THE ROLE OF PENICILLIN BINDING PROTEINS IN THE RESISTANCE OF NEISSERIA GONORRHOEAE

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INTRODUCTION

Neisseria gonorrhoeae, an organism which previously was found to be extremely sensitive to penicillin and other antibiotics is becoming increasingly resistant. As penicillin is still used in many areas as first line antibiotic therapy for the treatment of gonorrhoea it has become imperative to draw attention to this fact.

This study demonstrates the appearance of resistant *Neisseria gonorrhoeae* strains in Bloemfontein and the dramatic increase in these strains. The mechanisms of resistance are demonstrated as well as the role which is played by the alteration of penicillin binding proteins.

CONTENTS

LIST OF ABBREVIATIONS

1.	CA	CHOCOLATE AGAR
2.	Cfu/POINT	COLONY FORMING UNITS PER POINT
3.	CMR	CHROMOSOMAL MUTATION RESISTANCE
4.	CMRMG	CHROMOSOMALLY MEDIATED RESISTANT *NEISSERIA GONORRHOEAE*
5.	d H_2O	DISTILLED WATER
6.	DST	DIAGNOSTIC SENSITIVITY TEST AGAR
7.	I	INTERMEDIATE
8.	IM	INNER MEMBRANE
9.	kD	KILO DALTON
10.	MIC	MINIMAL INHIBITORY CONCENTRATION
11.	MNYCM	MODIFIED NEW YORK CITY MEDIUM
12.	MTMM	MODIFIED THAYER MARTIN MEDIUM
13.	MW	MOLECULAR WEIGHT
14.	*N. GONORRHOEAE*	*NEISSERIA GONORRHOEAE*
15.	NON-PPNG	NON-PENICILLINASE PRODUCING *NEISSERIA GONORRHOEAE*
16.	OM	OUTER MEMBRANE
17.	OMP	OUTER MEMBRANE PROTEIN
18.	PAGE	POLYACRYLAMIDE GEL ELECTROPHORESIS
19.	PBP	PENICILLIN BINDING PROTEIN
20.	PID	PELVIC INFLAMMATORY DISEASE
21.	POMP	PRINCIPAL OUTER MEMBRANE PROTEIN
22.	PPNG	PENICILLINASE PRODUCING *NEISSERIA GONORRHOEAE*
23.	Rf	RELATIVE MOBILITY
24.	S	SENSITIVE
25.	STD	SEXUALLY TRANSMITTED DISEASES

CHAPTER I

LITERATURE REVIEW

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LITERATURE REVIEW

1.1 THE PATHOGENICITY OF NEISSERIA GONORRHOEAE

In 1879 Albert Neisser (1) described Gram-negative diplococci occurring intracellularly in the polymorphonuclear leucocytes of man. In 1885 Bunn was the first to achieve culture *in vitro* of these organisms (1,2). These *Neisseria gonorrhoeae* are the causative agents of gonorrhoea which is the most prevalent sexually transmitted disease (3).

1.2 PATHOGENESIS OF THE DISEASE

Neisseria gonorrhoeae causes a purulent discharge of the genito-urinary tract in both sexes. Man is the only known host of this organism (1) except for recent reports of successful experimental infection of chimpanzees (4). The healthy carrier state is unknown. Some cases of chronic infection are clinically inapparent and may serve as reservoirs of infection.

The gonococci adhere to mucous membrane receptors by means of pili and enter the mucosal cell by endocytosis. In the polymorph the phagosome and lysosome are prevented from fusing so that the neisseria may escape

killing by the enzymes of the phagocyte. In addition *Neisseria gonorrhoeae* possesses a protease specifically directed against IgA antibodies present on mucous surfaces (5). Gonorrhoea is an acute infectious disease with a three to four day incubation period. Initial infection affects the anterior urethra in males and the urethra and cervix in females. If this infection remains untreated it may spread along the genital tract and establish a chronic infection.

The organism is susceptible to drying, low temperatures and the absence of nutrients, and consequently rarely survives other than by direct transmission. Cases have been documented where transmission has ostensibly occurred via damp towels or fingers (4).

1.3 INFECTION IN MALES

In the male, gonorrhoea presents as a purulent infection of the urethra with suppurative inflammation and a purulent discharge. When untreated, the infection extends along the mucous surfaces to the prostrate gland, seminal vesicles and epididymis. It may invade the peri-urethral tissue producing an inflammatory reaction, abcesses and subsequent stricture. Rectal infection (proctitis) is another complication in both women and homosexual men.

1.4 INFECTION IN THE FEMALE

The infections are initially frequently asymptomatic in the female leading to serious complications if not treated. When untreated the fallopian tubes and ovaries may become infected causing pelvic inflammatory disease (PID) - a debilitating infection which can cause partial or complete damage to the fallopian tubes resulting in infertility, ectopic pregnancies, rupture and may cause the necessity of the surgical removal of the tubes. Further complications include infections of the vestibular glands (Bartholonitis), endometrium (endometritis) and fallopian tubes (salpingitis), proctitis or even infection spreading to the peritoneal cavity (1,2,4,6).

In genetically predisposed individuals or with infection by highly virulent strains, blood invasion occasionally may result from primary untreated gonococcal infection and arthritis and tenosynovitis may occur. Acute gonococcal endocarditis, acute leptomeningitis and pharyngitis occur as rare complications. Although *Neisseria gonorrheae* has been cultured from joint fluid in arthritis, gonococcal arthritis may be caused by hypersensitivity (reactive arthritis). Transference of the organism by fingers may cause secondary gonococcal conjunctivitis.

Rarely, a septic gonococcal dermatitis may be present accompanied by arthritis, arthralgia and fever. Scanty maculopapular skin lesions may be present mostly on extremities or around joints. This condition is more common in females than in males (7).

1.5 INFECTION IN CHILDREN

In children a vulvo-vaginitis may occur. Rectal involvement may also be noticed mainly from sexually abused children and rarely, in children from crowded unhygienic institutions. Ophthalmia neonatorum, an acute purulent conjunctivitis which may lead to blindness, may occur in newborn children from an infected mother (5).

Repeated infections and relapses are the rule rather than the exception. Resistance to infection does not appear to develop as part of the disease process. A variety of antibodies can be demonstrated but these are either highly strain specific or have little protective value. Even the IgA on the mucosal surface seems inadequate to prevent reinfection (5).

1.6 DEVELOPING RESISTANCE OF NEISSERIA GONORRHOEAE

Since the late 1930s, when sulphonamides were used successfully, *Neisseria gonorrhoeae* has slowly developed resistance. Between 1936 - 1946 the proportion of strains highly resistant to sulphonamides in many countries increased from less than 10% to 90%. Sulphonamide treatment was replaced by penicillin therapy. Although gonococci were highly susceptible to penicillin initially, relatively resistant strains were selected through worldwide use of this antibiotic for the treatment of gonococcal disease. Twenty years ago 300 000 units of penicillin were usually sufficient to cure a case of gonorrhoea while today 4,8 million units is the advocated dose and is not always successful (3, 8).

Since the mid 1950's gonococci have shown reduced sensitivity to penicillin and other antibiotics. This low level of penicillin resistance was not enzymatic i.e. not due to the presence of beta-lactamase, and required an increase in the recommended dosage of penicillin to 4,8 million units. Penicillin resistant strains were not encountered until 1957 but have since increased especially in the past decade. Strains of gonococci not possessing beta-lactamase and having a low level resistance to penicillin may still be treated with a high dosage of penicillin with satisfactory results.

In 1976 penicillin resistant strains of *N. gonorrhoeae* posessing the beta-lactamase enzyme (PPNG) were detected for the first time in the United Kingdom, USA and thereafter in various parts of the world (9). These strains showed high level penicillin resistance and contained an R factor which coded for the synthesis of beta-lactamase. Gonococci contain several plasmids (6) and at least one small plasmid (MW 4.5 x 10^6) carries the gene for beta-lactamase production. These plasmids are transmissible and may have been acquired from other organisms. The appearance of beta-lactamase producing strains prompted many worldwide studies to establish the incidence in various regions. Almost without exception the results demonstrated an increase in the incidence of PPNG everywhere. Despite this increasing incidence of penicillin resistance many areas still use penicillin as the first line therapy for gonorrhoea.

1.7 MECHANISMS OF RESISTANCE

The antibacterial activity of beta-lactam antibiotics on Gram negative bacteria depends on three factors.

(a) Permeability

The ability of the antibiotic to cross the outer membrane permeability barrier.

(b) Stability to beta-lactamase.

(c) **Chromosomal**

The affinity of the essential penicillin binding proteins located in the cytoplasmic membrane for the antibiotic (10, 11, 12, 13).

1.8 ALTERED PENICILLIN BINDING PROTEINS (PBP) IN *STREPTOCOCCUS PNEUMONIAE*

For many years *Streptococcus pneumoniae* was found to be universally sensitive to penicillin. The first report of a penicillin resistant clinical isolate appeared in 1967 (14,15).

During 1977 a number of penicillin resistant strains of *Streptococcus pneumoniae* were isolated in South Africa from children suffering from meningitis, pneumonia and bacteremia (16, 17, 18, 19, 20). These resistant strains did not produce beta-lactamase (21) and were intrinsically resistant. Recent epidemiological surveys have suggested that the overall susceptibility to penicillin of clinical isolates of *Streptococcus pneumoniae* has changed significantly. Intermediate resistant isolates [Minimal inhibitory concentration (MIC) 0,1 - 1,0 mg/l] have been associated with treatment failure (22, 23, 24, 25, 26).

Studies showed several types of major alterations in the PBP profiles of these resistant strains as compared to those of a penicillin sensitive laboratory strain (MIC 0,006 mg/l) (16).

1.9 ALTERED PENICILLIN BINDING PROTEINS IN N. GONORRHOEAE

Increased resistance to antimicrobial agents commonly used in the treatment of *N. gonorrhoeae* infections is of great concern worldwide. Resistance to penicillin is generally attributed to either chromosomal mutation (CMR) resulting in intermediate levels of resistance (MIC for penicillin 0,15 - 0,5 mg/l) or to the acquisition of plasmids that mediate the production of penicillinase (PPNG) usually resulting in high levels of resistance (MIC $\geq$ 2,0 mg/l). Penicillinase producing *N. gonorrhoeae* isolates were first reported in 1976 in Britain and the USA (9) and in Johannesburg and Durban (South Africa) in 1977 (27, 28). Until June 1984 no PPNG isolates were reported in Bloemfontein despite extensive routine screening of *N. gonorrhoeae* isolates for penicillinase production.

A study conducted during the second semester of 1984 (29) revealed that 80,6% of all gonococcal isolates were completely susceptible to penicillin (MIC = 0,06 mg/l). Fifteen percent showed intermediate resistance (MIC 0,15 - 0,5 mg/l) and 4% were resistant. An alarming

observation in this study was that although most intermediately resistant and resistant isolates produced beta-lactamase, 2 of the resistant isolates did not produce detectable levels of beta-lactamase, indicating that chromosomally mediated penicillin resistance was on the increase. Chromosomally mediated penicillin resistance amongst non beta-lactamase producing isolates of *N. gonorrhoeae* might be due to either diminished permeability of outer membrane (OM) porins or to altered PBP.

1.10 AIM

1. The developing resistance of both PPNG strains of *N. gonorrhoeae* as well as non-PPNG producing strains, which was reported worldwide as well as in South Africa, established the need for epidemiological studies to be conducted in Bloemfontein.

2. As altered PBPs were responsible for increased resistance in South African strains of *Streptococcus pneumoniae*, this study was undertaken to establish the role of PBP in the resistance of local strains of *N. gonorrhoeae*.

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CHAPTER II

Page

THE BACTERIAL CELL WALL AND THE ACTION OF PENICILLIN

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THE BACTERIAL CELL WALL AND THE ACTION OF PENICILLIN

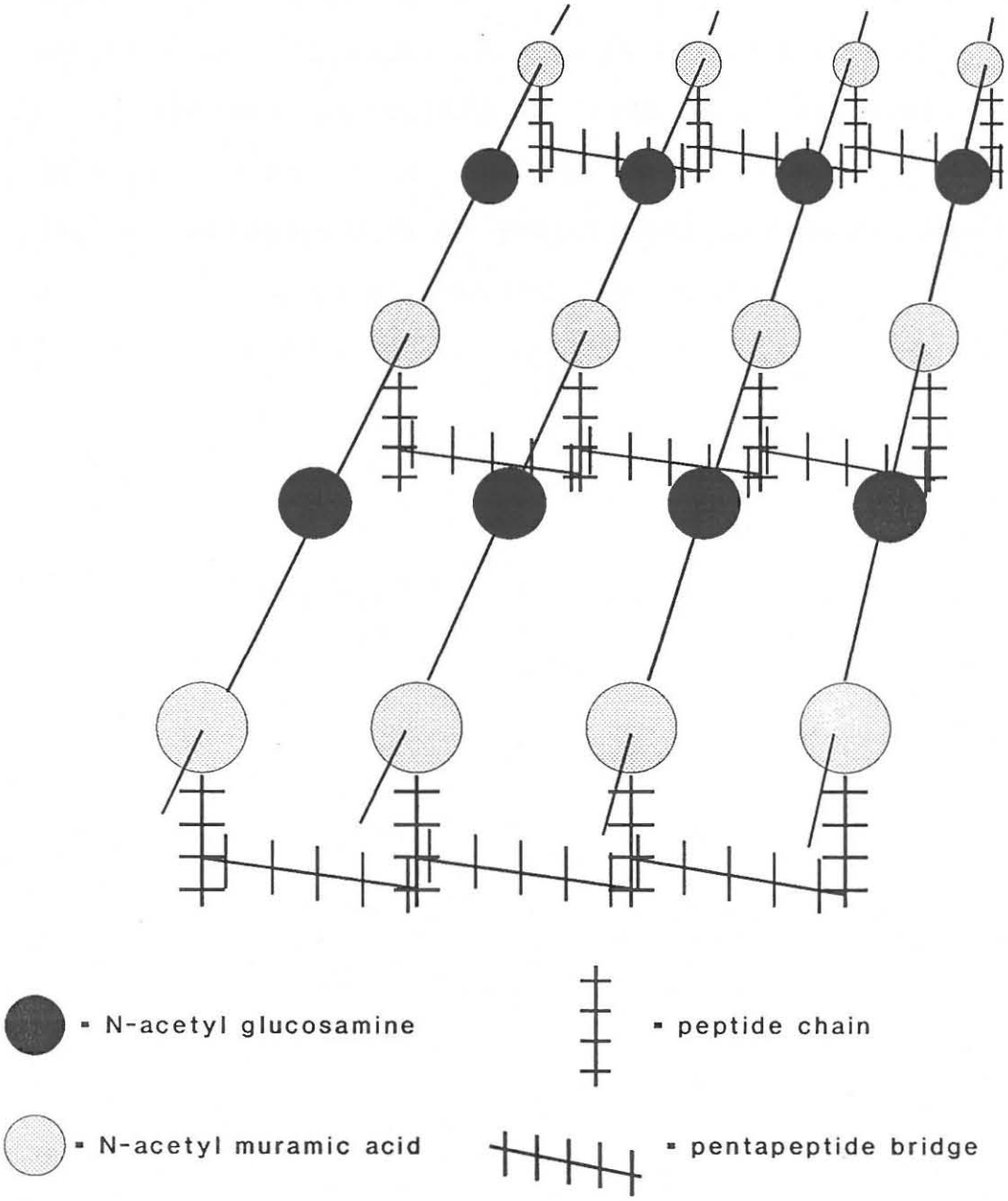
2.1 THE CELL WALL

The bacterial cell has a high internal osmotic pressure and is enclosed by a rigid outer layer, the cell wall, which maintains the shape of the micro-organism. The cell wall is composed of a chemically distinct complex polymer mucopeptide (peptidoglycan, murein; figure I) consisting of polysaccharides and a highly cross linked polypeptide. The polysaccharides regularly contain the amino sugars N-acetyl glucosamine and N-acetylmuramic acid linked alternately into a chain (figure II). N-acetylmuramic acid molecules each carry a short tri-, tetra- or pentapeptide side chain containing amino acids:

D and L alanine
D glutamic acid and either
L lysine or in the Gram-negative cell wall diaminopimelic acid

The rigidity of the wall is maintained by the cross-linking of the peptide chains through pentaglycine bonds as a result of transpeptidation reactions carried out by several enzymes.

