toxic antigens proved to be a problem. The inactivation of native pneumolysin was attempted by a number of means: formaldehyde treatment, S-carboxymethylation and oxidation. (It has since become apparant that the reason that oxidation failed to diminish the haemolytic activity of the pure protein substantially was that the oxygen-mediated inhibition of a pneumolysin molecule is probably a consequence of the formation of a disulphide bond

between its single cys residue and the cys of some other, contaminating (non-pneumolysin) protein (Alouf, 1980); pure preparations of pneumolysin are therefore stable to oxygen). One way in which the problems associated with chemical inactivation were avoided in the case of pneumolysin was the use of a specifically-altered toxoid version of the protein produced by recombinant DNA techniques. Such techniques might be further exploited to produce other specifically-modified derivatives of the protein which are as immunogenic as GPL but have even less toxicity. Work along these lines is currently proceeding in the laboratory of Dr. G. Boulnois at the University of Leicester, U.K., and will be further discussed in Chapter 6.

Experiments reported in the present Chapter indicate that native neuraminidase is not a protective immunogen in mice, but that some protection is provided by neuraminidase which has been partially inactivated by appropriate treatment with formaldehyde. When a toxin such as neuraminidase is used as an immunogen while retaining some or all of its activity, its protective effects may be partially countered or overcome by its toxicity. The most rigorous formaldehyde treatment which did not strongly reduce the immunogenicity of the enzyme reduced its activity against the artificial substrate MUAN by only 60%. (While it is difficult to assess the degree to which this may reflect a reduction in its activity against whichever of its possible substrates may be important *in vivo*, there is some indication that formaldehyde treatment reduces the toxicity of the enzyme in mice). Since partial inactivation of neuraminidase produces a poorly protective immunogen, it is possible that more complete inactivation might give better protection, provided that the immunogenicity of the protein could be maintained. However, chemical methods of enzyme inactivation, such as treatment with formaldehyde, are far from ideal because they are non-specific. A method which targets specifically the active site of the enzyme would be preferable. Site-directed mutagenesis of

the gene coding for pneumococcal neuraminidase could accomplish such specific inactivation. The first step in this direction, the cloning of the pneumococcal neuraminidase gene, has now been achieved in the author's laboratory (Berry *et al.*, 1988).

In the studies reported here, autolysin was administered as an immunogen without modification. The results provide the first direct evidence that the enzyme plays a role in pneumococcal pathogenesis. (At the time of writing, these results have been submitted for publication as part of a larger study involving also an investigation of the virulence of *lytA*$^-$ strains of *S. pneumoniae*). While immunization with autolysin did afford mice a significant degree of protection against challenge, the degree of protection was not large (2.2 days survival for controls was increased to 3.3 days for immunized mice). Certainly, it was not of the magnitude that would have been expected if the anti-autolysin induced in the mice had been able to inhibit the release of a large proportion of the pneumolysin, neuraminidase, and the other toxic products, such as peptidoglycan and LTA, generated by the pneumococci during infection. The degree to which the release of each of these toxins is inhibited by anti-autolysin *in vivo* is not known. This is a problem which should certainly be investigated by double-immunization experiments. A first step would be to immunize mice with both autolysin and GPL to test whether the protective effects of these immunogens is additive.

2. Immunization.

The only adjuvant used in the present study was Freund's; there is now available a variety of adjuvants (e.g. Seppic's Montanide ISA, available from Tall Bennett, Moorabin, Aust.) which are claimed to be less toxic than

Freund's and capable of more efficiently promoting immune responses. Different adjuvants and routes of immunization should be tested, with particular attention to those which are acceptable for use with humans.

3. Challenge.

A standard dose of pneumococci, consisting of about 5×10^6 viable type-2 organisms, was used in all challenge experiments, this having been determined as the minimum dose required to ensure, reliably, the death of $\geq$ 95% of non-immunized control mice and is about 100 times the intranasal LD_{50}. At such a dose, few immunized mice survived either, and the degree of protection afforded by the various immunogens was assessed by the prolongation of survival time after challenge. An alternative way of proceeding would be to reduce the challenge dose so that a higher proportion of the mice survived, and then score the numbers of mice deemed to have recovered completely (e.g. those that lived longer than 14 days after challenge) in control and immunized groups.

