THE PENICILLIN BINDING PROTEINS AND BETA-LACTAMASES OF NEISSERIA GONORRHOEAE

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A thesis submitted in partial fulfilment of the requirements of The Nottingham Trent University for the degree of Doctor of Philosophy

August 1993

The Nottingham Trent University in collaboration with Wellcome Foundation Limited

DECLARATION

This work has not been accepted in substance for any other degree, and is not concurrently being submitted in candidature for any other degree.

This is to certify that the work presented in this thesis was carried out by the candidate. Due acknowledgement has been made of all assistance received.

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ACKNOWLEDGEMENTS

I would like to thank all who have been involved with this project.

Special thanks must go to my supervisor Dr S. J. Hammonds for his help, advice and encouragement throughout both the practical stages and during the writing of the thesis. Thanks also to Dr B. Scanlon and Dr E. Billet for their helpful suggestions concerning this work on several occasions. Thank you to Wellcome Foundation Limited for their financial support of the project and to the staff of Wellcome Diagnostics for their friendly assistance given on the many visits made to Beckenham.

I would like to thank Dr M. Gaston especially for his time and enthusiasm over the three years spent guiding me on practical aspects of the study.

Many thanks are due to Anne Lane and Andrew Young at Beckenham and Ted Willis and Sharon Thornton at Dartford for their help in the polyclonal antibody production.

A special thanks to Tim Easterbrook and Dr. Julie Holland for their friendly advise during the early part of the project. Thank you also to Diane, Satwinder and sadly, the late Jim Tricket and for a short time Rob, Zoe and Lisa for putting up with me and for the fun we had on many social events especially the reviews! I wish them all success in their future careers.

Thank you to Mrs M. Martin and prior to her arrival, Martin, for the many photographs of gels and blots and particularly to Maggie for the professional presentation of my poster and figures in this thesis.

Thank you to Mrs D. J. Dancer and Ms L.Brazier for their patience, time and skilful production of this work.

Finally, I should like to dedicate this thesis to my wife, Erica, and to thank her for her support and understanding during the ups and especially the downs during this project.

Abstract

Several strains of *Neisseria gonorrhoeae* have been characterised and minimum inhibitory concentrations of benzyl penicillin and methicillin against each strain were determined. Three penicillin binding proteins (PBPs) labelled with ^{3}H-penicillin were separated and visualised using traditional fluorography.

Antisera against beta-lactam conjugates were prepared and subsequently characterised using ELISA. The antisera raised against the methicillin conjugate were used in Western blots to probe for methicillin bound to PBPs from sensitive and resistant strains of gonococci. PBPs 1 and 2 bound methicillin and were visualised using the anti-methicillin sera. The reduced affinity for methicillin of PBPs 1 and 2 in chromosomally resistant strains was demonstrated by Western blotting. This correlates with a reduced level of binding of radio labelled penicillin to PBPs of these strains as detected by fluorography.

Affinity chromatography with beta-lactam ligands was used to isolate and purify PBPs from detergent solubilised membranes of both a sensitive and resistant strain of *Neisseria gonorrhoeae*. PBP3 proved difficult to solubilise compared with the other two PBPs. All 3 PBPs bound avidly to the ligands: 6-amino penicillanic acid and 7-amino cephalosporanic acid. The bound PBPs were difficult to elute, PBP3 was removed from both ligands in small amounts using rigorous conditions.

A beta-lactamase was purified from *Neisseria gonorrhoeae* and

partially characterised. Antibodies were prepared against this protein using material cut from polyacrylamide gels. The antibodies recognised the beta-lactamase from *Neisseria gonorrhoeae* in Western blots and also cross reacted with beta-lactamase from *Escherichia coli* (R-TEM) and *Bacillus cereus* type I. This suggests that proteins have regions of amino acid homology which can be recognised by the antibodies. The potential use of the anti-methicillin and anti gonococcal beta-lactamase antibodies to detect chromosomally mediated resistance and beta-lactamase mediated resistance respectively, is discussed.

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ABBREVIATIONS

7-ACA	7-Amino-Cephalosporanic Acid
AIDS	Acquired Immunodeficiency Syndrome
6-APA	6-Amino-Penicillanic Acid
ATP	Adenosine Triphosphate
BSA	Bovine Serum Albumin
Cpase	Carboxypeptidase
DGI	Disseminated Gonococcal Infection
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic acid
DPG	Di-phosphatidylglycerol
EDC	N'-(3-dimethylaminopropyl)-carbodiimide hydrochloride
ELISA	Enzyme Linked Immunosorbent Assay
Epase	Endopeptidase
EDTA	Ethylene diamine tetra-acetic acid
HIV	Human Immunodeficiency Virus
HSA	Human Serum Albumin
HPLC	High Performance Liquid Chromatography
IgA	Immunoglobulin type A
IgE	Immunoglobulin type E
IgG	Immunoglobulin type G
IgM	Immunoglobulin type M
KDO	Keto-deoxy-D-manno-octulosonic acid
LOS	Lipo-oligosaccharide
LPS	Lipo-polysaccharide
MRSA	Methicillin Resistant *Staphylococcus aureus*
MIC	Minimum Inhibitory Concentration
MW	Molecular Weight
OD	Optical Density
PBP	Penicillin Binding Protein
PBS	Phosphate Buffered Saline
pCMB	Per-chloromercuribenzoic acid
Pc^r	Cryptic plasmid
PG	Peptidoglycan
NADH	Nicotinamide Adenine Dinucleotide (reduced)
NAG	N-Acetyl Glucosamine
NAM	N-Acetyl Muramic Acid

Spp	Species
STD	Sexually Transmitted Disease
TEMED	N,N,N′,N′-Tetramethylethylenediamine
TMB	Tetra-methyl-benzidine
Tn	Transposon
Tpase	Transpeptidase
UDP	Uridine di-phosphate
UMP	Uridine mono-phosphate

Chapter 1

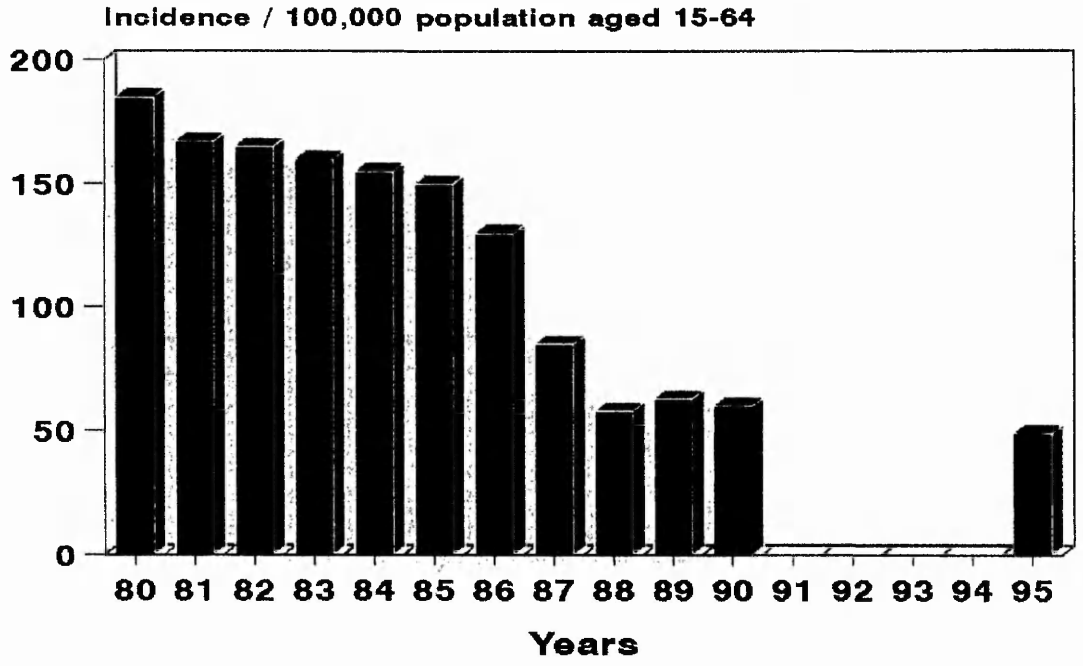
MAIN INTRODUCTION

1.1 Gonorrhoea: Epidemiology of a sexually transmitted disease.

1.1.1 Incidence and importance of the disease.

Neisseria gonorrhoeae the aetiological agent of gonorrhoea was first described by Albert Neisser in 1879 who observed the organism in urethral pus. The disease is still a major problem in the world today despite the first successful use of anti-microbial chemotherapy using sulphonamides in the 1930's (Tramont and Boslego, 1988).

The actual incidence of gonorrhoea is thought to be double the number of reported cases. The incidence of new cases of gonorrhoea in England has fallen steadily from 180 cases per 100,000 population in 1981 to about 60 per 100,000 population in 1988 in the age range 15-64 (**Figure 1.1**). However figures for 1989-1990 show a small increase in the number of cases (data from Health of the Nation, 1992). This reversal of the downward trend for gonorrhoea in 1989 is the first since 1973 (Catchpole, 1992) and is reflected in the increase in gonorrhoea in homosexual males in England between 1989-1990 (Waugh, 1991). A similar increase has also been reported in the USA (Handsfield and Schwebke, 1990). The decrease in incidences since 1981 is thought to be due to a modification of human sexual behaviour, especially in homosexual men (Carne *et al.*, 1987) since the first diagnosis of AIDS and HIV infection. The small rise in gonorrhoea is



1995 target: 49 new cases of gonorrhoea

Figure 1.1 New cases of gonorrhoea at genitourinary clinics in England 1980-1990 and the target for the year 1995. (Health of the Nation , 1992)

possibly due to firstly a relapse in the behaviour of those who had changed their practices in response to the threat of HIV infection and secondly, part of the rise may be due to transmission within a new cohort of younger people who lack in sexual experience and education (Catchpole, 1992).

The reported number of people in England with HIV infection to March 1992 was 15,133, the actual incidences are likely to be higher. Since HIV may not progress to the symptoms of AIDS for several years after initial infection, compared with gonorrhoea which usually presents symptoms within days after infection, it is therefore considered as a better indication of any changes in the sexual behaviour of a population which may have an effect on HIV transmission. Thus a decrease in numbers of reported cases of gonorrhoea may indicate a change in behaviour and attitudes of people to sexually transmitted diseases as a whole and therefore a fall in incidences of all STD's including HIV (Health of the Nation, 1992).

1.1.2 Epidemiology of resistance.

The epidemiology of penicillin resistant *Neisseria gonorrhoeae* has recently been studied by Ison and Easmon (1991). Out of 1589 strains isolated from a London hospital, 840 were penicillin sensitive (MIC < 0.5 mg/l) 100 were penicillin resistant (MIC ≥ 1mg/l) and 649 were penicillinase producing resistant strains.

1.2 Characteristics of *Neisseria gonorrhoeae*.

Neisseria gonorrhoeae is a member of the Neisseriaceae family

which includes another pathogen, *Neisseria meningitidis* and other non-pathogenic species, *Neisseria sicca, N. subflava, N. flava* and *N. perflava* (Knapp, 1988). *Neisseria gonorrhoeae* is a Gram negative diplococcus, size 0.6-1.0μm (Sleigh and Timbury, 1986). The characteristic "bean-shaped" cells are arranged with flattened surfaces together and are often located within or associated with polymorphonuclear leucocytes in clinical samples containing pus. Cells are piliated or non-piliated and colonies are opaque or transparent. A nomenclature based on relative size, opacity and sharpness of edge demarcation is used to identify colonial types. Kellogg *et al.* (1968) described four different colonial variations in *Neisseria gonorrhoeae*. Gonococci are fastidious and have exacting growth requirements, *in vitro* the organism requires an enriched medium with a defined supplement (Kellogg *et al.*, 1963). An improved liquid and solid medium was developed by MacFarlane and Elias-Jones (1980) to support growth of the gonococcus in routine isolation laboratories. Recent studies have used a proteose peptone based medium with a supplement of Isovitalex containing various amino acids, vitamins and minerals (Dougherty *et al.*, 1980). Optimum growth is achieved between 35-37°C in moist aerobic conditions with a carbon dioxide level of 5-10%. In the case of clinical isolates, heated blood agar is routinely used and selective media for example Thayer-Martin, containing colistin, vancomycin and nystatin (Thayer and Martin, 1964) are of value in the isolation of gonococci from other organisms. Gonococci have survived for up to a week on charcoal swabs in transport medium containing anaerobic salts solution, agar and methylene blue (Carlson *et al.*, 1980).

The organism is oxidase positive and can be distinguished from other neisserias by the fact that it produces acid from glucose (but no other carbohydrates) in a rapid carbohydrate utilisation test (Young *et al.*, 1976). The gonococcus has a fairly complex antigenic structure and antibodies directed against it cross react with the antigens in other Neisseria species present in the pili, cell wall lipopolysaccharide (LPS) and outer membrane proteins. Antibodies from sera of infected children and adults were used in an immunoblotting study to detect cross-reaction between major antigens of *Neisseria polysaccharea*, *N. lactamica* and *N. meningitidis*. There was a significant decrease in the antibodies directed against these common antigens following absorption of the sera with the three *Neisseria* species (Cann and Rogers, 1989). Immunoblotting with patient sera to *Neisseria gonorrhoeae* antigens was used by Hadfield and Glynn (1984) to detect antibodies to gonococcal antigens in sera of patients with gonorrhoeae. Immunoglobulin G (IgG) was found predominantly to be against lipopolysaccharide. A monoclonal antibody prepared to *Neisseria gonorrhoeae* lipopolysaccharide was found to cross react with LPS from *Neisseria meningitidis* in an ELISA (Apicella *et al.*, 1981).

The disease is normally diagnosed by isolation and identification of *Neisseria gonorrhoeae* using direct microscopy and carbohydrate utilisation tests. A fluorescent monoclonal antibody for the identification of cultures of *Neisseria gonorrhoeae* has been described (Ison *et al.*, 1988). It is also possible to detect antibodies in patients' serum by means of an ELISA using whole

cells attached to the plate using poly-l-lysine (Ison *et al.*, 1981).

1.3 Antibiotic sensitivity testing and treatment of gonorrhoea.
Antibiotic susceptibility testing of *Neisseria gonorrhoeae* isolates is important for the determination of resistance. Antimicrobial susceptibility testing has recently been reviewed by Doern and Jones, 1988. Chromosomal resistant isolates of penicillin can be detected by direct susceptibility tests and these should be performed on beta-lactamase negative isolates which fail to respond to penicillin. The effect of different media on susceptibility testing has been studied by Woodford and Ison (1988). Four media were compared, all of which showed good MIC agreement with beta-lactams but there was a discrepancy using gonococcal base agar and 1% Isovitalex with tetracycline and erythromycin antibiotics. Since the emergence of beta-lactamase producing strains of gonococci (Ashford *et al.*,1976 ; Percival *et al.*, 1976) diagnostic tests have become of importance in the evaluation of effective antibiotic regimens (Doern and Jones, 1988).

Benzyl penicillin administered in a single intramuscular dose together with probenecid to delay renal excretion, is a satisfactory treatment for combating gonococcal infection. However ampicillin, administered orally, is the preferred drug (Anderson *et al.*, 1969). Tetracycline and spectinomycin are the usual drugs to treat complicated infection or are the choice in the event of hypersensitivity to penicillin in the patient or

resistance to penicillin in the bacteria. However, some strains are resistant to tetracycline due to gene mutations affecting the outer membrane and ribosome function (Heritage and Hawkey, 1988).

1.4 Pathogenicity of *Neisseria gonorrhoeae* infection and disease characteristics.

The sexually transmitted disease is primarily an infection of the urethral tract in males and of the urethral tract and endocervix in females with asymptomatic infection in females being a dangerous reservoir of infection. An indirect ELISA using outer membrane proteins, from several serotypes in *Neisseria gonorrhoeae,* as a plate antigen for detecting gonococcal antibodies has been evaluated as a method for diagnosis of gonorrhoea in asymptomatic individuals (Brodeur *et al.*, 1982). The organism in women may spread to the cervix, Bartholin's glands, uterus, uterine tubes, ovaries and peritoneum. In men the bacteria may infect the prostate gland, seminal vesicles, epididymis and testes. Infection of the pharynx and rectum is not uncommon. Disseminated infection is a relatively rare complication and is readily treated with penicillin, however, following the initial bacteraemic stage serious complications such as endocarditis can occur.

Neonates can present with ophthalmia neonatorum; a conjunctivitis following infection from the mother at birth. Rapid systemic antibiotic treatment together with topical applications to the eye are necessary to prevent complications leading to blindness.

1.5 Gram negative cell envelope.

Neisseria gonorrhoeae possesses the typical Gram negative envelope (**Figure 1.2**), consisting of an inner or cytoplasmic membrane composed of phospholipids and proteins.

1.5.1 Cytoplasmic membrane.

The cytoplasmic membrane has the basic structure of the fluid mosaic model with a phospholipid bilayer containing integral proteins (Singer and Nicholson, 1972). The phospholipids are phosphatidylethanolamine, phosphatidylglycerol (PG) and diphosphatidylglycerol (DPG). In gonococci the cytoplasmic membrane has a lower proportion of DPG (cardiolipin) and a higher proportion of PG compared with the outer membrane (Wolf-Watz *et al.*, 1975). The cytoplasmic membrane of all Gram negative bacteria contains relatively less saturated fatty acids compared with the outer membrane (Lugtenberg and Peters, 1976). The proteins including penicillin binding proteins (PBPs) are grouped together in the phospholipid bimolecular layer giving the so-called mosaic structure. The membrane contains the usual complement of enzymes ie succinate, lactate, NADH-dehydrogenases and ATPase (Morse *et al.*, 1977). Recent work, reviewed by Spratt in 1988, showed that PBP1 and PBP3 from *Escherichia coli* are essentially periplasmic enzymes that are only anchored in the cytoplasmic membrane at their amino termini. Whereas PBP5, a low molecular weight PBP, is attached in the membrane at the carboxylic acid end and the penicillin binding domain is in the periplasm.

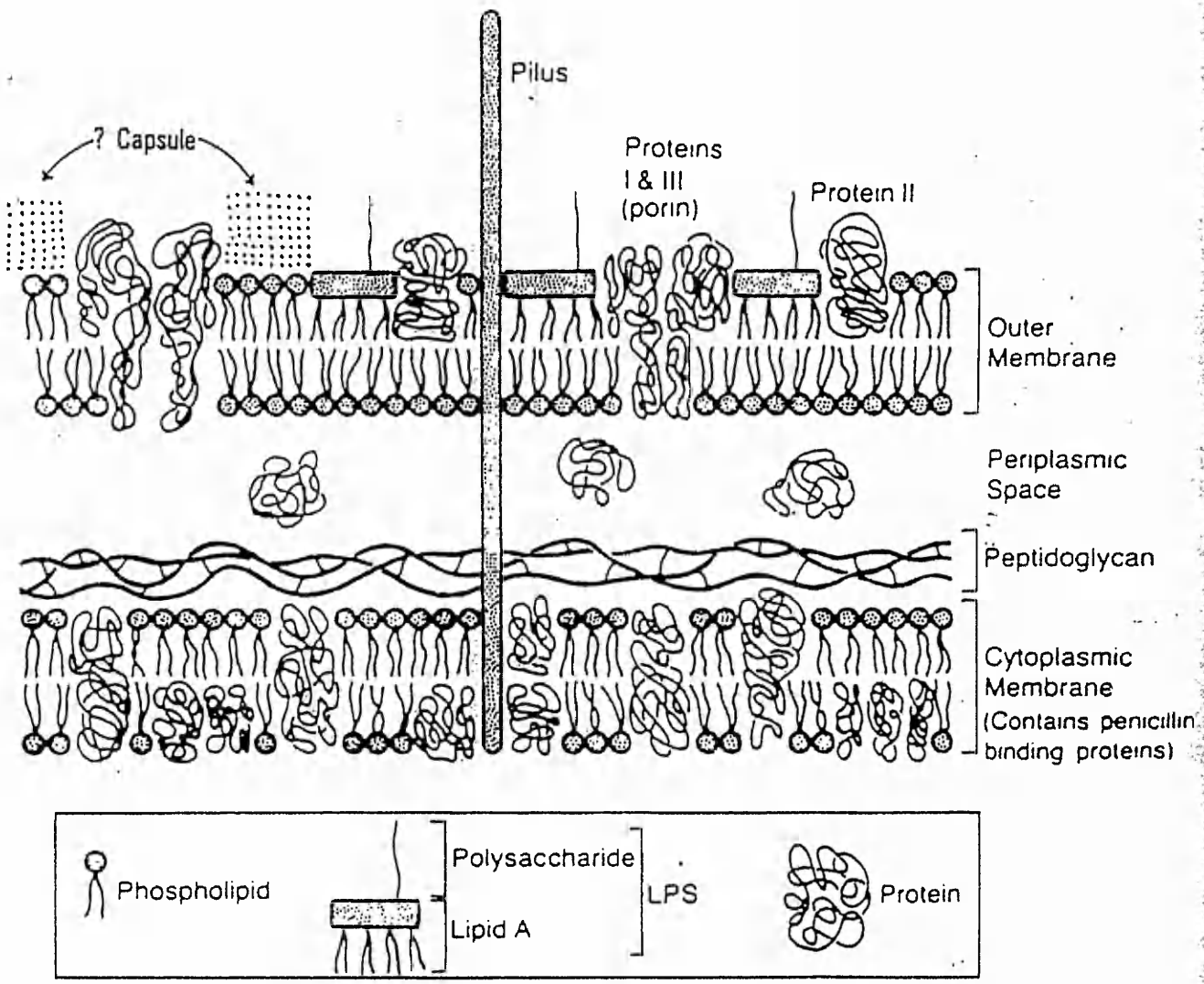


Figure 1.2: Cell wall of *Neisseria gonorrhoeae* (Hook and Holmes, 1985).

lps (lipopolysaccharide)

1.5.2 Periplasm.

The periplasm which contains the peptidoglycan (discussed in detail in section 1.5.3) occupies around 25% of the cell volume (Morse and Lysko, 1980). This compartment contains various specific proteins (Nessel and Heppel, 1966) such as beta-lactamase and nutrient binding proteins, which prevent leakage through the outer membrane.

1.5.3 Peptidoglycan.

A layer of peptidoglycan is responsible for conferring the shape and maintaining the rigidity of the cell. Peptidoglycan in Neisseriae, which forms 1-2% of cell dry weight (Hebeler and Young, 1976) is typical of Gram negatives being of the chemotype I (Scheifer and Kandler, 1972; Wolf-Watz *et al.*, 1975). It is composed of alternating sugars of N-acetyl-glucosamine (NAG) and N-acetyl-muramic acid (NAM) joined by a beta 1-4 glycosidic bond. Attached to NAM in the three-dimensional lattice of peptidoglycan is a tetrapeptide of both D and L amino acids comprising L-alanine, D-glutamic acid, diaminopimelic acid and D-alanine. (**Figure 1.3**) Precursors of peptidoglycan synthesis are located in the cytoplasm. First, NAM and NAG are each joined to a molecule of uridine diphosphate (UDP) (Park and Strominger, 1957). A penta-peptide composed of the tetra-peptide and one additional D-alanine is bound to the NAM-UDP. The nucleotide is released and the sugar units, NAM first, are joined to a lipid carrier in the cytoplasmic membrane called undecaprenyl phosphate via a pyrophosphate attached to NAM. The sugars become coupled together, the energy for which is derived from the release of the

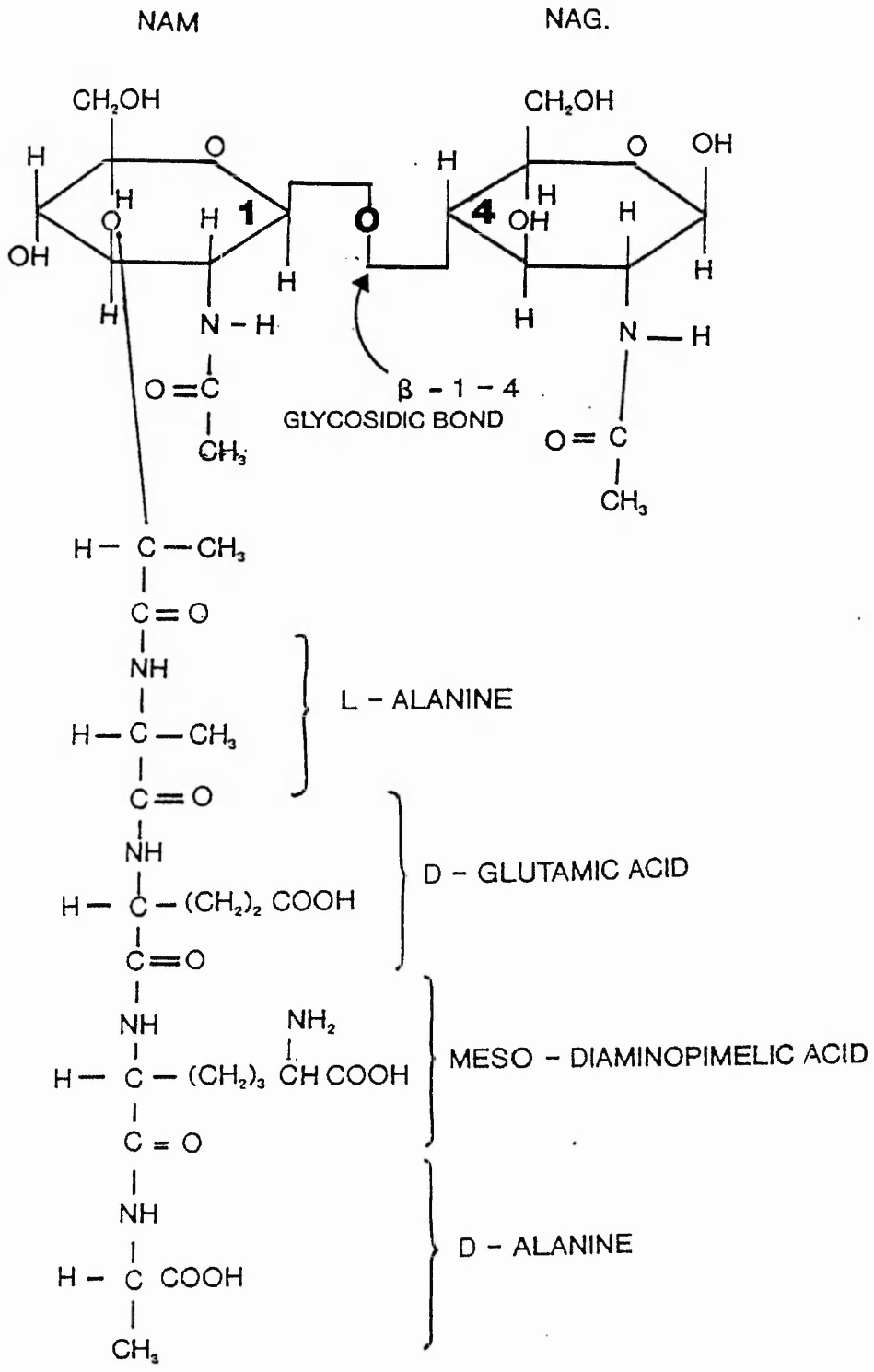


Figure 1.3: Peptidoglycan subunit structure found in *Neisseria gonorrhoeae*.

nucleotides, to form a disaccharide penta-peptide containing a beta-1-4 glycoside bond (**Figure 1.4**). The joining of the unit to the carrier in the membrane affords translocation of the now lipid-soluble unit across the membrane to the cell wall at the region of growth. The unit is joined to the linear glycan strand between the NAM in the pre-existing PG and NAG of the entering disaccharide penta-peptide unit. The lipid carrier is simultaneously released and a pyrophosphorylase cleaves a phosphate from the carrier which then collects another NAG-NAM peptide unit from the cytoplasm (**Figure 1.4**). Finally the linear peptidoglycan strand is cross-linked to the rest of the network by the PBPs or penicillin enzymes in the cytoplasmic membrane.

1.5.4 Outer membrane.

The outer membrane of *Escherichia coli* bacteria is 7.5 nm thick (Glavert and Thornley, 1974) and has been extensively reviewed by De Maargd and Lugtenberg,(1987). All outer membranes contain protein, phospholipids and lipopolysaccharide in variable amounts (Lugtenberg and Van Alphen, 1983). Lipopolysaccharide is located on the outside together with proteins which are attached by hydrophobic interactions and divalent cations. Some of these proteins are porins, aqueous pores through the membrane, typically consisting of 3 porin molecules. They permit the transmembrane diffusion of small hydrophilic molecules such as beta-lactam antibiotics. Outer membranes permit the permeation of solutes by this hydrophilic route or a hydrophobic use (Nikaido, 1976). Hydrophobic compounds pass through the phospholipid bilayer by passive diffusion. In gonococci passage

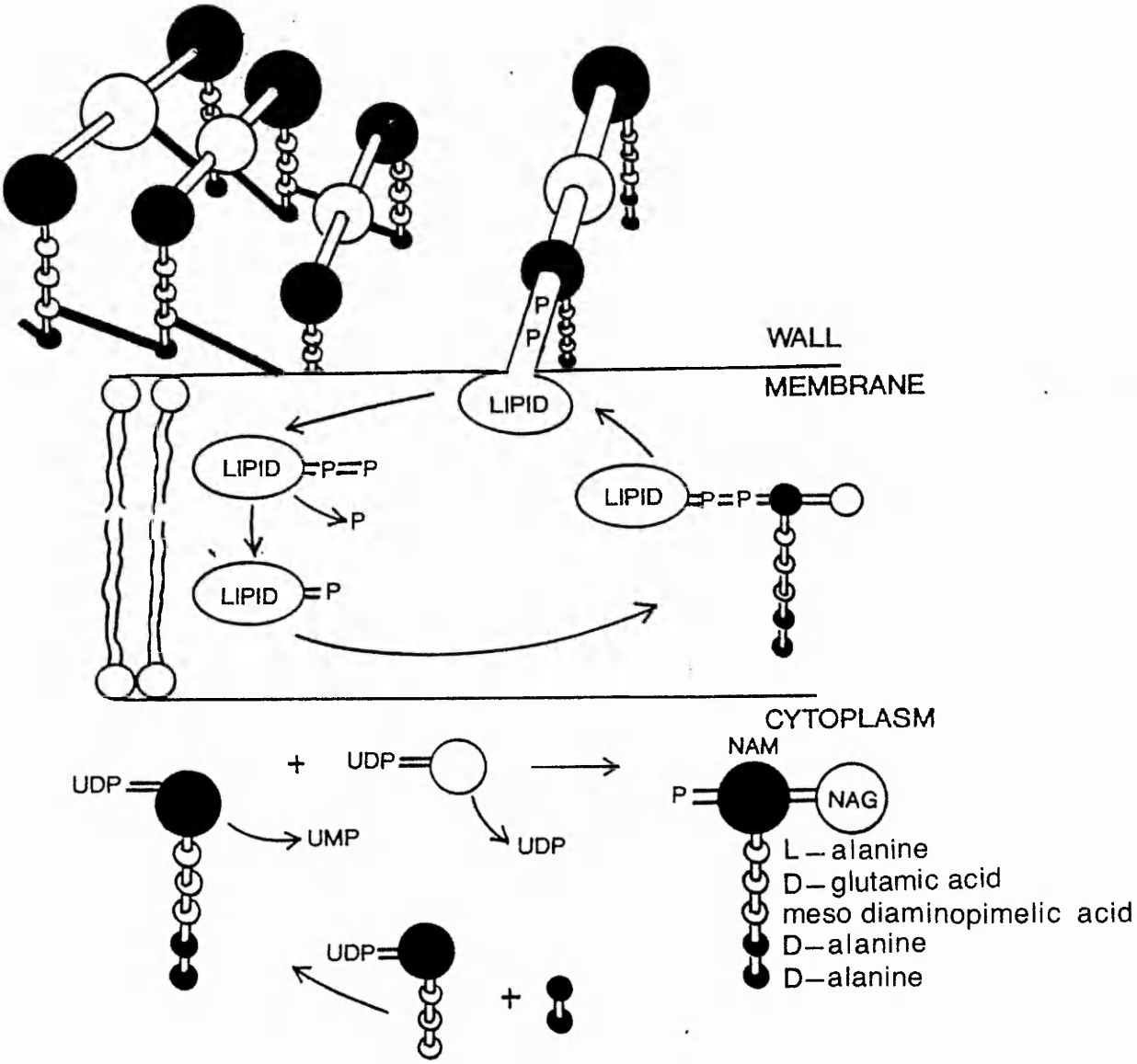


Figure 1.4: Peptidoglycan synthesis in *Neisseria gonorrhoeae*.

of hydrophobic fatty acids (Miller *et al.*, 1977) and steroids (Lysko and Morse, 1980) renders the organism sensitive to low inhibitory concentrations of these compounds. Recently a third pathway has been described; the self-promoted uptake pathway, which involves the displacement of divalent cations in the LPS of Gram negative bacteria by antibiotics such as polymyxin B and streptomycin (Hancock,Raffle and Nicas, 1981). This leads to displacement of the LPS and allows uptake of these antibiotics. On the inner face is a phospholipid layer with lipoproteins covalently attached to the peptidoglycan. In gonococci there is evidence for a phospholipid bilayer in the outer membrane (Lysko and Morse, 1981).

1.5.4.1 LPS.

The LPS in *Neisseria gonorrhoeae* lacks the O-antigenic side chains present in many Gram negatives and hence is termed lipo-oligosaccharide (Grifiss *et al.*, 1988). However it possesses lipid A and core oligosaccharide composed of several monosaccharides and keto-deoxy-D-manno-octulosonic acid (KDO). EDTA chelates with $Mg2^{+}$ ions which cross-bridges adjacent LPS polyphosphates. This leads to instability and breakdown of the LPS (Leive 1965). The LPS is endotoxic and in a fallopian tube model has been shown to mediate the loss of cilia and cause death in adjacent uninfected cells (Gregg *et al.*, 1981).

1.5.4.2 Pili

Pili serve for the attachment to mucosal surfaces in the host (Schoolnik *et al.*, 1984). Piliated strains have been shown to be

more virulent in several animal models compared with non-piliated strains (McGee *et al.*, 1981). Protein II in the outer membrane is also involved in attachment to mucosal surfaces.

Pili protrude from the outer membrane of *Neisseria gonorrhoeae* and comprise multiple units of pilin protein of molecular weight 19kDa (Buchanan *et al.*, 1978). Pilin is very important in terms of pathogenesis of the organism and thus have been the subject of interest for a vaccine. Field studies in to a pilin vaccine however, were unsuccessful (Tramont, 1989). The carboxy end of the pilin molecule shows antigenic variability from strain to strain whereas the amino end has regions of interstrain homology (Segal *et al.*, 1985; Schoolnik *et al.*, 1984). Also a single strain can produce pili of differing antigenic composition (Britigan *et al.*, 1985; Heckels *et al.*, 1984). Differing colonial forms can be observed by growing gonococci on translucent agar and viewing under stereo-microscope (Kellogg *et al.*, 1963). The differences in the form of the colonies is dependent on the numbers of pili on the cells. Clinical isolates are which are piliated give P^{+} or P^{++} colonies (formerly T1 and T2) but within 24 hours of culture the non-piliated cells predominate giving P^{-} (formerly T3 and T4) colonies. Non-piliated strains are avirulent when inoculated into humans (Kellogg *et al.*, 1963). The change from P^{+}, P^{++} to P^{-} is under chromosomal control (Brittigan *et al.*, 1985).

1.5.4.3 Outer membrane Proteins I , II and III.

Protein I (molecular weight 32-36kDa) is the major protein (30-40%) in outer membranes, is associated with LPS and acts as a

porin (Blake and Gotschlich, 1983) through which hydrophilic substances can pass through the otherwise hydrophobic outer membrane. Swanson (1979) reported the existence of two forms of protein I molecules (protein IA and IB). The two sub-classes; IA and IB can be distinguished in a common coagglutination assay using six monoclonal antibodies against the proteins. Depending on the reaction to the panel of antibodies the strain can be classified as a serovar (Tam *et al.*, 1982; Knapp, 1988). A study using peptide mapping of tryptic fragments from labelled protein I demonstrated that protein IA molecules contain the antigens classified as serogroup WI and protein IB molecules contain the antigens common to the serogroups WII and WIII (Sandstrom *et al.*, 1982).

Protein II is a collection of several proteins with molecular weights 20-28kDa (Swanson 1982). An immunological investigation using rabbit polyclonal sera by Swanson and Barrera (1983) concluded that all gonococcal PII moieties have common antigenic determinants which are not generally accessible on the cell surface to antibodies. However the surface-exposed antigens of different PII constituents are generally immunologically different from each other. The protein II group are termed the opacity proteins since they make the colonies appear opaque under a stereomicroscope (Swanson 1982). The proteins can vary in amount and presence; one strain may have none whereas another may have up to six protein II types (Black *et al.*, 1982). Presence of protein II has been associated with virulence and adherence to human neutrophils and loss of protein II prevents phagocytic

killing by neutrophils (Virgi and Heckels, 1986).

Protein III, with a molecular weight of 30-31kD, has no interstrain antigen variation and is found in close association with protein I and LPS (Blake and Gotschlich, 1983). A monoclonal antibody to protein III was used in immunoprecipitation and Western blots to detect protein III in several strains of *Neisseria gonorrhoeae* (Swanson *et al.*, 1982). The authors concluded that the protein III is a surface-exposed antigen common to all strains of *Neisseria gonorrhoeae*. Immunoprecipitation of protein I and III with whole antigonococcal sera in the presence of protein and sepharose in similar ratios suggested the two proteins may form heteropolymers in the gonococcal outer membrane. Little is known of the function of protein III but it has been found to stimulate blocking antibodies which decrease serum bactericidal action against *Neisseria gonorrhoeae* (Rice *et al.*, 1986). Protein III is analogous to outer membrane A in *Escherichia coli*. Other outer membrane proteins include those which are similar to the *Neisseria meningitidis* iron utilisation and transport proteins with molecular weights from 37 to >800kD (West and Sparling, 1985).

1.6 Vaccines.

Studies on a gonococcal vaccine have been ongoing for many years, however to date there is no successful preventative vaccine. The most recent work on immunization against gonorrhoea has been reviewed by Tramont (1989). The basis of a good vaccine requires

a detailed working knowledge of the infection and because there is no animal model potential vaccines must be tried on human volunteers. Possible vaccine candidates have included pili (because pili are essential for attachment to mucosal surfaces), protein I, protein II, lipo-oligosaccharide, major iron-regulated protein, H.8 antigen, capsule and IgA protease. A pilus vaccine administered to human volunteers afforded protection against a challenge from the strain from which the vaccine was derived (Brinton *et al.*, 1978). A significant proportion of human volunteers developed antibodies to pili after parenteral administration of purified pili, in a large study however no overall protection was afforded (Tramont *et al.*, 1985). It has been suggested that other cell membrane antigens should now be investigated although the antigenic variation within these antigens may hamper the search for a suitable candidate (Tramont, 1989).

1.7 Function of Penicillin Binding Proteins (PBPs).

Neisseria gonorrhoeae has three PBPs. The PBP1 has transpeptidase activity and it binds to the penultimate D-alanine in the penta-peptide with the release of the terminal alanine. The transient acyl-enzyme intermediate is active and the enzyme links the carboxylic acid group of the D-alanine to the epsilon-amino group (Schleifer and Kandler, 1972) of diaminopimelic acid on an existing peptidoglycan strand forming a peptide bond with the release of the enzyme (**Figure 1.5**). Observations by Rosenthal *et al.*, 1980, showed that the gonococcal peptidoglycan is only about

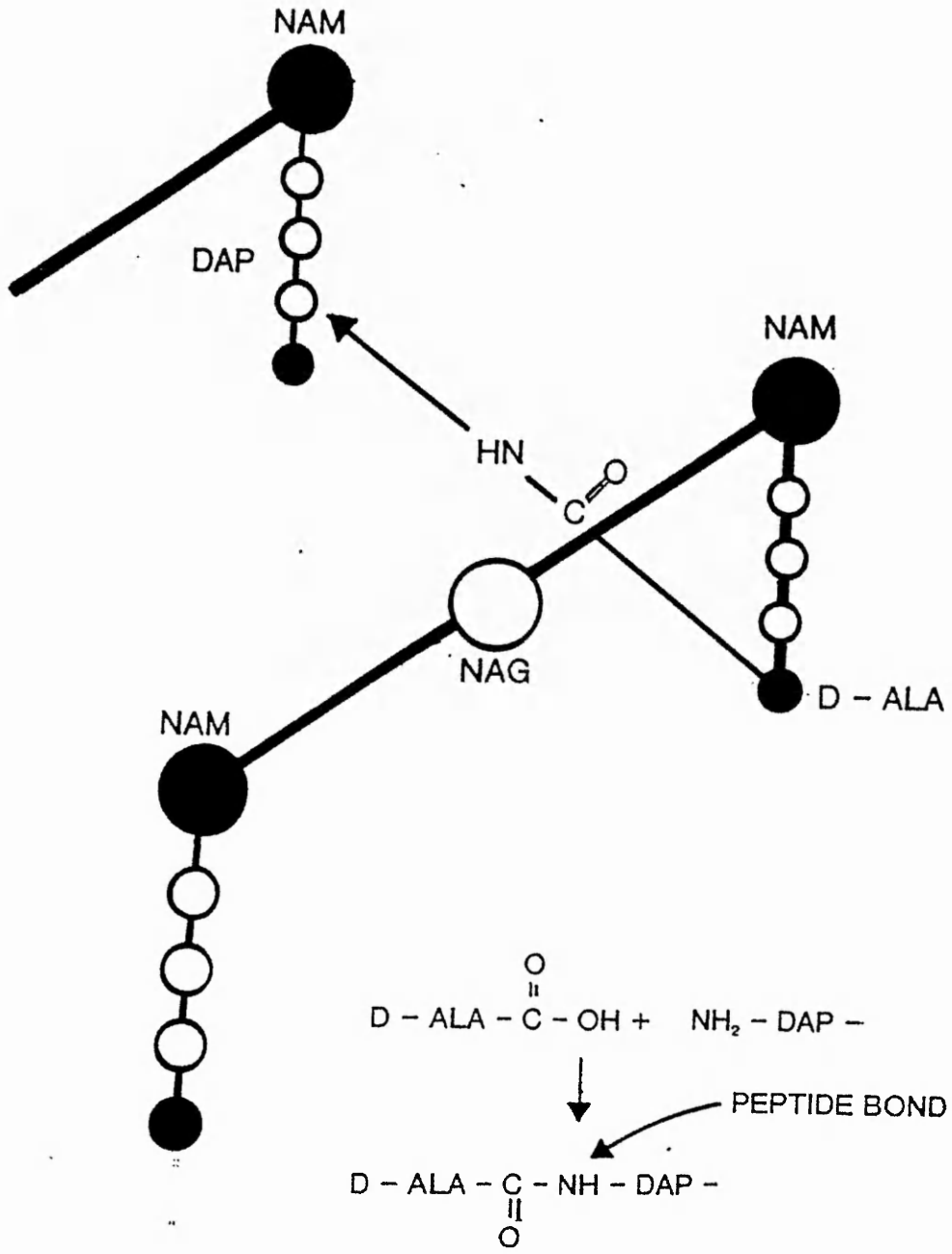


Figure 1.5: The final cross-linking of tetra-peptides (transpeptidation) in the peptidoglycan of *Neisseria gonorrhoeae*.

40% cross-linked but chemical analysis has proved that all the terminal D-alanines have been removed. This is brought about by the action of a carboxypeptidase enzyme, PBP2 which removes all D-alanines not involved in the cross-linkage reaction. Thus, the degree of cross-linked peptidoglycan is controlled by the balance of transpeptidases (Tpases) and carboxypeptidases (Cpases) (Blumberg and Strominger, 1974). Other studies by Blundell *et al.*, 1985 proved that peptidoglycan in *Neisseria gonorrhoeae* is extensively O-acetylated. O-acetylation of the sugar units is a post synthetic modification of the peptidoglycan and Dougherty (1985) and it is thought that such groups may contribute to the rigidity of the peptidoglycan network or possibly act as a substrate level control for the turnover of peptidoglycan by the active hydrolase system. The differences in the degree of O-acetylation of peptidoglycan in strains with decreased susceptibility to penicillin was later explained by the reduced activity of the carboxypeptidase. The function of PBP3 is not known and it does not seem to be involved in resistance. However, it has been suggested that non-essential PBPs may be involved in the sequestration of beta-lactams (Livermore, 1988)

1.8 Penicillins

1.8.1 The discovery of penicillin.

The observation by Alexander Fleming in 1928, that *Penicillin notatum* produced a substance, later named penicillin, which inhibited bacterial growth on agar plates, was not fully exploited at the time. This was due to the advent of sulphonamide therapy. However, Falk and Chain isolated penicillin from the

mould and set up clinical trials to measure the efficacy against staphylococcal and streptococcal infections. The research into bulk production of the antibiotic was carried out by workers in America who used *Penicillium chrysogenum* to produce larger amounts of the drug.

1.8.2 Spectrum of activity of Penicillin.

Penicillin is still used today to treat infections caused by *Staphylococcus*, *Streptococcus*, *Corynebacterium*, *Bacillus*, *Clostridium*, *Actinomyces*, *Treponema* and the Gram negative *Neisseria*. Many new beta-lactams have been developed by altering the side chain R-group attached to 6-amino penicillanic acid (reviewed by Robinson, 1988). These semi-synthetic drugs have been useful in treating infections by bacteria which have developed resistance to the parent drug benzyl-penicillin. The discovery of 6-APA came about during experimentation on semi synthetic penicillins using p-amino-benzyl-penicillin as a starting compound. It was found that 6-APA, with weak antibacterial action was produced by fermentation of *Penicillium chrysogenum* in the absence of a side chain precursor (Batchelor *et al.*, 1959). The first penicillins to be derived from 6-APA were the acid stable phenethicillin and propicillin which were better absorbed than the orally administered penicillin V. However the production of ampicillin (active against Gram negative and Gram positive species) and methicillin in 1961 proved the significance of 6-APA as a precursor of drugs with improved pharmaco-kinetic and pharmaco-dynamic properties. Methicillin was the first penicillin which was stable to beta-

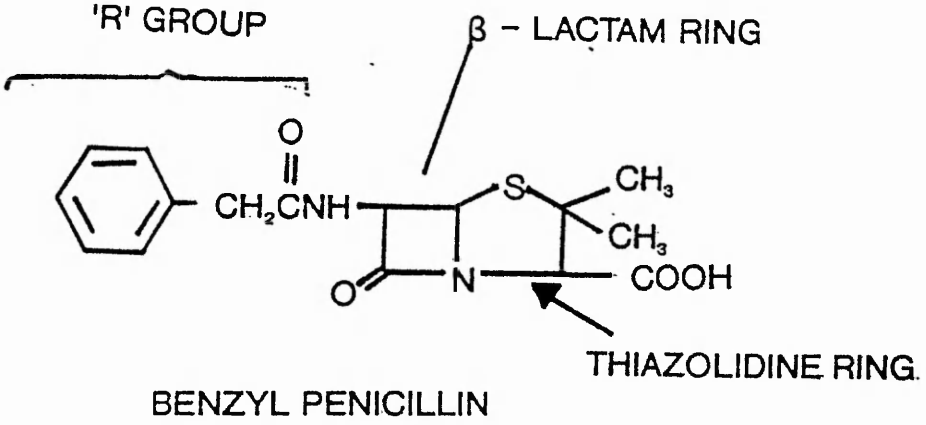
lactamase hydrolysis due to low affinity of the enzyme for the drug. However methicillin could not be administered by mouth and the affinity for PBPs was low. This led to the synthesis of isoxazoyl penicillins such as oxacillin and cloxacillin with higher in vitro activity than methicillin and better absorbed by mouth. Carbenicillin was the first penicillin with activity against infections caused by *Pseudomonas aeruginosa* (Hugo and Russell, 1987).

1.8.3 Structure of Penicillin.

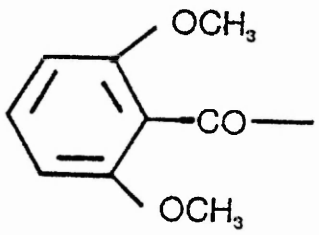
Penicillin is a beta-lactam antibiotic (**Figure 1.6**), containing a highly reactive beta-lactam ring containing the peptide bond which is acylated by PBPs. It also consists of a thiazolidine ring and a variable R-group which in some penicillins confers beta-lactamase stability eg. methicillin. (**Figure 1.6**). However methicillin has been superseded by other beta-lactamase stable drugs with improved anti-bacterial and pharmacological properties (Rolinson, 1988).

1.8.4 Mode of action of penicillin.

The effect of penicillin on the inhibition of cell wall biosynthesis was first reported by Duguid in 1946. The drug was found to cause spheroplast production and subsequent bursting of them in normal hypotonic media. The destruction of peptidoglycan by lysozyme led to the formation of spheroplasts and this observation reinforced the belief that penicillin also interferes with peptidoglycan. Wise and Park, 1965 and Tipper and Strominger, 1965, demonstrated that penicillin inhibited the



BENZYL PENICILLIN

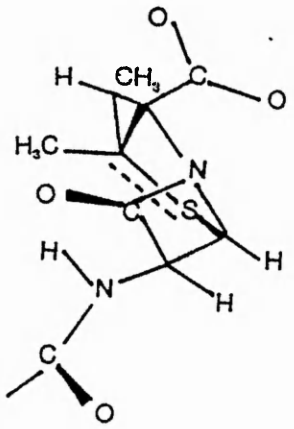


'R' GROUP IN METHICILLIN

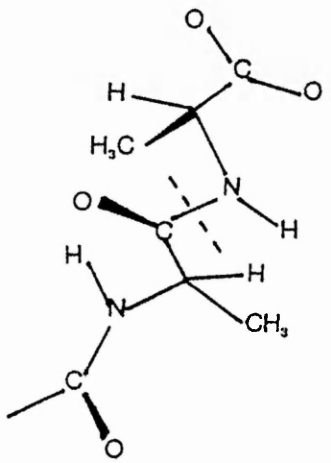
Figure 1.6: Molecular structure of benzyl-penicillin and methicillin

final crosslinking of peptidoglycan. The **figure 1.7** shows the molecular structure of benzyl-penicillin (formed from L-cysteinyl-D-valine and the natural substrate of PBPs D-alanyl-D-alanine. Tipper and Strominger, 1965 postulated that penicillin is a structural analogue of the terminal D-alanyl-D-alanine of the penta-peptide. The marked dotted line shows the peptide bonds which are broken in the acylation of the PBPs by penicillin and D-alanyl-D-alanine. A substrate requirement which is common to beta-lactams is the presence of a negative charge at the 3′ position in the penicillins provided by the COO^- and the 4′ position in cephalosporins. The beta-lactam binds to the enzymes to form an inactive penicilloyl-enzyme complex which is comparable with the natural active enzyme substrate intermediate which is formed as part of the terminal stages of peptidoglycan synthesis.

The bond between penicillin and penicillin-sensitive enzymes is now known to be a carboxylic ester between the carbon 7 of the beta-lactam ring and the hydroxyl group of a serine residue at the active site of the enzyme (Kozarich *et al.*, 1977). It was originally thought to be a thioester linkage but work by Kozarich on the hydroxylaminolysis of PBPs showed that release of PBPs from beta-lactams by neutral hydroxylamine is enzymatically catalysed and the bond was characteristic of a carboxylic ester. The serine residue involved in penicillin binding has been identified as serine 36 by amino acid analysis of the active sites of Cpases from *Bacillus* spp. and *Escherichia coli* PBP5. Certain class A beta-lactamases from *Staphylococcus aureus*, *Bacillus cereus*, *Bacillus licheniformis* and *Escherichia coli* have



PENICILLIN



D – ALANYL – D – ALANINE

Figure 1.7: Atomic structure of benzyl-penicillin and D-alanyl D-alanine. The ------ denotes the bond which is cleaved during acylation by PBPs.

been analysed for amino acid sequence homology (Ambler, 1980). Considerable homology was noted between the active sites of the Cpases and the amino terminal portion of the class A beta-lactamases especially in the region of serine 36 of the Cpases ie low molecular weight PBPs (Frere and Joris, 1985).

1.9 Penicillin sensitive enzymes may have a common origin.

Some PBPs, including *Escherichia coli* PBPS 5 and 6, which have D-alanine carboxypeptidase action have a half-life of 2 to 5 minutes at 37°C with certain beta-lactam antibiotics. The bound penicilloyl moiety is enzymatically hydrolysed releasing penicilloic acid (the product of beta-lactamase hydrolysis) Tamura *et al.*, 1976. Afterwards the catalytic activity of the PBP is resumed. Such observations suggest a common structural and evolutionary origin of beta-lactamases and PBPs. The beta-lactamases of *Bacillus cereus* type 1, *Bacillus licheniformis* RTEM, *Streptomyces albus* G (Samraoui *et al.*, 1986; Dideberg *et al.*, 1987) and the D, D-carboxypeptidase of *Streptomyces* R61 have been crystallised (Kelly *et al.*, 1989) and the 3-dimensional structures show similarity over several regions of tertiary structure although total amino acid homology is low. Again this is indicative of a common but distant evolutionary origin for beta-lactamases and PBPs.

1.10 Effect of Penicillin on PBP function

The consequences of penicillin action including morphological effects has been most extensively researched in *Escherichia coli*. Workers have looked at the effects of different beta-lactams on

PBPs. The use of PBP-deficient or defective mutants has given information about the role of individual PBPs and the lethal or non-lethal effects of beta-lactams on these PBPs has been elucidated.PBPs can be studied in membrane extracts or whole cells using SDS-PAGE to visualise the proteins. Radioactive penicillin bound to PBPs can be visualised by fluorography (Laskey and Mills, 1975). The individual PBPs have been isolated and purified using affinity chromatography (Blumberg and Strominger, 1972) whereby PBPs bound covalently to 6-aminopenicillanic acid on sepharose can be removed using neutralised hydroxylamine. **Table 1.1** is a summary of the seven PBPs in *Escherichia coli* K12.

Brian Spratt (1977) investigated the effects of over 30 different beta-lactams on *Escherichia coli* K12. Four major effects were observed:

(i) Inhibition of cell division and formation of long filaments.

(ii) Formation of filament with a central bulge.

(iii) Inhibition of cell elongation resulting in lysis after formation of a spheroplast.

(iv) Formation of spherical cells

Mecillinam selectively binds to PBP2 and causes the formation of large spherical cells which after a while stop dividing and eventually lyse. Binding of antibiotics to PBP3 and inhibition of its normal physiological function results in loss of septa, stops cell division and causes the filamentation of the cells. If PBP 1A and 1B are inhibited cell elongation ceases. PBP 1A can

Table 1.1: *Escherichia coli* PBPs

Protein	**Mol.wt.**	**% total PBPs**	**Morphological effects of inactivation**	**Function/enzyme activity**
1A	90,000	6	not essential for growth if PBP1B present normally	compensates for 1B if deficient **/Tpase**
1B	87,000	2	inhibition of cell elongation and lysis	peptidoglycan crosslinkage/ **Major Tpase**
2	66,000	0.7	large cells	maintenance of rod shape
3	60,000	2	filamentation	cell septum formation
4	49,000	4	none	regulates cross linkage/**Tpase Cpase, Epase**
5	42,000	65	none	regulates cross linkage/ **Cpase**
6	40,000	21	none	regulates cross linkage/ **Cpase**

Key: Tpase-Transpeptidase; Cpase-Carboxypeptidase; Epase-Endopeptidase.

take over the role of PBP 1B, as the major transpeptidase enzyme, if 1B is deficient as in certain mutants. Inactivation of both PBP 1A and 1B is necessary for rapid cell lysis. At low concentrations of most penicillins and cephalosporins cell division ceases, but cell growth continues and they elongate and become filamentous. Eventually the cells lyse unless the medium is isotonic. Increasing the antibiotic concentration inhibits concentrations of antibiotics rapidly inhibit elongation causing cell lysis and spheroplast production.

PBPs 1A, 1B and 3 have two possible areas of homology with each other and with PBPs 5 and 6 and the TEM beta-lactamase. Site directed mutagenesis has been used to convert the active site serine 307 in PBP3 (Keck *et al.*, 1985) to a cysteine residue, the result being that the mutant PBP3 causes a malfunction in cell division (Broome-Smith, Hedge and Spratt, 1985).

X-ray crystallographic studies have given a picture of the 3-dimensional configuration of two soluble D,D-Cpases secreted by *Streptomyces* R61 (Kelly *et al.*, 1989; Knox and Pratt, 1990) and *Streptomyces albus* G (Dideberg *et al.*, 1987; Jacob *et al.*, 1990). The former also has Tpase activity and has a serine residue at the active site at which beta-lactams bind to the enzyme. There are two major areas of secondary structure; one of five helices and the other of five strands of beta sheets with three helices covering the faces of the beta sheets. The binding site was deduced using cephalosporin C.

1.11 Genetics of the Gonococcus

1.11.1 Transformation.

Neisseria gonorrhoeae is readily genetically transformed but the organisms must be piliated for this to occur (Sparling, 1966). *Neisseria gonorrhoeae* is an extremely autolytic species the release of DNA by competent cells is dependent only on the presence of glucose and mono- or divalent cations in the medium (Biswas *et al.*, 1977).

Gonococci can also be transformed with plasmid DNA. A hybrid plasmid pFA14 of the conjugative plasmid pFA2 and the beta-lactamase Pc^r plasmid pFA3 has been used to transform competent cells which produce penicillinase encoding plasmids without any deletions (Biswas *et al.*, 1982).

1.11.2 Conjugation.

Antibiotic-resistance plasmids are transferred in gonococci by conjugation. However chromosomal genes cannot be transferred by conjugation (Cannon and Sparling, 1984). The plasmids Pc^r encoding beta-lactamase are transferred by the conjugative plasmid 24Mda (Biswas *et al.*, 1980; Roberts and Falkow, 1977; Sox *et al.*, 1978); pili are not necessary for conjugation (Einstein *et al.*, 1977) since mating pair formation by cell to cell contact is not affected by the presence or absence of pili. However, conjugation of Pc^r plasmids is reduced if either parent expresses a 28kDa form of outer membrane P II (Biswas *et al.*, 1980)
A physical map of the *Neisseria gonorrhoeae* chromosome has recently been constructed. The apparent size of the genome was

2219 Kbases, (Dempsey *et al* 1991).

1.11.3 Plasmids in *Neisseria gonorrhoeae*.

Plasmids are extra chromosomal double stranded, circular pieces of DNA which multiply independently of, although often at the same time as, the bacterial chromosome.They are generally less than 1/20 of the bacterial chromosome (Roberts, 1989).

1.11.3.1 Cryptic Plasmids.

Most gonococci carry the cryptic plasmid of 2.6 MDa, which was first described in 1972 (Engelkirk and Schoenhard, 1972). In one survey 96% of clinical isolates were found to contain the cryptic plasmid (Roberts *et al.*, 1979), which has no known phenotypic expression. However, the plasmid DNA has been fully sequenced (Korch *et al.*, 1985) and it has been found in other Neisseria species (Elwell and Falkow, 1975). The cryptic 2.6 Mda plasmid was found to be present in all the beta-lactamase producing strains in table 3.1 (information was supplied by the Gonococcus Reference Laboratory, Bristol). However, the plasmid content of the other strains was not known.

1.11.3.2 Conjugative Plasmids.

The 24.5 Mda conjugative plasmid was first described in 1975 (Elwell and Falkow 1975) and has been located in both clinical isolates of penicillinase-producing and non-penicillinase-producing strains (Roberts *et al.*, 1979). It is not present in all strains but may coexist with the cryptic and penicillinase plasmids. It can mobilise itself and both the smaller beta-

lactamase plasmids to other strains of *Neisseria gonorrhoeae* (Roberts and Falkow, 1977; Sox *et al.*, 1978; Biswas *et al.*, 1980).

The conjugative plasmid can also mobilise transfer of the beta-lactamase plasmids (but not itself) to *Escherichia coli* and other Neisseria species (Genco *et al.*, 1984).

1.11.3.3 25.2 MDa Tet M Conjugative Plasmids

Johnson and Morse (1988) described a 25.2 MDa Tet M plasmid which confers tetracycline resistance on gonococci. It shares a lot (>60%) of it's DNA sequence with the 24.5 MDa plasmid (Morse *et al.*, 1986) and this suggests that it may be derived from the 24.5 MDa plasmid containing the transposed Tet M determinant which encodes tetracycline resistance. Like the 24.5 MDa conjugal plasmid, the 25.2 MDa plasmid also mobilises itself and beta-lactamase plasmids to suitable recipient strains (Roberts and Knapp, 1988).

1.11.3.4 Gonococcal beta-lactamase plasmids

Beta-lactamase plasmids in pathogenic Neisseria species have recently been reviewed by Dillon and Yeung (1989). *Neisseria gonorrhoeae* which produce a TEM-type beta-lactamase (Elwell *et al.*, 1977; Bergstrom *et al.*, 1978) usually contain a Pc^r plasmid to be either between 3.2 - 3.4 Mda or between 4.4 - 4.7 Mda. The 3.2 -3.4 Mda plasmid was isolated in the UK and linked epidemiologically to strains in West Africa, whereas strains producing the 4.4 - 4.7 Mda plasmid were isolated in North

America and were linked to East Asia (Cannon and Sparling, 1984; Roberts *et al.*, 1977). Both plasmids encode 40% of the transposon Tn2 (Fayet *et al.*, 1982; Sanchez-Pescador *et al.*, 1981) commonly located on R-plasmids of Enterobacteriaceae and *Haemophilis influenzae*. Molecular analysis of the two plasmids has shown them to be identical except that the African plasmid has a 2.1kb deletion fragment compared with the Asian one (Roberts *et al.*, 1977). Gouby *et al.* (1986) reported the isolation of a 6.6 kbase plasmid encoding a TEM1 beta-lactamase in *Neisseria gonorrhoeae* in France. This was derived from the African plasmid 5.1kb containing an insertion of 1.1 kbases.

A 4.9 kbase (3.05 Mda) plasmid was isolated from *Neisseria gonorrhoeae* in an outbreak of gonorrhoeae in Toronto (Yeung *et al.*, 1986). A 4.6 kbase (2.9 MDa) plasmid isolated from a gonococcal strain in the Netherlands had a similar restriction endonuclease map to the Asian plasmid (4.4 - 4.7 MDa) (Van Embden *et al.*, 1985). Brett (1989) detected a 6.5 MDa plasmid which encoded beta-lactamase in 2 isolated strains of *Neisseria gonorrhoeae*. A multiple resistance plasmid was isolated from a strain of the commensal *Neisseria sicca* which encoded resistance to streptomycin, penicillin and sulphonamide (Rotger *et al.*, 1986).

1.12 *Neisseria gonorrhoeae* PBPs

Neisseria gonorrhoeae possesses three PBPs (Dougherty *et al.*, 1980 and Barbour 1981). The reported molecular weights are PBP1 87-90,000 daltons, PBP2 59-63,000 daltons and PBP3 44-48,000

daltons. In 1978 Rodriguez and Saz extracted membranes from sensitive and resistant gonococci and detected 8 penicillin proteins by slab gel electrophoresis. PBP patterns of both disseminated gonococcal infection (DGI) and non-disseminated gonococcal infection strains were compared using fluorography (Nolan and Hilderbrandt, 1979). The authors concluded that PBPs do not undergo characteristic changes in the DGI strains. Both Dougherty,1980, and Barbour, 1981, carried out PBP assays with ^{14}C penicillin in whole cells and isolated membranes. In both preparations and in all strains tested the same pattern of three PBPs was observed. However, strains with a higher MIC showed a decreased binding to PBPs 1 and 2. The labelling of PBP 3 did not alter. The percentages of total radioactive penicillin bound to individual PBPs in isolated sensitive membranes was 7% - PBP1, 41% - PBP2 and 52% - PBP3, in whole cells the figures were 24, 22 and 54% respectively (Barbour, 1981). A 15 minute incubation time at 37°C was necessary to saturate the PBPs. A 90 minute chase period with excess cold penicillin failed to cause release of bound radioactive label (Dougherty *et al.*, 1980). Neither did neutral hydroxylamine or 2-mercaptoethanol stimulate the release of label contrary to that reported in other systems (Kozarich, 1977).

1.13 Selective inhibition of gonococcal PBPs compared with those of *Escherichia coli*.

Barbour (1981), investigated the competitive binding of cephaloridine, mecillinam, cephalexin and benzyl-penicillin to

the PBPs of *Neisseria gonorrhoeae*. Cephaloridine produced spheroplasts from lysing bacteria whereas cephalexin and mecillinam, caused enlargement of cells which remained intact. Benzyl-penicillin produced spheroplasts and large cells. Similar morphological responses to beta-lactams in *Neisseria gonorrhoeae* were observed by Lorian and Atkinson, (1975) and Westling-Haggstrom *et al.*, (1977). Penicillin and cephaloridine bound to all three PBPs and induced spheroplast formation whereas cephalexin and mecillinam did not bind to gonococcal PBP, at their MICs and the cells remained intact. PBP 1B from *Escherichia coli*, and PBP 1 from *Neisseria gonorrhoeae* have a similar affinity for cephaloridine which causes spheroplast formation in both species. Since PBP 1B in *Escherichia coli* has a role in cell wall growth and peptidoglycan cross-linkage (Tamaki *et al.*, 1977), Barbour suggested that the gonococcal PBP 1 had an analogous function. Cephalexin binds to gonococcal PBPs 2 and 3 but mecillinam to PBP 2 at their MICs causing production of large cells. This is in contrast to *Escherichia coli* where PBP 2 selectively binds mecillinam producing filamentous cells. A laboratory mutant of *Escherichia coli* was resistant to mecillinam, at 30°C or less, due to production of an altered PBP 2 which had a decreased affinity for the beta-lactam. The fact that mecillinam bound to only PBP 2 in *Neisseria gonorrhoeae* suggested that PBPs 1 and 3 were not necessary targets for beta-lactam action. PBP 3 of *Neisseria gonorrhoeae* bound benzyl penicillin more readily than the other two PBPs and was also 50% saturated with cephaloridine at a concentration significantly below the MIC. PBP 3 in *Neisseria gonorrhoeae* certainly did not

appear to be an important target since there was no decrease in the binding to this protein in a comparatively resistant strain (such a strain probably evolved from a sensitive strain, Maier *et al.*, 1977). This is similar to the PBPs 5 and 6 in *Escherichia coli* where there is also evidence (Matsuhashi *et al*, 1978) that these two PBPs are not an important target of beta-lactam activity.

1.14 Resistance of *Neisseria gonorrhoeae* to beta-lactam antibiotics and other antimicrobial agents.

Penicillin resistance is associated with one of two main mechanisms (Cannon and Sparling, 1984).

(i) Mutations at genetic loci

(ii) Production of beta-lactamase.

Resistance to other antimicrobial agents is due to several other genetic loci in *Neisseria gonorrhoeae*.

1.14.1 Chromosomally-mediated penicillin resistance

1.14.1.1 Low-level resistance to penicillin.

Dougherty *et al.*(1980) produced a set of isogenic transformants by stepwise transformation of a sensitive strain (MIC 0.007μg/ml) with DNA from a non-isogenic resistant strain (MIC 2μg/ml). Three transformants corresponding to three distinct levels of resistance were assigned genetic loci after determination of MICs to penicillin, erythromycin, rifampin and tetracycline based on a method by Sparling *et al.*, 1976. The three transformants were *penA* (transformant 1, MIC 0.06μg/ml), *penA mtr* (transformant 2, MIC 0.12μg/ml), *penA mtr penB* (transformant 3, MIC 0.5μg/ml). Low

level resistance MIC 0.06μg/ml controlled by the *penA* locus correlated with a decrease in the affinity of PBP 2 for penicillin (Sparling *et al.*, 1975; Dougherty, 1985a; Cannon and Sparling, 1984).

The decrease in the affinity of PBP 2 for penicillin was shown to be related to the control of O-acetylation, since a fully penicillin sensitive strain treated with its MIC of penicillin contained less O-acetylated peptidoglycan (Dougherty, 1983a; Dougherty, 1985b). Low concentrations of beta-lactam antibiotics cause a decrease in O-acetylation of peptidoglycan in growing *Neisseria gonorrhoeae* (Blundell and Perkins, 1981). A *penA* transformant treated with its MIC shows binding of penicillin to PBP 1 and 3 which leads to a rapid decrease in the cross-linkage of the peptidoglycan. Since binding to PBP 3 in both sensitive and resistant strains is the same, a role for PBP 1 in transpeptidation was suggested (Dougherty, 1985a). O-acetylation of peptidoglycan was found to be a post-synthetic modification (Dougherty, 1983b; Lear and Perkins, 1983). This observation corroborated reports that in *Proteus mirabilis* O-acetylation is carried out after incorporation of nascent peptidoglycan into the pre-existing network (Gmeiner and Kroll, 1981).
The O-acetylation of peptidoglycan was possibly a mechanism of ensuring the integrity of the network (Dougherty, 1985b) or a substrate-level control of the active hydrolase system in the gonococcus (Sinha and Rosenthal, 1980).

Reverse phase HPLC of muramidase-digested peptidoglycan from

penicillin sensitive and resistant strains revealed differences in the relative concentrations of cross-linked muropeptide species depending on the MIC of the strain to penicillin. Garcio-Bustos and Tomasz (1990) used HPLC to analyse the peptidoglycan of penicillin resistant and susceptible clinical pneumococci. They concluded that the altered PBPs conferring resistance to penicillin by reduced affinity for the drug also changed the substrate preference of the peptidoglycan synthesis enzymes. They found higher levels of branched peptides in the resistant strains compared with more linear peptides in the susceptible strains.

