THE EFFECT OF SUBMINIMUM INHIBITORY CONCENTRATION OF ROCEPHIN(R) ON TWO ISOLATES OF PSEUDOMONAS AERUGINOSA

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A thesis submitted to the Postgraduate School, Ahmadu Bello University, Zaria in partial fulfillment Of the requirements for the award of the degree of MASTER OF SCIENCE IN PHARMACEUTICAL MICROBIOLOGY.

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DECLARATION

I hereby declare that the work reported in this thesis was carried out by me under the supervision of Dr. J. A. Onaolapo and Dr. P. F. Olurinola, both of Department of Pharmaceutics & Pharmaceutical Microbiology. It has not been presented in any previous application for higher degree. The work of other investigators are acknowledged and referred to accordingly.

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CERTIFICATION

This thesis entitled "The Effect of Subminimum Inhibitory Concentration of Rocephin (R) on Two Isolates of Pseudomonas aeruginosa" by Jamiu Oladapo Salami meets the regulations governing the award of the degree of MASTER OF SCIENCE of Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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TO:

OLAMIDE

"My Precious Little Joy"

and

'DOJA

"My Darling Wife"

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ABSTRACT

THE EFFECT OF SUBMINIMUM INHIBITORY CONCENTRATION OF ROCEPHIN(R) ON TWO ISOLATES OF PSEUIOMONAS AERUGINOSA

The effect of subminimum inhibitory concentration (Sub M.I.C) of Rocephin(R) (Ceftriaxone) was tested on two isolates (a clinical isolate and a wild type) of Pseudomonas aeruginosa. The concentration tested has little or no effect on the growth rate of these isolates. The M.I.C of Rocephin(R) against the clinical isolate was l80ug/ml and that agaiist the wild was 300ug/ml. The subMIC used was 7.5ug/ml for the clinical and 12.5ug/ml for the wild (which were 1/24 of the M.I.Cs).

It was found that there is a strain-dependent optimum antibiotic concentration below the M.I.C for maximal filamentation. The clinical isolate showed greater filamentation with increasing antitiotic concentration up to a point (l50ug/ml) whereas the wild type showed increase in length of filament with lowering antibiotic concentration, with optimum at about 40ug/ml while being at the same time more resistant than the clinical isolate.

The antibiotic untreated cells were more phagocytosed by fresh human polymorphonuclear

neutrophils (PMN) in the exponential phase than in the stationary phase for both isolates. Relatively, the clinical isolate was more susceptible to phagocytic uptake than the wild type in the exponential phase. However, this relative susceptibility was reversed in the stationary phase. The two isolates were moderately hydrophobic with the clinical isolate being more hydrophobic than the wild type.

When the cells were treated with Rocephin$^{(R)}$ at a concentration of 1/24 of M.I.C, there was an increase in phagocytic uptake of both isolates at both phases of growth and the observed effect of phase of growth on relative susceptibility was maintained. The subMIC also increased cell surface hydrophobicity of both isolates, with increase in the wild type being more than that of the clinical isolate at both exponential and stationary phases of growth. However, antibiotic treatment reversed the order of relative hydrophobicity observed in the untreated cells.

Serum increased cell surface hydrophobicity of both isolates at both phases of growth but the increase at the stationary phase was not as pronounced

as in the exponential phase. When the cells were treated with Rocephin and serum together there was a general increase in the hydrophobicity at both phases of growth, greater than when the cells were treated with either the antibiotic or serum alone. However, this combination nullified the reversal of relative hydrophobicity between the isolates observed with Rocephin$^{(R)}$ alone, in favour of the clinical isolate.

When the ability of the isolates to adhere to surfaces was tested, the clinical isolate as found to adhere less than the wild type in the exponential phase, while in the stationary phase the clinical isolate adhere more. However, the effects of Sub M.I.C. of Rocephin$^{(R)}$, **serum** individually and the combination of the two appeared to be dependent on the cells, phase of growth and the test material.

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Chapter One

1. INTRODUCTION

1.1 History of Antibiotic Chemotherapy

The concept of "antibiosis" a word first coined by Vuillemin in 1889 (Weinstein, 1985) to denote antagonism between living creatures generally, is almost as old as the science of microbiology. In fact, the application of antibiotic therapy without recognizing it as such is even older. For instance, the Chinese were aware, over 2500 years ago, of the therapeutic properties of mouldy curd of soybean applied to carbuncles, boils and similar infections (Weistein, 1985). The specific term "antibiotics" was however, first described by Waksman in 1942 (Waksman & Lechevalier, 1962) as substances produced by micro-organisms antagonistic to the growth or life of others in high dilution.

The earliest form of antimicrobial chemotherapeutic agents for curing infections were natural plant products, followed by an era of synthesis and then again to natural plant products (Garrod & O'Grady, 1971). The first investigators to recognise the clinical potentialities of microorganisms as therapeutic agents were Pasteur & Joubert (Weinstein, 1985). In their report of 1877, they noted that life

destroys life among the lower species even more than among higher animals and plants. This led to systematic search for an organism which could attack pyrogenic Cocci and Dubos in 1939 (Garrod & O'Grady, 1971) discovered tyrothricin (gramicidin + tyrocidine) formed by Bacillus brevis. Although this agent was toxic to man, the work however, influenced Florey and his colleagues to embark on a study of naturally-formed antibacterial substances. Penicillin, earlier discovered accidentally by Fleming in 1929 was the second agent on their list. It however, took several years before penicillin was fully purified, its structure ascertained and its large-scale commercial production achieved.

The discovery of penicillin by Fleming was followed by the therapeutic revolution produced by sulphonamides, with the modern era of chemotherapy of infection starting with the clinical use of sulphanilamide in 1936 while the "golden age" started with the production of penicillin in 1941. The production of penicillin was followed by a sporadic discovery of many other antibiotics from natural sources with an active period spanning from 1944 to 1956 with the discovery of such agents as:

Streptomycin in 1944, Polymyxins in 1947, Chloramphenicol, the first broad spectrum antibiotic was discovered in 1947 & 1948 in two independent laboratories. Tetracycline was discovered in 1948, Erythromycin in 1952 and Cephalosporin C in 1955, 1956. A substantial number of antibiotics have now been identified with nearly 100 of them now developed to clinical stage. They differ markedly in physical, chemical and pharmacological properties, antibacterial spectra and mechanisms of action (Weinstein, 1985), with penicillin, the first to come into general therapeutic use, still being in many ways the best.

After the natural penicillins like benzylpenicillin (Penicillin G), phenoxymethylpenicillin (Penicillin V) came the semi-synthetic penicillins and cephalosporins, through (in the case of the penicillins) the elucidation of the penicillin 'nucleus' i.e. the 6-aminopenicillanic acid (6-APA) obtainable from Penicillin G or V by the action of amidase. The availability of the 'nucleus' allowed for the attachment of a great variety of side chains resulting in compounds, of which those in therapeutic use possess one or more of such advantages as: (a) acid resistance, (b) resistance to penicillinase and (c) broader spectrum.

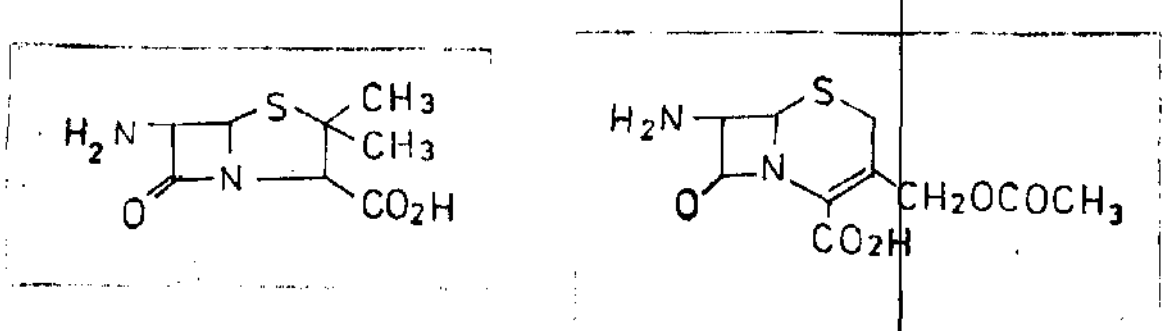
The cephalosporins constitute a group of antibiotics of many generations with a host of promising new derivatives which are generally used in chemotherapy. They are also beta-lactam antibiotics closely related to the penicillins. The cephalosporins were first described by Florey (1955) and later by Abraham (1962), they are formed by Cephalosporium acremonium. The extract from the culture was found to contain three quite distinct antibiotics namely: cephalosporin N (a penicillin, adicillin), cephalosporin P (an antibiotic of steroid structure similar to Fucidin) and cephalosporin C.

Cephalosporin C has the same side chain (derived from D-alpha-aminoadipic acid) as cephalosporin N, fused with a dihydrothiazine beta-lactam ring system (the 7-aminocephalosporanic acid or 7-ACA), has moderate activity and a high degree of penicillinase resistance. Substitution of other side chains on the 7-ACA 'nucleus' resulted in the cephalosporin family with enhanced activity. Cephalosporins are resistant to penicillinase but sensitive to cephalosporinase, have molecular weight of about 420. They are freely soluble in water and relatively stable. They are active in concentration of 10ug/ml or less against gram-positive organisms including

penicillinase producing staphylococci and against many gram-negative bacteria.

Although cephalosporins have close structural relationship with penicillins but because of the chemical difference in drug nuclear structure, their antigenicity differs. Hence most individuals who are

Fig. 1.1: Nuclear ring structures in penicillins and cephalosporins.



6-aminopenicillanic acid (6- APA)

7-aminocephalosporanic acid (7- ACA)

hypersensitive to penicillins can tolerate cephalosporins (Jawetz et al, 1982). In fact, it is said that only about 10% of penicillinalergic patients are sensitive to cephalosporins, although some cases of primary allergy to cephalosporins have been reported in patients not known to be penicillin sensitive (O'Callaghan & Muggleton, 1972). Cephalosporins also have low toxicity potentials.

Penicillins and Cephalosporins are known to form spheroplasts (which may lyse in isotonic media) under appropriate conditions at concentrations dependent on individual minimum inhibitory concentration (M.I.C). They are also known to inhibit the third stage in bacterial cell wall synthesis and in normal nutrient media results into very elongated cells (filaments) in cultures of cells like Escherichia coli and Proteus mirabilis (O'Callaghan & Muggleton, 1972). Such filaments are apparently normal in every way except that they do not divide into cells of normal length but continue to elongate up till as much as 50 times the parent cells and this is in agreement with the observation of Pennington and O'Grady (1967) that inhibition of the cross-linking reaction itself is not necessarily lethal. A high antibiotic concentration results in spheroplasts and although filamentation preceeds spheroplast formation, the two steps are not necessarily sequential. Filaments are formed at all pH values but spheroplasts are not formed above pH 7.5 (Grula & Grula, 1962).

Structural manipulations about the 7-ACA has been of great value in the development of cephalosporins. It appears that substitutions at position 7

of the beta-lactam ring are associated with alteration in antibacterial activity while substitutions at position 3 of the dihydrothiazine ring are associated, with changes in metabolism and the pharmacokinetic property of the drugs (Huber et al, 1972). For instance based on the view that cephalosporins with great activity share active hydrogen(s) on the alpha-carbon of the side chain acyl, various beta-ketoacid substitutions led to cefotiam and cefsulodin that could control a wide range of pathogenic bacteria (Morita et al, 1980). It was also suggested by Hamilton-Miller (1976) that relatively small changes in structure may alter markedly the bacteriolytic activity of lytic-deficient agents that cause filamentation and also replacement of sulphur atom (S) by oxygen atom (O) at position 1 in the cephem ring has been found to result in 4-16 fold increase in antibacterial activity though with concomitant increase in sensitivity to beta-lactamase to some extent, which was presumed to be due to increase in chemical reactivity of the beta-lactam ring system. However, two types of substituents were found to protect oxacephem from this hydrolysis and this protection was specific for the type of beta-lactamase produced by the gram-negative bacteria. Hence with this

knowledge a product (6059-S) with stable and broad spectrum of activity was developed (Yoshida, 1980).

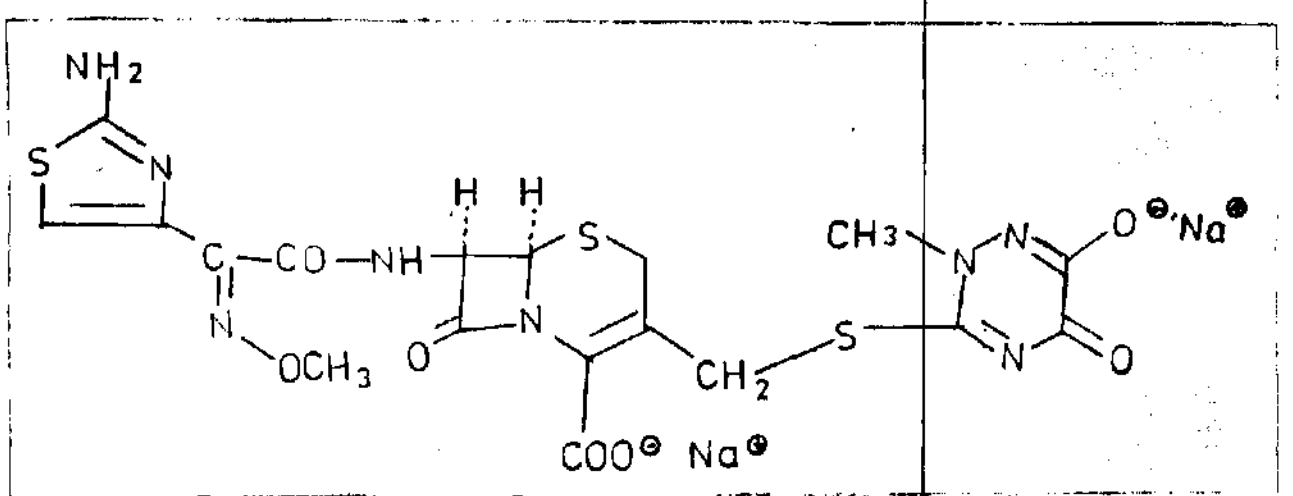
The observation by Hamilton-Miller (1976) that lytic-deficient cephalosporins caused filamentation and that this lytic deficiency depended on the bacterial species challenged has been expanded upon by Ryan and Monsey (1981) and they concluded that filamentation is not a determining factor in therapeutic efficacy since six commercially available cephalosporins investigated were found similar with respect to the major properties that contribute to successful therapy i.e. M.I.C, pharmacokinetics, in-vivo kill rate and ED_{50} value while differing in their abilities to induce filamentation.