The route of challenge could also be varied. Intranasal challenge was chosen for this study because it mimics the normal route of infection leading to bacteraemic pneumonia. However, it does not mimic disease states such as pneumococcal otitis media or meningitis. Other workers (O'Toole *et al.*, 1971) have suggested that neuraminidase in particular may contribute to the development of pneumococcal meningitis, increasing the severity of the condition once infection has been established. Autolysin may also play an important role in meningitis, since components of the pneumococcal cell wall induce a substantial degree of meningeal inflammation (Tuomanen *et al.*, 1985b). The contribution of such enzymes to meningitis would be difficult to investigate by immunization/challenge studies in an animal

model, even using an intracranial route of challenge, because, when the meninges are not inflamed, immunoglobulins are present in the cerebrospinal fluid only in concentrations so low that they would be unlikely to protect an animal from challenge (Rahal and Simberkoff, 1982).

A huge, multivariate analysis covering all possibilities would clearly be impossible. Nevertheless, the results of the relatively simple study which is presented in this Chapter lead to important conclusions and provide a strong indication of the general direction which future investigations might profitably follow.

One major result of this study was to provide the first direct evidence that pneumolysin, neuraminidase and autolysin are all virulence determinants in *S. pneumoniae*, i.e. that they each have a direct involvement in the pathogenesis of the organism. Previous evidence to this effect had been circumstantial only.

A second conclusion is that strongly inhibitory titres of circulating antibody directed against any one of these proteins (or F-Neu and PL in combination) are not in themselves sufficient to provide complete protection against challenge, at least in the present model. Nevertheless, the results obtained here certainly suggest that inactivated versions of pneumolysin, and, in particular, toxoid versions of this protein produced by recombinant DNA techniques, should be considered very seriously as additives to capsular polysaccharides in future clinical trials of pneumococcal vaccines in humans.

CHAPTER SIX

DISCUSSION

The work presented in this thesis (and the larger project of which it forms a part) was designed with reference to a (partially speculative) model of pneumococcal pathogenesis, which will now be described. Immunological details of the extremely complex interplay of the various mediator systems, inflammatory cells and toxic products will be omitted. However, the outline which remains will be sufficient to place the current work in its larger context, and indicate the contribution it has made towards an understanding of the molecular mechanisms involved in the pathogenesis of pneumococcal disease.

A] MODEL OF PNEUMOCOCCAL PATHOGENESIS

1. Colonization and Invasion.

Pneumococcal disease in humans requires, as a first step, that the organism should colonize the mucosal surfaces of the upper respiratory tract of its host. The source of infection is usually aspiration of nasopharyngeal secretions from a previously-infected person (i.e. droplet infection).

In order to begin colonization, the bacterium must first adhere to the mucosal surface strongly enough to counter those host defences, including

immune functions and mechanical factors such as flows of secretion, and ciliary activity, which act to remove it. General models of bacterial attachment have been reviewed by Freter and Jones (1983).

Adhesion of the pneumococcal cell appears to be inhibited by its polysaccharide capsule (Andersson *et al.*, 1982). Because of this, binding of the pneumococcus may be especially dependent upon the actions of specific adhesins, the nature of which has been examined recently by Beuth *et al.*, (1987) and Andersson *et al.*, (1988). *Streptococcus pneumoniae* appears to attach to human pharyngeal epithelial cells through the mediation of bacterial adhesins (probably proteins or glycoproteins) anchored to the pneumococcal cell wall. Pneumococcal adhesins appear to interact specifically with N-acetyl-D-glucosamine / D-galactose or N-acetyl-neuraminic acid moieties of glycoconjugate receptors on host-cell surfaces. Since the N-acetyl neuraminic acid moieties at least should be susceptible to cleavage by the pneumococcal neuraminidase, it must be assumed that, at this early stage of pathogenesis, the release of this enzyme is rigorously restricted by the bacterium.