The role of PBP 2 was elucidated by Dougherty (1985b). It was found that inhibition of PBP 2 by penicillin led to increased cellular concentrations of disaccharide penta-peptides and none were O-acetylated. The lack of Cpase activity in PBP 2 caused the accumulation of the penta-peptides. It was suggested that these un-processed penta-peptides were poor substrates for the post synthetic O-acetylation reaction.

1.14.1.2 PBP 2 gene(*penA*)mutations

Studies by Dowson *et al.* (1989) showed that an extra aspartate 345a codon was located in the *penA* gene of all strains with MICs $\geq$ 0.016μg/ml. DNA from a low level resistant strain could transform a truly sensitive strain to an increased level of resistance. It was concluded that all strains with this low-level resistance and also all strains with high level resistance would contain altered forms of the *penA* gene encoding a lower affinity

PBP 2. This was confirmed by use of a constructed oligonucleotide probe corresponding to the *penA* gene regions which differed between penicillin sensitive and resistant strains. These strains of MIC greater than or equal to the low-level resistant strain had altered *penA* genes of one or the other classes of *penA* genes (class A and B *penA*^{r}) identified by nucleotide sequencing by Spratt (1988) suggesting a clonal origin for these genes. Laboratory studies on *Escherichia coli* have shown that PBPs with reduced affinity for penicillin have not been achieved by a single amino acid substitution (Hedges and Spratt, 1985a) but by sequential multiple amino acid insertions and substitutions in the penicillin sensitive transpeptidase domain which gradually reduces the affinity of the PBP for penicillin (Hedges and Spratt, 1985b).

A major step in the development of lower affinity *Neisseria gonorrhoeae* PBP 2 to penicillin was due to the insertion of the extra aspartate 345a codon in the penicillin transpeptidase domain of the *penA* gene (Brannigan *et al.*,1990). Site-directed mutagenesis was employed to insert the aspartate 345a codon into a penicillin sensitive *penA* gene. The resulting PBP 2 had a decreased affinity for penicillin and the DNA from the strain itself could transform a sensitive strain to an increased level of resistance. Removal of the aspartate 345a codon prevented this transformation of a sensitive strain to an increased resistant strain. It was concluded that this amino acid insertion was therefore in part but not totally accountable for decreased affinity of PBP 2 for penicillin.

1.14.1.3 Multiple transferable resistance

The presence of the *mtr* locus encoding multiple transferable resistance causes the reduced cellular uptake of several drugs including penicillin, detergents, fatty acids and dyes (Maier *et al.*, 1975; Sarrubi *et al.*, 1975). The *penA mtr* transformant constructed by Dougherty *et al.* (1980) did not produce a further change in PBP 2 affinity for penicillin compared with the *penA* transformant. The *mtr* locus is not linked to other genetic loci in the gonococcus (Cannon and Sparling, 1984) and the phenotypic expression of the *mtr* locus results in increased amounts of a 52 kDa outer membrane protein and increase in the peptidoglycan crosslinking (Guymon *et al.*, 1978). However, no difference in the composition of cell envelope phospholipids and lipopolysaccharides was observed which confirmed reports by Wolf-Watz *et al.* (1975) who only found moderate differences in the fatty acid distribution of lipid A and phospholipids. The outer membrane as a barrier to the penetration of antibiotics was investigated in a sensitive *penA mtr* and *penB* transformant of *Neisseria gonorrhoea* (Scudamore *et al.* 1979). Only small outer membrane barriers were detected for actinomycin D and benzyl penicillin. The resistant transformant had no observed change in the penetration of these antibiotics compared with the penicillin sensitive strain. These observations led the authors to conclude that mechanisms other than outer membrane penetrability were responsible for the acquired low-level resistance due to *penA mtr* and *penB*.

The phenotypic expression of *mtr* is suppressed by the *env* locus

which increases the antibiotic sensitivity of *Neisseria gonorrhoeae* (Sarubbi *et al.*, 1975).

1.14.1.4 The *penB* locus

Mutations at the *penB* locus cause low-level resistance to penicillin and tetracycline (Dougherty *et al.*, 1980; Sparling *et al.*, 1975). However, the phenotypic expression of *penB* is dependent on the presence of *mtr* (Sparling *et al.*, 1975).

1.14.1.5 The *tem* locus

Tem is a modifier gene (Warner *et al.*, 1980) as defined by Bryan (1961) which has no phenotypic expression of its own but results in low-level resistance to penicillin and tetracycline similar to *penB*.

In summary low-level resistance to penicillin is due to an interaction of the genetic loci *penA*, *mtr*, *penB*,and *tem*. Resistance to five antibiotics is brought about by various gene combinations in *Neisseria gonorrhoeae Tet*, *mtr*, *penA* and *penB* are defined as polygenes (Bryan, 1961) encoding resistance to penicillin, tetracycline, ampicillin, nafcillin and doxycycline in combination with the modifier genes *tem* and *pem* (Warner *et al.*, 1980).

1.14.1.6 *pem* modifier gene

Pem another modifier gene in combination with *penA* and *penB*, *mtr* and *tem* account for the high level of resistance to penicillin and tetracycline in *Neisseria gonorrhoeae* (Warner *et al.*, 1980).

The high level of resistance to penicillin, MIC ≥ 2μg/ml, is associated with the reduced affinity of PBPs 1 and 2 in *Neisseria gonorrhoeae* (Dougherty, 1986).

Genetic transformation of a *penA mtr penB* transformant strain with DNA from a highly resistant clinical isolate led to the isolation of two classes of high level resistant transformants, suggesting that two genes were involved. One type of transformant had a PBP 1 affinity which resembled the DNA donor and the other type had an intermediate affinity PBP 1 (for penicillin) which was lower than the *penA mtr penB* recipient strain but higher than the resistant DNA donor strain (Dougherty, 1986).

Other genetic loci which encode for resistance to antibiotics include the *amp* genes.*Amp A*, *B*, *C* and *D* are alleles of the *amp* gene which, when combined, encode for a stepwise increase in MIC of ampicillin (Jones *et al.*, 1985). Decreased sensitivity to spectinomycin (*spc*), streptomycin (*str*), chloramphenicol (*cam*), fusidic acid (*fus*) and rifampin (*rif*) is due to loci encoding the resistance to these antibiotics which affect ribosomal protein function (Sparling *et al.*, 1976; Cannon and Sparling, 1984).

1.15 Summary of PBP function in *Neisseria gonorrhoeae*

In summary, PBP1 is a transpeptidase like PBP1 in *Escherichia coli*, PBP2 is a carboxypeptidase and is a homologue (40%) of *Escherichia coli* (Spratt, 1988). PBP3 has no role in gonococcal resistance to beta-lactams. PBP3 and 44,000 dalton surface-exposed peptidoglycan binding protein are the same proteins

(Shafer and Judd, 1991). The authors purified the 44kD protein from sarkosyl insoluble membrane material and found it covalently bound radioactive penicillin. In *Escherichia coli* PBP3 has two functions; an amino-terminal penicillin insensitive transglycolase domain and a carboxy terminal penicillin sensitive transpeptidase domain. The Tpase domain in gonococci is between 266-581 amino acids and the serine 310 is the active site residue acylated by beta-lactams (Keck, 1985). Spratt (1988) also suggested that part of the region which codes for the penicillin sensitive domain with transpeptidase activity had been exchanged with the homologous portion of a closely related species. Dowson *et al.* (1990) observed that PBP2B genes in *Streptococcus pneumoniae*, which encode for a PBP2B decreased penicillin affinity, is similar to the PBP2B in penicillin resistant viridans streptococci which also encodes a PBP with a reduced affinity for penicillin. They suggested that this was due to horizontal gene transfer from penicillin resistant pneumococci to the viridans streptococci.

1.16 Beta-lactamase production in *Neisseria gonorrhoeae*.

Penicillinase producing *Neisseria gonorrhoeae* were first reported in 1976 following treatment failure in patients being administered benzyl penicillin for gonorrhoea (Ashford *et al.*, 1976; Percival *et al.*, 1976; Phillips, 1976). The beta-lactamase hydrolyses the cyclic amide bond in the beta-lactam ring of benzyl penicillin, ampicillin and cephaloridine but has low activity against methicillin and oxacillin (Heffron *et al.*, 1977). *Neisseria gonorrhoeae* produce a TEM-type beta-lactamase

(Elwell *et al.*, 1976; Bergstrom *et al.*, 1978) typical of that found in enteric bacteria. TEM is derived from the name "Temoniera" ; the name of a young girl in Athens from whom an ampicillin resistant *Escherichia coli* strain was isolated (Datta and Kontomichalou, 1965). The ampicillin resistance and beta-lactamase production could be transferred from this strain to a recipient *Escherichia coli* strain. This indicated that a resistance factor (R-factor) was involved. Such strains usually contain a plasmid (Pc^{r}) reported to be either between 3.2-3.4 Mdaltons of 4.4-4.7 Mdaltons. These plasmids cause the strain to exhibit an MIC from 10-100μg/ml.

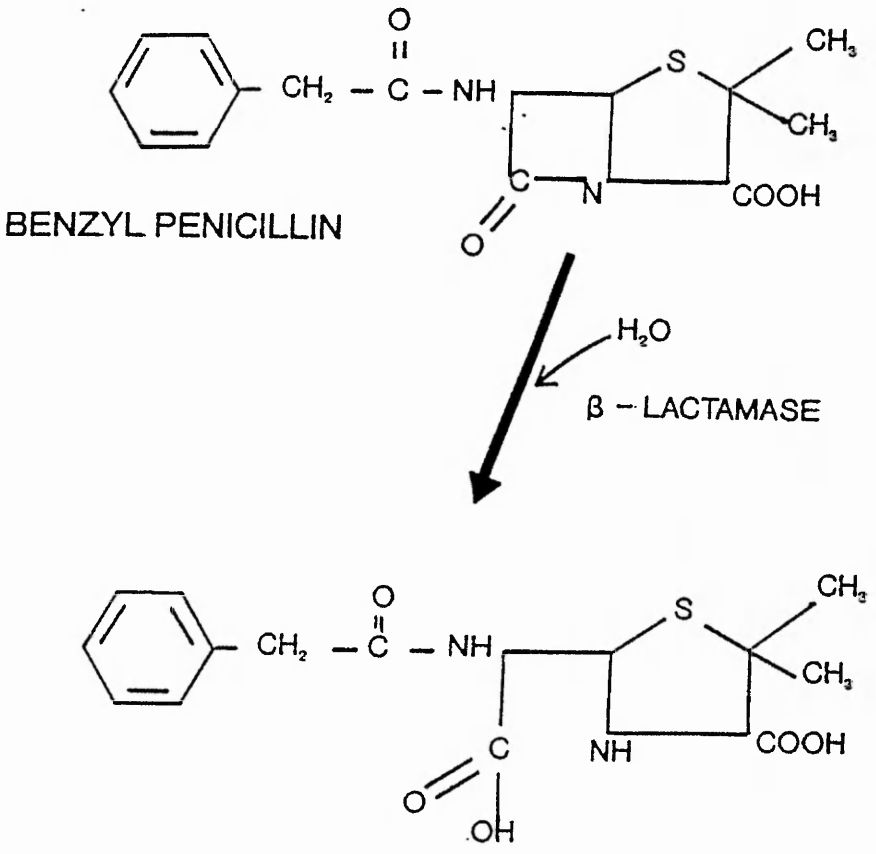
1.17 Structure of TEM beta-lactamase.

Antisera to TEM1 beta-lactamase cross reacts with TEM2 probably due to a number of common amino acids since the genes of these two enzymes have been shown to be largely homologous (Heffron *et al.*, 1975). TEM2 was first isolated from *Pseudomonas aeruginosa*. It has a similar substrate profile to TEM1 but differs electrophoretically (Sykes and Richmond, 1971). Monoclonal antibodies to TEM1 plasmid-mediated beta-lactamase were produced by Mofin *et al.* (1987). They characterised 28 murine monoclonals which recognised TEM1. The study showed that common epitopes were shared by at least nine biochemically different beta-lactamases encoded by plasmids. TEM1 beta-lactamase was the first Gram negative beta-lactamase recognised (Datta and Kontomichalou, 1965) and sequences for the amino acids and gene nucleotides have been determined (Ambler and Scott, 1978; Sutcliffe, 1978), respectively. Beta-lactamase producing *Neisseria gonorrhoeae*

first appeared in South-East Asia and Africa simultaneously and soon became worldwide in distribution. The active site sequence of amino acids for several beta-lactamase (Ambler, 1980) including RTEM in *Escherichia coli* is now known. Using gene hybridisation techniques the molecular basis for beta-lactamase action is being studied. TEM2 which differs from TEM1 by a single amino acid due to one nucleotide change, hybridised with a gene-size probe against a TEM1 fragment containing the active site (Boissinot, 1987). The three dimensional structures are known for the class A beta-lactamases including that of *Staphylococcus aureus* and other beta-lactamases of classes B and C. They all contain four similar amino acid sequences which, when the protein is folded and these four "motifs" are brought close together, generate the active site at the junction of an alpha domain and an alpha-beta domain of five stranded beta sheets, protected by additional alpha helices on both faces (reviewed by Ghuysen, 1991). All the beta-lactamases, the low molecular weight PBPs and the penicillin sensitive domains of high molecular weight PBPs contain these same four motifs in their amino acid sequences.

1.18 Beta-lactamase hydrolysis of penicillin

Beta-lactamases hydrolyse the cyclic amide bond of the penicillin beta-lactam ring causing the production of bacterially active penicilloic acid, (**Figure 1.8**). Consider the reaction; penicillin P binds to enzyme E forming a non-covalent complex EP. This acylates the enzyme and a covalent complex E-P is formed. The penicillin is subsequently released as an inactive substance X.



$$E + P \underset{K-1}{\overset{K_1}{\rightleftharpoons}} EP \xrightarrow{K_2} E - P \xrightarrow{K_3} E + X$$

Figure 1.8: Beta-lactamase hydrolysis of benzyl-penicillin to penicilloic acid.

If constants K-1/K1 and K3 have low values compared with K2 the penicillin behaves as an inhibitor. If however K-1/K1 is low in value and K2 and K3 are high, then the penicillin is a good substrate for the enzyme and is inactivated by it. Since the product of hydrolysis is acidic many detection assays make use of this fact with the use of indicator dyes or starch-iodine. However, chromogenic cephalosporin Nitrocefin (O'Callaghan, 1972) is a useful rapid method now available. This test uses the feature of colour change from yellow to red on hydrolysis, by beta-lactamases, for rapid detection and assay of such enzymes.

1.19 Classification of beta-lactamases

Several different classifications for beta-lactamases have been described. Ambler (1980) based 3 classes of enzyme on amino acid sequences. Class A contains *Staphylococcus aureus*, *Bacillus licheniformis*, *Bacillus cereus* and *Escherichia coli* TEM and PBR322 enzymes. The important amino acid is serine 70 or 44 which is acetylated by penicillin resulting in hydrolysis. Class C shares a common lysine with class A enzymes two amino acids downstream from the important serine. Class C enzymes are chromosomally-mediated and include *Pseudomonas aeruginosa* and *Escherichia coli amp* C gene beta-lactamases. Class B enzymes are quite rare, they are metalloenzymes and they destroy drugs containing the 7-methoxy group as well as carbapenems. These enzymes are inhibited by chelating agents that bind zinc.

The Richmond and Sykes (1973) classification provides grouping of enzymes based on substrate and inhibition profiles, molecular

weights and isoelectric points. The rate of substrate hydrolysis is measured against the rate for benzyl penicillin or cephaloridine depending on whether the enzyme is basically a penicillinase or cephalosporinase. **Table 1.2** shows six classes of beta-lactamases including information on whether the enzyme is plasmid or chromosomally mediated. Some of the beta-lactamases are constitutive like the TEM beta-lactamase in gonococci, others are inducible such as that in *Staphylococcus aureus*. *Bacillus licheniformis* produces a beta-lactamase after the induction of the penicillin sensory transducer BLAR (Zhu, 1990). This organism produces a membrane-bound and a secretory form of the beta-lactamase. The BLAR protein is thought to have evolved from a beta-lactamase which has lost the deacylation activity, has no carboxypeptidase activity and is fused to a trans-membrane transducer. All Gram negative beta-lactamases are found in the periplasmic space. Preparation of the crude enzyme is therefore possible following sonication to break open the cells and centrifugation to deposit insoluble protein. In the Richmond and Sykes (1973) classification scheme *Neisseria gonorrhoeae* beta-lactamase is a type III enzyme.

1.20 Beta-lactamase inhibitors

Beta-lactamase inhibitors can be reversible or irreversible. Inhibitors of beta-lactamases, for example methicillin bind at or close to the active site. Such inhibitors are poor substrates for the enzyme and although they are bound with high affinity, the enzyme slowly hydrolyses them. Irreversible inhibitors are

Table 1.2: Richmond-Sykes Classification of Beta-lactamase with a sixth class added for *Bacteroides*

TYPE I

Enzymes:- active principally against cephalosporins,
:- inhibited by isoxazoyl penicillins,
:- partial inhibition by carbenicillin,
:- no inhibition by clavulanic acid and sulbactam.

Molecular weight: 24,000-44,000 daltons.

Genetic origin : Chromosomal

Hydrolysis : Amp 100, Carb 5, Clox 0, Cer 150-8,000.

Trivial names : P99 of Enterobacter, Sabath-Abraham enzyme from *Pseudomonas aeruginosa*.

Found in : *Enterobacter*, *Morganella*, *Proteus vulgaris*, *Providencia*, *Pseudomonas*, *Klebsiella*, *Serratia* and *Citrobacter*.

TYPE II

Enzymes:- active against penicillins,
:- inhibited by isoxazoyl penicillins and clavulanic acid,
:- not inhibited by carbenicillin.

Molecular weight: 25,000-30,000 daltons.

Hydrolysis : Amp 150, Carb 40, Clox 0, Cer 10.

Found in : *Proteus mirabilis* and *Escherichia coli*.

TYPE III

Enzymes:- active against penicillins and cephalosporins,
:- low activity against carbenicillin,
:- inhibited by parachloromercuribenzoate (pCMB), isoxazoyl penicillins, clavulanic acid and sulbactam.

Molecular weight: 17,000-29,000 daltons.

Genetic origin : Plasmid.

Hydrolysis : Amp 110, Carb 10, Clox 0, Cer 75.

Trivial names : TEM1, TEM2, SHV1 and HMS.

Found in : *Escherichia coli*, *Haemophilus*, *Neisseria*, *Salmonella*, *Shigella*, *Pseudomonas*. Most common enzyme worldwide.

TYPE IV

Enzymes:- active against penicillins and cephalosporins,
:- inhibited by clavulanic acid and sulbactam
:- not inhibited by isoxazoyl penicillins, carbenicillin and pCMB.

Molecular weight: 18,000-25,000 daltons.
Genetic origin : Chromosomal.
Hydrolyses : Amp 150, Carb 50, Clox 20, Cer 70.
Trivial names : K-1.
Found in : *Klebsiella*.

TYPE V

Enzymes:- active against penicillins, cephalosporins and isoxazoyl penicillins,
:- inhibited by clavulanic acid.

Molecular weight: 12,000-32,000 daltons.
Genetic origin : Plasmid.
Hydrolysis : Amp 200, Carb 90-250, Clox 200, Cer 10-50.
Trivial names : OXA 1.2.3. and PSE 1.2.3.4.
Found in : *Escherichia coli*, *Pseudomonas*, *Serratia*.

TYPE VI

Enzymes:- active against cephalosporins, penicillins, cefamandole, cefuroxime and cefotaxime.
:- not active against cefoxitin,
:- inhibited by cloxacillin and carbenicillin.

Hydrolyses: Pen 3, Amp 1.5, Carb 0.5, Cer 100.
Found in : *Bacteroides*.

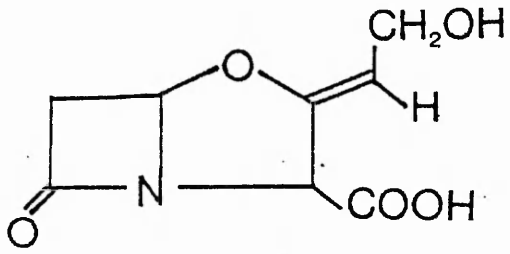
KEY

All hydrolysis rates relative to penicillin (100) except for TYPE VI where it is measured relative to cephalosporin.
Pen = Penicillin, Amp = Ampicillin, Carb = Carbenicillin, Clox = Cloxacillin, Cer = Cephaloridine.

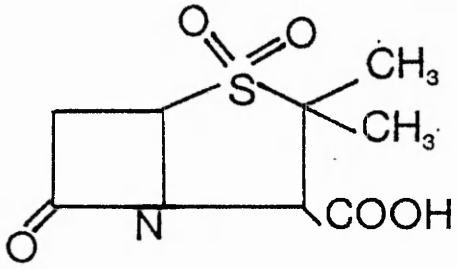
usually more effective than reversible ones since they often cause eventual destruction of the enzyme.

Clavulanic acid, sulbactam and aztreonam are all inhibitors of beta-lactamases, the first two are used to block enzyme hydrolysis of therapeutically important antibiotics (**Figure 1.9**). Clavulanic acid is derived from *Streptomyces clavuligerus* and was the first suicide inhibitor to be reported (Brown *et al.*, 1976; Reading and Cole, 1977). The evidence suggested that after initial binding of the enzyme to the inhibitor, that a number of transiently inhibited forms of the enzyme were present. However, the clavulanic acid, itself a beta-lactam was eventually destroyed with loss of enzyme activity, (Fisher *et al.*, 1978). Different amounts of the inhibitor were needed to inhibit various beta-lactamases. Sulbactam was developed and found to be more effective against cephalosporinases than clavulanic acid, although it was found to have a similar mode of action to clavulanic acid (Brenner, 1981). Neither of the aforementioned inhibitors are effective against inducible chromosomally-mediated cephalosporinases but aztreonam is one example of such an inhibitor that is useful against this class of enzymes. Aztreonam was developed as an antibiotic and although it is eventually hydrolysed by the enzyme, this occurs after several bacterial cell divisions (Bush, 1988).

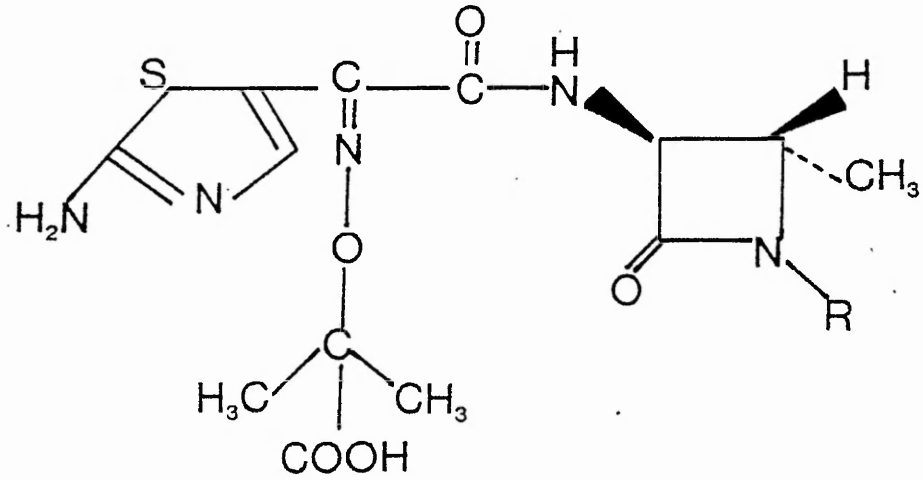
Other inhibitors possess anti-bacterial properties as well. Clavulanic acid binds avidly to PBP2 in *Escherichia coli* causing characteristic swelling of the cells and lysis leading to



CLAVULANIC ACID (a)



SULBACTAM (b)



AZTREONAM (c)

Figure 1.9: Molecular diagrams of three beta-lactamase inhibitors.

spheroplast formation (Spratt, Jobanputra and Zimmerman, 1977). Sulbactam binds most effectively to PBP1A in *Escherichia coli* (Labia *et al.*, 1986) and less avidly to PBPs 1B and 2. Clavulanic acid demonstrates weak anti-bacterial activity against Enterobacteriaceae, Gram positive and anaerobic bacteria. Moderate activity against *Haemophilus influenzae* was reported (Neu and Fu,1978) but the inhibitor is reported to be most active against penicillinase producing *Neisseria gonorrhoeae* (for 75% of the isolates tested the MIC was 0.1 μg/ml.) (Miller, 1978; Van Klingeren and Van Wignagaarden, 1981; Neu and Kwung, 1978. The anti-bacterial activity of sulbactam is also greatest against *Neisseria gonorrhoeae* (English, 1978). Augmentin is a drug in current clinical use which comprises a 2:1 combination of amoxycillin and clavulanic acid, it has been used to treat penicillinase-producing *Neisseria gonorrhoeae* effectively (Key, 1985).

1.21 Justification and aims of this study.

Gonorrhoea is still a major sexually transmitted disease in the world today. Recent statistics have shown a reversal in the declining trend of incidences of the disease from 1989 (Catchpole, 1992). It has been suggested that gonorrhoea incidence may be used as an indication of the incidence of other sexually transmitted diseases especially HIV infection (Health of the Nation, 1992). Gonorrhoea incidence in a population is a suitable indicator of any change in behaviour and attitudes to all sexually transmitted disease. This is because diagnosis of the infection is relatively simple, accurate and symptoms present in the patient early after the initial contact with an infected individual in contrast to a person infected with HIV who may not show any outward signs of the infection for many years after infection. Diagnosis as soon as possible after infection is essential to prevent the spread of the organism.

Traditionally *Neisseria gonorrhoeae* is diagnosed by direct microscopy and culture at sexually-transmitted-disease clinics or by general practitioners. The presence of intracellular Gram negative diplococci is used as a presumptive diagnosis of gonorrhoea. Culture of the organism is necessary for antibiotic sensitivity testing to be carried out. Other methods for the diagnosis of gonorrhoea have recently been reviewed (Ison, 1990). Both polyclonal and monoclonal antibodies can be used to demonstrate gonococcal antigen in clinical samples using techniques such as ELISA and immunofluoresence. A diagnostic ELISA, Gonozyme (Abbott Laboratories) using polyvalent antiserum

to gonococcal antigens has been extensively tested (Danielson *et al.*,1983) and is almost as sensitive as the Gram stain (87 - 100%) and has a specificity of 99.4 - 100% when tested on a high risk population of symptomatic men. However the test is not as sensitive (60-100%) and specific (71-100%) in women. DNA probes have been used for diagnosing gonorrhoea. A *Neisseria gonorrhoeae* specific sequence of DNA is labelled and used as a probe for a sample which may contain gonococcal DNA, to which it will hybridise. To date the cryptic plasmid (Roberts *et al.*, 1979) and chromosomal DNA (Donegan *et al.*,1989) specific to *Neisseria gonorrhoeae* have been used for probes. However, this technique using radiolabels which takes time for the development of results, requires expensive reagents and expertise in the methods, is already available using non-radioactive DNA-probes (Sprott, Kearns and Neale, 1989). A enzyme activity strip called Neisstrip is a rapid diagnostic method for detection of beta-galactosidase and prolyl-and gamma-glutamyl aminopeptidases which is accurate and easy to use (Dealler *et al.*, 1991). *Neisseria gonorrhoeae* are negative for beta-galactosidase but positive for one or both of the other two enzymes.

The newer tests at the present time have no advantage over the Gram stain and culture methods in widespread routine practice. However, in legal cases where medical evidence to prove gonococcal infection is important, the use of these alternative methods to corroborate the evidence from the conventional tests is of greater importance. It is therefore important that research and development into these newer tests continues so that viable

alternative diagnostic tests are realised.

Perhaps a more important problem at the present time is the widespread resistance of *Neisseria gonorrhoeae* to a variety of antimicrobial agents (Cannon and Sparling, 1984). It is imperative that the sensitivity of the causative strain to potential therapeutic antibiotics be determined if successful treatment of the disease is to achieved. *Neisseria gonorrhoeae* can become resistant to beta-lactam antibiotics by a decrease in the affinity of the PBPs for the drug or due hydrolysis of the antibiotic by a plasmid-mediated beta-lactamase. At present the cultured organism is tested for sensitivity to a variety of antibiotics. Resistance due to beta-lactamase production can be determined using the chromogenic cephalosporin Nitrocefin (O'Callaghan *et al.*, 1972). In contrast detection of resistance due to a chromosomal mutation encoding reduced affinity PBPs is not at present possible. The use of DNA probes for this type of resistance was recently discussed (Towner, 1992). However, at present such probes are limited to the detection of identified structural genes eg. beta-lactamases, as it would be difficult to detect all possible mutations arising from random spontaneous mutation of genes on the bacterial chromosome. An oligonucleotide probe was used to detect the *penA* gene in chromosomally-resistant *Neisseria gonorrhoeae* (Dowson *et al.*, 1989). Such technology is not readily available and it would have to be developed so that a diagnostic kit was produced to enable the routine laboratory to carry out the procedure. DNA probes have been described for the detection of gonococcal beta-lactamase plasmids in *Neisseria*

gonorrhoeae (Perine *et al.*,1985). An alternative microbiological assay for the detection of beta-lactamase has been described (Thickett and Winstanley, 1991) which utilises a penicillin-sensitive strain of *Staphylococcus aureus*. The principle of the assay is that an agar plate containing an inhibitory concentration of penicillin is seeded with *S.aureus* and the test organisms are spotted onto the plate. If the organisms produce beta-lactamase there is a satellite of *S.aureus* growth. The results correlated well with the nitrocefin test for *Neisseria gonorrhoeae*.

In this study there is an investigation of the PBPs and beta-lactamase of *Neisseria gonorrhoeae*. An alternative method of detection of both chromosomally and beta-lactamase mediated resistance was tested and compared with the conventional methods. A method using anti-beta-lactam conjugate antibodies was used to probe for penicillin bound to PBPs in *Neisseria gonorrhoeae* in Western blots with different sensitivities to penicillin. The method was evaluated as a procedure for the detection of reduced-affinity PBPs which were responsible for increased MICs to penicillin antibiotics. The use of such antibodies to detect penicillin bound to PBPs in *Streptococcus pneumoniae* and *Escherichia coli* has been described (Hakenbeck, 1986). However, there is no report in the literature for the use of probe antibodies to penicillin to detect PBPs or antibiotic resistance in *Neisseria gonorrhoeae*. In addition antibodies to a TEM-like beta-lactamase from *Neisseria gonorrhoeae* were prepared and used in a Western blotting method to detect the enzyme in strains

which were beta-lactamase positive by the nitrocefin test. The correlation between the two methods was 100% for the small number of strains tested. This method was evaluated as an alternative method for the detection of beta-lactamase mediated resistance in *Neisseria gonorrhoeae*. Chapter 2 details the production of beta-lactam conjugates, the preparation of antibodies to them and analysis of their specificity. Chapter 3 demonstrates the use of these antibodies as probes for beta-lactam antibiotic bound to the PBPs of *Neisseria gonorrhoeae*. The problems of the isolation and the purification of native PBPs is also discussed in some detail. Finally, chapter 4 discusses the purification of a TEM-like beta-lactamase from *Neisseria gonorrhoeae*. The production of antibodies to this enzyme is explained and the use of the anti-sera to visualise the beta-lactamase in both crude and partially purified extracts by Western blotting is illustrated. Partial characterisation of the isolated beta-lactamase and its immunological relatedness to other species' beta-lactamases is mentioned.

Chapter 2

PRODUCTION OF ANTIBODIES TO BETA-LACTAMS

2.1 INTRODUCTION

2.1.1 Allergy to Penicillin

Penicillin is one of the least toxic antibiotics in use today but allergy to the drug is not uncommon. A person who is allergic to penicillin may develop a rash with intense itching and development of such a symptom during therapy would indicate allergy to penicillin and would necessitate the use of alternative antibiotics. Occasionally the allergy causes the patient to develop anaphylactic shock which if left untreated can be fatal. People who have been treated with penicillin have been found to have antibodies against penicillin-protein conjugates. Some people who have not been exposed to penicillin therapeutically also have low levels of such antibodies and it has been suggested that these may be associated with exposure to small amounts of penicillin. This exposure may come from the air, for example in production plants growing *Penicillium* species for antibiotic production, or from the milk of cattle undergoing treatment with penicillin for mastitis. Ward *et al.* (1975) suggested the possibility that residues of penicillin antibiotics may be present in meat and dairy products from animals treated with penicillin. However, Dewdney and Edwards (1984) concluded that there is little evidence that penicillin-protein conjugates in cow's milk are allergenic. The presence of anti-penicillin

antibodies does not automatically mean that a person will be allergic to penicillin.

When a person is exposed to penicillin it is assumed that the penicillin finds its way into the blood stream where it may bind to lysyl groups in proteins. Sensitization may then occur due to the production of IgE antibodies which bind to basophils and mast cells in tissues. If the person is then exposed to penicillin for a second time the cell-bound IgE antibodies bind the antigen causing degranulation of the cells with the release of vasoactive amines such as histamine and other mediators. This is known as an immediate Type I hypersensitivity reaction. A much less common type of hypersensitivity (type II cytolytic) involves the penicillin hapten binding to proteins in the cell membranes of blood cells. This causes production of IgG, IgA and IgM antibodies against the penicillin-protein complex. On subsequent exposure to penicillin the penicillin binds to the cells and the antibodies then react with the penicillin-protein complex on the cell causing complement fixation and cell lysis. The ability of penicillin to bind to body proteins making it immunogenic can also lead to a "serum sickness" type III hypersensitivity reaction. The presence of the antigen leads to the formation of IgG antibody in smaller quantities than normal so the circulating antigen concentration remains high. The reaction usually occurs 7 to 14 days after exposure with symptoms such as rash, fever, and aches and pains in the joints (Roitt 1984, Bowman and Rand 1980).

2.1.2 Anti-penicillin antibodies in man

Levine and Ovary (1961) investigated the antigens which cause the production of penicillin antibodies in man. They produced a set of penicillin-protein conjugates by reacting D-benzyl-penicillenic acid with various proteins in solution at pH 7.5-8.0. They found that this resulted predominantly in a mixture of diastereoisomers of Σ-N-(D-α-benzyl penicilloyl)-lysine groups due to the penicillenic acid reacting with the Σ-amino groups of lysine in proteins. Lafaye and Lapresle (1988) discovered that patients who had been treated with penicillin had serum antibodies to penicilloyl groups which were attached to albumin. The sites of attachment were not readily accessible to the antibodies except after pronase digestion. The amount of penicilloylation was related to the dose of penicillin received. Lafaye and Lapresle (1987) also showed that human serum albumin had two sites for attachment of penicilloyl groups.

Christie *et al*. (1988) showed by ELISA that the concentration of IgM and IgE antibodies against benzyl penicilloyl determinants were higher in patients who claimed to be allergic to penicillin compared with healthy volunteers. Similar results were obtained by Daxun and Stadler (1989) who used a dot-immuno-binding assay for penicillin-specific antibodies in patients with or without allergy to penicillin. Again the concentration of IgM and IgE antibodies against penicillin were higher in allergic patients. Antibodies from rabbits immunised with benzyl penicillin alone, or with benzyl penicillin and normal rabbit serum, were found to react mainly with the diastereoisomeric mixture of penicilloyl

groups. When patients allergic to penicillin were challenged with these penicilloyl groups in a skin test 50% produced a characteristic wheal and erythema reaction. The antigenic penicilloyl groups have also been found in the serum of patients undergoing penicillin treatment. It has been observed both *in vivo* and *in vitro* that the carbonyl group of beta-lactams can react with primary amino groups of proteins to form penicilloyl-protein derivatives (Levine 1963, Dewdney 1977).

2.1.3 Beta-lactam-protein conjugates

Penicillin-protein conjugates can readily be made by mixing an excess of antibiotic in alkaline solution with proteins such as bovine serum albumin (BSA) or human serum albumin (HSA), or with poly-l-lysine (Figure 2.1). The degree of substitution of lysine groups on the protein can be assessed by measuring the decrease in the number of primary amino groups by amino assay (Kenchington 1960) or by measuring the increase in the number of penicilloyl groups by reacting *p*-chloromercuribenzoic acid with the penicillin to form a penamaldate (Levine 1962).

2.1.4 Penicillin antigens

Lee et al. (1985) investigated the effect of the number and density of penicilloyl hapten groups in protein conjugates on the immunogenicity of such conjugates. They were working on the use of a radio-immunoassay for the measurement of penicilloylated proteins in body fluids. They concluded that the assay of unknown samples using a standard curve prepared from "standard conjugates" was inappropriate since the hapten density and

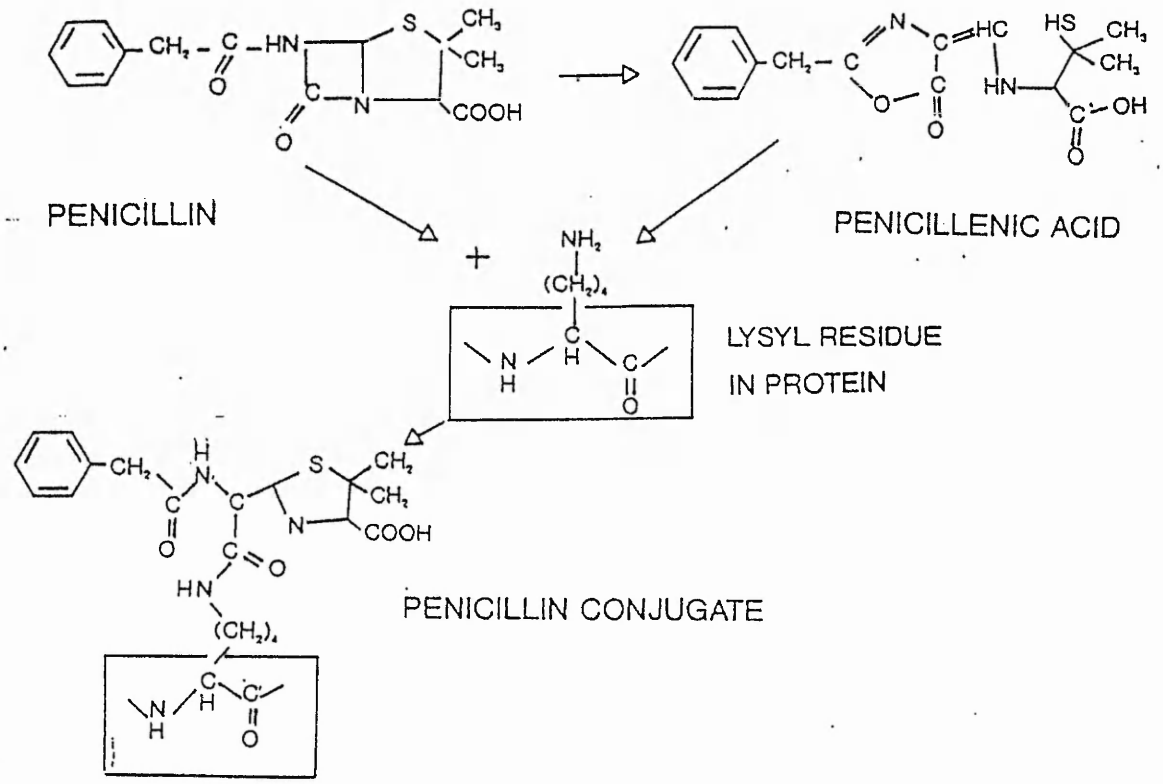


Figure 2.1: The formation of penicilloylated protein by the covalent bonding of penicillin or penicillenic acid with primary amino groups in a protein.

distribution have a significant effect on the immunogenicity and antigenicity of the conjugate.

Monoclonal antibodies against benzyl penicillin-protein conjugates were prepared by de Haan *et al.* (1985). They produced three different antibodies which could recognise distinct epitopes on the penicilloyl determinant. These included one which recognised the side group of penicillin, a second which recognised the thiazolidine ring, and a third which recognised the new antigenic determinant formed as a result of the penicillin binding to the protein carrier (Figure 2.2). Thus at least three epitopes can be recognised by antibodies formed against benzyl penicilloyl groups.

Fukushima *et al.* (1987) prepared mouse monoclonal antibodies which were class IgE and which were found to react with benzyl penicilloyl-hapten, benzyl penicillin polymer, cephalothin hapten, and cephalothin polymer. They determined that the antibodies were mainly directed against the phenylacetyl portion of the benzyl penicilloyl group using affinity chromatography.

Harle and Baldo (1990) used a radio-immunoassay to detect penicillin- reactive IgE antibodies. They used quantitative hapten inhibition studies to detect IgE-binding regions on different penicillin molecules. The authors concluded that the major allergenic determinants were α-amino benzyl (ampicillin) and benzyl (penicillin) side-chain groups and the thiazolidine and beta-lactam rings.

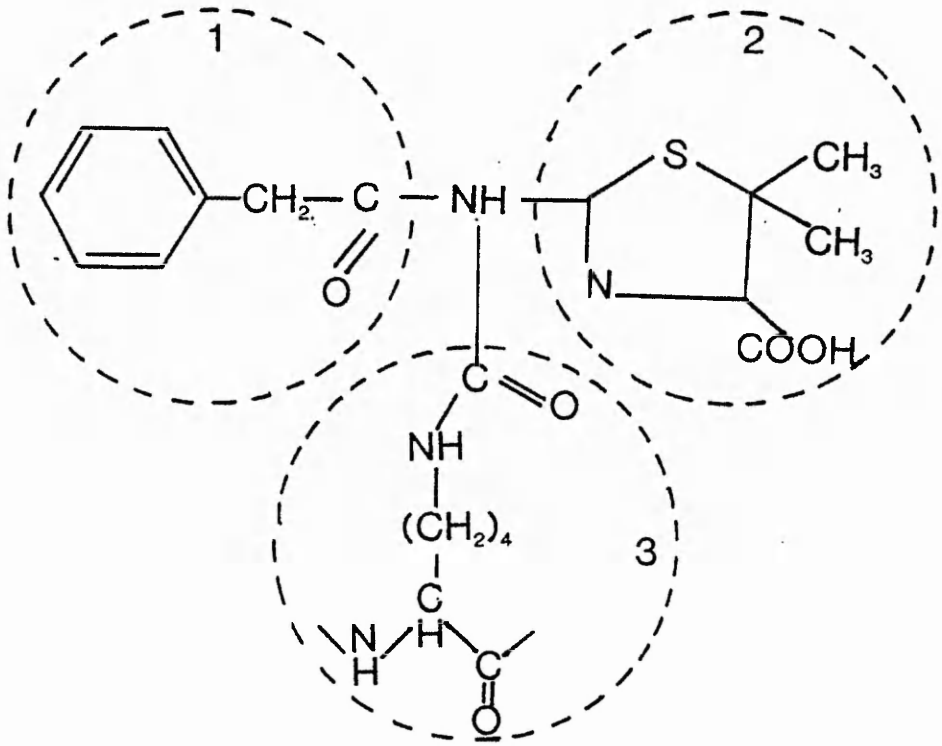


Figure 2.2: Epitopes on a penicilloylated protein:

1) the side-chain of the penicillin,
2) the thiazolidine ring,
3) the new epitope formed as a result of conjugation.

2.1.5 Aims and summary of the present study

One of the aims of this research was to make antibodies against beta-lactam antibiotics which could subsequently be used to probe cells and cell membranes of penicillin-treated *Neisseria gonorrhoeae* for penicillins bound to the penicillin-binding proteins (PBPs). Such a method could be a useful alternative to the usual method of fluorography used to detect the PBPs. A similar method has been described by Hakenbeck *et al.* (1986) to probe for the PBP-penicilloyl complexes in the cell membranes of Gram positive bacteria and also in whole cells of *Escherichia coli*. These techniques are discussed in more detail in Section 3.1 of Chapter 3.

To help achieve these aims three protein-beta-lactam conjugates were prepared using a method similar to that of Lee *et al.* (1985) and these were used to immunise three sets of rabbits to provide the specific antisera sought. A BSA-penicillin conjugate was synthesized using bovine serum albumin (molecular weight 66,000) as the carrier and benzyl penicillin as the penicillin. Poly-l-lysine-penicillin was made using the polypeptide poly-l-lysine as an alternative carrier since it contained the primary amino groups to which penicillin is known to attach. This conjugate was also useful for the ELISA as the plate antigen to detect penicillin-specific antibodies in the antisera raised against the other conjugates. The third conjugate was BSA-methicillin. This was produced since it has a different side-chain to benzyl penicillin (Figure 2.3), a factor in itself which may determine antigenicity, but also because the side-chain confers beta-

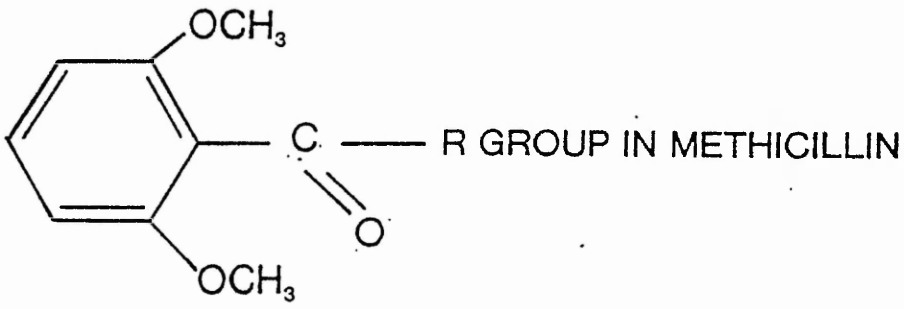


Figure 2.3: Diagram of the side-chain of methicillin.

lactamase stability on the antibiotic. It was thought that this property might prove useful in probing the PBPs of beta-lactamase-producing *Neisseria gonorrhoeae*.

The resulting antisera from the immunised rabbits, and affinity-purified antibodies from these antisera, were tested using an enzyme-linked immunosorbent assay (ELISA) to determine the specificity of the anti-beta-lactam and other antibodies produced. Inhibition assays were performed on crude antisera and affinity-purified antibodies to find out if the antibodies were directed against the native penicillin or part of the new antigenic determinant formed as a consequence of conjugation of penicillin to the carrier.

2.2 MATERIALS AND METHODS

2.2.1 Production of protein-beta-lactam and poly-l-lysine-beta-lactam conjugates

Three conjugates were made using a method similar to that of Lee *et al.* (1985). The conjugates were:

a) bovine serum albumin-benzyl penicillin,

b) bovine serum albumin-methicillin,

c) poly-l-lysine-benzyl penicillin.

All four chemicals used were obtained from Sigma. The poly-l-lysine used had a mean molecular weight of 38,000.

In each case 100mg of the carrier and 500mg of the beta-lactam were dissolved in 5ml of 0.5M sodium carbonate buffer at pH 10. The solutions were filtered through a 0.45μm Millipore filter then incubated at 37°C for 24 hours. The solutions were then dialysed through Visking dialysis tubing (Gallenkamp) against 3 x 10 litre changes of 10 mM ammonium bicarbonate solution to remove unconjugated antibiotic. After dialysis the solutions were lyophilised and stored at -20°C.

2.2.2 Protein assay

This was adapted from Lowry *et al.* (1951). On the day of the assay 2ml of 2% copper sulphate solution, 2ml of 4% potassium tartrate solution, and 98ml of 3% sodium carbonate in 0.1N sodium hydroxide solution were mixed together to give 'reagent A'. Just before use Folin-Ciocalteau reagent (BDH) was diluted 1:1 in distilled water. The standard protein solution used was bovine serum albumin (BSA) at 1mg/ml, stored at -20°C.

The standard BSA solution was diluted in distilled water over the range 20-100μg/ml to give a total volume of 500μl of each dilution. 5ml of 'reagent A' were added to duplicate standard dilutions and samples of the conjugates also diluted in distilled water. After 10 minutes at room temperature 500μl of diluted Folin-Ciocalteau reagent were added and the solutions were thoroughly mixed. After 30 minutes incubation at 30°C the absorbance at 650nm was determined using a Phillips PU8625 spectrophotometer.

2.2.3 Penamaldate assay

To determine the approximate degree of penicilloylation of the proteins a penamaldate assay as described by Levine (1962) was used. In this assay 0.1ml of a $1.5x10^{-2}$M solution of sodium *p*-chloromercuribenzoic acid (PCMB) was added to 5ml of a $2x10^{-5}$M conjugate solution in 0.05M carbonate buffer at pH 9.2. The absorbance of the solution was read at 285nm between 5 and 15 minutes after mixing using a Phillips PU8625 spectrophotometer. The absorbance value was corrected for the contribution to the absorbance of the final concentration of $3x10^{-4}$M PCMB by subtracting 0.06 from the value. The value was also corrected for dilution by multiplication by 1.02. The penicilloyl amine concentration (M) was calculated by dividing the corrected optical density by the molar extinction coefficient for penamaldate under the above conditions ($2.38x10^{4}$).

2.2.4 SDS-PAG electrophoresis of the beta-lactam conjugates

An attempt was made to determine the molecular weights of the

conjugates to see if they were significantly higher than the expected molecular weights of the unconjugated carriers which would provide further evidence for extensive penicilloylation and allow a more accurate estimation of the degree of penicilloylation. SDS-PAGE and a Western-blotting technique using an anti-penicillin antiserum were used as it was believed that this would identify the conjugated carriers as distinct from the unconjugated carriers.

BSA, BSA-penicillin, BSA-methicillin, poly-l-lysine, and poly-l-lysine-penicillin at concentrations of 50μg per gel lane were electrophoresed on a 10% sodium dodecyl sulphate polyacrylamide gel, along with a set of Rainbow molecular weight markers, and subsequently blotted onto nitrocellulose. The details of this technique may be found in Chapter 3 Section 3.2.14. The nitrocellulose blots were probed with a rabbit antiserum against BSA-methicillin (rabbit 8043 3rd test bleed) at a 1/30 dilution in skimmed milk buffer. This antiserum had been shown to react with penicilloyl groups as well as methicilloyl groups on the carriers (see Chapter 3).

2.2.5 Immunisation of rabbits with beta-lactam-protein conjugates

For each conjugate three New Zealand white rabbits were used. Each rabbit was bled prior to the first injection. The sera from these bleeds, labelled 'prebleeds', were stored in small volumes at -20°C. At each administration the rabbits were each injected subcutaneously with 1mg of conjugate in 1ml of saline plus Freunds complete adjuvant (1:1) spread over four sites (ie 4 x

0.25ml). The first test bleed was taken 14 days later, then each rabbit was given a further dose of 1mg conjugate in 1ml of saline again spread over four sites. The second test bleed was taken 10 days later. The sera were monitored for antibodies against the beta-lactam-conjugates used to immunise them and also against poly-l-lysine-penicillin using an ELISA technique as described in Section 2.2 6. Further injections and test bleeds were performed as necessary. When a maximum titre had been obtained the rabbits were killed and then bled out. The antisera were stored at -20°C in small volumes.

2.2.6 Enzyme-linked immunosorbent assays (ELISA)

2.2.6.1 Development and optimisation of ELISAs

An established ELISA technique as used in our laboratory (adapted from Hackenbeck 1986) was used with the buffers and incubation times described in Section 2.2.6.2. In order to optimise the binding of reagents to the solid phase the effects of antigen concentration and temperature of incubation were investigated. In these investigations 0.5% skimmed milk was used as the blocking agent. The second test-bleed serum from rabbit 6997 immunised with BSA-penicillin was used and in each experiment the other variables were kept constant. Details of these other variables are given in the results.

1) The optimum concentration of the coating antigen was investigated using a range of concentrations of BSA-penicillin from 2μg/well to 0.125μg/well.
2) The effect of temperature (4°C, 20°C and 37°C) on the

antigen-coating, blocking and primary antibody-binding steps was investigated. In each of these cases the temperature of incubation of the peroxidase-conjugate binding was kept constant at 4°C. The times incubation were as detailed in the optimised ELISA technique (section 2.2.6.2).

During these investigations it was observed that increasing the concentration of skimmed milk in the blocking buffer from 0.5% to 3% considerably reduced the level of non-specific binding of the primary antibody. This was not investigated any further in specific experiments but the effect was so clear that 3% skimmed milk was used in the blocking buffer for all further assays after this optimisation.

After these optimisation investigations were completed the assays which had been done on antisera prior to this time were repeated with the optimum technique described in Section 2.2.6.2.

2.2.6.2 Specific details of the optimised assay technique used

Penicillin-protein conjugate was bound to Nunc ELISA plates at 1μg per well in 100μl of 50mM carbonate buffer pH 9.6 overnight at 37°C. The wells were washed six times with 0.85% saline containing 0.05% Tween using a Titretek plate washer. Each well was then exposed to 100μl of 3% skimmed milk (Tesco) in 50mM carbonate buffer pH 9.6 for 3 hours at 37°C to block any remaining free binding sites. The wells were washed as before then dilutions of sera in 66mM phosphate buffer pH 7.4 were added at 100μl per well and the plates incubated at 37°C for 1½ hours.

The wells were washed as before then 100μl of a 1/5000 dilution of goat anti-rabbit-IgG-peroxidase conjugate (ICN) in 66mM phosphate buffer pH 7.4 were added to each well and the plate incubated at 4°C for 1½ hours. The wells were then washed as before and 100μl of a freshly prepared solution of 0.05% tetra-methyl-benzidine (TMB) (Wellcome) in 2.2% trisodium citrate with 0.01% hydrogen peroxide were added to each well and the plate left at room temperature in the dark for 20 minutes. The blue colour development was terminated by the addition of 50μl of 2M sulphuric acid to each well. The absorbance at 450nm of the solution in each well was read using a Titretek Multiscan plate reader blanked on air.

All ELISA test plates included control wells containing (i) all reagents except the antigen, (ii) all reagents except the primary antibody, and (iii) all reagents except the antigen and primary antibody. All assays included an assay of the relevant pre-bleed serum. The absorbances for the test sera and the pre-bleed sera were corrected by subtracting the absorbances for the antigen-free control (i) and then the absorbances for the test sera were corrected by subtraction of the absorbances for the corresponding pre-bleed sera.

In assays where the maximum absorbance formed a plateau (ie antibody saturation was achieved) the dilution which gave 50% absorbance was calculated and expressed as the $-\log_{10}$ of the serum dilution (the titre). Where this calculation was not possible the curves were compared visually except where explained

otherwise in the results.

2.2.6.3 Competitive ELISA techniques

In the competitive ELISAs a range of concentrations of the competitive antigen (200mg/ml to 0.0002mg/ml) were prepared in 66mM phosphate buffer pH 7.4 and each dilution was preincubated with an equal volume of a dilution of the test serum in the same buffer at 37°C for 1½ hours before adding to the assay plate. Other conditions were as in Section 2.2.6.2.

2.2.7 Affinity purification of anti-beta-lactam antibodies

Several affinity columns were made using CNBr-activated Sepharose 4B (Pharmacia). Four column supports were made using the following ligands:

a) 6-amino penicillanic acid (6-APA),

b) BSA-penicillin conjugate,

c) poly-l-lysine-penicillin conjugate,

d) BSA-methicillin conjugate.

It was expected that ligand (a) would bind antibodies directed against the beta-lactam ring and the thiazolidine ring, whereas the others would bind antibodies directed against the side-group in addition.

About 1.14g of dry CNBr-Sepharose was washed and reswollen with 1mM hydrochloric acid on a sintered glass filter connected to a vacuum line giving an approximate volume of 4ml of swollen Sepharose. The ligand to be coupled was dissolved in coupling buffer (0.1M sodium bicarbonate buffer containing 0.5M sodium

chloride pH 8.3). Approximately 4.5mg of ligand was used per ml of swollen Sepharose. The ligand and Sepharose were mixed end-over-end in a universal bottle overnight at 4°C. Excess unbound imidocarbonate groups on the Sepharose were blocked by transferring the Sepharose to 0.2M glycine pH 8 for 2 hours at room temperature. Finally non-absorbed protein was washed off with alternate washes of coupling buffer at pH 8.3 and 0.1M acetate buffer with 0.5M sodium chloride pH 4. The Sepharose was stored at 4°C in phosphate buffered 0.15M saline (PBS) pH 7.2 with 0.02% sodium azide added to inhibit microbial growth. The non-absorbed protein was assayed by protein assay (Lowry *et al.* 1951) to determine the degree of coupling of the ligand.

To purify the antibody 2ml of the antiserum was diluted with 8ml of PBS and mixed with the corresponding specific ligand-coupled Sepharose overnight at 4°C. A small glass column was poured and equilibrated with PBS until no further protein, monitored by measuring the absorbance of the washings at 280nm, could be detected. Two populations of antibodies were eluted by addition of two successive eluants:

a) 20ml of 0.1M glycine-HCl buffer pH 2.5 ("low affinity" antibody),

b) 20ml of 0.1M glycine-Hcl buffer pH 2.5 + 10% dioxane ("high affinity" antibody).

The low pH buffer is a deforming agent which causes changes in the ionic interactions between antibody and antigen and alters the shape of the bound antibody causing a decrease in its affinity for the antigen on the Sepharose. A "low affinity"

antibody is released in this elution and its activity is almost fully regained by raising the pH to neutrality. The addition of a solvent such as dioxane to the buffer reduces the hydrophobic interactions between the antibody and the antigen on the gel causing the release of further "high affinity" antibodies. However, this treatment causes much irreversible denaturation of the eluted antibodies. Antibodies with high affinity antigen binding sites are the essential constituents of a high-titred antiserum (Hudson and Hay 1991).

The fractions from each 20ml elution were pooled and immediately adjusted to pH 8 with solid Tris. The two pools were dialysed against 5 x 1 litre changes of PBS pH 7.2 and then the volumes were reduced by ultrafiltration using an Amicon cell with a 10,000 molecular weight membrane.

2.3 RESULTS AND DISCUSSION

2.3.1 BSA-beta-lactam and poly-l-lysine-beta-lactam conjugates

The conjugates were assayed for protein using the Lowry assay (a typical standard curve for the Lowry assay is shown in Figure 2.4), and for penicilloyl groups using the penamaldate assay as described in Sections 2.2.2 and 2.2.3. Table 2.1 shows the estimated protein concentration of the samples in mg/ml, and the estimated penicilloyl amine concentration in moles/mg of protein. From these values and the estimated molecular weights of BSA (taken from the reference literature as 66,000) and of poly-l-lysine (provided by the suppliers as an average of 38,000) the approximate number of moles of beta-lactam bound per mole of protein has been calculated and these values are also shown in Table 2.1.

PAG electrophoresis and Western blotting were performed on the conjugates as described in Section 2.2.4 (these results are not shown due to deterioration of the blot). This investigation showed that the methicillin-conjugate anti-serum used stained the unconjugated BSA which gave a dense band at a molecular weight of about 67,000 as determined from the molecular weight standards. This staining of the unconjugated BSA suggests that antibodies which recognise BSA alone may be present in the antiserum as well as indicating possible non-specific binding of the antibodies to the BSA. There was also in the unconjugated BSA lane a diffuse band at a much higher molecular weight than the BSA which may also have been stained non-specifically by the antiserum (which was used at a relatively high concentration).

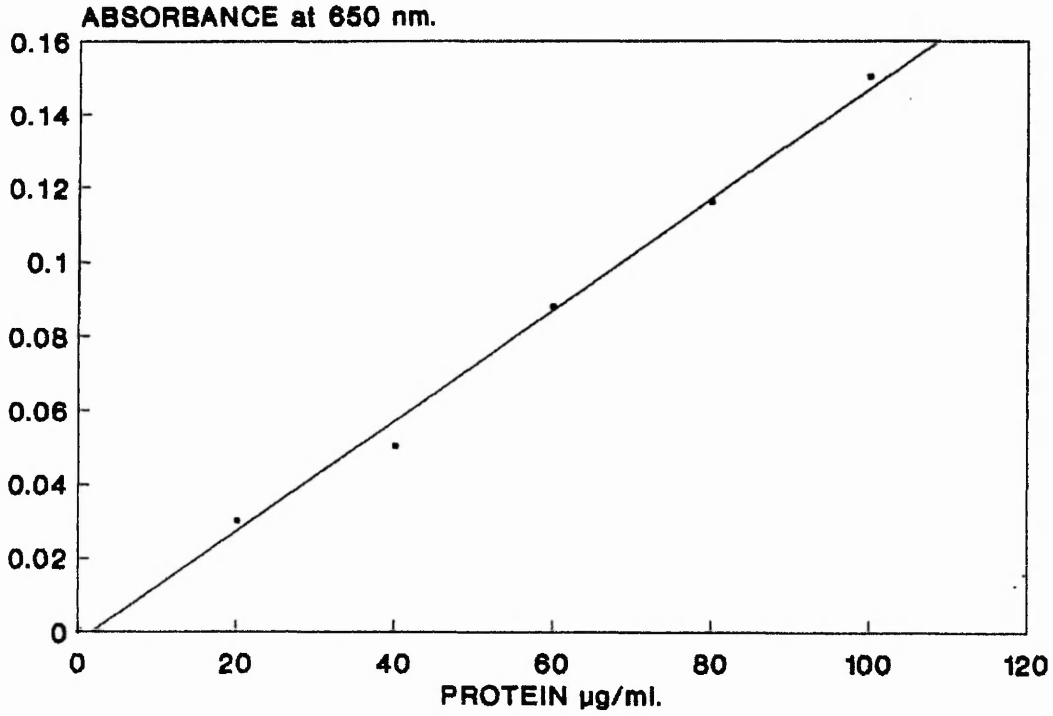


Figure 2.4: A typical standard curve for the Lowry protein estimation. Concentrations of BSA from 20 to 100µg/ml were assayed as described in Section 2.2.2 and the absorbances plotted against the concentration.

Table 2.1: Analysis of the three beta-lactam conjugates showing protein concentration estimated by the Lowry method, the penicilloyl concentration per mg protein estimated by the penamaldate assay, and the calculated number of penicilloyl groups per mole of carrier.

Conjugate	Protein conc^{n}. (mg/ml)	Penicilloyl conc^{n}. (moles/mg protein)	Penicilloyl groups per mole of carrier
BSA-penicillin	1.50	3.25×10^{-8}	2.14
BSA-methicillin	0.81	1.58×10^{-7}	10.40
Poly-l-lysine penicillin	0.33	2.8×10^{-7}	10.64

This might suggest that the specific staining of the BSA-beta-lactam conjugates in the other lanes may be obscured by the staining of the BSA. However, the stained bands in the BSA-beta-lactam lanes were in an intermediate molecular weight position as compared with the bands in the BSA lane so the results were retained as an indication of the relative molecular weights of the conjugates compared with the unconjugated BSA. The BSA-methicillin and BSA-penicillin conjugates stained slightly less densely than the BSA and these were spread over a molecular weight range of 90,000 to 200,000. In these two lanes there was no significant band at the molecular weight of the unconjugated BSA which suggests that little or no unconjugated BSA remained after the conjugation process. The antiserum did not bind to the unconjugated poly-l-lysine nor to the poly-l-lysine-penicillin conjugate.

The molecular weights could not be determined accurately from these results. Although it was recognised that the molecular weights given earlier would be under-estimates of the true values for the conjugates (since they did not take into account the penicilloyl groups) they were still used as they allowed a comparative assessment of the degree of penicilloylation of the carriers as shown in Table 2.1. The exercise also provided evidence that the carriers had been conjugated and that this conjugation affected the migration properties in PAGE possibly due to their effect on the molecular weights.

The number of penicilloyl groups per mole of protein was highest

for the poly-l-lysine penicillin and for the BSA-methicillin and the numbers of penicilloyls on the BSA-penicillin was much lower. Since the penicilloyl groups react with the lysine groups on the carrier through the carbonyl group of the beta-lactam ring (Levine and Ovary 1961) it would be expected that the poly-l-lysine would bind a large number of such groups. The observed binding is much less than the theoretical maximum for poly-l-lysine with an average molecular weight of 38,000, and Hackenbeck *et al.* (1986) achieved binding of 1 penicillin to every 5 lysine groups compared with 1 penicillin to every 24 lysine groups approximately found in the present study. The reason for this inefficiency in the conjugation is not known. The binding of methicillin to BSA was much more efficient than the binding of penicillin to BSA. It is possible that methicillin may be more reactive due to the presence of the different side-chain.

In general, though, the results showed that the carriers were conjugated with the beta-lactams used and were suitable for use in the immunisation of animals and in further investigations.

2.3.2 ELISA optimisation

2.3.2.1 Effect of antigen concentration

Figure 2.5 shows the results obtained for the ELISA of anti-BSA-penicillin antiserum 6997 (second test-bleed) against various concentrations of BSA-penicillin as the plate antigen. It is clear from the charts that the absorbances reach a maximum at an antiserum dilution of 100. At this concentration the correlation between antigen concentration and absorbance is not perfect as

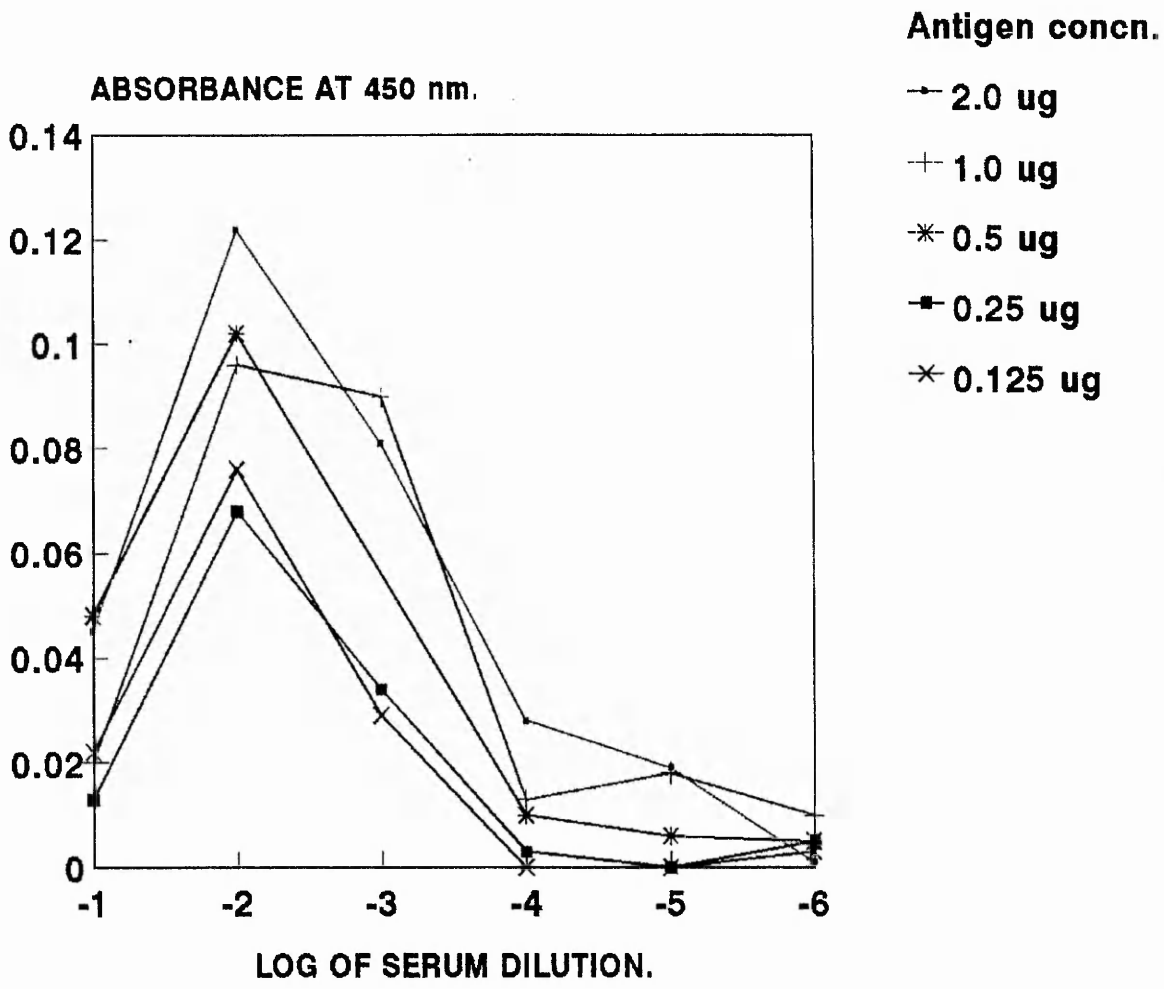


Figure 2.5: ELISA on the second test-bleed antiserum from rabbit 6997 tested against various concentrations of BSA-penicillin as the plate antigen. The conditions are as described in section 2.2.6.1. Absorbance values are corrected for absorbance given by the antigen-free controls and absorbance given by the pre-bleed serum.

can be seen from the chart and the absorbance values were very low. The reason for this is not known but it may be due to variation introduced by the many reaction stages involved in the assay and the need to correct the absorbance values with antigen-free controls and pre-bleed. However, there is a downward trend of absorbance related to decreasing antigen concentration. The original ELISA technique used a concentration of 1μg/well for the antigen-coating solution and the experiment showed that the 2μg/well was not markedly different from this concentration, so 1μg/well was accepted as the optimum.

2.3.2.2 Effect of temperature of incubation

Figure 2.6 shows the results obtained for the ELISA of anti-BSA-penicillin antiserum 6997 (second test-bleed) against BSA-penicillin as the plate antigen and with the antigen-coating, blocking, and primary antibody-binding steps carried out at temperatures of 4°C, 20°C and 37°C. In all cases the peroxidase-conjugate-binding step was carried out at 4°C. The chart shows that the absorbances for 37°C and 20°C assays are almost the same, whereas the absorbances for the 4°C assay are much lower. There could be several explanations for this such as reduced antigen-binding or reduced primary antibody-binding at 4°C. However the results clearly showed that the higher temperatures gave higher absorbance values so 37°C was chosen as temperature-controlled incubators at this temperature were readily available.