As with penicillin derivatives, the semi-synthetic cephalosporins vary in effectiveness and stability and are classified in terms of generations based on the general features of antimicrobial activity. The first generation cephalosporins, epitomised by cephalotin and cefazolin, have good activity against gram-positive bacteria and relatively modest activity against gram-negative microorganisms. The second generation cephalosporins e.g. Cefamandole, Cefuroxime etc, have somewhat increased activity

against gram-negative microorganisms but are much less active than third generation. The third generation cephalosporins e.g. Cefotaxime, Ceftriaxone etc, are generally less active than first generation against gram-positive cocci, but are much more active against the enterobacteriaceae including penicillinase-producing strains. A subset of the third generation cephalosporins e.g. Ceftazidine, Cefsulodin, Cefoperazone etc, is also active against Pseudomonas aeruginosa (Neu, 1982; Garzone et al, 1983a, 1983b & Schumacher, 1983).

Cephalosporins having notable activity against Ps. aeruginosa include: Cefuroxime, Cefotaxime, Ceftriaxone, Moxalactam and though comparable, they vary in their degree of effectiveness. Of the third generation cephalosporins, cefotaxime was the first to become available in United States, it is highly resistant to bacteria beta-lactamases and has good activity against many gram-positive and gram-negative aerobic bacteria with a half-life of about 1 hour. Ceftriaxone (Rocephin$^{(R)}$) is said to have in-vitro activity similar to that of Ceftizoxime and Cefotaxime with a half-life of about 8 hours being the outstanding feature (Mandell & Sande, 1985). Rocephin$^{(R)}$ is said

to have a similar activity with Cefuroxime and Cefotaxime against Ps. aeruginosa (M.I.C_{90} 16mg/L) (Wise et al, 1980). It also has a comparable volume of distribution with other marketed cephalosporins but a much slower clearance which therefore results in the long plasma half-life earlier mentioned and its renal clearance of unchanged drug is not dose-dependent (about 65%) (Stoeckel et al 1981).
Its activity is relatively unaffected by serum despite a high protein binding of 95% (Wise et al, 1980). In patients with normal serum creatinine, Rocephin$^{(R)}$ serum levels ranged from a peak of 40-200mg/L (mean of 75) and a trough of 16-70mg/L (mean of 42) for a dose of one gram intravenously twice daily (Eron et al, 1983). Rocephin$^{(R)}$ has the following chemical structure:.



(Z)-(6R,7R)-7-(2-(2-amino-4-thiazolyl)-2-(methoxy -imino) acetamido)-3-[(2,5-dihydro-6-hydroxy-2-methyl-5-Oxo-as-triazine-3-yl) thiol methyl-8-Oxo-5-thia-1-azabicyclo [4.2.0] Oct-2-ene--2-carboxylic acid disodium salt.

Neu et al (1981) found that Rocephin(R) was stable to most plasmid-mediated beta-lactamases but was hydrolysed by some Enterobacteria, Proteus and Bacteroides species and beta-lactamases of chromosomal origin. They also noticed that alteration of pH and use of various media did not change the inhibitory levels. It was however found that inoculum size affected M.I.C level considerably especially from between 10^5-10^7 CFU/ml. Angehrn et al (1980) also noted that inoculum size affects M.I.C and minimum bactericidal concentration (M,B.C) and that this effect was strain dependent. Some strains of Ps. aeruginosa were found to be resistant to Rocephin(R).

Rocephin(R) and other cephalosporins like penicillins act by selectively inhibiting bacterial cell wall synthesis through inhibition of the terminal cross-linking of the linear glycopeptides i.e. the transpeptidation reaction (Jawetz et al, 1982) by

attaching to receptors some of which are transpeptidase enzyme thereby inhibiting its action. After inhibition of transpeptidation comes the removal or inactivation of an inhibitor of autolytic enzymes in the cell wall thereby resulting into cell lysis if the environment is isotonic through activation of the lytic enzymes. The remarkable lack of toxicity of penicillins and cephalosporins to mammalian cells is probably due to the absence of bacteria-type cell wall with its peptidoglycan in animal cells.

1.2 Pseudomonas aeruginosa

The genus pseudomonas are bacteria of considerable scientific and practical importance. Their widespread occurence in soil and water can be considered as an indication of their involvement in the natural process of mineralisation of organic matter. The group include aetiological agents of important plants and animals diseases. They are unicellular rods, straight or curved (occasionally) but not helical, measuring 0.5-1 um by 1.5-4 um (though the maximum of 4 um is sometimes exceeded as in some strains of Ps. putida, a fluorescent plant pathogen). They are motile by means of one or more polar flagella (Palleroni, 1975). They are gram-negative and non-spore forming cells deriving

their metabolic energy from respiration and never from fermentation or photosynthesis. They are also chemoorganotrophs, although some are facultative chemolithotrophs capable of using hydrogen gas as an energy source. They are aerobic, using molecular oxygen as terminal oxidant but some members can live anaerobically in media containing nitrate. Members of the group are often pigmented, some producing water soluble fluorescent or pyocyanin pigments that diffuse into the media while others produce carotenoid pigments that remain associated with the cells and which are responsible for the yellow or orange colour of the colonies of such organisms. Some species e.g. Ps. aeruginosa (which is normally an occasional pathogen) have become widespread in clinical specimens due to their resistance to deleterious agents including antibiotics and this resistance has become a considerable concern in medical practice.

All microorganisms termed occasional pathogens are either saprophytes or commensals and are capable of causing severe pathological effects when they gain access to tissues with poor antimicrobial defences, or to patients whose resistance is impaired by illness or by certain forms of medical treatment. Such

infections are termed "opportunistic" (Lowbury, 1975). Due to the biological advantage of being resistant to a wide range of antibiotics that are usually active against the more pathogenic bacteria, opportunist pathogens become selected in hospitals where antibiotics are much used. Ps. aeruginosa is particularly typical of the opportunists and although virtually harmless to healthy, uninjured subjects (except in infants), it appears to be more pathogenic than S. aureus for example, in the anterior chamber of the eye and more prone to cause fatal infections in patients with severe burns. It also presents special hazards in patients requiring instrumentation (e.g. catheterization) because equipments that are difficult to sterilize or disinfect are more liable to contamination by it than by many other opportunists e.g. Staphylococcus aureus or Haemophilus influenzae that do not multiply (while many strains do not even survive for long) on moist inanimate environment (probably an advantage offered by its ability to grow as a saprophyte in water with minimal nutrient additives) (Lowbury, 1975). Hence patients treated with such equipments, who may also be receiving antibiotics that select a variety of opportunist pathogens or receiving steroid hormones,

immunosuppressive drugs etc. become more susceptible to infection by Ps. aeruginosa.

The species Ps. aeruginosa is very well defined and readily recognizable by a number of simple characters. For instance, the cultures have a colour resembling copper rust or verdigris, the cells are straight rods of average thickness of 0.5um and length ranging from 1.4um-3um and are mostly motile, typically with one polar flagellum per cell. Production of pyrocyanin and fluorescent pigments are characteristic of the species (Palloreni, 1975). Most strains have characteristic sweetish smell (described variously as being "fruity" or like the odour of trimethylamine). All strains grow fast at $37^{o}C$, a character that differentiates Ps. aeruginosa from other fluorescent pseudomonas. However, while $37^{o}C$ is the optimum growth temperature, strains of the species can actually grow between $5^{o}C$ - $42^{o}C$ while some even grow at $44^{o}C$.

Members of the species Ps. aeruginosa are nutritionally versatile, with individual strains being capable of utilizing 80 or more organic compounds. They are absolute aerobes except in media containing nitrate (which they reduce to

gaseous nitrogen) and biochemically they give positive response to Catalase, Oxidase and arginine dihydrolase reactions (catalase being somehow diagnostic in being faster in the genus than in any other catalase positive organism), gelatin is hydrolysed while starch is not. They produce acid from glucose, arabinose, xylose and glycerol but not from maltose, lactose and dulcitol. In peptone water media, acid may be produced from glucose but rarely from other carbonhydrates (Palleroni, 1975; Wilson & Miles, 1975). The organisms can easily be cultivated and they grow well on a selective medium that inhibits enterobacteria as well as staphylococci and streptococci e.g. nutrient agar at pH 7.8 containing 0.03% cetrimide.

1.3 Bacterial Cell Envelope and Effect of Antibiotics on it

The bacterial cell is usually encased by the cell envelope which differs in terms of gross morphology between gram-negative and gram-positive bacteria. The cell envelope is made up of the cell wall and the cytoplasmic membrane and it regulates the complex molecular traffic between the cell and its environment. The bacterial cell wall is a complex, partly inelastic structure which protects the cell from osmotic swelling and also conditions the micro environment of the cytoplasmic membrane.

The cell wall is very important to the organism and this can be demonstrated by treatment with enzymes or certain antibiotics. For instance, the enzyme Lysozyme which is an important component of the host defence mechanism against invading bacteria and is contained in leucocytes and many body fluids e.g. serum, tears etc, if added to a bacteria culture degrades the peptidoglycan resulting in cell lysis. This is very spectacular in gram-positive cells where the enzyme has direct access to the peptidoglycan layer (Hammond et al, 1984). Similarly, many antibiotics effect their lethal action by inhibiting a number of enzymes involved in peptidoglycan assembly, resulting into a progressive weakening of the cell wall until it can no longer resist the internal turgor pressure, causing bulges to develop at the site of cell division which may enlarge until the cells ultimately burst.

Drug penetration of the cell envelope, especially by antibiotics depends to a considerable extent on the properties, hydrophilicity or hydrophobicity of the drug molecule and on the composition of the outer membrane (OM) of gram-negative bacteria. Proteinaceous pores in the OM allow the entry of low molecular weight hydrophilic agents into

the cell (Nikaido & Vaara, 1985). In contrast, hydrophobic molecules enter by passage across the phospholipid component of the OM which is normally shielded from the environment by an intact lipopolysaccharide and/or porin proteins (Russell & Furr, 1986).

Since the cell envelope serves as a "control valve" for molecular movement in and out of the bacterial cell and also since most drugs, in particular antibiotics, must penetrate the cell envelope to reach their metabolic "targets" within the cell, the structure of this cell envelope thus plays a vital role in determining bacterial sensitivity to antibiotics.

Consequent to the seeming importance of the cell envelope, a lot of work has been done to elucidate its structure and the effect of various chemotherapeutic agents on its components and how these affect the barrier function of cell envelope.

1.3.1 The Cell Envelope

The cytoplasmic membrane has a relatively consistent chemical composition and molecular architecture while the cell walls vary widely as supported by the gram-positive/negative staining

techniques, from the chemically simple walls of some gram-positive species e.g. Micrococcus lysodeikticus, to the very complex multilayered walls of smooth strains of gram-negative bacteria (Costerton et al, 1974).

The cell wall of gram-positive bacteria is made up principally of a thick layer of peptidoglycan (PDG) closely associated with the cytoplasmic membrane and this is sometimes covered by an extracellular slime or capsule layer in the envelope of some species. On the other hand the cell wall of gram-negative bacteria contains a comparatively less substantial amount of PDG, not as closely associated with the cytoplasmic membrane as in gram-positive cells and most notably a second membrane to the exterior of the PDG called the outer membrane. Also extracellular slime or capsule layer is sometimes present in the cell envelope of some species. In addition, the gram-negative cell wall has a well defined periplasmic space as contrasted to the narrow "periplasmic space" seen separating the gram-positive cell wall from the cytoplasmic membrane on cell fixation and which has been shown to contain some degradative enzymes (Ghosh et al, 1968; Ghosh et al, 1971; Okabayash et al, 1974).

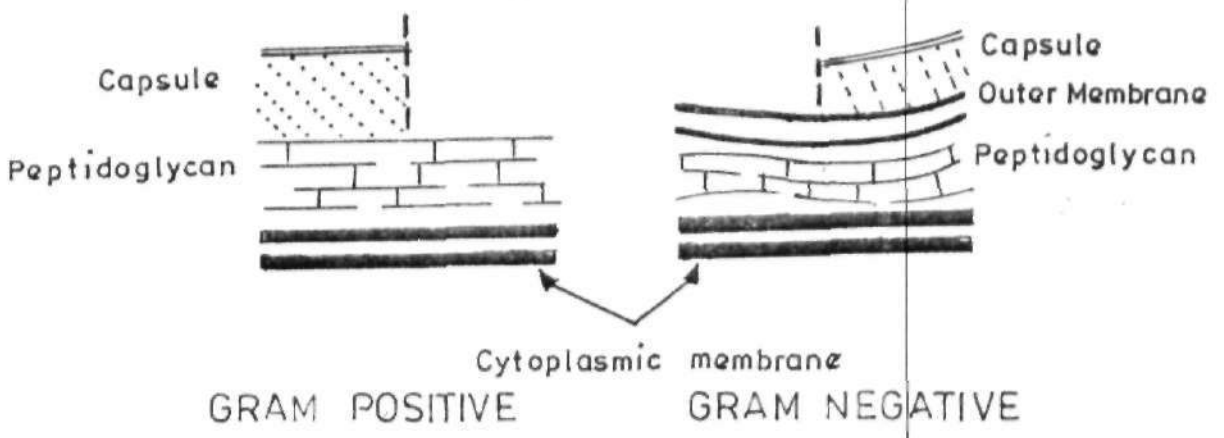


Fig. 1.2: Diagramatic Representation of the Cell Surface Surface of Gram-positive and Gram-negative Bacteria.