One way in which the host may defend itself against bacterial attachment is by secreting oligosaccharides which match those of the recognized glycoconjugates and act as competetive inhibitors of adhesion. Other defences may include free, bactericidal long-chain fatty acid surfactants (which have been investigated by Coonrod and co-workers; see, e.g. Coonrod, 1987), and secretory immunoglobulins, in particular IgA1 and IgA2 which are synthesized by plasma cells lying within the mucous membrane under the epithelium lining the respiratory tract. The pneumococcus resists the action of human IgA1 by releasing a protease specific for the hinge region of its heavy chain, cleaving the molecule and inactivating it (see Mulks *et al.*, 1980a and Plaut, 1983 for reviews). The closely related

immunoglobulin IgA2, which has a different amino acid sequence in the target region of its heavy chain, is not cleaved. The reason there appears to be no specific bacterial mechanism for inactivating this immunoglobulin is not known, although it has been suggested that IgA2 may be a late evolutionary development by humans enhancing their resistance to colonization by organisms which produce an IgA1-protease. Kilian and Reinholdt (1987) have suggested that one benefit which the bacterium may gain from the cleavage of IgA1 may stem from its subsequent binding of the Fabα fragments of this molecule which are generated by the protease; being monovalent, the Fabα fragments are unable to induce bacterial agglutination, and are ineffective at inhibiting the colonization process, but, being bound to epitopes on the bacterial cell surfaces, they will protect the bacterium from the immune system by blocking the further access of intact antibody molecules. All clinical isolates of *S. pneumoniae* appear to be IgA1-protease positive (Plaut, 1983: review), and this, combined with the high specificity of the enzyme for a component of the human immune system, suggests that it is an important virulence protein for *S. pneumoniae* in human hosts. If secretory immunity to IgA1-protease (i.e. IgA inhibiting the ability of the protease to cleave IgA1!) could be induced in humans by vaccination, significant protection against pneumococcal colonization might well result. However, this possibility could not be examined in the present study simply because there is no appropriate animal model in which immunization / challenge studies might be undertaken: IgA1-protease does not appear to cleave any of the animal immunoglobulins which have been tested so far (Plaut, 1983, review).

If the pneumococcus is able to overcome host defences at the mucous membrane surfaces, it may proceed to colonize these membranes, and live with the host as a benign parasite. This is not an uncommon occurrence with human hosts. Indeed, early studies attempting to establish the

pathogenicity of *S. pneumoniae* were confused by the frequency of its asymptomatic carriage. Following some breach in the host's defences, however, the status of the infection may change. The breach may be a consequence of mechanical trauma to the membrane, or damage induced by some other micro-organism, such as a respiratory virus, or perhaps, in the presence of a very high dose of the pneumococcus, the inflammatory response of the host's own immune system may result in tissue damage. The pneumococcus can invade the deeper tissues of its host by a number of routes, and a number of tissues may be the primary site of infection. The present work is largely concerned with bacteraemic lobar pneumonia, but general features of the model presented here will apply to other pneumococcal infections.

Once within the host's body, the bacterium's first line of defence against the hostile response of the immune system is its polysaccharide capsule. Both the chemical composition and the quantity of capsular polysaccharide which a pneumococcal strain produces will affect its virulence; among strains with the same capsular serotype, those producing the largest amount of polysaccharide appear to be the most virulent, and those that produce the least are the least virulent (McLeod and Krauss, 1950). The effectiveness of encapsulation as protection is also attested by the fact that encapsulated bacteria in general are the most prevalent in causing serious bacterial infections in humans world-wide (Lee, 1987: review). Polysaccharide capsules are relatively poor antigens and activators of complement which probably have their protective effect for the bacterium by partially masking the more highly antigenic determinants of the cell wall. (Non-encapsulated (rough) strains of pneumococcus are highly susceptible to direct opsonization, and are hence avirulent). The interaction of pneumococcal cell surface components with antibody, C-reactive protein (CRP) and the various components of the complement

system is a fascinating and complex story which has been reviewed by Johnston (1981), Winkelstein (1981, 1984), Brown *et al.*, (1982) and Hostetter (1986). No attempt will be made to summarize it here, except to make two points. Firstly, even though components of the complement system can penetrate the capsule and bind to the cell wall, their direct bactericidal activity there appears to be highly inefficient. Secondly, even though the presence of the capsule may not prevent the binding of complement, CRP or antibody to the cell wall, it may prevent this binding from resulting in true "opsonization" of the bacterium by blocking access of phagocytic cells to the activated surface.

Capsular polysaccharide can direct the production of antibody, but, in the previously non-immune host, this is probably protective only relatively late in the course of infection (Winkelstein, 1984).

Apart from the passive protection which is afforded by a capsule, the pneumococcus appears to produce a number of molecular species which it can deploy actively to disable or distract the host immune system, and it is with respect to these that the results reported in this thesis have particular relevance.