Fig. I

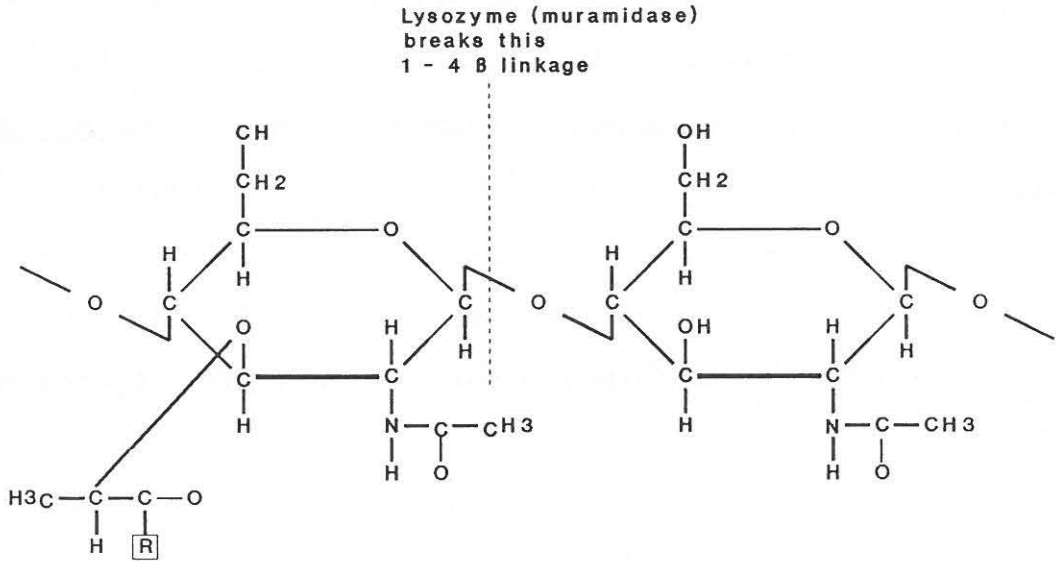


the structure of cell-wall mucopeptide

(1)

The Gram-negative cell contains lipopoly- or oligo- (in neisseria) saccharide which constitutes endotoxin. The cell wall and in particular its basal mucopeptide component is the target of action of many antibiotics. Bacteria growing in the presence of one of these antibiotics acting on cell wall synthesis form defective cell walls, undergo lysis and die. Beta-lactam antibiotics e.g. penicillins and cephalosporins act by inhibition of cell wall synthesis (1, 2).

Fig. II



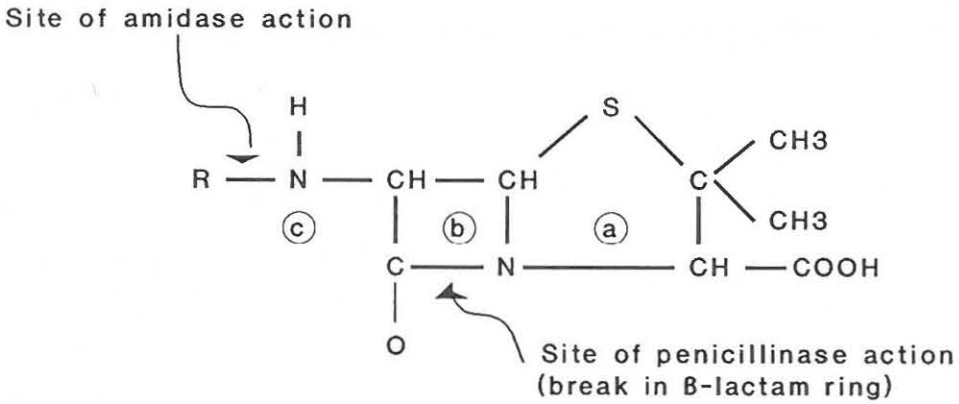
THE MUCOPEPTIDE STRUCTURE OF THE BACTERIAL CELL WALL.

(1)

2.2 PENICILLIN

An antibiotic is a chemical substance totally or partially produced by micro-organisms and toxic at low concentrations to micro-organisms. In 1928 Alexander Fleming discovered that bacteria growing in the presence of a contaminating mould, *Penicillium notatum*, were lysed (3). Penicillin was later isolated in 1941 by Chain and Florey. From the mould Penicillium, 6 amino penicillanic acid (figure III) is isolated on a large scale. This makes it possible to synthesize penicillin-like compounds by coupling the free amino group to free carboxyl groups of different radicals (1) (figure IV).

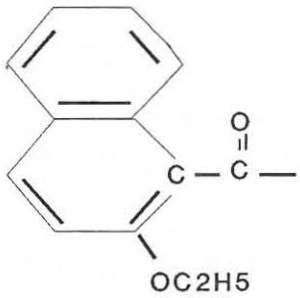
Fig. III



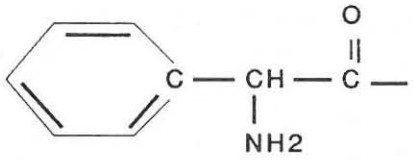
6-AMINOPENICILLANIC ACID

(4)

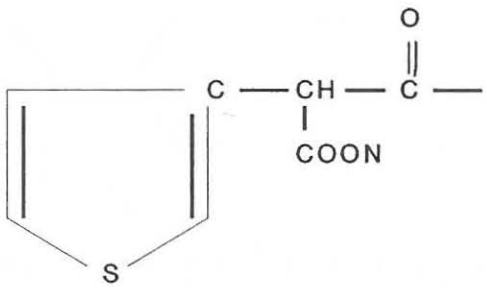
Fig. IV



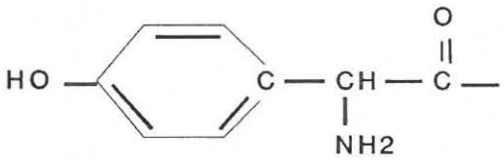
Nafcillin (ethoxynaphtamidopenicillin)



Ampicillin (alpha-aminobenzylpenicillin)



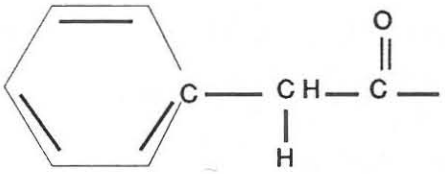
Ticarcillin



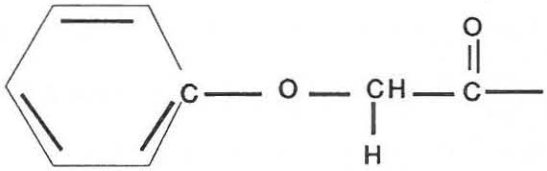
Amoxicillin

STRUCTURES OF SOME PENICILLINS

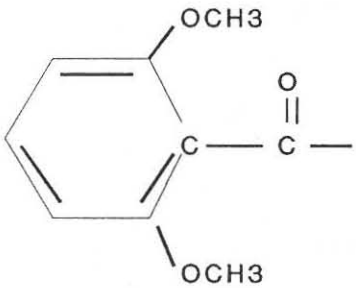
Fig. V



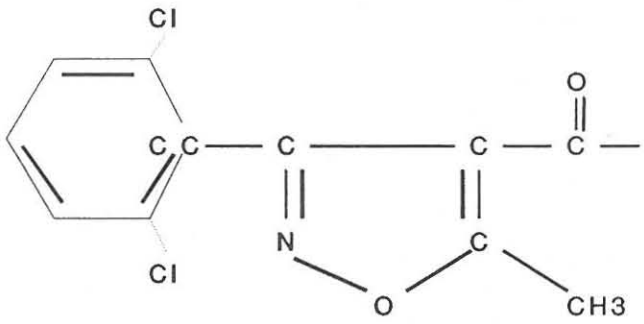
Penicillin G (benzylpenicillin)



Penicillin V (phenoxymethyl penicillin)



Methicillin (dimethoxyphenylpenicillin)



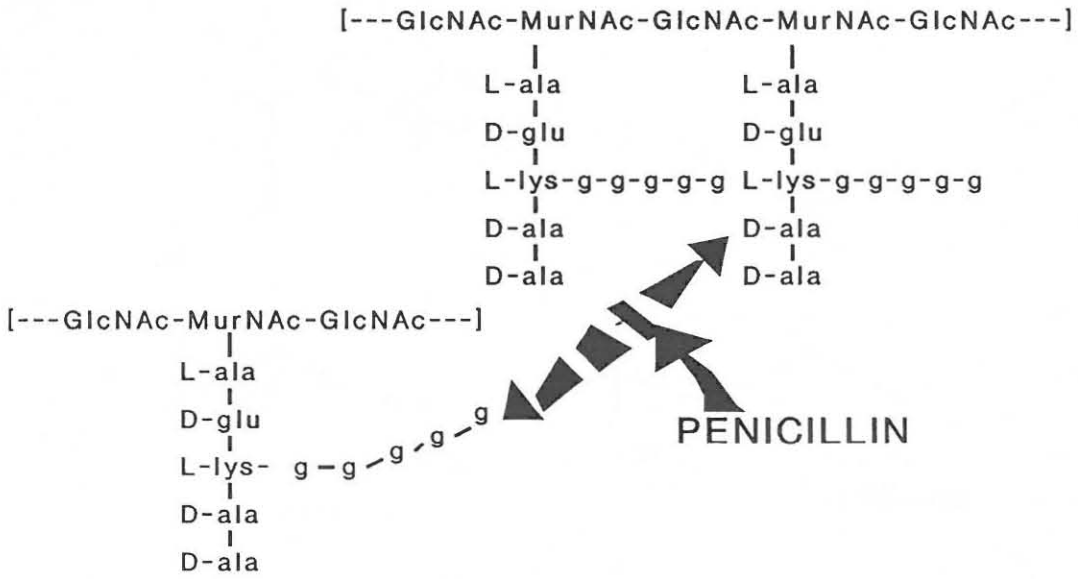
Oxacillin;
Cloxacillin (one Cl in structure);
Dicloxacillin (2 Cls in structure);
Flucloxacillin (one Cl, one F);
(Isoxazolyl penicillins)

All penicillins and cephalosporins (beta-lactam drugs) are selective inhibitors of bacterial cell wall synthesis. During the first stage the antibiotic is bound to cell receptors i.e. Penicillin binding proteins (PBPs).

Penicillin prevents synthesis of the cell wall by inhibition of transpeptidation of the side chain on the peptide bridge thereby preventing cross binding of the peptidoglycan chain. The cross linking enzymes (transpeptidases) are inhibited by penicillin.

According to the hypothesis of Stromminger *et al.*, the structure of penicillin is similar to that of the D-alanyl-D-alanine terminus of the peptide side chain of peptidoglycan (2, 5, 6, 7, 8, 9).

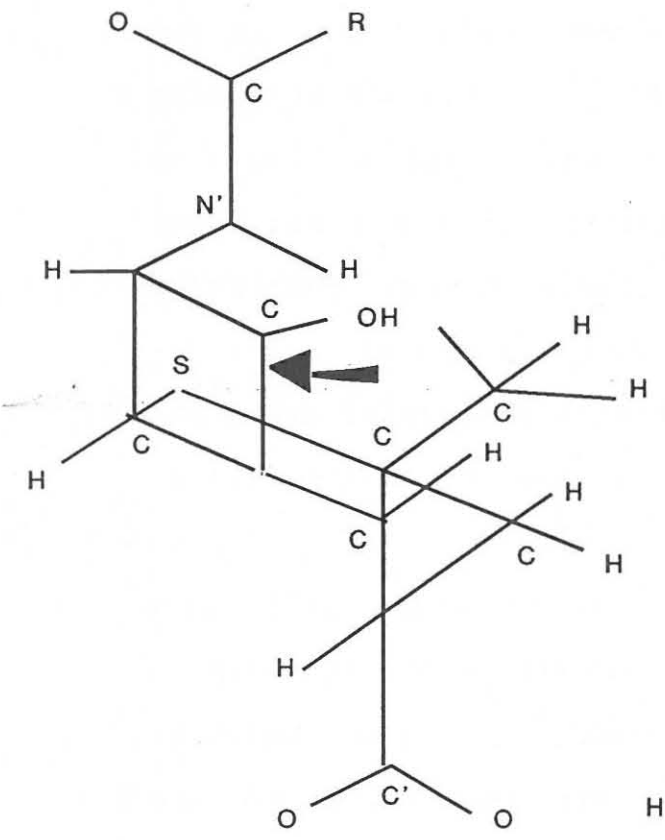
Fig. VI



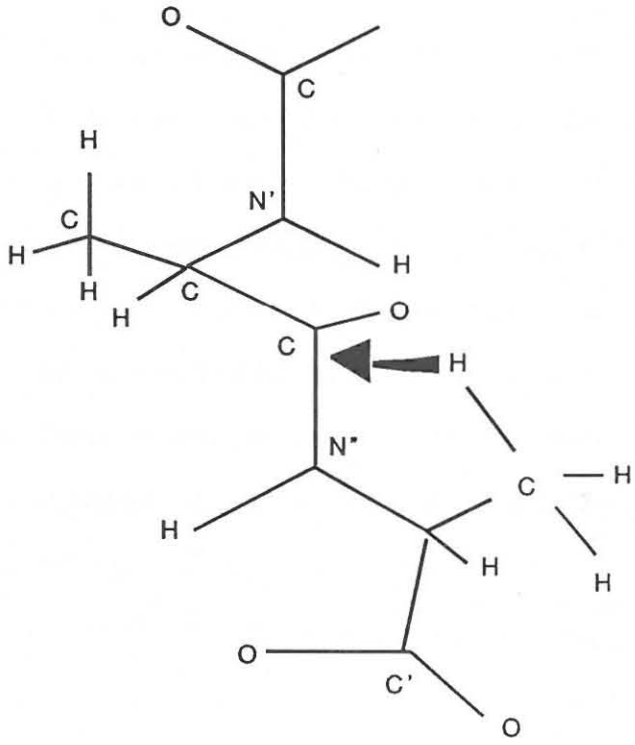
formation of cross-links in murein and the point of penicillin action in detail.

(2)

Fig. VII



PENICILLIN



D-ALA-D-ALA

THE RESEMBLANCE OF PART OF THE PENICILLIN STRUCTURE TO THE BACKBONE OF D-ALA-D-ALA IS INDICATED, WITH THE ARROWS AT THE BONDS BROKEN DURING COVALENT ATTACHMENT TO THE ENZYME INVOLVED.

(2)

It is postulated that the penicillins and cephalosporins occupy the D-alanyl-D-alanine substrate site of the transpeptidase enzyme. After penicillin molecules have attached to the receptors, peptidoglycan synthesis is inhibited and final transpeptidation is blocked. The inhibitors of the autolytic enzymes are inactivated, resulting in cell lysis. Organisms with defective autolysis function are inhibited but not killed by beta-lactam antibiotics and are termed tolerant (10, 11, 12).

Penicillin binds to the cell receptors or penicillin binding proteins (PBPs) some of which are enzymes involved in transpeptidation reactions. From three to six (or more) PBPs are present per cell (e.g. *Staph. aureus* - 4, *E. coli* more than 7). Some PBPs are targets for lethal action of beta-lactam antibiotics. These are generally high molecular weight protein while others are not. PBPs are under chromosomal control and mutations may alter their numbers or affinity for beta-lactam drugs (10, 11).

Goodell *et al* (13) describe the effects of benzylpenicillin on the cell envelope of *Neisseria gonorrhoeae*. The murein synthesis was halted due to the inhibition in the final stage. The rate of turnover of glucosamide (an amino acid found only in the murein in the peptide side chain) increased and lipids and lipopolysaccharides were shed into the medium. There

was a loss of lipid from the cell. The protrusion of the cell membranes as well as holes in the murein cell wall were evident by electron microscopy. A rapid loss of motility was noted.

2.3 RESISTANCE TO PENICILLIN

Is therefore encountered:

(1) **if the enzyme beta-lactamase is present.** This is plasmid controlled and can be induced. Beta-lactamases attack the amide bond in the beta-lactam ring, disrupting the molecule and causing it to become ineffective. Beta-lactamases play an important role in the resistance of many bacteria to penicillins and cephalosporins.

(2) **in the case where lack or alteration of penicillin binding proteins occurs.** Penicillin molecules bind to the receptors (PBPs) and inhibit peptidoglycan synthesis thereby blocking the final transpeptidation stage.

(3) **in the presence of permeability barriers** i.e. decreased permeability of the outer membrane thereby preventing the antibiotic from reaching its target sites.

(4) due to tolerance. Organisms with defective autolysin function are inhibited but not killed by beta-lactam drugs and are termed "tolerant" (11, 12).

(5) if L-forms or mycoplasmas are present. Penicillin inhibits cell wall synthesis. L-forms or mycoplasmas being cell wall deficient are not affected by this agent.

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CHAPTER THREE

CHAPTER THREE

MECHANISMS OF RESISTANCE

3.1.1 THE PERMEABILITY BARRIER

The outer membrane of Gram positive organisms is composed of peptidoglycan with co-valently linked teichoic acid and teichuronic acid as well as capsular proteins and carbohydrates located externally to the peptidoglycan layer.

Beta-lactam antibiotics can readily penetrate the Gram positive cell wall which does not present a significant barrier (1, 2, 3, 4, 5). In the Gram negative organism a second membrane is covalently linked with lipoprotein bridges to the peptidoglycan. This outer membrane presents a physical barrier to the penetration of antibiotics and acts as a selectively permeable barrier which prevents or restricts beta-lactam antibiotics from reaching their target sites i.e. the penicillin binding proteins (6, 7, 8, 9).