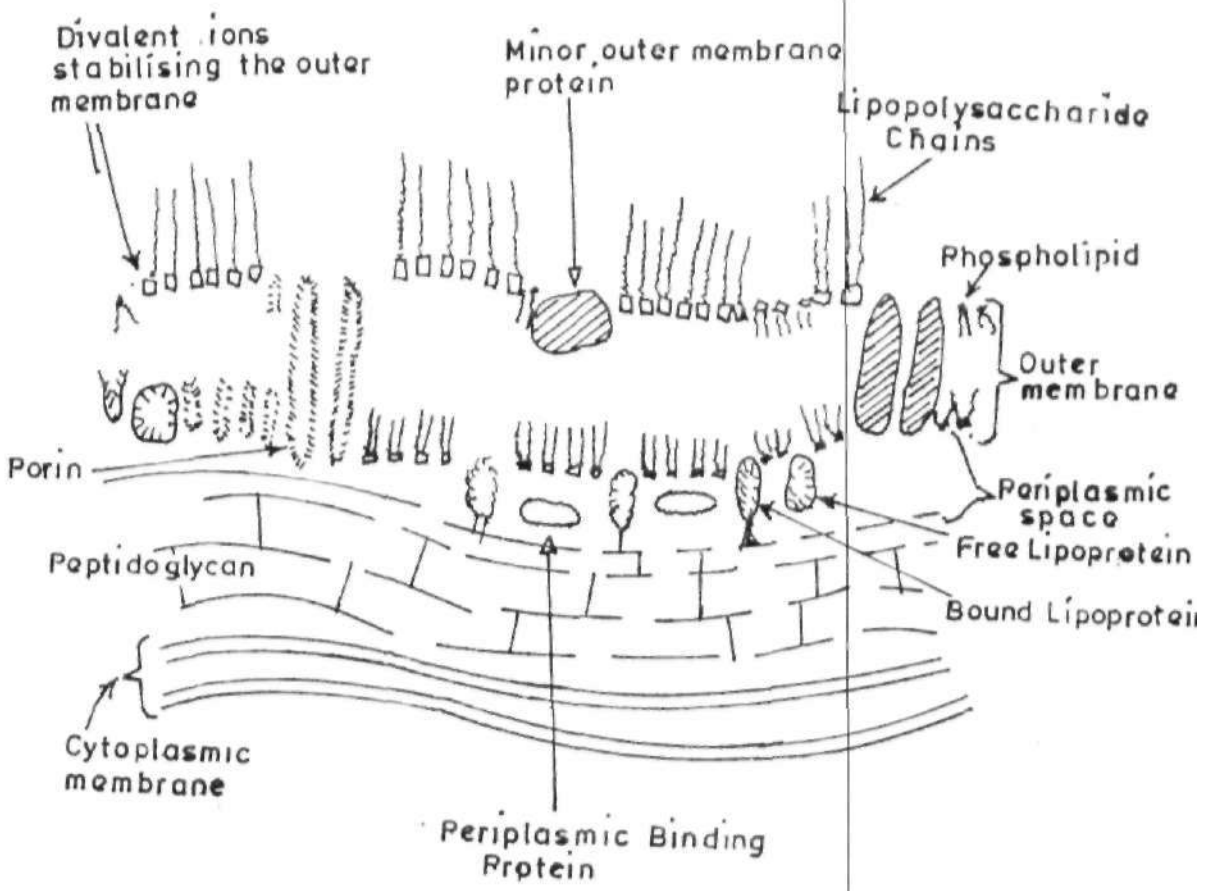


Fig. 1.3: Representation of the Envelope of Gram-negative Bacteria.

It is however noteworthy that in live cells, the content of such observed "periplasmic space", including the enzymes, would be present in the spaces between peptidoglycan fibres and immediately outside the cytoplasmic membrane (Costerton & Cheng, 1975).

1.3.1.1 The Cytoplasmic Membrane

The cytoplasmic membrane in both gram-positive and negative bacteria and the outer membrane, in gram-negative cells consist of lipid/protein bilayers but beyond this superficial resemblance, few structural or physiological similarities exist between the two membranes, reflecting the differing roles of the membranes in the life of the bacterium.

The cytoplasmic membrane contains the enzyme systems of the electron transport chain (cytochromes) and oxidative phosphorylation, systems for the active transport of solutes and excretion of waste products plus the synthetic apparatus necessary for the production of exterior layers (Hammond et al, 1984). It is made up essentially of proteins and phospholipids. The protein profile is complex consisting of 40-100 distinct protein bands. The cytoplasmic and outer membranes have relatively similar phospholipid content with about 60% of the total cell envelope phospholipid

being present in the outer membrane. However, a number of membrane enzymes are functionally dependent on the presence of phospholipid and these include the membrane-bound adenosine triphosphatase (Hammond et al, 1984).

The phospholipids form a liquid hydrophobic bimolecular leaflet whose limited penetrability by polar molecules is affected by changes in chain length and degree of saturation of their component fatty acids (Machtiger & Fox, 1973; McElhaney et al, 1973). The fluidity of the bulk phospholipids, is modified by proximity to proteins (Jost et al, 1973) which "float" in the phospholipid phase and some of these proteins appear to be the permeases that effect facilitated diffusion and active transport across the membrane (Kaback & Hong, 1973; Tourtellotte & Zupnik, 1973). The cytoplasmic membrane (CM) which has little tensile strength is in most cases adpressed against the inelastic peptidoglycan structure of the cell wall by osmotic forces (Costerton & Thompson, 1972) while in gram-negative cells, points of adhesion have been observed between the CM and the PDG (Bayer, 1968; Olsen et al, 1974).

The cytoplasmic membrane is a penetrability barrier, the effective "pore size" of which is so small that even passage of ions like potassium is

restricted (Thompson et al, 1970). Hence antibiotics must pass the membrane via the transport systems or by intercalation among the hydrophobic phospholipid molecules. However, some antibiotics capable of this intercalation often damage the membrane and thus have the membrane as their site of action e.g. polypeptide antibiotics like Polymyxin and Bacitracin (Cerny & Teuber, 1971; Weinberg, 1967).

The cytoplasmic membrane is the primary barrier in intrinsic resistance of Ps. aeruginosa to stre ptomycin, tetracycline (Tseng & Bryan, 1974) and to actinomycin D (Cheng et al, 1973). Changes in the cytoplasmic membrane has been known to confer tetracycline resistance on bacterial cell. This is thought to involve deletion of a membrane protein (Chopra et al, 1974) or the addition of a new protein to the membrane (Levy & McMurry, 1974). Hence the molecular make-up of the cytoplasmic membrane is important.

1.3.1.2 The Peptidoglycan

The bacterial cytoplasm is enclosed by the partly inelastic cell wall which is a highly structured layer of great mechanical strength and is responsible for maintaining the shape and integrity of the cell.

The rigid part of the cell wall is made up of a unique highly cross-linked aminosugar polymer called the peptidoglycan (PDG) which determines its strength.

Peptidoglycan is the major component of the gram-positive cell wall (Salton, 1964) with other molecules such as teichoic acids, teichuronic acids and lipids being interspersed with the basic PDG structure. These other molecules however, do not form coherent or continous structures within the cell wall (Millward & Reaveley, 1974). In fact 50-80% of the gram-positive cell wall is PDG while the rest is made up of a variety of polymers depending on the organism. In gram-negative bacteria, PDG makes up only 10-20% of the weight of the wall, and does not contain integrated polymers similar to the teichoic acids, it is covalently linked by lipoprotein molecules to the outer membrane.

The PDG can be isolated from majority of outer membrane components by heating the wall fraction with an anionic detergent like sodium dodecyl sulphate (SDSP), followed by extensive washing to remove the detergent. Removal of covalently linked lipoprotein will however, involve treatment with proteolytic enzymes. Although there is considerable variation in

detailed composition of PDG among different organisms, the basic structure is the same.

Peptidoglycan consists of a network of linear polysaccharide chains (glycan strands) of up to 200 dissacharide units in length, cross-linked by short peptide chains. The glycan strands are made up of alternating units of N-acetylglucosamine and N-acetylmuramic acid joined by a 1,4-beta-glycosidic linkage.

The synthesis of PDG into the cell wall of an actively growing cell is a highly organised and complex series of events taking place in a definite pattern such that the protective and shape determining functions of the cell wall are not jeopardized. Precursors to PDG during synthesis are synthesized in the cell in a sequence divisible into three stages namely: (a) synthesis of the precursors in the cytoplasm. The precursors synthesized are the sugar nucleotides: UDP-N-acetylglucosamine and UDP-N-acetylmuramylpentapeptide. (b) The transfer of the precursors to a lipid carrier molecule (undecaprenyl phosphate) which transports them across the membrane and (c) insertion into the wall and coupling to existing PDG.

Many antibiotics inhibit PDG synthesis and it is known that all stages of PDG biosynthetic pathway are susceptible to inhibitors as exemplified by table 1.1. A very important group of antibiotics, both clinically and as biochemical probes, are the beta-lactams, especially the penicillins and cephalosporins. When bacteria are exposed to beta-lactams a small amount of the antibiotic becomes covalently linked to the cells and the bound molecules are responsible for damaging and ultimately killing the cell.

Table 1.1: Some antibiotics which interfere with the synthesis of peptidoglycan.

Antibiotic	Site of Action
Phosphonomycin	Inhibits synthesis of UDP-N-acetylmuramic acid from UDP-N-acetylglucosamine and phosphoenol-pyruvate.
D-cycloserine	Inhibits conversion of L-alanine into D-alanine by alanine racemace and coupling of two D-alanines by D-alanine: D-alanine ligase.

Antibiotic	Site of Action
Bacitracin	Prevents de-phosphorylation of undecaprenyl pyrophosphate thereby inhibiting the lipid carrier cycle.
Vancomycin	Binds to acyl-D-alanyl-D-alanine region of the lipid-linked precursors preventing incorporation into the wall.
beta-lactam antibictics	Prevent cross-linking by inhibition of transpeptidase/carboxypeptidase.

The antibiotics are bound to transpeptidases and carboxypeptidases, thereby inhibiting the enzymes. Hence the cells continue to grow without being able to produce cross-linked PDG. Thus abnormal cell shapes develop and the cells eventually stop growing. The precise fate of the cells depends on the nature and concentration of the antibiotic used and the organism itself. However, lysis is common but filamentation and other unusual shapes can persist without lysis.

It is believed that the enzymes bind beta-lactams because the latter are structural analogues of D-alanyl-D-alanine terminal portion of the pentapeptide linked to N-acetylmuramic acid in uncross-linked PDG

(i.e. nascent PDG). This is because the reactive and susceptible beta-lactam bond of the antibiotic molecule lies in a similar position as the peptide bond of D-alanyl-D-alanine. Hence the enzymes probably mistake the antibiotic molecules for their genuine substrate, cleave the beta-lactam bond, thereby becoming inactive due to the relative stability of the antibiotic-enzyme intermediate formed. Such intermediate complexes are called penicillin binding proteins (PBPs) and at least seven different PBPs have been identified in cells of Escherichia coli and having molecular weight between 40K - 90K. Most abundant are the low molecular weight PBPs 5 & 6, having carboxypeptidase activity and while they may control the degree of cross-linking of the PDG, they are not considered essential for the viability of the cells. Other PBPs except PBP4 are essential. PBP 1b has transpeptidase and transglycozylase activity, while the activities of PBPs 1a, 2 & 3 are yet to be established.

It seems that PBPs 1a, 1b, 2 & 3 have distinct functions within the cells in the different enzymic activities that produce PDG. PBPs 1a and 1b appear to be vital in maintaining the integrity of the cells and possibly in controlling extension of the PDG in

the side walls during cell growth. Selective inhibition of 1b leads to cell lysis while PBP2 is involved in maintaining the rod shape in E. coli and its inhibition results in osmotically stable oval forms. PBP3 is involved in septum formation, selective inhibition of its activity resulting into filamentation. Such filaments are not osmotically fragile and do not normally undergo lysis.

Penicillin G binds to all the PBPs and the one responsible for cell death depends on concentration used but is most likely to be 1b, since cells are observed to undergo lysis rapidly. However, other beta-lactam antibiotics do not necessarily have the same affinities for all the PBPs as penicillin G. In fact most of them bind to all the PBPs but show some preference for certain PBPs at low concentrations. Hence their morphological effects depend on the concentration used and can vary between two extremes of lysis and filamentation, while a combination effect results in abnormal cell shapes.

Beta-lactam antibiotics also trigger various autolytic enzymes in the wall and this also plays an important part in the eventual destruction of the cell.

To the exterior of the PDG in the envelope structure, there could be extracellular slime or capsule layer depending on the organism among gram-positive cells while in gram-negative bacteria there is yet another highly organised bilayer called the outer membrane to which the PDG is associated by covalently bonded lipoprotein.

1.3.1.3 The Outer Membrane (OM)

The OM of the gram-negative bacteria is an effective permeability barrier to many drug molecules especially antibiotics. The existence of this barrier is implied by the retention of enzyme in the periplasmic space (Lindsay et al, 1973) and by the exclusion of externally-added lysozyme (Sanderson et al, 1974). The outer membrane has extremely limited enzymic activity with no role at all in electron transport (Hammond et al, 1984) but it however, has a distinguishing novel series of proteins and lipopoly-saccharide (LPS) and this probably has to do with the specialised functions of the membrane. The membrane's impermeability to hydrophobic compounds and high molecular weight hydrophilic compounds accounts for the resistance of gram-negative bacteria to a variety of antibiotics and antimetabolites by preventing accumulation of lethal concentrations of the agents at their respective sites.

The gram-negative OM is also important for the survival of the organism in an animal host i.e. it helps the bacterium overcome the emulsifying action of bile salts and enzymes of the gut while also aiding resistance to phagocytosis by white blood cells and the complement-directed bactericidal action of serum.