2. Pneumolysin.

Pneumolysin binds specifically to cholesterol in host cell membranes. At relatively high concentrations there it can aggregate to form micropores, resulting in lysis of the cell. At sub-lytic concentrations, it may inhibit the activities of the cell, perhaps by disturbing membrane functions. Pneumolysin purified during the course of the present work has been used in a series of experiments (Paton and Ferrante, 1983; Paton *et al.*, 1984; Ferrante *et al.*, 1984; Nandoskar *et al.*, 1986) demonstrating a variety of

detrimental effects which the toxin can induce in components of the human immune system *in vitro*.

Pneumolysin was shown to have the ability to activate the classical complement pathway, bringing about local depletion of serum opsonic activity. In addition, some activated cleavage products of the complement system cause aggregation of polymorphonuclear leukocytes and leukostasis *in vivo*, a process which may cause immunopathologic damage to the host during infection (Winkelstein, 1984: review). As will become apparent, the misdirection, remote activation and depletion of host immune factors, and possible concurrent damage to host cells, is a recurring theme in this discussion of active pneumococcal defences. As early studies using impure preparations of pneumolysin introduced into rabbit eyes have suggested, (Johnson and Allen, 1975), pneumolysin has the potential to cause acute inflammation. Particularly interesting in this regard is recent evidence (G. Boulnois, personal communication) that a region of the pneumolysin molecule appears to mimic the structure of CRP, an acute-phase reactant which binds to phosphoryl choline in the pneumococcal cell wall and can activate the complement cascade there. Site-specific mutations in the part of the gene coding for the CRP-analagous region of pneumolysin produce mutant proteins which fail to activate complement. Clearly, it is the CRP-analogous region which gives pneumolysin its ability to activate and deplete complement.

Before the results reported in the present study, there was no direct evidence that pneumolysin was a virulence factor in the pneumococcus. The present work has shown that circulating mouse antibody which is capable of inhibiting the haemolytic activity of pneumolysin *in vitro* is significantly protective *in vivo* against experimental infection by *S. pneumoniae*, extending the survival time of mice challenged intranasally with the

organism by a factor of about two. (The importance of pneumolysin has more recently been confirmed in the author's laboratory (Berry *et al.*, 1989b) by virulence studies with pneumococcal strains in which the pneumolysin gene has been specifically inactivated. The virulence of such strains was substantially reduced from that of their otherwise isogenic parent).

While these results suggested that pneumolysin had potential as a vaccine antigen, the toxicity of the protein presented a problem to which chemical modification proved an unsatisfactory solution. However, at this stage, Dr. G. Boulnois and coworkers at the University of Leicester, U.K., were able to supply the recombinant source of a mutant version of pneumolysin in which the cysteine residue at the putative active centre of the protein (position number 428 in the amino acid sequence) had been specifically replaced by a glycine residue. In the present study, it was determined that the specific haemolytic activity of this mutant protein, GPL, was only about 0.6% that of native pneumolysin, and that the toxicity of GPL in mice had also been greatly reduced. A method for rapidly purifying GPL in relatively large amounts was developed, and immunization / intranasal challenge trials were undertaken using it. GPL proved highly antigenic, directing the production of antiserum which inhibited the haemolytic activity of pneumolysin *in vitro* with about the same efficiency as anti-pneumolysin serum. Finally, GPL proved at least as protective an immunogen as pneumolysin.

The latest development in this line of research has arisen from experiments directed at a sequence of four amino acid residues (predicted from the nucleotide sequence of the cloned gene: Walker *et al.*, 1987) which begins five residues away from the single cysteine residue towards the C-terminal of the pneumolysin molecule. This sequence, which contains three tryptophan residues, must impose a gross structural deformation on the

polypeptide backbone of the molecule. Also, occurring, as it does, so close to the putative active site of the enzyme (the cys residue which was converted to gly to produce GPL) it must contribute very significantly to the three-dimensional structure of the active site. Modifications to one or more of these trp residues might be expected, therefore, to have drastic effects on the activity of the protein. Several trp mutants of pneumolysin have now been generated (G. Boulnois, personal communication), and some of these indeed appear to have greatly reduced haemolytic activities. At the time of writing, they are about to be supplied to the present author for further studies on their toxicities, immunological cross-reactions, and assessment as protective immunogens. The ultimate aim of this work is to identify and purify mutant versions of PL having effectively no toxicity but retaining sufficient immunological cross-reactivity with native pneumolysin to direct the production of protective anti-pneumolysin in the serum of human vaccinees.