The beta-lactam antibiotics pass through pores in the outer membrane formed by specific proteins known as porins. The ability of the penicillin or cephalosporin to cross the outer membrane is linked to their hydrophobicity. An increase in the hydrophobicity of the

beta-lactam antibiotic tends to decrease the rate of diffusion (10).

Alterations in the outer membrane porins contribute to resistance.

3.1.2 PENETRATION OF THE OUTER MEMBRANE (OM)

The OM of *N. gonorrhoeae* is generally highly permeable to antibiotics (11). In contrast to *E. coli*, gonococci possess a relatively poor permeability barrier to penicillin. It is postulated that factors in addition to outer membrane porin barriers are responsible for increased resistance (12). The low level of ^{14}[C] penicillin binding by preparations of *N. gonorrhoeae* cell walls seem to exclude it as an important site for penicillin binding (11, 13). Membranes from susceptible strains did however bind more penicillin than membranes from resistant strains (13). Gram negative organisms are capable of making OM alterations as well as penicillin binding protein changes. Very few differences were found in the overall chemical composition of the cell envelope of sensitive and resistant strains (13, 14). There was no difference in the composition of phospholipids and lipopolysaccharides. Low level of non specific resistance to antibiotics was accompanied by a seven fold increase in a 52 kD molecular weight (MW) outer membrane protein (OMP), a disappearance of a principal OMP (POMP) at 36,90

kD and the appearance of a new POMP at 39,40 kD. These small changes point to a more important mechanism of resistance (15).

In a further study, the cell envelope of one resistant and two sensitive strains showed only moderate differences in fatty acid composition (11).

The OM does not contribute too much of a penetration barrier towards antibiotics, only possible low level resistance from decreased penetration. It has been claimed that low level multiple antibiotic resistance may be due to a decreased OM permeability (11).

3.2.1 BETA-LACTAMASE

The first beta-lactamase producing strains of *N. gonorrhoeae* were detected in 1976 (16, 17). Epidemiologists worldwide conducted surveys to establish the incidence of penicillinase producing strains of *N. gonorrhoeae* (PPNG) as these strains demonstrated a high level penicillin resistance. An increase in these resistant strains would limit the efficacy of penicillin in the treatment of gonorrhoea.

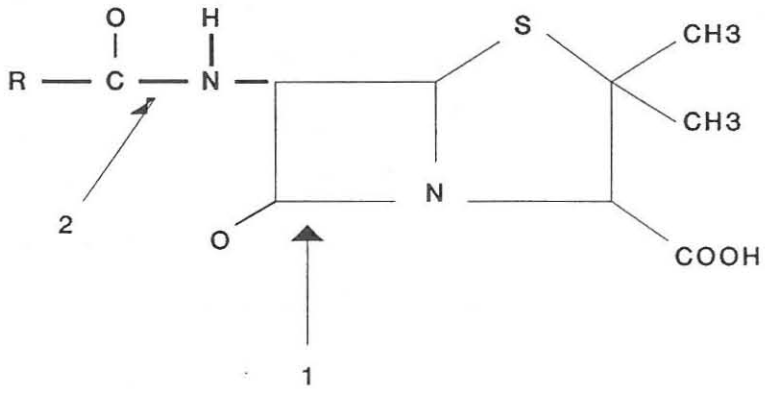
3.2.2 ACTION OF BETA-LACTAMASE

Gonococci contain several plasmids and at least one small plasmid (mw 4,5 x 10^6 kD) carries the gene for beta-lactamase production. These plasmids are transferable between organisms and may have been acquired from other beta-lactamase containing organisms. PPNG contain an R-factor which codes for the synthesis of beta-lactamase (18).

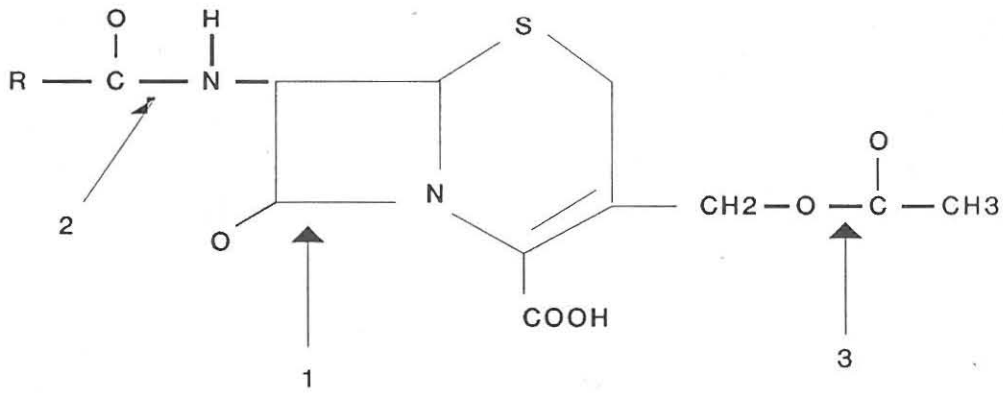
Commercially available beta-lactam antibiotics fall mainly into two groups, namely the penicillins and the cephalosporins. These antibiotics are susceptible to enzymatic modification and degradation i.e. by beta lactamase activity.

Fig. VIII

1. PENICILLINS



2. CEPHALOSPORINS



1. β-LACTAMASE
2. ACYLASE
3. ESTERASE

SITES FOR ENZYMATIC ATTACK ON PENICILLINS AND CEPHALOSPORINS

(20)

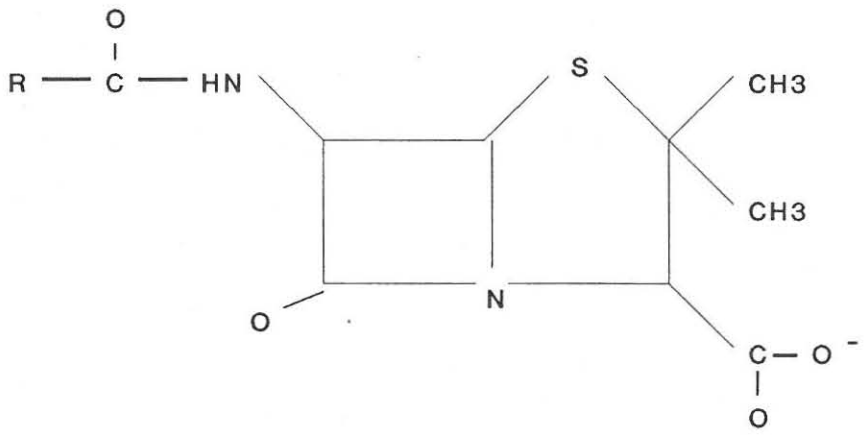
Penicillins and cephalosporins may also be degraded by other enzymes e.g. amidase or acylase enzymes, however from a clinical point of view the most important of the degradation enzymes are the beta-lactamases.

Penicillins and cephalosporins are distinguished from other antibiotics by their beta-lactam ring in the nucleus of the antibiotic molecule (figure VIII). The integrity of this structure is essential for the antibacterial activity of the antibiotic. Beta-lactamases attack the amide bond in the beta-lactam ring, disrupting the molecule and causing it to become ineffective. Beta-lactamases therefore play an important role in the resistance of many bacteria to penicillins and cephalosporins. Once the beta-lactam ring of penicillin or cephalosporin is hydrolysed by a beta-lactamase, penicilloic acid is produced (figure IX). Antibacterially inactive penicilloic acid has two acidic groups where the previous compound had only one. These can readily be detected and assayed (19).

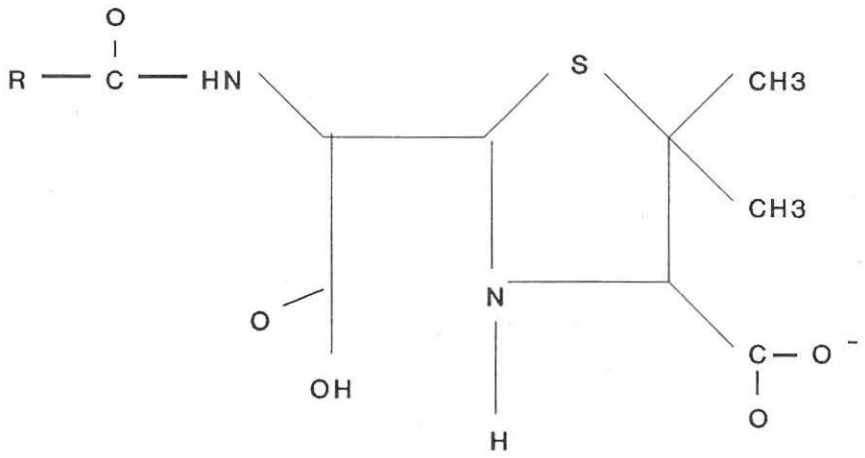
Many different types of beta-lactamases have been described.

Beta-lactamase from Gram negative organisms are different from those produced by Gram positive organisms. They are often produced in much smaller amounts and are inducible, although often found at much lower levels after induction.

Fig. IX



BASIC PENICILLIN STRUCTURE



BASIC PENICILLOIC ACID STRUCTURE

(20)

In Gram negative organisms the beta-lactamase is often cell bound and located internal to the outer membrane of the cell wall. In the periplasmic space the cell wall acts as a permeability barrier for many antibiotics. This location often makes enzyme detection difficult necessitating sonification of the cells. The resulting suspension is examined for beta-lactamase activity. The level of beta-lactamase, if inducible may be increased by growing the relevant organism in the presence of 100 mg/l of benzyl penicillin (20).

CLINICAL SIGNIFICANCE

Beta-lactamases inactivate penicillin and cephalosporin to antibacterially inactive substances. Most Gram negative organisms are capable of producing beta-lactamase, yet many are sensitive to beta-lactam antibiotics. The rate of antibiotic inactivation is dependent upon many factors and the presence of beta-lactamase alone does not signify a penicillin or cepholosporin resistant organism. The organisms possess an outer cell membrane which can act as a penetration barrier. Combined lack of outer cell permeability and beta-lactamase activity may determine the ultimate resistance of the organism (20).

DETECTION OF BETA-LACTAMASE

The chemical changes in a sensitive substrate which take place after hydrolysis of the beta-lactam bond in the penicillin or cephalosporin compound demonstrate the presence of beta-lactamase.

This may be demonstrated in various ways, namely:

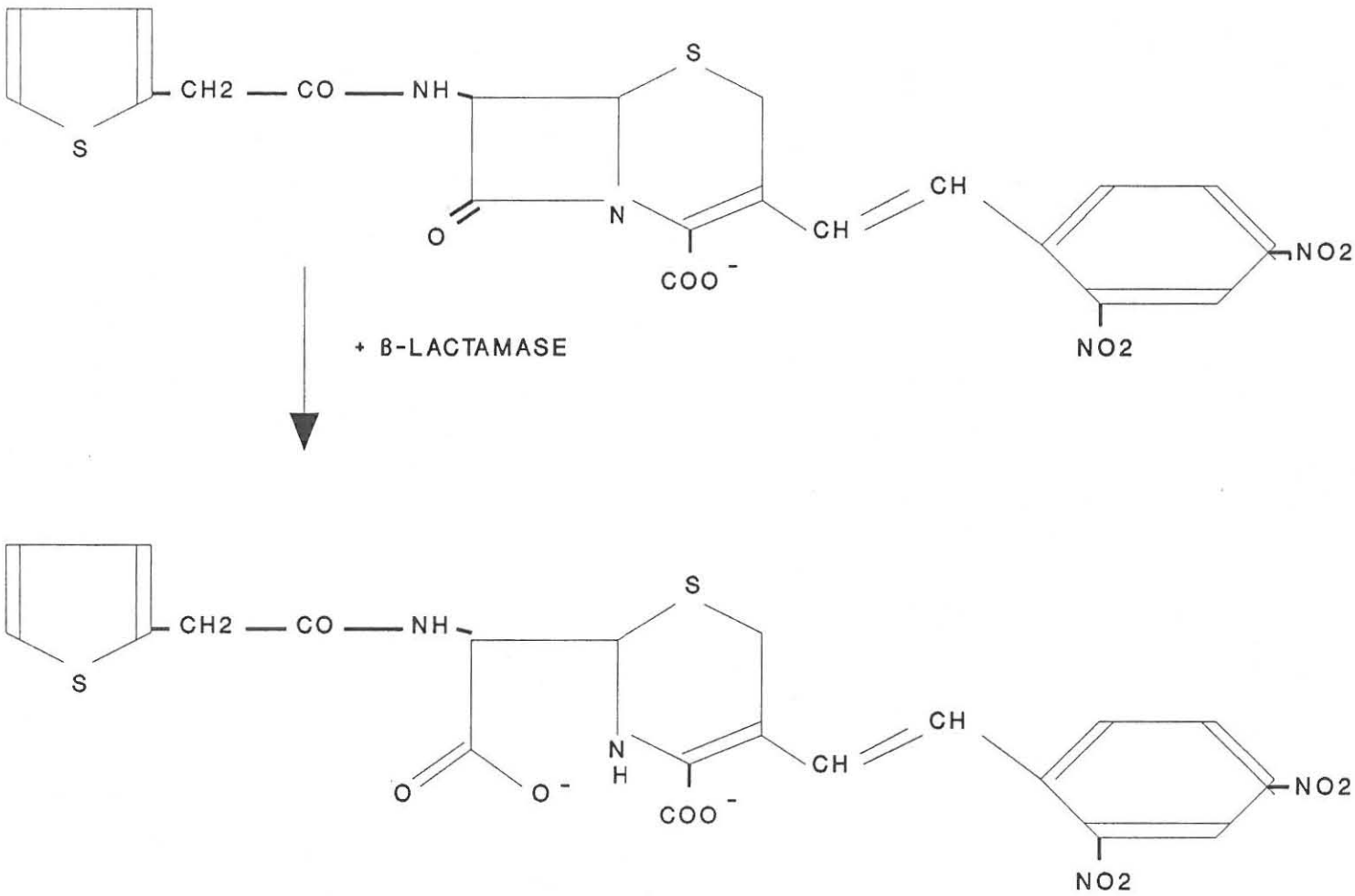
(a) The formation of an extra carboxyl group to give a dibasic acid compound can be detected by using pH indicators or by its ability to reduce iodine.

(b) The loss of antibacterial activity of the compound can be detected by microbiological assay techniques.

(c) An electron shift in the chromogenic cephalosporin molecule gives rise to the formation of coloured particles.

Various methods to detect beta-lactamase activity are available however one of the most sensitive and simple methods for both Gram positive and Gram negative organisms is by using chromogenic cephalosporin 87/312 (Glaxo Research Ltd). The hydrolysis of chromogenic cephalosporin (figure X)results in the molecule undergoing a distinct colour change (19).

Fig. X



ENZYMATIC HYDROLYSIS OF CHROMOGENIC CEPHALOSPORIN 87/312

(20)

THE IODOMETRIC METHOD

Iodine is reduced from a blue starch iodine complex to a colourless compound. This method is less reliable for Gram negative organisms and only Gram negative organisms producing large amounts of beta-lactamase give reproducibly positive results. This method has however proved successful in the detection of beta-lactamase from *Haemophilus influenzae* and *N. gonorrhoeae*. It is specific for beta-lactamase production but is described as the least sensitive method for beta-lactamase detection (20).

CONCLUSION

The detection of cell bound beta-lactamases of Gram negative organisms can present problems. It may be necessary for the organisms to be disrupted e.g. by sonication in order to release the cell bound beta-lactamase to interact with the substrate. The outer membrane of Gram negative organisms play an important part in the whole cell beta-lactamase reaction.

Beta-lactamase producing strains of *Haemophilus influenzae* and *N. gonorrhoeae* are easily detected due to the abscence of an antibiotic barrier. Although expensive, the chromogenic cephalosporin 87/312 is regarded as the most sensitive and simple method to use (19, 20, 21, 22, 23).

3.3.1 CHROMOSOMAL RESISTANCE

Until recently the genetic determination of resistance in penicillin resistant gonococci was chromosomal, as penicillinase producing strains were only detected in 1976. Resistance can be acquired *in vitro* through the transformation of DNA (24) and is associated with the reduced permeability of the bacterial cell envelope and reduced binding of penicillin to the cell membrane (25, 26, 27, 28).

Penicillin resistance in *N. gonorrhoeae* may therefore be due to either

(a) **destructive mechanisms** e.g. beta-lactamase production which leads to enzymatic destruction

(b) **production of permeability barriers** - chromosomal (29)

(c) **change of target site** - arising from a series of mutations in the penicillin binding proteins of *N. gonorrhoeae* which results in a decreased affinity for penicillin as a substrate (29, 30) (chromosomal).

There is strong evidence that permeability barriers are linked with beta-lactamase production (31).

Resistant non-PPNG strains were reported as early as 1957 and were associated with treatment failures at the dosages of penicillin then employed (32, 33). These strains showed an ever increasing frequency worldwide (34). The upper level of chromosomal resistance, until recently, rarely exceeded 0,5 - 1,0 mg/l although more resistant strains were prevalent in the Far East (34). Strains reported from Canada had a MIC as high as 50 mg/l, yet did not produce beta-lactamase (33). These chromosomally resistant strains often show cross resistance to other antibiotics. They conspicuously lack the R-plasmid and/or conjugative plasmid so characteristic of PPNG (29).