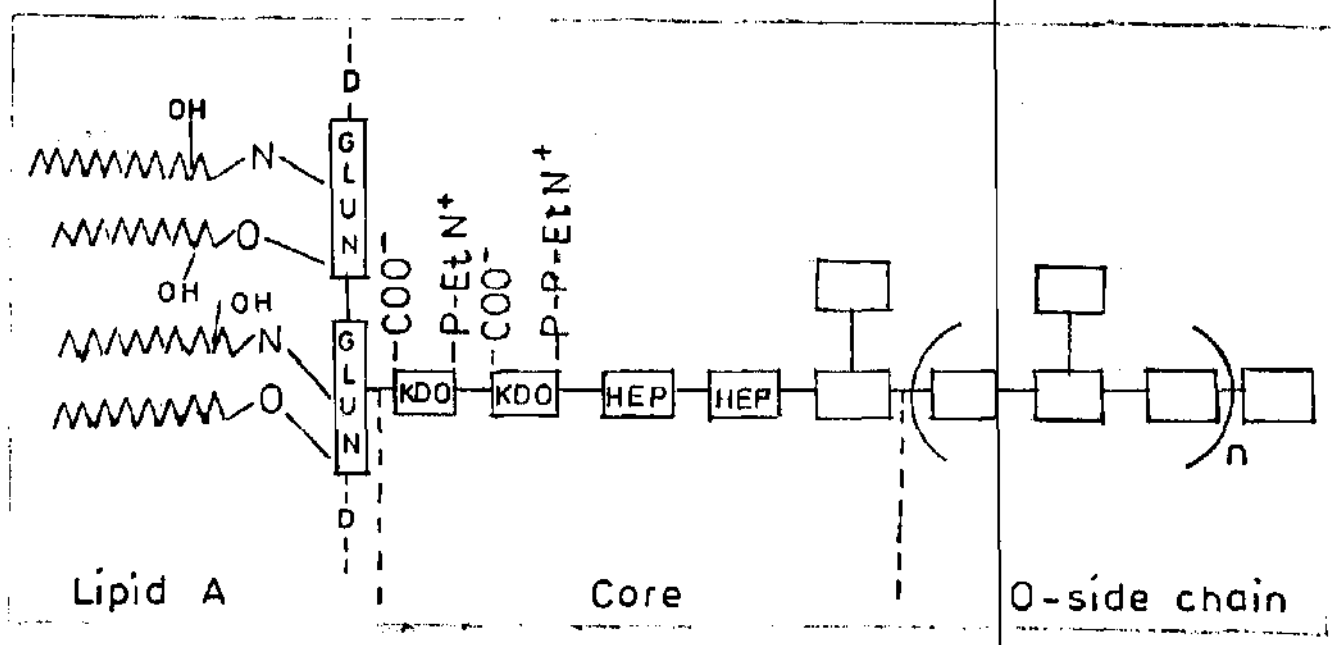
A probe into the barrier function of the OM was initiated with the finding that treatment with ethylene diamine tetra acetic acid (EDTA) notably increased the permeability of the envelope. It was found that viable EDTA-treated cells retained normal growth rates and undisturbed synthetic and cytoplasmic membrane functions but are however, more vulnerable to a variety of antimicrobial agents and enzyme substrates like phospholipase and lysozyme (normally excluded from the cells) to which they were hitherto insensitive. In the presence of the organic buffer, tris-hydroxymethylaminomethane, EDTA is thought to withdraw magnesium ions from the outer membrane, resulting in release of specific OM components and greatly increasing the permeability of the membrane (Hammond et al, 1984). This fact is supported by some workers' observations that the

stability of the OM-PDG complex is maintained by divalent cation bridges (DePamphilis, 1971; Schnaitman 1971a, 1971b) and lipoproteins (Costerton et al, 1974). Also of relevance is the observation by Meadow (1975) that the complexing lipoprotein is much reduced or even absent in Ps. aeruginosa and divalent cation bridges are presumably vital in the stabilizing role. This probably explains the very high sensitivity of the organism to EDTA since a very low concentration is said to lyse the cell (Hammond et al, 1984). However, the effect of EDTA is reversed by addition of excess divalent ions, thus preventing the loss of OM components like : between a third and a half of the LPS component, about 5-20% of phosphatidylethanolamine and a little protein in enteric organisms and this observation is another pointer to the stabilizing role of divalent ions.

Despite great structural and physiological diversity found amongst gram-negative bacteria, they seem to share a common pattern in the structure of their OM. The structures that make up the OM include : lipopolysaccharide (LPS), proteins, lipoproteins and phospholipids. The term LPS quite often is applied exclusively to the endotoxic O-antigens from "smooth" gram-negative bacteria and the corresponding products

from "rough" strains and mutants. The LPS has a classical model as originally proposed by Luderitz and Westphal (Hammond et al, 1984) consisting of three covalently linked regions namely : Lipid A, the core and the O-side chains, each with a distinctive composition and biological function.

Fig. 1.4: Structure of Lipopolysaccharide



The lipid A region is integrated into the OM bilayer and is made up of fatty acid chains linked to glucosamine. Extending outwards from the lipid A is the core region made up of short carbonhydrate chain and from the core region, in smooth strains is a longer carbonhydrate polymer, the O-side chain.

The lipid A composition is relatively similar within a wide range of gram-negative. So also are the sugars in the core region but the O-side chain where present, show gross differences in composition and structure, often within a single species. O-side chain polysaccharide synthesis is mediated by a number of cytoplasmic membrane-bound enzymes where it is synthesized before being transported and incorporated on the outer surface of the OM.

Of the OM components, the LPS appear to be vital in the permeability barrier protective function in that various workers observed that the sensitivity to most antibiotics was increased in LPS-deficient "rough" mutants of E. coli (Lazdunski & Shapiro, 1972; Sekiguchi & Iida, 1967; Tamaki & Matsuhashi, 1973) and Salmonella typhimurium (Lindsay et al, 1973; Sanderson et al, 1974) and this sensitivity was found to be proportional to the degree of LPS deletion in S. typhimurium (Sanderson et al, 1974).

Also there are some cells whose penetrability by antibiotics is affected by R-plasmid-mediated changes in the OM e.g. penicillin-resistant E. coli (Curtis & Richmond, 1974), cells of Ps. aeruginosa resistant to gentamycin and carbenicillin (Korfhagen et al, 1975). Apart from these genetic factors, the

nutritional status of some gram-negative bacteria has been found to influence their OM permeability by Lysozyme (Klainer & Russell, 1974) and by antibiotics (Walker & Durham, 1975). Similarly, Schnaitman (1974) found that the number and the proportions of OM proteins vary in response to nutritional changes.

The OM contains a limited number of proteins compared to the cytoplasmic membrane and in bacteria like E. coli and S. typhimurium, these heterogenous proteins can now be classified in a standard nomenclature based upon the genetic loci controlling the production of the proteins (designated Omp A-F) (Hammond et al, 1984). Apart from the major OM proteins and lipoproteins, there are other proteins termed minor proteins, many of which are inducible or derepressible and under appropriate environmental conditions may reach levels in the OM comparable with those of the major proteins. Hence the OM should not be regarded as a structure of invariable composition. Proteins located at the cell surface often serve as receptors for bactericidal proteinaceous antibiotics called bacteriocins and bacteriophages (Hammond et al, 1984).

Some of these apparently important proteins include : lipoprotein (7.5 K), Omp A or protein d (33 K or 28 K), Omp C or protein C (36 K) and Omp F or protein b (37 K). These proteins constitute 70-80% of the proteins in the OM (Rossouw & Rowbury, 1984). Omp C and Omp F proteins have been termed porins or matrix proteins and are PDG-associated transmembrane proteins which form water filled pores for the permeation of low molecular weight hydrophilic molecules. Omp A or protein d is also trans-membrane and is required for optimal growth in certain cultural conditions.

It has been found that a group of resistance plasmids of the N-incompactibility group reduced the amount of OM porin (Omp F equivalent) thereby increasing resistance to ampicillin, chloramphenicol, ethionine and copper ion. All these agents use the non-specific proteinaceous pores formed by Omp C and Omp F (Rossouw & Rowbury, 1984). Reduced Omp F level is accompanied with reduced LPS to a lesser extent but this change is thought to be associated with the appearance of phospholipid in the outer leaflet of the OM and this appearance of phospholipid and the reduced Omp F level are thought to jointly alter the OM permeability in favour of hydrophobic agents.

This observation is in total agreement with that by Russell and Furr (1986) who found that removal of part or much of the LPS and the absence of one or more porins would modify the cellular response to hydrophobic inhibitors, since phospholipid would now appear in the outer leaflet of the OM in the form of a classical membrane structure.

However, plasmid encoded membrane protein resulting in increased permeability for hydrophobic agents may not necessarily have the same effect on the cytoplasmic membrane because since some of these plasmids are capable of coding for more than one protein, the cytoplasmic membrane may itself have been altered hence an increased penetration of hydrophobic agent may not be matched with decreased resistance as noted by Rossouw and Rowbury (1984).

From the foregoing, it is evident that the OM of gram-negative bacteria is an important penetration barrier to certain antibiotics and its structure is influenced by genetic as well as nutritional factors in addition to chemical and physical agents.

1.3.2 Effect of Antibiotics on Cell Envelope

Antibiotics can exert their various effects on the bacterial cell by interacting with the cell

envelope at different levels e.g. the OM or the cytoplasmic membrane. For example penicillin binds rapidly and irreversibly to sensitive organisms with most of the bound antibiotic on the cytoplasmic membrane where it inhibits transpeptidase enzyme. Penicillin and its derivatives as well as cephalosporins are known to bind to the same site(s) with consequent inhibition of growth.

Inhibition of growth can also result from exposure to some antibiotics that interfere with the early stages of PDG synthesis especially of precursors in the cytoplasm e.g. Streptomycin, Tetracycline, Chloramphenicol, Erythromycin etc (Park et al, 1974).

Other antibiotics act on the OM component e.g. the phospholipids, LPS etc. For instance, polypeptide antibiotics e.g. polymyxin B act on membrane phospholipid although this action is subject to access of the antibiotic to the membranes. Polymyxin B is known to act on the phosphate group on the phospholipid (Pache et al, 1972). Also LPS or endotoxin which is also a polyphosphate interacts with polymyxin, thereby becoming inactivated (Rifkind, 1967). In conformity with these facts, Tsang & Brown (1980) observed that polymyxin B altered the antigenic components through

loss of both antigenic protein and LPS component effected by the complexing as well as the degradative effects of the antibiotic on the LPS component of the OM. In fact, even polymyxin-resistant bacteria (determined by the classical lack of effect on growth rate and viability) are believed to be altered by the drug. For example, after exposure to polymyxin, the organisms become susceptible to killing by desoxycholate to which they were previously resistant (Sud & Feingold, 1972) and they similarly become susceptible to serum-killing. Another peptide antibiotic EM 49 is said to cause formation of numerous protrusions or blebs on the surface of E. coli with apparent release of membrane vesicles from cells and consequent release of OM fragments into the surrounding (Rosenthal et al, 1976).

Gentamycin, an aminoglycoside antibiotic was found to bind to the core region of the LPS in Ps. aeruginosa and the level of binding appear to be related to the amount of organic phosphate associated with the LPS (Day, 1980). This interaction is thought to affect the barrier function of the cell envelope. Also some cephalosporins at sub M.I.C. levels cause some cultures to loose their characteristic gel-like viscosity with exposure of OM antigens which were

otherwise masked (Kadurugamuwa et al, 1985a) and this could be important in complement-mediated serum killing and phagocytosis.

Protein A, a component of the cell wall outer layer impairs opsonization by serum complement and thereby delays phagocytosis by polymorphonuclear (PMN) leucocytes. Sub M.I.Cs of Clindamycin and Fusidic acid reduced the amount of protein A in S. aureus thereby rendering it more susceptible to phagocytic uptake and killing (Veringa & Verhoef, 1976; Gemmell & O'Dowd, 1983). Diphenylamine was also observed to effect phenotypic conversion of E. coli from serum-resistant to serum-sensitive due to a decrease in the amount of O-antigen in the cell wall and along with the conversion there was an inhibition of synthesis of surface polysaccharide (Feingold, 1969).

1.4 Role of Cell Envelope in Bacterial Virulence and Pathogenicity Determination

The terms virulence and pathogenicity are interchangeable and can be described as the ability of a bacterium to express disease symptoms which could be mild or fatal. For a bacteria specie to be virulent it should be able to : (1) adhere to and invade the host tissue, (2) multiply in the host, (3) survive in the host by being able to overcome the obstacles posed

by host's response to infection such as antibody formation, phagocytosis, serum bactericidal action, depletion of vital nutrients etc and administered drugs. To achieve all these a lot of events take place involving adhesion, invasiveness, hydrophobicity and phagocytosis all of which are surface phenomena thus indicating the vital role of bacterial cell envelope in bacterial pathogenicity. For instance, the lipopolysaccharide (LPS) is known to carry the O-antigen specificity of the bacterium, responsible for endotoxic activity of the cell and is thus involved in the pathogenicity of many gram-negative bacteria (Ward, 1980) while it also protects the cell surface from easy access by hydrophobic drugs and molecules. Also Sansano Jr. (1985) noticed that bacterial smooth phenocopy was much more resistant to the bactericidal activity of normal human serum than the rough phenotype, an implication for the effect of the capsular component of the cell envelope. Other cell surface components that participate in virulence determination include peptidoglycan, proteins and pili (Aguero et al, 1984).

1.4.1 Involvement of Cell Envelope in Bacterial Adhesion

Adhesion has been used broadly to describe any initial interaction between bacteria and host tissues,

regardless of the mechanisms or components involved and bacterial adhesion, thus imprecisely defined, must be a pre-requisite for colonisation and infection. However, the term adhesion should be used for the specific binding of bacteria to epithelial cells at the site of infection (Eden et al, 1983) and it is probably primarily a result of interactions between surface molecules of bacteria and host cells.

Generally, the first step in the adhesion of bacteria to host cells or to different polymeric substrates is directly related to the surface tension of bacteria, with the initial attachment being frequently followed by a firm irreversible adhesion (Marshall, 1972). This latter selective process is mediated by polymeric bridges.

After a consideration of various published data, Rutter (1980) concluded that four mechanisms can operate in the adhesion of organisms to surfaces. These are : (a) non-specific attachment at the primary minimum, (b) non-specific attachment at the secondary minimum, (c) polymer bridging and (d) polymer consolidation. The non-specific interactions involve charged groups and in this respect, both gram-positive

and gram-negative bacteria are negatively charged due mainly to an excess of carboxyl and phosphate groups at their surfaces. Also it seems the interactions between the charged components of the organisms and other surfaces (substrates) are mediated by ions from the local environment and such interactions will persist for a sufficient length of time to allow more specific linkage to occur (Ward, 1980). Normally, the overall physico-chemical surface properties of bacteria do not favour the initial non-specific attachment, however, some structures of the bacterial surface can overcome the repulsive electrostatic forces (Heckels et al, 1976).