3. Neuraminidase.

Neuraminidase is a second toxin which probably forms part of the pneumococcal armoury against host defences. Neuraminidase released from pneumococcal cells during infection has the potential to cleave N-acetyl-neuraminic acid (and possibly other) residues from the glycoprotein cell-surface receptors of host cells, possibly impeding the function of the cell. Such loss of surface markers might even result in the loss of the body's self-recognition of the affected cells, resulting in the misdirection of the host's immune responses towards its own cells. Neuraminidase may also deactivate immunoglobulins by cleaving their carbohydrate moieties (Ward and Kunkel, 1983; Ginsberg, 1985: reviews).

Unfortunately, there is little direct evidence for any of these functions *in vivo*, other than detection of circulating NANA in the cerebro-spinal fluid of some human patients with pneumococcal meningitis (O'Toole *et al.*, 1971), and studies on the loss of glycoproteins from the surface of organs removed from mice dying after inoculation with partially-purified preparations of neuraminidase (Kelly and Greiff, 1970).

Prior to the present study, research on pneumococcal neuraminidase was hampered by the lability of the enzyme, its apparant multiplicity of forms, and the consequent impossibility of purifying it. The present work has indicated the likely existence, within the pneumococcal cell, of proteolytic activity apparently with a degree of specificity for neuraminidase, associating with it through early stages of purification, and digesting it, by discrete steps, from its parent MW 107K form into a number of smaller species, at least some of which retain neuraminidase activity (at least against the artificial, fluorogenic substrate MUAN). The function of the putative neuraminidase-protease *in vivo* has not been determined. Effective inhibitors of neuramninidase proteolysis (PMSF and EDTA) were identified, and, by use of these, undegraded neuraminidase was obtained for the first time, and used for immunization / challenge studies.

The toxicity of the enzyme proved to be a problem which could be only partially overcome by careful, non-specific chemical inactivation, and, while the results obtained here represent the first direct confirmation that neuraminidase is a virulence protein in *S. pneumoniae*, they also suggest that the potential of the protein as a protective immunogen is limited unless specifically-mutated toxoid versions of the enzyme, analagous to GPL and related versions of pneumolysin, can be manufactured by recombinant DNA techniques. Unfortunately, the pneumococcal gene for neuraminidase has so far proved to be extremely unstable in *E. coli* hosts,

and, while a large part of the structural gene for the enzyme has been cloned into *E. coli* by workers in the author's laboratory and has been shown to produce an enzymically active product (Berry *et al.*, 1988), subsequent attempts to manipulate it, with the aim either of eventually generating a toxoid version of neuraminidase or a specifically-mutated neuraminidase-negative strain of pneumococcus for virulence studies, have not yet succeeded.

Another complicating factor in the assessment of neuraminidase as a virulence factor and possible protective immunogen has been the possibility that the animal challenge model used in the current study may not be appropriate for this toxin which may conceivably have a more important function in pneumococcal meningitis than in bacteraemia.

Clearly, there is still much work to be done on pneumococcal neuraminidase, but current information suggests that it may well warrant the effort.

4. Autolysin.

In the present model of pneumococcal pathogenesis, it is suggested that the bacterium's toxic defences remain largely cell-associated while the cell is intact. Pneumolysin has been shown to be a strictly cytoplasmic protein (Johnson 1977), a conclusion which was confirmed in the present study. In the case of neuraminidase, some researchers have suggested that the enzyme is actively secreted, but in the present work, a rough parallel between extracellular pneumolysin and neuraminidase in cell cultures of a number of pneumococcal strains suggested that this is not the case, and that neuraminidase is either cytoplasmically located, like pneumolysin, or strongly associated with the cell surface. This has recently been confirmed

in the author's laboratory by experiments investigating the release of neuraminidase from defined *lytA*$^-$ mutants which do not release neuraminidase unless exogenous autolysin is added (A. Berry, personal communication). The question of whether or not IgA1-protease is actively secreted was not addressed in the present study.