2.3.3 Production of antisera against the conjugates

The three rabbits immunised with the poly-l-lysine-penicillin

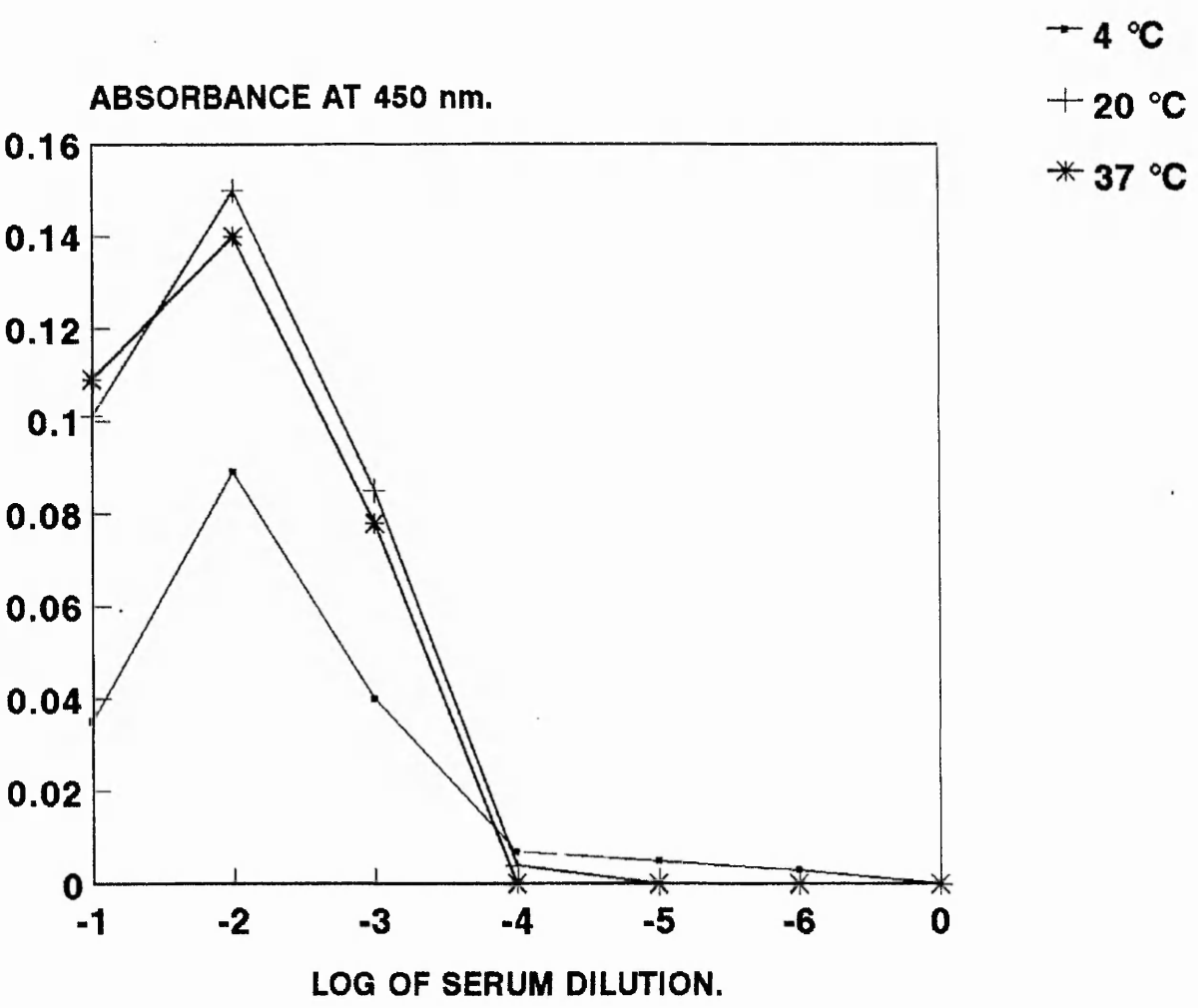


Figure 2.6: ELISA of the anti-BSA-penicillin antiserum 6997 (second test-bleed) against BSA-penicillin as the plate antigen carried out at temperatures of 4°C, 20°C and 37°C. The conditions are as described in Section 2.2.6.1 and 2.2.2.2. Absorbance values are corrected for the absorbance given by the antigen-free controls and the absorbance given by the pre-bleed serum.

conjugate did not produce detectable antibodies. The fact that none of the rabbits in this group responded to the immunogen suggested that it may be due to lack of immunogenicity of the conjugate, or perhaps due to suppression of the antibody response in some way by the conjugate. These three rabbits were re-immunised with the BSA-penicillin conjugate to provide a reserve of antiserum. The titration curves for these animals are not shown as little use was made of the antisera.

Figures 2.7a-f show the ELISA titration curves for the 14-day, the 24-day and the 52-day test-bleed antisera from the rabbits immunised with BSA-penicillin and BSA-methicillin. The results for the assays of each antiserum against its own specific immunogen are shown. The later test-bleeds were also assayed but these were taken much later to check on the antibody concentration which had invariably fallen by the time of the fourth test-bleed. The animals were reimmunised one or more times with the same immunogen as had been administered previously resulting in increases in the antibody titres (these results are not shown as they make the charts confusing and do not lead to any conclusions different from those described below).

All the rabbits immunised with the BSA-beta-lactam conjugates produced antibodies which reacted with the specific immunogen administered to the animal. The BSA-methicillin induced a higher antibody response within the first 14 days than was induced by the BSA-penicillin indicating that this conjugate was a stronger

Figures 2.7a-f: These figures show the results of the ELISA tests used to monitor the development of antibodies in the antiserum of rabbits immunised with the conjugates. The results for the first three test-bleeds for each rabbit are shown. The method used was as described in Section 2.2.6.2. The absorbances were corrected for non-specific binding by subtraction of the absorbance of the antigen-free control and then subtraction of the absorbance of the equivalent dilution of the pre-bleed as described in Section 2.2.6.2.

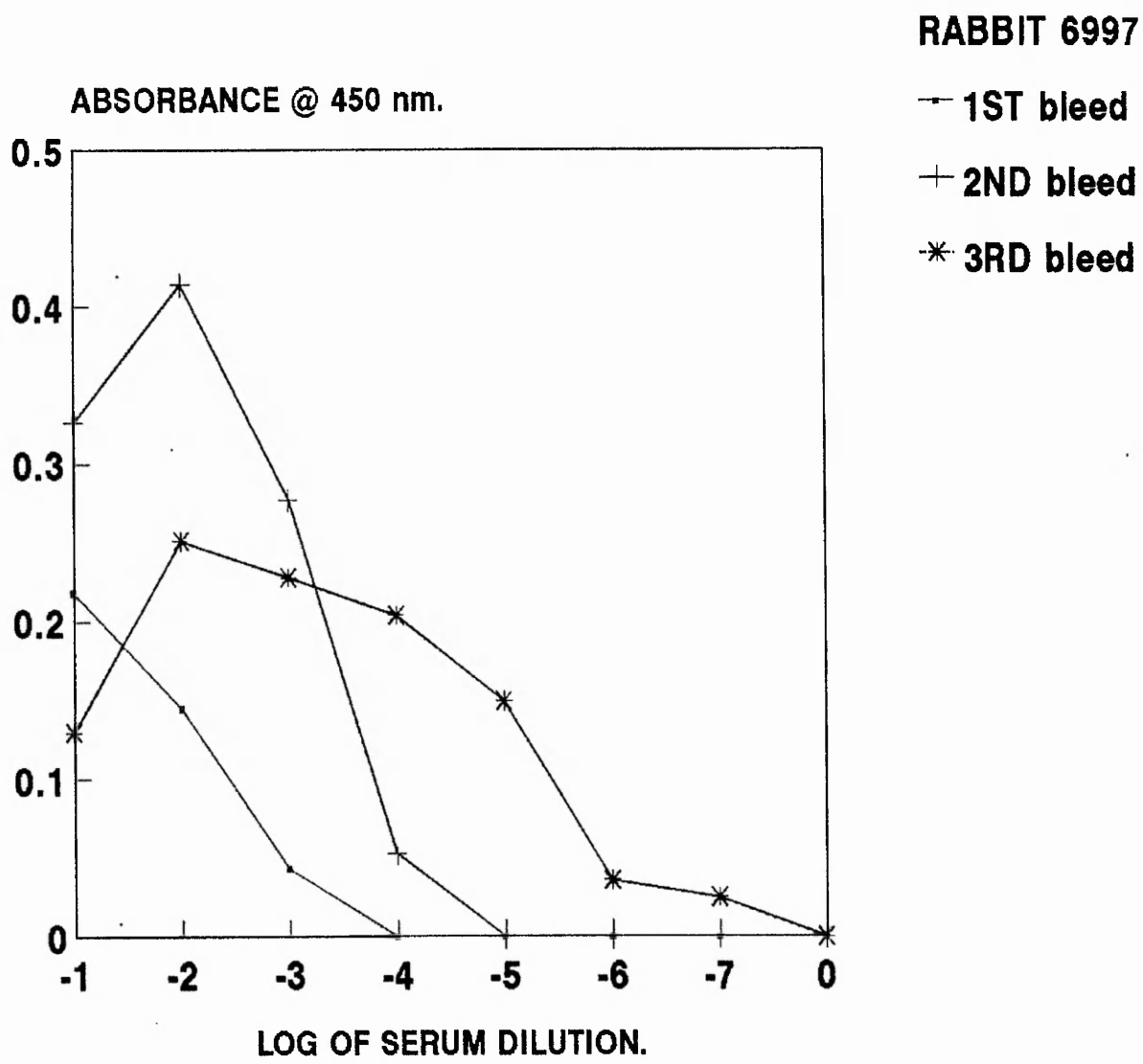


Figure 2.7a: ELISA of the 14-day, 24-day and 52-day test-bleed antisera from rabbit 6997 immunised with BSA-penicillin and assayed against BSA-penicillin as the plate antigen.

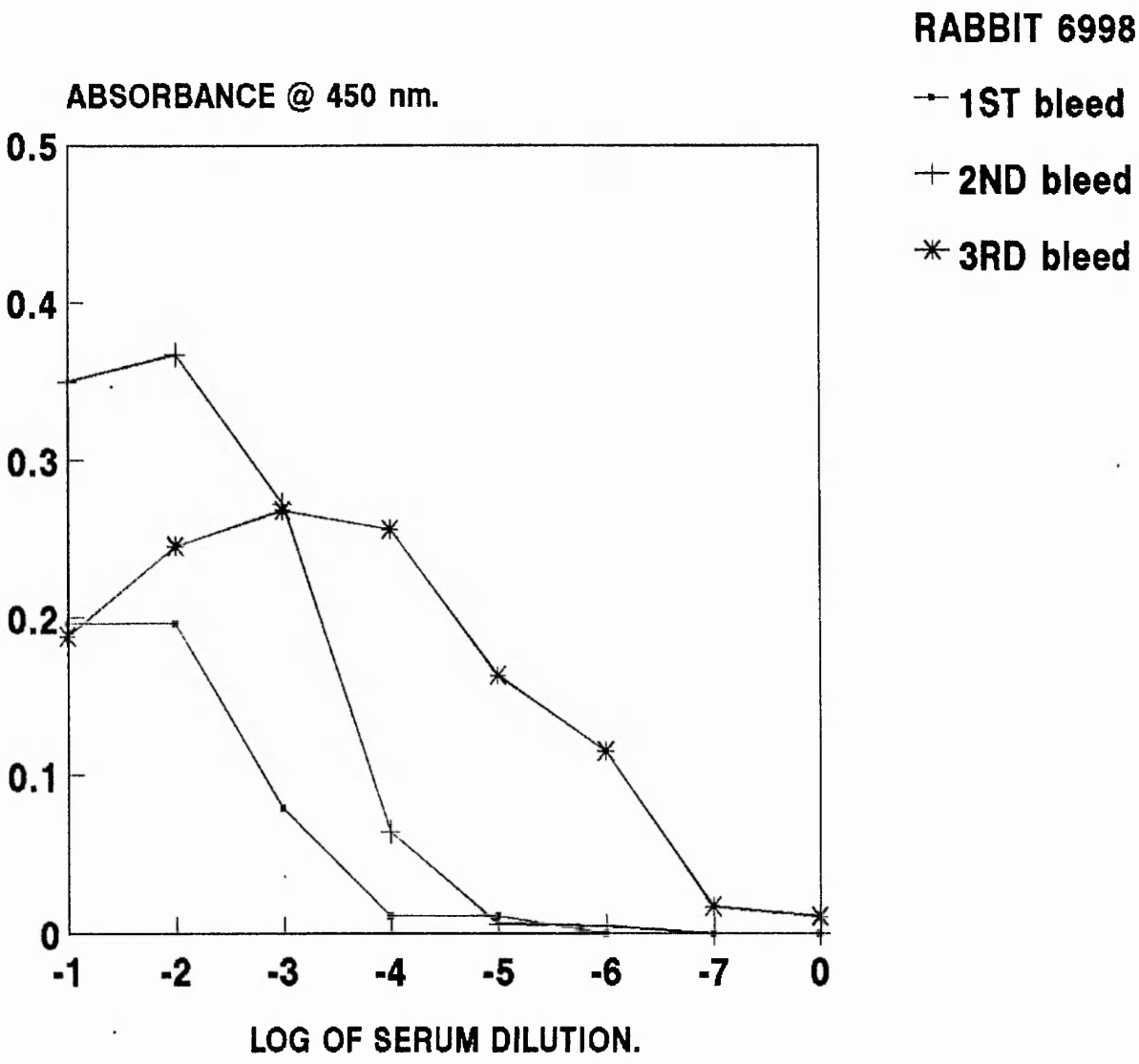


Figure 2.7b: ELISA of the 14-day, 24-day and 52-day test-bleed antisera from rabbit 6998 immunised with BSA-penicillin and assayed against BSA-penicillin as the plate antigen.

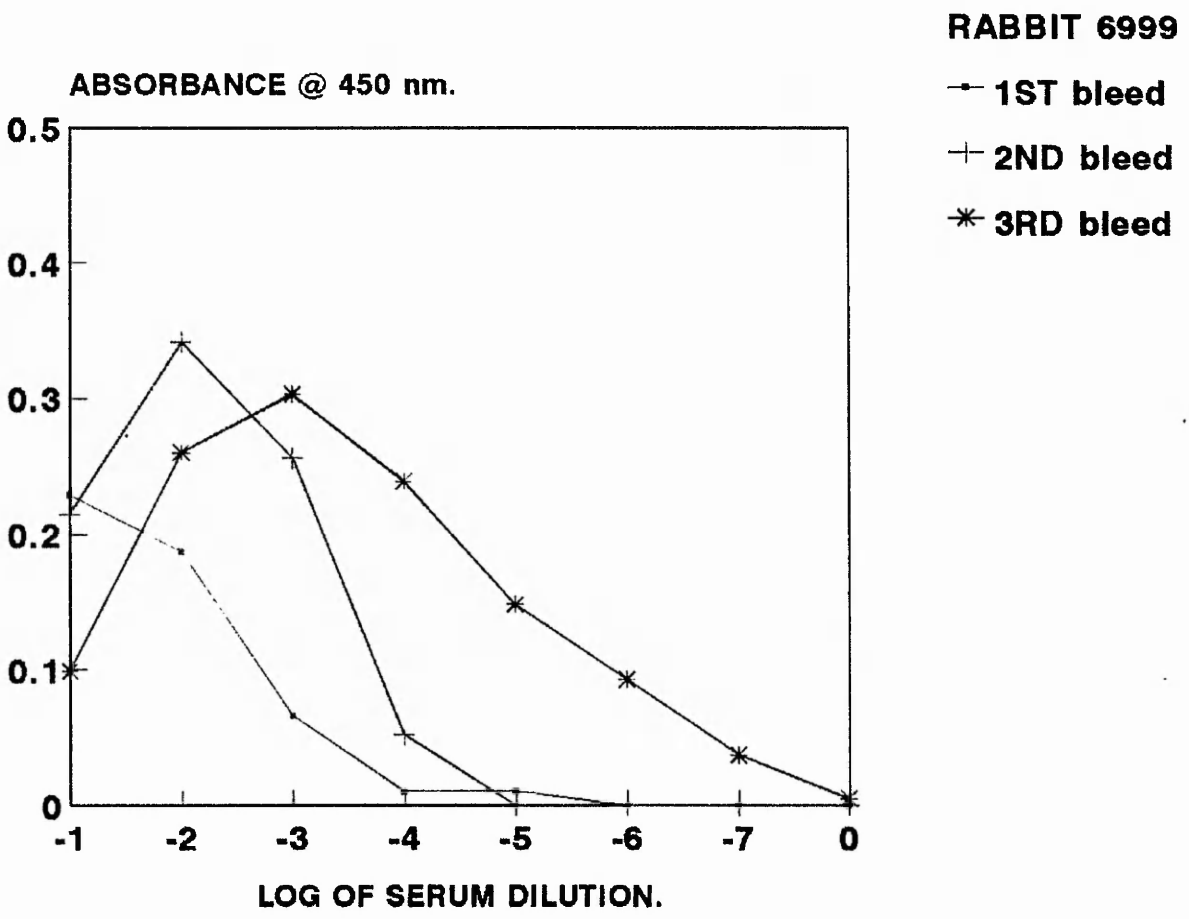


Figure 2.7c: ELISA of the 14-day, 24-day and 52-day test-bleed antisera from rabbit 6999 immunised with BSA-penicillin and assayed against BSA-penicillin as the plate antigen.

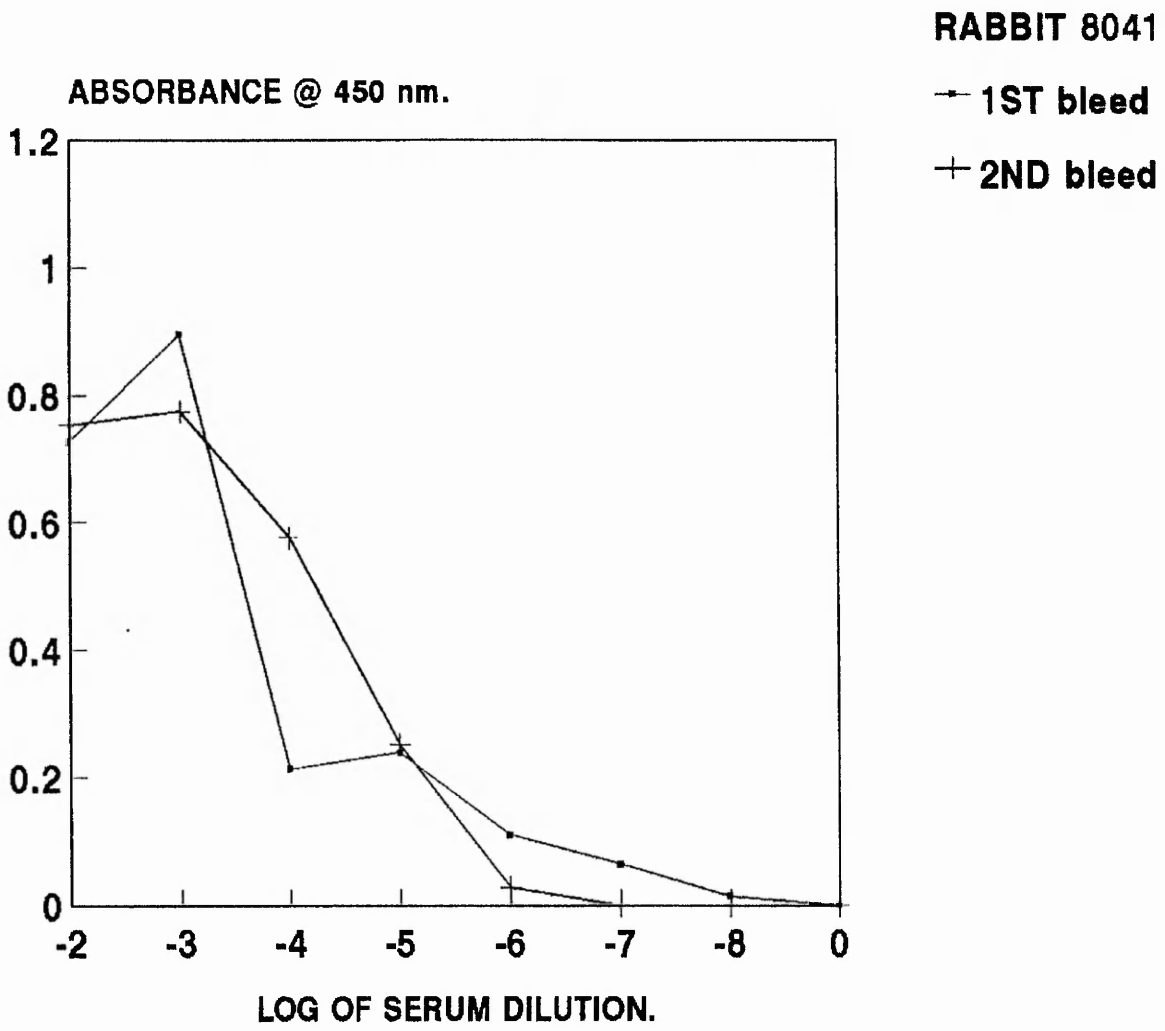


Figure 2.7d: ELISA of the 14-day and 24-day test-bleed antisera from rabbit 8041 immunised with BSA-methicillin and assayed against BSA-methicillin as the plate antigen. This rabbit died before the 52-day test-bleed could be taken.

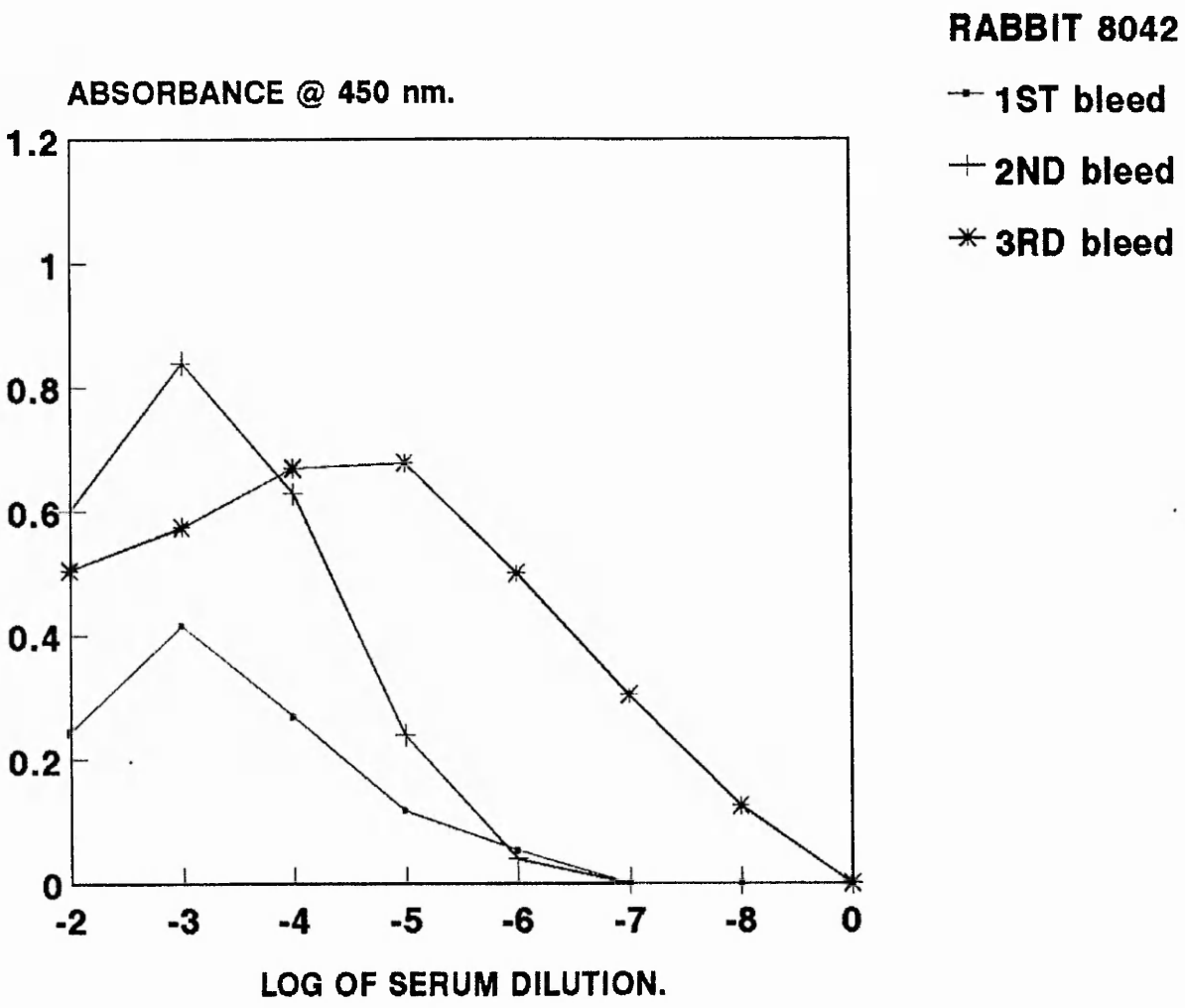


Figure 2.7e: ELISA of the 14-day, 24-day and 52-day test-bleed antisera from rabbit 8042 immunised with BSA-methicillin and assayed against BSA-methicillin as the plate antigen.

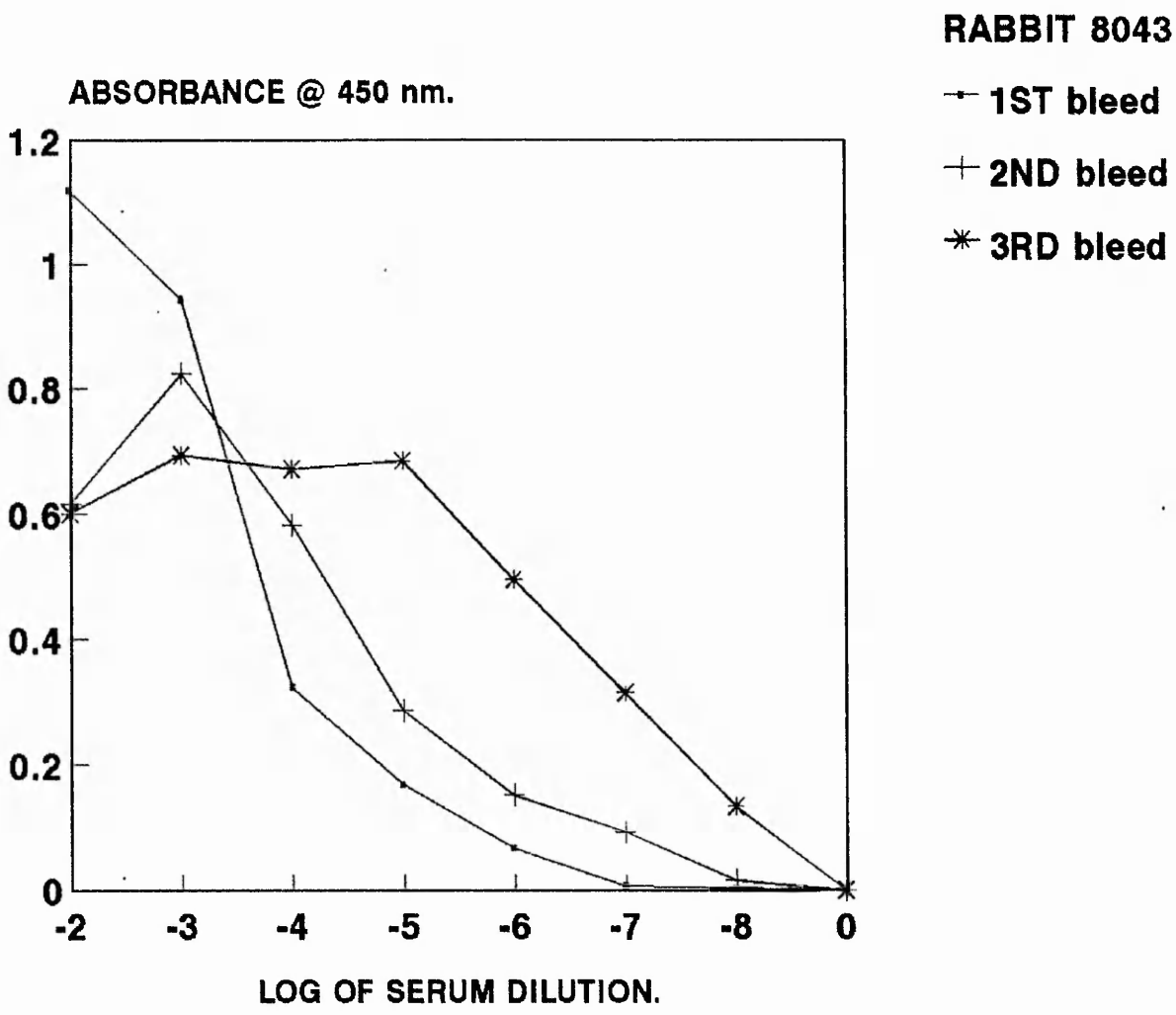


Figure 2.7f: ELISA of the 14-day, 24-day and 52-day test-bleed antisera from rabbit 8043 immunised with BSA-methicillin and assayed against BSA-methicillin as the plate antigen.

immunogen. This may correlate with the fact that the BSA-methicillin was conjugated with more beta-lactam groups than the BSA-penicillin (Table 2.1).

2.3.4 Investigations into the specificity of the antisera.

2.3.4.1 Specificity of the anti-BSA-penicillin antisera.

A number of experiments were performed on the BSA-penicillin antisera to try to determine the specificity of the antibodies, and in particular, to provide evidence that specific antibodies against the beta-lactam epitopes were present.

2.3.4.1.1 Cross-reactions of the anti-BSA-penicillin antisera with the carriers and conjugates in standard ELISA tests.

Each of the second test-bleed antisera from rabbits 6997, 6998 and 6999 were titrated by ELISA using the standard technique described in Section 2.2.6.2 against the following antigens on the plates:

a) BSA,
b) BSA-penicillin
c) poly-l-lysine,
d) poly-l-lysine-penicillin.

The titration curves are shown in Figures 2.8 a-c. The results show that antisera from rabbits immunised with BSA-penicillin react well with poly-l-lysine-penicillin as the plate antigen, but react weakly with unconjugated poly-l-lysine. This suggests that the antisera contain antibodies specific for the penicilloyl epitopes. The reaction with BSA-penicillin is similarly strong but the interpretation of this result is confused by the fact

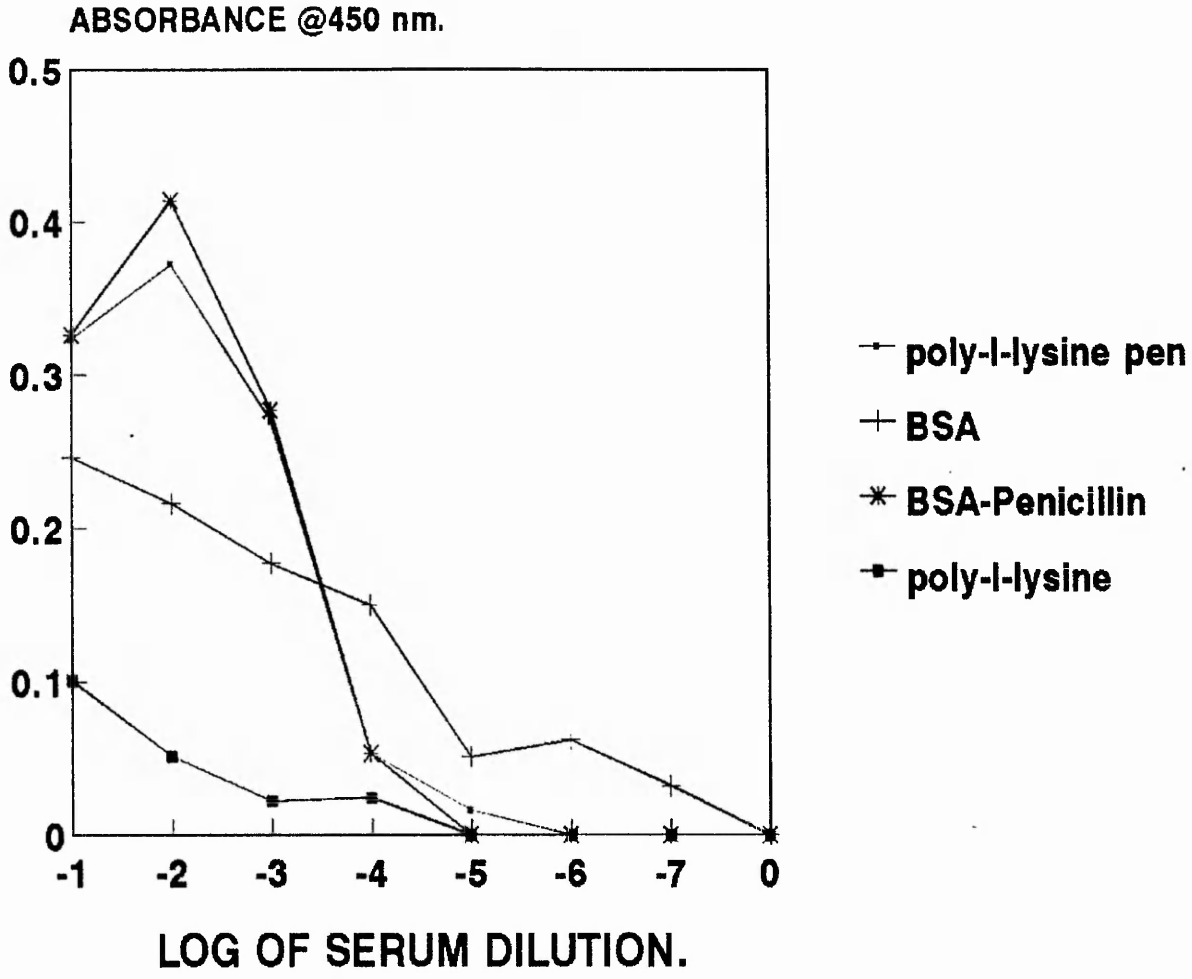


Figure 2.8a: ELISA of the second test-bleed antiserum from rabbit 6997 immunised with BSA-penicillin and tested against BSA, BSA-penicillin, poly-l-lysine, and poly-l-lysine-penicillin as the plate antigen. Method as described in Section 2.2.6.2.

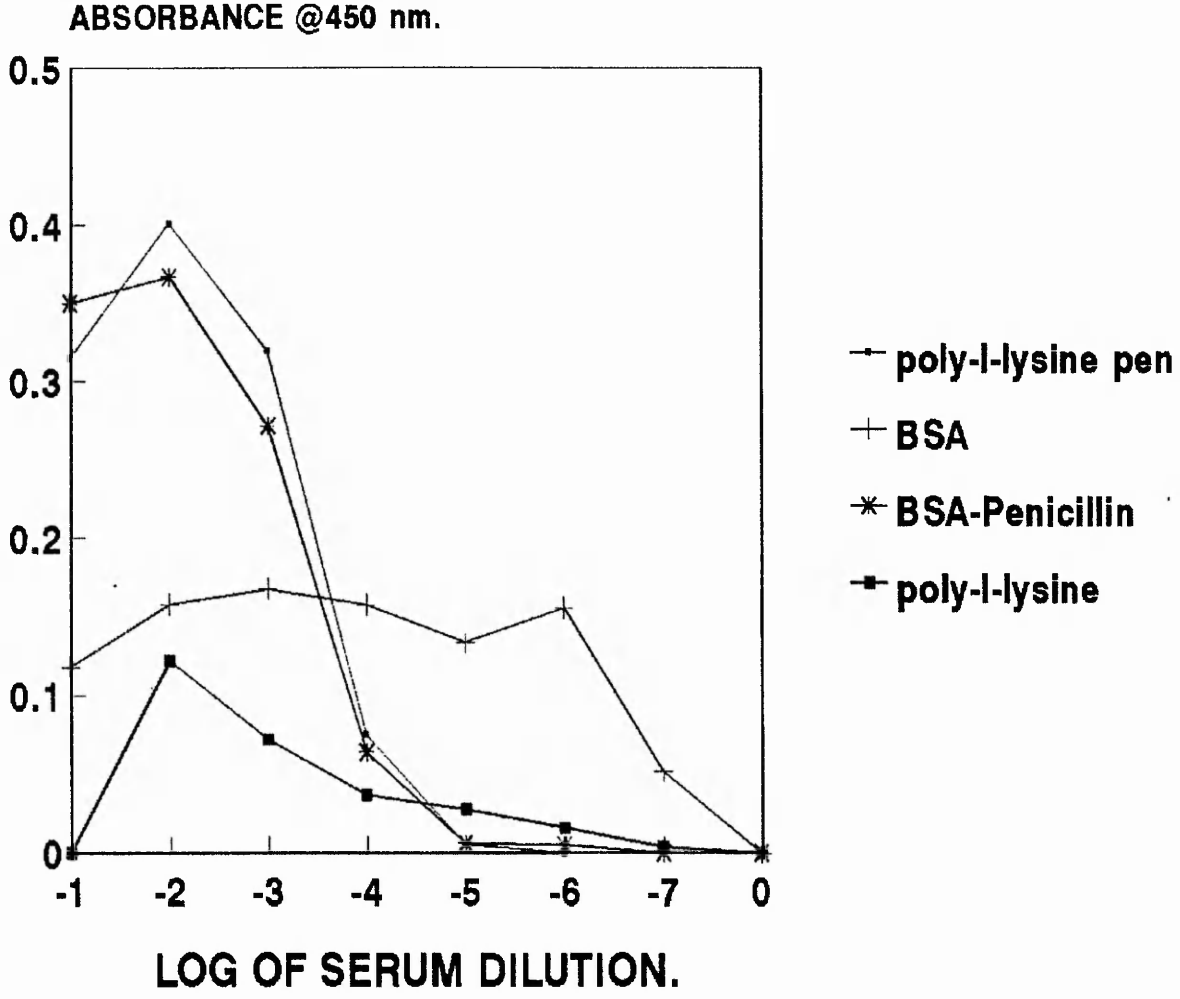


Figure 2.8b: ELISA of the second test-bleed antiserum from rabbit 6998 immunised with BSA-penicillin and tested against BSA, BSA-penicillin, poly-l-lysine, and poly-l-lysine-penicillin as the plate antigen. Method as described in Section 2.2.6.2.

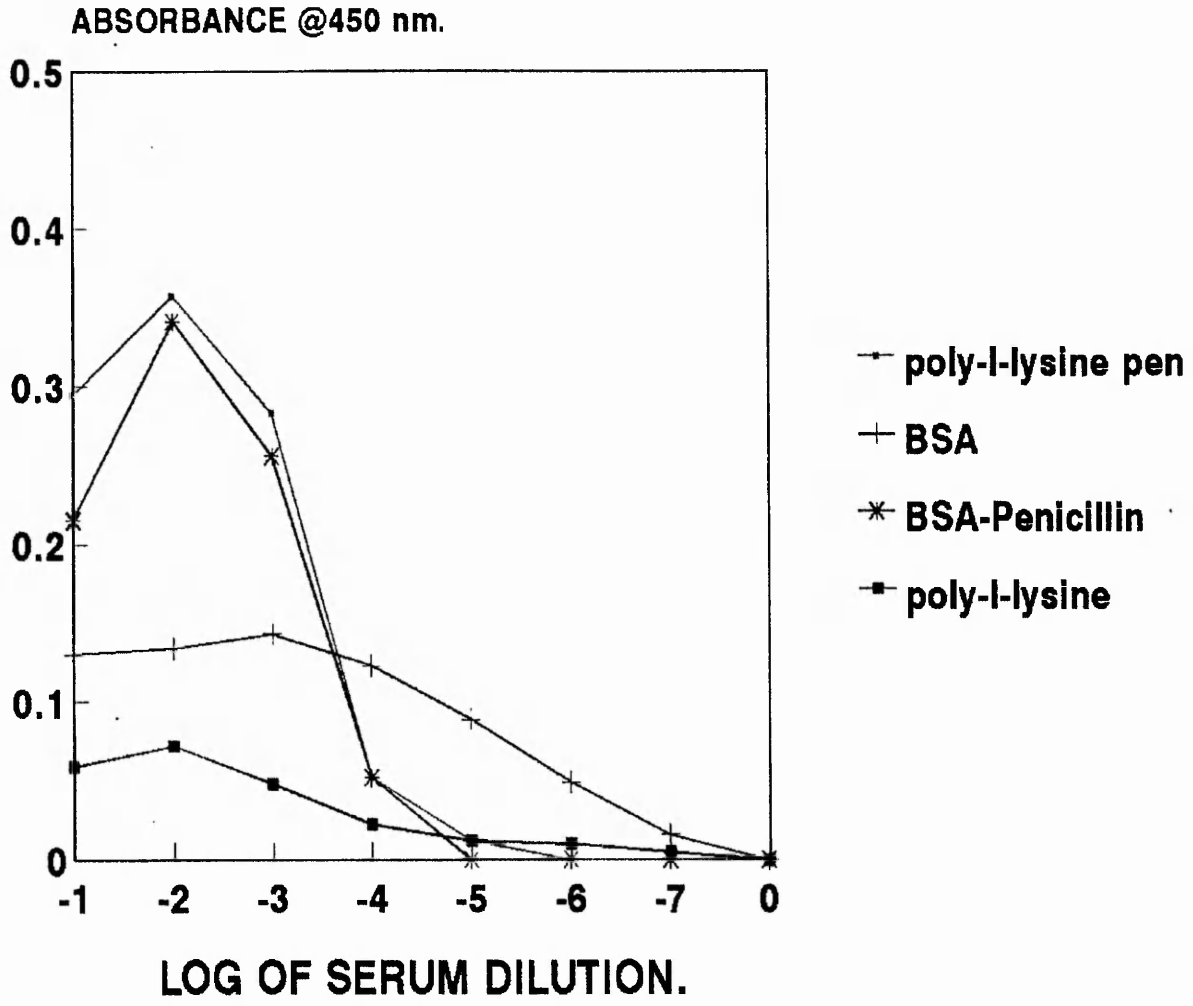


Figure 2.8c: ELISA of the second test-bleed antiserum from rabbit 6999 immunised with BSA-penicillin and tested against BSA, BSA-penicillin, poly-l-lysine, and poly-l-lysine-penicillin as the plate antigen. Method as described in Section 2.2.6.2.

there is a significant reaction with the unconjugated BSA. This suggests that the antiserum contains antibodies against the BSA epitopes as well as against the penicilloyl epitopes.

2.3.4.1.2 Competitive inhibition ELISA tests on the anti-BSA-penicillin antisera

The second test-bleed antisera from rabbits 6997, 6998 and 6999 immunised with BSA-penicillin were tested in competitive inhibition ELISAs using BSA-penicillin and poly-l-lysine-penicillin as the plate antigen and benzyl penicillin as the potential inhibitor. An antiserum dilution of 10^{-2} was chosen for these assays as this dilution was just saturating (ie just at the peak of the absorbance curve) and hence would give a good positive reaction while being sufficiently sensitive to show any inhibition. The titrations of the three antisera are shown in Figure 2.9 a and b.

The Figures show that at a concentration of 100mg/ml the benzyl penicillin significantly reduced the binding of the anti-BSA-penicillin antibodies in all the antisera to both the BSA-penicillin and the poly-l-lysine- penicillin. This suggests that a proportion of the antibodies in the sera can recognise the benzyl penicillin hapten. The inhibition was only observed with the highest concentration of benzyl penicillin which suggests that the assay was relatively insensitive, possibly due to the fact that high concentrations of antisera were used.

A variation of the competitive inhibition ELISA was used to try

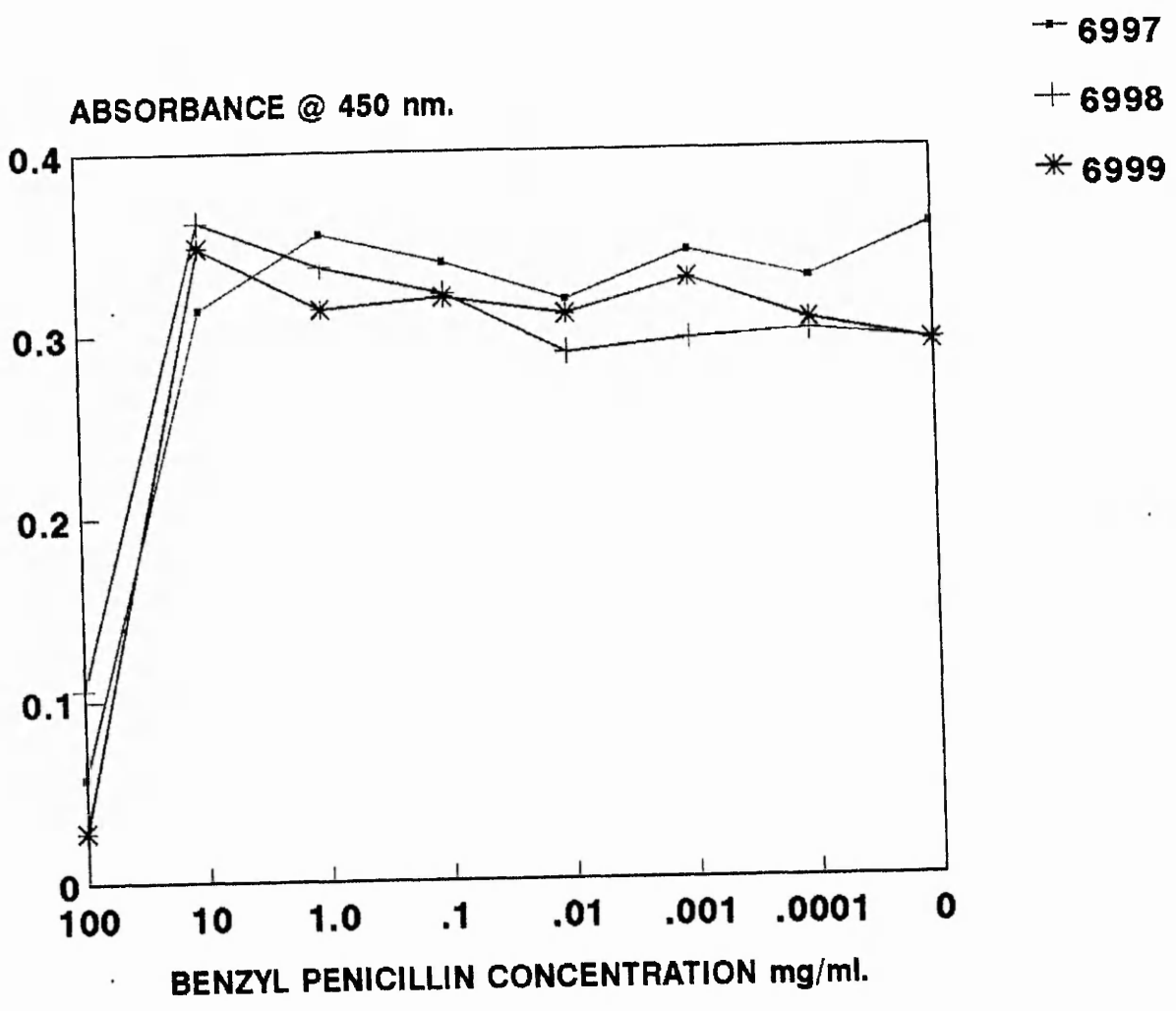


Figure 2.9a: Competitive inhibition ELISA on a 10^{-2} dilution of the second test-bleed antisera from rabbits 6997, 6998 and 6999 immunised with BSA-penicillin. Each antiserum was pre-incubated with an equal volume of a solution of benzyl penicillin at the concentrations shown then assayed against poly-l-lysine-penicillin as the plate antigen. Method as described in Section 2.2.6.3.

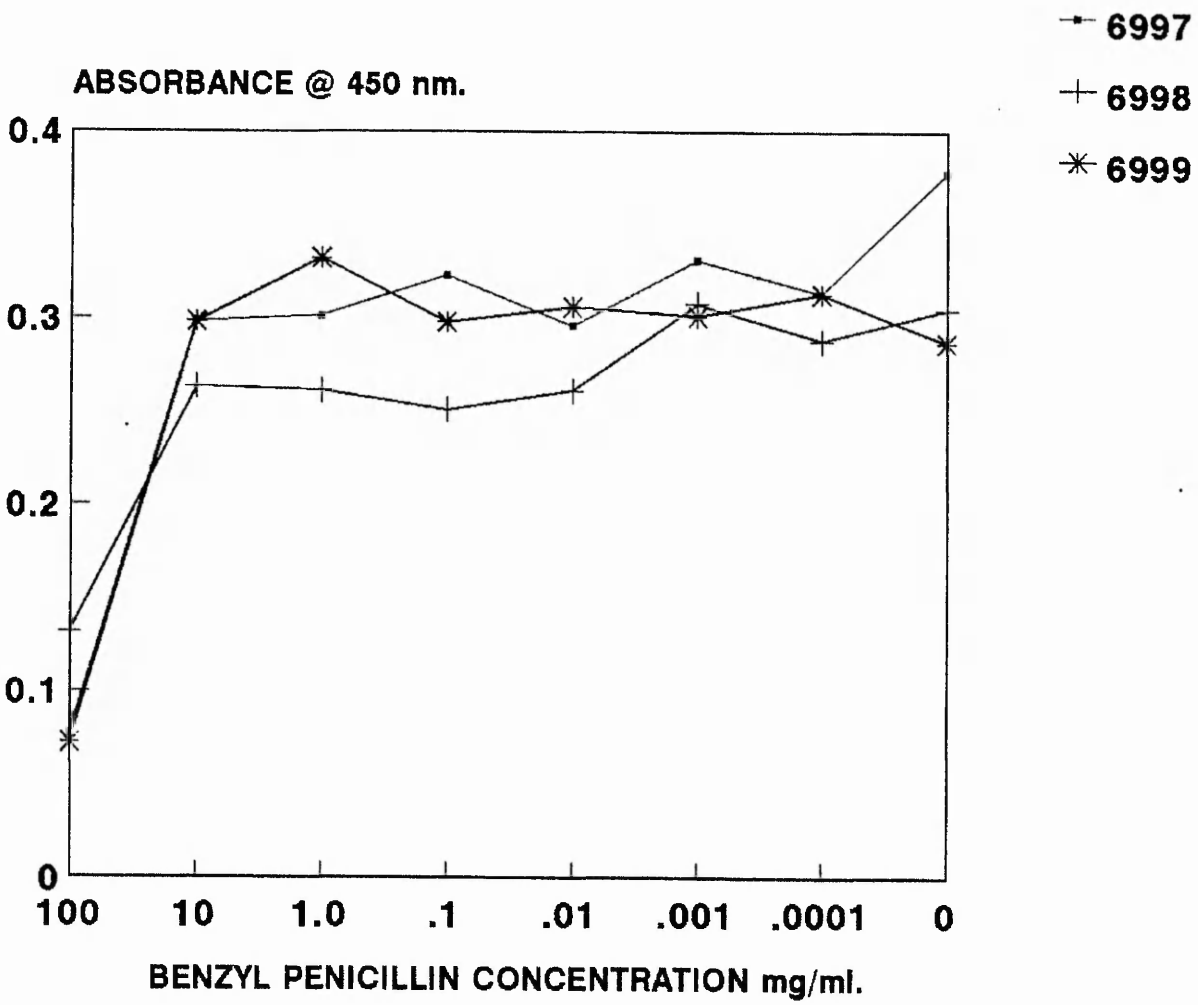


Figure 2.9b: Competitive inhibition ELISA on a 10^{-2} dilution of the second test-bleed antisera from rabbits 6997, 6998 and 6999 immunised with BSA-penicillin. Each antiserum was preincubated with an equal volume of a solution of benzyl penicillin at the concentrations shown then assayed against BSA-penicillin as the plate antigen. Method as described in Section 2.2.6.3.

to confirm the inhibition observed. In this assay a series of dilutions of the same antisera as above were each pre-incubated with 100mg/ml of benzyl penicillin then assayed against poly-l-lysine-penicillin as the plate antigen. All other conditions were as in the standard ELISA method. These titrations are shown in Figure 10. The results show that the pre-incubation with benzyl penicillin has caused a very considerable reduction in the absorbances compared with the control buffer. These results again indicate that a proportion of the antibodies in the antisera can react with benzyl penicillin.

2.3.4.2 Specificity of the anti-BSA-methicillin antisera.

A number of experiments were also performed on the BSA-methicillin antisera to try to determine the specificity of the antibodies, and in particular, to provide evidence that specific antibodies against the beta-lactam epitopes were present.

2.3.4.2.1 Cross-reactions of the anti-BSA-methicillin antisera with the carriers and conjugates in standard ELISA tests.

The second test-bleed antisera from rabbits 8041, 8042 and 8043 immunised with BSA-methicillin were assayed against the following plate antigens:

a) BSA-methicillin,

b) poly-l-lysine-penicillin.

The titrations are shown in Figures 2.11 a-c. These antisera were not tested against BSA nor against poly-l-lysine as by the time they were being tested the competitive inhibition ELISA had been developed and was providing more definite results which

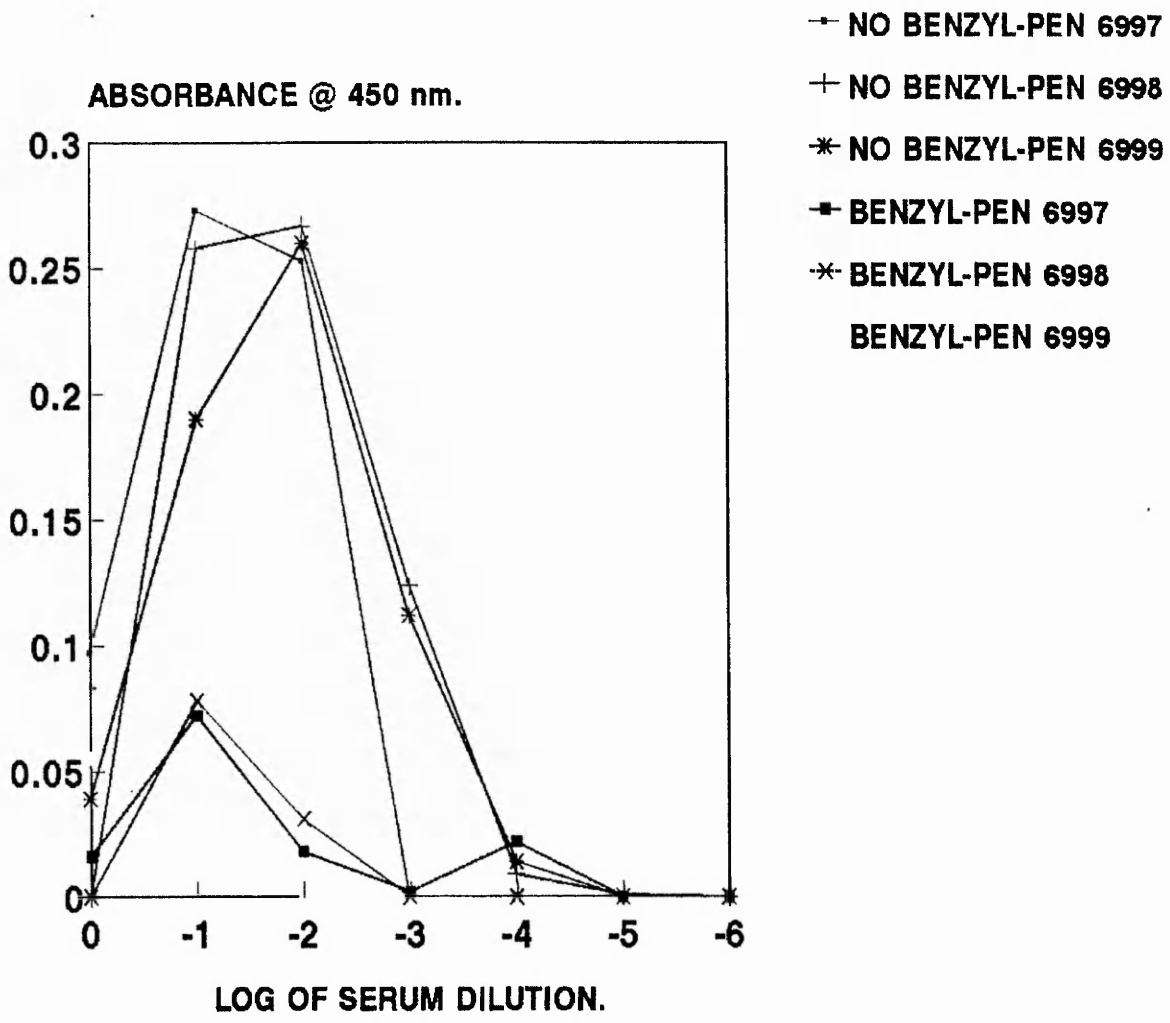


Figure 2.10: Competitive inhibition ELISA on serial 10-fold dilutions of the second test-bleed antisera from rabbits 6997, 6998 and 6999 immunised with BSA-penicillin. Each antiserum dilution was pre-incubated with an equal volume of a solution of 100mg/ml of benzyl penicillin or phosphate buffer pH 7.4 as a control then assayed against poly-l-lysine-penicillin as the plate antigen. Method as described in Section 2.2.6.3.

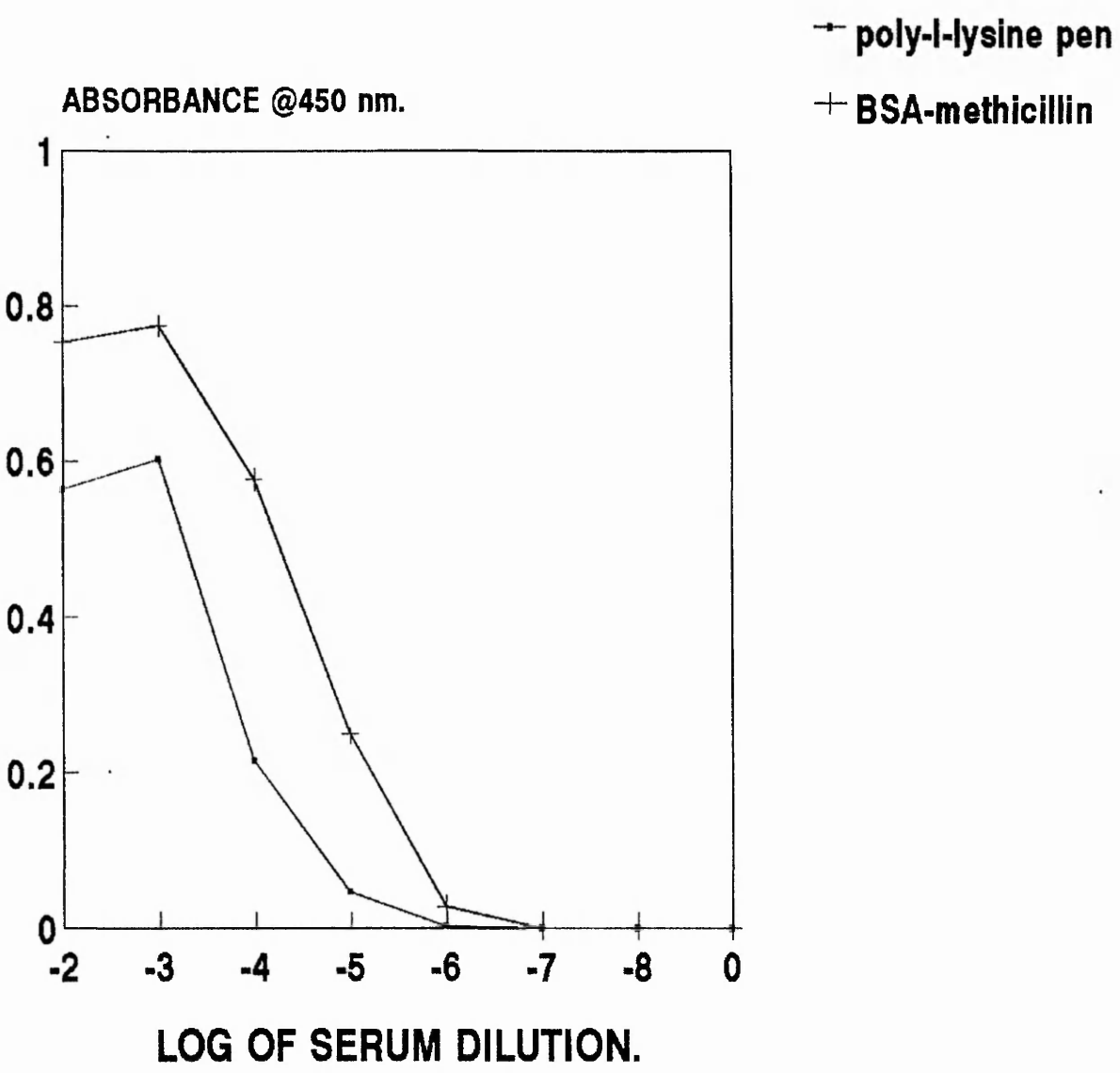


Figure 2.11a: ELISA of the second test-bleed antiserum from rabbit 8041 immunised with BSA-methicillin and tested against BSA-methicillin and poly-l-lysine penicillin as the plate antigen.

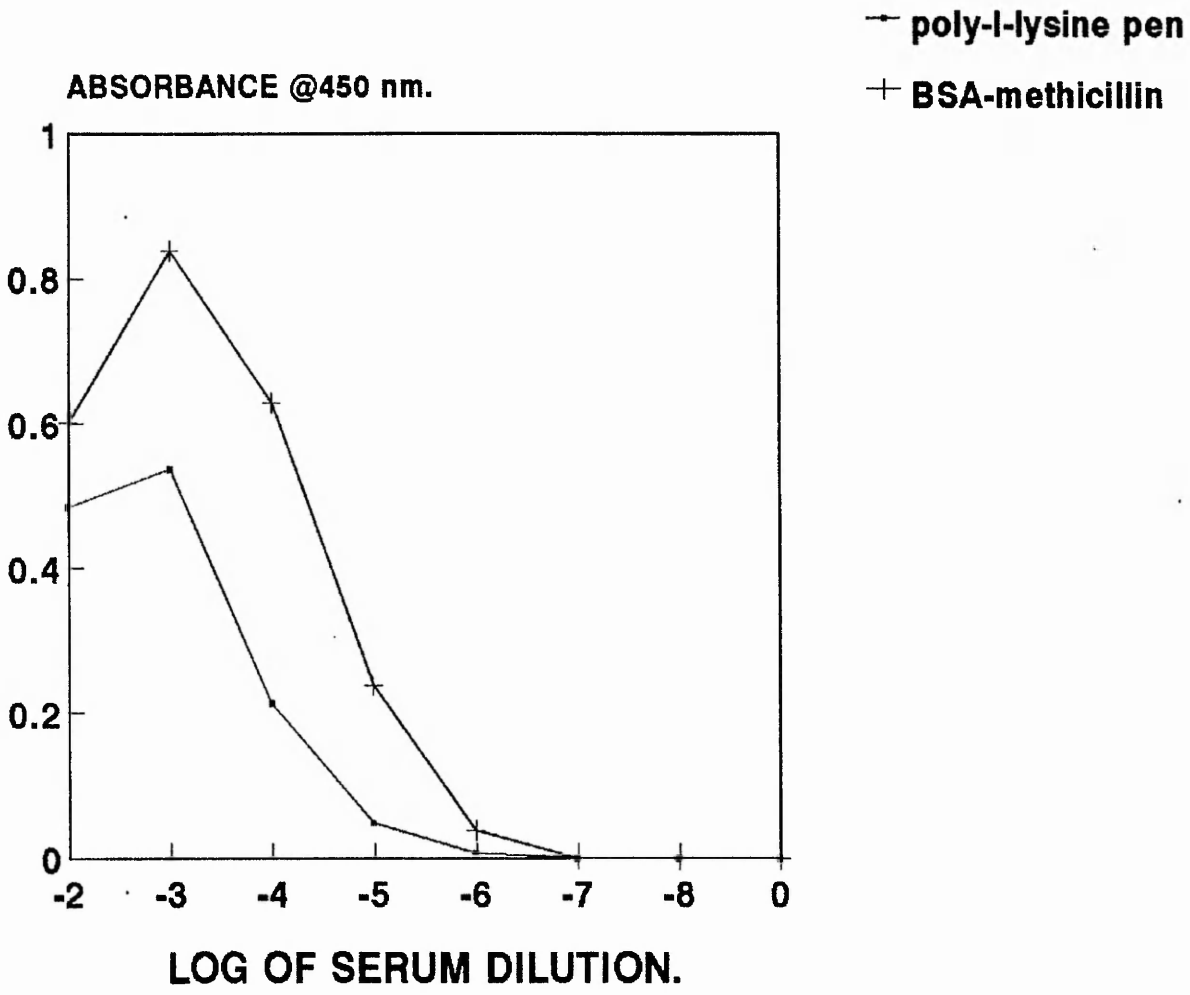


Figure 2.11b: ELISA of the second test-bleed antiserum from rabbit 8042 immunised with BSA-methicillin and tested against BSA-methicillin and poly-l-lysine-penicillin as the plate antigen.

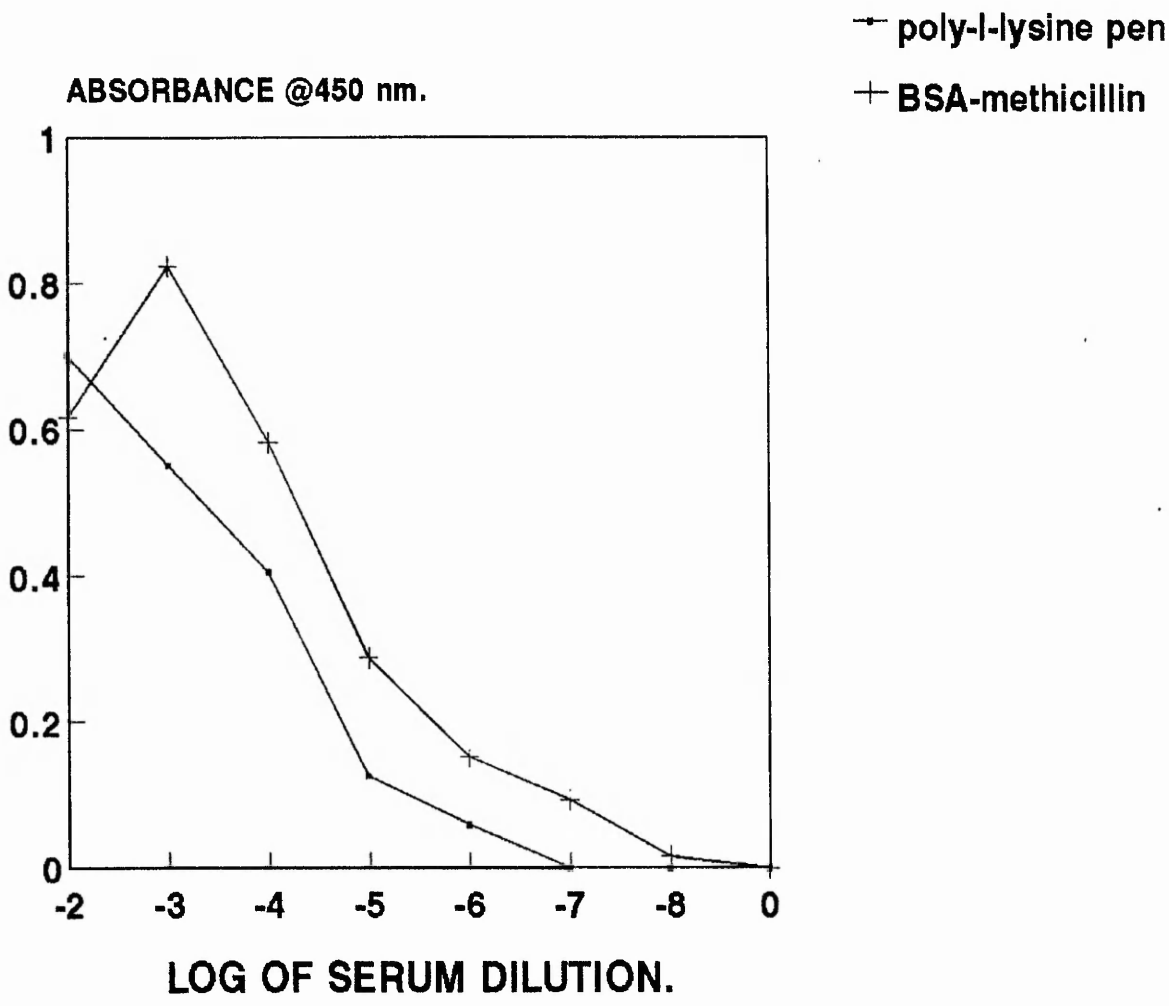


Figure 2.11c: ELISA of the second test-bleed antiserum from rabbit 8043 immunised with BSA-methicillin and tested against BSA-methicillin and poly-l-lysine-penicillin as the plate antigen.

superseded the direct ELISA tests. For the purpose of interpreting these direct ELISA results the assumption is made that these three antisera would have given similar reactions with BSA and poly-l-lysine as did the anti-BSA-penicillin antisera.

The notable observation from these three charts is the fact that the anti-BSA-methicillin antisera have reacted well with the poly-l-lysine penicillin as the plate antigen. If it can be assumed that these antisera would have given an insignificant reaction with unconjugated poly-l-lysine then the results indicate that a proportion of the antibodies in these anti-BSA-methicillin antisera cross-react with penicillin. Possibly this cross-reaction is with the penicillin nucleus and/or its derivative in the conjugate.