As for specific interactions involving polymers, many polymers have been implicated e.g. LPS (Wolpert & Albersheim, 1976; Kamberger, 1979a, b), capsule (Dazzo, 1979). These are in cases where the binding lectin is on the substrata. In constrast, adhesion of E. coli to human epithelium cells is dependent on a mannose-binding protein (lectin) located on the bacterial surface (Ofek et al, 1977). Leban & Whitmoyer (1979) also suggested the involvement of capsular material and slime layer in adhesion while pili have also been demonstrated to be clearly involved in adhesion. In fact, it is known that

piliated cells will attach to a wide variety of surfaces and depending on the surface, the same type of bacterium may adhere in either a specific or non-specific way (Ofek et al, 1977).

Further indication for the involvement of cell envelope in bacterial adhesion can be found in the report of ØGAARD et al (1985) on strains of Ps. aeruginosa, in which they were able to establish that there were at least two adhesive factors. One was extracellular, with its activity reduced or lost on subcultivation and is neutralised by non-specific factors in serum while the other was cell wall-bound, with activity retained on subcultivation and is inhibited by immunoglobulin.

Thus bacterial adhesion depends to some extent on the components and nature of the bacterial surface as well as some physico-chemical activities taking place at the surface, all suggestive of the vital role of the cell envelope in bacterial adhesion.

1.4.2 Hydrophobicity As A Cell Surface Phenomenon

Hydrophobicity is an expression of how oil-loving or how compatible with oily materials a substance is. The interaction of pathogenic bacteria with cells of the host results from the balance of

several attractive and repulsive forces operating between the complex surface components of procaryotic and eucaryotic cells (Ofek & Beachey, 1980). Bacterial hydrophobicity is considered important for cell aggregation and for bacterial adhesion to animal cell membranes during colonisation and in phagocytosis (Malmqvist, 1983). Hydrophobic interaction have also been implicated in adherence of bacteria to non-wettable plastics (Gerson & Scheer, 1980; Rosenberg, 1981), to hydrocarbons (Rosenberg et al, 1980a; Rosenberg et al, 1980b) and in the partitioning of bacteria at the water: air interface (Dahlback et al, 1981). A lot of other studies have been done on hydrophobicity. For instance, McBride et al (1984) comparing hydrophobic strains of Streptococcus mutans with variant strains that were less hydrophobic found that hydrophobic cells contained high molecular weight proteins absent in cells with reduced hydrophobicity. The proteins were found in purified cell walls suggesting that they are located on the bacterial surface.

The report of Parker & Munn (1984) supports the association of hydrophobicity with bacterial cell surface. They found the cell surface of strains of Aeromonas salmonicida possessing an additional surface

protein (A-protein) to be more hydrophobic than strains devoid of this protein.

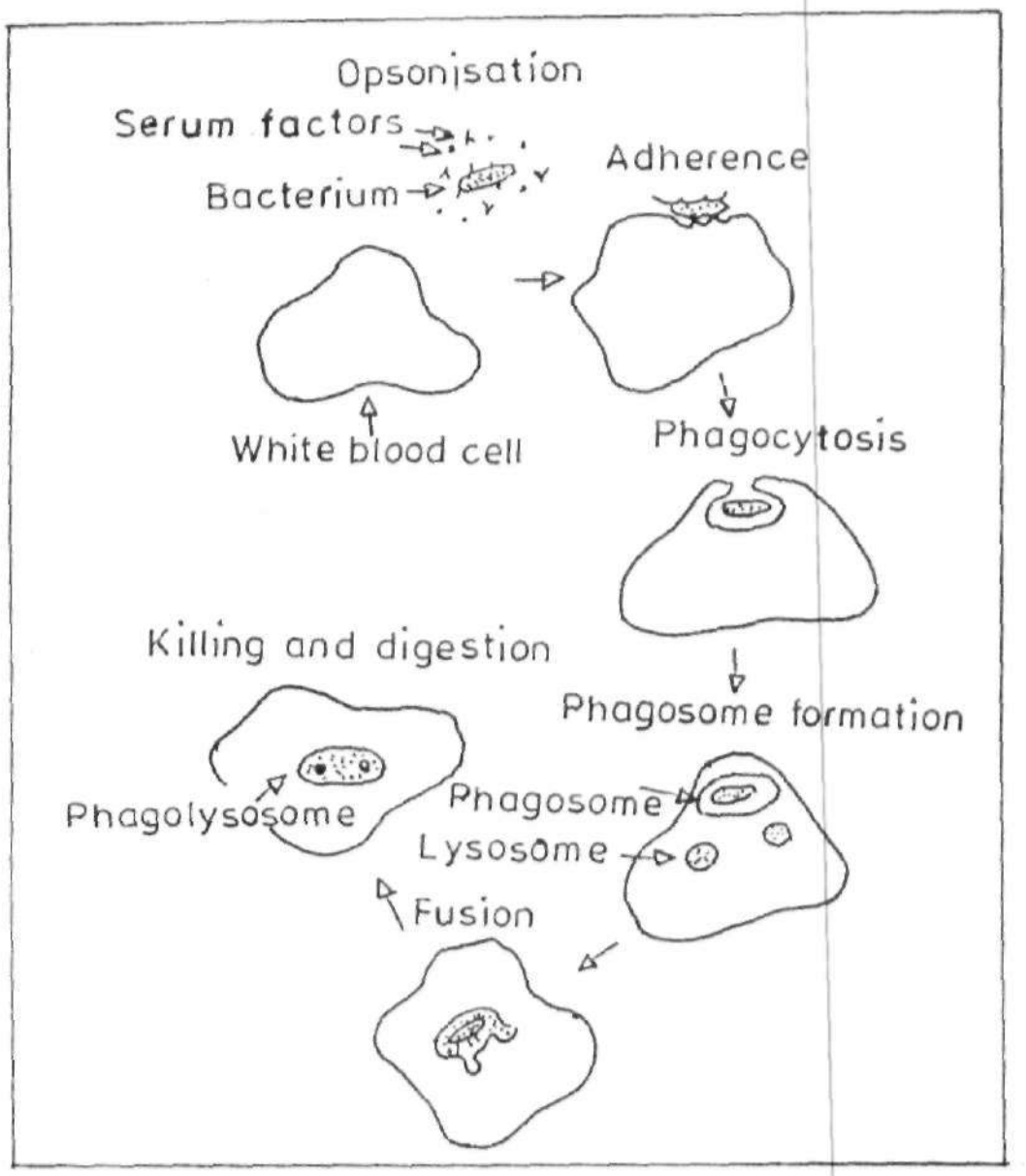
That proteins are involved in determining hydrophobicity can be established through enzyme treatment using Trypsin or pepsin. Ofek et al (1983) noticed that Streptococcus pyogenes cells in which the proteins have been degraded lost their hydrophobicity. It was further observed that hydrophobicity as measured by adherence to hydrocarbon is an index of the available lipophilic residue on the cell surface which are either hydrophobic regions of surface protein structures, or more likely glycolipids complexed with and oriented by surface proteins (Ofek et al, 1983). Tylewska et al (1980) suggested that M protein is involved in determing the hydrophobic property of group A streptococci but Miorner et al (1983) believed that lipoteichoic acid (LTA) is the major cell wall component responsible for surface hydrophobicity of these bacteria. However, both M protein and LTA have been associated with adherence of group A streptococci to epithelial surfaces (Ellen & Gibbons, 1972). Beachey (1975) also believed that the LTA in the cell wall may be oriented in such a way that the hydrophobic lipid moiety extends from the cell surface and would be available for reaction with hydrophobic

receptors on epithelial cells. Similarly, Jonsson & Wadstrom (1983) working on strains of S. aureus observed that protein A and probably other surface proteins and amphiphilic molecules like LTA contribute to the high surface hydrophobicity of the organism.

1.4.3 Phagocytosis and Bacterial Cell Envelope

Phagocytosis is the process by which bacteria are ingested and killed by white blood cells such as polymorphonuclear (PMN) leucocytes and macrophages. It is phylogenetically the oldest and most fundamental defence mechanism of the host against foreign invaders. However, before bacteria are engulfed a lot of forces come into play i.e. contact between bacteria and PMNs (adhesion). The fitness between bacteria adhesins and target cell receptors has been shown to influence interaction with human PMN leucocyte. For instance, mutants of a hydrophilic E. coli strain required mannose sensitive (MS) adhesins for binding to and activation of the PMN leucocytes (Eden et al, 1984). They also thought that the mechanism of binding which improves epithelial adhesion, may prevent binding to PMN leucocytes, thus improving the survival of the bacteria.

Fig. 1.5: The process of phagocytic uptake and digestion.



Presence of certain proteins do have effect on bacteria sensitivity to phagocytosis. For instance, the OM protein Trat 5 which is encoded by plasmid R6-5 is said to reduce E. coli sensitivity to phagocytosis by macrophages. This was thought to be by antagonising opsonisation through reduced deposition and altered distribution of C3 (i.e. third component of complement) (Aguero et al, 1984). Similarly, M protein has been implicated in altered or uneven C3 distribution associated with resistance to phagocytosis in group A streptococci (Jack-Weis et al, 1982). Various K-antigens in E. coli are known to reduce the activation of the complement system (Verweij-Van Vught et al, 1984) while virulence of Vibrio vulnificus has also been associated with the possession of an antiphagocytic surface antigen (Kreger et al, 1981).

The degree of bacterial cell surface hydrophobicity is known to correlate at least qualitatively with their susceptibility to phagocytosis. Van Oss & Singer (1966) observed that hydrophobic surfaces are particularly prone to physical adsorption of immunoglobulins. Thus the more hydrophobic bacteria and particles are, the more readily aspecifically they adsorb immunoglobulins and would in the presence of complement become phagocytosed. However, extremely

virulent bacteria endowed with hydrophilic capsules resist phagocytosis until specific anti-capsular antibodies have been formed (Van Oss, 1975).

Also there could be aspecific adsorption of immunoglobulins without phagocytosis as in some encapsulated bacteria. For instance, IgG adsorption by an encapsulated strain and a regular non-encapsulated strain of S. aureus were the same, the encapsulated strain were hardly phagocytosed while that of the non-encapsulated strain was quite strong (Van Oss, 1975). It was therefore thought that aspecific adsorption of immunoglobulins by the encapsulated strain took place somewhere inside the capsule such that the adsorbed immunoglobulins were spatially unavailable for opsonizing duties. With removal of the capsule, this strain still adsorbed the same amount of IgG but now strongly phagocytosed. Hence only those phenomena taking place at the very surface of the microorganisms appear to be the crucial ones in deciding their engulfment or non-engulfment.

1.5 Effect of Antibiotics on Bacteria Sensitivity to Phagocytosis

In the rationale for antimicrobial therapy, apart from organisms' sensitivity, site of infection etc, the possibility of interaction between chemobiotic and immune systems must be considered. This is so

considering the increasing number of infections due to resistant organisms which are the consequence of frequent clinical use of immunosuppresant drugs including some antibiotics (Ripa et al, 1986).

Many antibiotics have effect on the PMN-microbe interaction. Firstly, if the antibiotic is unable to penetrate the phagocyte (because they vary greatly in their ability to enter normal phagocytes e.g. penicillin G and cephalosporins are poorly absorbed by phagocytes, while the more lipid-soluble antibiotics e.g. rifampin and chloramphenicol are well absorbed) or is inactivated within the phagocyte, then surviving intracellular microbes may be protected from destruction. Although when absorbed by PMN, there could be synergism in the killing effect of PMN and antibiotic on facultative intracellular organisms, however, absorption does not always predict activity (Hand, 1984) and this may be due to a number of possible factors like : (a) intracellular concentration, (b) the agents intrinsic antibacterial properties (i.e. mechanism of action, type of activity, whether inhibitory or bactericidal) and (c) effects of the intracellular environment (i.e. pH, protein binding, enzymes, whether aerobic or anaerobic conditions etc) on biological activity of the antibiotic and

susceptibility of the organisms. Secondly, antibiotics may alter phagocyte functions (i.e. chemotaxis, phagocytosis or microbicidal activity) by a direct effect on the phagocytic cells. Thirdly, antibiotics may alter the microbe without killing them, thus changing their susceptibility to phagocytosis and killing (Yourtee & Roots, 1984). This is an observation supported by Lorian and Atkinson (1984) who reported that antibiotic-induced filaments of gram-negative bacilli were much more susceptible to bactericidal activity of PMN than were the normal bacilli. However, this change in susceptibility is not always positive. For instance, it has been shown that low concentrations of antibiotics effect subtle ultrastructural changes in bacterial cell wall and in biosynthesis of various virulence factors e.g. M protein on Streptococcus pyogenes (Gemmell et al, 1982), protein A on S. aureus (Gemmell & O'Dowd, 1983) etc and going by Gemmell's (1984a) report, drugs that affect cell wall biosynthesis may or may not potentiate phagocytosis depending on whether filamentation does occur or not at sub M.I.C level. Even at therapeutic level some drugs are known to decrease killing by PMN e.g. tetracycline, trimethoprin (Hand, 1984) whereas some others improve killing e.g. cephalotin, chloramphenicol, penicillin etc (Hand, 1984).

The alteration in the integrity of bacterial cell envelope as a result of antibiotic effects, in relation to susceptibility to host defence mechanisms become manifest if one considers bacterial pathogenicity determining factors e.g. hydrophobicity, adherence, serum-susceptibility etc, most or all of which are interrelated. Over the years, clinical observations and experimental results indicated that serum resistance is an important determinant of pathogenicity in a number of infections caused by gram-negative bacteria (Taylor et al, 1979). For instance, by decreasing the amount of O-antigen in the cell wall and inhibiting synthesis of surface polysaccharide, Diphenylamine was able to effect a phenotypic conversion of E. coli strain from serum-resistant to serum-susceptible (Feingold, 1969). Also through a more rapid opsonization (requiring both antibody and complement) of clindamycin treated S. aureus cells, phagocytosis by PMN was enhanced (Veringa & Verhoef, 1976). This is thought to involve the exposure of a greater number of receptor sites for C3b due to reduced cell wall protein A content in the treated cells (Gemmell & O'Dowd, 1983).