As well as cytoplasmic, soluble proteins, there is a second class of pneumococcal products known to have direct toxic effects on the host. These materials are cell-associated (while the pneumococcus remains intact) because they form structural components of the living bacterial cell. Peptidoglycan and teichoic acid from the cell wall and lipoteichoic acid (LTA) from the plasma membrane are all known to have highly inflammatory effects if solubilized and released into the host (e.g. see Chetty and Kreger, 1980, 1981). When freed from the shielding provided by the polysaccharide capsule of the intact pneumococcal cell, the highly antigenic determinants of the cell wall fragments and LTA present inappropriate targets for the host immune system, having both the capacity to bind strongly to host cells and trigger the destructive complement cascade there, sometimes leading to the death of the host cell (Hummell and Winkelstein, 1986; Weinreb *et al.*, 1986; Tuomanen *et al.*, 1987b).

Both toxic cytoplasmic and toxic structural components of the pneumococcus are released from the bacterial cell by the action of the pneumococcal N-acetyl-muramic acid - L-alanyl hydrolase: i.e. autolysin. While related cell-wall hydrolases are common in other bacterial species (probably playing a role in maintaining the dynamism of the cell wall structure) possession of a cell-wall hydrolase so efficient that it readily induces the self-destruction of static-phase cells in culture is a feature peculiarly characteristic of *S. pneumoniae*. Recent studies on possible roles for the pneumococcal autolysin *in vivo* established only that it may

have some involvement in the separation of daughter cells after cell division, but have failed to provide any strong evidence that this is a function critical enough to require the possession of a potentially suicidal enzyme. Indeed, these studies have shown that *lytA*$^-$ mutants appear to lack no functions necessary for normal growth *in vitro* (Sanchez-Puelles *et al.*, 1986; Ronda *et al.*, 1987). In the present model, the major role proposed for autolysin is to act during infection as the catalyst for releasing the various, powerfully toxic, immune-inhibiting substances discussed above. Possession of a thick, protective capsule of polysaccharide may reduce the efficiency of the active secretion of toxic cell products by the pneumococcus. Furthermore, in a fluid environment such as the blood, gradual secretion is probably a less efficient means of raising the local concentration of toxins than is the sudden, autolytically-catalysed release of intracellular contents. The release of toxins by autolytically-induced sacrifice of a proportion of the invading population of cells will be worthwhile if the remaining cells thereby gain a sufficient degree of protection from host immune defences, for, despite the loss of some individual cells, the strategy will confer substantial benefit on the infecting pneumococcal *genotype*. (The present model suggests that an important consequence of antibiotic therapy using β-lactam bactericidal agents, which have their effect by stimulating autolysin-induced cell killing, may be the sudden release of a burst of toxic products, which may be sufficient to cause irreparable damage to the host even as the bacterial cells are destroyed. It is known that some patients treated for pneumococcal disease sometimes die even after the apparant eradication of the organism by β-lactam antibiotic treatment: Austrian, 1981: review; see also Tuomanen *et al.*, 1985a,b).

Until the immunization / challenge studies reported in this thesis, there was no direct evidence for the importance of autolysin to virulence.

The results presented here indicate that the induction of serum antibody capable of inhibiting the autolysis of pneumococcal cells (at least *in vitro*) provides mice with a partial protection against experimental pneumococcal bacteraemia. The degree of protection provided did not appear to be commensurate with the pivotal role proposed for autolysin in the present model, being less than that provided by immunization with pneumolysin. However, one may surmise that the efficiency of circulating anti-autolysin at preventing cell lysis may be somehow compromised *in vivo* so that higher antibody titres than those currently attained may be necessary for effective protection. Alternatively, the antibody must be delivered more effectively to the location in the body where the consequences of cell lysis are most critical. In any case, a potential for the use of autolysin as a protective immunogen in humans is certainly indicated, particularly since it is unlikely to have any toxic effects on vaccinees.