3.3.2 COMPARISON BETWEEN PPNG AND NON-PPNG STRAINS

PPNG	NON-PPNG
1) Discovered in the late 1975 or early 1976.	Discovered as early as 1957.
2) Produce beta-lactamase which is responsible for penicillin in-activation	Do not produce beta-lactamase
3) Possess a resistance plasmid which codes for beta-lactamase	No resistance plasmid found. Penicillin resistance develops through chromosomal mutation
4) An additional transfer plasmid may also be present	No transfer plasmid is found.
5) Usually show a total resistance to penicillin	The resistance is usually partial. Strains may respond to a high dose of penicillin

(29)

If the rate of entry of beta-lactam into the cell is equal to or less than the rate of hydrolysis, the organism triumphs hence the importance of barrier associated with enzymatic resistance.

3.3.3 PENICILLIN BINDING PROTEINS (PBP)

A third type of resistance which has been shown to occur in both Gram positive and Gram negative organisms involves modification of one or more penicillin binding proteins to prevent or decrease interaction of the antibiotic and protein while retaining the enzymatic activity of the penicillin binding protein. This type of intrinsic resistance should be distinguished from tolerance where the sensitivity of the PBP is unchanged and the antibiotics are bacteristatic rather than bactericidal because of changes in the autolytic activity (1).

PBPs are bacterial proteins (35, 36) in the cytoplasmic membrane, that bind penicillin and other beta-lactam antibiotics covalently. Individual species of bacteria have a characteristic set of PBPs each with a different affinity for penicillin. These proteins are thought to be natural enzymes involved in the cross linking of murein (37). At least three types of PBPs have been distinguished, one type especially involved in the generalised cross linking that occurs at many sites in the

periphery of the cell, a second may participate in the special cross linking at the septum between separating daughter cells. A third appears to function at sites where the murein turns corners to determine the cell shape (37).

The widespread use of beta-lactam antibiotics which readily penetrate the outer membrane and are highly resistant to hydrolysis, provides a considerable selection pressure for organisms resistant to their antibacterial activity by virtue of changes in PBP affinity. There is evidence that such organisms are being found among clinical isolates and that intrinsic resistance will become an increasing problem.

Altered PBP may be responsible for the high level of resistance seen clinically with some organisms that are usually very sensitive to penicillin. Highly penicillin resistant non beta-lactamase producing pneumococci have been isolated (5, 38, 39).

The first organisms to show intrinsic resistance due to decreased affinity of a PBP were mutants of *E. coli* resistant to mecillinam, isolated in the laboratory (1, 14, 40).

Acquisition of resistance was accompanied by a decrease in the affinity of PBP-2. *N. gonorrhoeae* provides a good example of the development of resistance by changes in PBP

affinity. Highly penicillin resistant strains of *N. gonorrhoeae* with intrinsic resistance, negative beta-lactamase reactions and altered PBP have been isolated with increasing frequency (14).

According to Lorian (1) in clinical terms these organisms are currently overshadowed by the beta-lactamase producing strains. It remains to be seen how long this will be the case with the constant increase in chromosomally resistant strains being detected.

All bacteria examined contain multiple PBPs (41). *Staph. aureus* has only four PBPs but some Gram negative bacilli like *E. coli* have seven or more. Some PBPs are lethal targets for the action of beta-lactam antibiotics i.e. mainly high molecular weight PBPs, while others are not.

The study of PBPs in bacterial membranes has led to significant progress in understanding beta-lactam antibiotic action (5).

As these antibiotics bind to their receptors in a covalent manner, the antibiotic enzyme complexes can be identified in the following way:

(1) Radiolabelled penicillin or cephalosporin is bound to the bacterial cell envelopes that are prepared by sonication and differential centrifugation.

Most PBPs are integral membrane proteins found in the inner membrane (IM) in Gram negative organisms and thus require treatment with non ionic or mild anionic detergents for solubilzation in active form (35).

(2) The different proteins are separated by electrophoresis on polyacrylamide gels. The protein covalently bound to radioactive antibiotic is detected by autoradiography (42, 43, 44).

(3) In a given organism PBPs are numbered in order of decreasing apparent molecular weights, which usually ranges from 140 to 40 kD, although PBPs as small as 25 kD have been detected (45).

(4) There is not necessarily a relationship between equivalently numbered PBPs of two unrelated organisms although taxonomically related bacteria have similar PBP patterns (46, 47).

(5) Most bacteria contain between 1 000 to 10 000 total PBP molecules per cell, with PBPs comprising approximately 1% of the total membrane protein (36).

(6) Particular PBPs vary greatly in their relative abundance e.g. *E. coli* (43) PBP 2 accounts for ± 1% of the organism's penicillin binding activity as compared to ± 90% of the total penicillin binding activity for *B. stearothèrmophilus* PBP 5 (48).

Spratt (49) made great contributions to the understanding of the role of PBPs in the resistance of beta-lactam antibiotics. Penicillin sensitive enzymes can be quantitated and their sensitivities to inhibition by beta-lactam antibiotics monitored.

Multiple PBPs can be routinely detected in the membranes of *E. coli* K12 (43, 49) and other Gram negative bacilli (46).

Only those of highest molecular weights (PBP 1a, b, 2 + 3) are lethal targets for beta-lactam antibiotics. The remaining proteins were found to be dispensable (5, 37, 38). Inhibition of any or all of the essential binding proteins eventually results in cell death following a characteristic morphological change in the bacterial cell (50). Five PBPs were detected in *B. subtilis*. PBP 2 was found to be the killing site (51).

3.4 REPORTED ROLE OF PBP IN THE RESISTANCE OF *NEISSERIA GONORRHOEAE*

Reports of chromosomally mediated resistant *N. gonorrhoeae* (CMRNG) are more frequent (52, 53, 54, 55). Studies on recent CMRNG have concentrated primarily on epidemiology and very little has been reported on possible resistance mechanisms (30) especially in South Africa.

N. gonorrhoeae has three major PBP with molecular weights (MW) of approximately

PB1 87 - 90 kD
PB2 59 - 63 kD
PB3 44 - 48 kD

(PBP 1 87 kD, PBP 2 59 kD, PBP 3 44 kD) (56)
(PBP 1 90 kD, PBP 2 63 kD, PBP 3 48 kD) (14)

Rodriquez and Saz (13, 56) reported several more bands however the concentration of penicillin used was much in excess of the MIC of the organisms studied. Bands have been produced by using penicillin concentrations much higher than required to saturate PBP 1 - 3.

Noland and Hildebrandt (56, 57) also reported more than three bands in membranes prepared from overnight plate cultures. Autolysis may have been more pronounced than in

broth cultures. Additional bands may represent non specific binding of penicillin to fragments of proteolysed PBP or other protein. An additional band at 34 kD was seen in whole cell preparations but not in inner membranes (56). This may represent degradation products or a subunit of a high MW PBP or altered PBP. No bands were seen in outer membrane (OM) preparations.

PBP 1 and 2 had reduced affinity for benzyl penicillin in resistant strains as compared to sensitive strains. The affinity for PBP 3 was unaltered (14). In sensitive strains PBP 2 has a higher affinity for benzyl penicillin. If PBP 1 and 2 are killing targets, the first steps in the acquisition of resistance is to produce an altered PBP 2 with an affinity for penicillin equal to or less than that of PBP 1. This is followed by a further mutation resulting in an alteration of PBP 1. Minimum inhibitory concentrations will increase and the organisms will no longer be eradicated with penicillin (58).

Relative resistance was associated with decreased binding to PBP 2 and to a lesser extent PBP 1 (56).

PBP 3 of *N. gonorrhoeae* is not an important target of beta-lactam activity. The undiminished binding to PBP 3 in a relatively resistant strain suggests that PBP 3 is not a critical target (14, 59, 60).

Strains varied in the amount of penicillin binding to PBP 1 and 2 but not to PBP 3. Although PBP 3 bound some beta-lactam antibiotics at concentrations below the MIC, PBP 1 and 2 appeared to be more important targets of beta-lactam action against *N. gonorrhoeae* (14).

Similar reports were made by Rodriquez and Saz (1, 12, 13, 14). PBP 1 and 2 of *N. gonorrhoeae* appear to be killing targets and the affinity of PBP 2 for beta-lactam antibiotics is greater than PBP 1 (58).

Resistance to benzyl penicillin must occur with a decrease in the affinity of PBP 2. This produces a strain which has only a low resistance level, since killing still occurs by the inactivation of PBP 1. Higher levels of resistance can be obtained by a decrease in the affinity of PBP 1, as well as mutations that decrease OM permeability (58). Further mutations that decrease the affinity of PBP 1 and 2 will accumulate and result in the increase in the MIC of these intrinsically resistant strains and eventually lead to their no longer being susceptible to beta-lactam antibiotics (58). It is only when both PBP 1 and 2 are modified that higher levels of resistance occur (i.e. > 0,5 mg/l) (1).

PBP 3 appeared to be more strongly labelled in resistant organisms than susceptible organisms (14).

Studies with genetic transformants showed that alterations in PBP 2 accompanied acquisition of resistance to intermediate levels of penicillin (pen A). Changes in PBP 1 were associated with the highest level of resistance controlled by PEN and TEM genes (14).

Rodriguez *et al.* and Nolan *et al.* (13, 57) found that PBPs of gonococci vary between strains.

Dougherty *et al.* (14) found PBP patterns to be highly reproducible. Local and geographically diverse strains did not vary. They therefore postulated that differences in methodologies used e.g. growth methods, electrophoresis procedure etc. may account for this.

Dougherty *et al.* (13, 14) conducted a study using eight high level resistant non beta-lactamase producing strains. These strains were obtained from patients on whom penicillin therapy had failed (52). Similar alterations in PBP were noted.

An increase in the concentration of labelled penicillin above 5 mg/l caused the appearance of additional radioactive bands on the fluorograms. Most had MWs lower than those of PBP 3. All of these bands seemed to be due to non specific labelling, since they were not eliminated by pretreatment of gonococcal membranes with an excess of

non radioactive penicillin. As expected the treatments eliminated the three true gonococcal PBPs (14).

As with a gradual change in PBP and resistance patterns seen in *Streptococcus pneumoniae*, similar changes may be encountered in *N. gonorrhoeae* (4). The gonococcus joins the pneumococcus on the list of bacterial species for which one mechanism of resistance in clinical isolates is altered binding to the target (14, 39, 56, 61, 62). Alteration in the cell envelope may also contribute (15, 60).

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CHAPTER FOUR

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BLOEMFONTEIN SURVEYS

4.1 INTRODUCTION

The increasing penicillin resistance of *N. gonorrhoeae* caused by both PPNG and non-PPNG strains has been described worldwide, as well as in Southern Africa. This fact established the need for epidemiological studies to be conducted in Bloemfontein.

Gonococcal urethritis is more common in black males than non-gonococcal urethritis (1, 2). In 1982 a study was undertaken at a Bloemfontein clinic for sexually transmitted diseases (STD) (unpublished study). Ninety two percent of black males presenting with urethritis were shown to have gonorrhoea. Examination of hundreds of local *N. gonorrhoeae* strains from 1981 - 1983 failed to detect any PPNG strains. Up to mid 1984, no PPNG strains had been detected in Bloemfontein in spite of routine screening of isolates for beta-lactamase production (1). A further study was undertaken in 1984 to determine the incidence, if any of PPNG, as well as penicillin resistance levels in Bloemfontein.

4.2 PATIENTS

Two hundred black males presenting with urethritis at a Bloemfontein clinic for STD during July - September 1984 were investigated. The clinic was visited on one afternoon per week and only patients who had not received antimicrobial therapy in the preceeding four weeks or patients without other concomitant lesions were included in the study.

4.3 METHODS

4.3.1 SPECIMEN COLLECTION

Urethral swabs were taken with calcium alginate tipped swabs (calgiswab type 1 Inolex).

Gram and methylene blue stains were made from direct smears of the urethral discharge. Chocolate blood Agar(CA), Modified Thayer Martin Medium (MTMM) and Modified New York City Medium (MNYCM) were inoculated with material obtained from each patient.

The inoculated plates were transported to the laboratory within one hour in a candle extinction jar. Plates were incubated at $\pm$ 36^{o}C in an atmosphere of 5 to 10% CO_2 for 48 hours.

The Gram stained smears were examined immediately for the presence of leucocytes and intracellular Gram negative diplococci. All patients were treated with penicillin G after examination at the clinic.

4.3.2 CHOCOLATE AGAR

Blood agar base no 2 (Difco 0696 - 17)	19,7 g
Agar	3,5 g
d H_2O	500 ml

Mix well. Sterilize at 121°C for 15 minutes.
Add ± 40 ml sterile sheep blood to hot agar. Mix well and pour plates (3).

4.3.3 MODIFIED THAYER MARTIN MEDIUM

A selective medium for the isolation of *N. gonorrhoeae* and *Neisseria meningitidis*.

- Transgrow basal medium with Trimethoprim (Gibco M 4900)
- Haemoglobin powder (Gibco 1401559)
- 10 ml VCN (vancomycin, colistin, nystatin solution)
- (Gibco 1402497)
- 10 ml CVA Enrichment (Gibco 1401582)

METHOD

(a) Haemoglobin solution

Haemoglobin 2 g

dH_2O 192 ml

Mix and filter through a few layers of gauze to remove undissolved particles. Sterilize at 121°C for 15 mins.

(b) **MEDIUM**

Transgrow basal 19 g

dH_2O 200 ml

Dissolve. Sterilize at 121°C for 15 minutes. Cool to 50°C.

Add:

Sterile haemoglobin solution	192 ml
VCN solution	4 ml
CVA solution	4 ml

Mix well. Pour plates.

Prepare fresh plates every 14 days. (3)

4.3.4 NEW YORK CITY MEDIUM

Gonococcus agar base (Oxoid CM 367)	18 g
dH_2O	425 ml

Sterilize at 121°C for 15 min

Cool to 50°C

Add:

lysed horse blood	50 ml
yeast autolysate supplement (oxoid SR105)	15 ml
LCAT supplement	10 ml

lincomycin colistin sulphate amphotericin B

trimethoprim lactate (Oxoid SR95)

Mix well

Pour plates

Prepare fresh plates every 14 days.

Culture medium for *N. gonorrhoeae* must be highly nutritious, often enriched with lysed horse blood, yeast autolysate or ascitic fluid. Addition of antibiotics makes the medium more selective for gonococci and facilitates their detection by inhibiting overgrowth of genital commensals also present in the average clinical specimen. Unfortunately up to 5% of gonococci will fail to grow on selective media because of their susceptibility to vancomycin.

4.3.5 GRAM STAIN

Flood the slide with methyl violet for ± 3 minutes.

Rinse with tap H_2O. Cover with Lugol's iodine for ± 1 minute.

Rinse with tap H_2O. Decolourize rapidly (5 - 10 seconds) with acetone.

Rinse with tap water. Counterstain with carbolfuchsin ± 3 minutes.

Rinse with tap H_2O. Dry.

Gram positive - blue

Gram negative - red

(3)

4.3.6 METHYLENE BLUE STAIN

Flood the slide with methylene blue ± 2 minutes.

Rinse and dry. (3)

4.3.7 CYTOCHROME OXIDASE TEST

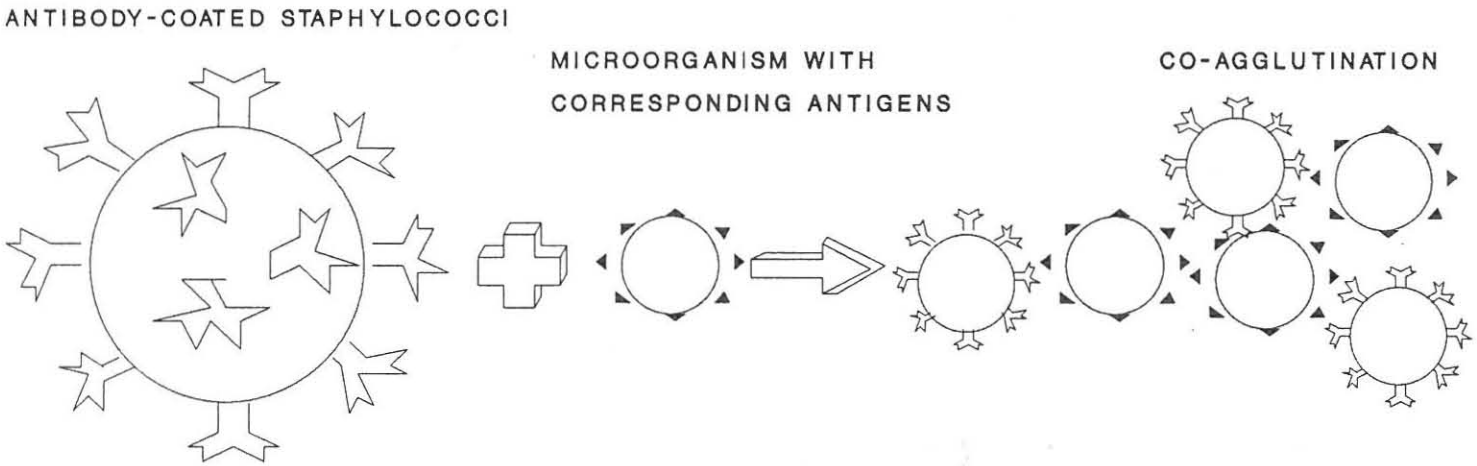
Drop 2 - 3 drops of 1% tetramethyl-p-phenylenediamine dihydrochloride onto a piece of filter paper. Smear the test organism onto the reagent with the aid of a platinum loop or glass rod. A positive reaction is shown by the development of a purple colour within 10 seconds (3).