SubMIC of Mecillinam while producing ovoid or round cells (inhibition of PBP2) also converted serum

resistant E. coli to serum sensitive (Taylor et al, 1981). Since increased serum susceptibility results in increased susceptibility to phagocytic uptake in most cases, production of oval form in this case would not necessarily be expected to give increased phagocytic uptake, taking cognisance of the observation of Gemmell (1984a) earlier mentioned. Even though increased serum susceptibility is supposed to yield cells of increased surface hydrophobicity which would ordinarily be expected to increase phagocytic uptake, this conflict, however, exemplifies the complexity of the pathogenicity factorial interlink.

1.6 Why The Project

The indiscriminate use of antibiotics and the frequency of human infections due to resistant organisms is a disturbing situation. Widespread use of antibiotics has been reported to increase cases of antibiotic resistance in gram-negative organisms (Al-Jebouri & Al-Meshhadani, 1985), just as low blood level of antibiotics due to either withdrawal of therapy or non-compliance may lead to resistance training. Also antibiotic therapy has been implicated in transfer of R-factors in the gut (Anderson et al, 1973). The gram-negative organisms are of importance here because they represent a large reservoir of resistance factors which are transmissible.

It has also been shown that antibiotic-resistant bacteria of animal origin can be transmitted to man (Johnston et al, 1983), just as human infection can result from contaminated drinking water resulting from river water contaminated with such pathogens like S. typhimurium or Vibrio cholerae (Al-Jebouri & Al-Meshhadani, 1985). Hence in what looks like a vicious ecological cycle, the human host is the point for increasing resistance and scope-widening for R-plasmid carriers. It is known however, that human infections with resistant microorganisms are difficult to treat due to limitation in choice of antibiotics.

The constant widespread use of antibiotics in animal husbandary, Veterinary and human medicine coupled with the current knowledge of transfer of antibiotic resistance makes it necessary to monitor the susceptibilities of known pathogenic bacteria to antibiotics. Of more importance is, the manner in which antibiotic is being abused in the society and the implication of low serum antibiotic concentration in resistance training and transfer of R-plasmids. This prompted the use of sub M.I.C of one of the commonly used antibiotics, ceftriaxone (Rocephin$^{(R)}$) in this work against one of the casual but potentially dangerous pathogens Ps. aeruginosa.

Chapter Two

2. MINIMUM INHIBITORY CONCENTRATION (M.I.C.) DETERMINATION AND MORPHOLOGICAL EFFECT OF SUB M.I.C. OF ROCEPHIN(R) ON PSEUDOMONAS AERUGINOSA

2.1 Determination of M.I.C. of Rocephin(R) against five Isolates of Ps. aeruginosa

2.1.0 Introduction

The minimum inhibitory concentration is a measure of the sensitivity of an organism to an antimicrobial agent and it is the least concentration of the agent that will inhibit its growth. It can be determined using liquid or solid media.

The M.I.C values determined by both methods have been found to be affected by the composition of the media used. For example, the M.I.C of gentamycin against Ps. aeruginosa was found to vary in different media (Waterworth, 1969), and that there was a direct relationship between the M.I.C and the magnesium content of the agar. Stokes & Waterworth (1972) also suggested the use of 25% serum broth when using the broth dilution method in order to account for the effect of serum binding of some antibiotics.

Inoculum size is also very important during M.I.C determination. Unduely large inoculum sizes

result in misleading high M.I.C values. A few suggestions have been recommended for reliable results :

(a) use of a drop of a 1:100 dilution of an overnight culture for inoculating the test media (Stokes & Waterworth, 1972).

(b) use of a drop of an overnight culture diluted to 10^5 - 10^6 viable organisms per millilitre to inoculate the test media (Bristol Laboratory, 1976).

Other factors that can affect M.I.C value are : (a) incubation time, (b) growth phase of the organisms, (c) protein binding and (d) incubation temperature. To obtain reliable results, these factors must be adequately controlled.

2.1.1 Materials

Rocephin$^{(R)}$ was obtained from a commercial store while four clinical isolates of Ps. aeruginosa (isolates A-D) were obtained from Ahmadu Bello University Teaching Hospital, Zaria. A wild isolate (E) was obtained from stagnant water around the premises of the Faculty of Pharmaceutical Sciences, A.B.U., isolated, purified and characterised by subjecting it to biochemical tests i.e. sugar fermentation and oxidase test. Mueller-Hinto (MH) broth (Oxoid) was obtained from the departmental stock.

2.1.2 Methods

M.I.C was determined using the serial and arithmetic dilution methods.

Serial dilution:

Single strength and double strength MH broth were prepared according to manufacturer's specification. The two strengths were then dispensed 2.5ml each into tubes and sterilized at 121°C for 15 minutes according to manufacturer's recommendation. Then a 200ug/ml stock solution of Rocephin(R) was prepared and filtered into a sterile bottle using membrane filter under aseptic conditions.

A serial dilution of Rocephin(R) was prepared using six tubes in a row (the first tube containing double strength MH broth), this was prepared in triplicate for each of the five cultures to be used. 2.5ml of stock solution was added to every first tube, mixed well on Whirl's mixer and 2.5ml was withdrawn and added to the next tube, repeating the process until the sixth tube where 2.5ml was withdrawn after mixing and discarded leaving all six tubes with 2.5ml single strength MH broth but containing different concentrations of Rocephin(R).

1:100 dilutions of overnight cultures of isolates A-E were prepared and using a pasteur pipette, 2 drops were added to all the tubes for each isolate, mixed well and then incubated at 37°C for 18 hours.

Arithmetic dilution:

Double strength MH broth was prepared and dispensed 1.25mls to each tube and sterilized at 121°C for 15 minutes. Then a 600ug/ml stock solution of Rocephin(R) was prepared and filtered.

Tubes containing medium were then arranged in triplicate for each isolate and for eight dilutions of agent. Then 1.25ml stock solution of agent was added to all tubes 1, thereby diluting the MH broth to single strength and the agent 1:2. Then calculated volumes of stock solution were added to other tubes and made up to 2.5ml with sterile distilled water such that the tubes contained: 300ug/ml, 250ug/ml, 200ug/ml, 180ug/ml, 150ug/ml, 120ug/ml, 100ug/ml and 40ug/ml of Rocephin(R) respctively.

1:100 dilutions of overnight cultures of isolates A-E were prepared and the tubes were inoculated with two drops of the diluted cultures. The inoculated media were then mixed and incubated at 37°C for 18 hours.

2.2 Growth Curves of Isolates A-E

2.2.1 Materials and Method

Single strength MH broth was prepared and dispensed 30ml into each of 100ml conical flasks and sterilized at 121°C for 15 minutes.

The optical density (O.D) of the overnight cultures was taken on a Spectronic 20 Spectrophotometer at 470nm. The overnight cultures were then harvested by centrifugation at 3000 rpm for 15 minutes using a Gallenkamp centrifuge. The harvested cells were washed once with warm MH broth and resuspended in the same broth to a uniform $O.D_{470nm}$.

Five flasks containing sterile 30ml MH broth maintained at bath temperature of 37°C were labelled A-E and inoculated with 1ml of their respective resuspended washed cells at 5 minutes intervals, starting the bath shaker after the first inoculation at a speed of 80 oscillations/minute (80 opm). Each flask's culture $O.D_{470nm}$ was taken at its time zero-minute after inoculation. Subsequent O.D. readings for each flask were taken after every 30 minutes interval. Cultures were diluted appropriately after the cells have grown beyond $O.D_{470nm}$ 0.3 before taking O.D readings. O.D readings were taken until the cells entered the stationary phase of growth.

2.3 Growth Characteristics of Isolates C&E Under the Influence of Sub M.I.C of Rocephin(R)

2.3.1 Materials and Method

Overnight cultures in MH broth were harvested by centrifugation at 3000 rpm for 15 minutes. The harvested cells were washed once with warm MH broth and resuspended in the same broth. A 500ug/ml Rocephin(R) stock solution was prepared and sterilised.

Four conical flasks containing sterile 30ml MH broth maintained at bath temperature of 37°C were inoculated with 1ml of the resuspended washed cells. The shaker bath was maintained at a speed of 80 opm. The O.D of each culture was taken at a timed interval starting from time zero-minute. Each culture sample was returned into the flasks after O.D reading. When the O.D approached 0.3, culture samples were diluted before taking subsequent O.D readings and such diluted cultures were discarded.

When the organisms had entered the exponential phase of growth, a slide of each culture was fixed and stained. Calculated volumes of Rocephin(R) stock solution were added to the test culture such that the final volume contained 1/8, 1/16 and 1/24 of M.I.C in flasks containing the test cultures

respectively (M.I.C was 180ug/ml for isolate C and 300ug/ml for isolate E from previous experiment). This time was denoted as zero-minute antibiotic-action time. More slides were prepared from each culture including the control at 30 minutes, 90 minutes and the terminal part of the exponential phase (180 minutes), after addition of Rocephin(R).

The above procedure was carried out for each of the two isolates, using Rocephin(R) stock solution containing 800ug/ml in the case of isolate E. The slides were then examined microscopically.

2.4 Results

2.4.1 The Minimum Inhibitory Concentration of Rocephin(R) to the Ps. aeruginosa Isolates

Serial dilution: There was growth at the highest concentration used in all the tubes.

Arithmetic dilution:

Isolate	M.I.C (ug/ml)
A	300
B	200
C	180
D	200
E	300

2.4.2 Growth Curves of Isolates A-E

The growth curves of the five isolates in MH broth are as shown in Fig. 2.1. This is a semi-log plot and the O.D in logarithmic scale measures the corresponding bacterial population or cell mass at the various times of growth.

Considering the starting O.D, among the clinical isolates, isolates C and D had comparable growth curves but isolate C's growth curve was better defined compared to D (see also Table 2.1 for doubling time). Isolate E grew very well.

Considering these growth characteristics in conjunction with M.I.C results, isolates C & E were chosen for further investigations.

2.4.3 Growth Characteristics of Isolates C&E under the influence of Sub M.I.C of Rocephin(R)

2.4.3.1 Growth curves

The growth pattern of the two isolates under the influence of 1/8, 1/16 and 1/24 of M.I.C are as shown in Fig. 2.2 for the clinical isolate (isolate C) and Fig. 2.3 for the wild type (isolate E).

From Fig. 2.2 it can be seen that Rocephin(R) at the concentrations used had little effect on the

Fig. 2·1

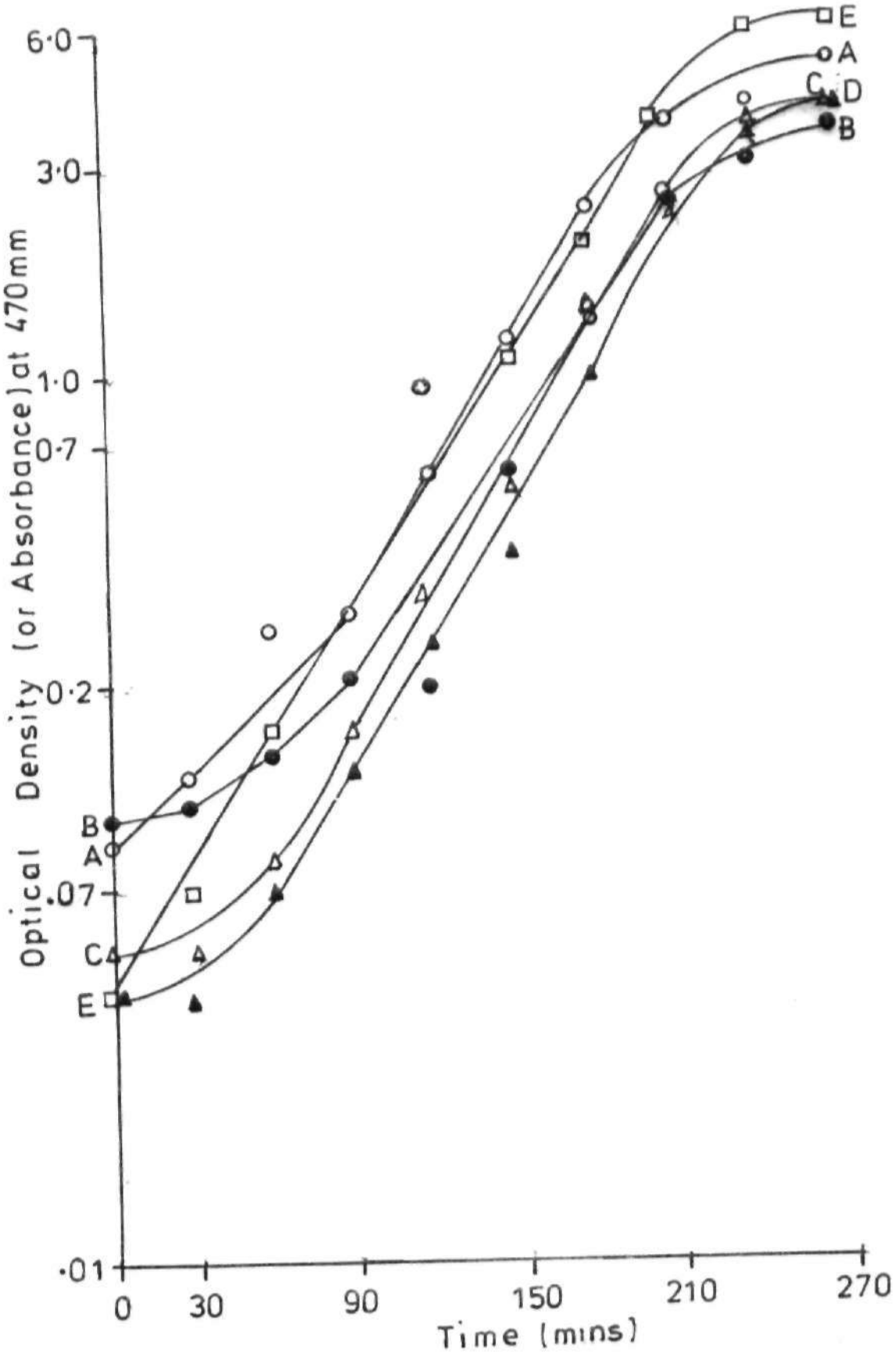

6·0
3·0
1·0
0·7
0·2
·07
·01
Optical Density (or Absorbance) at 470mm
E
A
C
D
B
B
A
C
A
E
0
30
90
150
210
270
Time (mins)