2.3.4.2.2 Competitive inhibition ELISA tests on the anti-BSA-methicillin antisera.

Figures 2.12 a and b show the titration curves for the competitive inhibition ELISA tests performed on the anti-BSA-methicillin antisera. A dilution of 10^{-3} was used for these assays as this dilution was just at antibody saturation point in the ELISA titration curves (ie it just gave the maximum absorbance). Figure 2.12a shows that up to 100mg/ml of BSA failed to inhibit the reaction of the antisera with the plate antigen BSA-methicillin. This provides evidence that the anti-BSA component of the antisera is a minor one. Figure 2.12b shows that methicillin at concentrations of 100μg/ml and 10μg/ml, and possibly at 1μg/ml and 0.1μg/ml inhibited the reaction of the

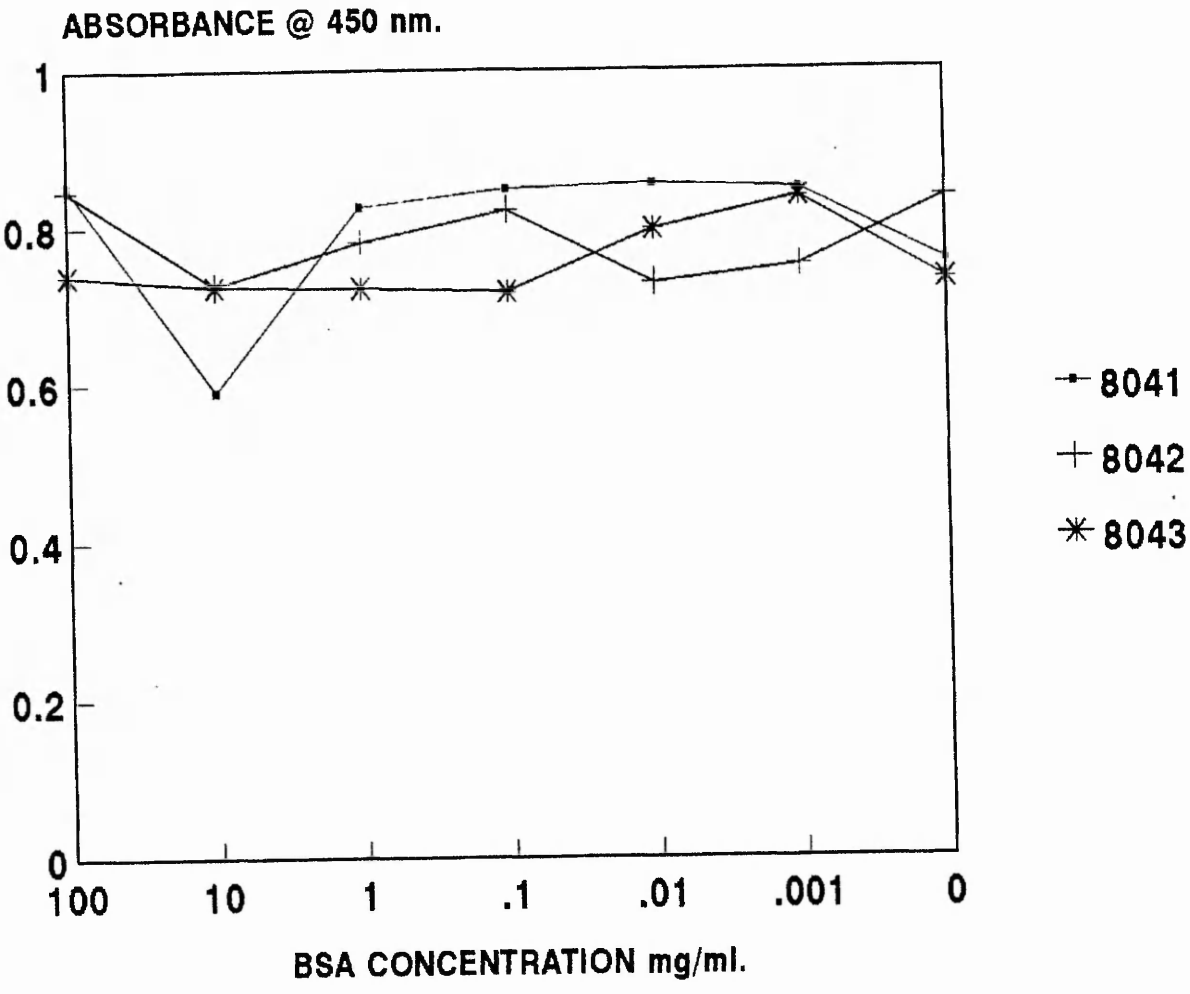


Figure 2.12a: Competitive inhibition ELISA on a 10^{-3} dilution of the second test-bleed antisera from rabbits 8041, 8042, and 8043 immunised with BSA-methicillin. Each antiserum was pre-incubated with an equal volume of a solution of BSA at the concentrations shown then assayed against BSA-methicillin as the plate antigen. Method as described in Section 2.2.6.3.

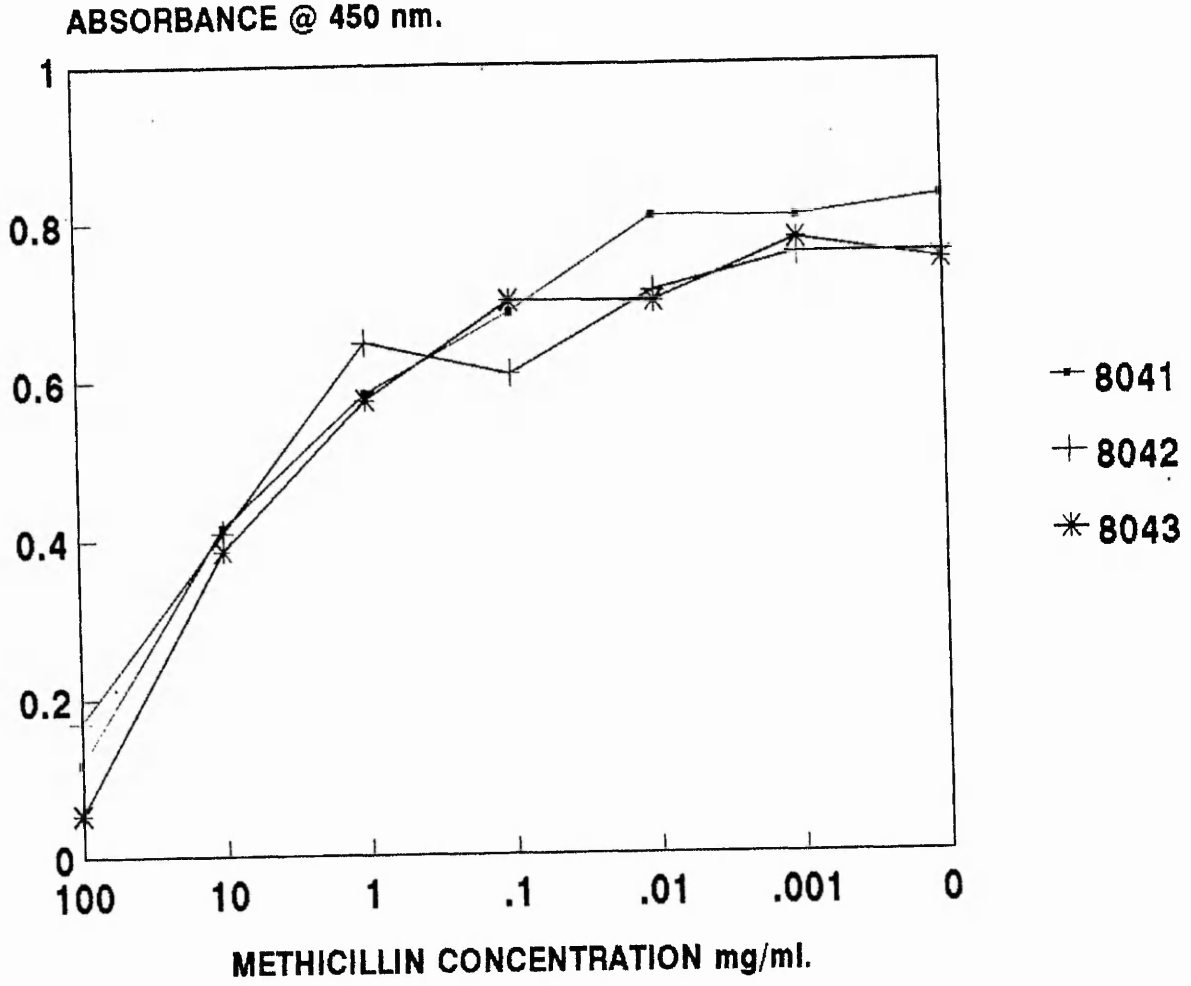


Figure 2.12b: Competitive inhibition ELISA on a 10^{-3} dilution of the second test-bleed antisera from rabbits 8041, 8042 and 8043 immunised with BSA-methicillin. Each antiserum was preincubated with an equal volume of a solution of methicillin at the concentrations shown then assayed against BSA-methicillin as the plate antigen. Method as described in Section 2.2.6.3.

antisera with the plate antigen BSA-methicillin. This suggests that the anti-BSA-methicillin antibodies have reacted with the methicillin during preincubation and this has prevented them from binding to the plate antigen. The fact that methicillin blocked the reaction of the antisera with BSA-methicillin while BSA did not suggests that a significant proportion of the antibodies in the antisera are directed against the methicillin epitopes.

2.3.5 Affinity purification of anti-methicillin antibodies

The anti-BSA-methicillin antisera were chosen for affinity purification as these antisera had higher titres than the anti-BSA-penicillin antibodies and the competitive inhibition ELISA tests suggested that a significant proportion of the antibodies were specific to the methicillin epitopes. BSA-methicillin was used as the ligand on the affinity column as the competitive inhibition ELISA tests described before had suggested that anti-BSA antibodies were not significant in these antisera.

2.3.5.1 Protein estimations from the affinity purification.

The protein concentration of the BSA-methicillin conjugate attached to the CNBr-sepharose was determined using the Biorad protein assay (Section 4.3.2). Approximately 17.48mg of bound protein was measured which represented an estimated 99% of the added conjugate suggesting a high efficiency of binding. The "low affinity" and "high affinity" antibody populations were each ultra-filtered back to 10ml to enable their concentrations to be compared with one another. Out of the original 10ml of diluted antiserum (protein concentration not measured) 1.7mg of protein

was measured in the "low affinity" antibody pool and 0.67mg of protein was measured in the "high affinity" antibody pool. Individual fractions were not collected in this procedure but the monitoring traces of absorbance at 280nm are shown in the Appendix.

2.3.5.2 Investigations on the affinity-purified antibodies.

2.3.5.2.1 ELISA of affinity-purified anti-BSA-methicillin antibodies.

Figure 2.13 shows the titration curves of the original antiserum and the two populations of affinity-purified antibodies against BSA-methicillin as the plate antigen. The eluted antibody populations showed much less non-specific binding to the antigen-free control than the original antiserum although this is not seen in the figure as this shows the corrected absorbances. This suggests that the affinity-purified populations are "cleaner" and hence less likely to give problems with non-specific reactions than the original antisera. The "low affinity" antibody pool had a higher titre than the "high affinity" antibody pool ELISA. This may be due to the fact that the "low affinity" pool had more than double the protein concentration of the "high affinity" pool but it may also imply that the antiserum contained more "low affinity" antibodies, perhaps as a consequence of chemical damage during elution.

2.3.5.2.2 Competitive inhibition ELISA tests on the affinity-purified antibodies.

The affinity-purified antibodies were tested for competitive

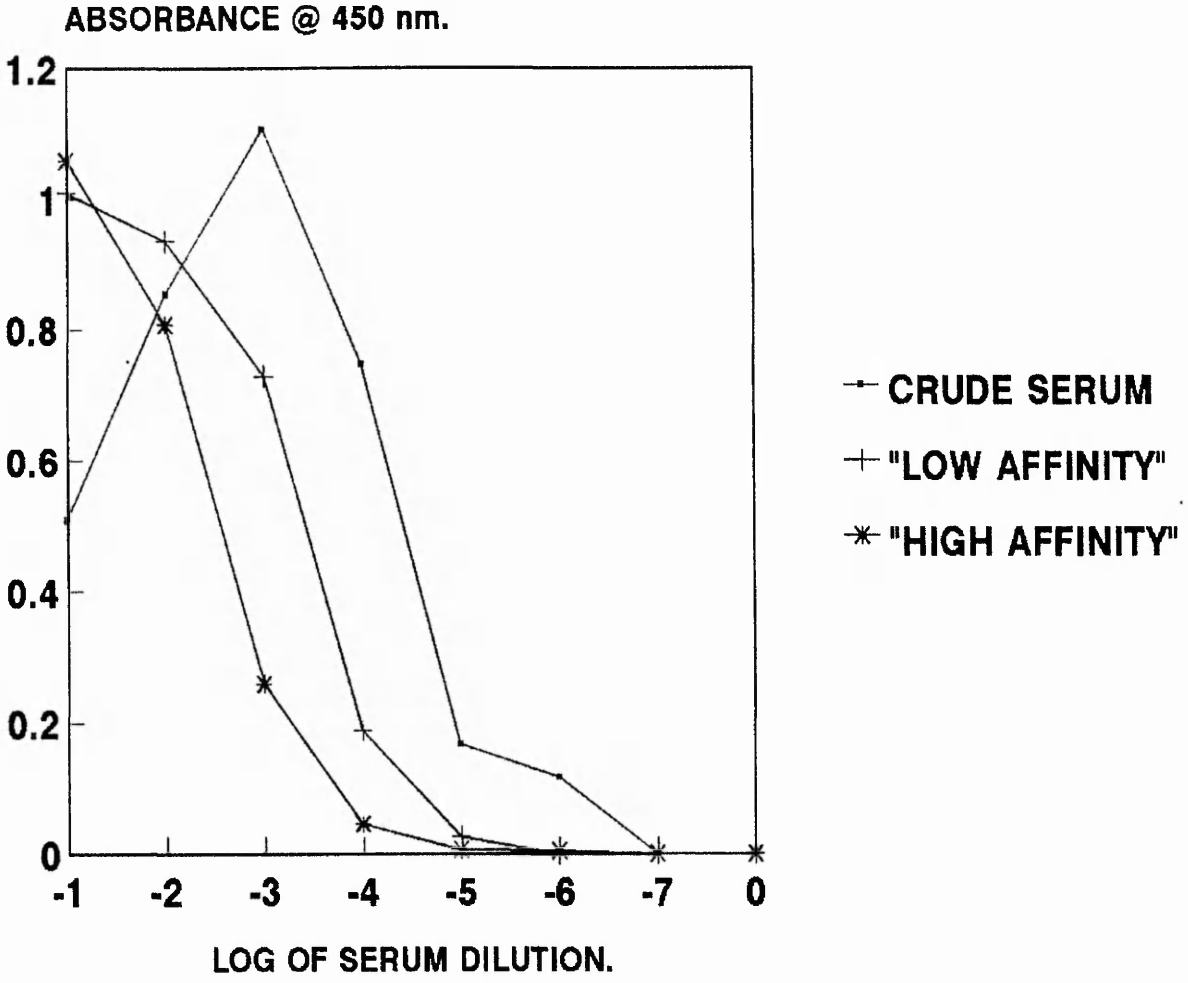


Figure 2.13: ELISA of the second test-bleed of antiserum 8043 and the "low affinity" and "high affinity" antibody pools purified from it against BSA-methicillin as the plate antigen.

inhibition by a number of inhibitors in an ELISA test against BSA-methicillin as the plate antigen. The results are shown in Figures 2.14a-c.

Figure 2.14a shows the charts for the competitive inhibition ELISA using BSA as the inhibitor. At the concentrations of BSA used no inhibition was observed.

Figure 2.14b shows the charts for the competitive-inhibition ELISA using methicillin as the inhibitor. These results show that both the "high affinity" and the "low affinity" antibodies were inhibited by preincubation with methicillin. This inhibition was almost 100% at the highest methicillin concentration. From the graphs, it was estimated that the concentration of methicillin required to give 50% inhibition of the low and high affinity antibodies was approximately 20 and 2 mg/ml respectively. This suggested that the low affinity antibodies were acting like high affinity antibodies, since a higher concentration of methicillin was needed to inhibit the binding of the low affinity antibodies to the plate antigen compared with the high affinity antibodies.

Figure 2.14c shows the charts for the competitive-inhibition ELISA using benzyl penicillin as the inhibitor. These results show that both the "low affinity" and the "high affinity" antibodies were inhibited by preincubation with benzyl penicillin but to a lesser extent (approximately 1000-fold) than with methicillin as shown in the previous chart. This suggests that although some antibodies have been isolated which can bind to

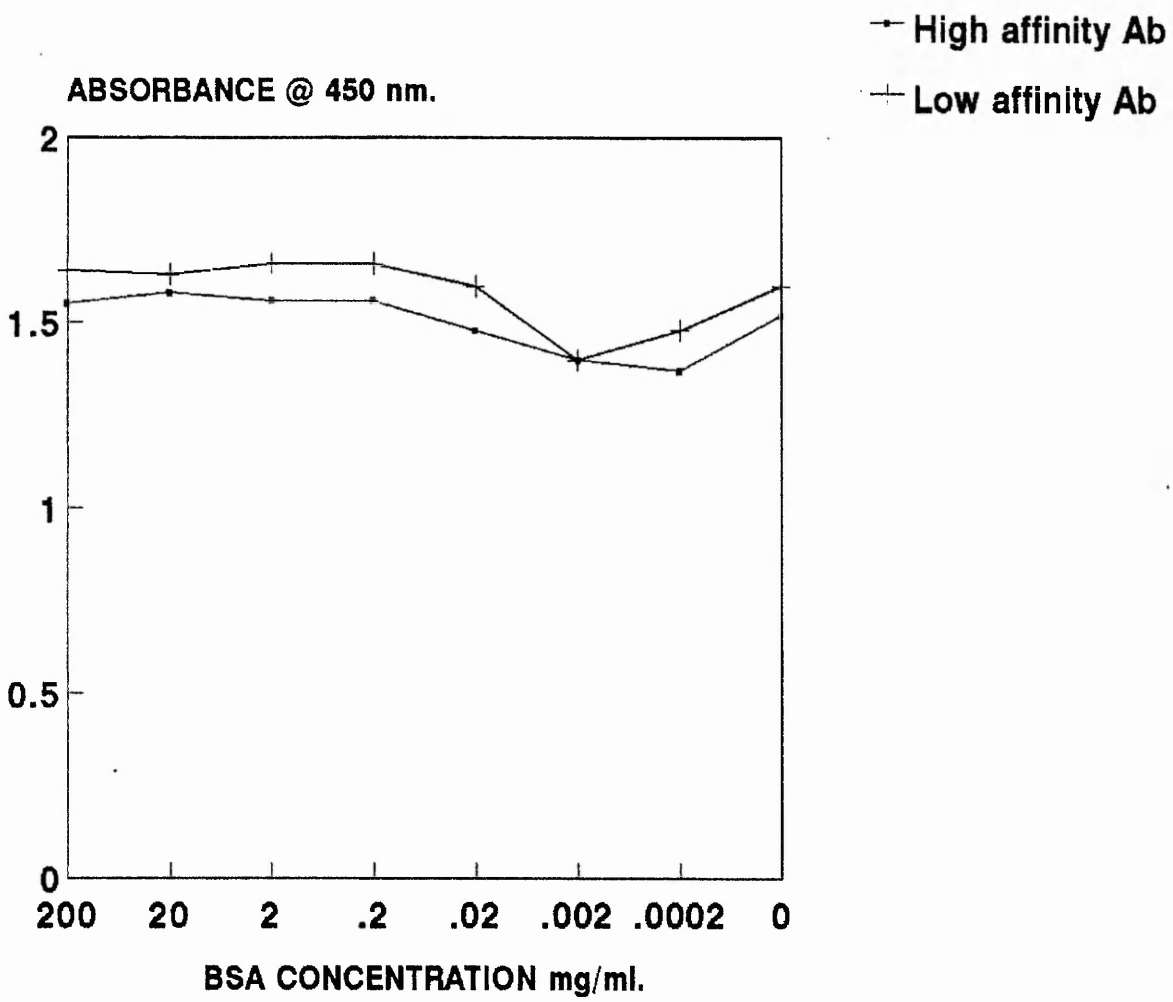


Figure 2.14a: Competitive inhibition ELISA of the affinity-purified "low affinity" and "high affinity" anti-BSA-methicillin antibody pools diluted 1/100 in 66mM phosphate buffer pH 7.4 using BSA-methicillin as the plate antigen and BSA as the inhibitor.

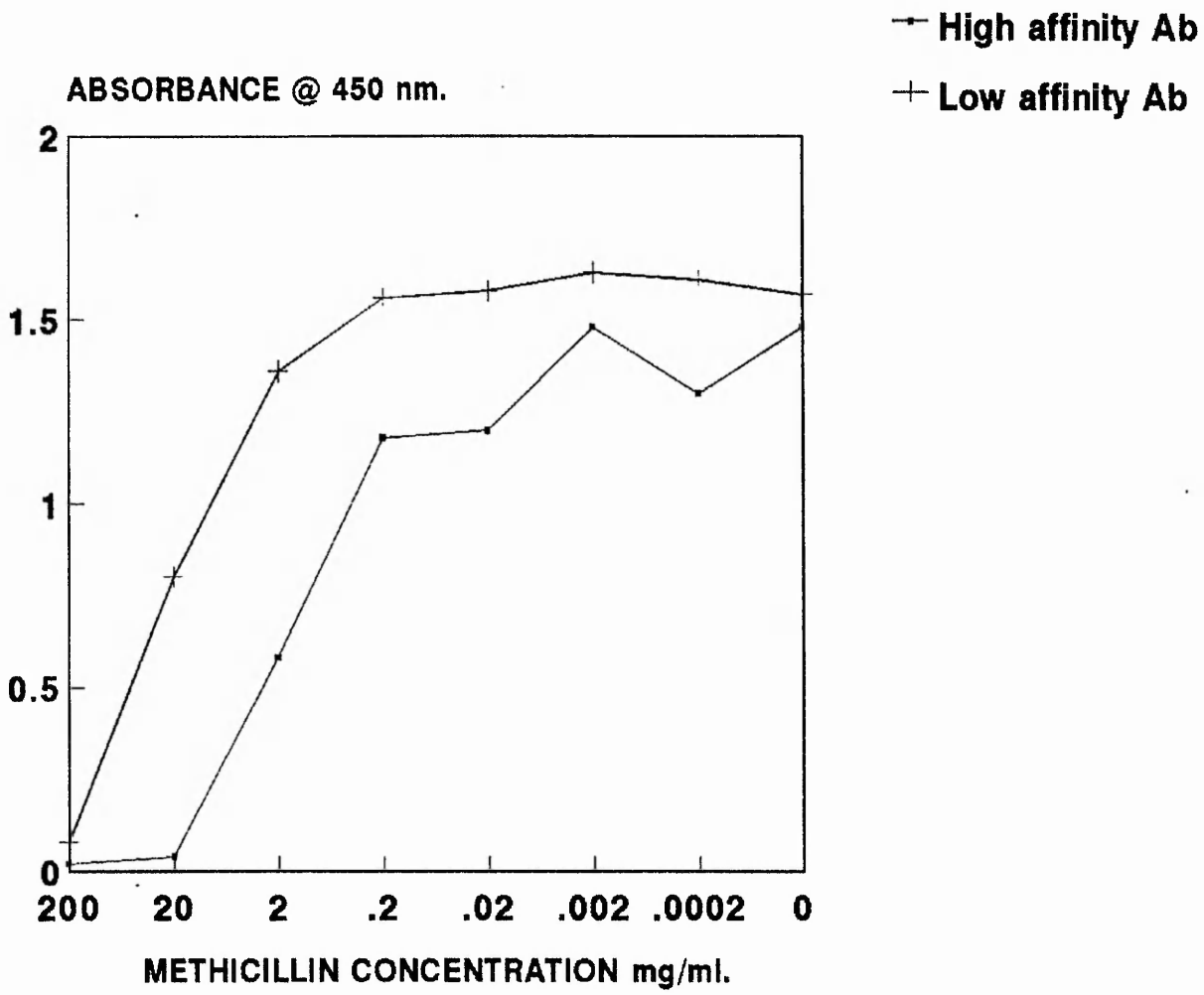


Figure 2.14b: Competitive inhibition ELISA of the affinity-purified "low affinity" and "high affinity" anti-BSA-methicillin antibody pools diluted 1/100 in 66mM phosphate buffer pH 7.4 using BSA-methicillin as the plate antigen and methicillin as the inhibitor.

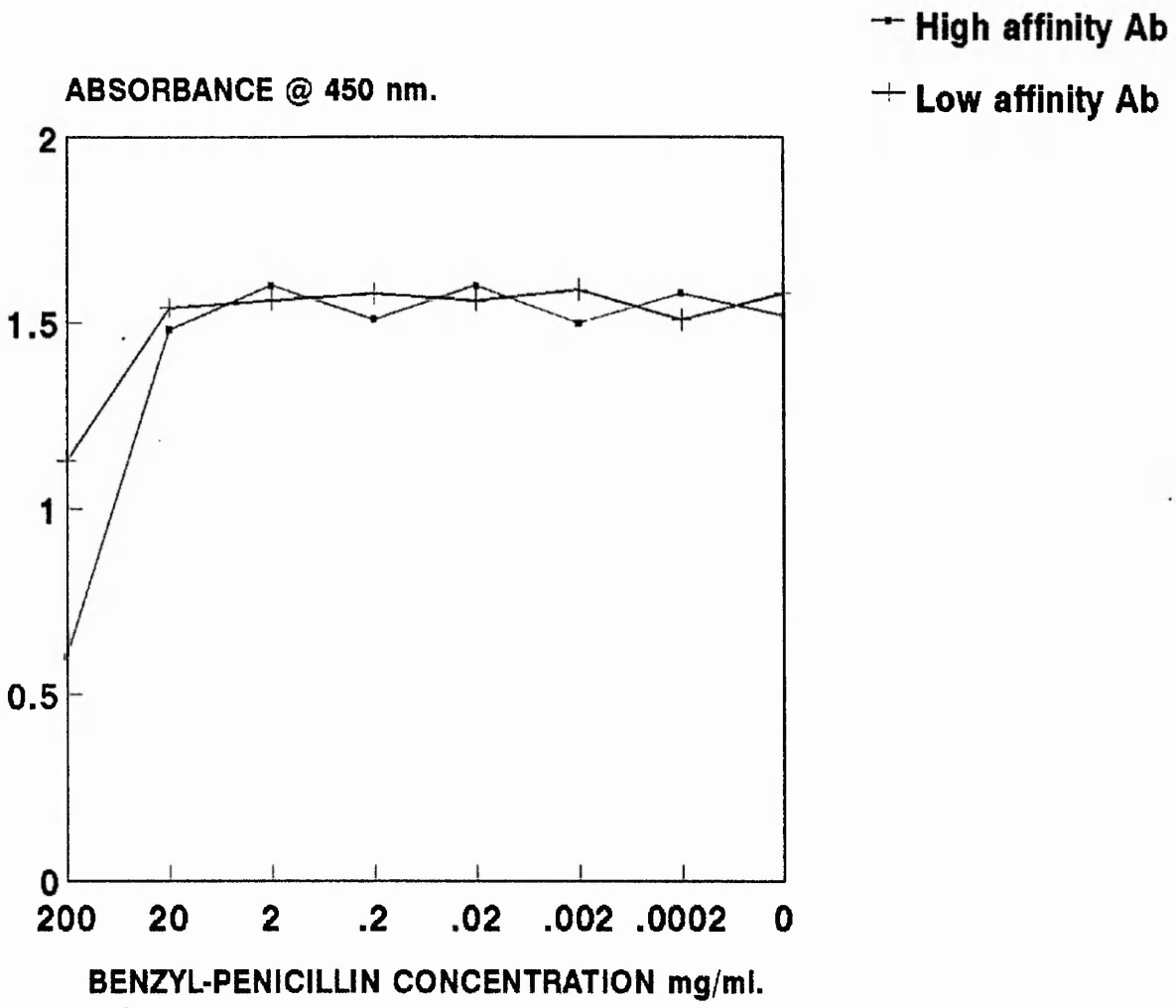


Figure 2.14c: Competitive inhibition ELISA of the affinity-purified "low affinity" and "high affinity" anti-BSA-methicillin antibody pools diluted 1/100 in 66mM phosphate buffer pH 7.4 using BSA-methicillin as the plate antigen and benzyl penicillin as the inhibitor.

both methicillin and benzyl penicillin most of them can only bind to methicillin. This suggests that the pools contain some antibodies which recognise the common beta-lactam and thiazolidine ring as well as others which recognise the methicillin side-chain.

2.4 SUMMARY AND CONCLUSIONS

Three beta-lactam conjugates were prepared according to a method described by Lee *et al.* (1985). Calculation of the number of penicilloyl groups attached per mole of protein carrier demonstrated that conjugation of both benzyl penicillin to BSA and to poly-l-lysine and of methicillin to BSA had been achieved. The poly-l-lysine-penicillin and BSA-methicillin conjugates had greater numbers of attached beta-lactam residues than the BSA-penicillin. All three beta-lactam conjugates were considered suitable for polyclonal antiserum production.

A Western blot of the conjugates and the native carriers probed with a dilution of anti-BSA-methicillin antiserum demonstrated that the antibodies recognised the protein carrier (BSA) and also the conjugated BSA, but not the poly-l-lysine and poly-l-lysine conjugate. The results showed that the conjugation to BSA was efficient since no unconjugated BSA band was observed in the BSA-beta-lactam lanes, however, accurate molecular weight estimation of the conjugated carriers was not possible by this method. An alternative approach would have been to assess the molecular weights by staining the SDS-PAGE gel with Coomassie blue but the diffuse nature of the bands would also have been visible by Coomassie blue staining and would have presented the same problem.

A standard ELISA method was used to assess antibody production in the rabbits immunised with the beta-lactam conjugates. Both the BSA-methicillin and the BSA-benzyl-penicillin conjugates

induced antibodies in all the rabbits immunised. The poly-l-lysine penicillin conjugate, which failed to elicit an immune response, contained the highest number of beta-lactams bound per mole of carrier and provided an alternative plate antigen against which the BSA-beta-lactam antisera could be tested. The two sets of antibodies in the third test-bleeds raised against the BSA-beta-lactam conjugates gave positive reactions in ELISAs against their immunogens up to $1/10^{+7}$ dilution for the BSA-penicillin and $1/10^{+8}$ dilution for the BSA-methicillin conjugates. The higher titre of the antisera produced against the BSA-methicillin conjugate may be due to the higher density of beta-lactam molecules present per mole of carrier in this conjugate compared with the BSA-benzyl-penicillin conjugate.

ELISAs were performed to determine whether antibodies to the beta-lactam moiety of each conjugate were present. ELISAs using the native carriers BSA and poly-l-lysine showed that serum antibodies did recognise the carriers but that the signal was lower than that obtained using the beta-lactam conjugates. The BSA-penicillin antiserum bound to the poly-l-lysine-penicillin conjugate and to the BSA-penicillin conjugate equally well. This suggested that a significant amount of the antibody population recognised the beta-lactam epitopes. Cross-reaction of this antiserum with the BSA-methicillin conjugate was not tested although this would have indicated whether the antibodies were specific for benzyl penicillin or for the common beta-lactam epitope and the new antigenic determinant due to conjugation. However, the BSA-methicillin antibodies were found to bind to

BSA-penicillin conjugate as well as to BSA-methicillin conjugate although differences in the titres obtained suggested that some of the antibodies were specific for the methicillin. These specific antibodies were possibly against the side-chain determinant which is unique to methicillin.

The inhibition ELISAs provided evidence to support the standard ELISA data and information on specificity. Both sets of antisera at the dilutions tested were not inhibited by the native carriers at the concentrations used. However, high concentrations of beta-lactam inhibited the antibodies' binding to the plate antigens. One drawback of this approach was that the antibody concentration used was not limiting and thus a large amount of antibody needed to be inhibited to prevent binding to the plate antigens. However, the dilution of the antisera used in these inhibition ELISAs was chosen from the maximum absorbance titres achieved in the test-bleed titration curves. This dilution was used so that a significant reduction in the absorbance value would be realised over the range of inhibitor concentrations used. The BSA-methicillin antiserum inhibition ELISA profiles suggested that the 10^{-3} dilution of antisera used for inhibition was more limiting in antibody content than the BSA-penicillin antisera.

Affinity purification of the BSA-methicillin antisera was carried out to purify antibodies which recognise the methicillin epitopes including the side-group determinant unique to methicillin. Purification was achieved providing two antibody populations which gave very low non-specific absorbances compared with the

crude antiserum. However, the end-point titre for the purified antibody populations was about 100-fold less than that for the crude antiserum. Inhibition ELISAs of the purified antibodies demonstrated that a large proportion of the antibodies specifically recognised the methicillin rather than the penicillin. This suggests that the side-group determinant in methicillin stimulated the production of antibodies as well as the common beta-lactam ring and the link to the carrier molecule.

It was recognised that the use of the immunogen as the purification ligand would probably lead to isolation of antibodies to the carrier as well as to the beta-lactam. However, the level of purified anti-BSA antibodies which were inhibited by free BSA in the inhibition ELISA was negligible suggesting that this was not a problem. A ligand of poly-l-lysine-methicillin would possibly have given the same result. Using 6-APA as the ligand might have isolated antibodies to the beta-lactam core but the antibodies to the specific side-group would not then have been isolated. These side-group determinant antibodies were considered to be of importance in a possible reagent to probe for the binding of methicillin to PBPs where the side-group is not involved in the covalent bond to the PBPs and may be the only determinant accessible to the anti-beta-lactam antibodies.

Chapter 3

DETECTION AND PURIFICATION OF PENICILLIN-BINDING PROTEINS

3.1 INTRODUCTION

3.1.1 Methods for the detection of PBPs by fluorography

Traditionally penicillin-binding proteins (PBPs) have been visualised on fluorographs using tritiated benzyl-penicillin or ^{14}C-benzyl-penicillin. Either whole cells or isolated membranes are incubated with radioactive antibiotic before terminating the reaction by addition of detergent. Spratt (1977) studied the properties of PBPs from *Escherichia coli* using fluorography. He terminated the binding of penicillin by the addition of excess non-radioactive penicillin and the anionic detergent sodium lauryl sarcosinate. The detergent was found to denature the PBPs and prevent the enzymic loss of bound penicillin. It also selectively and completely solubilised the inner membrane, containing the PBPs, allowing the insoluble outer membranes to be removed by centrifugation. The membrane proteins were separated in 10% acrylamide slab gels using a discontinuous buffer system based on the method of Laemmli and Favre (1970). The gel was stained and destained and prepared for fluorography using the method described by Bonner and Laskey (1974). The six PBPs in *Escherichia coli* were detected by this method. 2-mercaptoethanol was found to stimulate the release of bound penicillin from the two lowest molecular weight PBPs. Dithiothreitol and hydroxylamine also induced the release of antibiotic from most of the proteins.

Similar methods were used by Barbour (1981) and Dougherty *et al.* (1980) for the detection of the three PBPs located in the cytoplasmic membranes of *Neisseria gonorrhoeae*. They were investigating the properties of PBPs in penicillin-sensitive and intrinsically penicillin-resistant strains. This has been discussed in more detail in Chapter 1.

3.1.2 Use of antibodies to detect PBPs

Hakenbeck *et al.* (1986) used antibodies raised in rabbits against penicillin-protein conjugates as a probe for penicillin-binding proteins in *Streptococcus pneumoniae* and other Gram positive bacteria. He also labelled some of the PBPs in whole cells of *Escherichia coli* with benzyl penicillin before preparing membranes from them and probing the penicillin-PBP complexes with anti-penicillin antiserum. The antibodies could only recognise PBPs bound with penicillin or 6-amino-penicillanic acid. Not all of the PBP-penicillin complexes bound the antibodies but those which did were immunostained quantitatively depending on how much penicillin was bound. Although he could not explain why some PBPs did not bind the antibodies he concluded that the penicilloyl-PBP complex had to be denatured for antibody-binding to occur since there was insignificant precipitation of native penicilloyl-PBP with the antiserum in the presence of protein-A-sepharose. The explanation for this may be that after boiling the protein in sample buffer containing a reducing agent and SDS, disulphide bonds would be reduced and the PBP would unfold possibly exposing the binding site for penicillin which would then be more accessible for antibodies. In the native state the PBP would

probably contain the beta-lactam hidden within the protein. The known three-dimensional structures of two PBPs, that of the D-alanyl-D-alanine Zn^{2+} peptidase of *Streptomyces albus* G (Dideberg *et al.* 1987, Jacob *et al.* 1990) and the serine enzyme of *Streptomyces* R61 (Kelly *et al.* 1989, Knox and Pratt 1990), suggest that the beta-lactam lies in the catalytic cavity.

3.1.3 Use of antibodies to study conformation of PBPs

Several studies have been undertaken in which antibodies against PBPs have been prepared in order to study the immunological conformation of the proteins and in certain cases to compare them with related proteins, eg beta-lactamases.

Hakenbeck (1986) studied the PBPs of penicillin-susceptible and penicillin-resistant pneumococci. In pneumococci the highest molecular weight PBP (PBP1A) of susceptible strains is not detectable in resistant strains. However the authors detected three PBPs immunologically related to PBP1A using antibodies prepared against PBP1A. The three proteins detected were of lower molecular weight than the PBP1A.

In one resistant strain PBP2B, which was not detectable by fluorography, was visualised using antiserum which recognised PBP2B in a sensitive strain. It was concluded that the lower antibiotic affinity of PBP2B was accountable for the lack of its detection in the resistant strain. Another resistant strain was found to contain a higher molecular weight PBP3 using antibodies raised against PBP3 from a sensitive strain.

O'Hara and Reynolds (1987) raised a polyclonal antiserum to PBP2' in methicillin-resistant *Staphylococcus aureus* (MRSA). This antiserum also recognised a similar PBP in methicillin-susceptible strains. The antiserum, when pre-absorbed with detergent-solubilised membranes from a sensitive strain to remove antibodies to this susceptible-strain PBP, could be used to detect PBP2' in truly methicillin-resistant *Staphylococcus aureus*.

Harrington *et al.* (1989) used a monoclonal antibody to MRSA PBP2' to purify this protein from detergent extracts of the MRSA membranes. The antibody reacted in an ELISA and in immunoaffinity chromatography with detergent-solubilised membranes from a resistant strain but not with PBP2' in SDS-PAGE-analysed membranes of the same strain. However, a second monoclonal against the protein did prove useful in detection of this PBP2' in membranes of resistant strains separated by SDS-PAGE and subsequently Western-blotted, although this second monoclonal antibody reacted weakly to PBP2' in ELISA and was not suitable for purification of the PBP by affinity chromatography. This was perhaps due to the first antibody recognising epitopes on the three-dimensional structure of the native protein which would be destroyed in SDS-PAGE. The second monoclonal conversely may have reacted with epitopes of smaller polypeptides in the PBP which are only accessible to the antibody after denaturation.

Den-Blaauwen *et al.* (1989) prepared monoclonal antibodies against native membrane-bound PBP1B of *Escherichia coli* in order to

investigate the membrane topology, spatial organisation and enzymic activities of this protein.

Den-Blaauwen *et al.* (1990) used four monoclonal antibodies against completely-formed PBP1B of *Escherichia coli* to determine the location of epitope areas of these antibodies by assessing the patterns of binding to incompletely-formed (truncated) PBP1B molecules extracted from within cells. Only one of the four monoclonals recognised the incomplete PBP1B epitopes. This, the authors suggested, was probably due to the PBP1B not assuming its full native conformation before being translocated across the membrane. In a related study Den-Blaauwen and Nanninga (1990) studied the topology of PBP1B from *Escherichia coli* and four antigenic determinants using monoclonal antibodies against PBP1B and peptidoglycan to visualise epitopes by immuno-colabelling electron microscopy. PBP1B appeared to colabel with peptidoglycan. Further investigation using monoclonal antibodies against four different epitopes of PBP1B revealed that approximately 1400 PBP1B molecules per cell were labelled in cells grown in broth. Using immuno-colabelling with monoclonal antibodies in pairs and competitive antibody-binding inhibition it was deduced that four epitopes make up a cluster of antigenic determinants which occupy under half of the surface of the PBP1B.

Hakenbeck *et al.* (1991a) used antibodies directed against PBP1A or PBP2B of *Streptococcus pneumoniae* to compare the PBPs of 55 strains from three continents. Several different PBP profiles were revealed suggesting that resistance due to reduced affinity

PBPs in the strains was evolving independently in different parts of the world. Hakenbeck (1991b) used both polyclonal and monoclonal antibodies against *Streptococcus pneumoniae* PBPs to show the antigenic variability of PBP1A and PBP2B. In different strains there were differences in electrophoretic mobility of PBP1A possibly resulting from a single amino acid change which would affect the overall charge of the protein, depending on whether the amino acid was positively or negatively charged.

Zijderveld *et al.*(1991), using polyclonal antisera to PBP1B, found that *Escherichia coli* PBP1B can form a 140kDa complex which is not held together with disulphide crosslinks. The complex was still able to bind penicillin. The complex, which was a PBP1B dimer, was found to be more strongly associated with the cell envelope than the monomer. Studies which involved the fusion of PBP1B to beta-lactamase suggested that the dimerisation part of the protein was in the amino-terminal moiety of the protein.

Chalkley *et al.* (1991) used polyclonal and monoclonal antibodies raised against PBP1A and PBP2B from a penicillin-susceptible strain of *Streptococcus pneumoniae* to visualise similar or related PBPs in other species of streptococci by Western blotting. From the observation that the PBPs in penicillin-resistant pneumococci were variable it was suggested that there were genes homologous to the pneumococcal PBP1A and PBP2B genes in viridans streptococci. Furthermore, genetic transfer experiments showed that penicillin resistance determinants could be transformed from viridans streptococci to the pneumococcus.

Ligozzi *et al.* (1991) used polyclonal antibodies raised against PBP5 of *Enterococcus hirae* to detect immunologically related proteins in *Enterococcus faecium* and *Enterococcus faecalis*. Several strains of each species contained a membrane protein which reacted with the antiserum.

This summary has shown that several workers have raised monoclonal and polyclonal antibodies to PBPs of various species of bacteria. They have assessed the immunological relatedness of these proteins using techniques such as Western blotting. Antibodies against PBPs from a sensitive strain have been used to probe for PBPs from a resistant strain and anti-PBP antibodies have also been used to study PBPs in several strains of one species or different species within a genus. However, no work has been reported on the production of antibodies to PBPs from *Neisseria gonorrhoeae*.

3.1.4 Extraction and purification of PBPs

Antibody production usually involves the isolation and purification (to some degree) of the antigen. Isolation and purification of the PBPs from *Neisseria gonorrhoeae* has not been reported before.

PBPs have been purified from many species of bacteria using covalent affinity chromatography. Blumberg and Strominger (1972) isolated five PBPs from the membranes of *Bacillus subtilis*. They prepared an affinity column of 6-amino-penicillanic acid bound to Sepharose-4B in an acid-catalysed condensation reaction.

Carbodiimides promote condensation between a free amino and a free carboxyl group to form a peptide link by acid-catalysed removal of water. The carbodiimide is hydrated forming an isourea. Thus, using such a reaction, the primary amino group on 6-amino-penicillanic acid can be attached to the carboxyl group on Sepharose leaving the beta-lactam ring intact. It is essential that the ring is not hydrolysed since covalent attachment of PBPs to the carboxyl group of the 6-amino-penicillanic acid is the basis of purification.

The degree of coupling of 6-amino-penicillanic acid to the Sepharose can be monitored by the hydroxamate assay based on the method for the colourimetric determination of benzyl penicillin described by Boxer and Everett (1949). The principle of the assay is that the carbonyl group of the intact beta-lactam ring reacts with hydroxylamine to form hydroxamic acid. This in turn combines with ferric ions to give a brown-red complex. This is in contrast to the penamaldate assay described in Chapter 2 which measures penicilloyl groups on protein present as cleaved beta-lactam rings.

In the affinity purification method the membrane proteins, including PBPs, are solubilised in a non-ionic detergent such as Triton-X100 and passed through the affinity column. Isolated PBPs can be removed by adding neutralised hydroxylamine which cleaves the penicilloyl-PBP bond. The hydroxylaminolysis of the covalent penicilloyl-PBP bond is enzymatically catalysed. Kozarich *et al* (1977) showed that denaturation of the complex with SDS, heat or

trichloroacetic acid inhibited the release of PBPs from penicillin. Treatment of the denatured penicillin-PBP complex with sodium borohydride or buffer at pH 12 caused the non-enzymic release of the penicillin. This suggested that a carboxylic ester bond was involved.

Purification of PBPs from *Escherichia coli* has been achieved, using a similar method, by several workers including Amanuma and Strominger (1980) and Curtis and Strominger (1981).

3.1.5 Summary and aims of the present study

In this study several strains of *Neisseria gonorrhoeae* are characterised according to their susceptibility to penicillin and methicillin. Fluorography is performed on representative strains in PBP assays using the scintillant reagent Amplify (Amersham) in the radioactive gels to improve the sensitivity of the fluorography. The fluorography of PBPs is investigated for use as a method for determining resistance of strains shown by a decrease in binding of penicillin to PBPs and also to compare with the non-radioactive method of detecting antibiotic-labelled PBPs using antisera against beta-lactam antibiotics. An account is given of the detection of beta-lactams bound to PBPs in strains of *Neisseria gonorrhoeae* differing in their susceptibility to the antibiotics and an attempt is made to explain the differences in terms of the intrinsic resistance of the PBPs, altering the ability of the PBP to bind antibiotic. Affinity chromatography is used to isolate and purify PBPs from several strains of *Neisseria gonorrhoeae* of different penicillin

sensitivities. Various ligands and elutants are investigated for the removal of the PBPs from the bound penicillin.

3.2 MATERIALS AND METHODS

3.2.1 Sources of bacterial cultures used

Freeze-dried cultures of *Neisseria gonorrhoeae* strain I were obtained from the culture collection of Wellcome Diagnostics Ltd, Beckenham, Kent. Freeze-dried cultures of all the other strains used were obtained from the Gonococcal Reference Laboratory, Public Health Laboratory, Kingsdown, Bristol. Stock cultures of each strain were freeze-dried and stored at 4°C. Fresh growing cultures were prepared from freeze-dried stocks each time a large volume culture was set up for experimental work. In this study the cultures were purified from isolated colonies without selection for piliated or non-piliated variants as all the cultures obtained were laboratory sub-cultured stocks which were expected to be non-piliated (Kellogg *et al.* 1963).

3.2.2 Culture media and maintenance of cultures

All cultures were grown and maintained on Difco GC base agar or broth of the following composition:

Proteose peptone	15 g/l
Corn starch	1 g/l
Dipotassium hydrogen phosphate	4 g/l
Potassium dihydrogen phosphate	1 g/l
Sodium chloride	5 g/l
(Agar	10 g/l)

The agar medium was purchased in complete dehydrated form and rehydrated by dissolving 14.4g in 400mls of distilled water and autoclaved at 121°C for 15 minutes. The final pH was 7.2 ± 0.2. Isovitalex (BBL) was added to a concentration of 1% (v/v) just

before pouring the agar into sterile plastic petri dishes. Cultures were streaked on the agar medium and incubated at 37°C in an atmosphere of 10% carbon dioxide in air in a moist incubator. Growing cultures were subcultured at least every 48 hours.

Cultures in liquid medium were used to grow cells for *in vivo* penicillin-binding experiments and for the preparation of membranes for *in vitro* experiments. The liquid medium was made from the individual components at the concentrations listed above for the agar medium except that the agar was omitted. The medium was autoclaved at 121°C for 20 minutes in 20ml volumes in universal bottles or in 600ml volumes in 1 litre screw-capped flasks. Before use Isovitalex supplement was added to a concentration of 1% (v/v) and sterile 6% sodium bicarbonate solution was added to give a final concentration of 0.2%.

3.2.3 Characterisation tests on cultures

3.2.3.1 Gram stain

Gram stains were performed on cultures regularly to confirm the purity of the cultures. The method used was based on one described by Collee *et al.* (1989).

3.2.3.2 Oxidase test

The cultures were tested for oxidase using 1% aqueous N,N,N′,N′-tetramethyl-paraphenylene-diamine-dihydrochloride (freshly prepared). A culture of *Pseudomonas aeruginosa* was used as a positive control and a culture of *Staphylococcus aureus* as a

negative control. Colonies of *Neisseria gonorrhoeae* were picked off plates with a glass rod and smeared onto filter paper wetted with the reagent. The appearance of a blue-purple colour was taken as a positive reaction.

3.2.3.3 Acid production from sugars

All strains were tested for production of acid from sugars using a rapid carbohydrate utilisation test (Young *et al*. 1976). Each strain was tested for acid production from glucose, maltose, sucrose, fructose and lactose. In each case 20μl of a 10% aqueous solution of the sugar was measured into a clean glass tube followed by the addition of 100μl of salt solution (5.2mM potassium phosphate with 0.8% potassium chloride and 0.1% phenol red). A densely turbid suspension of each strain was prepared in the same salt solution and 40μl was added to each sugar solution. After mixing the tubes were incubated at 37°C for 20 minutes. Production of acid was indicated by the formation of a yellow colour in the solution, whereas if no acid was produced the solution remained red.

3.2.3.4 Antibiotic sensitivity tests

Cultures of all strains were tested for sensitivity to benzyl penicillin and methicillin using agar media containing ranges of concentrations of each antibiotic.

Plates of agar medium to which had been added filter-sterilised antibiotic solutions to give a range of final concentrations of 0.01μg/ml to 50μg/ml were prepared. Single colonies of each

strain were resuspended in broth medium and incubated with shaking at 37°C for 4 hours to produce an actively growing culture. A 10μl drop of a 1/50 dilution of each broth culture was spotted onto the surface of each plate. The plates were incubated at 37°C overnight. The plates were examined for growth of the cultures in the area of the spots and the amount of growth compared with that on a control plate containing the same medium with no antibiotic. The results were expressed as the minimum inhibitory concentration (MIC) of the antibiotic defined as the lowest concentration which totally inhibited the growth of the bacterium. Each culture was tested in triplicate to confirm the MIC.

3.2.3.5 Test for beta-lactamase production

Cultures of all strains were tested for beta-lactamase production using nitrocefin (Glaxo). Nitrocefin is a chromogenic cephalosporin which when hydrolysed by beta-lactamase undergoes a rapid colour change from yellow to red. To prepare the reagent 1mg of nitrocefin was dissolved in 0.1ml of dimethyl sulphoxide (DMSO) and then made up to 2ml with 66mM sodium phosphate buffer pH 7.4 to give a stock solution of 0.5mg/ml. The stock solution was stored at 4°C in a dark bottle for up to 1 week. The stock solution was diluted 1/10 in 66mM phosphate buffer pH 7.4 to give a working solution of 50μg/ml. The beta-lactamase was detected by the following two methods. Both methods are qualitative, testing only for the presence or absence of beta-lactamase. A quantitative method is described in Chapter 4.

(a) Plate method

A piece of filter paper was moistened with the diluted nitrocefin. Colonies of the bacteria were smeared onto the impregnated paper using a plastic disposable loop. Development of a red colour within 60 seconds indicated beta-lactamase activity.

(b) Broth method

A 10μl loopful of a broth culture of the bacterium was added to 50μl of the test solution in a clean glass tube. Development of a red colour within 60 seconds indicated beta-lactamase activity.

3.2.4 Membrane preparations

Bacteria were grown in batches of 7.21 in GC broth containing Isovitalex and bicarbonate supplements as described before. Cultures were grown at 37°C with vigorous shaking to an optical density of 0.5 at 650nm. This density had been found by experiment to correlate with the mid log phase of the culture (see Appendix 3). The cells were deposited by centrifugation at 6000g for 10 minutes at 4°C using a Europa 20 centrifuge. The pellet was resuspended in 20ml of ice-cold 20mM sodium phosphate buffer at pH 7.2 with 0.5% (v/v) 2-mercaptoethanol. The cells were broken in a French pressure cell (Aminco) at 10 tons. Unbroken cells were removed by centrifugation at 8000g for 20 minutes at 4°C. The membranes were deposited by centrifugation at 50,000g for 90 minutes at 4°C. The pink membrane pellet was washed twice and resuspended in 20mM phosphate buffer at pH 7.2

and stored at -70°C. The protein concentration of the membrane preparations was estimated by the method of Lowry *et al.* (1951) as described before. Some membrane preparations were made using an ultrasonicator (MSE) with a large (2.5cm diameter) sonicating probe. The cells were resuspended in the same buffer as for the pressure cell method described above and were sonicated in tubes in an ice bath for 5 x 30 second pulses with 60 seconds cooling between pulses.

3.2.5 Labelling of PBPs with tritiated penicillin

Whole cells were labelled as follow. The cells from 500μl of a log-phase cell suspension with an OD of 0.5 at 650nm were centrifuged and resuspended in 50μl of GC broth. Tritiated benzyl penicillin (14.5Ci/mmol) (Amersham) was added to give penicillin concentrations in the range 0.5μg/ml to 8.6μg/ml as detailed in the Figure legends and the suspension was left at 37°C for 15 minutes. The reaction was terminated by the addition of an equal volume of SDS-PAGE sample buffer and the sample was prepared for electrophoresis as explained in the next Section.

Membrane preparations were labelled by adding the tritiated penicillin to about 100μg of membrane protein in 50μl of 20mM phosphate buffer pH 7.2 and the suspension left at 37°C for 15 minutes. The reaction was terminated by the addition of an equal volume of SDS-PAGE sample buffer and the sample prepared for electrophoresis as described below.

3.2.6 SDS-PAG electrophoresis

All reagents were obtained from BDH and were 'Electran' grade. Slab gels 1.5mm thick were cast using a Biorad Protean II system. Resolving gels were routinely cast the day before use and stored at 4°C. Stacking gels were prepared on the day of use. The gel was prepared as follows.

	10% gel	**15% gel**
30% acrylamide	10.00ml	15.00ml
1% methylene-bis-acrylamide	1.73ml	2.60ml
1M tris-Hcl buffer pH 8.8	11.20ml	11.20ml
Distilled water	6.87ml	1.0ml

These reagents were combined in a Buchner flask and degassed using a vacuum pump for 15 minutes then the following were added (to both gel concentrations):

10% sodium dodecyl sulphate	300μl
Ammonium persulphate solution 150mg/ml freshly prepared	100μl
N,N,N',N'-tetramethylethylene diamine (TEMED)	15μl

The gel was poured into the casting plates carefully so as not to trap air bubbles, overlaid with butanol, and left to polymerise. The gel was then rinsed to remove the butanol and overlaid with 0.25M tris HCl-buffer pH 8.8 and stored at 4°C.

On the day of the experiment a 5% stacking gel was made as follows and poured on top of the resolving gel with a comb inserted:

30% acrylamide	1.67ml
1% methyl-bis-acrylamide	1.5ml
1M tris-HCl buffer pH 6.8	1.75ml
Distilled water	5.6ml

The solution was prepared and degassed as before for 15 minutes before the following were added:

10% SDS	100μl
Ammonium persulphate 150μg/ml (freshly prepared)	50μl
TEMED	10μl

Samples and standards were diluted in SDS-PAGE sample buffer composed of the following:

Distilled water	4.0ml
0.5M tris-HCl buffer pH 6.8	1.0ml
Glycerol	0.8ml
10% SDS	1.6ml
0.05% bromophenol blue	0.2ml

The samples were diluted 1:1 in sample buffer and 2-

mercaptoethanol was added to a final concentration of 10%. Samples were boiled for 3 minutes in a water bath prior to loading them on the gel with a Hamilton syringe (volume 50 - 100μl). Dilute protein samples, ie samples containing less than 1mg/ml of protein, were concentrated by precipitating the sample in 4 times the sample volume of ice-cold acetone immediately prior to the addition of SDS sample buffer. This procedure, described by Curtis and Strominger (1981) allowed the analysis of dilute protein samples by SDS-PAGE without the need to load large sample volumes on the gel.

Rainbow molecular weight standards (Amersham) were used on each gel. They were supplied in 50% glycerol and stored at -20°C. They contained the following proteins at 1mg/ml each:

Protein	**Molecular weight**	**Colour**
Myosin	200,000	Blue
Phosphorylase B	97,400	Brown
Bovine serum albumin	69,000	Red
Ovalbumin	46,000	Yellow
Carbonic anhydrase	30,000	Orange
Trypsin inhibitor	21,500	Green
Lysozyme	14,300	Magenta

5μl of the solution of standards was added to an equal volume of SDS-PAGE sample buffer and, after addition of 1μl of 2-mercaptoethanol, they were boiled and loaded onto the gel as described for the samples.

The gels were run using a 1000V power pack (LKB) at a constant current of 30mA through the stacking gel and 35mA through the resolving gel until the blue dye in the sample buffer was about 1cm from the gel edge.

The gel was electrophoresed in a running buffer containing:

Glycine	144g/l
Tris	30g/l
10% SDS	50ml/l

The gel was removed from the casting plates and fixed and stained in the following fixative/stain solution for 1 hour at room temperature on an orbital shaker, then destained with several changes of the following destain solution until the bands were clearly visible against the background. The destained gel was then prepared for fluorography as in the next section.

Fixative/stain solution

5 volumes methanol

1 volume acetic acid

5 volumes distilled water

0.1% Coomassie blue dye

Destain solution

7% acetic acid with 5% methanol in distilled water

3.2.7 Fluorography of SDS-PAGE gels

Destained gels were soaked in Amplify reagent (Amersham) for 30 minutes prior to drying the gel under vacuum using a Biorad Model 543 gel drier. The distance moved by each molecular weight marker was measured and its Rf-value calculated from the distance moved by the marker divided by the distance moved by the bromophenol blue dye. A graph of $\log_{10}$ of molecular weight against Rf-value was plotted and the molecular weights of the bands of interest were estimated from the graph. The dried gel was placed on a piece of pre-flashed Hyperfilm MP (Amersham) at -70°C for 3 to 7 days. The film was developed for 2 minutes in Kodak developer (LX-24) and fixed for 1 minute in Kodak fixative (FX-40).

3.2.8 Immunochemical detection of beta-lactams bound to PBPs

Log phase cells grown as described before (Section 3.2.4) were deposited by centrifugation in a Microfuge (MSE). The cells were resuspended in 20mM tris-HCl buffer pH 6.8 to an OD of 0.5 at 650nm. The beta-lactams were dissolved in the same buffer and added to the cells to a final concentration of 500μg/ml. Antibiotic-free controls (buffer only) were also set up. The cells were incubated at 37°C for 15 minutes then the reaction was terminated by the addition of SDS-PAGE sample buffer. The samples were prepared for electrophoresis on 10% SDS-PAG as before except that most experiments were carried out using an Atto mini-gel system. The conditions for mini-gel runs were 15mA constant current for the stacking gel and 20mA for the resolving gel. The gel was blotted onto nitrocellulose (Gelman Sciences) using a Hoeffer Wet-blot system at 66V for 2 hours. The transfer buffer

consisted of:

Tris	12.16g
Glycine	56.83g
20% methanol	1000ml
Distilled water to	5000ml
pH with HCl to 7.4	

The blot was stained for protein using Ponceau red before being incubated in 0.5% skimmed milk in blotting buffer overnight at room temperature.

Blotting buffer:

Sodium chloride	45.0g
Tris	6.0g
0.05% Tween	2.5ml
Distilled water to	5000ml
pH with HCl to 7.4	

Ponceau red stain:

2% Ponceau S in 30% trichloroacetic acid with 30% sulphosalicylic acid. This stock solution was diluted 1/10 in distilled water before use.

Dilutions of anti-beta-lactam antiserum (as specified in the Results section) in 0.5% skimmed milk in blotting buffer were incubated with the blot at room temperature for 1.5 hours. After 5 x 5 minute rinses in blotting buffer the blots were developed

in chloronaphthol substrate solution for 20 minutes at room temperature then the reaction was terminated by washing the blot in distilled water.

Chloronaphthol substrate solution:

1 x 30mg Chloronaphthol tablet (Sigma) dissolved in 10ml of ethanol and added to 40ml of sodium phosphate buffer, 66mM pH 7.4, containing 25μl of 30% hydrogen peroxide.

3.2.9 Methods for the isolation and purification of PBPs

3.2.9.1 Preparation of Affinity Chromatography Columns

About 15ml of ECH-Sepharose-4B (Pharmacia) was pipetted onto a double filter paper in a Buchner funnel. It was washed thoroughly with 0.5M sodium chloride (1 litre). The Sepharose was sucked dry and added to a small glass bottle containing 0.7 mmols of 6-amino-penicillanic acid (6-APA) in 10ml of distilled water at pH 4.5 (some of the 6-APA remained undissolved at this pH). N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) was dissolved in water at 10mg/ml and adjusted to pH 4.5. The EDC was added to the 6-APA/Sepharose mixture and mixed end over end at room temperature overnight. The 6-APA-Sepharose was then washed on a vacuum filter with distilled water and tested for bound 6-APA using the hydroxamate test as described below.

3.2.9.2 Hydroxamate test for beta-lactam bound to Sepharose

Reagents:

Neutralised hydroxylamine alcohol solution was prepared on

the day of the test by adding 1 volume of 5M hydroxylamine hydrochloride solution and 1 volume of alkali buffer solution (86.5g of sodium hydroxide and 10.3g of anhydrous sodium acetate in 500ml of distilled water) to 5 volumes of 95% ethanol.

Ferric ammonium sulphate in H_2SO_4 was prepared by dissolving 100g of ferric ammonium sulphate dodecahydrate and 46.7ml of concentrated sulphuric acid in distilled water to a final volume of 500ml.

Method:

1ml of the Sepharose was mixed with 3ml of neutralised alcohol hydroxylamine solution then after 3 minutes 1ml of the ferric ammonium sulphate in H_2SO_4 was added. The appearance of a red colour on the Sepharose indicated that the sepharose was conjugated with intact beta-lactam rings from the 6-APA.

3.2.9.3 Affinity purification of *Escherichia coli* PBPs.

The standard procedure used for affinity purification of PBPs was essentially a method which had been applied to the purification of *Escherichia coli* PBPs described by Curtis and Strominger (1981). In the present study the method was applied to membranes from *Escherichia coli* to confirm that it was being used correctly and then applied to *Neisseria gonorrhoeae*.

Reagents:

Solubilising buffer: 2% Triton X-100 (Pierce) and 1M sodium chloride in 100mM potassium phosphate pH 7.0.

Dilution buffer: 20mM potassium phosphate pH 7.0.

Washing buffers:

(i) 0.1% Triton-X100 and 1mM 2-mercaptoethanol in 50mM potassium phosphate pH 7.0.

(ii) As (a) with the addition of 0.1M sodium chloride.

(iii) As (a) with the addition of 2.0M sodium chloride.

Elution buffer: 0.4M neutralised hydroxylamine and 1% Triton-X100 in 50mM potassium phosphate pH 7.0.

Dialysis buffer: 1mM 2-mercaptoethanol and 0.1% Triton-X100 in 0.01M tris-HCl pH 7.5.

Ion-exchange buffers:

(i) 1% Triton-X100 in 0.01M tris-HCl pH 7.5.

(ii) 0.5M NaCl and 1% Triton-X100 in 0.05M tris-HCl pH 7.5.

Method:

Membrane protein adjusted to 12mg/ml was incubated with solubilising buffer, at the ratio of 20mg of protein to 1ml of solubilising buffer, at room temperature with gentle shaking for 30 minutes. The membrane/detergent mixture was centrifuged at 50,000g for 30 minutes to pellet insoluble protein. The supernatant containing crude solubilised PBPs was diluted with an equal volume of dilution buffer then mixed with the slurry of 6-APA-Sepharose for 30 minutes at room temperature with gentle agitation. Results of the PBP assays following this step suggested that all PBPs had attached to the 6-APA column within this time. The 6-APA Sepharose was then filtered with a vacuum and washed successively with 300ml each of the washing buffers (i), (ii) and (iii) to remove non-specifically-bound proteins. The non-adsorbed protein including the washings were saved for later assay to check for the presence of PBPs.

The washed 6-APA-Sepharose was poured into a small glass column. One column volume of the elution buffer was applied to the column and the equivalent volume of eluate collected. The elution buffer was left in contact with the sepharose for 30 minutes then a second column volume of elution buffer was applied to the column

and the equivalent volume of eluate collected. A third column volume of elution buffer was applied to the column and the equivalent volume of eluate collected. The three column volumes collected, containing the eluted protein, were pooled and dialysed at 4°C against 5 x 21 volumes of dialysis buffer to remove the hydroxylamine.

The protein solution was concentrated on a 0.5ml ion-exchange column as follows. Whatman DE-52 anion-exchange resin was washed in 10x concentrated ion-exchange buffer (i) and the pH of the resin adjusted back to the starting pH of 7. A small column of 0.5ml of resin was made in a glass pasteur pipette stopped with a plug of glass wool. The resin was equilibrated with ion-exchange buffer (i) then the dialysed protein solution was slowly dripped through the column. Any bound protein was eluted with the higher ionic strength ion-exchange buffer (ii).

Samples taken at each stage of the purification procedure were assayed for PBPs by fluorography using tritiated penicillin and for protein using a modified Lowry protein assay as described below.

3.2.9.4 Modified Lowry protein assay for samples containing detergent

This modification was based on a method described by Clark (1984). 1 volume of 4% sodium potassium tartrate solution and 1 volume of 2% copper sulphate solution were added to 20 volumes of 2% sodium carbonate in 0.1M sodium hydroxide solution to give

'reagent A'. Folin-Ciocalteu reagent (BDH) was diluted 1:3 with distilled water just before use. The bovine serum albumin standard solution of 500μg/ml was made up in a detergent buffer solution of 1% Triton-X100 in 50mM potassium phosphate pH 7.4.

Protein samples and BSA standard solutions of 0-100 μg/ml were diluted to a final volume of 200μl with the detergent buffer solution. 1ml of reagent A was added to each and the samples and standards were incubated at room temperature for 10 minutes. 1ml of 10% SDS in phosphate buffer pH 7.4 was added and mixed, followed by 100μl of diluted Folin-Ciocalteu reagent. The samples were mixed immediately and incubated at room temperature for 30 minutes then the absorbance at 700nm was measured. Samples and standards were assayed in duplicate the protein concentrations of the samples were estimated from a standard curve of absorbance plotted against protein concentration.

3.2.9.5 Solubilisation of *Neisseria gonorrhoeae* PBPs from membranes

Early experiments, using the procedure described above, showed that PBPs of *Neisseria gonorrhoeae* were not solubilised by the standard solubilising buffer used for *Escherichia coli* PBPs. The following experiment was performed to determine the optimum conditions for the solubilisation of *Neisseria gonorrhoeae* PBPs.

Membrane protein from a sensitive strain was adjusted to a concentration of 20mg/ml in 20mM potassium phosphate at pH 7.0. A solubilising buffer containing 5% Triton-X100 and 1M sodium

chloride in 100mM potassium phosphate at pH 7.0 was used. Four different volumes of the stock 5% detergent solution were tested, each against 1mg of membrane protein in a volume of 50μl of 100mM phosphate buffer pH 7.2. Each concentration was incubated at room temperature for 1 hour then adjusted to the same volume for centrifugation at 50,000g for 30 minutes to pellet the insoluble material. 50μl of each supernatant was assayed for PBPs by fluorography. The corresponding pellet from each sample was resuspended in 20mM potassium phosphate pH 7.0 and also assayed for PBPs by fluorography.

3.2.9.6 Elution of *Neisseria gonorrhoeae* PBPs from affinity columns

Experiments proved that the PBPs from *Neisseria gonorrhoeae* could not be eluted from the affinity column using the procedures described in Section 3.2.9.4. Consequently several variations of the elution procedure were tried:

(i) 5M hydroxylamine in 1% Triton X-100 in 50mM phosphate buffer pH 7.0 for 30 minutes at room temperature.

(ii) 3% hydrogen peroxide in 1% Triton X-100 in 50mM phosphate buffer pH 7.0 for 30 minutes at room temperature.

(iii) 1M ethylamine in 1% Triton X-100 in 50mM phosphate buffer pH 7.0 for 30 minutes at room temperature.

(iv) 1M hydroxylamine in 1% Triton X-100 in 50mM tris-HCl buffer pH 8.8 for 30 minutes at room temperature.

(v) Boiling in dialysis buffer for 10 minutes.

3.3 RESULTS AND DISCUSSION

3.3.1 Characterisation and antibiotic susceptibility

All the strains used were characterised as Gram negative diplococci which were oxidase positive and produced acid from glucose but not the other sugars tested. These results confirmed that the organisms were *Neisseria gonorrhoeae*.

Table 3.1 is a list of the strains tested showing the minimum inhibitory concentrations, in μg/ml, of benzyl penicillin and methicillin against each strain. The results for the nitrocefin test for beta-lactamase are also shown, together with a list of the plasmids present (information on the plasmid content was supplied by the Gonococcal Reference Laboratory, PHL, Bristol who supplied the original cultures). The strains are listed in order of sensitivity to benzyl penicillin, the most sensitive first.

Sensitivities to benzyl penicillin ranging from 0.01μg/ml to >10μg/ml were observed in the 26 strains of *Neisseria gonorrhoeae* tested. The most sensitive strains that have been reported (Dowson *et al* 1989) had an MIC of 0.004μg/ml with benzyl penicillin, 2.5 times more sensitive than the most sensitive strains tested in the present study. However, Dowson *et al*. suggested that strains with MICs between 0.004μg/ml and 0.03μg/ml approximately with benzyl penicillin were truly sensitive while strains with MICs of greater than 0.03μg/ml approximately had increasing levels of resistance. Of the strains in Table 3.1 the 9 strains with MICs of benzyl penicillin of 0.04μg/ml were considered to be sensitive as they were very close to this

Table 3.1: Benzyl penicillin (pen) and methicillin (meth) sensitivities of the strains used. Beta-lactamase was determined by the nitrocefin reaction. The plasmids were determined by the Gonococcal Reference Laboratory, Bristol.