4.3.8 CO-AGGLUTINATION BY MEANS OF THE PHADEBACT MONOCLONAL GC OMNITEST KIT (PHARMACIA DIAGNOSTICS AB)

The gonococcal omni reagent reacts with a gonococcal specific membrane protein called protein 1. When a sample containing gonococci is mixed with the gonococcal omni reagent, the specific protein 1 antigens of the cell bind to the corresponding specific monoclonal antibodies. In this way a co-agglutination lattice is formed which is visible to the naked eye.

Detailed methods enclosed in each test kit (4).

Fig. XI

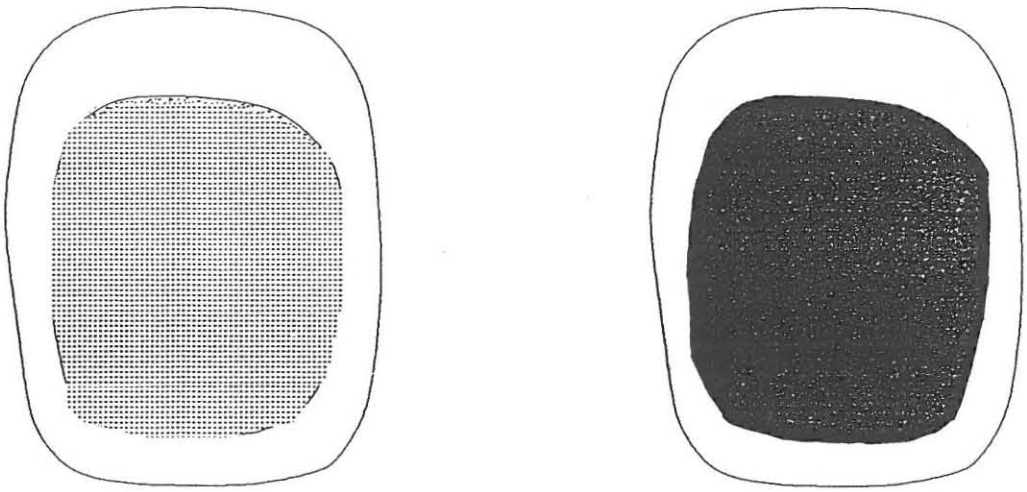


CO-AGGLUTINATION AS SEEN THROUGH AN ELECTRON MICROSCOPE
(X 10 000)

(4)

Fig. XII

APPEARANCE OF REACTION

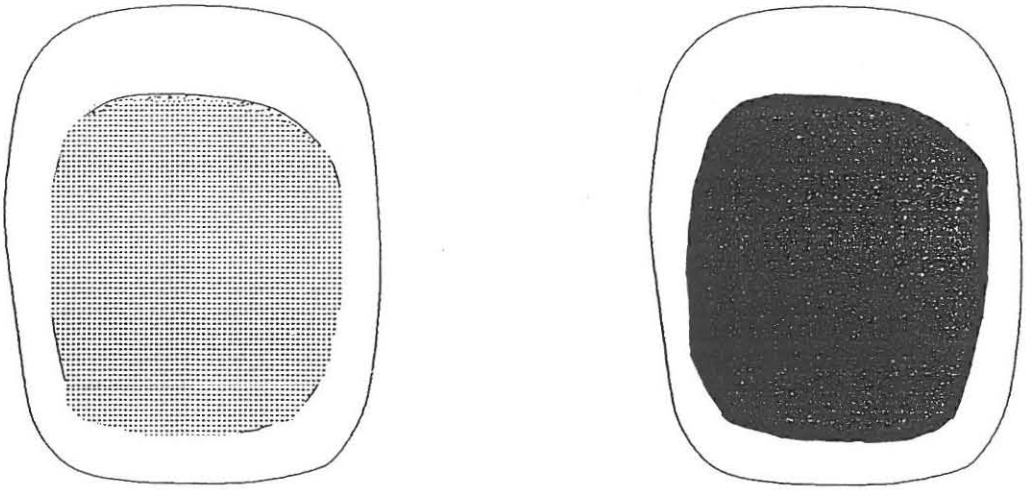


POSITIVE REACTION NO REACTION

THE CO-AGGLUTINATION REACTION

Fig. XII

APPEARANCE OF REACTION



POSITIVE REACTION NO REACTION

THE CO-AGGLUTINATION REACTION

Table 1. Sensitivity and specificity of the phadebact monoclonal GC omnitest kit

Positive reference method	Positive by co-agglutination
582	580
Sensitivity: 99,7%	
Negative by reference method	Negative by co-agglutination
186	186
Specificity: 100%	

4.3.9 BETA-LACTAMASE DETECTION USING CHROMOGENIC CEPHALOSPORIN 87/312 (GLAXO RESEARCH LTD)

The chemical changes in a sensitive substrate which takes place after hydrolysis of the beta-lactam bond in the penicillin or cephalosporin compound demonstrates the presence of beta-lactamase.

One of the most sensitive and simple methods using chromogenic cephalosporin results in the molecule undergoing a distinct colour change.

METHOD

Add a loopful of chromogenic cephalosporin to the test organism smeared on a glass slide. A red colour developing within 5 minutes shows a positive reaction (5,6).

All cultures of *N. gonorrhoeae* were tested for beta-lactamase production using chromogenic cephalosporin 87/312 as substrate (5).

4.3.10 VAN DE KLUNDERT TEST FOR BETA-LACTAMASE PRODUCTION:

STARCH IODINE REAGENT FOR BETA-LACTAMASE

Starch	0,05 g	
Potassium iodide	0,415 g	10 ml PO_4
Iodine	0,0032 g	buffer
Penicillin	0,01 g	

Suspend the starch in 6 ml 0,1 M PO_4 buffer (pH 7,0). Heat. The starch hydrolyses and is clarified. Add KI then I_2. Mix and add penicillin. Make up the volume to 10 ml with phosphate buffer. Using a microtitre plate add 50 µl test and 50 µl starch/iodine mixture. A clearing of the colour developing within 5 minutes shows a positive test. A negative reaction remains blue (7).

4.3.11 ANTIMICROBIAL SENSITIVITY TESTING

Antimicrobial sensitivity testing against the following antimicrobial agents was performed on all isolates using the Stokes disc diffusion method.

penicillin G	2 units
	10 units
tetracycline	10 mg/l
erythromycin	15 mg/l
cotrimoxazole	25 mg/l
ampicillin	10 mg/l
spectinomycin	100 mg/l

THE STOKES METHOD (6, 8)

The Stokes method was performed on a sensitivity testing medium i.e. Diagnostic Sensitivity test agar (DST agar) (Oxoid Cm 261) supplemented with lysed horse blood. The test organism was inoculated from the middle of the plate to give a semi confluent growth. The control organism i.e. *Staphylococcus aureus* NCTC 6971 was inoculated on the periphery of each plate. Not more than 6 antimicrobial discs were tested on each plate. Using the Stokes method the results are interpreted by comparing the zone size of the test organism with the zone size of the control organism which is set up on the same plate, at the same time and under identical conditions. Any

variations affecting the test will affect the control equally and will thus cancel any effect on the interpretation. The interpretation is by comparison of the zone sizes of the test and control organism. There are three categories:

Sensitive (S)
Intermediate (I)
Resistant (R)

the criteria of which have been developed by relating the results of the tests to the clinical effectiveness of the agents.

INTERPRETATION

Sensitive: Zone diameter equal to or wider than the control.

Intermediate: Zone diameter up to 3 mm smaller than the control.

Resistant: Difference in zone diameter more than 3 mm smaller than control.

MIC

Minimum inhibitory concentrations (MICs) of penicillin G were performed on all isolates by the agar dilution method using DST agar supplemented with 6% lysed horse blood. Antibiotic containing plates (range 0,001 mg/l - 16 mg/l) were inoculated with a multipoint inoculator ($\pm 10^4$ cfu/point).

METHOD

DST agar supplemented with 6% lysed horse blood was dispensed in 18 ml volumes. Stock penicillin solutions (10 x concentrated) were double diluted in 2 ml volumes. 2 ml of each antibiotic dilution was added to 18 ml agar and poured into a petridish (1:10 dilution of antibiotic). An antibiotic range of 0,001 mg/l - 16 mg/l was used. An overnight broth culture of each test organism was standardised to a 0,5 McFarland $BaSO_4$ standard. The test organisms were plated onto each antibiotic containing plate by means of a replicator. Plates were inoculated overnight in an atmosphere of 5 to 10% CO_2 at 37°C and then examined for growth. The minimum inhibitory concentration is the lowest concentration of antibiotic where no growth is visible. *Staphylococcus aureus* NCTC 6971 and plates without antibiotics were used as controls (9).

4.4 ISOLATION AND IDENTIFICATION OF NEISSERIA GONORRHOEAE

After 48 hours all plates were examined for the presence of *N. gonorrhoeae*. Colonies of *N. gonorrhoeae* were identified by colonial morphology and microscopy. The identification of *N. gonorrhoeae* was confirmed by a positive oxidase test as well as co-agglutination (Phadebact gonococcus test) (4,10).

4.5 RESULTS

A diagnosis of gonococcal urethritis was established in 92,7% of the patients. Three (2,15%) of the 139 culture positive cases were penicillinase producing strains. The finding of these PPNG strains was the first report of penicillinase producing strains of *N. gonorrhoeae* in Bloemfontein. Subsequent to this study PPNG have been isolated from other patients with gonococcal urethritis and neonates with conjunctivitis in the Bloemfontein area.

SUSCEPTIBILITY TO PENICILLIN

MICs of penicillin G were performed on 93 *N. gonorrhoeae* strains. Two of the three PPNG strains were included in this number. The results in table 2 show that 80,6% of the strains were fully sensitive to penicillin with MIC levels $\leq$ 0,06 mg/l. 15% showed intermediate resistance

with MIC levels 0,125 - 0,5 mg/l. 4,4% of the strains were fully resistant with MIC levels 4 - 8 mg/l. This last number includes the two PPNG strains as well as two resistant strains where penicillinase production could not be demonstrated.

Table 2

SUSCEPTIBILITY OF 93 *N. GONORRHOEAE* STRAINS TO PENICILLIN G

MICs for penicillin mg/l	Number of strains	Percentage
0,007 - 0,06	75	80,6
0,125 - 0,5	14	15,0
4 - 8	4*	4,4

* includes 2 PPNG strains

Table 2

SUSCEPTIBILITY OF 93 *N. GONORRHOEAE* STRAINS TO PENICILLIN G

MICs for penicillin mg/l	Number of strains	Percentage
0,007 - 0,06	75	80,6
0,125 - 0,5	14	15,0
4 - 8	4*	4,4

* includes 2 PPNG strains

4.6 DISCUSSION

92,7% of the black males presenting with purulent urethritis were diagnosed as having *N. gonorrhoeae* infection. In a previous study undertaken in Bloemfontein an incidence of 92% was found which supports the figure in the present study. Reports from Johannesburg and Durban have also shown that gonococcal urethritis is more common than non gonococcal urethritis in black males (2).

This was the first report of PPNG strains in Bloemfontein. Prior to this study hundreds of *N. gonorrhoeae* strains had been examined locally for the presence of penicillinase but all reports were negative. It was confirmed that these infections had been acquired from local contacts (who may have had contact with contacts from elsewhere). Intermediate resistance to penicillin G amongst *N. gonorrhoeae* isolates in South Africa has been reported previously. The finding that 15% of local strains exhibit intermediate resistance is similar to the figures of 14%, 19% and 23% reported from Durban in 1983, Johannesburg in 1978 and 1982 respectively (2, 11, 12). The majority of local strains were fully sensitive to penicillin. The significant proportion of intermediate penicillin resistant *N. gonorrhoeae* strains stresses the need for high dose therapy. At that stage it was suggested that, should the

prevalence of PPNG continue to rise, it may become necessary to review current recommendation regarding first line therapy of gonococcal infection in South Africa.

4.7 FOLLOW UP STUDY

A follow up study was conducted in Bloemfontein in 1986 to determine:

(a) if there was an increase in PPNG strains in the Bloemfontein area

(b) whether there was an increase in penicillin resistance.

The survey was conducted at the same clinic over a period of two months, October and November 1986. Specimens from 200 black males were obtained and cultured in the manner already described. Colonies of *N. gonorrhoeae* were identified and sensitivity testing performed as described.

Table 3

AGE DISTRIBUTION OF 200 PATIENTS

Age in years	Number	Percentage
< 20	11	5,5
21 - 24	70	35,0
25 - 29	58	29,0
30 - 34	25	12,5
35 - 39	20	10,0
> 40	16	8,0

Average age: 28 years

86,5% between the ages of 21 - 40 years

64,0% between the ages of 21 - 29 years

4.7.1 ANTIBIOTICS

All isolates (194) were tested by the Stokes disc method (8) against the following antibiotics:

penicillin	2 units
tetracycline	10 mg/l
erythromycin	15 mg/l
ampicillin	10 mg/l
cotrimoxazole	25 mg/l
spectinomycin	100 mg/l

The following findings were recorded:

tetracycline	1%	intermediate resistance	
erythromycin		no resistance	
cotrimoxazole	9,3%	intermediate resistance	(18/194)
	26,3%	resistance	(51/194)
spectinomycin		no resistance	

All isolates (40) showing penicillin resistance were further tested against

		mg/l
cephalothin	(30)	85% sensitive, 12,5% intermediate 2,5% resistance
cefoxitin	(30)	100% sensitive
cefotaxime	(30)	100% sensitive
kanamycin	(30)	37,5% sensitive, 32,5% intermediate 30% resistant
gentamicin	(10)	62,5% sensitive, 32,5% intermediate 5% resistant
tobramycin	(10)	65% sensitive, 27,5% intermediate 7,5% resistant

(6, 8)

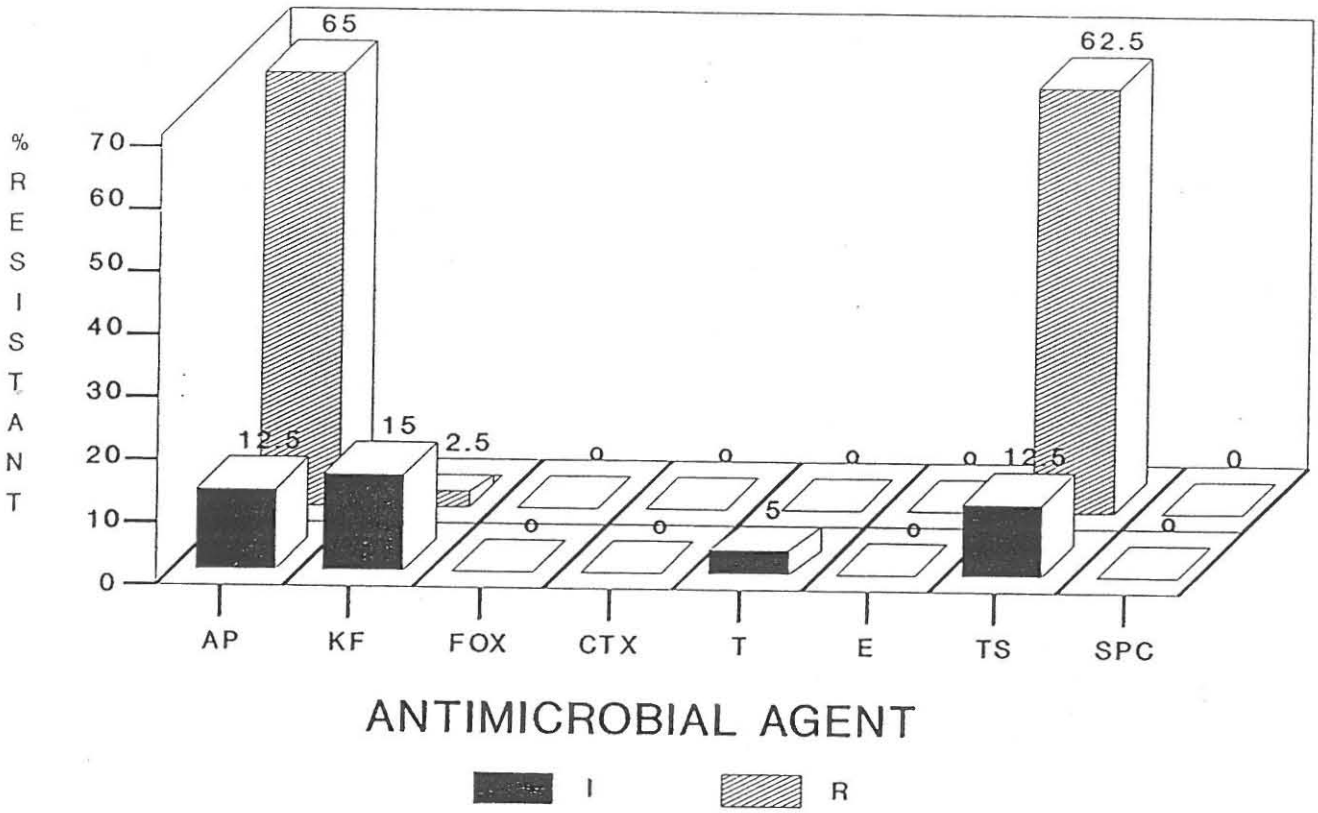


Fig XIII. Antibiotic susceptibilities of penicillin G resistant isolates.