FIG. 2·2

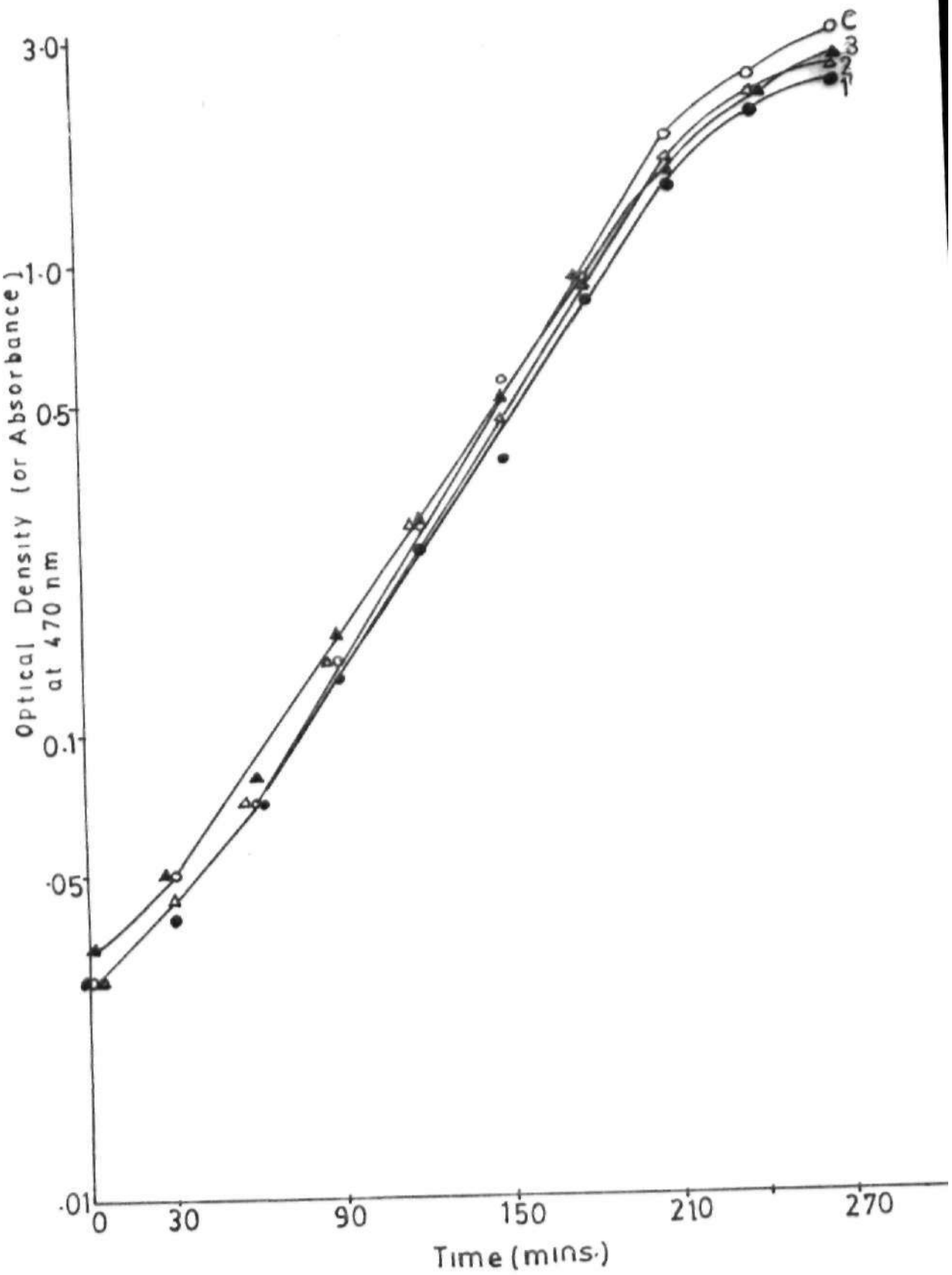

3·0
1·0
0·5
0.1
·05
·01
Optical Density (or Absorbance) at 470 nm
0
30
90
150
210
270
Time (mins.)

Fig. 2·3

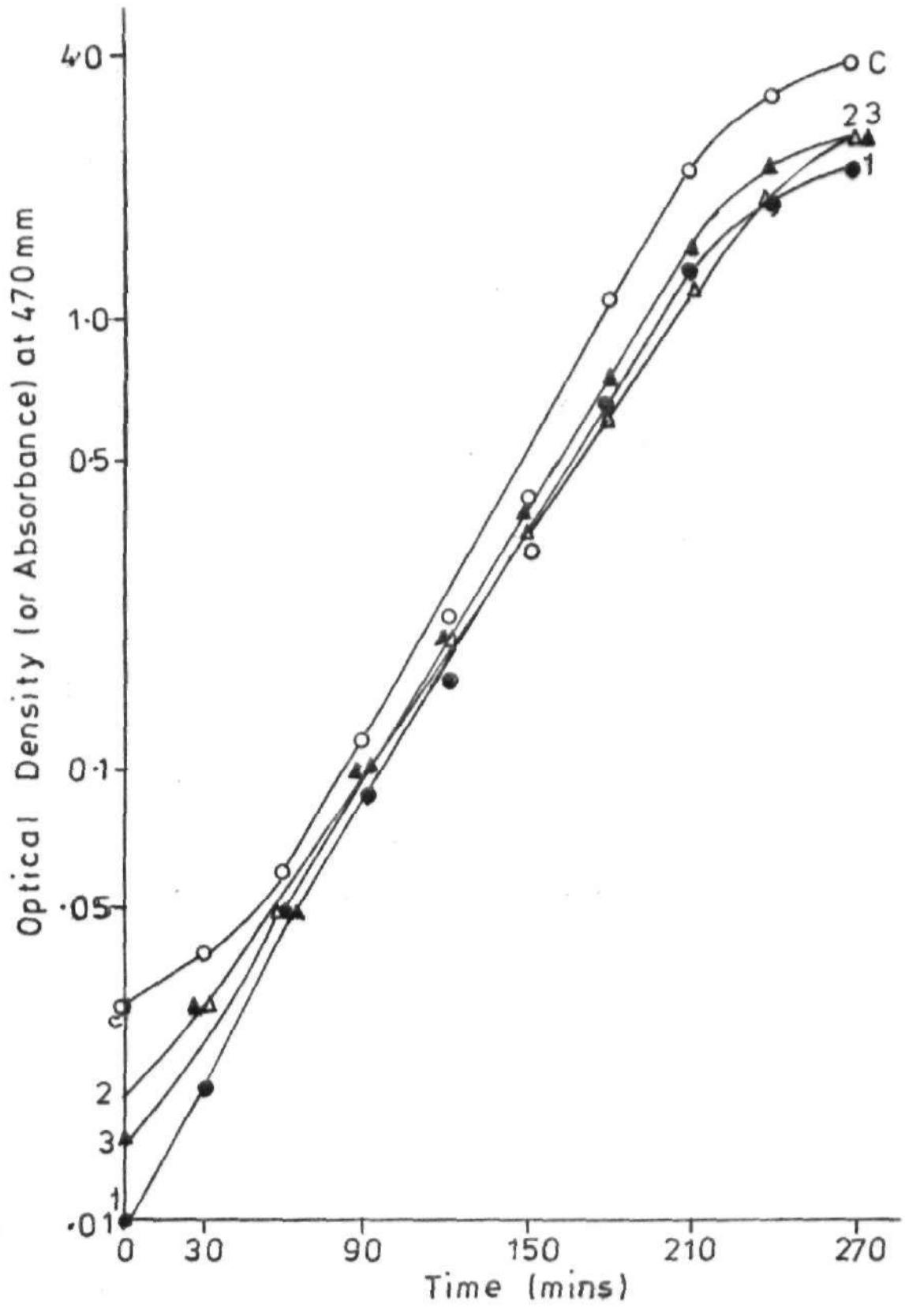

Optical Density (or Absorbance) at 470 mm
4·0
1·0
0·5
0·1
·05
·01
C
2
3
1
0
30
90
150
210
270
Time (mins)

Fig. 2·4

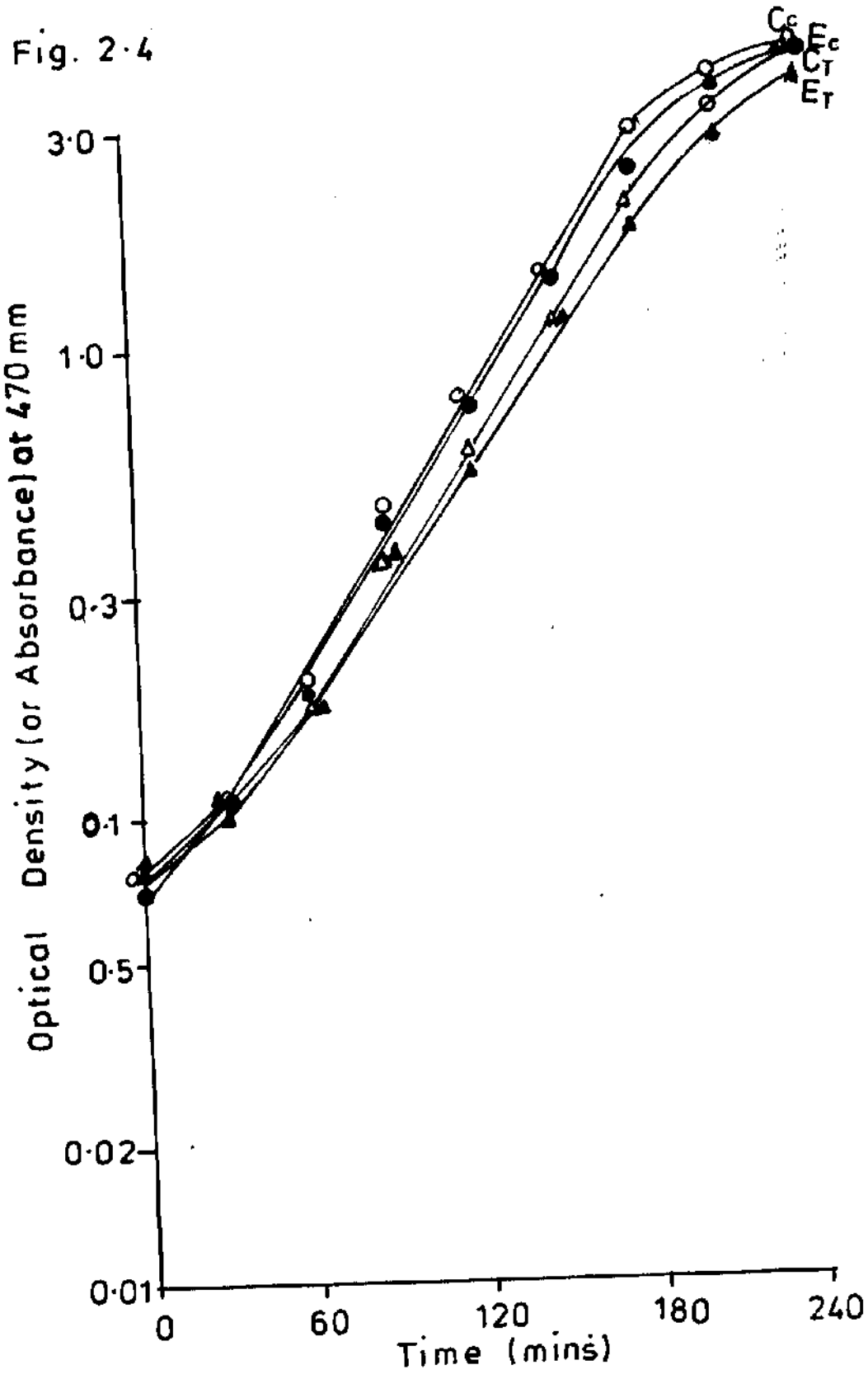

Optical Density (or Absorbance) at 470 mm
3·0
1·0
0·3
0·1
0·5
0·02
0·01
0
60
120
180
240
Time (mins)
Cc
Ec
CT
ET

Table 2.1: Doubling times of Rocephin(R) Treated and Untreated Isolates

Table for Fig. 2.1

Isolate	A	B	C	D	E
Doubling time (t_D) in min	30	34	30	31	32

Table for Fig. 2.2

Isolate C	C_c (control)	C_1 (1/8 M.I.C)	C_2 (1/16 M.I.C)	C_3 (1/24 M.I.C)
Doubling time (t_D) in min	32	35	33	36

Table for Fig. 2.3

Isolate E	E_c (control)	E_1 (1/8 M.I.C)	E_2 (1/16 M.I.C)	E_3 (1/24 M.I.C)
Doubling time (t_D) in min	29	31	34	31

Table for Fig. 2.4

Isolate	C_c (control)	C_T (1/24 M.I.C)	E_c (control)	E_T (1/24 M.I.C)
Doubling time (t_D) in min.	31	32	33	35

growth rate of the isolate with 1/24 of M.I.C showing the least effect with a curve very close to that of the control. Also from Fig. 2.3, 1/24 of M.I.C had very little if not no effect on the growth rate of the isolate, considering the starting O.D.

Fig. 2.4 shows the growth patterns of the two isolates with and without 1/24 of M.I.C. Rocephin(R) can be said to have little or no effect on the growth rate of the two isolates at a concentration of 1/24 of M.I.C (see also Table 2.1 for doubling time).

*1/24 of M.I.C was chosen for use in all other investigations, since we want a concentration that will not affect the growth rate.

2.4.3.2 Results of Morphological Effect of Sub M.I.C of Rocephin(R) on Isolates C and E

During the M.I.C determination, the effect of various concentrations of Rocephin(R) on the morphology of the isolates are as shown below:

Isolate C

At 40 ug/ml: A good majority of cells were almost unaffected but there were a few long filaments.

Isolate C

At 100 ug/ml: The filaments were longer than at 40 ug/ml.

At 150 ug/ml: Cells showed extensive filamentation with very few short and medium filaments and virtually no standard size cells.

At 300 ug/ml: Less filament was formed (even lower than that at 100 ug/ml) and most cells were in their normal size.

Isolate E

At 40 ug/ml: There were numerous extensively long filaments, many long ones and several aggregate cells (normal size each) in chains of different units (from 2-6 cells).

At 100 ug/ml: Many cells were about normal size but there were some long filaments.

At 150 ug/ml: Many more cells were about normal size and there were some short filaments and a few long ones.