Strain	MIC (μg/ml)		Beta-lactamase	Plasmids (MDa)
	Pen	Meth		
9372	0.01	0.04	-	-
9929	0.01	0.10	-	-
V	0.01	0.10	-	-
I	0.04	0.10	-	-
9941	0.04	0.04	-	-
9542	0.04	0.10	-	-
9880	0.04	0.10	-	-
W	0.04	0.10	-	-
X	0.04	0.20	-	-
Y	0.04	0.10	-	-
Z	0.04	0.20	-	-
9876	0.04	1.00	-	-
9881	0.20	2.50	-	-
9924	0.50	10.00	-	-
9487	1.00	>10.00	-	-
9569	1.00	>10.00	-	-
9631	1.00	>10.00	-	-
95	5.00	>10.00	-	-
9549	5.00	>10.00	-	-
9621	5.00	2.50	-	-
9744	5.00	10.00	-	-
13	>10.00	>10.00	+	2.6, 3.2, 24.5
5921	>10.00	>10.00	+	2.6, 3.2, 24.5
5907	>10.00	>10.00	+	2.6, 3.2
5982	>10.00	>10.00	+	2.6, 3.2
5909	>10.00	>10.00	+	2.6, 4.4, 24.5

0.03μg/ml limit and the difference was considered to be within the limits of experimental error for the type of MIC determination used. Stepwise increases in resistance to benzyl penicillin have been achieved experimentally by Dougherty *et al.* (1980) who prepared a set of isogenic transformants sensitive to MICs of 0.007μg/ml to 2μg/ml.

The definition of resistance to penicillin is not simple. Clinicians treating cases of gonorrhoea have three broad groups of sensitivity which they routinely use as criteria for determining treatment with benzyl penicillin. They consider isolates with MICs of 0.004μg/ml to 0.2μg/ml to be clinically sensitive, those isolates with MICs of 0.2μg/ml to 0.5μg/ml to be intermediate in sensitivity, and those isolates with MICs above 0.5μg/ml to be clinically resistant. The strains shown in Table 3.1 illustrate the whole range of sensitivities to benzyl penicillin. Five of the strains tested contained a plasmid-mediated beta-lactamase which rendered them totally resistant to benzyl penicillin (MIC >10μg/ml). The beta-lactamase-producing strains tested contained various plasmids as shown in Table 3.1 (information supplied by the Gonococcal Reference Laboratory, Bristol). All five strains contained a 2.6MDa cryptic plasmid which has no known phenotype. These five also contained either the 3.2 or the 4.4MDa plasmid both of which encode a TEM beta-lactamase. Three of the strains also contained the 24.5MDa conjugative plasmid which mobilises itself and the smaller beta-lactamase plasmids between strains of *Neisseria gonorrhoeae*.

With one exception all the strains were less sensitive to methicillin than to benzyl penicillin. Methicillin was the first beta-lactamase-stable semi-synthetic penicillin derived from the 6-amino-penicillanic acid nucleus. All the beta-lactamase-producing strains had low sensitivity to methicillin although all were sensitive to concentrations between 10μg/ml and 50μg/ml. Three strains representing penicillin sensitivity (strain I), chromosomally-mediated resistance (strain 95) and resistance due to beta-lactamase production (strain 13) were selected for the major investigations described in this chapter. These strains were chosen from the first set of cultures obtained from the Gonococcal Reference Laboratory, Bristol and from the Wellcome Foundation Ltd. The other strains were obtained later and tested for penicillin susceptibility as for the three chosen strains, but these other strains were then used only for Western blot analysis of PBPs.

3.3.2 Fluorography assays on PBPs from whole cells and from membranes

3.3.2.1 Determination of molecular weights of PBPs from fluorographs

The apparent molecular weights of the *Neisseria gonorrhoeae* PBPs in each experiment were determined using the Rainbow markers on the dried gel which was subsequently used to expose the film for fluorography. Figure 3.1 is a typical graph showing the log of the molecular weights of the standard proteins in the Rainbow markers mixture plotted against their Rf values following SDS-PAGE. The graph of the $\log_{10}$ of the molecular weights of the

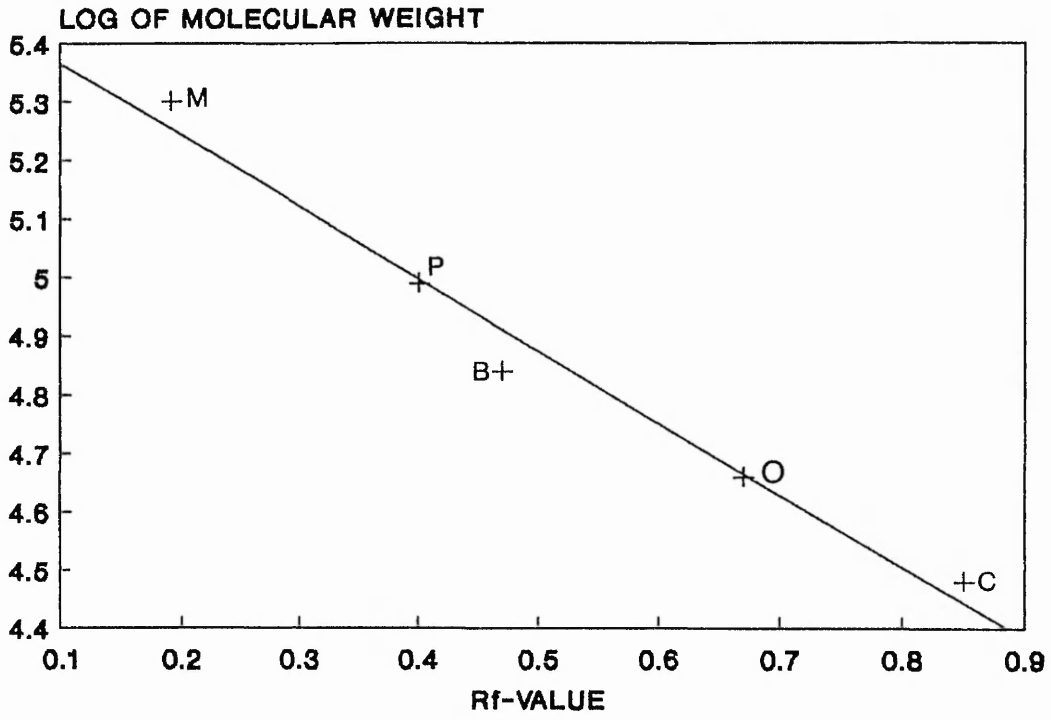


Figure 3.1: Log of the molecular weight of the protein standards (Amersham Rainbow markers) against Rf value following 10% acrylamide SDS-PAGE. The Rf value was calculated from the distance moved by the protein divided by the distance moved by the marker dye front.

Key: M (Myosin), P (Phosphorylase b), B (BSA), O (Ovalbumin) and C (Carbonic anhydrase).

standards against Rf values was plotted as explained in the legend to Figure 3.1. The developed fluorograph film was laid on top of the dried gel in exactly the same orientation and position as it was during development. The bands on the developed fluorograph were lined up with the bands on the dried gel and their Rf values calculated as described in the legend to Figure 3.1. The molecular weights were then determined from Figure 3.1. Only five of the seven protein standards were resolved on a 10% acrylamide gel.

3.3.2.2 PBPs in cells of a penicillin-sensitive strain

Cells of strain I with an MIC of 0.04μg/ml with benzyl penicillin were tested by fluorography using concentrations of 1.1, 2, and 3 μg/ml of tritiated penicillin. Figure 3.2 shows that three PBPs were present with apparent molecular weights of 82,000 (PBP1), 59,000 (PBP2) and 46,000 (PBP3) which compare favourably with the molecular weights reported by Dougherty *et al* (1980) and Barbour (1981). The labelling of the three PBPs increased with increasing amounts of tritiated penicillin and PBP3 gave the densest band at each concentration suggesting that it bound the most penicillin of the three PBPs. The affinity for penicillin appeared to be PBP3 > PBP1 > PBP2.

3.3.2.3 PBPs in cells of an intrinsically-resistant strain

Cells of the same sensitive strain I and the intrinsically-resistant strain 95 (penicillin MIC 5μg/ml) were exposed to 2.4μg/ml of tritiated penicillin. The fluorograph from this experiment is shown in Figure 3.3. Little, if any, penicillin

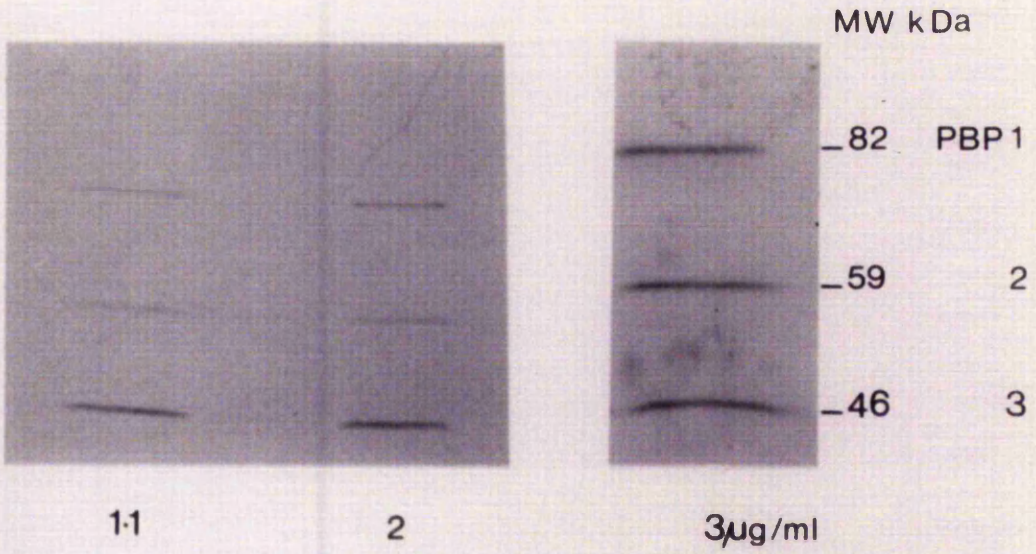


Figure 3.2: Fluorograph of membrane proteins from whole cells of the sensitive strain I exposed to tritiated penicillin at concentrations of 1.1, 2, and 3µg/ml as indicated. Labels 1, 2 and 3 mark the positions of bands thought to be PBP1, PBP2 and PBP3.

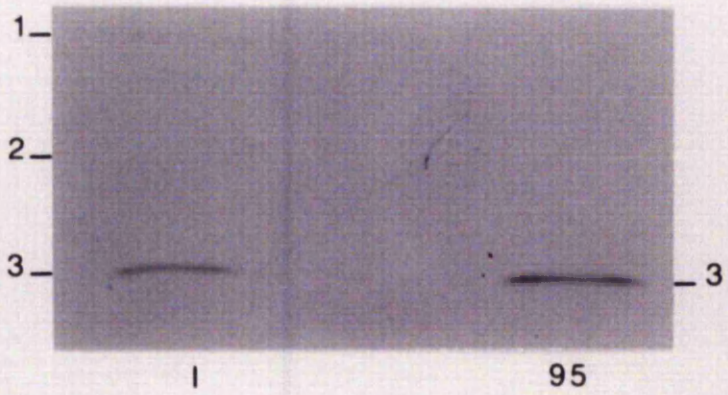


Figure 3.3: Fluorograph of membrane proteins from whole cells of sensitive strain I and intrinsically-resistant strain 95 exposed to 2.4μg/ml tritiated penicillin for 15 minutes at 37°C. Labels 1, 2 and 3 mark the positions of PBP1, PBP2 and PBP3.

bound to PBP1 and PBP2 of the resistant strain compared with the good binding to these PBPs in the sensitive strain. These results support the hypothesis that PBP1 and PBP2 in intrinsically-resistant strains have a decreased affinity for penicillin due to mutations in the genes encoding these proteins (Dowson *et al.* 1989). Conversely more penicillin bound to the PBP3 of the resistant strain than to the corresponding protein of the sensitive strain. The reason for this is not known.

3.3.2.4 PBPs in cells and membranes of a beta-lactamase-producing strain

Cells of the beta-lactamase-producing strain 13 (penicillin MIC >10μg/ml) were exposed to 3μg/ml of tritiated penicillin in whole-cell and membrane preparations and the resulting fluorographs are shown in Figure 3.4. The whole-cell preparation shows only PBP3 labelled at this concentration of penicillin compared with all three PBPs labelled in the membrane preparation. The whole-cell and membrane preparations for strain 13 were tested for beta-lactamase activity by the nitrocefin test and only the whole-cell preparation gave a positive result. It would be expected that the beta-lactamase would be lost from the membrane preparations since it is in the periplasmic space (Richmond and Sykes, 1973) However it would be trapped in the periplasmic space in whole-cell preparations where it would still be accessible to the beta-lactams, as the outer membrane does not present a permeability barrier to these molecules (Sykes and Percival 1978). It is possible that the beta-lactamase trapped in the periplasmic spaces in the whole-cell preparation may have

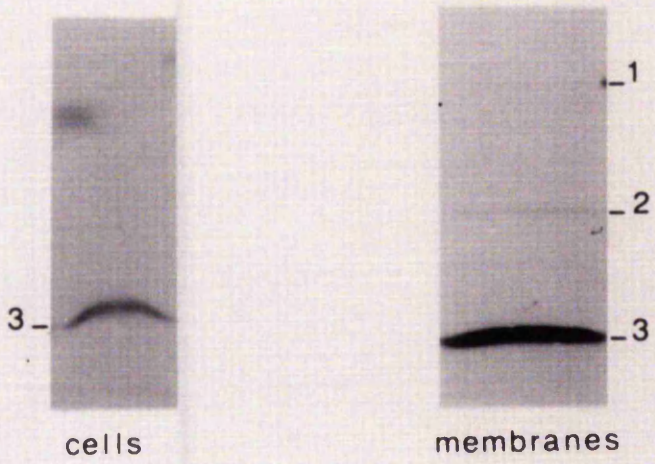


Figure 3.4: Fluorograph of membrane proteins of beta-lactamase-producing strain 13 from whole cells and from isolated membranes exposed to 3μg/ml tritiated penicillin for 15 minutes at 37°C. Labels 1, 2 and 3 mark the positions of PBP1, PBP2 and PBP3.

hydrolysed the tritiated penicillin during exposure and this would explain the different fluorograph patterns of whole-cell and membrane preparations in the beta-lactamase-producing strain. Thus a negative nitrocefin test, indicating a lack of beta-lactamase activity, would suggest that little or no hydrolysis of the penicillin would occur allowing the PBPs to bind the antibiotic.

The high degree of binding of penicillin to PBP3 in the whole cells and in the membranes may be due to PBP3 having a higher affinity for penicillin than the other PBPs. Dougherty *et al* (1980) and Barbour (1981) both observed that PBP3 was saturated with penicillin first of the three PBPs although binding of penicillin to this protein seems to have little or no effect on peptidoglycan synthesis.

3.3.2.5 PBPs in membranes of sensitive and resistant strains

Figure 3.5 shows a fluorograph of membranes of sensitive strain I and intrinsically-resistant strain 95 exposed to 1.1 and 3 μg/ml of tritiated penicillin. On exposure to 1.1μg/ml of penicillin less penicillin bound to PBP1 and PBP2 in the resistant strain 95 compared with the same proteins in the sensitive strain I. At the higher penicillin concentration level more penicillin bound to PBP1 and PBP2 in the resistant strain 95 than at the lower penicillin concentrations (the PBP1 band in strain 95 exposed to 3μg/ml is not clearly visible in the photograph but it was clearly visible in the original fluorograph). As in the whole-cell preparations the reduction in

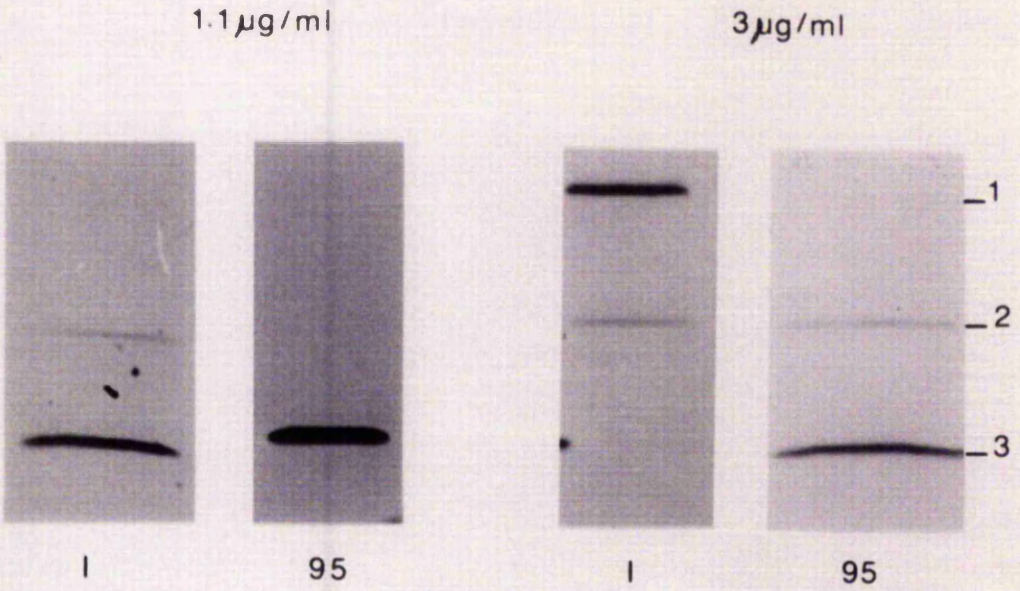


Figure 3.5: Fluorograph of membrane proteins from isolated membranes of the sensitive strain I and the resistant strain 95 exposed to 1.1 and 3µg/ml of tritiated penicillin. Labels 1, 2 and 3 mark the positions of PBP1, PBP2 and PBP3.

penicillin binding to PBP1 and PBP2 in strain 95 may be explained by mutations in the genes encoding them (Dowson *et al.* 1989).

The results in Figure 3.5 suggest that PBP3 is not involved in resistance to penicillin since, from the band intensity at 1.1μg/ml, PBP3 appears more strongly labelled in the resistant strain compared with the sensitive strain. Such a phenomenon was observed by Dougherty *et al.* (1980) who suggested that it may be due to a cellular increase in the amount of PBP3 in resistant strains. However, they did not observe a corresponding increase *in vivo* which they suggested may be due to a change in the permeability of the resistant bacteria. This was in contrast to the observations made in the present study where the labelling of PBP3 in whole cells as well as in membranes was usually higher in the resistant strains than the sensitive strains as shown in Figure 3.3.

In Figure 3.5 the very low intensity band for PBP3 in strain I at 3μg/ml is an atypical result and cannot be easily explained. It suggests that this PBP3 preparation has not bound the tritiated penicillin or, if it has, it has subsequently been lost before preparation of the fluorograph.

3.3.2.6 Competitive inhibition of PBPs with beta-lactams other than benzyl penicillin

Penicillin-binding protein assays with tritiated penicillin were also done in the presence of non-tritiated penicillin, methicillin, and the beta-lactamase inhibitor clavulanic acid to

see if they interfered with the binding of the tritiated penicillin. The conditions for the preincubation with non labelled competitors are described in the legends of Figures 3.6 and 3.7.

Figure 3.6 shows a fluorograph of a whole-cell preparation of the sensitive strain I showing the three PBPs clearly labelled. However, preincubation of the cells with non-tritiated penicillin or with methicillin in excess shows that subsequent binding of the tritiated penicillin is considerably reduced. The non-tritiated penicillin has totally inhibited the binding of tritiated penicillin to all the PBPs in this strain suggesting that they were completely saturated with the non-tritiated penicillin. However, the sample preincubated with methicillin shows two very weak bands in the positions of PBP1 and PBP3 (barely visible in the photograph but more obvious in the original fluorograph). This may suggest that these two PBPs are not saturated with methicillin under the conditions used. The MIC of methicillin against this strain was 2.5 times higher than that of penicillin which correlates with this result.

Figure 3.7 shows a fluorograph of whole cells of the sensitive strain I which shows the effect of non-radiolabelled clavulanic acid on binding of tritiated penicillin to the PBPs. In lane (a), which was loaded with a sample from cells treated with tritiated penicillin but with no other treatment, three PBPs are visible due to binding of tritiated penicillin (although the bands are rather weak on the photograph). In lane (b), which was loaded

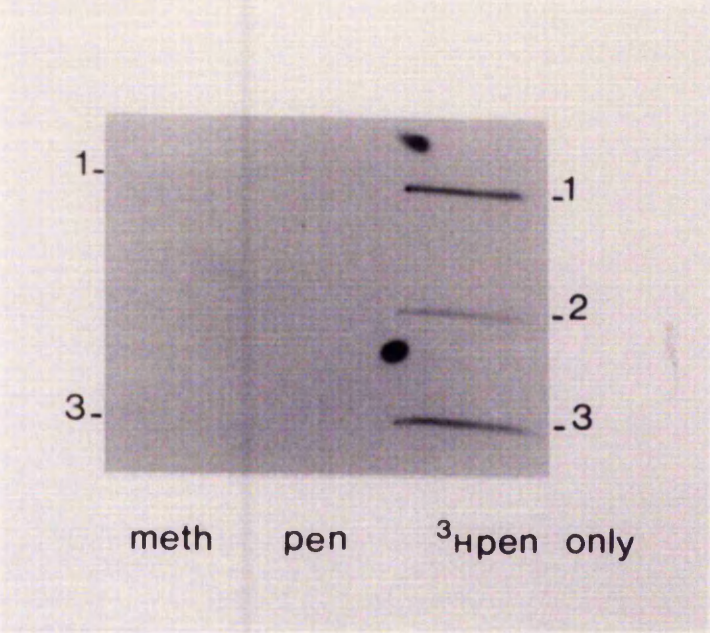


Figure 3.6: Fluorograph of membrane proteins from whole cells of sensitive strain I:

(a) exposed to 60μg/ml of non-radiolabelled methicillin for 15 minutes at 37°C prior to exposure to 1.2μg/ml of tritiated penicillin for 15 minutes at 37°C,

(b) exposed to 60μg/ml of non-radiolabelled penicillin for 15 minutes at 37°C prior to exposure to 1.2μg/ml of tritiated penicillin for 15 minutes at 37°C,

(c) exposed to 1.2μg/ml of tritiated penicillin alone for 15 minutes at 37°C.

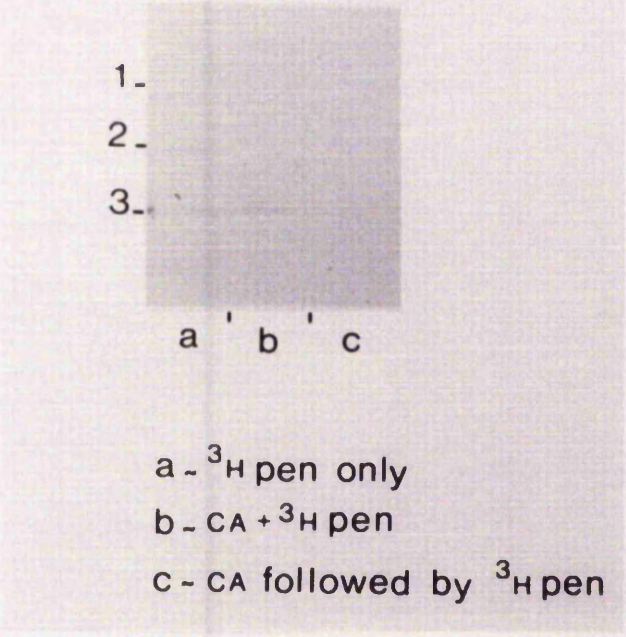


Figure 3.7: Fluorograph of membrane proteins from whole cells of sensitive strain I:

(a) exposed to 1.2μg/ml of tritiated penicillin for 15 minutes at 37°C,

(b) exposed to 40μg/ml of clavulanic acid and 1.2μg/ml of tritiated penicillin simultaneously for 15 minutes at 37°C,

(c) exposed to 40μg/ml of clavulanic acid for 15 minutes at 37°C followed by exposure to 1.2μg/ml of tritiated penicillin for 15 minutes at 37°C.

with a sample incubated with tritiated penicillin and non-radiolabelled clavulanic acid simultaneously, only a weak PBP3 band is visible. This suggests that the clavulanic acid may have competed successfully with the tritiated penicillin for the binding site on PBP1 and PBP2. In lane (c), which was loaded with a sample from cells preincubated with clavulanic acid before addition of tritiated penicillin, no radio-labelled PBP bands are visible at all. Again this is likely to be due to the clavulanic acid binding to the PBPs preventing the tritiated penicillin from binding. Clavulanic acid has potent antimicrobial activity against *Neisseria gonorrhoeae* (Miller 1978, Neu and Kweung 1978, van Klingeren and van Wijnagaarden 1981). This antimicrobial action is most likely due to inhibition of PBPs as with other beta-lactam antibiotics. Clavulanic acid binds most readily to PBP2 in *Escherichia coli*.

These results prove that the radioactive bands in the fluorograph are due to binding of tritiated penicillin to the proteins . Also that the binding site is not specific to benzyl penicillin as it will also bind methicillin and clavulanic acid.

3.3.2.7 Conclusions

PBP assays using tritiated penicillin in both membrane and whole cell preparations have been done on strain I (fully sensitive); strain 13 (beta-lactamase producer); strain 95 (intrinsically resistant). These have demonstrated differences in the PBP profiles between the three strains which were correlated with the MIC of penicillin for each strain. Methicillin was shown to compete with benzyl penicillin for the binding to PBPs.

3.3.3 Western blots of penicillin-binding proteins labelled with beta-lactams and probed with anti-beta-lactam conjugate antisera.

3.3.3.1 Anti-penicillin antisera

An experiment with membrane proteins from the sensitive strain I and the intrinsically resistant strain 95, incubated with or without penicillin and probed with anti-penicillin antiserum at a 1/50 dilution, was performed and the results are shown in Figure 3.8. The immuno-stained bands in each lane were of the same intensity whether penicillin was present or not. It was thought that the bands may have been non-specifically stained by the antibodies. When the antiserum was preincubated with gonococcal membrane protein, to absorb out any anti-gonococcal-membrane antibodies, and then used to probe the blotted proteins fewer bands were stained in the lower molecular weight regions (results not shown). This suggests that antibodies were present in the antiserum which could cross-react with gonococcal membrane proteins. However, no specific PBP-penicillin bands were stained. Other rabbit bleeds were tested but gave similar results. Further investigation using different concentrations of antisera and penicillin produced no improvement. Whole-cell blots were also tried to no avail.

One reason why the antibodies failed to recognise penicillin bound to the PBPs is that once bound the penicillin may be inaccessible to the antibodies. This is unlikely since the penicilloyl-PBP complex is denatured in detergent and 2-mercaptoethanol prior to blotting. Another explanation could be that the bound penicillin is changed such that the antibodies no

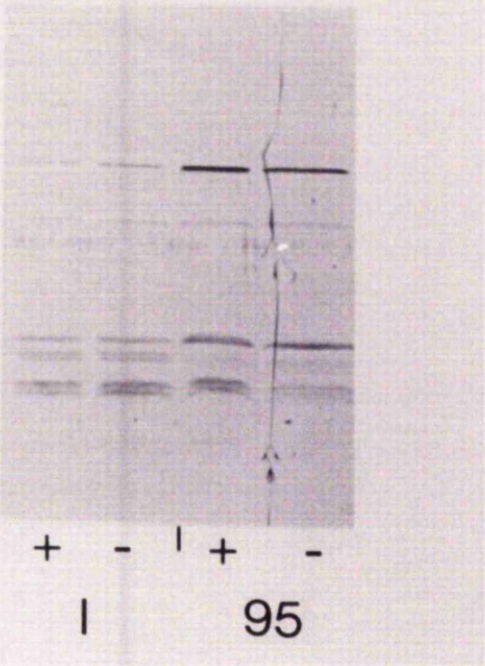


Figure 3.8: Western blot of membrane proteins from cells of the sensitive strain I and the intrinsically-resistant strain 95 incubated with **(+)** or without **(-)** 500μg/ml benzyl penicillin and probed with a 1/50 dilution of an anti-BSA-penicillin antiserum.

longer recognise it and therefore cannot bind to it. This might occur if the antibodies were directed against the penicilloyl bond to the BSA carrier as this may have a different epitope structure to that of the penicilloyl bond to the PBP. Also the avidity of the penicillin-specific antibodies was found to be very low (Chapter 2).

3.3.3.2 Antimethicillin antisera

More success was obtained with the anti-methicillin antiserum. Figure 3.9a shows a Western blot of extracts of whole cells of sensitive strain I incubated with and without methicillin and probed with anti-BSA-methicillin antiserum at dilutions of 1/10, 1/100, and 1/1000. Two bands, in the correct positions for PBP1 and PBP2, of molecular weights 81,850 and 59150 respectively, were labelled when the cells were incubated with methicillin. These bands did not appear when the cells were not incubated with methicillin nor when methicillin-treated cells were probed with non-immune pre-bleed serum. The possible PBP1 band is clearly labelled even down to a dilution of antiserum of 1/1000 and the possible PBP2 band is visible down to a dilution of antiserum of 1/100. No band was labelled specifically in the position of PBP3. This may possibly be due to an inability of anti-methicillin antibodies to bind to methicillin when it is bound to PBP3, or alternatively the PBP3 band may be masked by non-specific antibody-binding to other proteins on either side of it on the blot obscuring the specific binding. There is a considerable amount of non-specific binding of the pre-bleed and test-bleed sera to various membrane proteins on the blots making it

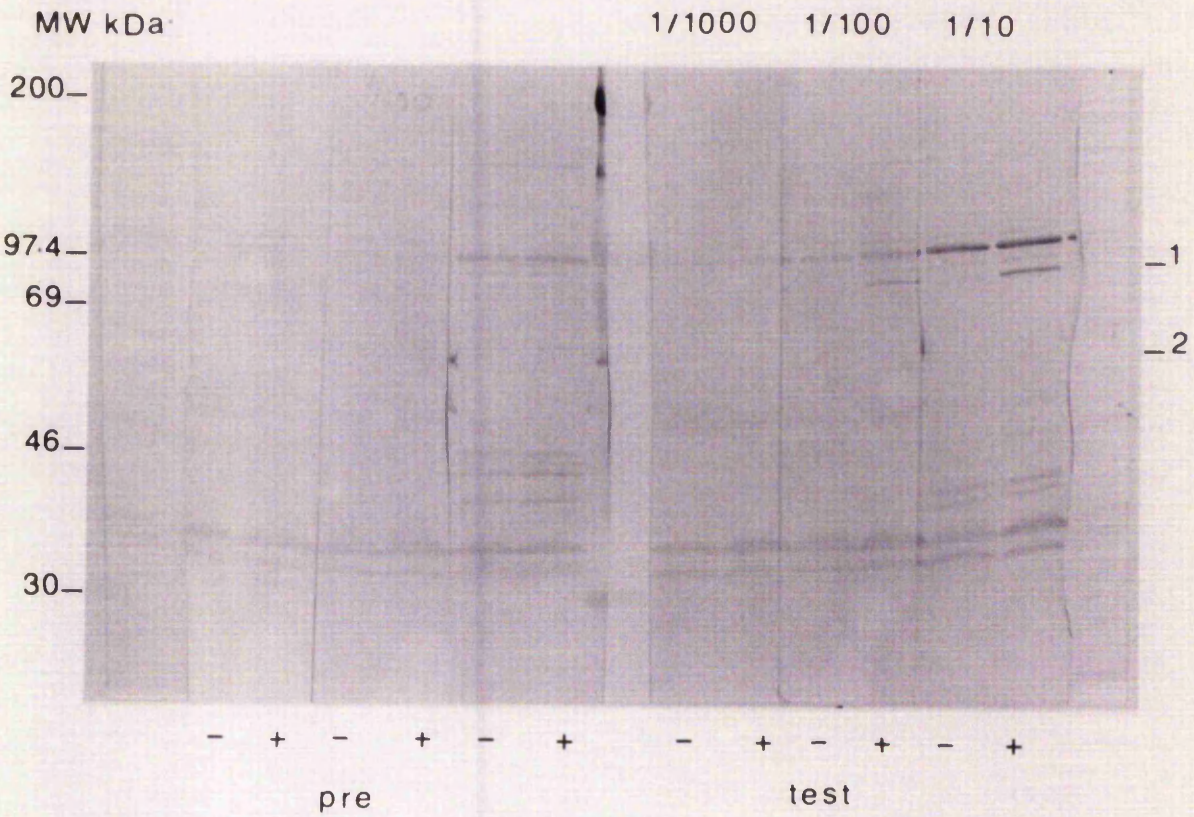


Figure 3.9a: Western blot of proteins from cells of the sensitive strain I incubated with **(+)** or without **(-)** 500µg/ml methicillin and probed with dilutions of 1/10, 1/100, and 1/1000 of anti-BSA-methicillin antiserum or the same dilutions of the corresponding pre-bleed serum. Labels 1 and 2 mark the expected positions of PBP1 and PBP2 located by their molecular weights.

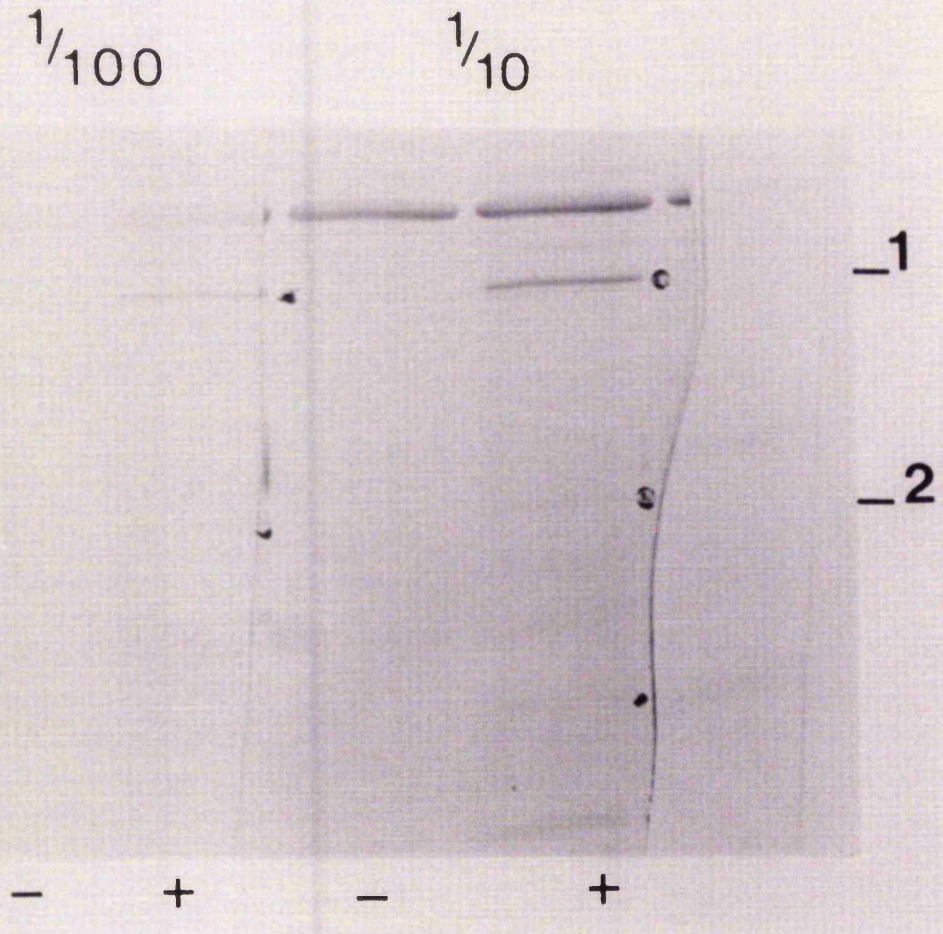


Figure 3.9b: A photographic enlargement of part of the blot shown in Figure 3.9a showing more detail of the lanes probed with the 1/10 and 1/100 dilutions of the anti-BSA-methicillin antiserum. The positions of two specifically-stained bands corresponding to molecular weights of (1) 81850 and (2) 59150 are indicated.

difficult to see the specific bands clearly. Figure 3.9b is a photographic enlargement of the part of Figure 3.9a showing the lanes probed with the 1/10 and 1/100 dilutions of the antiserum. The possible PBP1 band is located next to at least two other proteins which are also weakly stained by the antiserum in both the methicillin-labelled cells and the non-methicillin-labelled cells. Various experiments were tried to clarify the specific staining of the possible PBP bands including using a higher concentration of methicillin to label the cells, and using purified IgG from the anti-methicillin antiserum, but all failed to clarify the specific staining. It was possible that the rabbit antiserum used contained antibodies to commensal neisserias which cross-reacted with the membrane proteins in *Neisseria gonorrhoeae* but the use of antiserum absorbed with *Neisseria gonorrhoeae* membranes failed to remove the non-specific binding. It is also interesting to note that the anti-methicillin antiserum stained specific bands while the anti-penicillin antiserum did not (data not shown). This may be due to the fact that it had a higher concentration of antibodies, these may have had a higher affinity for the beta-lactams (as the results in Chapter 2 indicated).

Figure 3.10 is a blot of three strains of differing sensitivity, the sensitive strain I, the intrinsically resistant strain 95 and the beta-lactamase-producing strain 13, labelled with methicillin (as whole cells) and probed with a 1/100 dilution of the anti-BSA-methicillin antiserum. The corresponding pre-bleed serum was also used as a control. Bands in the correct positions for PBP1 and PBP2 are clearly labelled in the sensitive strain I and the

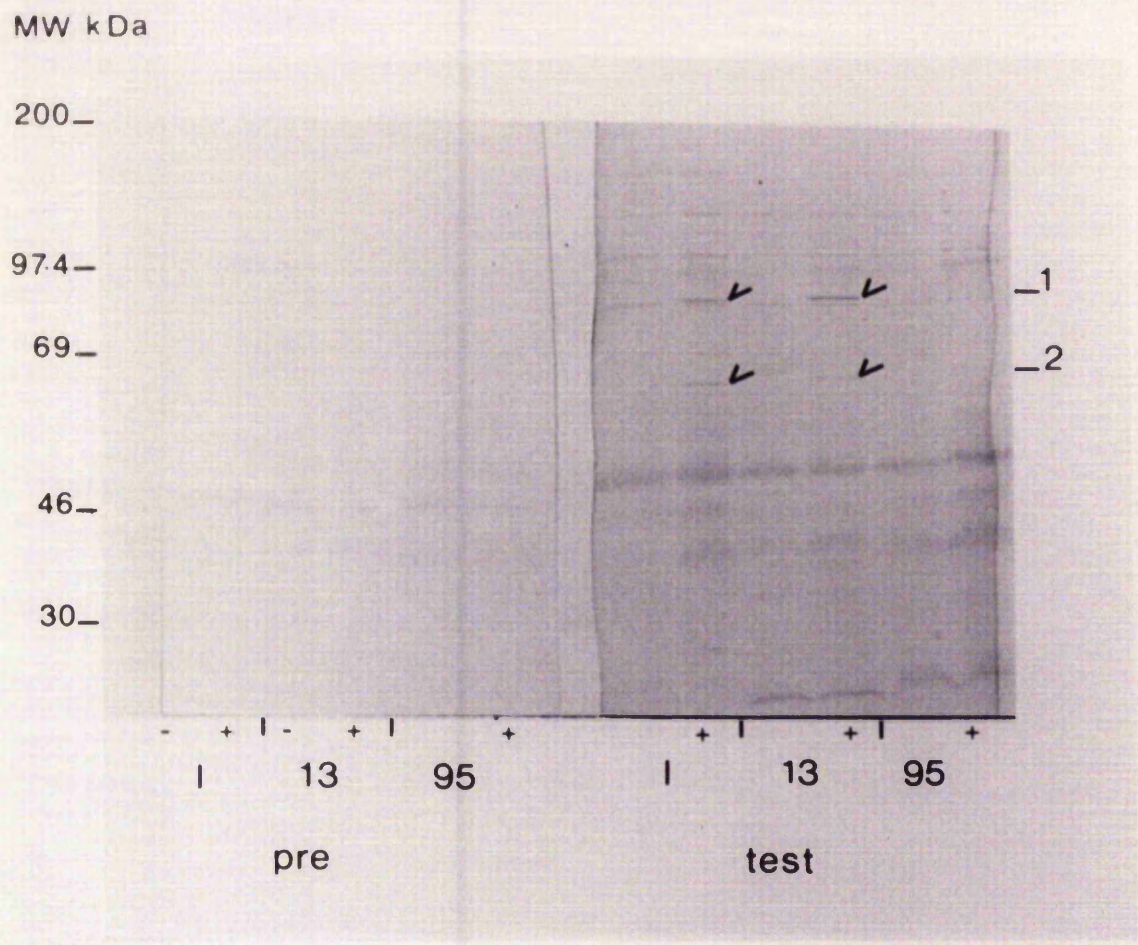


Figure 3.10: Western blot of the proteins from cells of sensitive strain I, beta-lactamase-producing strain 13, and intrinsically-resistant strain 95 incubated with **(+)** or without **(-)** 500μg/ml methicillin and probed with a 1/100 dilution of anti-BSA-methicillin antiserum or the corresponding prebleed serum. The arrows and the labels 1 and 2 mark the position of specifically-stained bands believed to be PBP1 and PBP2 respectively.

beta-lactamase-producing strain 13. These bands are not clearly visible in the intrinsically-resistant strain 95 (although a weak PBP2 band is present) which might be explained by the decreased affinity for beta-lactams of the PBPs of this type of chromosomally-resistant strain due to mutations in the genes encoding the PBPs (Dowson *et al.* 1989). Thus the specific-staining of the bands on the blots correlates well with the known sensitivity in the case of the sensitive strain I and the intrinsically-resistant strain 95. In the case of the beta-lactamase-producing strain 13 the bands believed to be PBP1 and PBP2 are also clearly stained in the blot but this strain is known to be totally resistant to benzyl penicillin and sensitive only to high concentrations of methicillin. This result is in contrast to the fluorography of whole cells of strain 13 with tritiated-penicillin where PBP1 and PBP2 were not labelled, apparently due to the beta-lactamase activity. In the Western blots methicillin was used which would not be hydrolysed by the beta-lactamase to the same extent as benzyl-penicillin. It is also interesting to note that the beta-lactamase producing strain 13 has PBPs which bind methicillin in the same ways as those of non beta-lactamase producing sensitive strains. This confirms the idea that the resistance of strain 13 is not due to a change in the PBPs but probably due to beta-lactamase production.

The specificity of the anti-BSA-methicillin antiserum for different beta-lactams was investigated by labelling the same three strains as above with methicillin, benzyl penicillin, ampicillin, cloxacillin and cephalexin at (each at 500μg/ml) and

probing with the anti-BSA-methicillin antiserum.

Figures 3.11 and 3.12 show the results. A high degree of non-specific binding of the antibodies caused some difficulty in interpreting the results but Figure 3.11 shows that in the sensitive strain I there are specific bands at the right positions for PBP1 and PBP2 which stain more densely in the methicillin-treated cells than in the non-treated cells. The same two bands appear in the beta-lactamase-producing strain 13. No equivalent specific bands can be seen in either of these strains labelled with either benzyl penicillin or with ampicillin.

Figure 3.12 shows that the sensitive strain I again has two bands in the correct positions for PBP1 and PBP2 specifically stained in the methicillin-treated cells compared with the non-treated cells. Similarly the beta-lactamase-producing strain 13 has the same two bands although the specificity of the reaction in this blot is not as clear as in the previous one. This blot also shows that the intrinsically-resistant strain 95 labelled with methicillin has no specifically-stained band in the right position for PBP1 but there is a specifically-stained band in the right position for PBP2, a result which is at variance with the previous result for this strain (Figure 3.10) which showed a very weak PBP2 band. The reason for this is not known.
Figure 3.12 also shows the results for all three strains labelled with cloxacillin (a penicillin) and cephalexin (a cephalosporin). This shows that a specific band has been stained in the right position for PBP1 in the sensitive strain I and the beta-

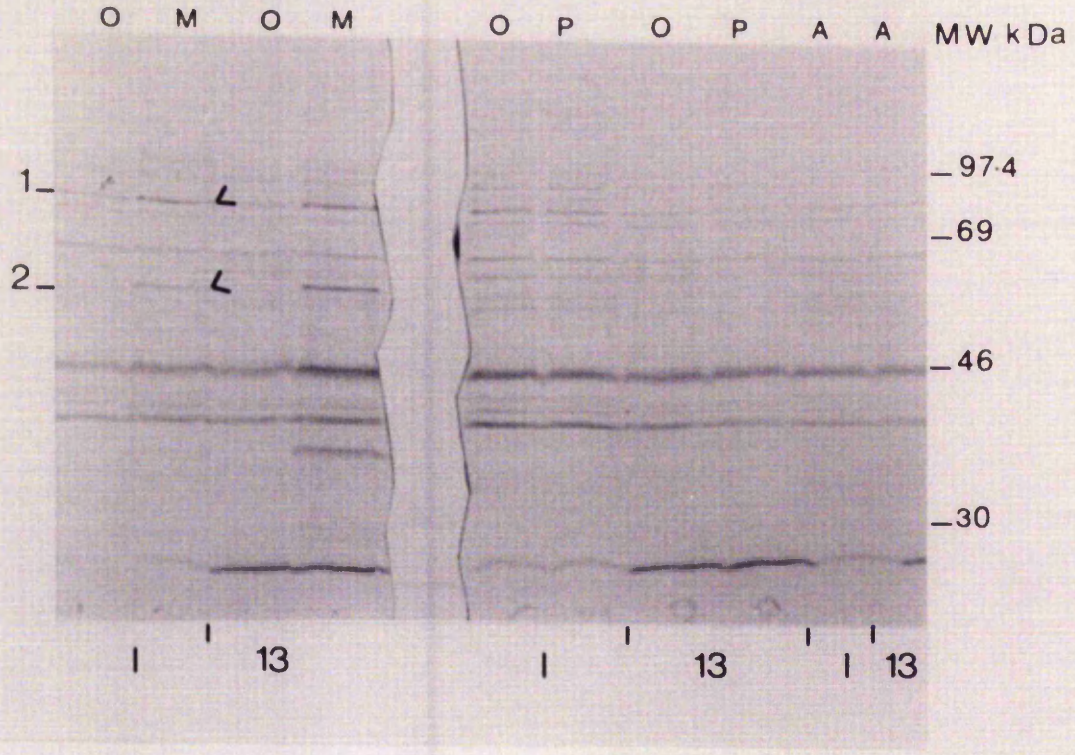


Figure 3.11: Western blot of the membrane proteins from cells of sensitive strain I and beta-lactamase-producing strain 13 incubated with 500μg/ml of methicillin **(M)**, benzyl penicillin **(P)**, ampicillin **(A)**, or no antibiotic **(O)** and probed with a 1/100 dilution of anti-BSA-methicillin antiserum. The arrows and the labels 1 and 2 indicate the positions of specifically-stained bands believed to be PBP1 and PBP2 respectively.

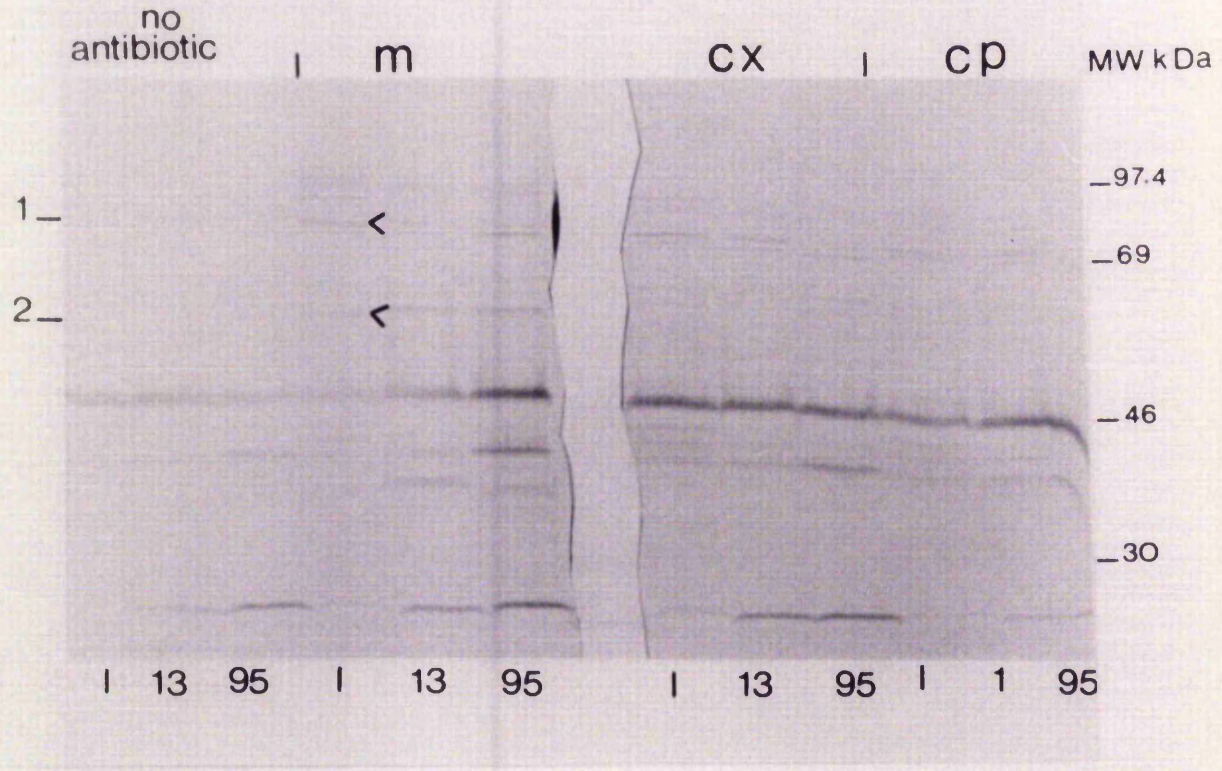


Figure 3.12: Western blot of membrane proteins from cells of the sensitive strain I, the beta-lactamase-producing strain 13 and the intrinsically-resistant strain 95 incubated with 500μg/ml of methicillin **(M)**, cloxacillin **(CX)**, or cephalexin **(CP)**, or no antibiotic, and probed with a 1/100 dilution of anti-BSA-methicillin antiserum. The arrows and the labels 1 and 2 indicate the positions of specifically-stained bands believed to be PBP1 and PBP2 respectively.

lactamase-producing strain 13 with both antibiotics, but no specific bands are visible in the right positions for any of the PBPs in the intrinsically-resistant strain 95.

Thus the anti-BSA-methicillin antiserum used seems to have recognised epitopes in PBPs labelled with methicillin, cloxacillin and cephalexin, but not in PBPs labelled with benzyl penicillin nor ampicillin. The cross-reaction between methicillin, cloxacillin and cephalexin is difficult to explain. It is possible that there are epitopes on the side-chains of the three antibiotics which are sufficiently similar to cause this degree of cross-reaction but this is not obvious from their structure. However, in contradiction the side chain of ampicillin ($C_6H_5NH_2CO$) is the same as the corresponding side group on cephalexin, therefore if the antibodies were recognising this group they would probably give immunostained bands in blots labelled with both antibiotics. It is also possible that the reaction of the antibodies with the side-chains of cloxacillin and cephalexin is non-specific although this is unlikely to be the case with methicillin as this was not stained by a pre-bleed serum (Figure 3.9a).

Figure 3.13, 3.14, 3.15 and 3.16 show a series of Western blots in which strains of different levels of sensitivity were labelled with methicillin then probed with anti-methicillin antiserum. Table 3.2 is a summary of the interpretations of these blots showing the presence or absence of specific bands in the right positions for PBP1 and PBP2 in each of the strains. In the strains sensitive to benzyl penicillin at MICs of 0.01μg/ml to

Figures 3.13, 3.14, 3.15 and 3.16 show a series of Western blots of membrane proteins from cells of various strains of *Neisseria gonorrhoeae* which have been incubated with 500μg/ml methicillin at 37°C for 15 minutes prior to SDS-PAG electrophoresis and blotting onto nitrocellulose as described in Section 3.2. A 1/100 dilution of anti-BSA-methicillin antiserum was used to probe the blots for methicillin bound to the proteins. Rainbow molecular weight markers were run on each gel to assess the blotting efficiency and to locate the positions of the PBP bands. The approximate positions of the markers are shown on each Figure.

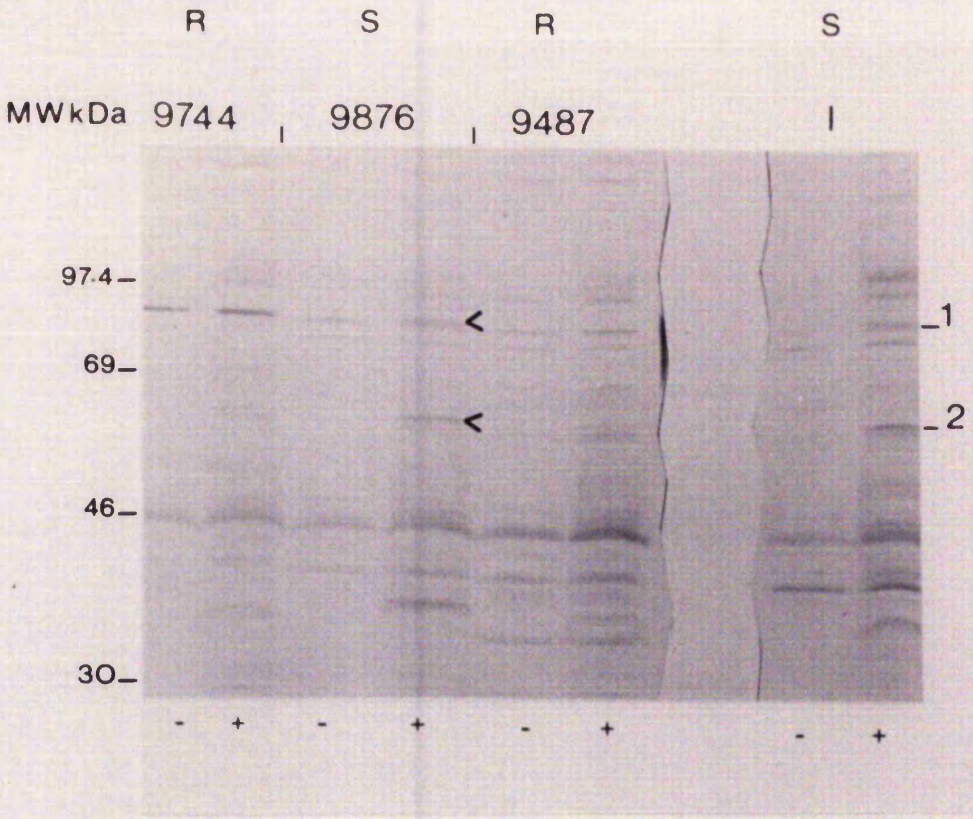


Figure 3.13: Western blot of membrane proteins from cells of various strains incubated with **(+)** or without **(-)** 500μg/ml of methicillin and probed with a 1/100 dilution of anti-BSA-methicillin antiserum. The arrows indicate examples of specifically-stained bands and the labels 1 and 2 mark the expected positions of PBP1 and PBP2 respectively.

Strain	**MIC with benzyl penicillin**
9744	5.00μg/ml
9876	0.04μg/ml
9487	1.00μg/ml
I	0.04μg/ml

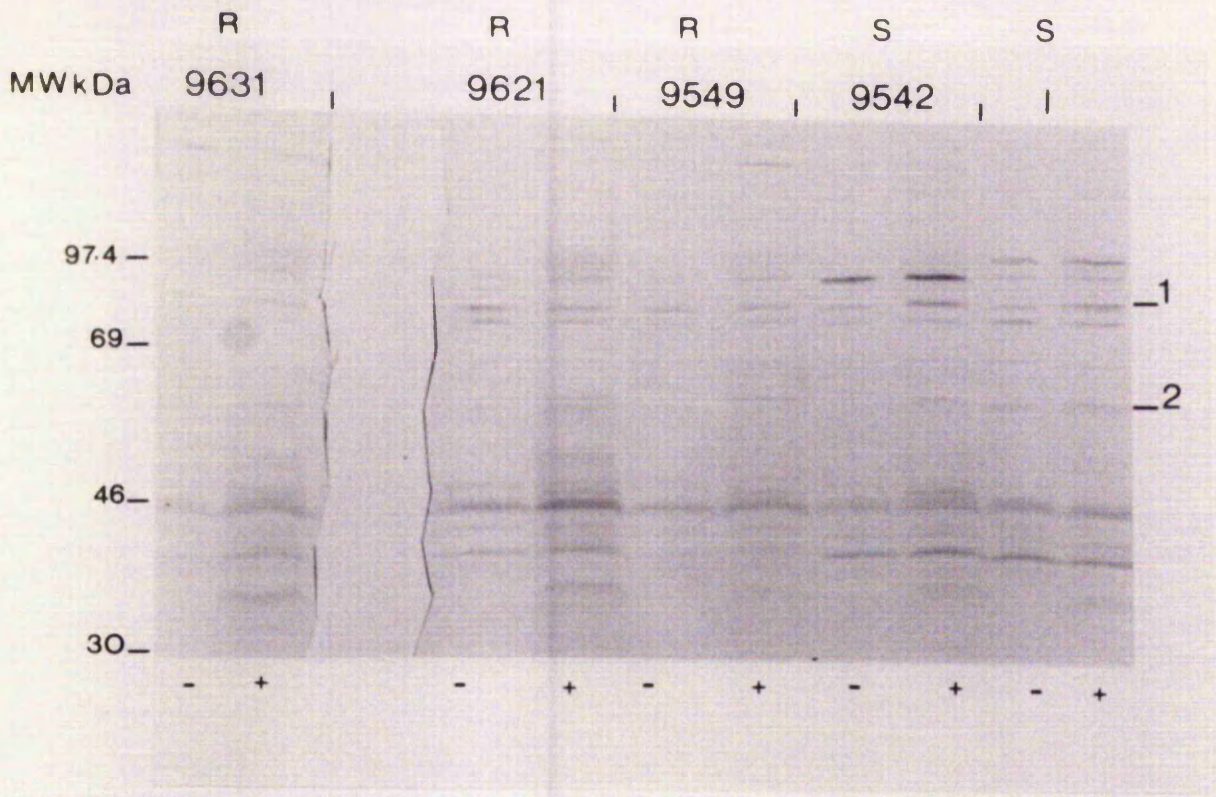


Figure 3.14: Western blot of membrane proteins from cells of various strains listed below incubated with **(+)** or without **(-)** 500μg/ml of methicillin and probed with a 1/100 dilution of anti-BSA-methicillin antiserum. The labels 1 and 2 mark the expected positions of PBP1 and PBP2 respectively.

Strain	**MIC with benzyl penicillin**
9631	1.00μg/ml
9621	5.00μg/ml
9549	5.00μg/ml
9542	0.04μg/ml
I	0.04μg/ml

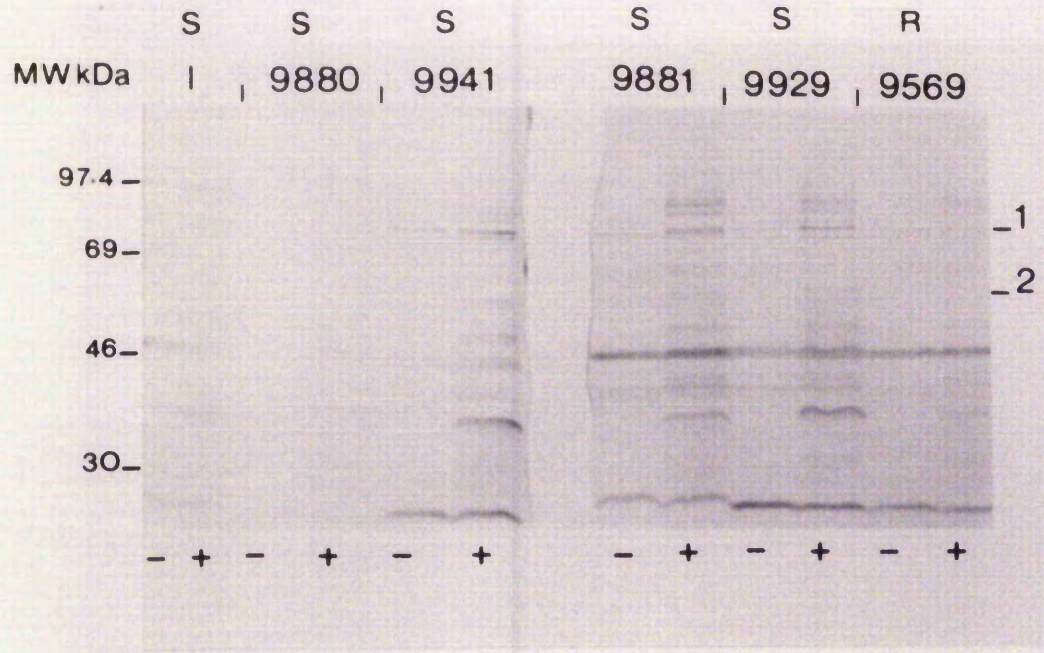


Figure 3.15: Western blot of membrane proteins from cells of various strains listed below incubated with **(+)** or without **(-)** 500μg/ml of methicillin and probed with a 1/100 dilution of anti-BSA-methicillin antiserum. The labels 1 and 2 mark the expected positions of PBP1 and PBP2 respecively.

Strain	**MIC with benzyl penicillin**
I	0.04μg/ml
9880	0.04μg/ml
9941	0.04μg/ml
9881	0.20μg/ml
9929	0.01μg/ml
9569	1.00μg/ml

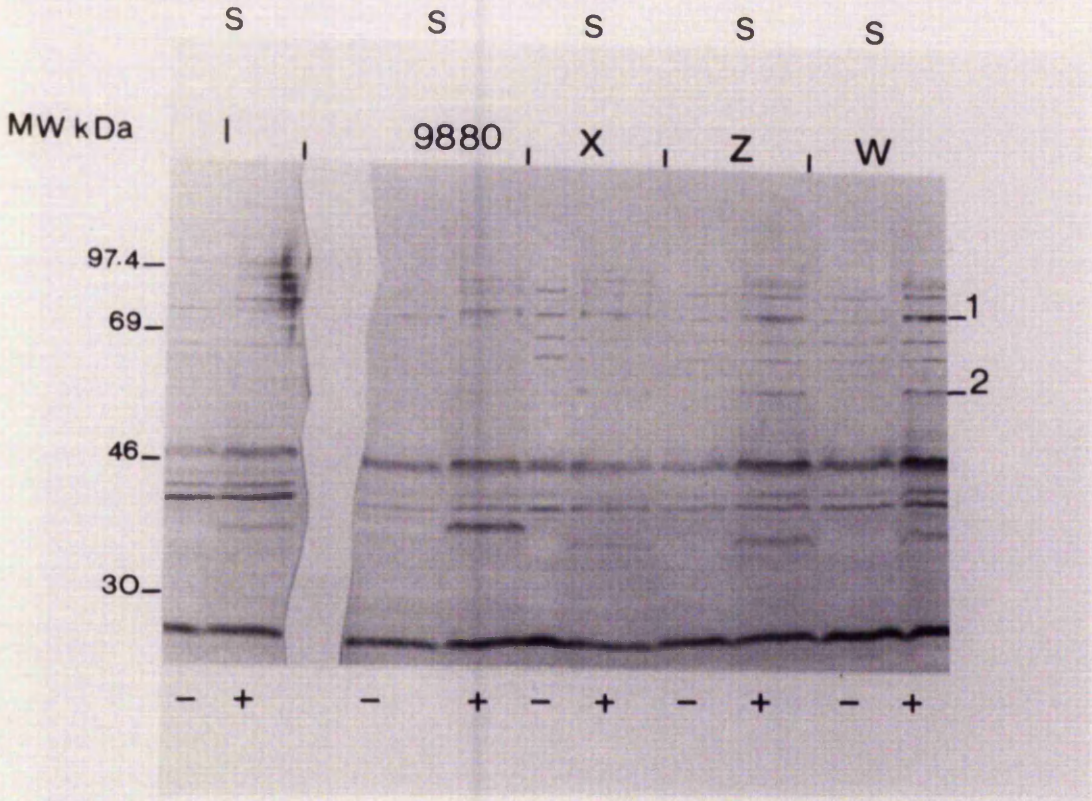


Figure 3.16: Western blot of membrane proteins from cells of various strains listed below incubated with **(+)** or without **(-)** 500μg/ml of methicillin and probed with a 1/100 dilution of anti-BSA-methicillin antiserum. The labels 1 and 2 indicate the positions expected for PBP1 and PBP2.

Strain	**MIC with benzyl penicillin**
I	0.04μg/ml
9880	0.04μg/ml
X	0.04μg/ml
Z	0.04μg/ml
W	0.04μg/ml

Table 3.2: A summary of the sensitivity patterns of the strains shown in Figures 3.13, 3.14, 3.15, and 3.16 and assessment of the blots for the presence or absence of specifically-stained bands in the correct positions for PBP1 and PBP2.

Strain	MIC (μg/ml) with penicillin	methicillin	PBP1	PBP2
9929	0.01	0.10	+	+
9941	0.04	0.04	+	+
I	0.04	0.10	+	+
9542	0.04	0.10	+	+
9880	0.04	0.10	+	+
W	0.04	0.10	+	+
X	0.04	0.20	+	+
Z	0.04	0.20	+	+
9876	0.04	1.00	+	+
9881	0.20	2.50	+	weak
9487	1.00	>10.00	-	+
9631	1.00	>10.00	-	+
9569	1.00	>10.00	-	+
9621	5.00	2.50	-	+
9744	5.00	10.00	-	+
9549	5.00	>10.00	-	+

0.04μg/ml specific bands in the right positions for both PBP1 and PBP2 are visible in the blots. In the strains resistant to benzyl penicillin at MICs of 1μg/ml to 5μg/ml a specific band in the right position for PBP2 but not one for PBP1 is visible in the blots. Strain 9881 may be regarded as a 'low-level resistant' strain in which the possible PBP1 band is much stronger than the possible PBP2 band. This would be typical of a strain with a *penB* mutation encoding for a PBP2 with a lower affinity for penicillin. The high degree of non-specific binding of antibody in these blots has made it difficult to correlate accurately the level of sensitivity with the intensity of staining of the bands. Some of the non-specific bands may be due to methicillin binding non-specifically to proteins other than PBPs because the bands are often more intense in the methicillin-treated samples than in the untreated samples. However, as shown earlier in Figure 3.9a many of the extra bands are present in the lanes probed with pre-bleed serum rather than specific antiserum. This may indicate previous exposure of the rabbits to commensal neisserias with cross-reacting membrane proteins. An experiment was carried out in which the antiserum was preabsorbed with *Neisseria gonorrhoeae* membrane proteins but this antiserum gave the same non-specific staining as the non-absorbed antiserum. Hakenbeck *et al.* (1986) experienced some problems due to antibodies raised against penicillin conjugates binding to extra protein bands in their blots but could not offer any explanation for it. Production of antisera against purified or partially purified PBPs would help to clarify these results. However it proved impossible to purify the PBPs from *Neisseria gonorrhoeae* by any variations of the

techniques used for other species despite a considerable effort (section 3.3.4) so such antisera could not be prepared. Also the use of radio-labelled methicillin to locate the specific methicillin-labelled bands in the blots would have helped to clarify the positions and labelling of the PBPs but radio-labelled methicillin was not available for this study.

Despite these problems, generally speaking an obvious difference between the sensitive and resistant strains could be observed using this technique. The most obvious difference was the lower intensity of staining of the supposed PBP1 band in resistant strains, while the lower affinity of the supposed PBP2 band in resistant strains was less obvious. This may be due to the fact that the sensitive strains tested in this study may themselves have been 'very low-level resistant' strains with slightly altered *penA* genes encoding a PBP2 with a slightly lower affinity for penicillin. Truly sensitive strains have penicillin MICs as low as 0.01μg/ml. Note that there is no significant difference between strains with MIC 0.01 and MIC 0.04μg/ml.