Convincing confirmation that autolysin is an important virulence factor in the pneumococcus has recently been provided by research involving the present author (Berry *et al.*, 1989a) using defined AL^- mutant strains of pneumococcus. Although there have previously been several reports in the literature of the identification of autolysin-deficient pneumococci (Garcia *et al.*, 1986a,c; Lopez *et al.*, 1986; Sanchez-Puelles *et al.*, 1986), there appear to have been no studies on the virulence of defined AL^- mutants, excepting only a recent, preliminary report by Pozzi *et al.* (1988). These workers insertionally inactivated the autolysin gene of a rough pneumococcus and then transformed it with DNA from encapsulated strains. Encapsulated autolysin-negative transformants were isolated after passage through mice, and shown to have LD_{50}s similar to those of the encapsulated parental strain. However, the possibility of unknown alterations to the mutant occurring during animal passage could not be excluded. In the

present case, *lytA*$^-$ mutants produced by insertion-duplication mutagenesis showed a marked reduction in virulence for mice: the intranasal and intraperitoneal LD_{50}s were roughly 10^2 and 10^5 fold greater, respectively, than those of the otherwise isogenic parental strain D39. Full virulence was restored, however, when autolysin production was reconstituted by back-transformation with the cloned autolysin gene. Random insertions of the cloning vector alone into the chromosome had been shown previously not to alter the virulence of D39, so the effects observed may therefore be directly attributed to autolysin deficiency. Most significantly, the reduction in virulence which was achieved by inactivation of the autolysin gene was more profound than that which had previously been achieved by inactivating the pneumolysin gene, which increased the intranasal and intraperitoneal LD_{50}s by only 10 and 100 fold, respectively (Berry *et al.*, 1989b).

Autolysin is clearly a virulence factor in the pneumococcus having an importance consistent with the role proposed for it in the model described here. This suggests that future research may well discover ways of improving its efficacy as a protective immunogen to the point where it will be worth assessing as a component of a vaccine for humans.

B] DIRECTIONS FOR FUTURE RESEARCH

1. General.

As indicated throughout the above summary of a working model for pneumococcal pathogenesis, the field is rich with possibilities for future study.

GPL has proven to be a highly promising immunogen; other mutant forms of pneumolysin are now available and warrant close investigation. Further advances in the study of neuraminidase will probably require construction of stable clones carrying the relevant gene. Availability of these could then lead to the construction of further clones expressing large amounts of specifically-mutated toxoid neuraminidase and defined neuraminidase-negative mutants for virulence studies. Results obtained with autolysin suggest that this is potentially the most promising of all the immunogens so far investigated in this study, but reasons for its present relatively poor efficacy will require close examination. Further immunization / challenge experiments using different regimes and combinations of antigens should also certainly be undertaken.

The shortcomings of current polysaccharide vaccines have suggested that polysaccharide might be supplemented with or replaced by appropriate protein immunogens. However, another possibility which should be carefully investigated concerns the covalent conjugation of polysaccharide with protein, and offers a chance to produce immunogens having the protective characteristics of both. This possibility is important enough to be considered here in some detail.

2. Protein-polysaccharide Conjugates.

Pneumococcal polysaccharides, like bacterial polysaccharides in general, are thymus-independent (TI) antigens (Basten and Howard, 1973). Characteristically, such antigens induce little response in immature immune systems, and fail to promote immunological memory even in immune systems which are fully competent. Thymus-dependent (TD) antigens, by contrast, are immunogenic in the young and can promote immunological memory (Snippe *et al.*, 1983). Therefore, in order for pneumococcal polysaccharides to provide effective long-term protection against disease, particularly in the young, they should ideally be converted from TI to TD antigens.

One way in which this conversion can be achieved is the covalent coupling of protein to the polysaccharide.

The pioneering work on protein - bacterial polysaccharide conjugates was undertaken by Avery and Goebel in 1929 (Avery and Goebel, 1929; Goebel and Avery, 1929) when they linked "serum globulin" to pneumococcal type-3 capsular polysaccharide, and to the repeating disaccharide unit of that polysaccharide. Ten years later, Goebel showed that, while purified type-3 polysaccharide itself had only poor immunogenicity in rabbits, and re-injection with it did not induce a booster effect, the protein-carbohydrate conjugate induced high levels of serum antibodies that increased in concentration with re-injection and were protective against challenge with the live organism (Goebel, 1939, 1940).

Since the 1940's, the covalent conjugation of bacterial polysaccharides to proteins has been fairly common (e.g. Jennings and Lugowski, 1981; Chu *et al.*, 1983; Anderson, 1983; Beuvery *et al.*, 1983a,b; Tsay and Collins, 1984; Schneerson *et al.*, 1984, 1986; Zigterman *et al.*, 1985; Porro *et al.*, 1985, 1986; Cryz *et al.*, 1986a,b; Kayhty *et al.*, 1987, in the 1980's

alone). The immunogenicity of the carbohydrate moiety has generally been shown to be enhanced by conjugation, and, more specifically, the characteristics of the carbohydrate have usually been observed to change from those of a TI to those of a TD antigen.