Ap, ampicillin; KF, cephalothin; Fox, Cefoxitin; CTX, cefotaxime; T, tetracycline; E, erythromycin; TS, cotrimoxazole; SPC, spectinomycin; I, intermediately resistant; R, resistant.

4.7.2 RESULTS

A diagnosis of gonococcal urethritis was established by microscopy and culture in 192/200 patients (96%) which concurs with the finding of 92% in the 1984 survey.

28/192* (14,7%) strains were penicillinase producing strains showing an increase from the level in 1984 (2,2%). The results showing increased resistance to penicillin were more alarming:

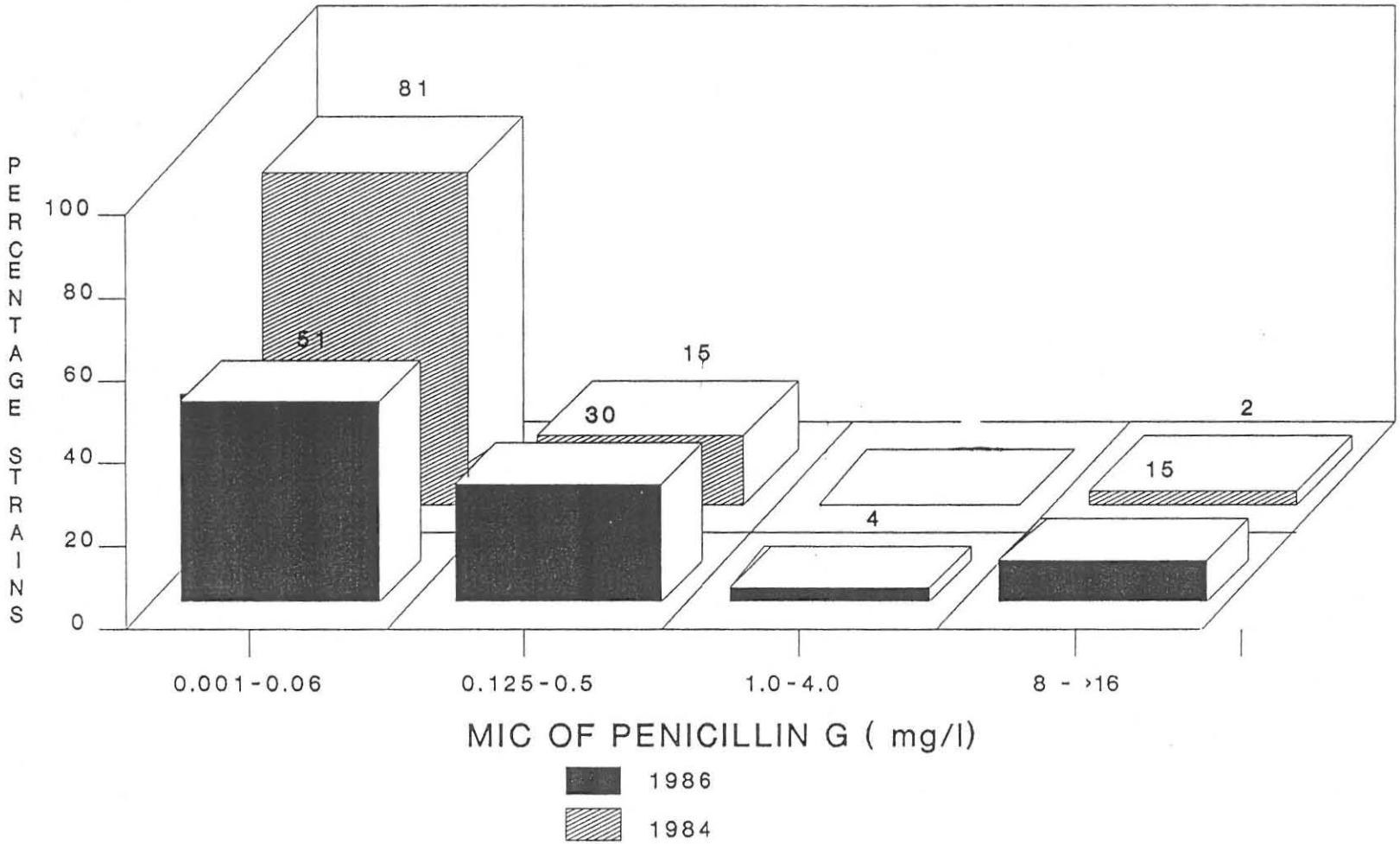
98 strains	(51,3%)	were fully sensitive to penicillin (MIC 0,001 - 0,06 mg/l)
57 strains	(29,8%)	showed intermediate resistance (MIC 0,125 - 0,5 mg/l)
8 strains (non-PPNG)	(4,2%)	were resistant to penicillin (MIC 1 - 4 mg/l)

The 28 PPNG strains (14,7%) showed high level penicillin resistance (i.e. MIC 8 $\geq$ 16 mg/l).

* One strain non-viable

Fig. XIV

SUSCEPTIBILITY TO PENICILLIN G



4.8 CONCLUSION

In Bloemfontein over an interval of 2 years:

(a) the incidence of PPNG has increased from 2,2% - 14,7%

(b) the intermediate resistance to penicillin (0,125 - 0,5 mg/l) increased from 15% - 29,8%

(c) more alarmingly the sensitivity to penicillin (MIC 0,001 - 0,05 mg/l) decreased from 80,6% to 51,3%

This study showed that, in agreement with worldwide reports, resistance caused by PPNG strains has increased. Easmon (13) stated that resistant non PPNG strains may prove to be a more difficult problem than PPNG. In Bloemfontein intermediate resistance was noticeable, with almost one third of the tested isolates showing intermediate resistance to penicillin.

As the intermediate resistance was not enzymatic (i.e. beta-lactamase) these results prompted further studies to demonstrate the strategy of resistance by these organisms. The important question that had to be addressed was:

What role, if any, did altered penicillin binding proteins play in the developing resistance of local strains of *N. gonorrhoeae*? (Chapter five).

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CHAPTER FIVE

ALTERED PBP IN N. GONORRHOEAE

5.1 INTRODUCTION

Increased resistance to antimicrobial agents commonly used in the treatment of *N. gonorrhoeae* infections is of great concern worldwide. Resistance to penicillin is generally attributed to either chromosomal mutation (CMR) resulting in intermediate levels of resistance (MIC for penicillin 0,15 - 0,5 mg/l) or to the acquisition of plasmids that mediate the production of penicillinase (PPNG) usually resulting in high levels of resistance (MIC $\geq$ 2,0 mg/l). Penicillinase producing *N. gonorrhoeae* isolates were first reported in 1976 in Britain and the USA (1) and in Johannesburg and Durban in 1977 (2, 3). Until June 1984 no PPNG isolates were reported in Bloemfontein despite extensive routine screening of *N. gonorrhoeae* isolates for penicillinase production.

A study conducted during the second semester of 1984 revealed that 80,6% of all gonococcal isolates were completely susceptible to penicillin (MIC = 0,06 mg/l). Fifteen percent showed intermediate resistance (MIC 0,125 - 0,5 mg/l) and 4% were resistant (4). An alarming observation in this study was that although

most intermediately resistant and resistant isolates produced ß-lactamase, 2 of the resistant isolates did not produce detectable levels of ß-lactamase. This indicates that chromosomally mediated penicillin resistance was on the increase. Chromosomally mediated penicillin resistance amongst non ß-lactamase producing isolates of *N. gonorrhoeae* might be due to either diminished permeability of OM porins or to altered PBPs. The objective of this study was to determine the contribution of altered PBP profiles in the resistance of *N. gonorrhoeae* to penicillin.

5.2 BACTERIAL ISOLATES

Selected strains of *N. gonorrhoeae* which were obtained from patients attending a local clinic for sexually transmitted diseases during a previous study were used (1986) (4). These organisms had been freeze dried and stored for further investigation. Minimal inhibitory concentrations (MICs) of benzyl penicillin were determined as described (5) and ß-lactamase production was determined by two methods as described (6, 7).

Isolates that were penicillin sensitive, intermediately resistant and resistant, were selected as follows:

1. Three sensitive isolates, MIC $\leq$ = 0,06 mg/l (isolates 18, 88 and 3025).

2. Two intermediately resistant isolates, MIC = 0,5 mg/l. Both were ß-lactamase negative (isolates 94 and 2547).

3. Three resistant isolates, MIC 2 - 4 mg/l. All were ß-lactamase negative (isolates 23, 37 and 68).

4. Two highly resistant isolates, MIC 64 and 256 mg/l respectively. Both these isolates produced ß-lactamase (Isolates 196 and 193).

5.3 **REAGENTS:**

5.3.1 **10 mM PHOSPHATE BUFFER (pH 7.0):**

Solution 1: 0,1M NaH_2PO_4: 15,601 g NaH_2PO_4 was dissolved in 1000 ml (d)H_2O.

Solution 2: 0,1M Na_2HPO_4: 14,196g Na_2HPO_4 was dissolved in 1000 ml (d)H_2O.

To make 10 mM phosphate buffer, 61 ml of solution 1 was added to 39 ml of solution 2 and made up to 1000 ml (d)H_2O (8, 9).

5.3.2 RADIOLABELLED PENICILLIN

Benzyl [^{14}C] penicillin (50 mCi/mmol; 50 µCi per vial) Amersham code CFA 244 was reconstituted by adding 1,45 ml distilled H_2O. This yielded a final concentration of 15,6 µg/ml benzyl [^{14}C] penicillin in labelling mixtures.

5.3.3 RESOLVING GEL BUFFER (Tris aminomethane)

Tris aminomethane	36,3 g
1N HCl	50 ml
Made upto 100 ml with (d)H_2O	
pH 8,8	

(8, 9)

5.3.4 STACKING GEL BUFFER

Tris aminomethane	6,0 g
Dissolved in 85 ml (d)H_2O	
pH was adjusted to 6,8 with 1N HCl	
and made up to 100 ml with (d)H_2O.	

(8, 9)

5.3.5 OVERLAY BUFFER

Resolving buffer	3,75 ml
10% sodium dodecyl sulphate (SDS)	0,3 ml
(d)H_2O	25,75 ml

(8, 9)

5.3.6 RUNNING BUFFER

Glycine	72 g
Tris aminomethane	15 g
SDS 2,5 g	
(d)H2O	5 000 ml
pH 8,2	

(8, 9)

5.3.7 GEL SAMPLE BUFFER

Stacking gel buffer (pH 6,8)	6,6 ml
SDS 3 g	
Glycerol	10 ml
0,02% bromophenol blue	10 ml
(d)H_2O	upto 100 ml

(8, 9)

5.3.8 STOCK STAIN

Coomassie brilliant blue R	2,0 g
(d)H_2O	200 ml
	(8, 9)

5.3.9 WORKING STAIN

Stock stain	62,5 ml
Methanol	250 ml
Glacial acetic acid	50 ml
(d)H_2O	upto 500 ml
	(8, 9)

5.3.10 DESTAINING SOLUTION

Methanol	2 500 ml
Glacial acetic acid	500 ml
(d)H_2O	2 000 ml
	(8, 9)

5.3.11 FIXING SOLUTION

Isopropanol	250 ml
(d)H_2O	650 ml
Glacial acetic acid	100 ml
	(8, 9)

5.3.12 ACRYLAMIDE-ACRYLAIDE STOCK SOLUTION (33%)

33% Acrylaide Solution	50 ml
Acrylamide	32 g
make upto 100 ml with (d)H_2O	

(8, 9)

5.3.13 GELS:

	10% gels	20% gels
Acrylamide-acrylaide Stock solution	9,37 ml	18,72 ml
(d)H_2O	12,97 ml	3,6 ml
*Separation buffer	7,50 ml	7,5 ml
TEMED	7,50 µl	7,5 µl
10% Ammonium persulphate	150 µl	150 µl

*1.5M Tris-aminomethane, 0,4% SDS, pH 8.8

(8, 9)

5.3.14 STACKING GEL STOCK

31,25 ml of the 33% stock solution was diluted to 100 ml with (d)H_2O (8, 9).

5.3.15 5% STACKING GEL

Stacking gel stock	10 ml
Stacking buffer	10 ml
TEMED	10 µl
10% Ammonium persulphate	20 µl

(8, 9)

5.4 METHODS:

5.4.1 GROWTH OF CELLS AND PREPARATION OF MEMBRANES:

All methods used for the preparation of membranes were with minor modifications essentially those of Spratt (10).

1. The selected freeze dried *N. gonorrhoeae* strains were reconstituted and each subcultured onto 5% sheep blood agar plates which were incubated at 37°C in an atmosphere of 5 - 10% CO_2 for 48 hours.

2. The cultures were twice subcultured and checked for purity before reculture onto 100 blood agar plates per strain.

3. After 48 hours all cultures were examined to ensure that pure growths were obtained on all plates.

4. Cultures were harvested and resuspended in 10 ml ice cold 10 mM phosphate buffer and the harvested cells were immediately placed on ice to prevent denaturation of any membrane protein.

5. The harvested cells were centrifuged at 6 000 x g for 6 minutes in a precooled SS34 Sorvall rotor.

6. The supernatants were discarded and the wet weights of the sediment were determined.

7. Each sediment was resuspended in 10 ml ice cold 10 mM phosphate buffer plus 0,72 ml 2-mercaptoethanol.

8. Each cell suspension was sonicated for 3 x 30 second pulses with 30 second intervals to disrupt the cells.

9. Unbroken cells were removed by centrifugation at 8000 x g for 20 minutes at 4^{o}C in an SS34 Sorvall rotor. The sediment containing unbroken cells was discarded.

10. The supernatant was decanted and centrifuged in a Beckman ultra centrifuge equiped with a 50 Ti rotor for 40 minutes at 4^{o}C at 39 100 rpm (100 000 x g) to pellet the cell membrane.

11. The membrane fractions were washed twice in ice cold phosphate buffer by centrifugation at 39 100 rpm for 40 minutes at 4^{o}C.

12. The protein content was measured according to the method of Lowry (11).

13. These preparations contained inner membrane, outer membrane and peptidoglycan and were labelled total membrane.

5.4.2 **DETERMINATION OF PROTEIN CONCENTRATION:**

Lowry method (11):

1. Reagent A: Hundred gram Na_2CO_3 was dissolved in 1l 0,5N NaOH.

2. Reagent B: One gram $CuSO_4 \cdot 5H_2O$ was dissolved in 100ml (d)H_2O.

3. Reagent C: Two grams potassium tartrate ($Na_2C_4H_4O_6 \cdot 2H_2O$) was dissolved in 100ml (d)H_2O.

Reagents prepared in steps 1, 2 and 3 could be stored indefinitely.

4. The spectrophotometer was turned on and allowed to warm up for 30 minutes.

5. Ten 16x150 mm test tubes were numbered. In each tube one of the following volumes of a 0.3 mg/ml solution of bovine serum albumin was pipetted carefully: 0,1, 0,2, 0,3, 0,4, 0,5, 0,6, 0,7, 0,8, and 1,0 ml.

6. The total volume of liquid in each tube was brought to 1,0 ml by adding an appropriate amount of (d)H_2O.

7. Fifteen ml reagent A, 0,75 ml reagent B, and 0,75 ml reagent C were mixed thoroughly in a 50 ml Erlenmeyer flask.

8. One ml of the solution prepared in step 7 was added to each of the tubes prepared above. The tubes were vortexed to mix them thoroughly.

9. The tubes were incubated for 15 minutes at room temperature.

10. While the tubes were being incubated, 5,0 ml 2N Folin-phenol reagent was added to 50 ml (d)H_2O in a 125 ml Erlenmeyer flask. The solution was mixed thoroughly.

11. At the conclusion of the incubation period (step 9), 3,0 ml of the solution made in step 10 was forcibly pipetted into each tube. The resulting solution was vortexed immediately. It is very important that the additional

mixing be accomplished in as short a time as possible. The addition to and mixing of each tube should be completed before proceeding to the next.