Figure 3.17 and 3.18 show the results obtained with strains which were resistant to benzyl penicillin due to the production of beta-lactamase. Table 3.3 is a summary of the interpretations of the blots indicating the presence or absence of stained bands in the positions of PBP1 and PBP2. All of these strains were sensitive to methicillin at relatively high concentrations (>10μg/ml). On these blots three non-beta-lactamase-producing strains were included for comparison. These were strain 9372

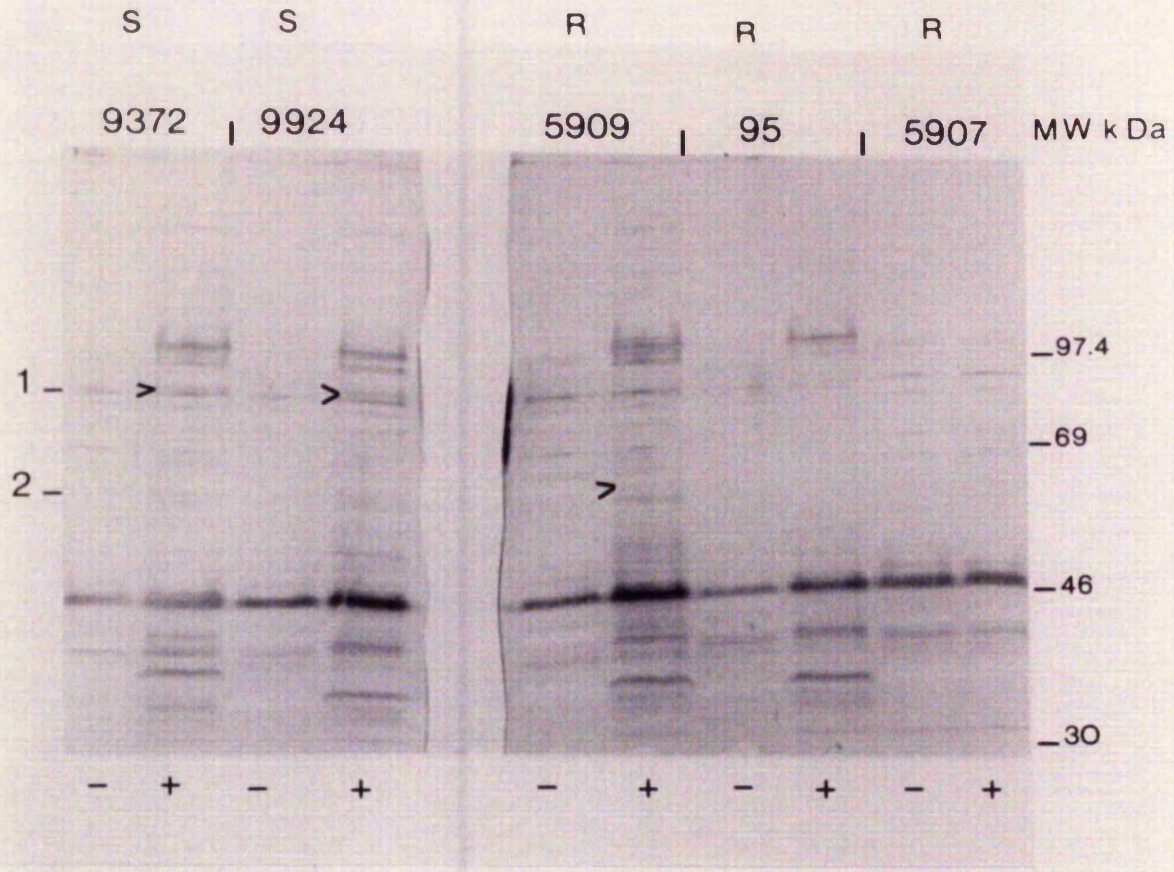


Figure 3.17: Western blot of the membrane proteins from cells of various strains including beta-lactamase producers. The cells were incubated with **(+)** or without **(-)** 500μg/ml of methicillin then probed with a 1/100 dilution of anti-BSA-methicillin antiserum. The arrows mark examples of specifically-stained bands and the labels 1 and 2 indicate the expected positions of PBP1 and PBP2 respectively.

Strain	**MIC with benzyl penicillin**	**Beta-lactamase**
9372	0.01μg/ml	-
9924	0.50μg/ml	-
5909	>10.00μg/ml	+
95	5.00μg/ml	-
5907	>10.00μg/ml	+

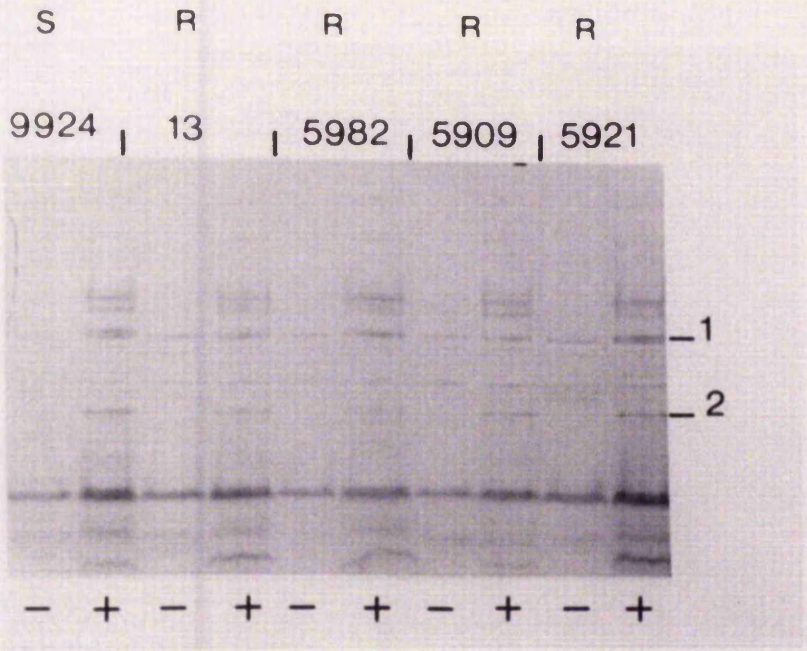


Figure 3.18: Western blot of membrane proteins from cells of various strains including beta-lactamase producers listed below incubated with **(+)** or without **(-)** 500μg/ml of methicillin and probed with a 1/100 dilution of anti-BSA-methicillin antiserum. The labels 1 and 2 indicate the expected positions of PBP1 and PBP2 respectively.

Strain	**MIC with benzyl penicillin**	**Beta-lactamase**
9924	0.05μg/ml	-
13	>10.00μg/ml	+
5982	>10.00μg/ml	+
5909	>10.00μg/ml	+
5921	>10.00μg/ml	+

Table 3.3: Summary of the sensitivity patterns of the strains shown in Figures 3.17 and 3.18 and assessment of the blots for the presence or absence of specifically-stained bands in the correct positions for PBP1 and PBP2.

Strain	ß-lac	MIC (µg/ml) with pen	meth	PBP1	PBP2
9372	-	0.01	0.04	+	+
9924	-	0.50	10.00	+	weak
95	-	5.00	10.00	-	-
5907	+	>10.00	10.00	-	-
5909	+	>10.00	>10.00	-	+
13	+	>10.00	>10.00	+	+
5982	+	>10.00	>10.00	+	+
5921	+	>10.00	>10.00	+	+

(penicillin MIC 0.01μg/ml) a fully sensitive strain which appears to have specifically-stained bands in the positions of both PBP1 and PBP2 labelled with methicillin, strain 9924 (penicillin MIC 0.5μg/ml) a low-level resistant strain which has a weakly stained specific band in the position of PBP2 and a clearly stained specific band in the position of PBP1, and strain 95 (penicillin MIC 5μg/ml) a high-level resistant strain which has no specific bands in the positions of PBP1 or PBP2. Of the beta-lactamase-producers, strains 13, 5982 and 5921 showed the labelling pattern found in the other sensitive strains previously tested, ie bands in the right positions for PBP1 and PBP2 both labelled. This suggests that these strains have the same PBPs as found in sensitive strains and their resistance is entirely due to the beta-lactamase. However, strain 5909 showed the labelling pattern previously found in the resistant strain (ie only one specific stained band in the position of PBP2) while strain 5907 had no specifically-stained bands in the position of any of the PBPs. Thus it is possible that these two strains may have an intrinsic chromosomally-mediated resistance as well as producing beta-lactamase. Further strains would have to be tested, using fluorography as well as Western blotting, to confirm this phenomenon.

Figure 3.19 shows a blot in which concentrations of methicillin from 0 to 500μg/ml of cell suspension were used to label cells of the sensitive strain I and the intrinsically-resistant strain 95. This blot was poorly developed for some unknown reason but the lowest concentration of methicillin at which the specific

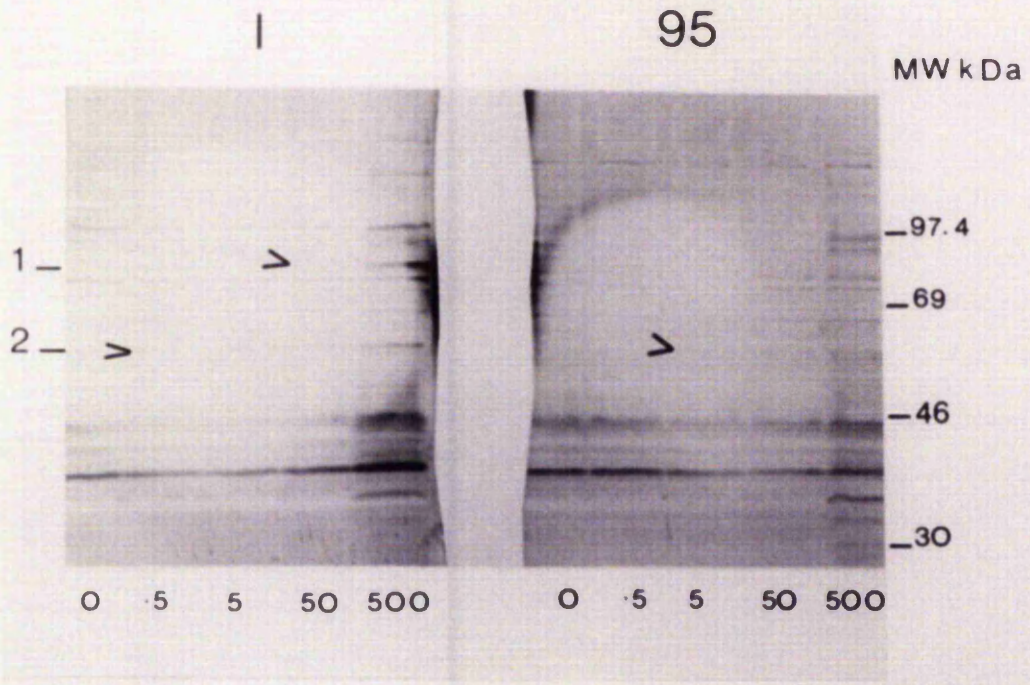


Figure 3.19: Western blot of the membrane proteins from cells of sensitive strain I and intrinsically-resistant strain 95 incubated with various concentrations of methicillin from 0 to 500μg/ml and probed with a 1/100 dilution of anti-BSA-methicillin antiserum. The arrows indicate the positions of bands believed to correlate with PBPs and the labels 1 and 2 indicate the expected positions of PBP1 and PBP2 respectively.

bands become labelled is clear.

Sensitive strain I PBP1 labelled at 50μg/ml and above,
PBP2 labelled at ≤0.5μg/ml and above,

Resistant strain 95 PBP1 labelled at 500μg/ml and above,
PBP2 labelled at 5μg/ml and above.

These results show that PBP2 is labelled by lower concentrations of methicillin than PBP1 in both strains. PBP1 in the resistant strain shows a very low affinity for methicillin possibly due to the presence of the *pem* and *tem* genes (Warner *et al.*, 1980). PBP2 in the resistant strain requires a higher concentration of methicillin to label it compared with the sensitive strain again possibly indicating a lower affinity for methicillin in this strain.

3.3.3.3 Conclusions

Anti-methicillin antibodies have been used to detect methicillin bound to proteins believed to be PBP1 and PBP2 in *Neisseria gonorrhoeae*. The method as it stands could not quantify the amount of methicillin bound to the PBPs but obvious differences in the binding of methicillin between sensitive and resistant strains were detectable.

3.3.4 Purification of PBPs by affinity chromatography

3.3.4.1 Modified Lowry protein assay

Figure 3.20 shows a typical standard curve for the Lowry protein

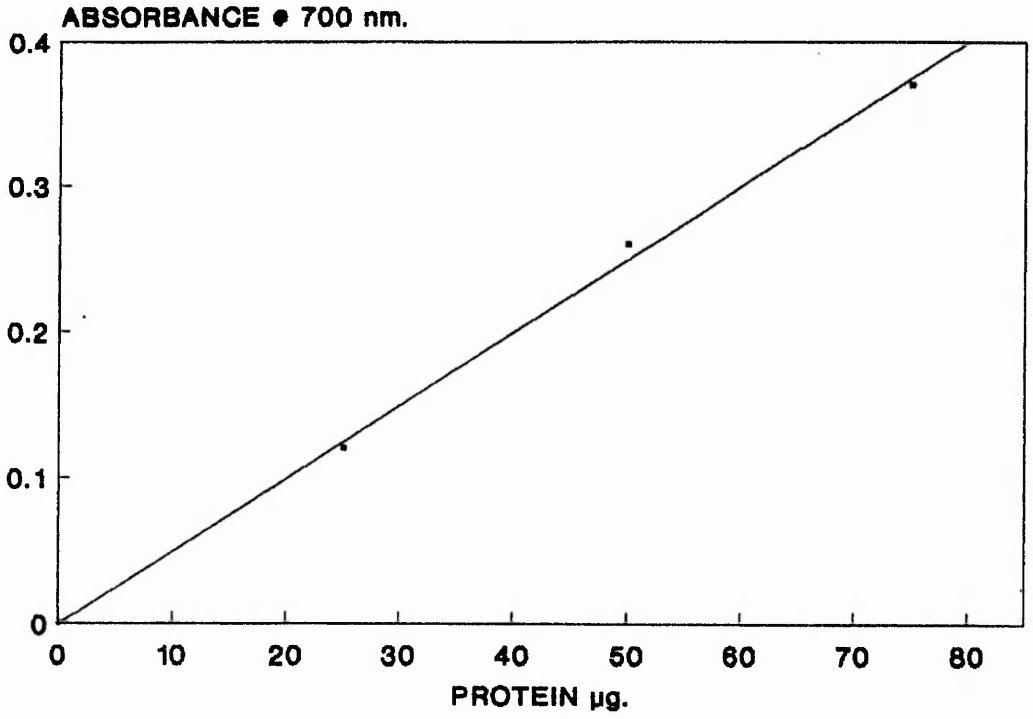


Figure 3.20: A typical standard curve of the modified Lowry protein assay for samples containing Triton X-100 showing absorbance at 700nm plotted against concentration of the standard protein bovine serum albumin.

assay modified for use with detergent-containing samples.

3.3.4.2 Affinity purification of the PBPs of *Escherichia coli* using 6-amino-penicillanic acid as the ligand.

The PBPs of *Escherichia coli* were purified on an affinity column of ECH-sepharose-4B with 6-amino-penicillanic acid as the ligand. 260mg of crude membrane protein was solubilised using 2% Triton X-100. Throughout the purification scheme samples were tested for protein and PBPs using SDS-PAGE and fluorography. Figure 3.21 shows the stained gel of the various stages of this purification and Figure 3.22 shows the fluorograph, from this stained gel, of the final purified PBP preparation. PBP1, PBP4, PBP5 and PBP6 were purified in relatively large amounts and they were clearly visible on the fluorograph. PBP5 and PBP6, which were most readily released from the ligand, were visible on the stained PAGE gel. PBP2 and PBP3 were very weak on the fluorograph but there are known to be lower numbers of molecules of PBP2 and PBP3 in the cell than of the other PBPs and they are also known to bind the lowest percentage of penicillin (Spratt 1977). The apparent molecular weights of the PBPs, determined from the fluorograph as described in the legend to Figure 3.22, are comparable to those reported by Spratt (1977) and both sets of values are shown in Table 3.4.

3.3.4.3 Affinity purification of the PBPs of penicillin-sensitive *Neisseria gonorrhoeae* using 6-amino-penicillanic acid as the ligand.

The method used to purify the PBPs of *Escherichia coli* was

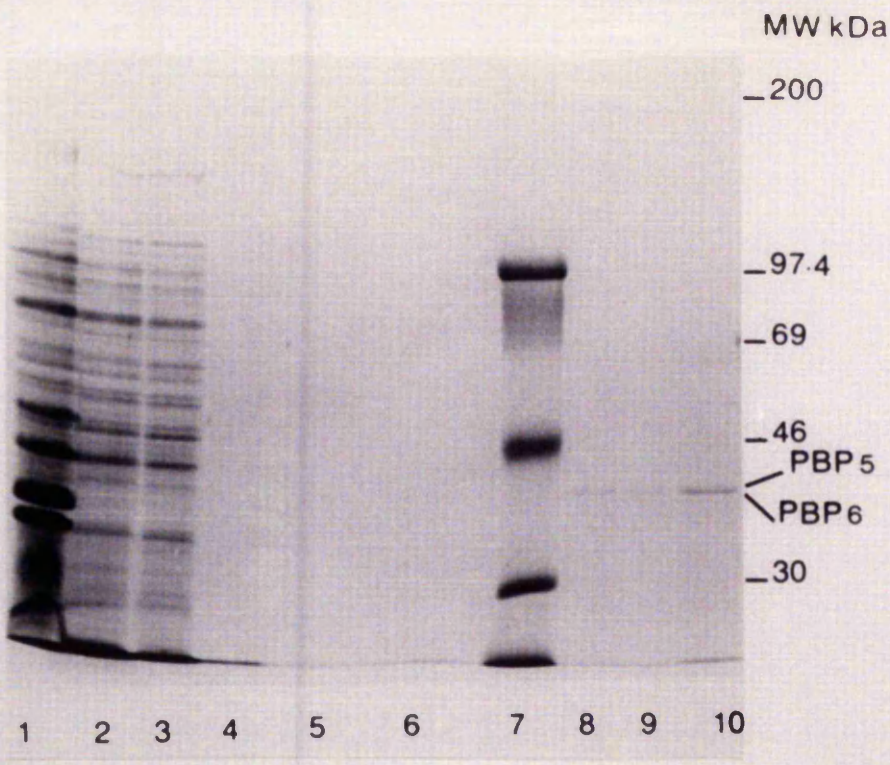


Figure 3.21: Stained SDS-PAGE gel of the samples listed below taken during the affinity purification of the PBPs of *Escherichia coli* on 6-APA Sepharose following solubilisation with 2% Triton X-100.

1: Crude membrane protein

2: Solubilsed membrane protein

3: Non-absorbed proteins from affinity column

4: Washing buffer 1

5: Washing buffer 2

6: Washing buffer 3

7: Molecular weight markers

8: Eluted dialysed protein from affinity column

9: Non-adsorbed protein from anion exchange column

10: Purified concentrated PBPs from ion-exchange column

The bands corresponding to PBPs 5 and 6 are labelled

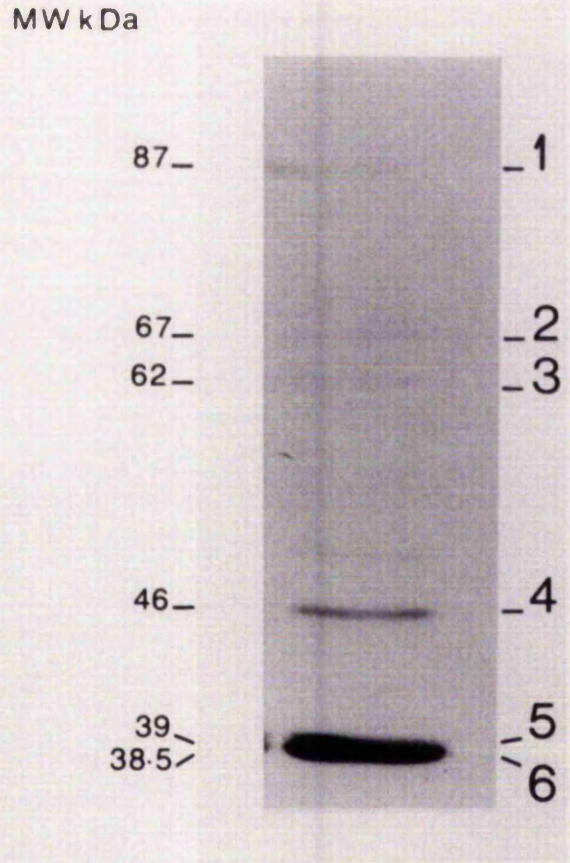


Figure 3.22: Fluorograph of lane 10 of the gel shown in Figure 3.21 labelled with 1ug/ml tritiated penicillin showing the affinity-purified PBPs of *Escherichia coli*. Labels 1-6 mark the positions of the PBPs.

Table 3.4: Molecular weights of the PBPs of *Escherichia coli* determined from the fluorograph in Figure 3.22 compare with those reported by Spratt (1977) determined by the same technique.

PBP	Molecular weight determined from Figure 3.22	Molecular weight reported by Spratt (1977)
1	87,000	91,000
2	67,000	66,000
3	62,000	60,000
4	46,000	49,000
5	39,000	42,000
6	38,500	40,000

applied to the purification of the PBPs of the penicillin-sensitive strain I of *Neisseria gonorrhoeae*. 283mg of membrane protein was solubilised in 2% Triton X-100 and passed through a column of 6-amino-penicillanic-acid-sepharose as for *Escherichia coli*. Elution was attempted with 0.4M and 0.8M neutralised hydroxylamine. Figure 3.23 shows the stained gel of the various stages of this purification and Figure 3.24 shows the fluorograph derived from it. A low molecular weight band (labelled X) can be seen on the fluorograph in the two lanes (9 and 10) loaded with the purified concentrated protein from the affinity column. This must be protein which has bound the tritiated penicillin but it is not the correct molecular weight for any of the known *Neisseria gonorrhoeae* PBPs. Since band X was not present in the affinity column load (lanes 1 and 2) it may be explained as the result of proteolytic breakdown of one of the PBPs, the protein product retaining the binding site for penicillin. An additional band was visible in lanes 9 and 10 in the Coomassie-stained gel which was not visible in the fluorograph. This band may be a protein which bound to the 6-amino-penicillanic acid non-specifically and was then eluted by the hydroxylamine.

Several possible explanations for this lack of isolation of the PBPs from *Neisseria gonorrhoeae* were considered. First it was noted that the hydroxamate test had proved that the sepharose used in the column was coated with 6-amino-penicillanic acid with active carbonyl groups. This was also confirmed by the success of the purification of the *Escherichia coli* PBPs. Second, lane 2 of the fluorograph shows that PBP3 was not solubilised at the

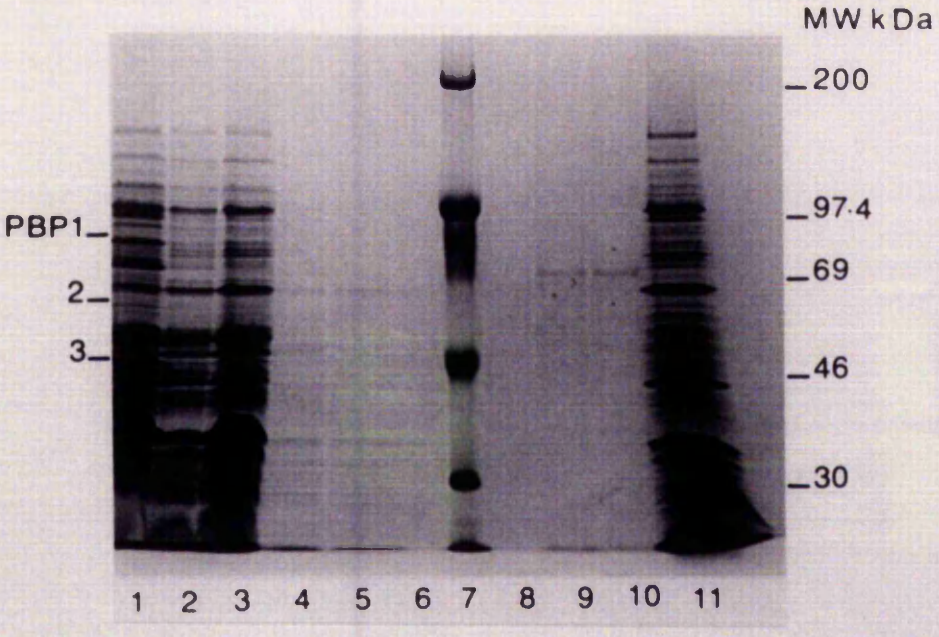


Figure 3.23: Stained SDS-PAGE gel of the samples listed below taken during the affinity purification of the PBPs of the sensitive strain I on 6-APA-sepharose following solubilisation with 2% Triton X-100. Labels 1, 2 and 3 refer to the expected positions of PBP1, PBP2 and PBP3.

1: Crude membranes

2: Solubilised membranes

3: Non-adsorbed protein from affinity column

4: Washing buffer 1

5: Washing buffer 2

6: Washing buffer 3

7: Molecular weight markers

8: Eluted dialysed protein from affinity column

9: Purified concentrated PBPs from ion-exchange column

10: As 9

11: Protein from log-phase cells

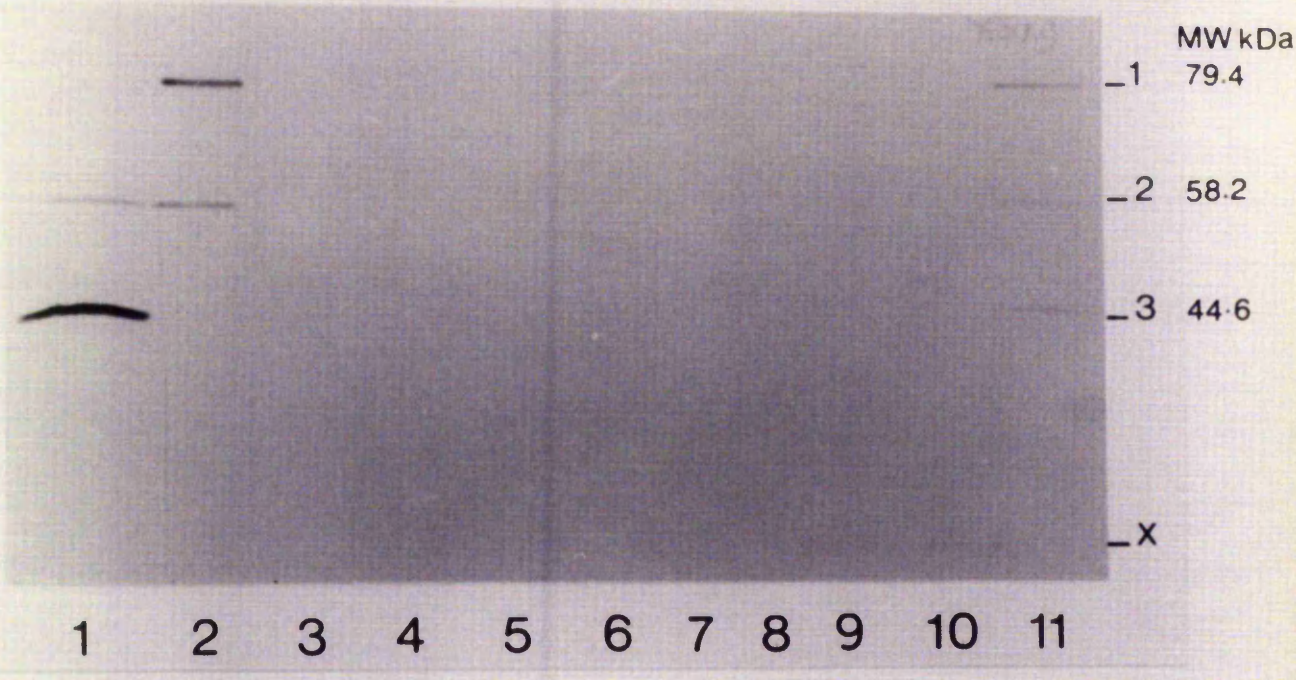


Figure 3.24: Fluorograph of part of the gel shown in Figure 3.23 labelled with 1μg/ml tritiated penicillin. Labels 1, 2 and 3 mark the expected positions of PBP1, PBP2 and PBP3. Label X marks an unidentified radio-labelled band referred to in the text.

1: Crude membranes

2: Solubilised membranes

3: Non-adsorbed proteins from affinity column

4: Washing buffer 1

5: Washing buffer 2

6: Washing buffer 3

7: Molecular weight markers

8: Eluted dialysed protein from affinity column

9: Purified concentrated PBPs from ion-exchange column

10: As 9

11: Proteins from log-phase cells

concentration of Triton X-100 used and this PBP3 was later found in the pellet of non-solubilised protein. However, lane 2 shows that PBP1 and PBP2 had been solubilised. Other detergents were not tried but higher concentrations of Triton-X were investigated (Section 3.3.4.7). Lane 3 shows that the non-adsorbed protein which passed through the column did not contain active PBPs suggesting that PBP1 and PBP2 had bound to the column. Subsequent washing with the washing buffers i, ii, and iii of increasing ionic strength, and elution with 0.8M hydroxylamine also failed to remove the PBPs. It was concluded that the PBP-penicilloyl bond was too stable to be broken by the hydroxylamine.

3.3.4.4. Elution of PBPs with different nucleophiles

Further attempts to remove the PBPs from the 6-APA-sepharose column were made using 5M hydroxylamine, 1M ethylamine, 3% hydrogen peroxide, boiling, and using higher pH buffers all failed to elute them. Consequently a different approach was tried to elucidate the problem. The elution of tritiated penicillin from the PBPs in isolated free membranes was attempted. Neutralised hydroxylamine at concentrations of 0.5M, 1M and 2M was added to samples of membranes bound with tritiated penicillin and incubated for ½, 1 and 2½ hours. Subsequent fluorography of the samples showed that PBP1 had not bound the penicillin but PBP2 and PBP3 had done so. However, the tritiated penicillin was not released from these PBPs by the elution procedures tested (results not shown).

3.3.4.5 Location of PBPs during affinity chromatography

The pellet from the previous purification (section 3.3.4.3) was re-extracted with 4% Triton X-100 to solubilise PBP3. Figures 3.25 and 3.26 show the stained gel and the corresponding fluorograph respectively. Lane 3 (of the fluorograph) shows that PBP3 and some of PBP2 are still unsolubilised in the 2% Triton pellet. Lane 4 (of the fluorograph) shows that 4% Triton solubilised PBP3 and PBP2, while lane 5 confirms that solubilisation, whilst complete for PBP2, was still not complete for PBP3. The position of PBP3 identified on the stained gel in Figure 3.25 appears to be coincidental with a single band suggesting that this protein is relatively abundant. The literature gives no information on the amount of PBP3 in the cell, however, it is known to bind 54% of the total bound penicillin (Barbour 1981) suggesting that PBP3 is more abundant than PBP1 and PBP2.

From these studies it was concluded that PBP1 and PBP2 of the sensitive strain I could be bound to 6-APA-sepharose but the PBPs had such a high affinity for the ligand that neither could be removed in their native form by any of the elutants tried.

3.3.4.6 Affinity purification of the PBPs of penicillin-resistant *Neisseria gonorrhoeae* using 6-amino-penicillanic acid as the ligand.

The chromosomally-resistant (intrinsically-resistant) strain 95 was selected for investigation in the expectation that its PBPs,

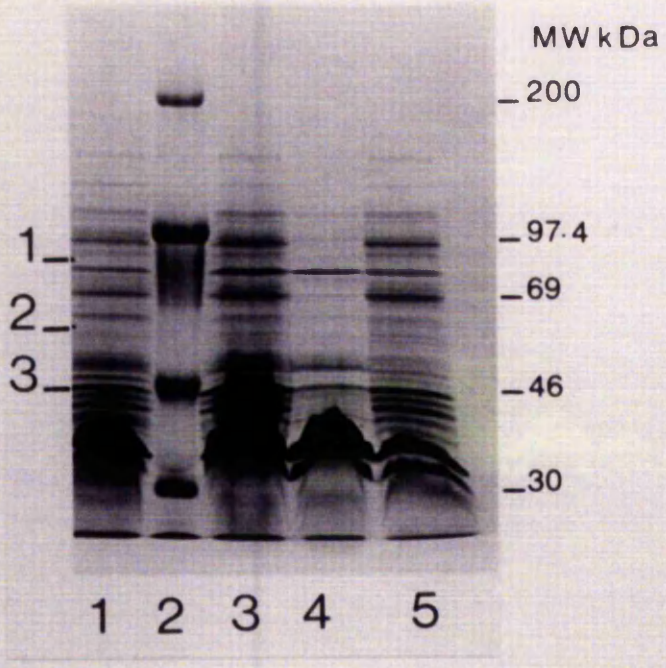


Figure 3.25: Stained SDS-PAGE gel of the samples listed below taken during the solubilisation of membranes from sensitive strain I with 2% and 4% Triton X-100. The labels 1, 2 and 3 mark the expected positions of PBP1, PBP2 and PBP3.

1: Crude membranes (the same preparation shown in Figs 3.23 and 3.24).

2: Molecular weight markers

3: Pellet following treatment of membranes with 2% Triton

4: Supernatant following re-extraction of the pellet in 3 above of membranes with 4% Triton

5: Pellet following re-extraction of the pellet in 3 above of membranes with 4% Triton

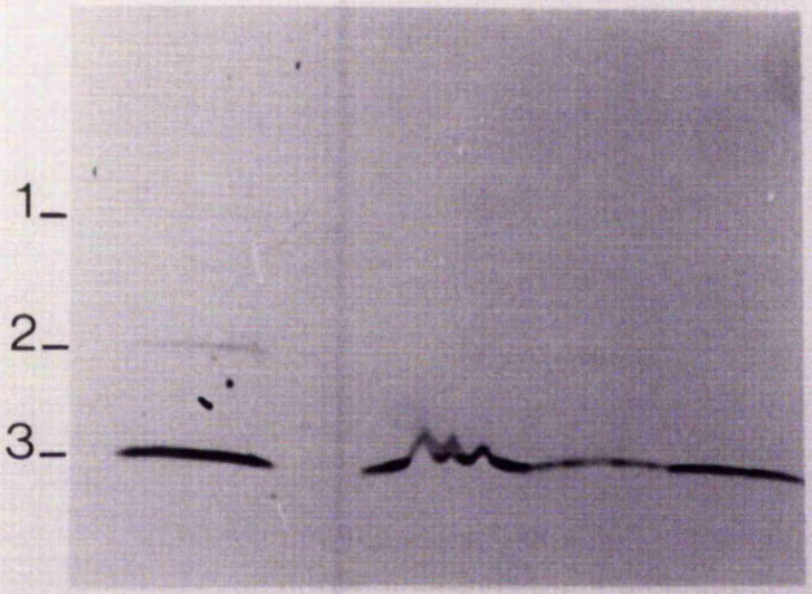


Figure 3.26: Fluorograph of part of the gel shown in Figure 3.25 labelled with 1μg/ml tritiated penicillin. The labels 1, 2 and 3 mark the expected positions of PBP1, PBP2 and PBP3.

1: Crude membranes

2: Molecular weight markers

3: Pellet following treatment with 2% Triton

4: Supernatant following treatment with 4% Triton

5: Pellet following treatment with 4% Triton

having a lower affinity for penicillin, would bind less avidly to the ligand. 433mg of membrane protein from strain 95 was solubilised in 5% Triton, applied to a 6-APA-sepharose column, the proteins eluted with 1M neutralised hydroxylamine, and concentrated by ion-exchange chromatography as described in Section 3.2. The results are shown in Figures 3.27 and 3.28. The two bands labelled X and Y in the stained gel (Figure 3.27) were eluted from the 6-APA column with 1M neutralised hydroxylamine. These protein bands correspond to the radioactive bands X and Y in lane 5 of the fluorograph shown in Figure 3.28. The molecular weight of X was 66,000 and that of Y was 47,800. The molecular weight of band Y was very close to, but slightly higher than, that of PBP3, and the molecular weight of band X was higher than that expected for PBP2. Subsequent investigation of this purification (results shown in Figure 3.28) showed that PBP3 proper was not solubilised with 5% Triton (lane 2) although this PBP was present in the untreated membranes (lane 1). Lane 4 shows that PBP3 remained in the pellet after solubilisation and therefore did not enter the affinity column. Investigation of the crude untreated membrane protein preparation (lane 1 Figure 3.28) showed that the two bands X and Y were present before purification. It is possible that bands X and Y are proteolytic products of PBP1 and PBP2 respectively which have retained the catalytic binding site for penicillin. The culture of strain 95 used was proved to be pure to eliminate the possibility that these bands were from a contaminating bacterium.

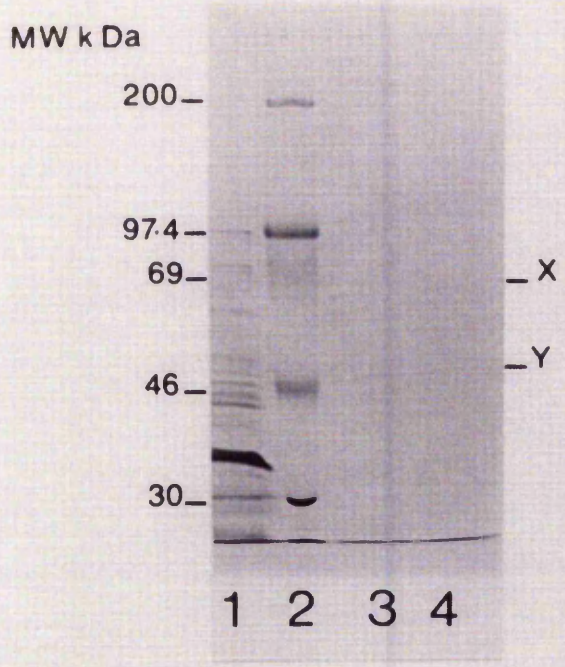


Figure 3.27: Stained SDS-PAGE gel of the samples listed below taken during an affinity purification of the membrane proteins of intrinsically-resistant strain 95 on 6-APA-sepharose. Labels X and Y mark bands referred to in the text and in the fluorograph in Figure 3.28.

1: Crude membranes

2: Molecular weight markers

3: Protein eluted with 1M hydroxylamine

4: As 3

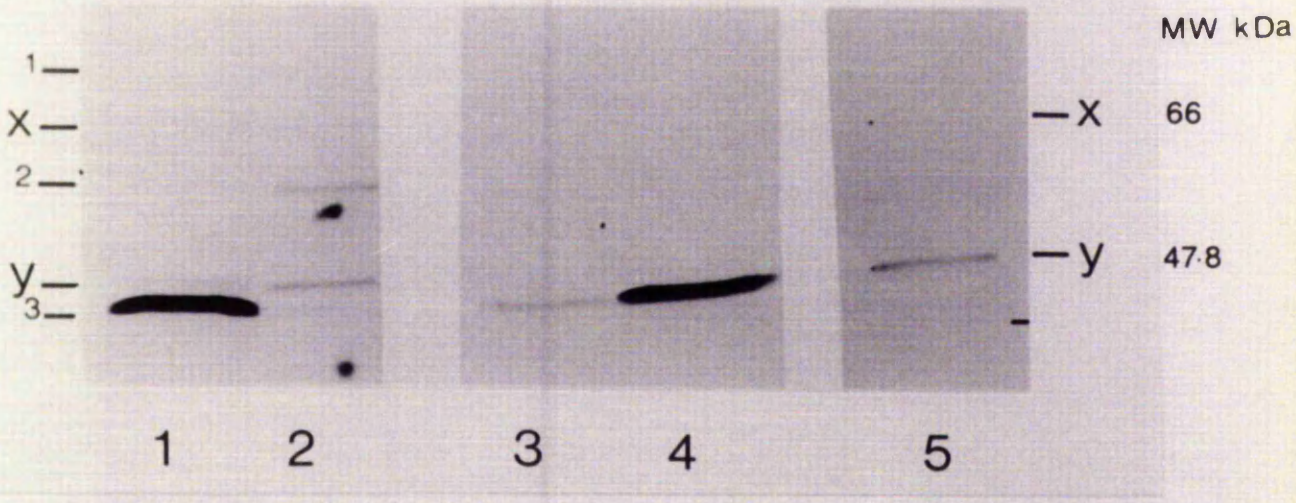


Figure 3.28: Fluorograph of the samples listed below labelled with 3μg/ml tritiated penicillin taken during affinity purification of intrinsically-resistant strain 95. Labels 1, 2 and 3 mark the positions of PBP1, PBP2 and PBP3. Labels X and Y mark the positions of radio-labelled proteins which do not correspond to any of the PBPs and which are referred to in the text.

1: Crude membranes

2: Supernatant following treatment with 5% Triton

3: Crude membranes (different batch) for comparison with 1

4: Pellet following treatment with 5% Triton

5: Bands X and Y eluted from 6-APA affinity column

3.3.4.7 Solubilisation of PBPs

Due to the problems of solubilising PBP3 an experiment was performed using different concentrations of Triton X-100 to solubilise dilute membrane suspensions from the sensitive strain I. 1mg of membrane protein was exposed to various concentrations of the detergent in a 50μl volume for 30 minutes. Figures 3.29 and 3.30 show the stained gel and the corresponding fluorograph respectively. The fluorograph in Figure 3.30 shows that increasing the Triton concentration increased the amount of all the PBPs solubilised. PBP1 and PBP2 were completely solubilised by the highest concentration of Triton tested (4%) but PBP3 was only partially solubilised. For all further experiments 5% Triton was used in a 400-500μl volume containing 20mg/ml of membrane protein.

3.3.4.8 Affinity purification of the PBPs of penicillin-sensitive *Neisseria gonorrhoeae* using D-alanyl-D-alanine as the ligand.

A column of D-alanyl-D-alanine-sepharose was tried for the purification of the PBPs from the sensitive strain I. D-alanyl-D-alanine is the natural substrate of the PBPs. Figures 3.31 and 3.32 show the stained gel and the fluorograph respectively. Lane 2 of the fluorograph (Figure 3.32) shows that all three PBPs were solubilised, however, lane 3 shows that some of the PBP3 was left in the pellet. Lane 4 shows that PBP1 and PBP3 were present in the washing from the column prior to elution and indicate that these PBPs were not completely bound. Elution with 0.4M hydroxylamine produced one PBP, visible on the fluorograph after ion-exchange concentration, which was either PBP3 itself or a

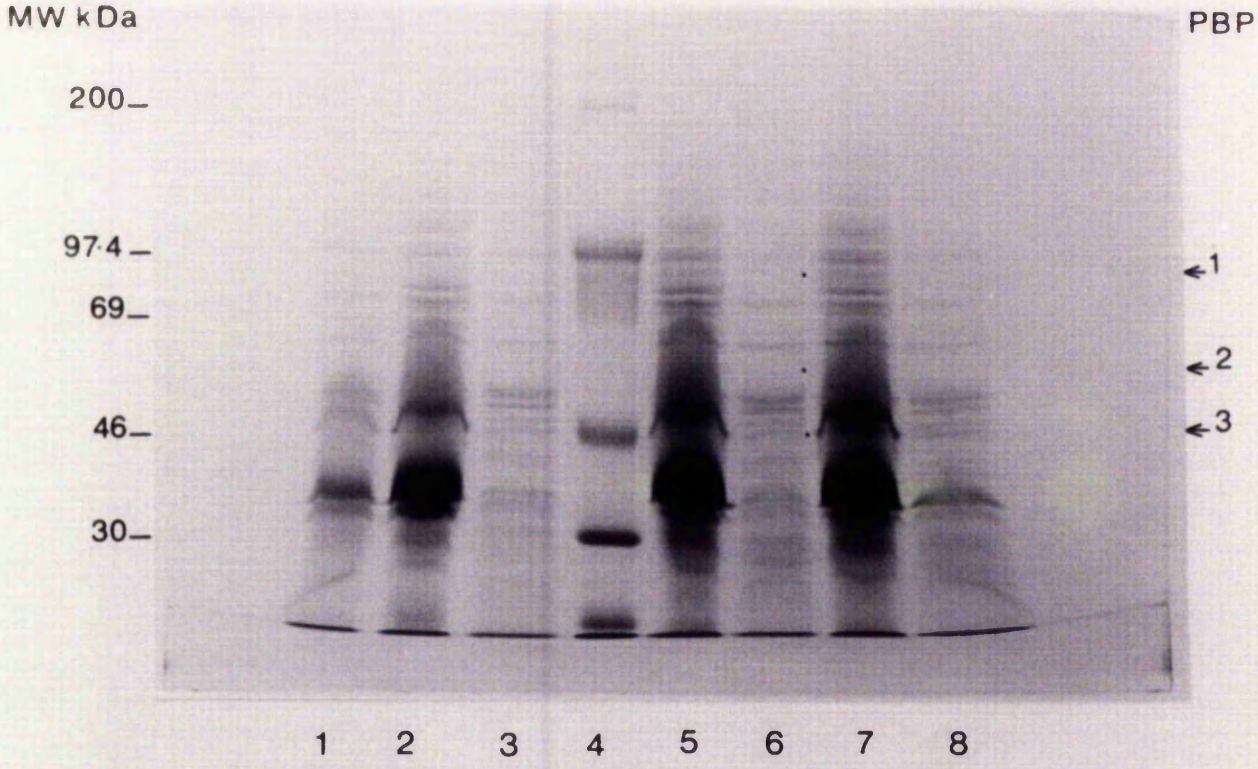


Figure 3.29: Stained SDS-PAGE gel of the samples listed below taken during an investigation of the solubilisation of membrane proteins of sensitive strain I with various concentrations of Triton X-100. The arrows mark the expected positions of PBP1, PBP2 and PBP3 located from the corresponding fluorograph shown in Figure 3.30.

1; Crude membranes

2: Pellet following treatment with 2.5% Triton

3: Supernatant following treatment with 2.5% Triton

4: Molecular weight markers

5: Pellet following treatment with 3.3% Triton

6: Supernatant following treatment with 3.3% Triton

7: Pellet following treatment with 4% Triton

8: Supernatant following treatment with 4% Triton

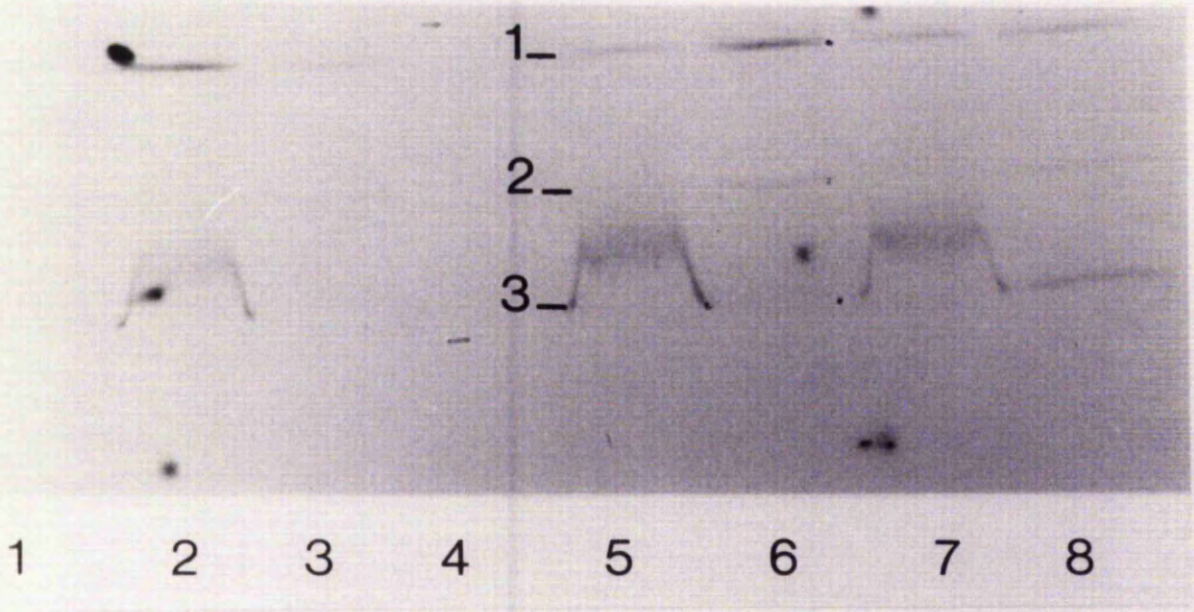


Figure 3.30: Fluorograph of part of the gel shown in Figure 3.29 labelled with 1μg/ml tritiated penicillin. Labels 1, 2 and 3 mark the positions of bands believed to be PBP1, PBP2 and PBP3.

1: Crude membranes

2: Pellet following treatment with 2.5% Triton

3: Supernatant following treatment with 2.5% Triton

4: Molecular weight markers

5: Pellet following treatment with 3.3% Triton

6: Supernatant following treatment with 3.3% Triton

7: Pellet following treatment with 4% Triton

8: Supernatant following treatment with 4% Triton

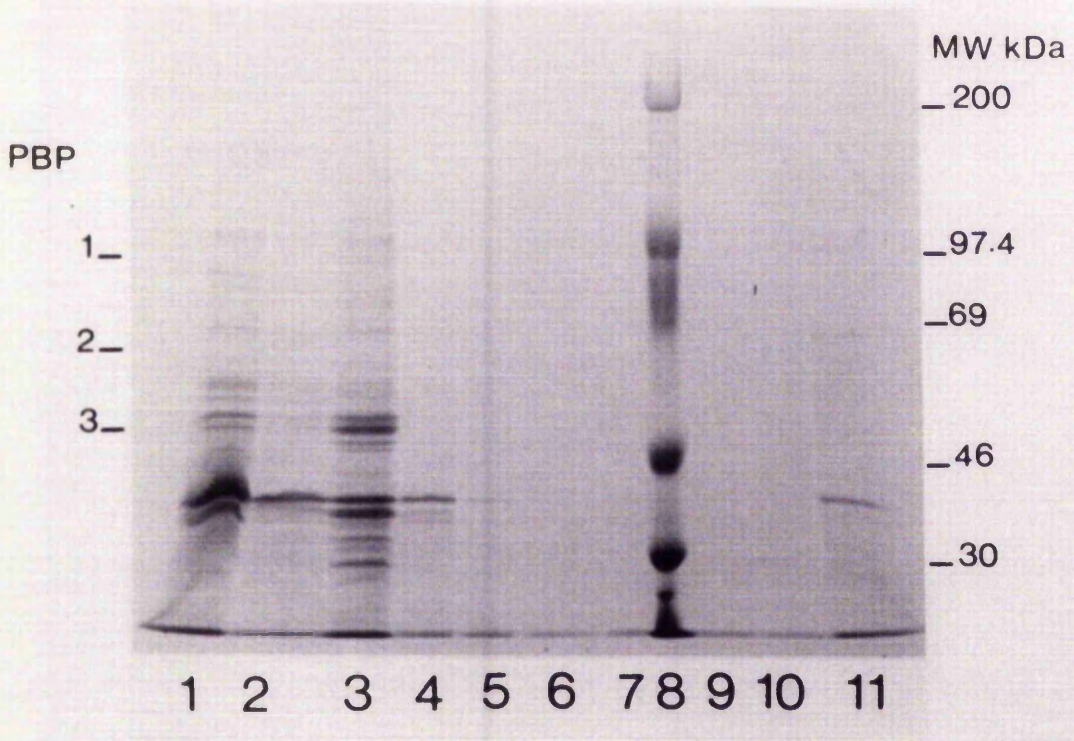


Figure 3.31: Stained SDS-PAGE gel of the samples listed below taken during affinity purification of sensitive strain I on D-alanyl-D-alanine-sepharose. Labels 1, 2 and 3 mark the positions of PBP1, PBP2 and PBP3 located from the fluorograph in Figure 3.32.

1: Crude membranes

2: Solubilised membrane supernatant

3: Solubilised membrane pellet

4: Non-adsorbed protein from affinity column

5: Washing buffer 1

6: Washing buffer 2

7: Washing buffer 3

8: Molecular weight markers

9: Eluted dialysed protein from affinity column

10: Non-adsorbed protein from ion-exchange column

11: Purified concentrated PBPs from ion-exchange column

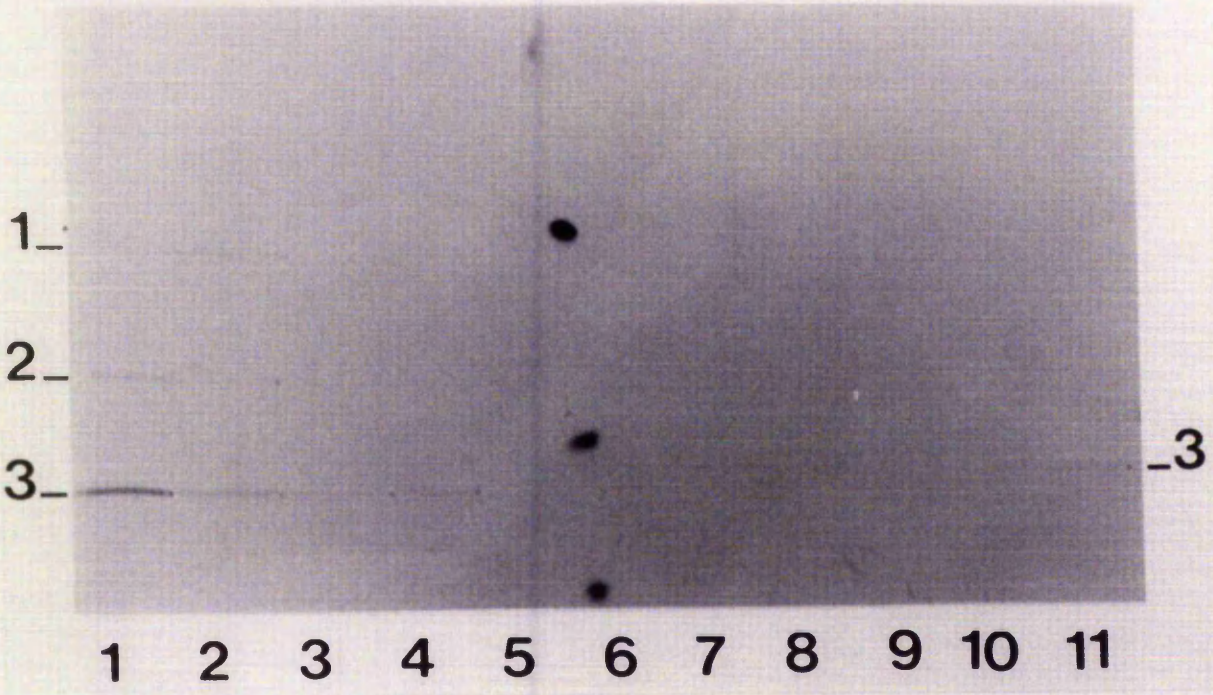


Figure 3.32: Fluorograph of part of the gel shown in Figure 3.31 labelled with 1μg/ml tritiated penicillin. Labels 1, 2 and 3 mark the positions of PBP1, PBP2 and PBP3.

1: Crude membranes

2: Solubilised membrane supernatant

3: Solubilised membrane pellet

4: Non-adsorbed protein from affinity column

5: Washing buffer 1

6: Washing buffer 2

7: Washing buffer 3

8: Molecular weight markers

9: Eluted dialysed protein from affinity column

10: Non-adsorbed protein from ion-exchange column

11: Purified concentrated PBPs from ion-exchange column

proteolytic product of PBP1 or PBP2. However, other proteins were eluted at the same time as shown in lane 11 of the stained gel (Figure 3.31).

3.3.4.9 Affinity purification of the PBPs of penicillin-sensitive *Neisseria gonorrhoeae* using 7-amino-cephalosporanic acid as the ligand.

Figures 3.33 and 3.34 show the stained gel and the fluorograph respectively from an affinity purification of PBPs from the membranes of the sensitive strain I using 7-amino-cephalosporanic acid (7-ACA) as the ligand. 7-ACA is the equivalent compound in the cephalosporins of 6-amino-penicillanic acid in the penicillins. Cephalosporins are less active against *Neisseria gonorrhoeae* and therefore the PBPs may have a lower affinity for 7-ACA. The results of the fluorograph show that all three PBPs were solubilised in this experiment, and all were bound to the 7-ACA-sepharose. However, none of the PBPs were eluted with 0.4M hydroxylamine.

A further investigation of this column involved incubating the column at room temperature overnight with 3M hydroxylamine and washing through the eluate which was then tested for PBPs by fluorography. A very small amount of PBP3 was released by this treatment (this result is not shown as the PBP3 band was very faint and did not photograph well). As a result of this experiment a further batch of sensitive-strain membranes were purified on a similar column and the PBPs eluted overnight with 5M neutralised hydroxylamine. The eluate was then tested for PBPs

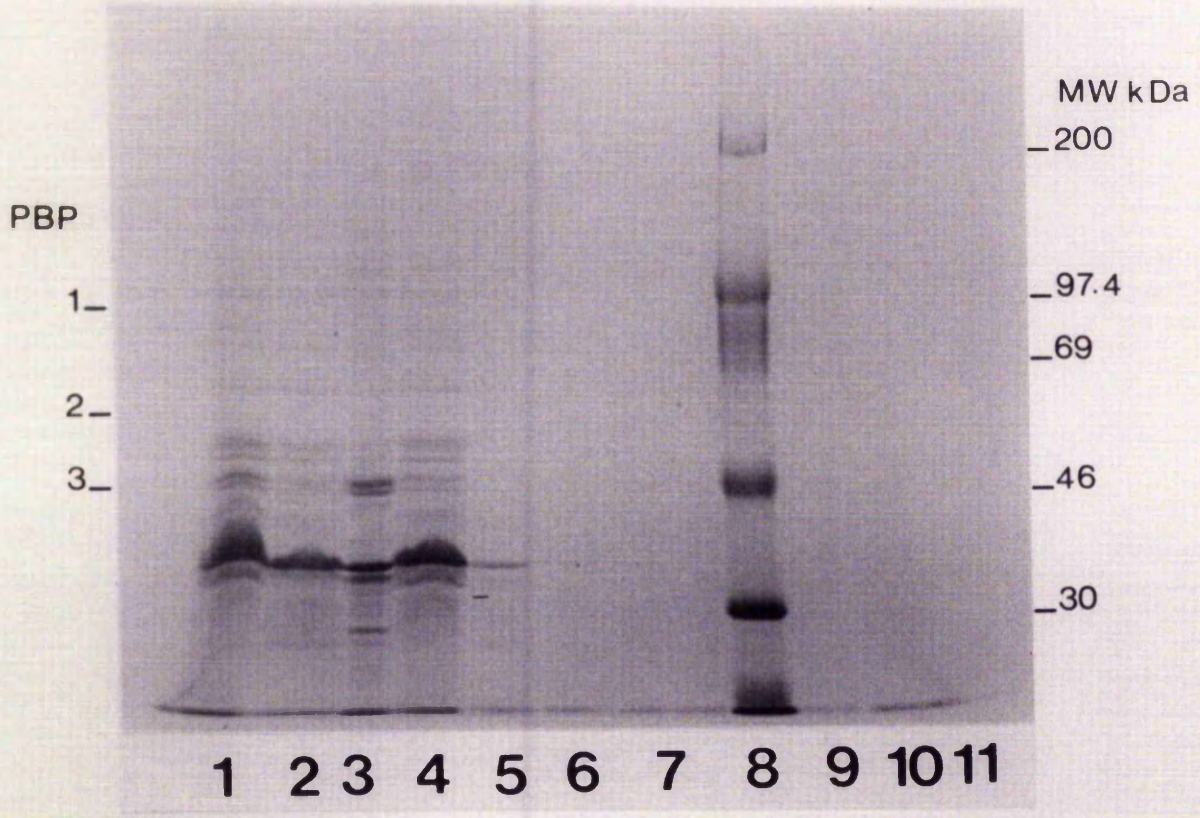


Figure 3.33: Stained SDS-PAGE gel of the samples listed below taken during affinity purification of membrane proteins of sensitive strain I on 7-ACA-sepharose. Labels 1, 2 and 3 mark the positions of PBP1, PBP2 and PBP3 located from the corresponding fluorograph in Figure 3.34.

1: Crude membranes

2: Solubilised membrane supernatant

3: Solubilised membrane pellet

4: Non-adsorbed protein from affinity column

5: Washing buffer 1

6: Washing buffer 2

7: Washing buffer 3

8: Molecular weight markers

9: Eluted dialysed protein from affinity column

10: Non-adsorbed protein from ion-exchange column

11: Purified concentrated PBPs from ion-exchange column

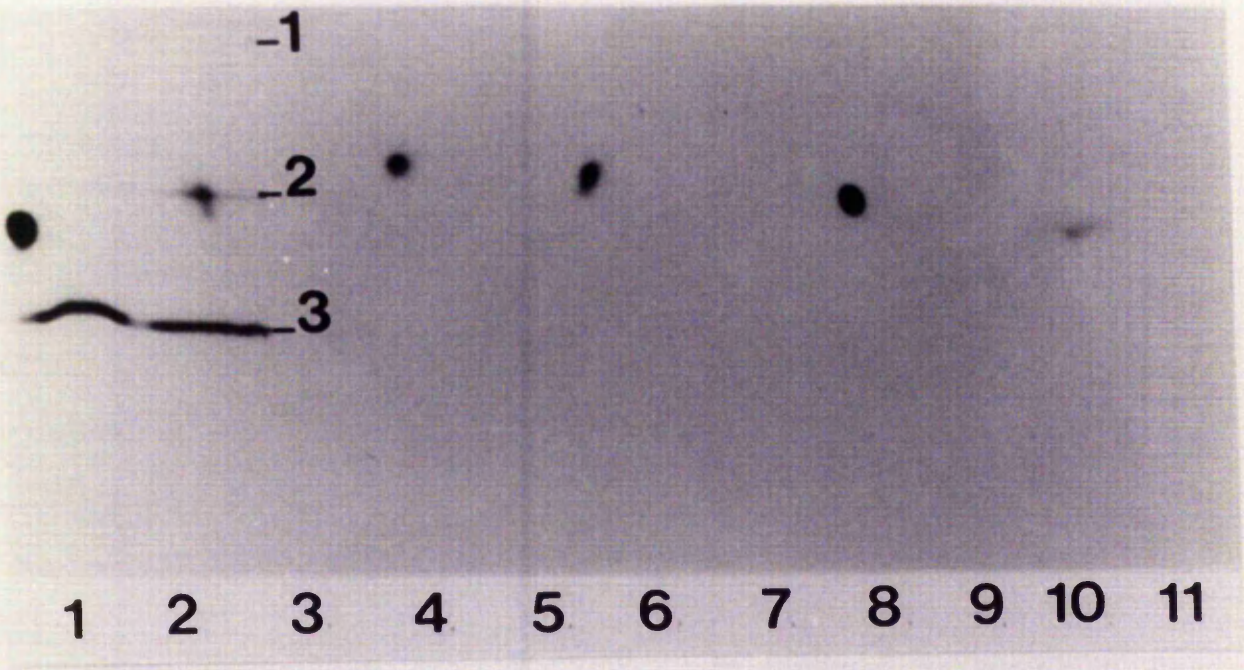


Figure 3.34: Fluorograph of part of the gel shown in Figure 3.33 labelled with 1μg/ml tritiated penicillin. Labels 1, 2 and 3 mark the expected positions of PBP1, PBP2 and PBP3.

1: Crude membranes

2: Solubilised membrane supernatant

3: Solubilised membrane pellet

4: Non-adsorbed protein from affinity column

5: Washing buffer 1

6: Washing buffer 2

7: Washing buffer 3

8: Molecular weight markers

9: Eluted dialysed protein from affinity column

10: Non-adsorbed protein from ion-exchange column

11: Purified concentrated PBPs from ion-exchange column

by fluorography. This fluorograph, Figure 3.35, shows that PBP3 was eluted.

3.3.4.10 Comparison of 7-amino-cephalosporanic acid and 6-amino-penicillanic acid as ligands for affinity purification of PBPs. Membranes from the sensitive strain I and the resistant strain 95 were applied to affinity columns of sepharose with the ligands 6-APA and 7-ACA and three elution conditions applied to each column (all incubated at room temperature).

1. 1M hydroxylamine for 1 hour.
2. 5M hydroxylamine for 1 hour.
3. 5M hydroxylamine overnight.

The eluates were tested for PBPs by fluorography. PBP3 was eluted from the 7-ACA column by eluting with 1M hydroxylamine for 1 hour, but it was eluted from 6-APA only after elution with 5M hydroxylamine overnight (results not shown). Cephalosporins have higher MICs against *Neisseria gonorrhoeae* than penicillins. This may be due to their PBPs having a lower affinity for the cephalosporin molecule. If the antibiotic is bound less avidly to the PBP this may account for the less vigorous elution conditions required to remove the PBPs from the 7-ACA-sepharose. An attempt was made to bind cephaloridine to sepharose-4B use as a low affinity ligand for PBP purification. Unfortunately no active cephaloridine could be bound using the carbodiimide condensation reaction. Hydroxamate assay of the treated sepharose showed that although active cephaloridine was present in solution, none could be detected bound to the sepharose.

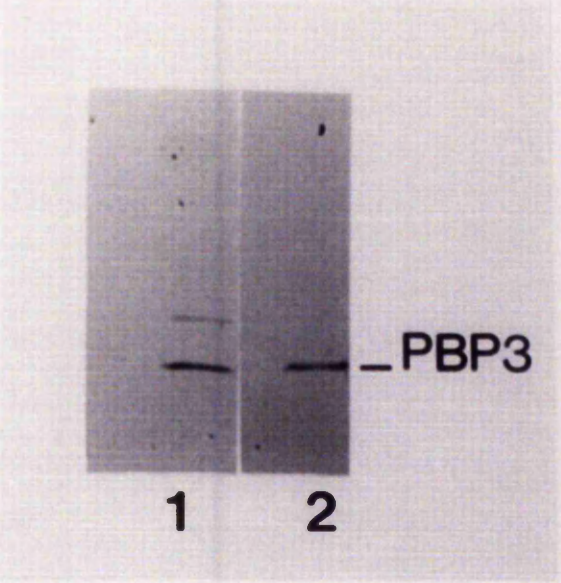


Figure 3.35: Fluorograph of samples labelled with 1μg/ml tritiated penicillin from affinity purification of the membrane proteins of sensitive strain I on 7-ACA-sepharose using 5M hydroxylamine to elute the protein. PBP3 is marked.

1: Solubilised membrane proteins (this lane shows PBP1, PBP2 and PBP3)

2: Purified concentrated PBP3 following affinity purification and ion-exchange chromatography.

3.4 Conclusions

PBP3 has been purified in small amounts from a sensitive and a resistant strain of *Neisseria gonorrhoeae* using 7-amino-cephalosporanic acid and 6-amino-penicillanic acid as ligands and rigorous conditions for elution. The lower-affinity ligand, 7-ACA, proved to be an easier medium from which to elute the PBP. PBP1 and PBP2 were only eluted in an altered form, perhaps as proteolytic breakdown products containing the active site for penicillin. These experiments have shown that the PBP-penicilloyl complexes in *Neisseria gonorrhoeae* are extremely stable compared to those in *Escherichia coli* making it difficult to purify the PBPs for further study.

3.5 DISCUSSION AND SUMMARY.

3.5.1 Detection of PBPs.

Twenty-six strains of *Neisseria gonorrhoeae* were identified, characterised and their sensitivities to methicillin and benzyl-penicillin were determined. Strains which were fully sensitive, low level resistant and high level resistant together with those which were beta-lactamase producing were selected for further study. Three strains I, 13 and 95 representing a sensitive, beta-lactamase producer and an intrinsically resistant strain were used for the PBP assays and the majority of the work on Western blotting. Strain I was not the most sensitive strain characterised although the MIC value for benzyl penicillin was very close to the value for a fully sensitive strain . The PBP assays using fluorography confirmed the altered penicillin binding to PBP's 1 and 2 in resistant strains (Dougherty *et al.*, 1980). However the comparison of the binding of the label to different PBP's was crude. The relative binding would have been more accurately demonstrated using a densitometer to quantify the label in each band. Also the day to day experimental variation in batches of cells and membranes made it sometimes difficult to compare results between different fluorographs. It would have been interesting to use the strains used by Dougherty *et al.* (1980), including the isogenic transformants, to compare the PBP profiles with the strains used in this study. Further suggestions for improvement of the fluorography would be to use radiolabelled protein marker standards so that relative mobilities of the PBPs could be compared with standards on the developed fluorograph. The experiments to show binding of methicillin to PBP was done

by a competition assay. This indirectly showed that *Neisseria gonorrhoeae* PBPs bind methicillin, however this could have been confirmed using a direct labelling of the PBPs with ^{3}H methicillin.

An alternative method for detecting PBPs was investigated using antibodies raised against protein beta-lactam conjugates. The anti-methicillin conjugate sera were shown to be useful in detecting the PBPs bound with methicillin in Western blots. The relative binding of the antibodies to the beta-lactam bound PBPs correlated with the amount of penicillin which bound to the individual PBPs in fluorography. Qualitative differences in the intensity of specific bands, thought to be methicillin bound PBPs 1 and 2, were noticeable between stains of different sensitivity to penicillin and methicillin. The ultimate experiment to confirm the location and presence of a PBP-beta-lactam band would have been to label PBPs with radioactive methicillin, perform blotting and subsequent autoradiography of the developed blot to confirm if putative PBP-methicillin bands lined up with a radioactive methicillin band. The fact that the anti-methicillin serum was able to detect PBP-beta-lactam bands and the anti-penicillin serum was not, could be due to several reasons. The antibodies which recognise the side group epitopes on the methicillin molecule may be the ones which bind to the methicillin PBP complex.The benzyl group antibodies may have been at too low concentration in the penicillin conjugate sera to bind to the comparable penicillin PBP complex. Data from competitive inhibition ELISAs in Chapter 2 suggested that a proportion (more

especially the methicillin-conjugate) of serum antibodies produced against both conjugates were specific for the specific side group determinants. It is possible that the antibodies against the core part of each beta-lactam are unable to bind to the epitopes when the beta-lactam-PBP complex is formed. This would suggest that core antibodies are not involved in the binding of PBP-beta-lactam bonds in Western blots. The results of blots using several beta-lactams would support this hypothesis as the cross reaction of the anti methicillin antibodies for other beta-lactams was low. Only weak binding to two other beta-lactams was observed suggesting that the core beta-lactam antibodies were not involved in the reaction.