Another important conclusion from the studies cited above has been that the protein moiety of the conjugate can remain antigenic. When the conjugate is to be used as a vaccine, there is therefore much to be gained by using a carrier protein which is itself a protective immunogen.

To date, the best characterised polysaccharide-protein conjugate vaccine has been a *Haemophilus influenzae* type b (Hib) polysaccharide - diphtheria toxoid conjugate. A conjugate containing these components was first prepared by Anderson in 1983. Recently, an Hib polysaccharide - diphtheria toxoid conjugate licensed for use in humans and manufactured by Connaught Laboratories was evaluated for clinical efficacy in an open trial involving 60,000 young children in Finland (Eskola *et al.*, 1987). While vaccine composed of Hib capsular polysaccharide alone had previously been shown to be ineffective in infants, the rate of short-term protection (up to nine months after final vaccination) provided by the conjugate vaccine in the Finnish study was 87%.

The strong suggestion that the pneumococcal polysaccharide vaccine might similarly be made more immunogenic by conjugation to appropriate proteins has been made recently by LaForce and Eickhoff (1988), and by Davies and Kumaratne (1988).

The covalent coupling of pneumococcal virulence proteins or their toxoid derivatives to capsular polysaccharides clearly has great potential to generate highly protective immunogens. The various conjugates should be produced and tested. A possibility particularly worth investigating is that

native neuraminidase, when covalently bound to polysaccharide, may be inactivated simply by steric hindrance of its active site while maintaining its antigenicity.

A note of caution should be sounded here, however. The production of protein-polysaccharide conjugates has inherent complications. Such conjugates are heterogeneous. Even when the protein and the polysaccharide and protein components are single (and a selection of capsular polysaccharide serotypes might be necessary in the present case) each of the various methods by which they might be coupled, as well as some of the variations of procedure within each particular method, will generate a different mix of product molecules having different characteristics. An interesting review of some of the problems which this may entail has recently been written by Penney (1987).

C] CONCLUSION

In the last few years, the field of pneumococcal research has progressed in a remarkable fashion. Following the development of antibiotics in the 1940's, there was a period when studies on the pneumococcus languished, and they did not recover for more than thirty years. The resurgence of interest which has taken place in the 1980's is a result, partly, of the increasing realization (prompted by the appearance of pneumococcal strains having multiple-resistance to antibiotics) that antibiotic therapy is not a complete solution to the problems of pneumococcal disease. It has also come about partly because the shortcomings of currently-available vaccines at

preventing pneumococcal infections have become increasingly apparant: pneumococcal polyvalent polysaccharide vaccines are too expensive for use in the Third World, are most efficient at protecting low-risk groups, and may confer no protection at all on some individuals in high-risk groups.

However, another very important stimulus to the "pneumococcal revival" has been the availability of increasingly-sophisticated techniques involving recombinant DNA, techniques which are, indeed, presently revolutionizing all aspects of molecular biology. For the first time we can now directly investigate the molecular mechanisms of pathogenicity, discriminating between its various components and assessing their relative contributions to disease states.

The present work forms a bridge between the old methods and the new, investigating both the attributes of proteins derived directly from the pneumococcal cell, and those of pneumococcal proteins, native in form or specifically-mutated into toxoid derivatives, which have been manufactured by recombinant strains of *E. coli* which host characterized fragments of pneumococcal DNA. As part of the present work, the importance of three pneumococcal proteins to the pathogenesis of the organism has been established, and first steps taken towards an assessment of these proteins as potential components of second-generation (protein-based) vaccines.

At the close of the first Chapter to this thesis, two major long-term goals of pneumococcal research were articulated. The first is the elucidation of the molecular mechanisms by which the pneumococcus damages and sometimes ultimately kills its host. The second major goal of pneumococcal research is to produce immunogens which are more effective than capsular polysaccharide alone in preventing pneumococcal infection in humans.

Although both these goals remain unattained, it is possible now to be confident that highly significant advances towards the prevention and alleviation of pneumococcal disease are presently in store. We have arrived at a particularly exciting stage in the history of pneumococcal research.

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