12. The absorbance of each sample was determined at a wavelength of 750 nm.

13. The colour of the samples remained stable for at least 45 minutes.

14. A Standard curve was constructed by plotting absorbance at 750 nm against mg/ml protein.

15. Test samples were assayed simultaneously using identical procedures to those used for the standard curve. The protein concentration of test samples were determined from the standard curve.

5.4.3 SEPARATION OF INNER AND OUTER MEMBRANES AND LABELLING WITH ^{14}C PENICILLIN (10)

1. The protein levels were adjusted to approximately 5 mg/ml with ice cold 50 mM phosphate buffer.

2. Forty µl ^{14}C benzyl penicillin was added to 400 µl of the total membrane and incubated at 30°C for 10 minutes.

3. The reaction was terminated by addition of 10 µl of cold penicillin (120 gms/l) and 20 µl 20% sarkosyl.

4. These reaction mixtures were incubated for 20 minutes at room temperature to dissolve inner membranes. Two hundred µl was removed and saved for later use.

5. The remaining membranes were sedimented by centrifugation at 100 000 x g (39 100 rpm) for 40 minutes at 10°C in a 50 Ti rotor.

6. The supernatant fluid contained the labelled inner membrane proteins and the pellet the labelled outer membrane proteins.

7. The sediment was resuspended in 2 ml of 1% sarkosyl and incubated for 5 minutes at room temperature and again centrifuged as before. This was labelled the washed outer membrane proteins.

5.4.4 MEASUREMENT OF BINDING OF BENZYLPENICILLIN BY COMPETITION WITH BENZYL [^{14}C] PENICILLIN (10)

1. Two hundred µl of non labelled inner membrane protein was added to 10 µl cold benzylpenicillin (120 gms/l) and incubated for 10 minutes at 30°C.

2. Twenty µl of 50 µCi/ml benzyl [^{14}C] penicillin was added and incubated for a further 10 minutes at 30°C.

3. The binding reaction was terminated by the addition of 5 µl cold benzylpenicillin (120 gms/l) and 10 µl of 20% sarkosyl.

(10)

5.4.5 ELECTROPHORESIS

Slab gel electrophoresis was performed on the labelled inner and outer membranes as described by Laemmli and Favre (12).

1. A 10 - 20% gradient acrylaide-acrylamide gel was run for approximately six hours. The powerpack was set to constant current at 60 mA. The voltage increased from 100 V to approximately 350 V during the run.

2. Ten to twenty µl sample volumes were loaded per slot to give 50-100 µg sample protein per slot.

Polyacrylamide gel electrophoresis was used for determining the molecular weights of protein. The molecular weight of membrane proteins was determined by comparing their electrophoretic mobilities with those of protein standards of known molecular weight.

The relative mobility (R_f) of the unknown protein is measured and is calculated by:

$$R_f = \frac{\text{distance migrated by protein from the origin}}{\text{distance migrated by marker dye}}$$

A standard curve was generated by plotting the R_f of each standard protein of the $\log_{10}$ of its molecular weight. The MW of an unknown protein can then be determined by finding its R_f on the standard curve and reading the $\log_{10}$ MW from the ordinate. The antilog of this number is the MW of the protein.

Table 4: Pharmacia low molecular mass calibration kit (Cat. no. 17-0446-01):

	Subunit MW	Source
phosphorylase b	94 000	rabbit muscle
albumin	67 000	bovine serum
ovalbumin	43 000	egg white
carbonic anhydrase	30 000	bovine erythrocytes
trypsin inhibitor	20 100	soyabean
alpha lactalbumin	14 400	bovine milk

Under a given set of electrophoretic conditions i.e. pH voltage, gradient, time and gel concentration, the electrophoretic mobility of a protein depends on its charge density, size and shape. Using SDS-PAGE protein molecular weights can be determined by comparison of the electrophoretic mobilities of unknown protein with the electrophoretic mobilities of the standard proteins of known molecular weights.

SDS denatures and interacts with a wide variety of proteins (13). Proteins with different charge, size and shape characteristics are reduced to SDS protein complexes of their constituent polypeptide chains. These SDS protein complexes have a constant charge per mass unit since the charge of the bound SDS masks the protein charge. SDS protein complexes have identical conformation which vary in size directly in relation to the protein subunit molecular weights (14).

Electrophoresis in a gel of increasing acrylamide concentration (gradient gel electrophoresis) offers advantages over electrophoresis in a gel of uniform concentration. The bands show improved sharpness and it is possible to resolve a wide range of protein sizes on the same gel.

In gradient gel electrophoresis the proteins migrate into regions of progressively higher gel concentrations and smaller average pore size. As the separation proceeds there is progressive decrease in protein mobility as the decreasing pore size increasingly restricts the migration of the protein. If the electrophoresis is continued until the protein migration has stopped, proteins of low charge will catch up to proteins of similar size but higher density. The influence of the protein's charge on its final migration position is minimised and the protein's final migration position is a function of its size. This allows molecular weights of unknown proteins to be determined by comparing their migration distances with those of standard proteins (15).

After the run the gels were stained in Coomassie brilliant blue, destained and soaked in 5% glycerol solution for 30 minutes to prevent cracking.

5.4.5.1 **STAINING**

1. Gels were fixed in fixing solution (described under 5.3.11 above) for 20 minutes.

2. Gels were stained for 2 hours in Coomassie brilliant blue working stain.

3. Destained in destaining solution for 1 hour.

4. Step 3 was repeated in fresh destaining solution until gel was clear.

(9)

5.4.5.2 DRYING

Glycerol soaked gels were placed onto Whatman filter paper, covered with porous cellophane film and all bubbles were removed by gentle rolling with a rubber roller. The gels were then dried at 60^{o}C for 2 hours in a Hoefer model SE 11600 slab dryer. After drying and to prevent cracking, the gels were cooled under vacuum for approximately 20 minutes before the vacuum was released. Gels used for fluorography were first soaked in Amplify^{TM} NAMP.100 (Amersham) with agitation for 30 minutes before drying as above.

5.4.6 FLUOROGRAPHY

1. The gels were fixed for 30 minutes in fixing solution (as described under 5.3.11 above). Alternatively stained gels could be used.

2. Gels were soaked in Amplify™ reagent for 30 minutes with constant agitation.

3. The gel was removed and dried under vacuum as described under (5.4.5.2) above.

4. The gel was brought in close contact with Hyperfilm™ (Amersham code RPN7). An X-ray cassette was used for this purpose. Films were stored at -70°C for 17 days and brought to room temperature before they were developed by standard methods for X-ray film.

5.4.6.1 PREFLASHING OF X RAY FILM (HYPERFILM™):

Preflashing was used to improve the sensitivity and linearity of the response of Hyperfilm™ (Amersham Code RPN7).

A battery operated photographic flash unit was used. An orange filter (Wratten 1 or 2) was taped over the flash window as well as a few layers of Whatman no. 1 filter

paper to give an absorbance of about 0,1 when the film was preflashed.

5.5 RESULTS

5.5.1 WET WEIGHT CELL YIELDS OF ISOLATES:

Growth of *N. gonorrhoeae* isolates on 100 blood agar plates yielded wet weights of between 2,1 g to 18,55 g wet packed cells. These yields were sufficient to give enough total membrane proteins for visible protein bands in all cases.

5.5.2 DETERMINATION OF PROTEIN CONCENTRATION

Washed total membrane protein yields for the different isolates were determined by the Lowry method (11) with bovine serum albumin fraction V as reference standard. Results are presented in table 5:

Table 5: Protein concentrations of total membrane preparations obtained for the isolates investigated:

Isolate number	Wet weight of packed cells (g)	Protein mg/ml	Penicillin resistance	MIC mg/l
23	4.64	12.0	R	4.00
2547	6.03	15.0	R/I	0.50
37	2.10	5.0	R	4.00
88	4.25	15.0	S	0.03
3025	4.33	14.0	S	0.03
68	4.80	7.5	R	2.00
18	18.55	23.0	S	0.06
94	5.98	26.0	R/I	0.50
193	11.48	29.0	R*	256.00
196	14.68	36.0	R*	64.00

* Beta-lactamase producing isolates.

S — Sensitive

I — Intermediate

R — Resistant

Small aliquots were stored at -80°C after suitable dilution and could be thawed when necessary for further extraction and examination.

5.5.3 RECOVERY OF MEMBRANE PROTEINS FROM PACKED CELLS

A linear relationship existed between the amount of membrane protein and the wet packed cell weight. However, the recovery was not completely qualitative as indicated by an r^2 value of 0.561. This is illustrated in fig. XV and table 5.

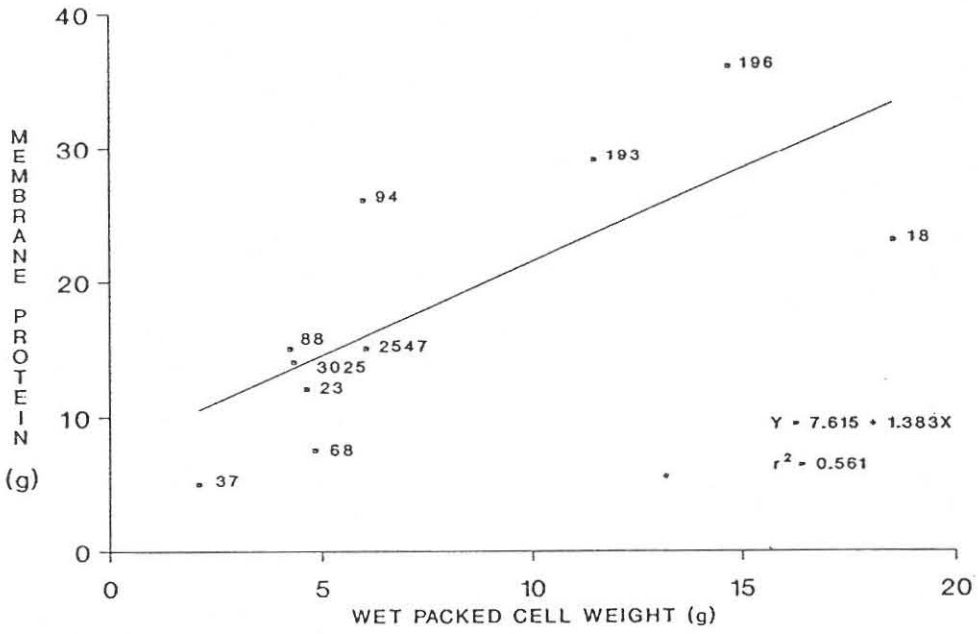


Fig. XV: Recovery of membrane proteins as a function of the wet packed cell weight.

5.5.4 DETERMINATION OF MOLECULAR WEIGHTS OF MEMBRANE PROTEINS

The MW of the unknown proteins were determined by comparing their electrophoretic mobilities with those of protein standards of known MW. A standard curve was generated by plotting the R_f of each standard protein against the $\log_{10}$ of its MW. The MW of any unknown protein was then read off the standard graph or calculated from the regression equation. A typical standard graph is represented in fig. XVI.

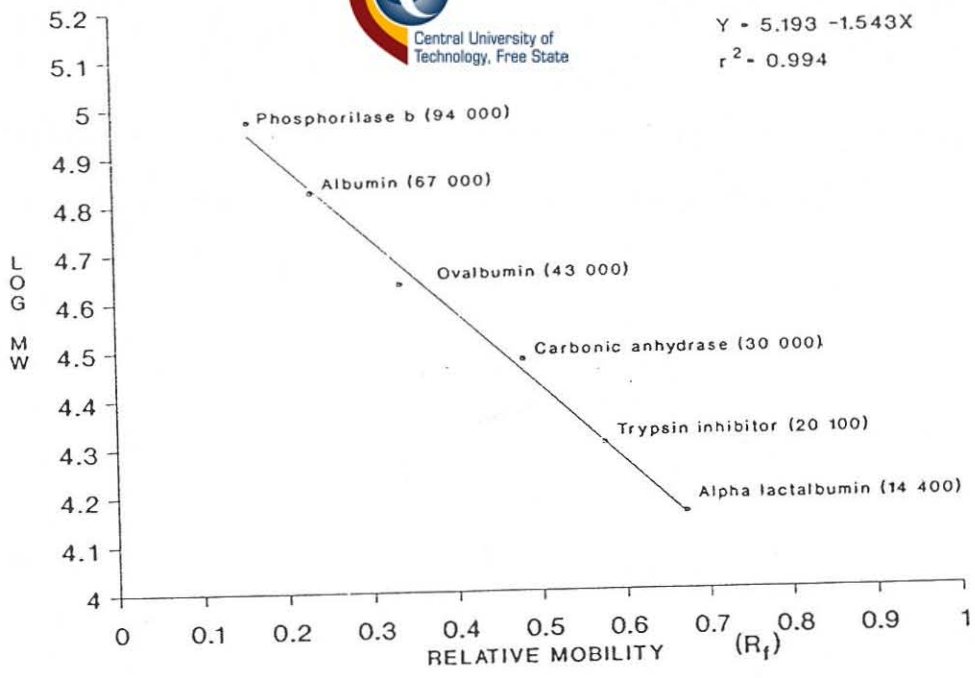


Fig. XVI: Standard curve for the determination of molecular weights of proteins. The MW of an unknown protein can be determined by finding its R_f on the standard curve and reading the $\log_{10}$ MW on the ordinate. Alternatively the $\log_{10}$ MW can be calculated from the regression equation.

5.5.5 BINDING OF ^{14}C PENICILLIN TO PBPs OF *N. GONORRHOEAE*

Slab gel electrophoresis was performed on the inner and outer membrane fractions as described by Laemmli and Favre (12). The acrylamide monomer gels were however replaced with acrylamide-acrylaide gels. This was done to eliminate cracking of the gels during drying. Benzyl[^{14}C]penicillin was used in conjunction with a fluorography method which was a modification of that of Bonner and Laskey (16) to illustrate binding of the

isotope to specific membrane proteins separated by electrophoresis (fig. XVII and fig. XVIII).