The main drawback of this technique was the very high non-specific binding of antibodies to blots. Various reasons for this phenomenon have been discussed and further work to resolve this problem was suggested. Important areas to investigate would be the concentration of antibiotic in the reaction assay mixture as penicillins are known to conjugate with a variety of proteins both *in vivo* and *in vitro* which may explain much of the high background staining. Another improvement would be to use a more purified extract of PBPs to cut down contaminating protein. However, the purification of *Neisseria gonorrhoeae* PBPs proved to be difficult as discussed below. Unfortunately too little purified PBP protein from *Escherichia coli* was obtained for Western blot analysis of beta-lactam PBP complexes. Overall the method of visualising beta-lactam bound PBPs using this Western blotting procedure was successful and constitutes a viable

alternative to the conventional methods already in use provided a more specific antiserum can be produced.

3.5.2 Purification of PBPs.

The purification of PBPs from *Neisseria gonorrhoeae* using the same published methods for *Escherichia coli* (Curtis and Strominger, 1980) was not straight forward. Many problems were encountered in the adaptation of the technique which was shown to be suitable for purification of *Escherichia coli* PBPs. Difficulties in the solubilisation of PBP3 were resolved by investigating the amount of detergent to solubilise the crude membranes. However, an alternative approach would have been to test other suitable detergents although technical literature suggests Triton X-100 to be most suitable. Due to the stability of the PBP-ligand complex various ligands and elutants were tested. The ligands with lower activity in terms of antimicrobial action in derivative antibiotics (ie 7-ACA) used for cephalosporin antibiotic synthesis proved to be better ligands from which the isolated PBPs could be released. However strong elutants were required to elute small amounts of PBP3 from 7-ACA. PBP 1 and 2 were only eluted in altered form. The very stable complex formed between *Neisseria gonorrhoeae* PBPs and penicillin reflects the usefulness of this drug for treating gonorrhoea since the penicillin must have high affinity for the target enzymes in the cell to be of value in treatment.

The strong bonds between the PBPs and the ligands caused difficulty in releasing the PBPs with nucleophilic elutants such as hydroxylamine. Alternative approaches would have been either

to use a sepharose with a shorter spacer arm which may have caused steric hindrance such that the PBP could not bind to the ligand with such high affinity, or to cleave the ligand-PBP complex chemically. Further purification of the PBPs could possibly be achieved using conventional techniques such as gel filtration and ion exchange chromatography etc. However, the use of non denaturing detergents to solubilise the enzymes from membrane lipids may cause operational problems. An assay for PBP containing fractions could involve radioactive labelling of samples followed by liquid scintillation counting to identify penicillin binding fractions. The main problem of these multiple step purifications is the necessity to start the procedure with large amounts of membrane protein. Rodriguez and Saz (1978) isolated penicillin binding fractions from *Neisseria gonorrhoeae* solubilised membranes in Tris-Nonidet detergent on DEAE-DE52 cellulose column.The problems met in the purification of *Neisseria gonorrhoeae* PBPs meant that crude materials were used in the visualisation of PBP-beta-lactam complexes in Western blots.

Chapter 4

PURIFICATION AND PROPERTIES OF THE BETA-LACTAMASE OF *NEISSERIA GONORRHOEAE*

4.1 INTRODUCTION

4.1.1 Beta-lactamase as a resistance mechanism

Beta-lactamases have long been known to play a significant role in the resistance of bacteria to penicillins and cephalosporins, the first report of such resistance being in a letter to Nature by Abraham and Chain (1940). The widespread use of penicillin in medicine has led to the emergence of resistant bacteria in patients who then failed to respond to treatment with the drug. A resistant strain of *Staphylococcus aureus*, for example, was found to produce a penicillinase which converted benzyl penicillin to the inactive penicilloic acid (Kirby 1945). This led pharmaceutical companies to synthesise new forms of penicillin which were stable to beta-lactamases. One of these new forms was methicillin, the first semi-synthetic penicillin to be derived from 6-amino-penicillanic acid, which was resistant to hydrolysis by beta-lactamase. However the use of these new penicillins caused bacteria to evolve other mechanisms of resistance not involving beta-lactamase. One such mechanism is the intrinsic resistance conferred by the extra penicillin-binding protein found in methicillin-resistant *Staphylococcus aureus* (Brown and Reynolds 1980). Another solution to the problem posed by the production of beta-lactamases by bacteria has been to develop inhibitors of the enzyme which can be used in conjunction with the original (beta-lactamase-susceptible)

penicillins. An example such a combination is Augmentin, a 2:1 mixture of amoxycillin and clavulanic acid. This mixture has been effective in treating disease caused by beta-lactamase-producing *Neisseria gonorrhoeae*.

Due to the importance of beta-lactamase as a mechanism of resistance much research has been undertaken into the origins, characterisation, properties and classification of these enzymes. Inevitably this kind of work requires the isolation and purification of the enzymes before a detailed study can be undertaken.

4.1.2 Detection and assay of beta-lactamases

The plasmid-mediated beta-lactamase in *Neisseria gonorrhoeae* is constitutive and can be detected readily in actively-growing cells and in soluble protein extracts from cells disrupted by ultrasonication. Various detection and assay techniques have been used but the most useful specific assay for beta-lactamase involves the use of nitrocefin, a chromogenic cephalosporin developed by Glaxo. This cephalosporin is 3-(2,4-dinitrostyrl)-(6R,7R)-7-(2-thienylacetamido)-ceph-3-em-4-carboxylic acid, E isomer) and it undergoes a rapid colour change from yellow to red (absorbance maximum at 486nm) when the amide bond in the beta-lactam ring is hydrolysed (O'Callaghan *et al*. 1972). This method provides a reliable fast method for the detection of beta-lactamase in cell cultures, isoelectric focusing gels, polyacrylamide gels and fractions following enzyme purification.

Sanchez-Pescador *et al.* (1988) described a rapid chemiluminescent nucleic acid assay for the detection of TEM-1 beta-lactamase-mediated penicillin resistance in *Neisseria gonorrhoeae* and other bacteria. The assay, which was 100 times more sensitive than commercially-available colourimetric assays, was based on oligonucleotide probes hybridising with target DNA sequences of beta-lactamase plasmids.

4.1.3 Purification of beta-lactamases

Various methods have been utilised to purify a variety of beta-lactamases. Dale and Smith (1971) purified a beta-lactamase specified by the resistance factor R-1818 in *Escherichia coli* and *Proteus mirabilis* using a combination of ammonium sulphate precipitation, ion-exchange chromatography and gel filtration chromatography. Purification of the *Escherichia coli* protein to one band on SDS-PAGE was achieved but the *Proteus mirabilis* enzyme was not completely purified by this method.

Baumann *et al.* (1989) used ion-exchange and gel filtration chromatography to purify a periplasmic cephalosporinase from *Rhodobacter sphaeroides*. Their final step of purification involved preparative gel electrophoresis.

Cartwright and Whaley (1984) reported a one-step purification method for beta-lactamase from crude cell extracts using affinity chromatography on boronic acid gels. The technique was used to purify the serine beta-lactamases from Gram-negative bacteria (*Pseudomonas aeruginosa, Pseudomonas maltophilia, Bacillus*

cereus, *Enterobacter cloacae* and *Klebsiella aerogenes*, the beta-lactamases from which were inhibited by boronic acids. Briefly inhibition assays of the hydrolytic activity of the enzyme to be purified were carried out using nitrocefin as a substrate for the beta-lactamase and *m*-amino-phenyl-boronic acid as the inhibitor. In those cases where the boronic acid inhibited the activity of the enzyme then the enzyme was regarded as suitable for purification by this technique. The affinity column contained phenyl boronic acid coupled to agarose via a spacer arm. A hydrophobic spacer arm was used to purify beta-lactamases with relatively low affinity for the ligand and a hydrophilic spacer arm for beta-lactamases with a high affinity for the ligand. 0.5M sodium chloride in triethanolamine hydrochloride buffer at pH 7 was used as the loading buffer and elution was then carried out with 0.5M sodium chloride in 0.5M sodium borate at pH 7. The elution relies on the fact that the beta-lactamase binds reversibly to the *m*-amino-phenyl-boronic acid and is removed when the borate breaks the bond between the beta-lactamase and the amino group of the *m*-amino-phenyl-boronic acid.

Eriquez and D'Amato (1979) purified the TEM-like beta-lactamase from *Neisseria gonorrhoeae* by affinity chromatography using a variety of beta-lactam ligands bound to Sepharose. They found that 7-amino-cephalosporanic acid and 6-amino-penicillanic acid bound to Sepharose via a five to eight carbon spacer arm were suitable ligands for purification. The similar TEM-1 beta-lactamase of *Escherichia coli* was purified by Bibi (1989) using a monoclonal antibody directed against the beta-lactamase. The

antibody was bound to Sepharose-4B and partially-purified beta-lactamase was applied to the column. The enzyme was eluted with benzyl penicillin or cloxacillin. A fast flow-rate was essential with both eluants for different reasons. With benzyl penicillin rapid hydrolysis of the penicillin occurred if the flow-rate was too slow which allowed the enzyme to rebind to the ligand. Cloxacillin on the other hand had a very high affinity for TEM-1 beta-lactamase (Km 13μM, Labia *et al.* 1979) so a fast flow-rate was necessary to ensure that the beta-lactamase was not irreversibly inhibited by the cloxacillin (Citri *et al.* 1976).

Following purification of the enzymes detailed studies on the characteristics and properties can be undertaken. One method used is that of iso-electric focusing. Matthew *et al.* (1975) used analytical iso-electric focusing for the detection and identification of beta-lactamases from Gram negative bacteria. They demonstrated that the presence of beta-lactamase in crude extracts could be visualised by this technique although a main band was often accompanied by a group of much fainter satellite bands. The reason for this was not known.

4.1.4 Antibodies against beta-lactamases

Antibodies against beta-lactamases can be used to differentiate between the different types of beta-lactamases found in bacteria and also to recognise common determinants in the different enzymes. Monoclonal antibodies have been prepared by Murakami and Yoshida (1985) against a cephalosporinase from *Pseudomonas aeruginosa*. They produced ten monoclonal antibodies which were

highly species specific showing no cross-reactions with nine other cephalosporinases nor four penicillinases from other Gram negative bacteria. Nine of the monoclonal antibodies increased the activity of the enzyme and only one inhibited it.

Bibi and Laskov (1990) prepared nine monoclonal antibodies against Class A beta-lactamases. They used a method for screening for low affinity antibodies by assaying for modifications in the catalytic and stability properties of the beta-lactamases in solution. Some of the antibodies stimulated the activity of the enzyme while others inhibited it. Two of the monoclonal antibodies were found to cross-react with different beta-lactamases in the same class suggesting shared epitopes.

Inoue *et al.* (1991) raised rabbit polyclonal antisera against *Klebsiella oxytoca* chromosomal beta-lactamase after isolation, purification and characterisation of the enzyme. The antibodies showed no cross-reactivity with other Gram negative beta-lactamases in neutralisation tests.

4.1.5 Aims of the present work

The aim of this study was to isolate and purify the TEM-1 beta-lactamase from *Neisseria gonorrhoeae*. The protein was used to immunise rabbits for the production of antibodies. The antisera was used to probe for beta-lactamase in Western blots of extracted proteins from *Neisseria gonorrhoeae* and other beta-lactamase-producing species to detect any antigenic cross-reaction between their enzymes.

The antisera was then evaluated for use as a reagent for the detection of beta-lactamase-mediated resistance in *Neisseria gonorrhoeae* as an alternative to chemical and spectrophotometric assays.

4.2 MATERIALS AND METHODS

4.2.1 Culture and extraction of beta-lactamase-producing strains

Beta-lactamase producing strain 13 was used for the major part of the work reported here. The beta-lactamase-producing strains of *Neisseria gonorrhoeae* were maintained in culture on GC Base agar (Difco) containing 1% Isovitalex (BBL) at 37°C in a moist incubator with an atmosphere containing 10% carbon dioxide in air. Large batches of cells were grown in 7.2 litre volumes of proteose peptone broth (Oxoid) containing 1% Isovitalex and 1.2g of sodium bicarbonate per 600ml. Flasks of broth were inoculated with 20ml of an overnight culture in broth of the required strain. The starter culture was checked for purity by Gram stain and assessed for beta-lactamase production by the Nitrocefin test (described in section 3.2.3.5). The flasks were incubated with vigorous shaking at 37°C for 20 hours then the culture was centrifuged at 5000rpm at 20°C to deposit the cells. The cells were washed twice in 0.85% sodium chloride then resuspended in 4-8ml of 20mM imidazole buffer at pH 6.5. The suspension was cooled to about 4°C and the cells subjected to 5 x 30 second bursts of ultrasonication on an MSE ultrasonicator on maximum power using a large (diameter 2.5 cm) probe. The cell suspension was cooled in an iced water bath for 1 minute between each 30-second burst. The extract was centrifuged at 50,000g at 4°C for 30 minutes. The supernatant containing the crude beta-lactamase was assayed for beta-lactamase with the Nitrocefin assay (described below in section 4.2.2) then stored at -20°C.

4.2.2 Nitrocefin assay for beta-lactamase

Nitrocefin, the chromogenic cephalosporin used in the beta-lactamase test, was also used as a substrate for assaying beta-lactamase activity. A stock solution of Nitrocefin of 0.5mg/ml, made up as described in section 3.2.3.5, was diluted to 50μg/ml in 66mM sodium phosphate buffer pH 7.4. Enzyme preparations (5μl volumes) were assayed by measuring the absorbance of the Nitrocefin solution at 486nm over a period of 1 minute after addition of the beta-lactamase solution. The rate of hydrolysis of the Nitrocefin was determined by plotting absorbance against time and finding the slope of the line. The molar extinction coefficient of Nitrocefin, 20,500 moles/litre (O'Callaghan *et al.*, 1972) was used to calculate the concentration and total number of units of beta-lactamase. The concentration was calculated as follows:

Rate of hydrolysis = X absorbance units/minute

$$= \frac{X}{\text{molar ext. coeff.}} \quad \text{moles/litre/minute}$$

$$= \frac{X}{20{,}500} \quad \text{moles/litre/minute}$$

$$= \frac{X \times 1000}{20{,}500} \quad \mu\text{moles/ml/minute}$$

1 unit of enzyme was defined as the amount of enzyme which gave a rate of hydrolysis of Nitrocefin of 1μmole per minute at 30°C at pH 7.4.

4.2.3 Soluble protein assay

Protein was assayed using the Biorad protein assay for soluble protein which is based on the observation that the absorbance maximum of a solution of Coomassie brilliant blue G-250 shifts

from 465nm to 595nm when binding to protein occurs (Bradford 1976). A stock solution (10mg/ml) of bovine serum albumin in distilled water was diluted to 25μg/ml in the same buffer as the samples. A standard curve was set up using concentrations of BSA of 0-12.5μg/ml x 0.8ml = 0-10μg protein. Samples were diluted in the same buffer as the standards (to the same volume of 0.8ml). Both standards and samples were prepared in duplicate. 200μl of neat reagent (containing dye, phosphoric acid and methanol as supplied by Biorad) was added to the 0.8ml volumes of samples and standards and the tubes were thoroughly mixed. The absorbances were read at 595nm between 5 and 60 minutes later. A graph of absorbance at 595nm versus μg protein was plotted and the unknown protein concentrations were estimated from it. This assay provided a fast reliable method for the determination of protein concentrations in fractions during the beta-lactamase purification. A typical standard curve of 0-10μg bovine serum albumin is shown in figure 4.1.

4.2.4 One-step affinity purification scheme for beta-lactamase

This was based on the method of Eriquez *et al.* (1979). 7-amino-cephalosporonic acid was bound to ECH-Sepharose in a carbodiimide condensation reaction as described in Section 3.1.4. A 10ml column of the 7-ACA-Sepharose was poured and equilibrated in starting buffer (0.05M tris-phosphate buffer pH 6.8 or 0.1M sodium acetate buffer at pH 4.4, depending on whether elution scheme a or b was being used) at 4°C. The beta-lactamase preparation was carried out as detailed in section 4.2.1 except that cells were resuspended in the appropriate starting buffer

for sonication instead of imidazole buffer. Samples of crude beta-lactamase (1-2ml containing about 10mg protein) were loaded onto the column. Two elution schemes (a and b, as below) were tried using five column volumes of elution buffer. A flow rate of 0.5ml/minute was used to collect 4ml fractions.

(a) 0.05M tris-phosphate buffer at pH 6.8 for equilibration and washing followed by the same buffer containing 0.8M sodium chloride for elution of the beta-lactamase.

(b) 0.1M sodium acetate buffer at pH 4.4 for equilibration and washing followed by 0.1M phosphate buffer at pH 6.8 for elution of the beta-lactamase.

4.2.5 Multiple-step purification scheme for beta-lactamase

This included anion-exchange chromatography, gel filtration, non-SDS-polyacrylamide electrophoresis and electro-elution.

4.2.5.1 Anion-exchange chromatography

Pharmacia Q-Sepharose Fast-flow with the partial structure $CH_2N^+(CH_3)_3$ was used. A 70ml glass column, with flow adaptors, was poured with the Sepharose and equilibrated at room temperature with 20mM imidazole buffer at pH 6.5. A Pharmacia Frac-100 system was employed with a peristaltic pump and fraction collector . The flow rate was adjusted to 1ml per minute. The column was loaded with crude filtered beta-lactamase using a flow adapter and washed extensively until the absorbance of the washings at 280nm was negligible. A gradient of 0 to 0.5M sodium chloride in 20mM

imidazole at pH 6.5 was commenced using a gradient former and applied over 5 column volumes. The absorbance at 280nm of collected fractions was monitored for protein and the fractions were screened for beta-lactamase activity by the Nitrocefin test (Section 3.2.3.5). Fractions which were found to contain active beta-lactamase by this test were then assayed by the Nitrocefin assay described in section 4.2.2. Protein concentration was estimated by the Biorad assay and the specific activity in units of enzyme per mg of protein was calculated. Fractions which had a higher specific activity than the crude beta-lactamase were pooled and ultrafiltered using 10,000 molecular weight cut-off membranes in an Amicon ultrafiltration cell.

4.2.5.2 Gel filtration

G50 Superfine Sephadex was boiled in 0.1M tris-HCl buffer with 0.05M sodium chloride at pH 7 and a 4ml column in a glass pipette was poured. Glass wool was used to plug the base of the column. The Sephadex was washed with 0.1M tris-HCl at pH 7 and then the column was loaded carefully with 200μl of enriched beta-lactamase after anion exchange chromatography and ultra-filtration (dialysed against 0.1M tris-HCl at pH 7). The column was eluted under gravity with 0.1M tris-HCl at pH 7 and 500μl fractions were collected. The fractions were assayed for beta-lactamase and protein as before. Fractions with active beta-lactamase were pooled and concentrated into 0.1M tris-HCl at pH 7 using an Amicon Centricon microconcentrator. The column was not calibrated with protein of known molecular weight.

4.2.5.3 Preparative non-SDS-PAGE

Large 7.5% polyacrylamide gels were prepared according to the following recipe:

Resolving gel

30% acrylamide	7.5ml
1% bis-acrylamide	1.3ml
1M tris-HCl pH 8.8	11.2ml
Distilled water	9.8ml

The solution was degassed for 15 minutes then the following added:

200mg/ml ammonium persulphate (freshly prepared)	156μl
TEMED	20μl

The gel was poured and overlaid with butanol until it had polymerised. It was stored at 4°C overlaid with 0.25M tris-HCl at pH 8.8 until needed.

On the day of the assay a stacking gel was prepared as follows:

Stacking gel

30% acrylamide	1.67ml
1% methylene bis-acrylamide	1.5ml
1M tris-HCl pH 6.8	1.75
Distilled water	5.6ml

After 15 minutes degassing the following were added:

200mg/ml ammonium persulphate (freshly prepared)	65μl
TEMED	10μl

A large comb was inserted and the gel left to polymerise.

The beta-lactamase sample was diluted 1:1 in sample buffer containing no SDS (Section 3.2.6) and loaded onto the washed 7.5% polyacrylamide gel. The gel was electrophoresed in a running buffer consisting of 28.2g/l glycine in 6g/l tris-HCl buffer at pH 8.3 using a constant current of 30mA through the stacking gel and 35mA through the resolving gel.

4.2.5.4 Detection of beta-lactamase in gels

A piece of Whatman filter paper (Number 54) soaked in a solution of 500μg/ml of Nitrocefin was laid over the gel covering one lane of the beta-lactamase sample. The other lanes on the gel were not stained. The rapid development of a pink-red colour in a band in the gel indicated the presence of beta-lactamase. The corresponding unstained gel region containing the active beta-lactamase was carefully cut out and prepared for electroelution. The rest of the gel was fixed and stained for protein.

4.2.5.5 Electrophoretic elution of protein

The Biorad electroeluter was set up according to the manufacturers instructions paying particular attention to exclude air bubbles from the glass tube containing the membrane. The gel slice was eluted for 3 hours at 10mA in the same tris-HCl-glycine buffer as was used for electrophoresis. After elution the eluted protein was quickly and carefully removed from the membrane and the eluted solution assayed for protein and beta-lactamase. A sample of the eluted protein was electrophoresed on a non-SDS-polyacrylamide gel and also on a 15% SDS-polyacrylamide gel to determine the purity of the preparation. The apparent molecular

weight of the beta-lactamase was estimated (from the SDS-polyacrylamide gel) using Amersham Rainbow molecular weight markers as described in Section 3.2.6.

4.2.6 Production of antisera against the beta-lactamase

4.2.6.1 Preparation of the antigen

The preparation used for immunisation consisted of the polyacrylamide gel slices containing the beta-lactamase. The band on the 7.5% non-SDS gel identified by the Nitrocefin filter paper test described earlier was cut out with a scalpel. The slice was frozen at -20°C then lyophilised in an Edwards vacuum freeze-drier for 12 hours according to the manufacturers instructions. The dried slice was ground into smaller pieces using a pestle and mortar and stored at -20°C in plastic ampoules.

4.2.6.2 Immunisation schedule

A pre-immunisation sample of blood (the pre-bleed) was taken from each of the three rabbits used for immunisation and the serum separated and stored at -20°C. Each rabbit then received a primary dose of 7 units of beta-lactamase from the freeze-dried stock resuspended in 1ml aluminium hydroxide adjuvant. This suspension was incubated at room temperature for 1 hour then broken up into a thick suspension with a syringe. Each rabbit was inoculated subcutaneously with 4 x 0.25ml. Fourteen days later a sample of blood was taken from each rabbit and the serum stored at -20°C. The next day each rabbit was inoculated with the same amount of beta-lactamase in the same volumes as the primary dose. Fourteen days later a further test bleed was taken followed the

next day by a further inoculation as before. A further test bleed was taken about 1 week later. In summary:

Day		
0	Prebleed	
1	1st dose	7 units in 4 x 0.25ml s/c
15	1st test bleed	
16	2nd dose	7 units in 4 x 0.25ml s/c
30	2nd test bleed	
31	3rd dose	7 units in 4 x 0.25ml s/c
38	3rd test bleed	

4.2.7 ELISA for beta-lactamase antibodies in antisera

An ELISA test, using partially-purified (by anion-exchange chromatography) beta-lactamase at a concentration of 500ng/well bound to the plates in 50mM carbonate buffer pH 9.6, was used to detect and measure anti-beta-lactamase antibodies in the antisera. Dilutions of the pre-bleed and test-bleed sera were assayed using the technique described in detail in Chapter 2.

4.2.8 Western blotting

Western blots were also used to detect antibody to beta-lactamase in the antisera. Beta-lactamase preparations of various degrees of purity (detailed in the results text and legends) were run on a 15% SDS-polyacrylamide gel, blotted onto nitrocellulose and stained with the anti-beta-lactamase antisera diluted in 66mM sodium phosphate buffer pH 7.4 at concentrations as detailed in the results legends. The detailed technique used was as described in Chapter 2. In some experiments samples of beta-lactamase from

species other than *Neisseria gonorrhoeae* were also used in Western blots to detect possible cross-reactivity between the beta-lactamases.

4.2.9 Renaturation of the beta-lactamase from SDS-polyacrylamide gels

A technique whereby the beta-lactamase resolved on 15% SDS-polyacrylamide gels could be renatured was developed. Samples of beta-lactamase in SDS-PAGE sample buffer were heated to 37°C or 100°C for 3 minutes before loading them onto the gel. The resolved gels were washed extensively in several changes of 10mM potassium phosphate buffer Ph 7, for at least 1½ hours at room temperature to remove the SDS. The gel was then assayed for active beta-lactamase using filter paper impregnated with Nitrocefin as described in section 4.2.5.4 before. The gel was photographed and blotted onto nitrocellulose as described before. The nitrocellulose was carefully marked in the exact location of the active beta-lactamase band and then immuno-stained with antiserum. Any immuno-stained bands were then examined for correlation with the marked position of the active beta-lactamase band.

4.2.10 Immuno-affinity purification of the beta-lactamase

A simplified procedure for purification of the beta-lactamase was developed. This used the antibodies prepared against the beta-lactamase since the one-step procedure did not work as well as expected and the multi-step procedure was time-consuming. This procedure involved two steps, anion-exchange chromatography

followed by immuno-affinity chromatography.

4.2.10.1 Purification of IgG antibodies by ion-exchange chromatography

IgG was purified from rabbit anti-beta-lactamase antisera using Whatman DE-52 ion-exchange cellulose. 100g of DE-52 was added to 500ml of 0.01M sodium phosphate buffer at pH 8 in a 1 litre beaker. The slurry was titrated back to pH 8 by the addition of 1M HCl. The slurry was left to stand for 30 minutes before the supernatant was removed, together with any fines. A further volume of the phosphate buffer was added to the slurry in the 1 litre beaker. This cycle of settling, decanting and resuspension was repeated twice more. The washed slurry was poured into a Buchner funnel containing two layers of filter paper and the cellulose was sucked almost dry with a vacuum pump. 50g of this equilibrated cellulose was mixed with 10ml of rabbit serum diluted to 40ml with distilled water. The slurry was stirred thoroughly every 10 minutes for 1 hour at 4°C. The liquid was removed from the cellulose using a Buchner funnel and vacuum pump. The cellulose was washed with 4 x 20ml of 0.01M phosphate at pH 8. The supernatant containing the IgG was concentrated to about 20ml by ultrafiltration and assayed for protein.

4.2.10.2 Preparation of immuno-affinity chromatography gel

CN-Br-activated Sepharose-4B (Pharmacia) was used to prepare an affinity medium with adsorbed anti-beta-lactamase IgG. About 1g of the Sepharose was washed and reswollen on a sintered glass filter with 200ml of 1mM HCl. 20mg of the purified IgG protein

was dialysed against coupling buffer (0.5M sodium chloride in 0.1M sodium bicarbonate at pH 8.3), added to the Sepharose in a universal bottle and mixed end-over-end at room temperature for 2 hours. The non-adsorbed protein was removed from the Sepharose by filtration and assayed for protein. The Sepharose was transferred to 0.2M glycine buffer pH 8 and left at room temperature for 2 hours, to block any remaining active groups. Finally the Sepharose was washed alternately in (i) 0.5M sodium chloride in 0.1M sodium acetate at pH 4, and (ii) coupling buffer 0.5M sodium chloride in 0.1M sodium bicarbonate at pH 8.3. The Sepharose was stored in the final coupling buffer.

4.2.10.3 Purification procedure

The crude beta-lactamase was first partially purified by anion-exchange chromatography as described before. The partially-purified beta-lactamase, containing about 45 units of beta-lactamase in 68ml phosphate buffered saline (PBS) (sodium chloride 8g/l, potassium chloride 0.2g/l, disodium hydrogen phosphate 1.15g/l, potassium dihydrogen phosphate 0.2g/l) at pH 7.4 was passed through the immuno-affinity Sepharose column under gravity. The column was washed extensively with PBS pH 7.4 then the protein was eluted with 10ml of each of (i) 0.1M glycine at pH 2.5 and (ii) 0.1M glycine at pH 2.5 with 10% dioxane. The eluted protein was adjusted immediately to pH 7 with solid tris before being dialysed against PBS at pH 7.4. The dialysed protein was concentrated by ultrafiltration and assayed for beta-lactamase and protein. Purified beta-lactamase was electrophoresed on 15% SDS-polyacrylamide gel, renatured and

tested for beta-lactamase activity as described before, then Western blotted with anti-beta-lactamase antiserum.

4.2.11 Inhibition assays

Beta-lactamase activity, in ion-exchange-purified protein, was also assayed after preincubation with dilutions of pre-bleed and test-bleed sera from rabbits immunised with beta-lactamase. The beta-lactamase was diluted 1:1 in 66mM phosphate buffer at pH 7.4 and assayed with Nitrocefin. Dilutions of the pre-bleed and test-bleed sera were made in the same buffer. At time 0 an equal volume of beta-lactamase solution was added to the serum dilutions. Samples were removed from each dilution at 15 and 30 minutes and assayed for beta-lactamase activity.

4.2.12 Determination of the molecular weight of the beta-lactamase

The molecular weight of the purified beta-lactamase was estimated by SDS-polyacrylamide gel electrophoresis as described before (Section 3.2.6). The molecular weight was also determined by gel filtration using a Superose-12 gel filtration column. Partially-purified beta-lactamase (0.2ml) was loaded onto the column in 20mM tris-HCl buffer at pH 7. The flow-rate was set at 0.3ml/minute and all the fractions were collected, monitored for protein by measuring the absorbance at 280nm, and assayed for beta-lactamase. Three protein molecular weight standards (200,000; 66,000 and 29,000) 1mg of each in 0.1ml, were passed through the column using identical conditions as used for the beta-lactamase sample.

4.2.13 Determination of the pH optimum for beta-lactamase activity

The optimum pH for the activity of the beta-lactamase was determined by measuring the activity of the enzyme at various pH values in the range 2.5 - 11.5. The following buffers were used:

20mM glycine-HCl pH 2.5 to 3.5

20mM sodium acetate-acetic acid pH 4.0 to 5.5

20mM sodium phosphate pH 6.0 to 7.0

20mM tris-HCl pH 7.5 to 8.5

20mM glycine-NaOH pH 9.0 to 10.0

20mM phosphate NaOH pH 11.0 to 11.5

The buffers were equilibrated at 30°C before measuring the rate of hydrolysis of Nitrocefin by 2.5μl of enzyme. The activity, units/ml was plotted against the pH and the optimum pH was deduced from the graph.

4.3 RESULTS AND DISCUSSION

4.3.1 Purification of the beta-lactamase

Purification of the beta-lactamase from strain 13 by the one-step method of Eriquez and D'Amato (1979) was not achieved using either of the elution buffers. The beta-lactamase bound well to the 7-ACA ligand but subsequent elution with the either of the two elution buffers resulted in the release of other proteins as well as the beta-lactamase (results not shown).

4.3.1.1 pH optimum and pI of the beta-lactamase

The effect of pH on the activity of the beta-lactamase from strain 13 was investigated and the results shown in Figure 4.1 where the enzyme activity (in units per ml) at 30°C is plotted against pH. The activity of the enzyme was highest between 7.0 and 7.5. This compares reasonably well with the value of pH 6.8 reported by Eriquez and D'Amato (1979).

The pI of the gonococcal beta-lactamase of strain 13 in crude supernatants (as described in section 4.2.1) was determined using a Pharmacia Phast system to give an indication of the pH which would be required for the anion exchange chromatography. Pre-formed gels containing ampholytes (with a Ph range of 3-9) were run according to the manufacturers instructions and the focused protein was stained using Nitrocefin (in filter paper method 4.2.5.4). The pI was approximately 5.4. Consequently a buffer with a pH of about 1 unit above this value was used in the anion-exchange chromatography to ensure binding of the enzyme to the positively-charged Q-Sepharose.

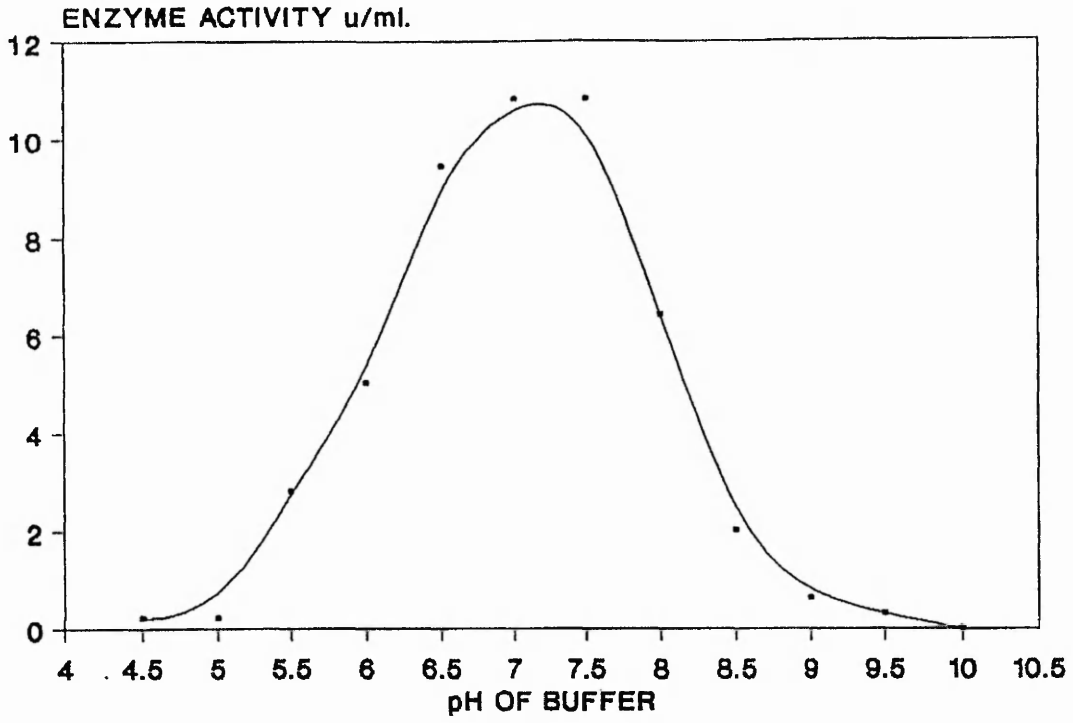


Figure 4.1: pH optimum of *Neisseria gonorrhoeae* beta-lactamase isolated from strain 13. The activity of the enzyme in crude supernatant extracts was measured using the Nitrocefin assay at different pH buffers as described in section 4.2.13.

4.3.1.2 Multiple-step purification of beta-lactamase

The multiple-step purification involved ion-exchange chromatography, gel filtration, non-SDS-PAGE and electroelution. The purification of the beta-lactamase from strain 13 is summarised in Table 4.1 which gives the enzyme activity in units/ml, the total activity, the volume of protein, the protein concentration, and the specific activity of the enzyme, at each stage of the purification. The total activity is calculated by multiplying the activity in units/ml by the volume of protein. A typical standard curve of the Biorad protein assay is given in Figure 4.2.

This procedure resulted in a highly purified beta-lactamase with a specific activity of 1833 units per mg of protein. The best purification that Eriquez and D'Amato (1979) achieved on a 7-amino-cephalosporanic acid column was 43.6 units per mg of protein (one unit of beta-lactamase activity was defined as that amount of enzyme which would hydrolyse 1μmole of beta-lactam antibiotic per minute at 25°C and pH 6.8). However, they recovered almost 80% of the active enzyme compared with only 5% in this purification schedule. The figures given in Table 4.1 at each stage of the purification are for the pooled fractions containing enzyme activity with a higher specific activity compared with the previous stage of the purification. Thus a higher yield may have been achieved if more of the enzyme preparation had been taken through at each stage of the purification. Significant activity, up to 40%, was lost in the ultrafiltration of the protein between the ion-exchange and the

Table 4.1: Multiple-step purification of the beta lactamase from *Neisseria gonorrhoeae* strain 13.

Step	Volume ml	Activity u/ml	Total Activity u	Protein Conc mg/ml	Specific Activity u/mg
1.	38.00	2.20	83.60	9.60	0.23
2.	23.40	2.30	53.80	0.08	28.70
3.	1.80	10.70	19.30	0.20	53.10
4.	0.50	8.80	4.40	0.0048	1833.00

1= Crude preparation

2= Post anion-exchange

3= Post gel filtration

4= Post electrophoresis and electroelution

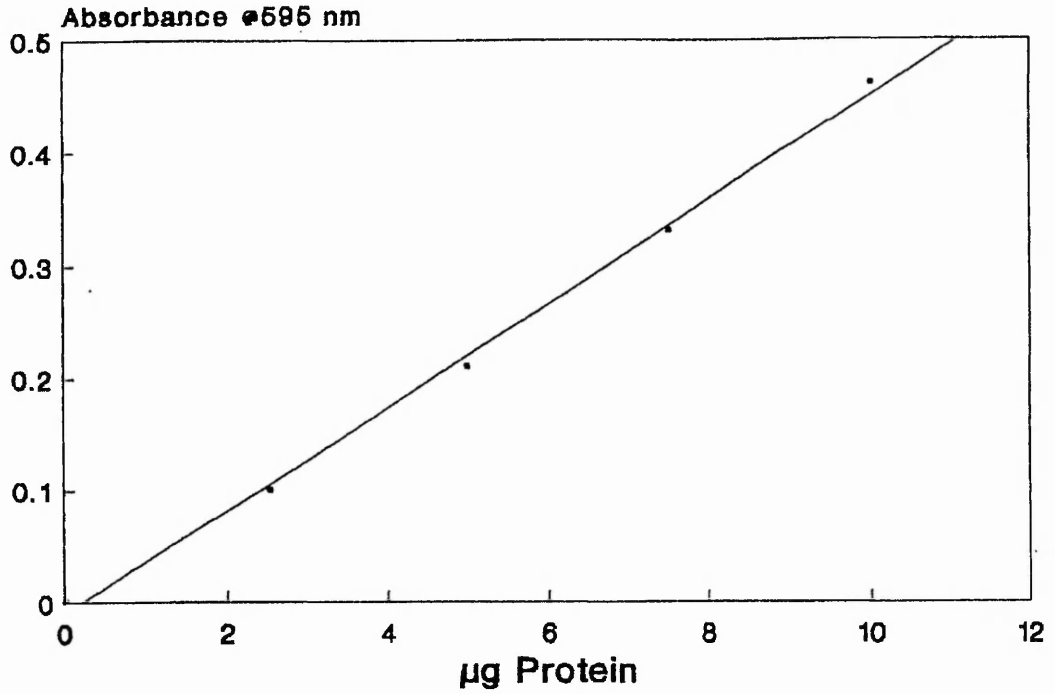


Figure 4.2: Standard curve for Biorad protein assay as described in Section 4.2.3.

gel filtration steps. This problem was later resolved by the use of very low absorbing membranes (Amicon). The final recovery of total active beta-lactamase from the electroeluted gel slice was only 23% of the total activity applied to the electrophoresis and electroelution stage. It is possible that the activity of the recovered enzyme was lost due to the electrophoretic process, or that most of the enzyme remained in the gel slice. The latter explanation is less likely since subsequent staining with Nitrocefin of the gel slice after it had been through the electroelution process revealed that very low activity remained in the gel. However, it can be calculated from the figures that only 0.67% of the total protein applied to the electrophoresis and electroelution stage was recovered suggesting that most of the protein was left in the gels.

The elution profile from the anion-exchange column is shown in Figure 4.3. It can be seen that the beta-lactamase was completely bound with no activity detected in the pre-elution washings. The beta-lactamase was eluted between 0.1M and 0.15M sodium chloride before the majority of the contaminating proteins. This accounts for the high purification achieved by the anion-exchange stage. The gel filtration stage (profile not shown) did not give a particularly high degree of purification but it was useful as it removed some high molecular weight proteins. Previous runs omitting this step had failed to produce preparations as pure as the one reported here. Repeating this stage with a more appropriate gel filtration medium of lower molecular weight range might possibly improve the purification.

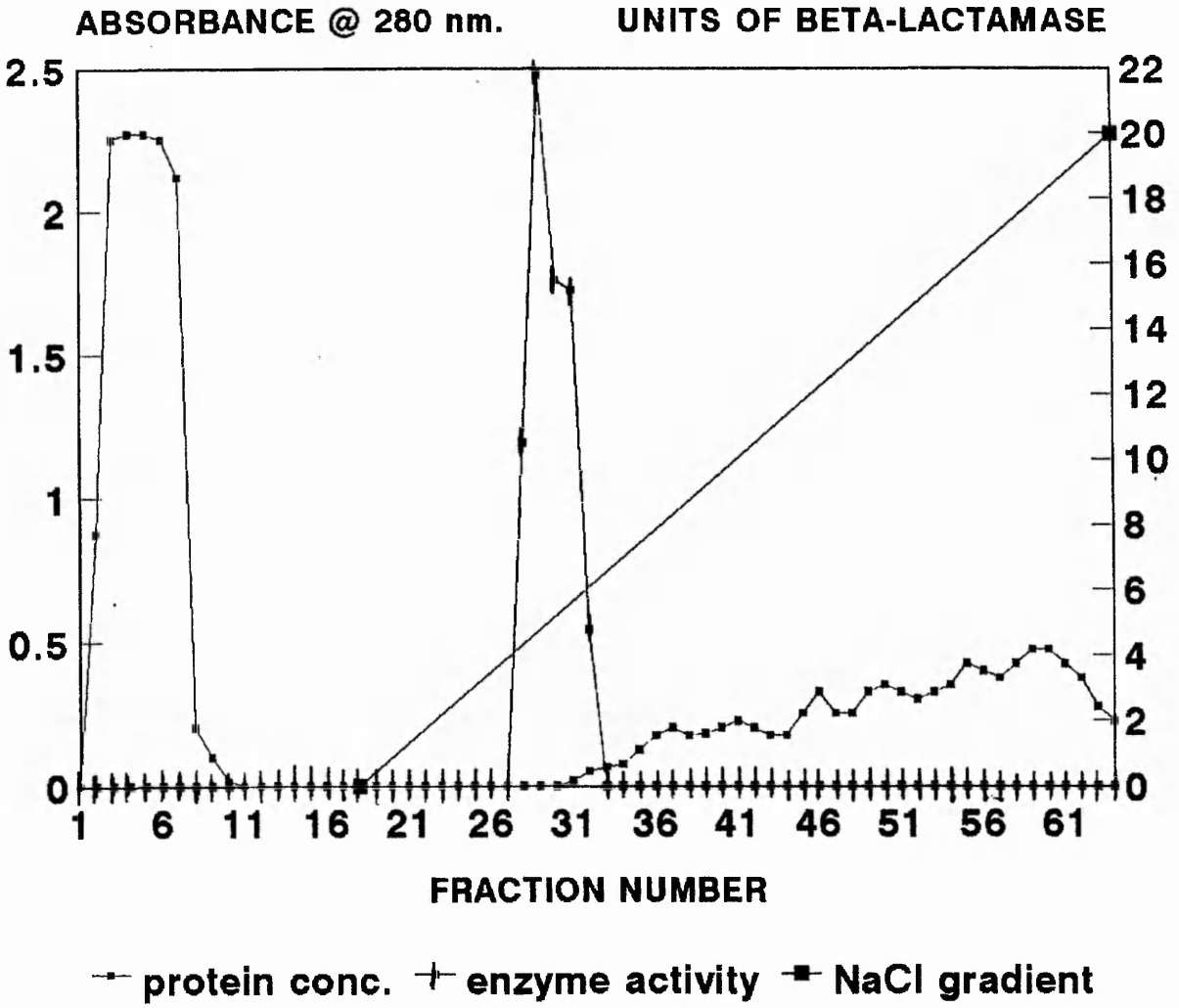


Figure 4.3: Elution profile of crude beta-lactamase from *Neisseria gonorrhoeae* strain 13 on Q-Sepharose anion-exchange resin. 38ml of crude cell extract supernatant was applied to a 70ml column. Non-adsorbed protein was washed through with the loading buffer 20mM imidazole at pH 6.5 until the absorbance at 280nm of the fractions was negligible. The bound protein was eluted with a 0 to 0.5M sodium chloride gradient in loading buffer.

The physical removal of the native beta-lactamase from non-SDS-polyacrylamide gels after electrophoresis was a significant step in purification since large amounts of concentrated beta-lactamase could be visualised by activity staining and then accurately excised from the gel. These gel slices were used for immunisation of rabbits for the production of anti-beta-lactamase antisera. The 15% SDS-PAGE (Figure 4.4) of the electroeluted protein from the gel slice shows one major band at 29,500 (estimated from the Rainbow molecular weight markers shown in Figure 4.5). Analysis of this protein band by non-SDS-PAGE yielded one active beta-lactamase band and subsequent protein staining with Coomassie blue stain confirmed this to be the only protein on the gel.

The molecular weight of the protein was confirmed by gel filtration using a Pharmacia Superose-12 column. The active beta-lactamase band was eluted at the same fraction position as one of the standard marker proteins with a molecular weight of 29,000. Eriquez and D'Amato (1979) reported the molecular weight of the beta-lactamase of *Neisseria gonorrhoeae* to be 25,000 as determined by their method of gel permeation chromatography. This amounts to a difference of about 14% between the molecular weights and this difference can't be explained easily as Weber and Osborn (1969) concluded that the molecular weights of polypeptides could be determined to within an accuracy of ±10% by SDS-PAGE. Repeated analysis of the protein in the present study consistently gave the same higher molecular weight value by SDS-PAGE and this confirmed the value by gel filtration.

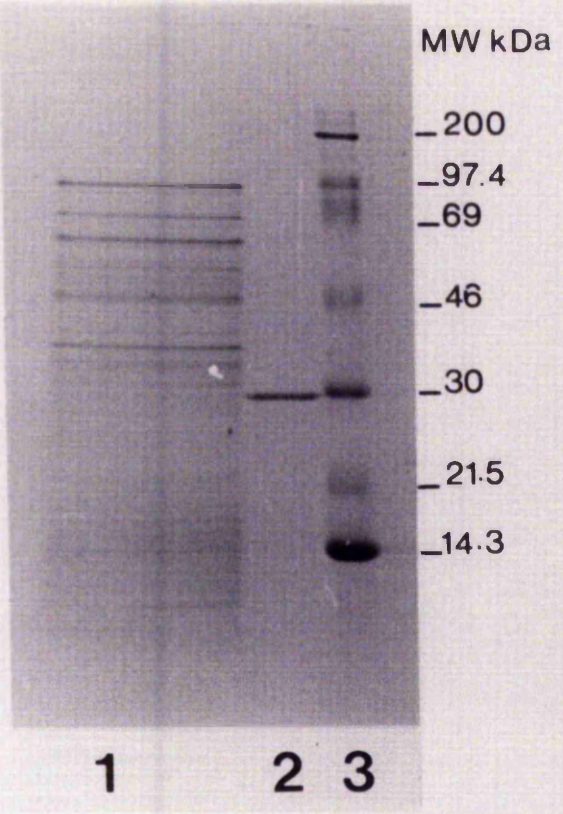


Figure 4.4: SDS-polyacrylamide gel of crude and purified beta-lactamase from *Neisseria gonorrhoeae* strain 13. Molecular weight markers are marked and labelled kD.

Lane 1: Crude cell extract supernatant.

Lane 2: Purified beta-lactamase.

Lane 3: Molecular weight markers.

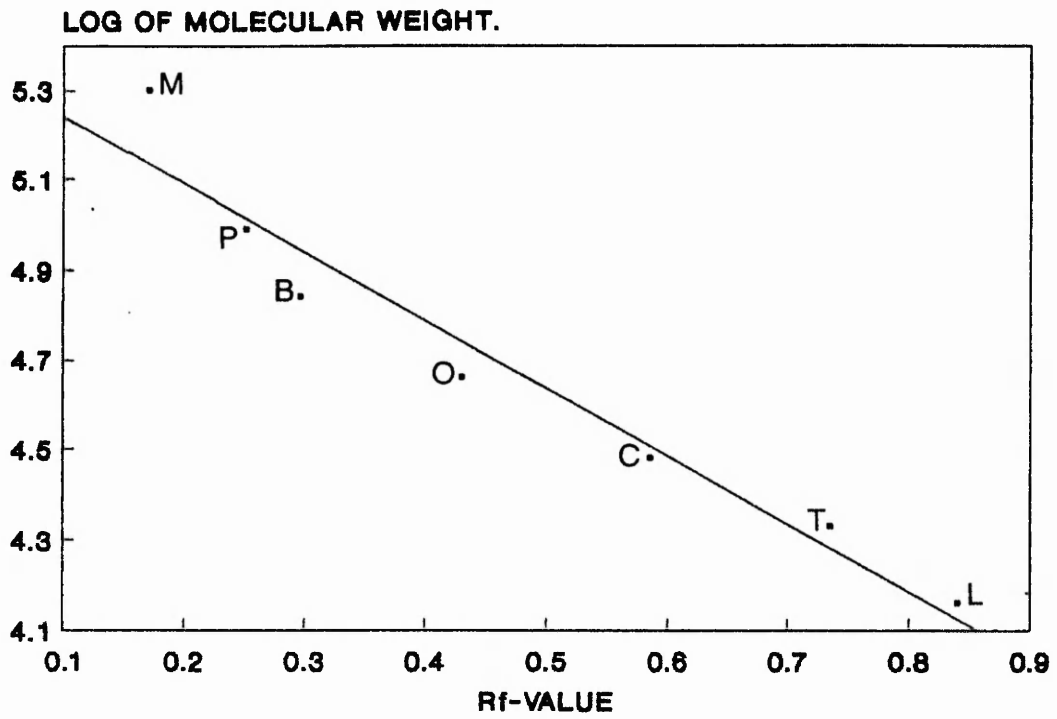


Figure 4.5: Rf values of Rainbow molecular weight markers (Amersham) following electrophoresis on 15% SDS-polyacrylamide gel plotted against the molecular weight. The Rf value is calculated from the distance moved by the protein divided by the distance moved by the marker dye front.

Key: M (Myosin), P (Phosphorylase b), B (BSA), O (Ovalbumin), C (Carbonic hydrase), T (Trypsin Inhibitor) and L (Lysozyme)

4.3.2 Antibodies to *Neisseria gonorrhoeae* beta-lactamase

4.3.2.1 Production of antibodies against the beta-lactamase

The rabbits immunised with the beta-lactamase in acrylamide gel slices using aluminium hydroxide as an adjuvant all responded (weakly) to the immunogen. Prebleed and test bleed sera were tested by ELISA (as described in section 4.2.7) against partially-purified beta-lactamase, by anion exchange chromatography, as the plate antigen. The pre-bleed and the first test bleed sera showed no detectable antibody response. Antibodies were first detected in the second test bleed and developed more strongly in the third test-bleed sera. Titrations of the third test bleed antisera from the three rabbits are shown in Figure 4.6. These titrations showed that antibodies which reacted with the partially-purified beta-lactamase had been produced and the sera were taken to be suitable for further analysis.

4.3.2.2 Western blotting

Samples of the second test bleed sera from all three rabbits were diluted 1/50 in 66mM sodium phosphate buffer pH7.4 and used to probe for the purified beta-lactamase in Western blots following 15% SDS-PAGE. The results are shown in Figure 4.7. Antiserum 947 gave an antibody-stained band (antiserum 948 also gave a very weak band) corresponding to the beta-lactamase at a molecular weight of 29,500.

Antiserum 947 gave the strongest immunostained band so this was tested in Western blots as a probe against log-phase beta-

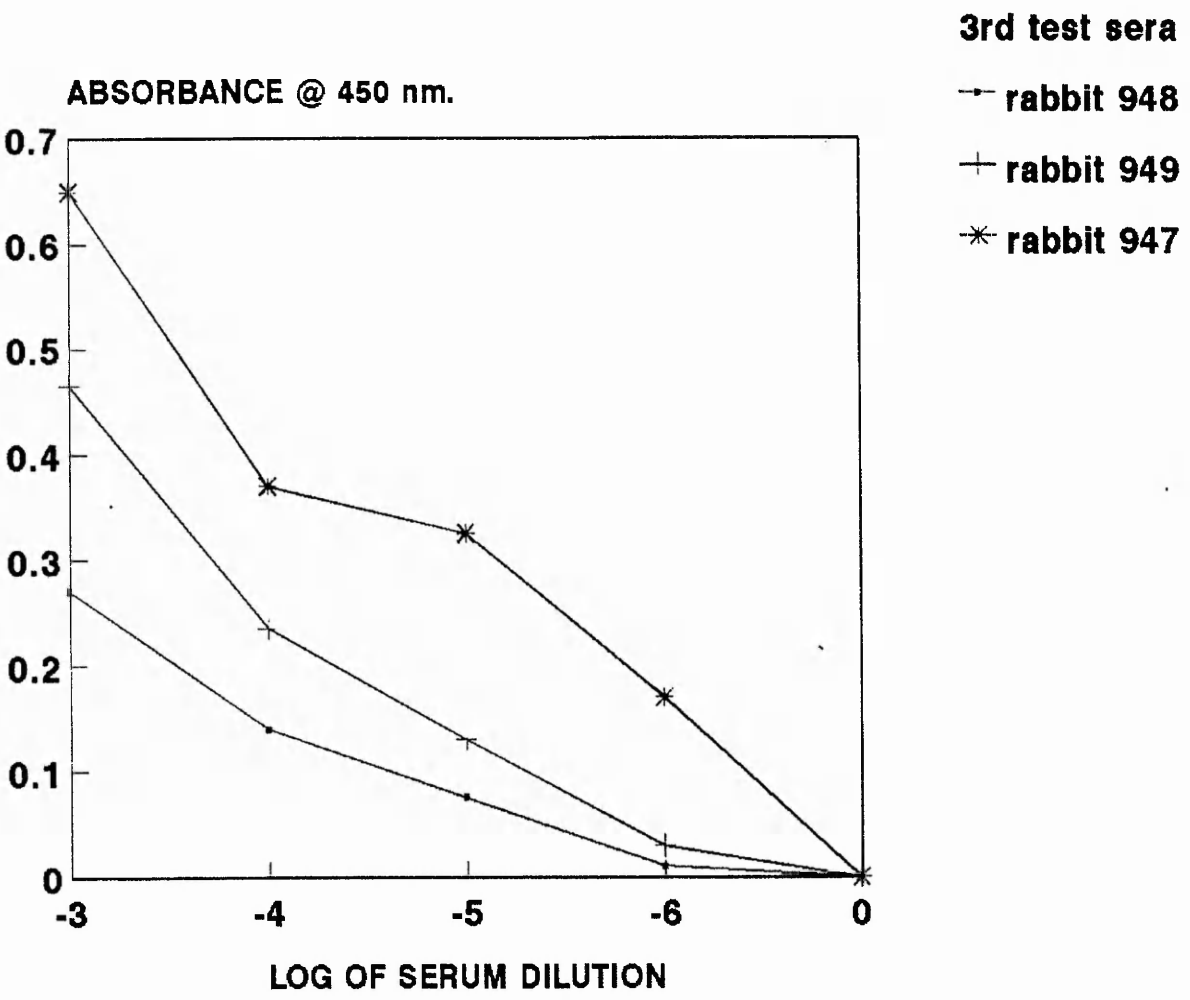


Figure 4.6: Results of ELISAs on the third test bleed antisera from rabbits 947, 948 and 949 immunised with purified beta-lactamase from *Neisseria gonorrhoeae* strain 13. Partially-purified beta-lactamase from the same strain was used as the plate antigen. Absorbance values were corrected as in section 2.2.6.2.

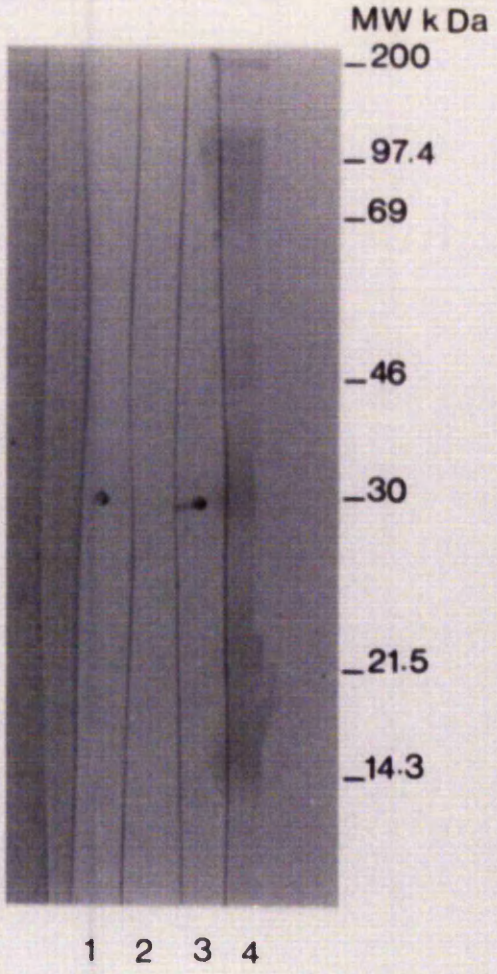


Figure 4.7: Western blot of purified beta-lactamase probed with a 1/50 dilution of the second test bleed antisera from rabbits 947, 948 and 949 immunised with purified beta-lactamase. The molecular weights of the standard proteins in lane 4 are marked.

Lane 1: Antiserum 949.

Lane 2: Antiserum 948.

Lane 3: Antiserum 947.

Lane 4: Rainbow molecular weight markers.

lactamase producing cells from strain 13, crude beta-lactamase (as described in section 4.2.1) and purified beta-lactamase. The results are shown in Figure 4.8. The two impure beta-lactamase preparations both gave two very strongly stained bands at around a molecular weight of 30,000 ie. close to the molecular weight for the beta-lactamase. The purified beta-lactamase gave a very weakly stained band close to the position of one of the two strong bands in the other lanes. The beta-lactamase in the two impure preparations may be present in too low a concentration to be detected by the antiserum. The two strongly labelled bands could possibly be due to the detection of contaminating proteins which were present in the immunising material in very low quantities, but stimulated the production of strong antibodies. However only one protein band was detected on SDS-PAGE gels of the immunising material which was thought to be the beta-lactamase as it gave a single activity band in a non-denaturing PAGE gel after staining with Nitrocefin. Alternatively one of the protein bands could have been the beta-lactamase and the other the beta-lactamase with a small portion of the protein having been cleaved during the process of electrophoresis. There is also a relatively strong band in lane 3 close to the dye front. The identity of this band is not known but it is unlikely that the immunising material contained this protein as it came from a cut gel slice (at the precise molecular weight of the beta-lactamase). However, it could possibly be a proteolytic product of the beta-lactamase protein.

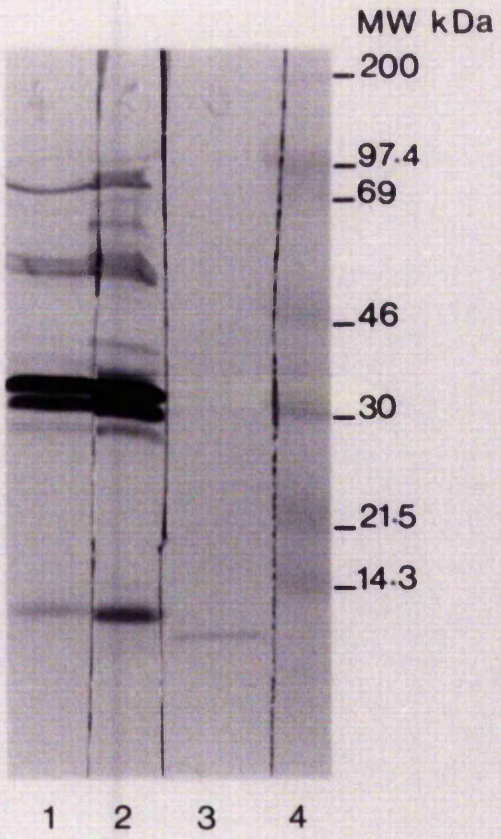


Figure 4.8: Western blot of various beta-lactamase preparations from *Neisseria gonorrhoeae* strain 13 probed with the second test bleed antiserum from rabbit 947 diluted 1/50 in 66mM phosphate buffer pH 7.4.

Lane 1: Log-phase cells.

Lane 2: Crude preparation of beta-lactamase.

Lane 3: Purified beta-lactamase.

Lane 4: Rainbow molecular weight markers.

The antiserum was also tested in Western blots against log-phase cells of the non-beta-lactamase-producing strain I and the beta-lactamase-producing strain 13 (both confirmed by the Nitrocefin test). The results are shown in Figure 4.9. Both strains gave the two strongly-stained bands at the molecular weight 30,000 position. Dilution of the antiserum decreased the background non-specific staining but the two strong bands were still stained even at 1/500. A sample of the penicillinase from *Bacillus cereus* was also tested and this gave a weakly-stained band at about the molecular weight 30,000 position in roughly the same place as expected for the purified beta-lactamase from *Neisseria gonorrhoeae* strain 13. Cross-reactions of the antisera with beta-lactamases from other species is discussed later.

Another blot of strain I and strain 13 was tested with a higher dilution of antiserum 947 (1/1000) and also with the same dilution of the corresponding pre-bleed serum. The results are shown in Figure 4.10. The two clearly stained bands at the molecular weight 30,000 position were again visible (although the higher molecular weight band was now dominant) in both strains with the 1/1000 dilution of the antiserum but were not detected by the pre-bleed serum. Therefore they must be regarded as specifically stained bands. The SDS-PAGE of the purified beta-lactamase used as the immunogen did not show either of these bands (Figure 4.4). In conclusion it would seem that non-immunised rabbits did not have antibodies against the two bands and were not apparently immunised with the proteins in these bands. However, as stated earlier these proteins may have been

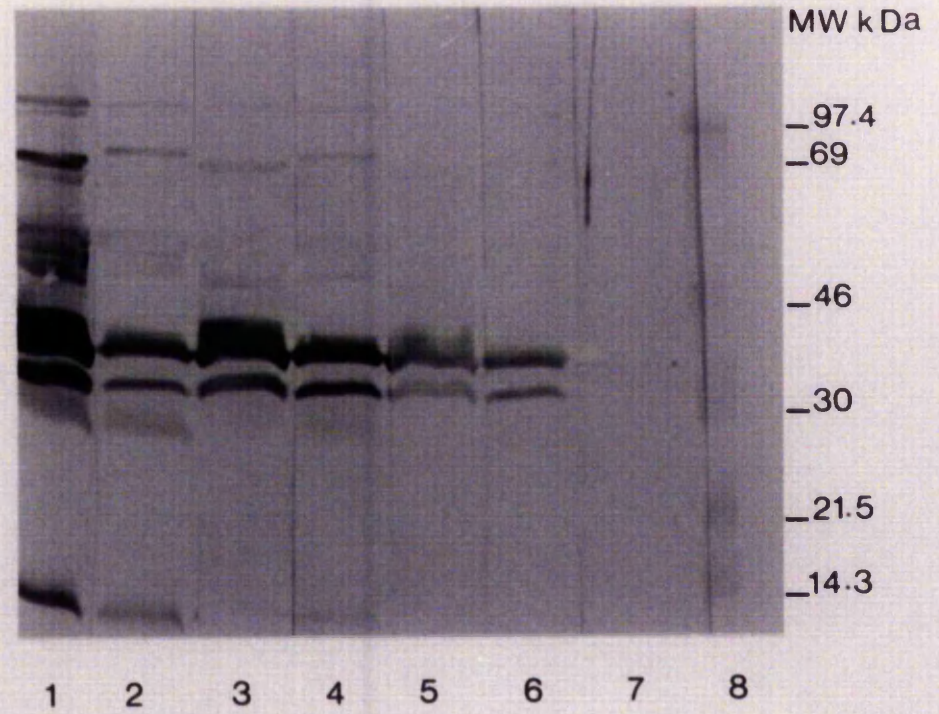


Figure 4.9: Western blot of cells of the beta-lactamase-producing strain 13, the non-producing strain I and the beta-lactamase from *Bacillus cereus* (Penase, Difco) The blot was probed with dilutions of antiserum 947.

Lane 1: Strain I with 1/50 dilution of antiserum.

Lane 2: Strain 13 with 1/50 dilution of antiserum.

Lane 3: Strain I with 1/200 dilution of antiserum.

Lane 4: Strain 13 with 1/200 dilution of antiserum.

Lane 5: Strain I with 1/500 dilution of antiserum.

Lane 6: Strain 13 with 1/500 dilution of antiserum.

Lane 7: Penase from *Bacillus cereus* I with 1/50 dilution of antiserum.

Lane 8: Molecular weight standards.

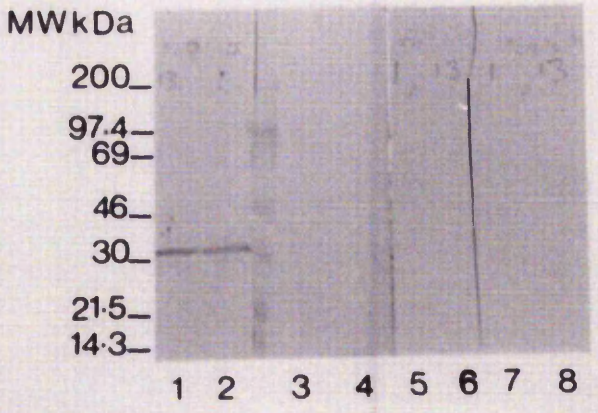


Figure 4.10: Western blot of cells of beta-lactamase-producing strain 13 and non-beta-lactamase-producing strain I probed with a 1/1000 dilution of antiserum 947 or the same dilution of the corresponding pre-bleed.

Lane 1: Strain 13 with 1/1000 dilution of antiserum 947.

Lane 2: Strain I with 1/1000 dilution of antiserum 947.

Lane 3: Strain 13 with no antiserum.

Lane 4: Strain I with no antiserum.

Lane 5: Strain 13 with no antiserum.

Lane 6: Strain I with no antiserum.

Lane 7: Strain 13 with 1/1000 dilution of prebleed serum 947.

Lane 8: Strain I with 1/1000 dilution of prebleed serum 947.

undetected but present in high enough concentration to induce antibody production.

An experiment was set up to renature beta-lactamase in SDS-polyacrylamide gels so that the beta-lactamase band could be stained with Nitrocefin and then correlated with the antibody-stained bands on a subsequent Western blot. Samples of soluble protein from beta-lactamase-producing and non-beta-lactamase-producing cells were prepared as described in Section 4.2.1. The samples were heated for 3 minutes 37°C or 100°C before loading onto 15% SDS-polyacrylamide gels as usual. After running the gel the SDS was washed from the gel by rinsing it in several changes of 5mM sodium phosphate buffer at pH 7.4. Subsequent staining of the gel with Nitrocefin located the active beta-lactamase in the producing strain only. The heating to 100°C had no detrimental effect on the activity of the renatured beta-lactamase. (Results not shown).

A similar experiment was performed using supernatants obtained by ultrasonication of a 20-hour culture of cells (strain 13). Insoluble protein was removed by centrifugation and the soluble protein was tested for beta-lactamase activity. Samples were electrophoresed on 15% SDS-polyacrylamide gels and renatured after electrophoresis as before. The gel was stained for beta-lactamase with Nitrocefin, photographed, laid onto a piece of nitrocellulose and the position of the stained beta-lactamase band marked on the nitrocellulose. The gel was then blotted onto the nitrocellulose and probed with anti-beta-lactamase antiserum. Figure 4.11 shows the gel stained for active beta-lactamase with Nitrocefin, and Figure 4.12. shows the corresponding immuno-

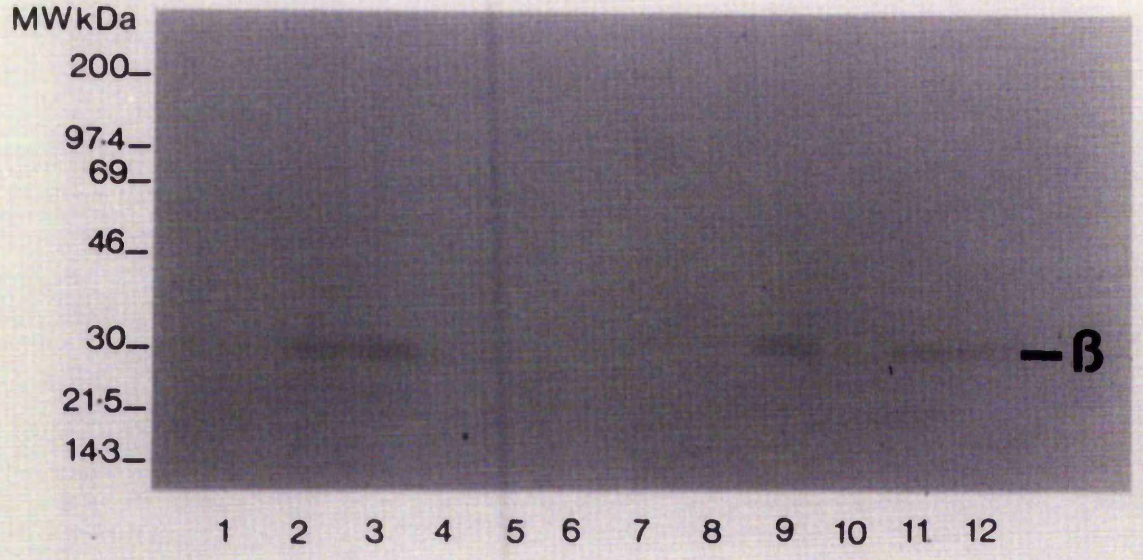


Figure 4.11: SDS-polyacrylamide gel of various strains of *Neisseria gonorrhoeae* stained for beta-lactamase activity with Nitrocefin. B indicates producers and the position of the active beta-lactamase bands.

1: Strain I
2: Strain 13 (B)
3: Strain 5909 (B)
4: Strain 5981
5: Strain 95
6: Strain 9569
7: Molecular weight markers
8: Strain I
9: Strain 13 (B)
10: Strain I
11: Strain 13 (B)
12: Strain 13 (B)

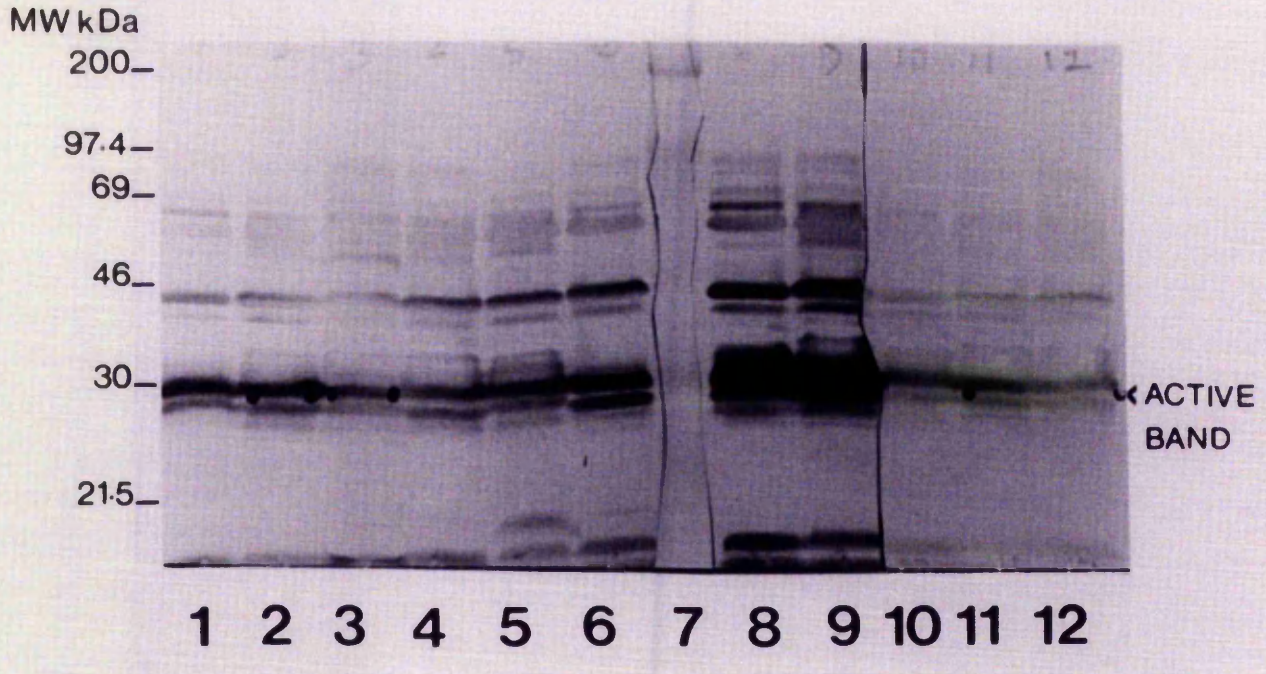


Figure 4.12: Western blot corresponding to the gel shown in figure 4.11. The blot was probed with a 1/1000 dilution of antiserum 947 (second test bleed) and various concentrations of peroxidase-conjugated antibody as detailed below.

1: Strain I 1/2500 c	7: Molecular weight markers
2: Strain 13 1/2500 c	8: Strain I 1/1000 c
3: Strain 5909 1/2500 c	9: Strain 13 1/1000 c
4: Strain 5981 1/2500 c	10: Strain I 1/5000 c
5: Strain 95 1/2500 c	11: Strain 13 1/5000 c
6: Strain 9569 1/2500 c	12: Strain 13 1/5000 c

c = conjugate

stained blot. Beta-lactamase activity was only detected in the beta-lactamase-producers as shown in Figure 4.11. Subsequent probing with anti-beta-lactamase antiserum resulted in the staining of many non-specific bands probably due to the use of too high a concentration of the peroxidase-conjugated antiserum as this was a new batch. The positions of the bands marked from the activity-stained gel were located roughly between two strongly-labelled bands at about the molecular weight 30,000 position. The two strongly-stained bands were present in all strains regardless of whether beta-lactamase activity had been detected or not. The antiserum at the dilution used seemed to be incapable of recognising and binding to the beta-lactamase bands in these preparations. It is possible that the concentration of the specific beta-lactamase band was too low to be detected by the antibodies.

The anti-beta-lactamase antisera bound very strongly to the two protein bands which appeared to lie just on either side of the active beta-lactamase band on SDS-polyacrylamide gels and subsequent Western blots. The binding of the antibodies was observed in non-beta-lactamase-producing strains as well as beta-lactamase-producing strains. The bands may be protein contaminants which were present in very low concentrations in the gel slices used for immunisation of the rabbits (and resulted in the production of sufficient antibody to stain the bands in Western blots. It is also possible that the two proteins could have been co-purified with the beta-lactamase although SDS-PAGE of the purified beta-lactamase showed only one band (figure 4.4).

The fact that one band has a higher molecular weight than the beta-lactamase suggests that this band at least is not a breakdown product of the beta-lactamase. The staining may also be due to the protein in the two bands being able to bind IgG molecules non-specifically.

4.3.3 Immunoaffinity chromatography of the beta-lactamase

An immunoaffinity column of Sepharose-4B with bound IgG against beta-lactamase was used to purify the anion-exchange purified beta-lactamase from strain 13. Figure 4.13 shows the 15% SDS-polyacrylamide gel of various samples taken during this purification stained with Nitrocefin as described in the last Section, and also the corresponding Western blot of the gel probed with anti-beta-lactamase antiserum. Lane 3 of the gel shows that a band with beta-lactamase activity, which corresponds exactly with a band immunostained with anti-beta-lactamase antiserum in the blot, has been eluted from the immunoaffinity column with glycine buffer. No further beta-lactamase was eluted with the glycine-dioxane buffer. Lanes 1 and 2 show that the two strongly-stained non-beta-lactamase bands noted earlier were not adsorbed to the column. So these bands, which are also present in the non-beta-lactamase-producing strains, can be recognised by anti-beta-lactamase antisera on blots but will not bind to IgG from the same serum on an affinity column. The reason for this phenomenon is not known. However, it may be due to the antibodies, once bound to the column support, being held in such an orientation that they can not bind to the protein to which the antibodies bind on Western blots. As with previous results it can

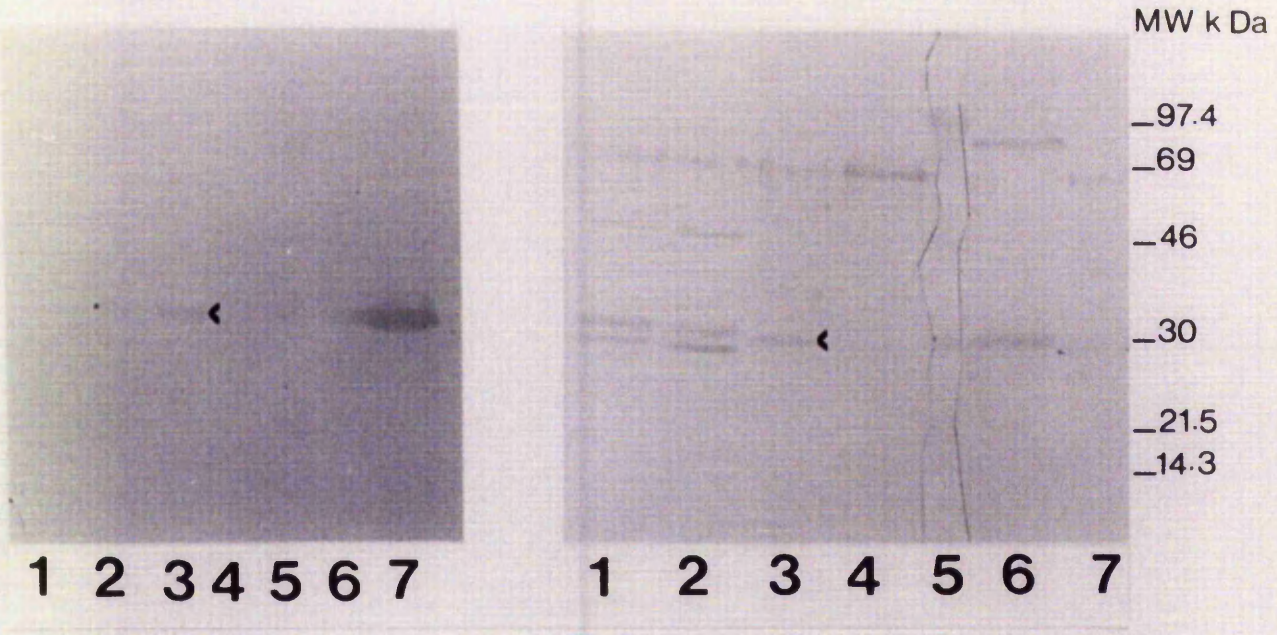


Figure 4.13: (Left) 15% SDS-polyacrylamide gel (renatured and stained with Nitrocefin) and (right) the corresponding Western blot (probed with a 1/200 dilution of the second test bleed antiserum 947) of samples taken during the immunoaffinity purification of the beta-lactamase of strain 13.

Lane 1: Non-adsorbed protein (10µl loaded)

Lane 2: Non-adsorbed protein (20µl loaded)

Lane 3: Glycine-eluted protein

Lane 4: Dioxane-eluted protein

Lane 5: Molecular weight standards

Lane 6: Crude beta-lactamase from strain 5909

Lane 7: Anion-exchanged partially-purified beta-lactamase from strain 5909

be seen that the beta-lactamase band is only visible on the immuno-stained blots when it is tested in a relatively concentrated form, in this case after elution from the affinity column. Lane 7 shows that a beta-lactamase partially purified by anion exchange chromatography from another beta-lactamase-producing strain (5909) gave a very strong activity band in the Nitrocefin-stained gel but only showed a weak antibody-stained band in the corresponding blot. This also shows that the antiserum used can recognise beta-lactamases from other strains as well as the strain 13 used as the immunogen. One further band of interest was noticed in lanes 1 to 4 of the blot. This band has approximately double the molecular weight of the beta-lactamase and reacts well with the anti-beta-lactamase antiserum 947. This band was found in the glycine-dioxane eluted fraction as well as in the beta-lactamase-containing glycine-eluted fraction. It is possible that this band is a non-active dimer of the beta-lactamase.

The beta-lactamase from strain 13 was also purified from a crude cell extract on the immunoaffinity column and the results for this purification are shown in Figure 4.14. After the single step of immunoaffinity purification of the crude beta-lactamase supernatant the glycine elution yielded a single protein which reacted with the anti-beta-lactamase sera on the blot and corresponded to an active beta-lactamase band after staining with Nitrocefin. These results demonstrate that the antibodies might also be used for a one-step immunoaffinity purification of beta-lactamase from *Neisseria gonorrhoeae*. Similar purified bands were

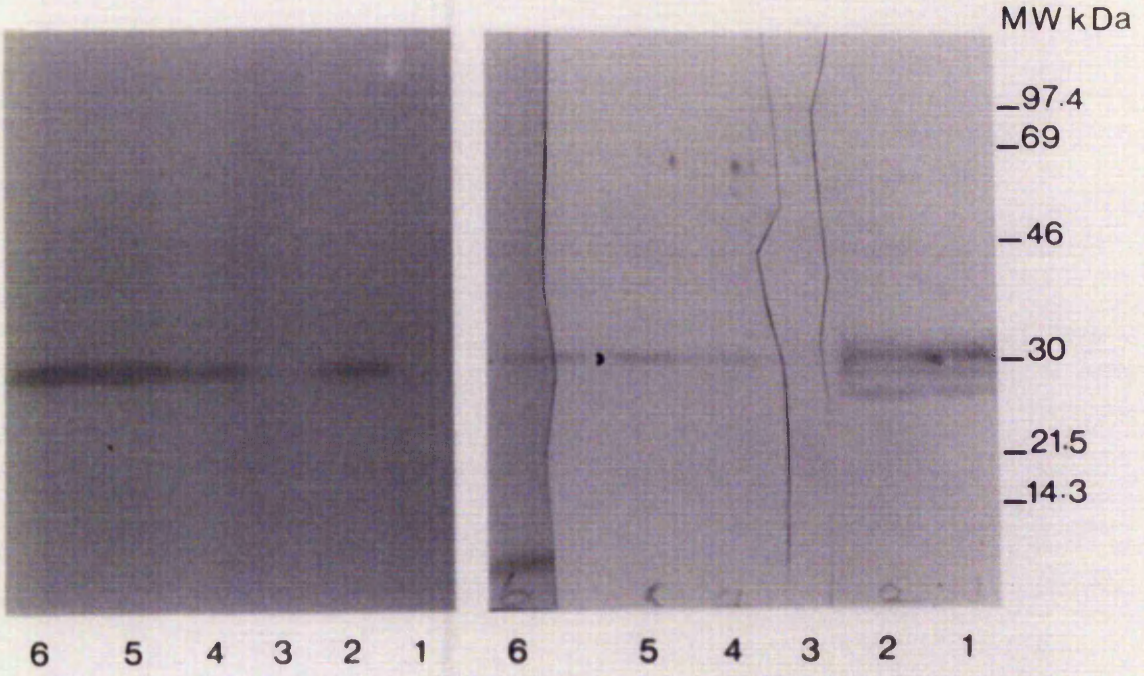


Figure 4.14: (Left) SDS-polyacrylamide gel (stained with Nitrocefin for beta-lactamase activity) and (right) the corresponding Western blot (probed with a 1/200 dilution of the second test bleed of anti-beta-lactamase antiserum 947) of various samples taken during the immunoaffinity purification of beta-lactamase from a crude cell protein supernatant of strain 13.

1: Crude protein supernatant from non-beta-lactamase strain I

2: Crude protein supernatant from beta-lactamase-producing strain 13

3: Molecular weight standards

4: Glycine-eluted beta-lactamase from strain 13

5: Glycine-eluted beta-lactamase from strain 13 2x concentrate

6: Amido black stained protein as in lane 4

obtained from the same immunoaffinity purification of beta-lactamase from strains 5909 and 5907. When a non-beta-lactamase-producer, strain I, was tested no beta-lactamase band was eluted.

4.3.4 Antigenic cross-reaction of beta-lactamases from *Neisseria gonorrhoeae* and other species

Rabbit 947, which had been immunised with purified beta-lactamase from *Neisseria gonorrhoeae* strain 13, was given two further inoculations of 75μg each of affinity-purified beta-lactamase. Subsequent ELISAs of the pre-boost and the post-boost sera, using commercial R-TEM beta-lactamase from *Escherichia coli* (ICN) at 500ng/well to coat the plate, showed an increase in titre of the anti-beta-lactamase antibodies from about 10^{-3} in the pre-boost to about 10^{-5} in the post-boost serum (results not shown).
Figure 4.15 shows the results of an ELISA of antiserum 947 raised against the purified beta-lactamase of *Neisseria gonorrhoeae* tested against the R-TEM beta-lactamase of *Escherichia coli* (ICN) bound to the plate. This demonstrated that the antibodies raised against the beta-lactamase from *Neisseria gonorrhoeae* also reacted with the beta-lactamase from *Escherichia coli*.

A 1/1000 dilution of the same antiserum 947 was pre-incubated with various concentrations of the beta-lactamase from *Escherichia coli* for one hour before addition of the mixtures to a plate coated with the *Escherichia coli* beta-lactamase. The results of this experiment are shown in Figure 4.16. Concentrations of the *Escherichia coli* beta-lactamase from 100μg/ml to 1μg/ml inhibited subsequent binding of the anti-

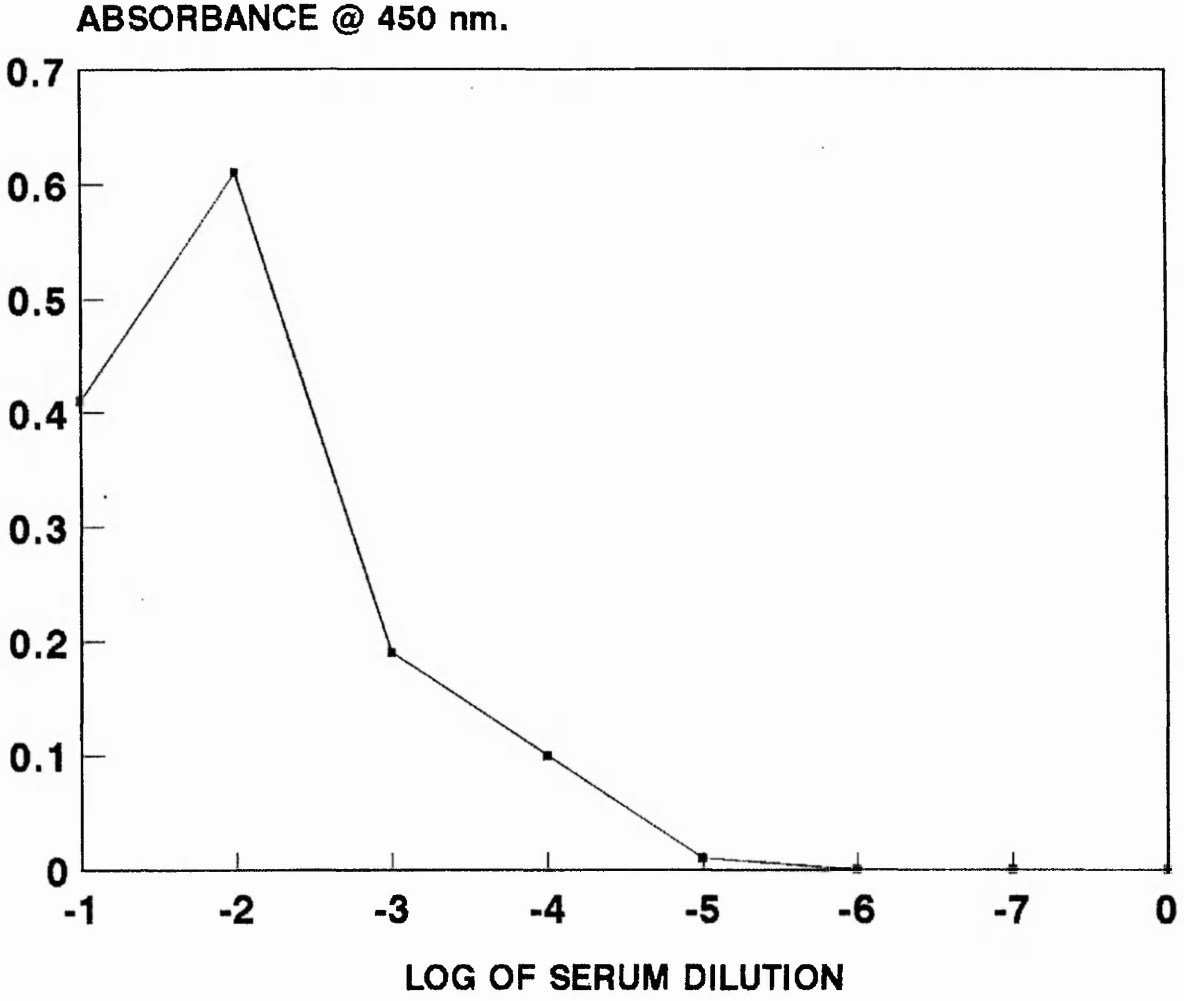


Figure 4.15: ELISA of antiserum 947 raised against the purified beta-lactamase of *Neisseria gonorrhoeae* strain 13 tested against the R-TEM beta-lactamase of *Escherichia coli* (ICN) as the plate antigen.

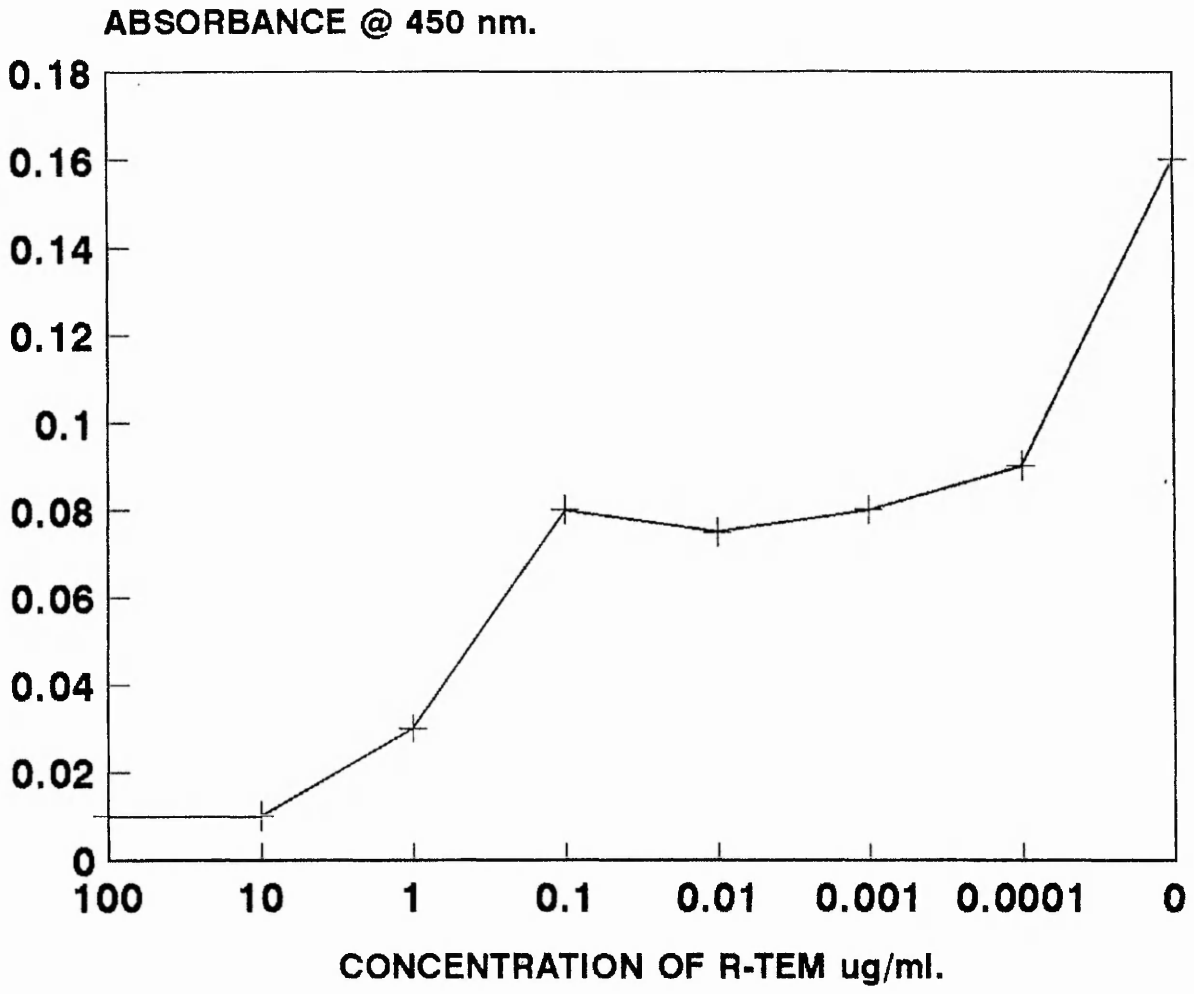


Figure 4.16: Competitive inhibition ELISA of a 1/1000 dilution of anti-beta-lactamase antiserum 947 raised against the purified beta-lactamase of *Neisseria gonorrhoeae* strain 13 pre-incubated with various concentrations of the R-TEM beta-lactamase from *Escherichia coli* (ICN) before testing the mixtures against the R-TEM beta-lactamase of *Escherichia coli* as the plate antigen.

gonococcal-beta-lactamase antibodies to the plate antigen by 94% and 54% respectively. From this result it was concluded that the beta-lactamases from both the species contained similar epitopes which could be recognised by the gonococcal beta-lactamase antiserum. Further cross-reactions between the antisera against *Neisseria gonorrhoeae* beta-lactamase and the beta-lactamases of other species using Western blotting will be discussed later.

Antisera against the beta-lactamases of *Escherichia coli* and *Bacillus cereus* (Nottingham Trent University) were tested in ELISAs against the *Escherichia coli* beta-lactamase and *Bacillus cereus* beta-lactamase (ICN) as well as the affinity-purified *Neisseria gonorrhoeae* beta-lactamase as the plate antigen. The results are summarised in Table 4.2. The antisera against the beta-lactamase of *Escherichia coli* and that of *Neisseria gonorrhoeae* both gave high titres (based on the 50% absorbance maximum) with their respective beta-lactamases. However, each of these two antisera tested against the beta-lactamase from the other species gave a titre at least 1000-fold lower. This suggests that there is an immunological similarity between these three beta-lactamases but they are not identical. The results for the *Bacillus cereus* serum (higher titre against *E.coli* beta-lactamase than against *Neisseria gonorrhoeae* beta-lactamase) would suggest that the *Bacillus cereus* enzyme is more similar to the *E.coli* beta-lactamase than the gonococcal one.

This similarity was confirmed in a Western blot of crude, post-ion-exchange, and affinity-purified beta-lactamase preparations

Table 4.2: The approximate titres of antisera raised against the beta-lactamases from three bacterial species tested against the beta-lactamases of *Escherichia coli* and *Neisseria gonorrhoeae* as the plate antigens.

	Titre of antiserum against the beta-lactamase of:		
Plate antigen:	B. cereus	E. coli	N.gonorrhoeae[a]
Escherichia coli beta-lactamase	10^{-6}	$>10^{-7}$	10^{-3}
Neisseria gonorrhoeae beta-lactamase	10^{-3}	10^{-4}	10^{-6}

[a]pre-boost 947

from *Neisseria gonorrhoeae* and the commercial beta-lactamase from *Escherichia coli* probed with anti-gonococcal-beta-lactamase antiserum 947 IgG fraction, post-boost antiserum 947, and the antiserum against the beta-lactamase of *Escherichia coli*. The results are shown in Figure 4.17. The IgG from the antiserum against *Neisseria gonorrhoeae* beta-lactamase failed to detect its corresponding beta-lactamase in the crude extract (lanes 1). However, there are faint bands in the right position for beta-lactamase in lanes 5, 8 and 11 which were probed with the same IgG preparation. This suggests that the anti-gonococcal beta-lactamase IgG can detect the beta-lactamase if it is present in purified concentrated form, and also shows that the antiserum cross-reacts with the *Escherichia coli* beta-lactamase (lane 11). The results show that the boosted antiserum against the gonococcal beta-lactamase did not detect beta-lactamase in the crude extract of *Neisseria gonorrhoeae* but it was able to detect the beta-lactamase in the partially-purified ion-exchange sample (lane 6) and also the affinity-purified gonococcal beta-lactamase (lane 9) and possibly also the *Escherichia coli* beta-lactamase (lane 12). This antiserum also stained non-specific bands in the affinity-purified sample of gonococcal beta-lactamase (lane 9). However, this batch of affinity-purified beta-lactamase was from an old affinity column and it was thought to contain some contaminating protein which would not otherwise be expected. The antiserum against the *Escherichia coli* beta-lactamase gave the strongest stained bands in the right position for beta-lactamase in all the preparations tested (lanes 3, 7, 10 and 13) although it also gave rise to some staining of non-specific bands in the

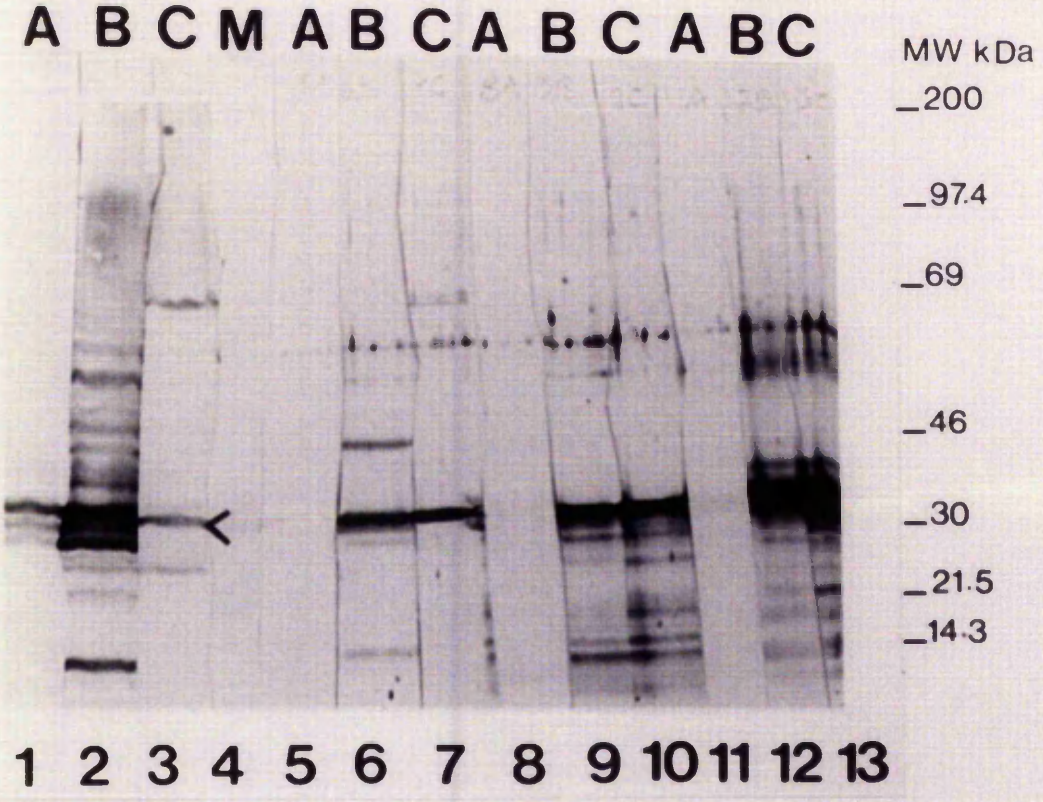


Figure 4.17: Western blot of various beta-lactamase preparations probed with various anti-beta-lactamase antisera.

A = Probed with anti-gonococcal beta-lactamase IgG

B = Probed with anti-gonococcal-beta-lactamase boosted antiserum

C = Probed with anti-*Escherichia coli* beta lactamase antiserum

M = Molecular weight standards

	Antiserum	Beta-lactamase preparation on blot
1:	A	Crude *N. gonorrhoeae* beta-lactamase
2:	B	As 1
3:	C	As 1
4:	M	Molecular weight standards
5:	A	Post-ion-exchange *N. gonorrhoeae* beta-lactamase
6:	B	As 5
7:	C	As 5
8:	A	Affinity-purified *N. gonorrhoeae* beta-lactamase
9:	B	As 8
10:	C	As 8
11:	A	Purified *Escherichia coli* beta-lactamase
12:	B	As 11
13:	C	As 11

affinity-purified gonococcal beta-lactamase sample (lane 10). There is clearly strong cross-reaction between the beta-lactamases of *Neisseria gonorrhoeae* and *Escherichia coli*.

Figure 4.18 shows the results of an SDS-polyacrylamide gel (stained for beta-lactamase activity) and the corresponding Western blot (probed with the anti-gonococcal-beta-lactamase antiserum) of the purified beta-lactamases of *Escherichia coli*, *Enterobacter cloacae*, *Bacillus cereus* I, *Bacillus cereus* II (from ICN Flow) and *Neisseria gonorrhoeae*. All the species gave a beta-lactamase band in the Nitrocefin-stained gel although there were obvious differences in the molecular weights of the *Enterobacter cloacae* and the *Bacillus cereus* II beta-lactamases. The anti-gonococcal-beta-lactamase antibodies used as the probe only bound significantly to the *Escherichia coli* beta-lactamase, weakly to the *Bacillus cereus* I beta-lactamase, and only very weakly to the *Neisseria gonorrhoeae* beta-lactamase in this blot. (The photograph does not show the bands as clearly as they were in the original blot). This result correlates with the cross-reactions seen in the ELISA test given earlier. The type I beta-lactamase from *Bacillus cereus* is related to the R-TEM beta-lactamase of *Escherichia coli* (Abraham and Waley, 1979) as it is a serine beta-lactamase and has similar amino acid sequences. The type II enzyme from *Bacillus cereus,* however is not a serine beta-lactamase and it requires Zn^{2+} as a cofactor. An extra band at molecular weight 60,000 was again immuno-stained in the blot suggesting the possibility of dimer of beta-lactamase. Possible reasons for the cross-reactions between the beta-lactamases of

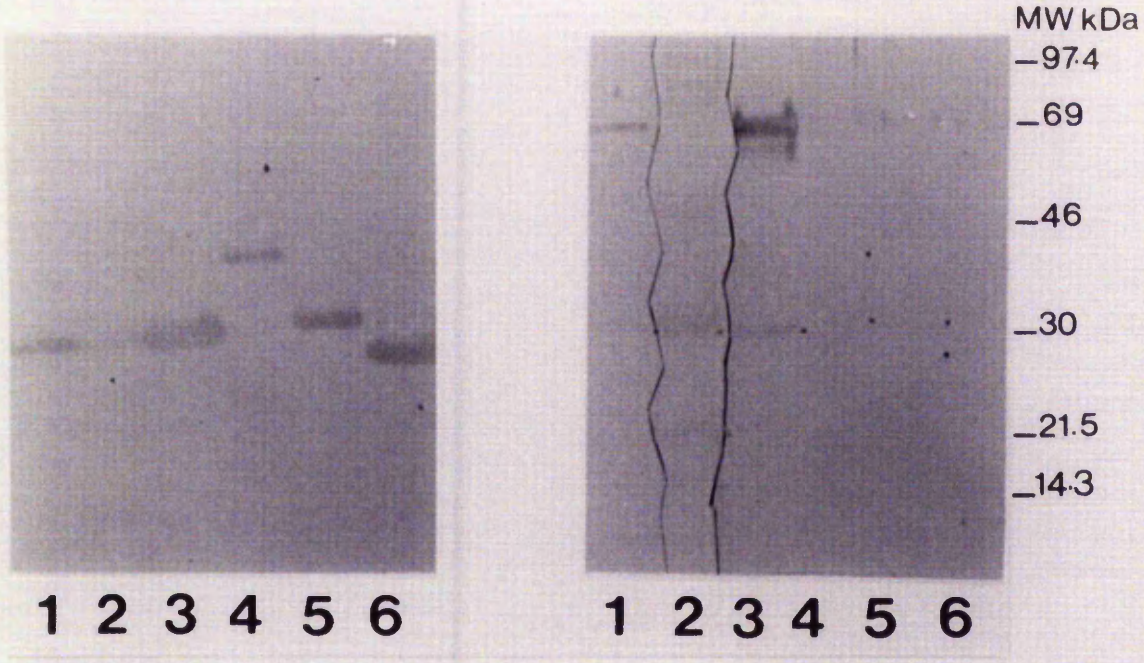


Figure 4.18: (Left) SDS-polyacrylamide gel stained for beta-lactamase activity with Nitrocefin, and (Right) the corresponding Western blot probed with a 1/50 dilution of anti-gonococcal-beta-lactamase antiserum 949, of the purified beta-lactamases of the species listed below.

1: *Neisseria gonorrhoeae*

2: Molecular weight markers

3: *Escherichia coli*

4: *Enterobacter cloacae*

5: *Bacillus cereus* I

6: *Bacillus cereus* II

the different species will be discussed later in relation to the classification of these enzymes.

4.3.5 Neutralisation of beta-lactamase activity by anti-beta-lactamase antiserum

An experiment was performed to assess whether the antiserum raised against the *Neisseria gonorrhoeae* beta-lactamase was able to inhibit the activity of this enzyme. The results are shown in Figure 4.19. A 1/2 dilution of the test bleed antiserum inhibited the activity by 78%. The same dilution of the prebleed serum was not tested but a 1/10 dilution of this serum gave a negligible reduction in activity. However, the 1/20 dilution of test and pre-bleed sera gave 40% and 20% inhibition of enzyme respectively. It is possible that the prebleed serum contained other serum proteins which could bind to the beta-lactamase and inhibit the hydrolysis of the Nitrocefin. The decrease in activity may be due to the antibodies binding to the enzyme in such a way as to distort the binding site for Nitrocefin. The results from this work contrast to that of Murakami and Yoshida (1985) who produced 10 monoclonal antibodies against cephalosporinase 9 of which increased the activity of the enzyme by 6% to 40% and only one reduced the activity by 50%. Monoclonal antibodies are much less likely to affect the activity as only a few antibodies are likely to be directed against the substrate binding site.

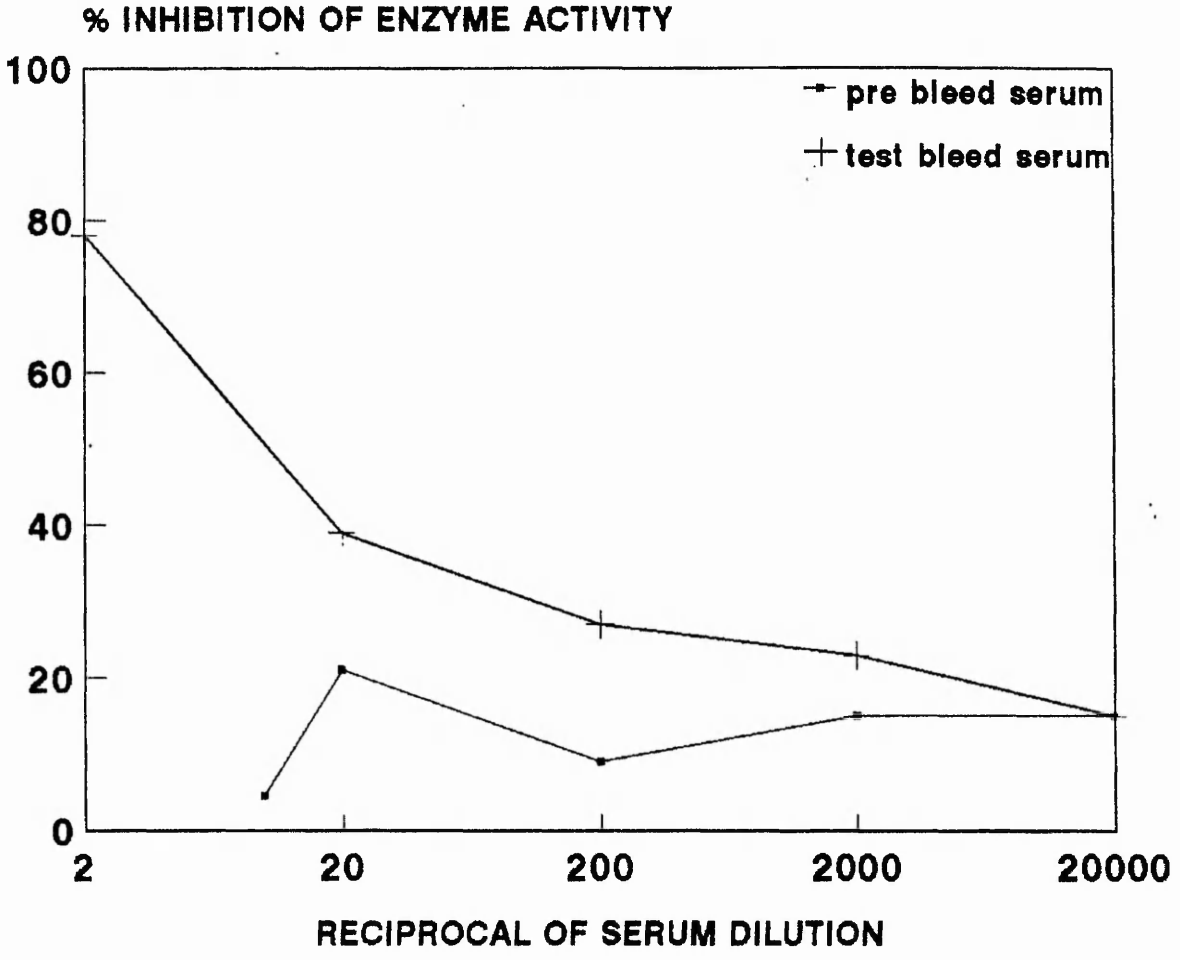


Figure 4.19: Inhibition of the activity of ion-exchange-purified gonococcal beta-lactamase by the pre-bleed and test-bleed of antiserum 947 diluted in 66mM phosphate buffer at pH 7.4. Equal volumes of the beta-lactamase preparation and the serum dilutions were mixed and incubated at 30°C for 15 minutes then assayed for rate of hydrolysis of Nitrocefin as described in Section 4.2.5.4. The percentage inhibition of activity was calculated from:

$$\frac{\text{Control rate - Rate in presence of serum}}{\text{Control rate}} \times 100$$

4.3.6 Inhibition of the gonococcal beta-lactamase by clavulanic acid

Clavulanic acid is a suicide inhibitor of beta-lactamase. It was found to bind to the beta-lactamase of *Neisseria gonorrhoeae* and reduce its hydrolytic activity in renatured polyacrylamide gels. Figure 4.20 shows the results of a renatured SDS-polyacrylamide gel of gonococcal beta-lactamase pre-incubated with various concentrations of clavulanic acid. As the clavulanic acid concentration was increased the amount of hydrolysed Nitrocefin produced was decreased. The corresponding blot probed with anti-gonococcal-beta-lactamase antiserum (not shown) did not show any difference in the amount of beta-lactamase at the different concentrations of clavulanic acid. The clavulanic acid bound to the beta-lactamase, being a comparatively small molecule, may be unlikely to distort the protein such that antibody-binding is reduced.

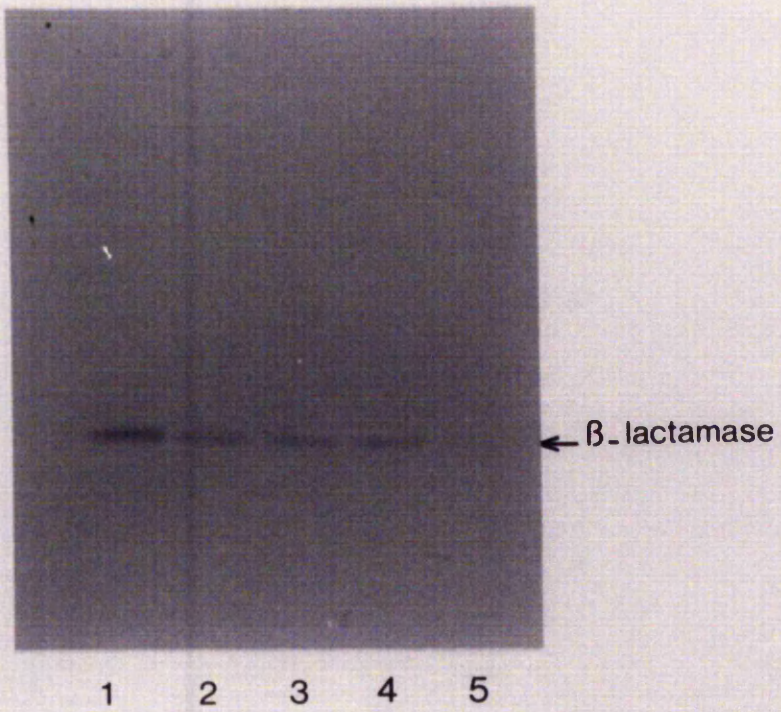


Figure 4.20: SDS-polyacrylamide gel, stained for beta-lactamase activity with Nitrocefin, of the beta-lactamase of *Neisseria gonorrhoeae* strain 13 pre-incubated with an equal volume of various concentrations of clavulanic acid.

1: 0µg clavulanic acid in 20µl

2: 25µg clavulanic acid in 20µl

3: 50µg clavulanic acid in 20µl

4: 100µg clavulanic acid in 20µl

5: 200µg clavulanic acid in 20µl

4.4 SUMMARY AND CONCLUSIONS.

The beta-lactamase of *Neisseria gonorrhoeae* was purified by a multiple-step schedule to give a single band on SDS-PAGE with an apparent molecular weight of 29,500-30,000. This value was higher than the reported of 25,000 (Eriquez and D'Amato, 1979). However, the value obtained in this study was consistently higher using SDS-PAGE and was confirmed using gel filtration chromatography. Polyclonal rabbit antiserum to this purified beta-lactamase was produced by immunisation of rabbits with polyacrylamide gel containing the beta-lactamase band. Antibodies in the antiserum bound to the beta-lactamase in ELISAs and reacted strongly, to give two intense bands, with soluble protein from both beta-lactamase-producing and non-beta-lactamase-producing strains, in Western blots. Beta-lactamase activity was detected, using Nitrocefin, in a band between these two bands, having renatured the beta-lactamase on the polyacrylamide gel. It was concluded that these two proteins were detected by the antisera possibly due to these proteins being present in the original immunising material at a low concentration. This would mean that these contaminating proteins had co-purified with the beta-lactamase through three different stages of purification. Immunoaffinity purification of ion-exchange-purified protein yielded a single band corresponding to the gonococcal beta-lactamase. This suggested that the antibodies which were reacting very strongly with the non-beta-lactamase bands on Western blots were unable to bind to these proteins when immobilised on the sepharose column. Antisera against the gonococcal beta-lactamase was found to cross-react with beta-lactamases from *Escherichia coli*

(strongly) and *Bacillus cereus* type I (weakly). The beta-lactamases of *Escherichia coli* and *Neisseria gonorrhoeae* are both TEM beta-lactamases (class III, Richmond and Sykes, 1973) encoded by a plasmid. The ease with which this plasmid can be transferred has led to this beta-lactamase being produced in at least 28 different species (Matthew, 1979). It is therefore likely that the antisera produced in this study would cross-react with the TEM beta-lactamase in all of these species. Further work to screen a variety of different species would confirm this.

However, the gonococcal beta-lactamase antiserum has been used to detect plasmid-mediated beta-lactamase resistance in partially-purified extracts of *Neisseria gonorrhoeae* soluble protein. (The information on the plasmid content of the beta-lactamase producers was supplied by The Gonococcus Reference Laboratory, Bristol and is given in Chapter 3). This is an alternative procedure to the biochemical methods of detecting beta-lactamase which through the Western blotting technique allows visualisation of the protein rather than biochemical detection. The method could be used to screen large numbers of isolates with unknown beta-lactamase content. The confirmation of the TEM type beta-lactamase in the isolates could be effected using characterised samples of gonococci containing the TEM enzyme. Any change in the molecular weight of the detected beta-lactamases would indicate the presence of a different enzyme. However, although this method does not require the use of the expensive Nitrocefin reagent the procedure is more involved and more time consuming than the biochemical detection of the beta-lactamase.

Chapter 5

FINAL DISCUSSION

5.1 Investigation of intrinsic resistance to beta-lactams in strains of *Neisseria gonorrhoeae* by Western Blotting.

5.1.1 Production and specificity of antibodies against beta-lactams.

One of the aims of this study was to prepare antibodies to beta-lactam antibiotics which could be used to probe for beta-lactams bound to *Neisseria gonorrhoeae* PBPs in Western Blots. Such a method was described by Hakenbeck *et al.* (1986) for the detection of PBP-penicilloyl complexes in *Streptococcus pneumoniae* cell membrane extracts and also in the whole cells of *Escherichia coli*. This method could be a useful alternative method to the traditional method of fluorography for the detection of PBPs. The beta-lactams had to be conjugated to carriers to render the molecules immunogenic, because the molecular weights of the unconjugated beta-lactams were only a few daltons and the molecules themselves would be unlikely to induce antibody production. The poly-l-lysine penicillin conjugate, although containing the highest number of antibiotic molecules per molecule of carrier, failed to induce an immune response. However, both the BSA-penicillin and BSA-methicillin conjugate rabbits produced antibodies to the conjugates.

The results presented in chapter 2 suggested that the anti-methicillin conjugate antiserum would be the best anti-serum for use in Western blotting. The anti-methicillin serum had a higher

titre of antibodies against the methicillin conjugate compared with the penicillin conjugate. This may be a reflection of the greater density of methicillin molecules present in the methicillin conjugate compared with the benzyl-penicillin conjugate.

De Haan (1985) raised monoclonal antibodies to benzyl penicillin conjugates which recognized three distinct epitopes: the side chain, the thiazolidine ring and the new antigenic determinant formed as a consequence of conjugation. Results are presented in this thesis (based mainly on inhibition ELISAs) to suggest that both the BSA-penicillin and the BSA-methicillin anti-sera contained antibodies directed against the beta-lactam ring of each conjugate. The methicillin conjugate antibodies were shown to react with the BSA-penicillin conjugate as well as the BSA-methicillin conjugate although a difference in titre suggested a proportion of the antibodies were specific to the side chain of methicillin. Both sets of sera contained antibodies to the native BSA carrier. However, at the dilutions tested, the antisera were not inhibited by the BSA at the concentrations used.

The antigenic determinant formed on binding of penicillin to PBPs is different from the determinant formed when penicillin is complexed to a carrier protein. The PBP beta-lactam complex contains the antibiotic bound via a serine at the active site of the PBP enzyme (PBPs normal function in the cell are as enzymes) whereas the conjugate protein is bound to penicillin or

methicillin via a lysine. Both complexes, once formed contain a cleaved beta-lactam ring.

The side group determinant antibodies present in the anti-penicillin and especially in the anti-methicillin sera were considered to be of importance in the probing of beta-lactam bound to PBPs in Western Blots. This is because the side-group, which is unchanged in the complex, is not involved in the covalent bond to the PBPs and may be the only determinant which is easily accessible to the anti-beta-lactam antibodies.

5.1.2 Use of anti-methicillin antiserum in Western Blots.

The anti-penicillin sera were unable to recognise and bind to PBPs bound with penicillin in Western Blots. The reasons for this have been discussed in some detail in Chapter 3. However, it is possible that the benzyl group determinant antibodies were at too low a concentration in the sera to bind to the PBP-penicillin complex. Unfortunately, as the cross reaction of anti penicillin conjugate antisera with the methicillin conjugate was not tried, an indication of the proportion of group determinant antibodies was not obtained. As explained earlier the anti-side-group determinant antibodies may be important in the recognition of beta-lactam PBP complexes by the sera. It is also possible that the antibodies which recognise the core determinant of the beta-lactam are unable to bind to the epitopes when the PBP-beta-lactam complex is formed. Observations by Hakenback (1986), suggested that the side group determinant is the dominant determinant recognised by anti-beta-lactam sera although

antibodies to the core determinants also bound to beta-lactam-PBP complexes in Western Blots.

The anti-methicillin serum shown to be useful in detecting PBPs of *Neisseria gonorrhoeae* bound with methicillin-PBP complexes, correlated with the relative binding of tritiated penicillin in fluorography. There were qualitative differences in the intensity of the specific bands in Western Blots. These bands which were possibly the PBPs 1 and 2 bound with the methicillin had different intensities which could be correlated with the strains sensitivity to methicillin and penicillin. Chromosomally-mediated resistant strains showed reduced binding of methicillin to PBPs 1 and 2 depending on the level of resistance. Beta-lactamase-producing resistant strains gave conflicting results; some of the strains gave a blot profile similar to a chromosomally resistant strain whereas others appeared to behave as fully sensitive. As explained this may be due to a combination of chromosomal resistance and beta-lactamase production in the former. More strains of this nature need to be tested together with fluorography profiles to enable a better understanding of this phenomenon. The main disadvantage of the method was very high non-specific binding of antibodies in the blot. A number of reasons for this have already been suggested. The relatively high concentrations of antibiotic used in this procedure probably causes binding to other proteins in the cell extract. The poor clarity of the blots makes it difficult to interpret the results. The refinement of the technique in order to improve the blots is necessary for this to be an alternative method to fluorography.

Solutions to this may be firstly, to reduce the beta-lactam concentrations in the assay mixture, secondly to enrich the PBP content perhaps by isolation of membranes and thirdly to increase the specificity of the sera by removing any anti-BSA antibodies from the sera prior to immuno-probing the blots. A relatively high concentration of methicillin was necessary to observe the PBP-methicillin bands in the blots compared with that of penicillin used in fluorography. It is thought that this was necessary due to lack of sensitivity of the method, since the anti-methicillin antibodies would bind to one methicillin-bound PBP to give the signal. However, in fluorography the beta-emission by the bound penicillin which causes fogging of the film is a cumulative process and therefore the system, although it may take several days, is more sensitive.

The immuno-probing method could be a useful alternative to the fluorographic detection of PBPs if a more specific antibody reaction can be achieved. Immuno-probing of antibiotic bound to PBPs could be used as an assay for the determination of resistance to antibiotics using small volumes of log phase cultures. Cold antibiotic would be used rather than radioactive label which is expensive and requires stricter safety procedures. The samples can be loaded (several at a time) on to polyacrylamide gels and electrophoresed within two hours. Blotting can be completed within a further two hours and immunostaining of the blot takes only five hours. Amplification of the gel is by the use of a secondary antibody system and so the result can be obtained within 24 hours compared with

fluorography which takes several days.

The anti-beta-lactam conjugate sera could also possibly be utilised in other ways. Antibodies which recognise the beta-lactam conjugate may also bind to other beta-lactam containing compounds such as clavulanic acid. Since this molecule is a suicide inhibitor the beta-lactamase should be irreversibly bound with the clavulanic acid. For example the beta-lactamase could be incubated with clavulanic acid and then purified using an anti-beta-lactam conjugate antibody. The antibodies could be immobilised on Protein A sepharose through the Fc part of the antibody molecule. The beta-lactamase bound with the clavulanic acid would be passed through the column to allow binding of the complex to the antibodies.After removal of non-specifically bound protein by extensive washing, the clavulanic acid-beta-lactamase complex could be released from the immunoaffinity column by the use of a deforming buffer such as pH 2.5 glycine. An investigation of neutralised hydroxylamine to break the bond between the clavulanic acid and the beta-lactamase may allow elution of the native beta-lactamase.

5.1.3 Purification of PBPs from *Neisseria gonorrhoeae* for antiserum production.

Purification of PBPs by affinity chromatography was attempted using a method described by Curtis and Strominger (1981) for the purification of PBPs from *Escherichia coli*. The PBPs were to be purified and used individually for antisera production in rabbits. The antisera would be of value in the probing of PBPs

in Western blots of *Neisseria gonorrhoeae* whole cell and membrane proteins. The cross reaction of the three PBPs with each other and with the beta-lactamase of the gonococcus could be determined by Western blotting. A purified antigen for the production of antisera to the PBPs would be preferable to the use of an impure antigen to avoid non-specific binding of non-anti-PBP antibodies to other proteins in ELISAs and Western blotting. The polyclonal antibodies against PBPs would have been to different epitopes within each PBP. These antibodies would be useful tools for the assessment of the immunological similarities between PBPs 1, 2 and 3 and help to identify any shared epitopes within each protein. Antibodies, for example to the active site penicillin binding domain of each PBP, may cross react with active site of the beta-lactamase which would demonstrate similarities between the two types of proteins.

The affinity isolation of PBPs from a penicillin sensitive and a penicillin resistant strain of *Neisseria gonorrhoeae* was achieved using 6-APA and 7-ACA ligand substituted sepharose. However, the high affinity of the *Neisseria gonorrhoeae* PBPs for the ligand prevented the elution of the PBPs from the column using the neutralised hydroxylamine buffer which eluted *Escherichia coli* PBPs in a similar method. High concentrations of the nucleophile were necessary to elute small quantities of PBP 3 from both a penicillin sensitive and a penicillin resistant strain. Altered forms of PBPs 1 and 2 were also released with same rigorous conditions. Insufficient material was obtained for the production of antiserum in rabbits.

The main reason for the lack of success of this purification method with *Neisseria gonorrhoeae* PBPs is probably due to strength of the covalent penicilloyl PBP complex. After detailed study of elutants and different ligands for the purification it was demonstrated that the lower affinity antibiotics were apparently more appropriate ligands for the subsequent release of the PBPs. Further investigations into the steric hindrance of the beta-lactam with the PBP binding site may allow for easier elution of the proteins from the ligand. Alternatively, a multiple step purification procedure as suggested in section 3.5 may be a preferable method. The success of the anti-methicillin antibodies prepared and used in probing PBP-methicillin complexes in Western blots suggests that the antibodies would possibly be useful in the immunoaffinity purification of methicillin-PBP complexes as outlined in section 3.1.2 for beta-lactamase purification.

5.2 Detection of beta-lactamase by Western blotting.

5.2.1 Purification of beta-lactamase.

The purpose of purifying the beta-lactamase of *Neisseria gonorrhoeae* was to raise antibodies to the protein which could be used to detect beta-lactamase mediated penicillin resistance in strains which had been analysed by SDS-PAGE and Western blotting. The antibodies could also be used to study the immunological relationship (if any) between the beta-lactamase and PBPs of this species.

A multiple step purification schedule was employed to purify the protein to a single band (as determined by SDS-PAGE) of apparent molecular of 29.5-30 kilo daltons. The final step of the purification of this protein was achieved by immunoaffinity purification using the prepared anti-beta-lactamase serum IgG bound to a CN-Br sepharose column. The final yield of purified enzyme was very low compared with published yield of Eriquez and D'amto, 1979. However, the method used in this study yielded a relatively pure beta-lactamase fraction with a higher specific activity (by Nitrocefin assay) than enzyme purified by Eriqez and D'amto. The anion-exchange purification was the most efficient stage, in the procedure, as supported by the rise in the specific activity. However, the gel filtration could have been improved using an alternative gel filtration medium with an exclusion limit closer to the molecular weight of the beta-lactamase. The final step of cutting the beta-lactamase from the PAG was a useful method of obtaining a concentrated sample of the active beta-lactamase.

5.2.2 Production and specificity of the antisera.

Polyclonal rabbit antiserum to the purified beta-lactamase was obtained by immunisation of the animals with concentrated beta-lactamase in the gel slice. Antisera reacted with *Neisseria gonorrhoeae* in ELISAs and Western blots. However, two strongly immunostained bands were labelled by the antibodies in the Western blots of protein from both non-producing and producing beta-lactamase strains. This result is not explicable, although several explanations have been discussed in chapter 4. After

further purification of the protein using anti-beta-lactamase serum on a sepharose column a single band corresponding to the active beta-lactamase was visualised on Western blots. The other two bands previously observed in both producing and non-producing beta-lactamase strains did not purify on the immunoaffinity column. It was concluded that a specific anti-gonococcal beta-lactamase antibody had been produced. The anti-gonococcal beta-lactamase sera were found to cross react with RTEM beta-lactamase from *Escherichia coli* and *Bacillus cereus* type I in ELISAs and Western blots. Both these class A enzymes and that from *Neisseria gonorrhoeae* have homologous amino acid sequences and therefore the cross reaction of the anti-gonococcal beta-lactamase antibody is understandable. No cross reactions with *Bacillus cereus* type II and *Enterobacter cloacae* beta-lactamases were seen in the blots.

5.2.3 Use of antisera in the detection of beta-lactamase.

The antiserum was used to detect beta-lactamase in strains of *Neisseria gonorrhoeae* analysed by SDS-PAGE and Western blotting. However, the blots were difficult to interpret when the protein was impure due to non-specific binding of the antibodies to other proteins on the blots. The reasons for this non-specific reaction are not clear since the immunoaffinity column only purified a single active beta-lactamase protein. It would suggest that antibodies were present in the serum which could recognise and bind to proteins on blots but which were unable to bind protein when immobilised on a sepharose column. The antibodies against the non-beta-lactamase proteins were possibly against unobserved

contaminating protein in the original immunising material. Analysis of the apparent single beta-lactamase band by two dimensional electrophoresis would possibly have shown the presence of the other proteins.

However, a specific anti-beta-lactamase antibody was produced which could bind to beta-lactamase from *Neisseria gonorrhoeae* and at least two species. This antibody could be used as an alternative method of detecting beta-lactamase mediated resistance in *Neisseria gonorrhoeae*, in place of the traditional biochemical detection systems. The main drawback of this immunological detection of resistance is the increase in the time involved and the relative complexity of this procedure compared with the biochemical assay. Alternatively the antibodies could be utilised in the purification of the beta-lactamase from crude extracts and thus would be a one step purification procedure.

5.3 Conclusions.

The aim the study was to investigate the PBPs and the beta-lactamase of *Neisseria gonorrhoeae*, involved in the intrinsic and beta-lactamase resistance to beta-lactam antibiotics respectively, using immunological techniques. Methods different from those in current use have been investigated, developed and evaluated as alternative procedures for detecting the two types of resistance in the gonococcus.

Anti-beta-lactam conjugate antisera were produced and used in Western blots to detect differences in the intrinsic resistance

of strains of *Neisseria gonorrhoeae* by observing the pattern of PBPs bound with beta-lactam antibiotic. This method was evaluated as an alternative to fluorographic detection of PBPs. The method was considered to be a successful alternative but the problems encountered with specificity of the anti-sera would require further investigation to improve the clarity and reproducibility of the results.

The anti-beta-lactamase sera were successfully used to detect beta-lactamase mediated resistance in strains of *Neisseria gonorrhoeae* by the detection of a specific beta-lactamase band in Western blots. However, the method again would need further improvement if it was to be used as an alternative to the current biochemical detection assays.

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APPENDIX 1

Names and Addresses for Suppliers of Chemicals, Reagents and Equipment.

Chemicals and Reagents

1. Amersham International Plc
   Lincoln Place
   Green End
   Aylesbury
   Bucks
   HP20 2TP

   (Tritiated penicillin, Amplify, Hyperfilm-MP)

2. BBL Microbiology Systems
   (Becton Dickinson UK Ltd)
   Between Towns Road
   Cowley
   Oxford
   OX4 3LY

   (Isovitalex)

3. BDH Ltd
   Broom Road
   Poole
   Dorset
   BH12 4NN

   (General purpose and Analar-R grade reagents)

4. Biorad Laboratories
   Caxton Way
   Watford Business Park
   Watford
   Herts
   WD1 8RP

   (Biorad Protein Assay Reagents)

5. Difco Laboratories Ltd
   PO Box 14B
   Central Avenue
   East Molesey
   KT2 OSE

   (GC Base dehydrated media)

6. Gallenkamp Express
Belton Road West
Loughborough
LE11 OTR

(Dialysis tubing)

7. Glaxo PLC
Greenford
Middlesex

(Nitrocefin)

8. ICN Biomedicals
Free Press House
Castle Street
High Wycombe
Bucks
HP13 6RN

(IgG peroxidase conjugated Antibody)

9. Oxoid
Unipath Ltd
Wade Road
Basingstoke
RG24 OPN

(Protease peptone)

10. Pharmacia LKB Ltd
Pharmacia House
Midsummer Boulevard
Milton Keynes
Bucks
MK9 3HP

(Affinity and ion exchange chromatography media)

11. Pierce and Warriner (UK) Ltd
44 Upper Worthgate Street
Chester
Cheshire
CH1 4EF

(Triton X-100 detergent)

12. Sigma Chemical Company
Fancy Road
Poole
Dorset
BH17 7NH

(General purpose chemicals including BSA and benzyl penicillin)

13. Whatman Labsales Ltd
PO Box 6
Twyfords
Reading
Berks
RG10 9NL

(DE-52 Anion exchange Resin)

Equipment

1. Amicon Ltd
Upper Mill
Stone House
Gloucs
GL10 2BJ

(Ultrafiltration membranes)

2. Aminco
Suppliers:
DG Electronics
16-20 Camp Road
Farnborough
Hampshire
GU14 6EW

(French pressure cell)

3. Biorad Laboratories
Caxton Way
Watford Business Park
Watford
Herts
WD1 8RP

(Gel drier, Protean II PAGE Tank and Electroeluter)

4. Corning
Ciba Corning Diagnostics Ltd
Halstead
Essex
CO9 2DX

(pH meter)

5. Europa Centrifuges
MSE
Sussex Manor Park
Crawley
West Sussex
RH10 2QQ

(Europa 25)

6. Flow Laboratories Ltd
Woodcock Hill
Harefield Road
Rickmansworth
Herts
WD3 1PQ

(Titretek- plate washer, Titretek Multiscan- plate reader)

7. Gelman Sciences Ltd
10 Harrowden Road
Backmills
Northampton
MN4 OEZ

(Nitrocellulose membrane)

8. Genetic Research Instrumentation Ltd
Gene House
Dunmow Road
Felsted
Dunmow
Essex
CM6 3LD

(Atto Mini gel system)

9. Hoeffer Scientific Ltd
Suppliers:
Biotech Instrument Ltd
183 Camford Way
Luton
Bedfordshire
LU3 3AN

(Wet Blot System)

10. LEEC
Suppliers:
Lab and Electrical Engineering Company
Colwick
Nottingham
Notts
NG4 2J

(CO_2 Incubator)

11. LKB/Pharmacia Ltd
Midsummer Boulevard
Milton Keynes
Bucks
MK9 3HP

(Powerpack LKB 2301 (1000 volts) Macrodrive, Frac 100 Chromatography system, Peristaltic pump and Gradient former)

12. MSE
Sussex Manor Park
Crawley
West Sussex
RH10 2QQ

(Centrifuges and Sonicator)

13. Pye Unicam Ltd
York Street
Cambridge
CB1 2PX

(Phillips Spectrophotometer)

APPENDIX II

Centrifuge Data

Europa 24

Rotor	Cat N° 43114-143	Cat N° 43115-113
Capacity	8 x 50ml	6 x 500ml
Type	Angle	Angle
Max speed	20,000rpm	10,000rpm
Max g	49,640g	18,000g

MSE 25

Rotor	Cat N° 43114-115	Cat N° 43115-110
Capacity	8 x 50ml	6 x 300ml
Type	Angle	Angle
Max speed	25,000rpm	14,000rpm
Max g	74,000g	33,400g

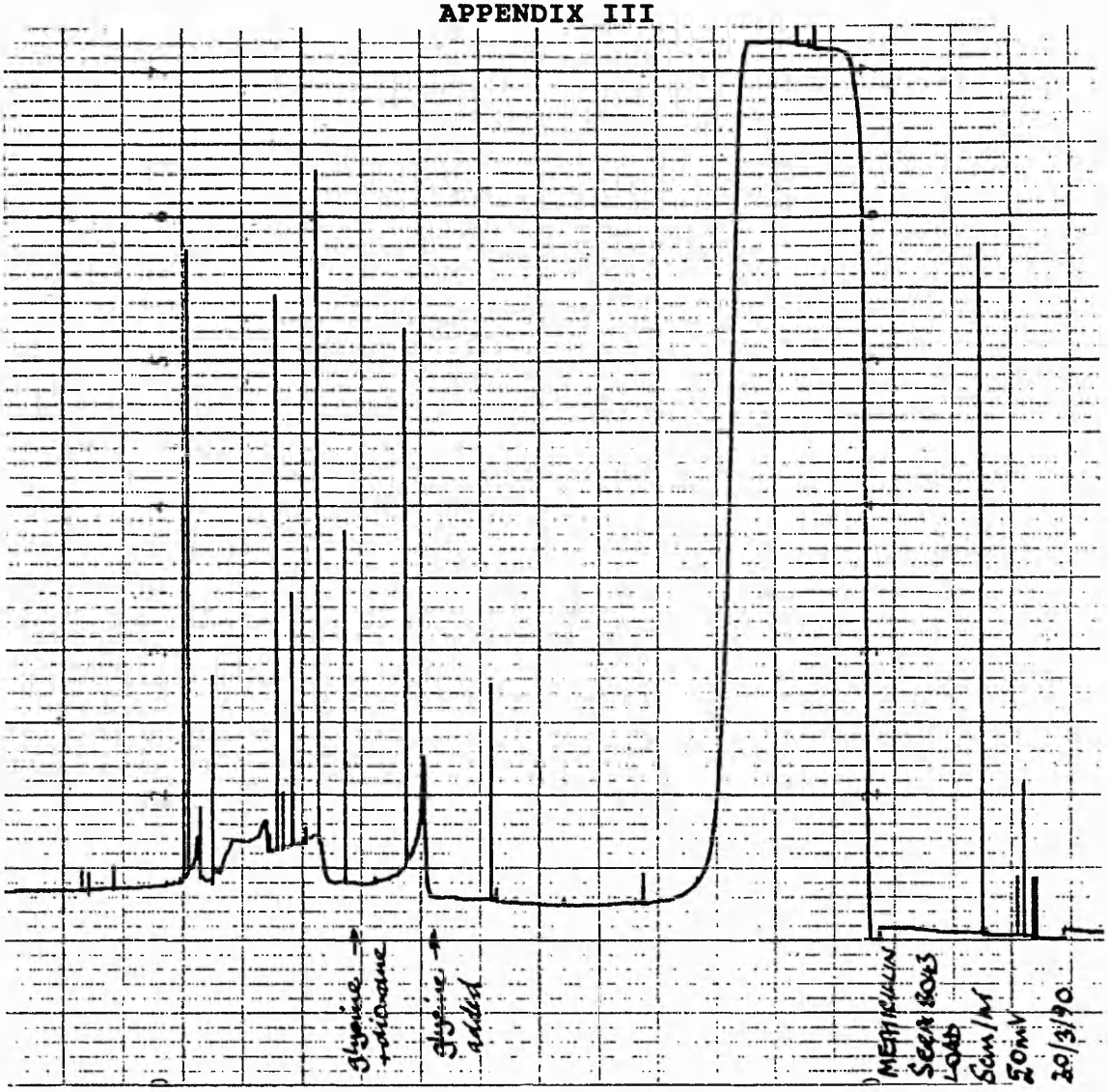
MSE Mistral 3000

Rotor	Cat N° 43122-105	Cat N° 43114-608
Capacity	56 x 15ml	24 x 15ml
Type	Swing out	Angle
Max speed	3,200rpm	6,000rpm
Max g	2375g	6030g

MSE Microcentaur

Capacity	12 x 1.5ml
Max speed	13,000rpm
Max RCF	13,400g

APPENDIX III



Protein monitor (measured @ 280nm) of anti-BSA-methicillin antibodies purified on an affinity column containing BSA-methicillin conjugate as the bound ligand. The protein monitor was not calibrated. The first large peak (right) is the load of 2ml diluted serum, the second and third peaks (right to left) are the protein eluted by addition of pH2.5 glycine and glycine + dioxane respectively. The vertical lines are due to electrical interference. All details of this purification schedule are given in section 2.2.7.