Fig XVII

PENICILLIN SENSITIVE STRAINS

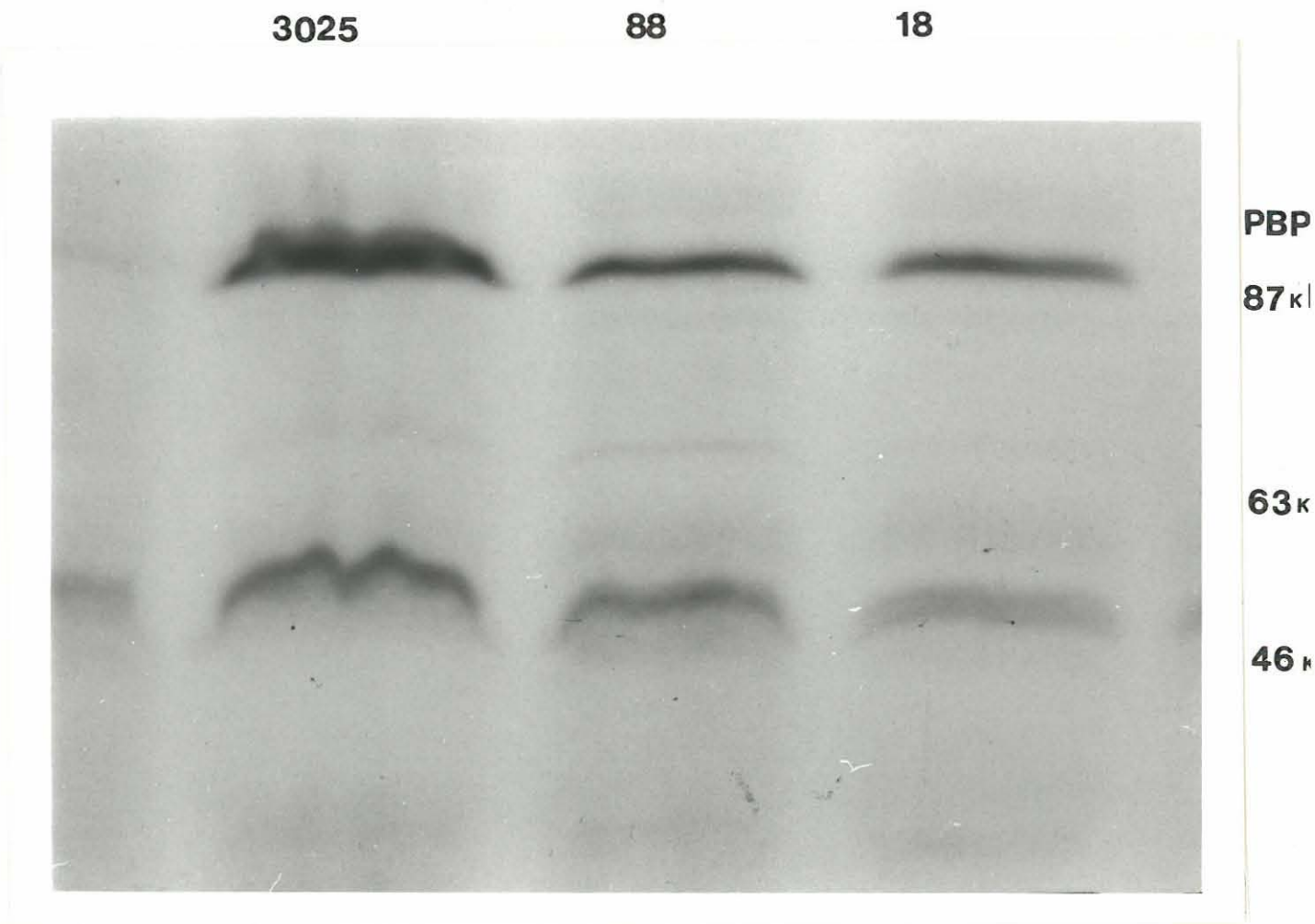
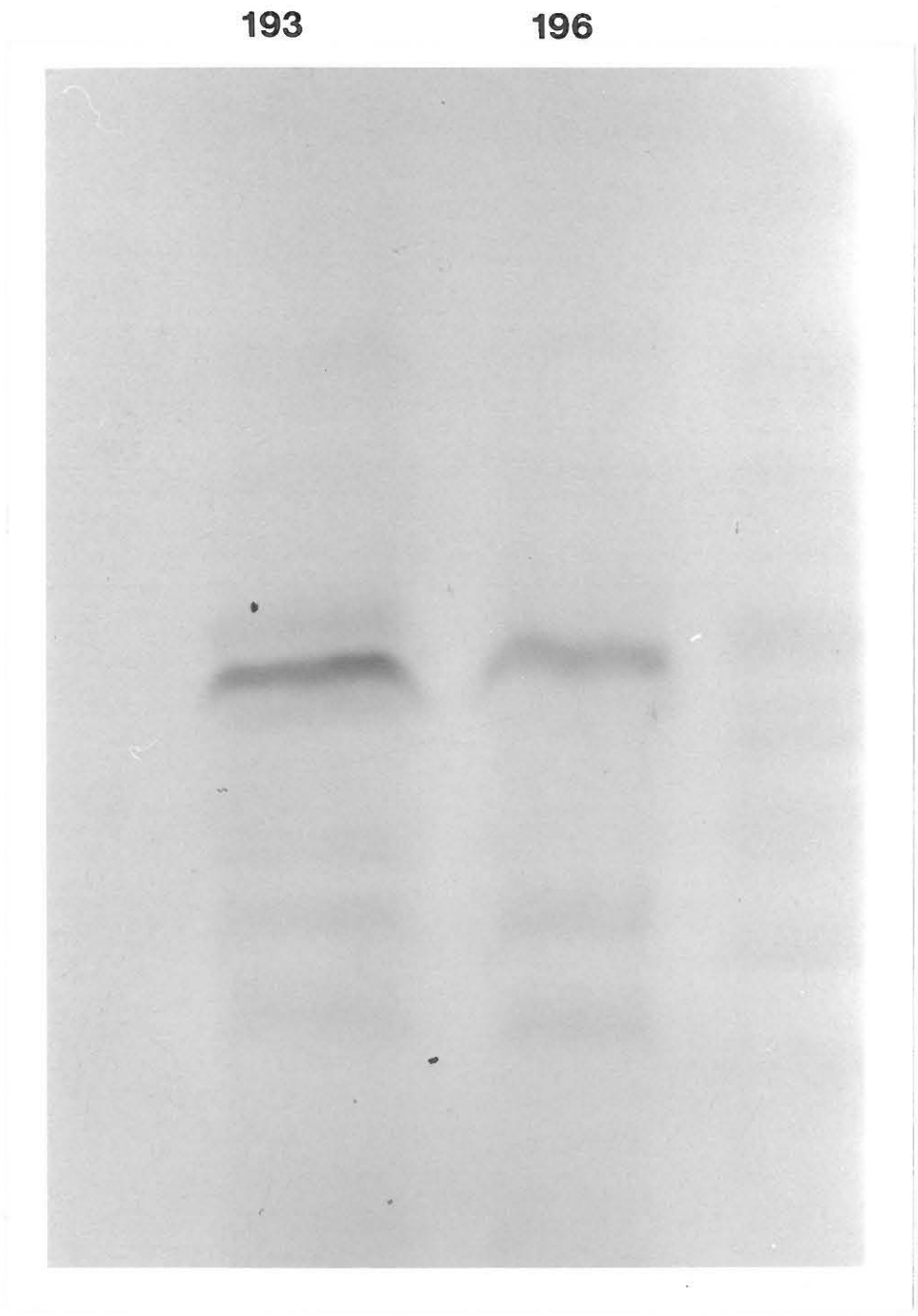


Fig XVIII

PENICILLIN RESISTANT STRAINS



Binding of ^{14}C penicillin to membrane proteins are represented diagrammatically in fig. XIX.

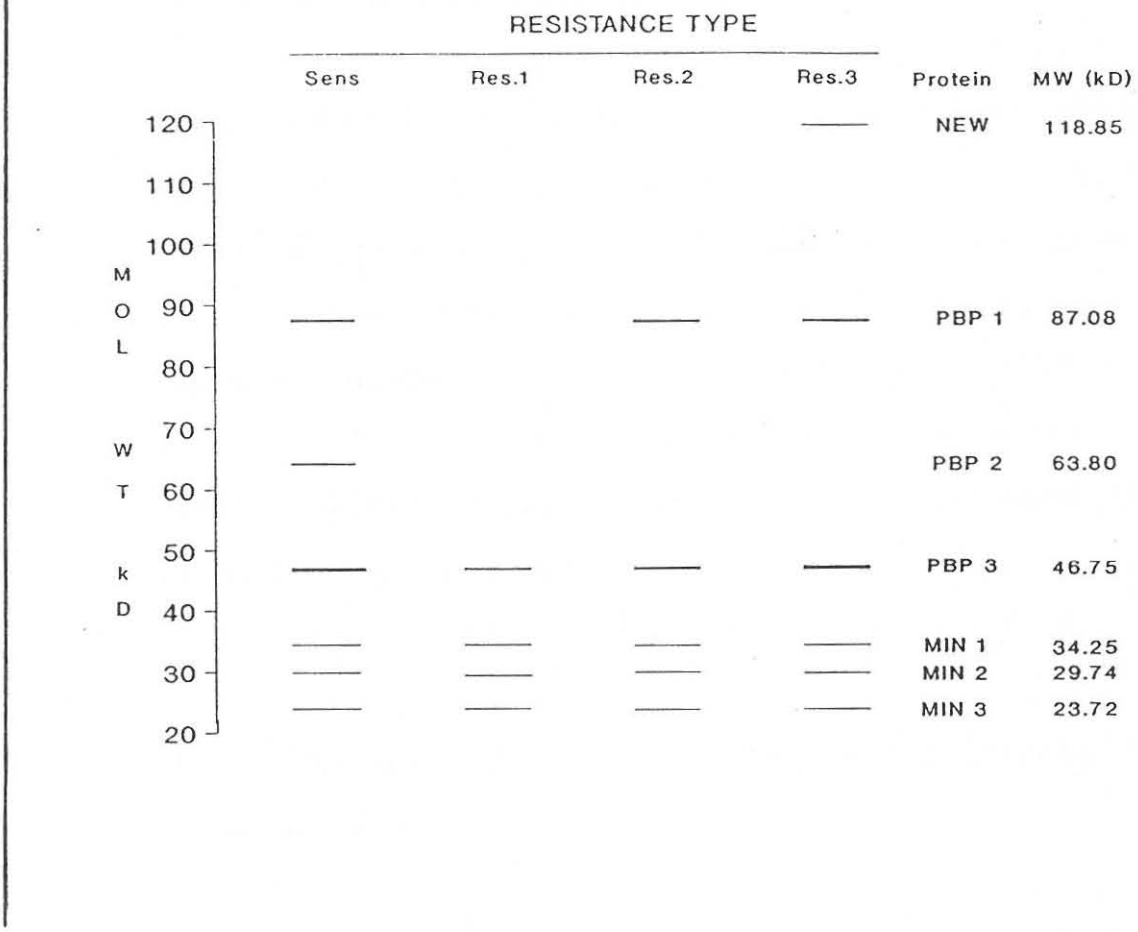


Fig. XIX: Schematic diagram illustrating variations in PBP profiles of sensitive and resistant *N. gonorrhoeae* isolates.

Apparent MW of the PBPs when assessed by the addition of MW standards to the gels were 87 080 (PBP 1), 63 803 (PBP 2) and 46 748 (PBP 3). An additional band representing a protein with an apparent MW of 118 850 was seen in resistance type 3. Very faint minor bands with apparent molecular weights of 23 716, 29 736 and 34 251 respectively were also detected in all resistance types. PBP 2 was absent from all resistant isolates. PBP 1 was absent only from resistance type 1 which was found in isolates 23, 37, 193 and 196. The first two isolates have MICs of 4 mg/l. The last isolate is a ß-lactamase producer with an MIC of 64 mg/l. Isolate 193, which is also a ß-lactamase producer, has an MIC of 256 mg/l. Resistance type 3, represented by isolates 68 and 94 with MICs of 2 and 0.5 mg/l respectively, exhibited an additional band with an apparent MW of 118 850.

No bands were seen in the sarkosyl-insoluble (outer membrane) fraction.

5.5.6 COMPETITION BETWEEN ^{14}C PENICILLIN AND UNLABELLED PENICILLIN FOR PBPs

When inner membrane protein was first incubated with cold benzylpenicillin at a final concentration of 2.8 mg/l for 20 minutes, followed by incubation with an excess of ^{14}C penicillin, no PBP bands could be detected. This indicates that cold penicillin, bound to

PBPs cannot be released by an excess of radio-active penicillin and that the binding of penicillin to PBPs is a specific interaction between penicillin and PBP.

5.6 DISCUSSION

Previous reports from Europe and America have demonstrated three major PBP in *Neisseria gonorrhoeae* with PBP 1 at approximately 90 000, PBP 2 at 63 000 and PBP 3 at 48 000. Alterations in PBP were detected in resistant strains showing decreased binding to PBP 2 and to a lesser extent PBP 1. No PBPs were detected from outer membrane preparations. With the exception of the extra band at 118 850 the results of this study were similar to previously reported results showing distinctly less binding to PBP 2 and decreased PBP 1 binding. The binding by PBP 3 remained constant.

The nature and function of the new PBP at 118 850 is at present unknown. It may represent an aggregate of lower MW PBPs or it may be an inner or perhaps an outer membrane protein that has become (a) accessible to penicillin in isolated membranes or (b) altered in its penicillin affinity. This additional PBP band has not previously been described in *N. gonorrhoeae*. Recent reports from Johannesburg (17, 18) described a similar finding in penicillin resistant *Streptococcus pneumoniae* isolates. A recently reported low MW PBP of 34 000 to

which no function could be allocated (19), was seen in all isolates irrespective of their penicillin resistance. The other two faint minor bands at 29 736 and 23 716 have not been described in other studies. Earlier studies (20, 21) indicated that more PBPs than the three major PBPs, i.e. PBP 1, PBP 2 and PBP 3 are present in *N. gonorrhoeae*. Failure to detect these minor PBPs (19) can be ascribed to experimental differences rather than to experimental artifacts as suggested by Barbour (19).

Judged by the intensity of the different bands on fluorographs of inner membrane proteins, PBP 3 binds the greatest proportion of labelled penicillin. However, since binding to this protein of *N. gonorrhoeae* has apparently no bearing on the reaction to penicillin, suggests that PBP 3 is not an important target for ß-lactam action. Similarly binding to PBP 1 appears not to be essential for effective ß-lactam action.

This study shows that as in other geographical areas altered PBPs do indeed play an important role in the penicillin resistance of South African strains of *Neisseria gonorrhoeae*. Two strains (MIC 64 and 256 mg/l respectively) possessing ß-lactamase activity showed altered PBPs in addition to altered PBP profiles, thus demonstrating that both these resistance mechanisms may be present simultaneously.

Penicillin resistance occurs in almost 50 % of *N. gonorrhoeae* isolates in Bloemfontein, with a considerable and increasing proportion due to altered PBPs. This observation seriously challenges the advisability of using penicillin as therapeutic agent of choice for the treatment of gonorrhoea in a third world setting. This problematic issue has been addressed by others (22, 23).

Widespread penicillin resistance and the apparent lack of awareness of the extent of this resistance and the implications thereof by some community health authorities and general practitioners alike may be contributory to therapeutic failures. Of particular concern in this regard is the relatively high levels of resistance due to altered PBPs.

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CHAPTER SIX

6.1 GENERAL DISCUSSION

The object of this chapter is to highlight the main points which prompted this study.

The pathogenesis of *Neisseria gonorrhoeae* infection is discussed in **Chapter one**. Gonorrhoea is thé most prevalent sexually transmitted disease with far reaching effects when left untreated (1). The organism was initially antibiotic sensitive but over time developed resistance to antibiotics such as sulphonamides and later to penicillin and even tetracycline (1, 2, 3). Various mechanisms of resistance are discussed including the emergence of penicillinase producing strains in 1976 (4).

In **Chapters two and three** the effect of penicillin on cell wall synthesis is discussed. Penicillin is bound to cell receptors *i.e.* to penicillin binding proteins (PBPs). Molecular alterations and associated alteration in function of these PBPs contribute to resistance (5, 6, 7, 8).

Everywhere an increase in ß-lactamase producing strains is noted. The emergence of penicillin-resistant strains of *Streptococcus pneumoniae* has been documented in South Africa. Altered PBPs were implicated in this resistance (8, 9, 10).

Chapter four discusses local surveys. Emergence of PPNG strains in Bloemfontein was noted in 1984. Follow up surveys detected not only an increase in PPNG strains but also in chromosomally resistant strains (11). Since altered PBPs had been implicated in the resistance of South African strains of *Streptococcus pneumoniae* this prompted the investigation to detect the role played by PBPs in the resistance of *N. gonorrhoeae*.

The procedures followed to demonstrate the PBPs in sensitive, intermediately resistant and resistant strains are described in Chapter five. Results indicate changes in PBP 1 and 2 in resistant organisms. These findings correspond to reports from other geographical areas.

A PBP at ~118,85 kD, not previously described, was demonstrated in two strains. Further studies will be necessary to determine the function of this protein.

Despite increasing penicillin resistance, penicillin still remains a favourite first line treatment mainly because it is inexpensive, easily available and easy to administer.

The findings of this study clearly and disturbingly indicate an increasing resistance amongst *N. gonorrhoeae* against penicillin, partly chromosomal in origin. This important fact implies alternative therapy should be sought. Various workers have favoured single low doses of

ceftriaxone or the new 4-fluoroquinolones, both of which are expensive. In the end the additional expense may be justified if this escalating resistance is to be combatted.

VD STRIKES SWAZIS

A REPORT BY A MEDICAL SURVEY TEAM WHICH STUDIED THE PREVALENCE OF AIDS AND OTHER SEXUALLY TRANSMITTED DISEASES IN SWAZILAND , SAID THE SURVEY HAD DISCOVERED A NEW STRAIN OF THE MOST COMMON VENEREAL DISEASE , GONORRHOEA , WHICH IS RESISTANT TO ALL KNOWN CURATIVE DRUGS. AT BEST NORMAL DRUGS PROVIDED ONLY A TEMPORARY CURE AFTER WHICH THE SYMPTOMS FLARED UP AGAIN.

THE STAR: DECEMBER 19, 1990:

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SUMMARY

This study illuminates the developing penicillin resistance of a previously highly penicillin sensitive organism, *Neisseria gonorrhoeae*. This resistance problem has occurred worldwide, and various South African surveys have produced similar results. Resistance due to the production of ß lactamase is discussed. The main aim of this study is to demonstrate that alterations in the positions and functions of penicillin binding proteins is an important and often overlooked mechanism of resistance.

The results clearly indicate that alterations in penicillin binding proteins play an important role in the resistance of *Neisseria gonorrhoeae*.

As penicillin still remains a favourite choice for first line treatment of gonorrhoea, the results obtained show that alternative therapy is indicated.

OPSOMMING

Hierdie studie beklemtoon die ontwikkeling van penisillien bestandheid by *Neisseria gonorrhoeae*, 'n organisme wat voorheen baie gevoelig vir penisillien was. Die bestandheidsprobleem kom wêreldwyd voor en soortgelyke resultate is met ander Suid-Afrikaanse studies behaal. Bestandheid weens die produksie van ß laktamase word bespreek. Die hoofdoelwit van hierdie studie is om aan te toon dat veranderings in die posisie en funksie van PBP belangrik is en dikwels oor die hoof gesien word as 'n meganisme van bestandheid. Hierdie resultate toon duidelik dat veranderinge in die PBP 'n belangrike rol by die bestandheid van *Neisseria gonorrhoeae* speel.

Omdat penisillien nog steeds 'n gewilde keuse by die eerste linie van behandeling van gonorrhoea is, toon die resultate dat alternatiewe behandeling aangewese is.