At 300 ug/ml: Many cells were about normal size and many also formed short filaments (though fewer than at 150 ug/ml) while a few were just about twice normal size.

The same trends were observed for the two isolates under accelerated growth conditions with much lower antibiotic concentrations. However, the magnitude of the differences observed was lower than in cultures grown under static conditions.

The observed morphological effects of sub M.I.Cs of Rocephin$^{(R)}$ on isolates C & E at various times of growth under accelerated growth conditions are as shown in figures 2.5 - 2.8.

Isolate C:-

30 minutes after addition of antibiotic

1/8 M.I.C: while most cells were about normal size, others have doubled in length.

1/16 M.I.C: most cells ranged from normal to twice normal size.

1/24 M.I.C: almost normal cells and a bit longer than normal cells.

90 minutes after addition of antibiotic:

1/8 M.I.C: numerous filaments, some as long as and others longer than in 1/16 (90 minutes) with slightly rough edges.

1/16 M.I.C: numerous filaments, no appendages and almost the same length (but a bit shorter than) as 1/24 (90 minutes).

1/24 M.I.C: Filaments are much shorter than in 1/24 (180 minutes), more conspicous and with undefined edges with appendages. Few other cells were about 2-3 times normal size.

180 minutes after addition of antibiotic:

1/8 M.I.C: virtually all the cells (just a very few exceptions) formed filaments about the length in 1/24 (180 minutes). However, the filaments did not have well defined edges.

1/16 M.I.C: long, moderately long to short filaments with a few normal and twice normal size cells.

1/24 M.I.C: most cells (almost but not all) formed fairly long filaments while few cells were normal or about twice normal size.

Isolate E:-

30 minutes after addition of antibiotic:

1/8 M.I.C: virtually all the cells have doubled in length with well defined edges.

1/16 M.I.C: many cells have doubled in length.

1/24 M.I.C: virtually all the cells have grown to 2-3 times normal size in length.

90 minutes after addition of antibiotic:

1/8 M.I.C: moderately long to short filaments (numerous) with rough edges or appendages. Scanty cells about normal size.

1/16 M.I.C: moderately long filaments (numerous), some with rough edges and some still with appendages or deposits (or probably bulges) at intervals along the length. Very few normal size and twice normal size cells.

1/24 M.I.C: moderately long to short filaments (numerous), almost fine edges (slightly rough) very few normal size cells.

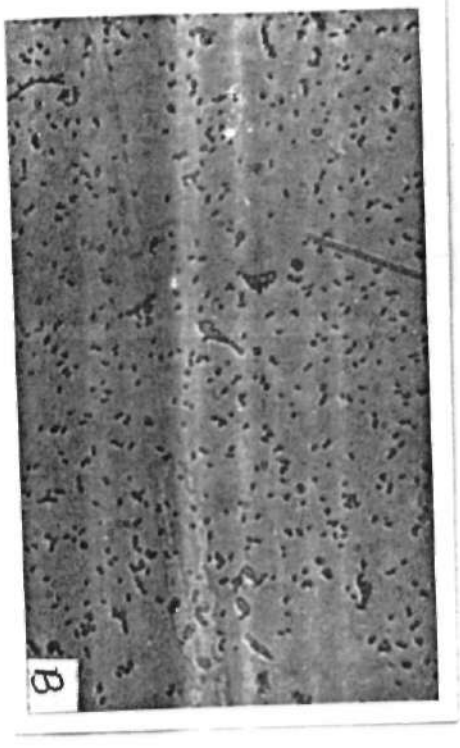
180 minutes after addition of antibiotic:

1/8 M.I.C: the filaments were very fine and numerous, as long as in 1/16 (180 minutes), no appendages. Also a few cells were normal size and about twice normal size in length.

1/16 M.I.C: the filaments were very long and seems to have some appendages.

1/24 M.I.C: the filaments seen were fat with rough edges and appendages.

Fig 2.5



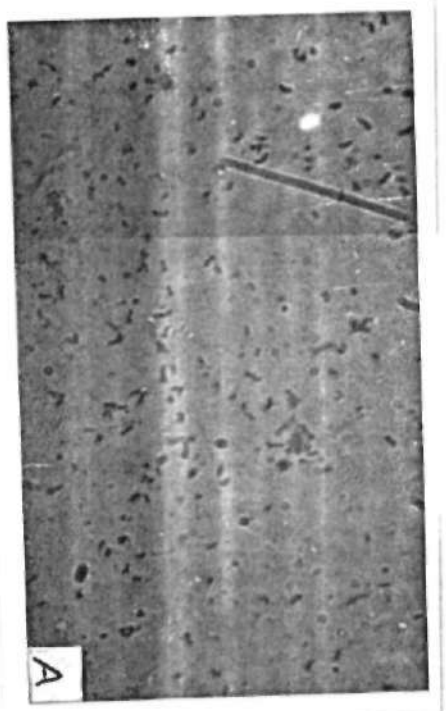
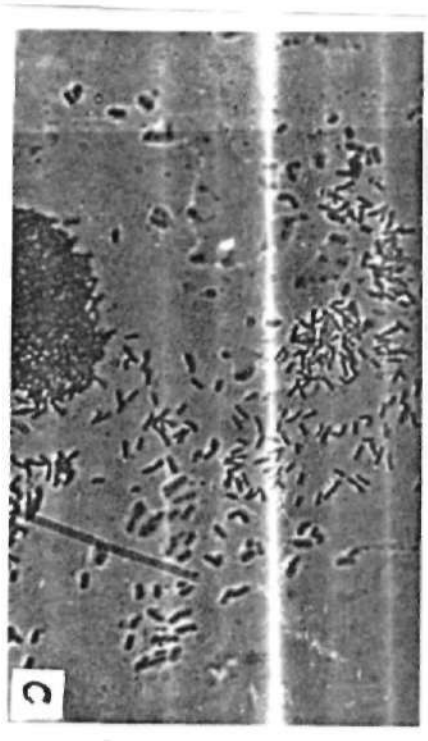


Fig 2.6a





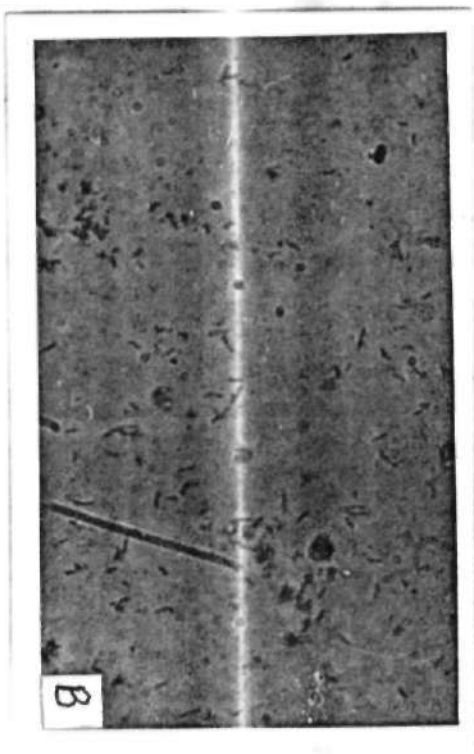


Fig 2.6b

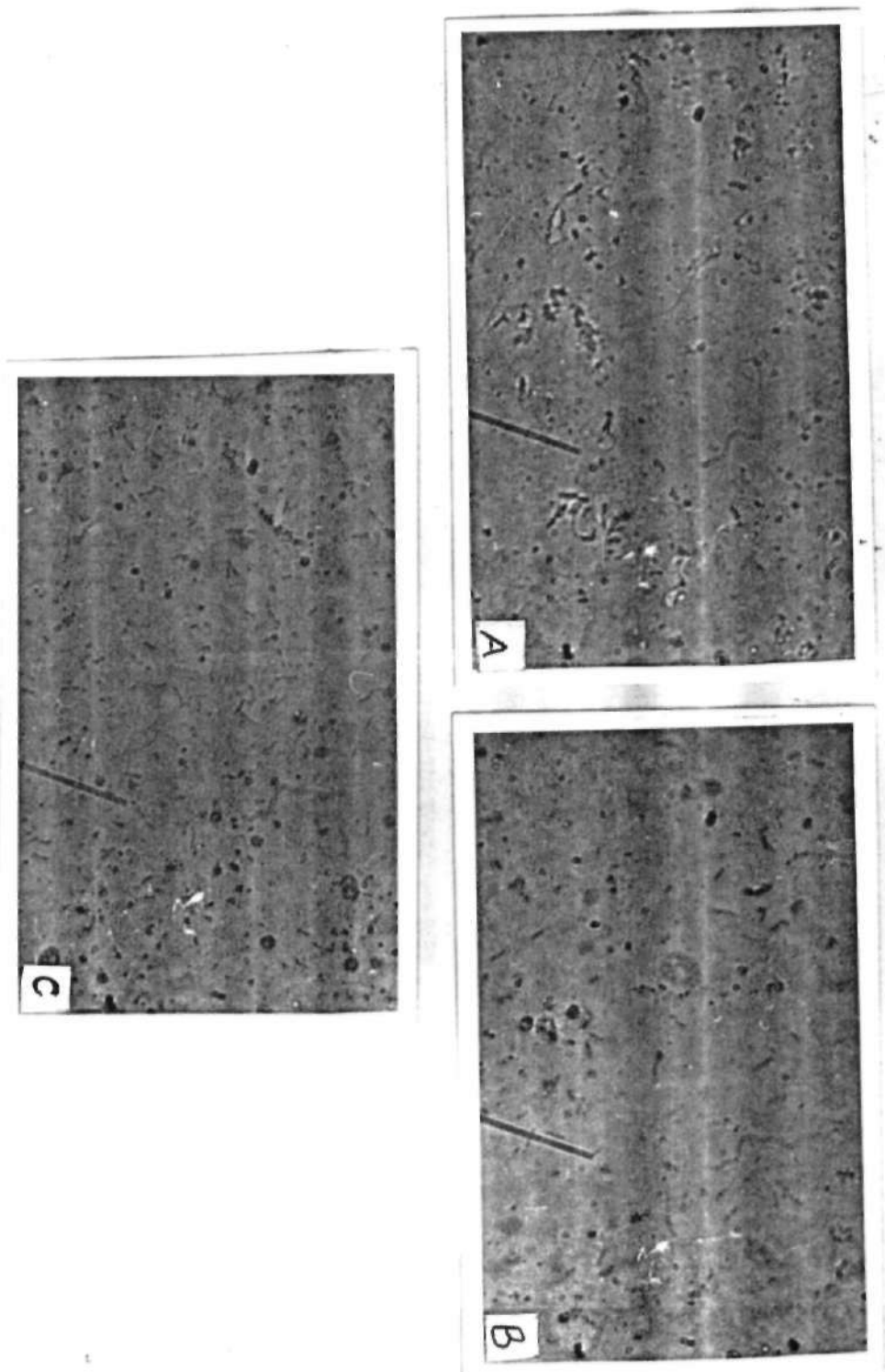
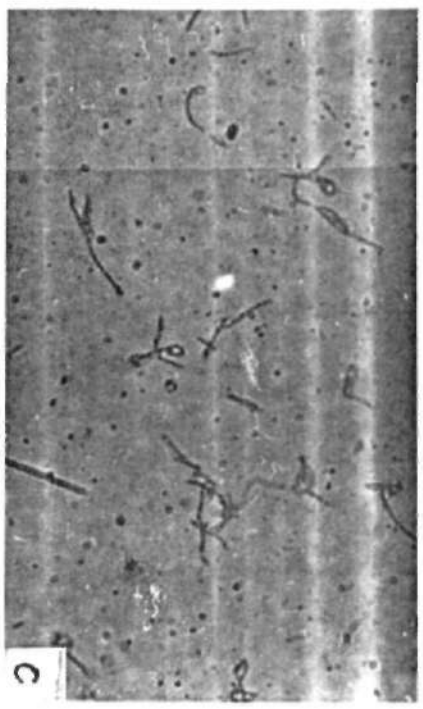
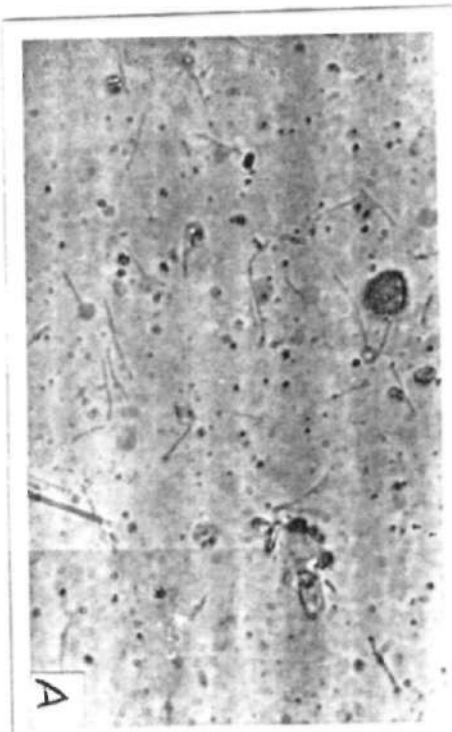


Fig 2.7a





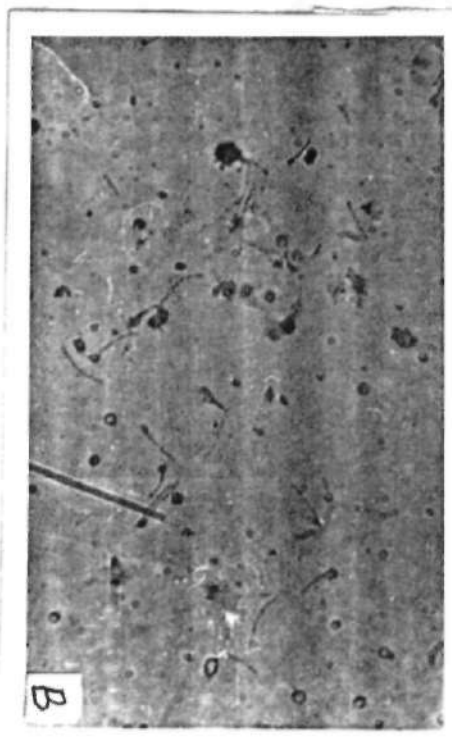


Fig 2.7b

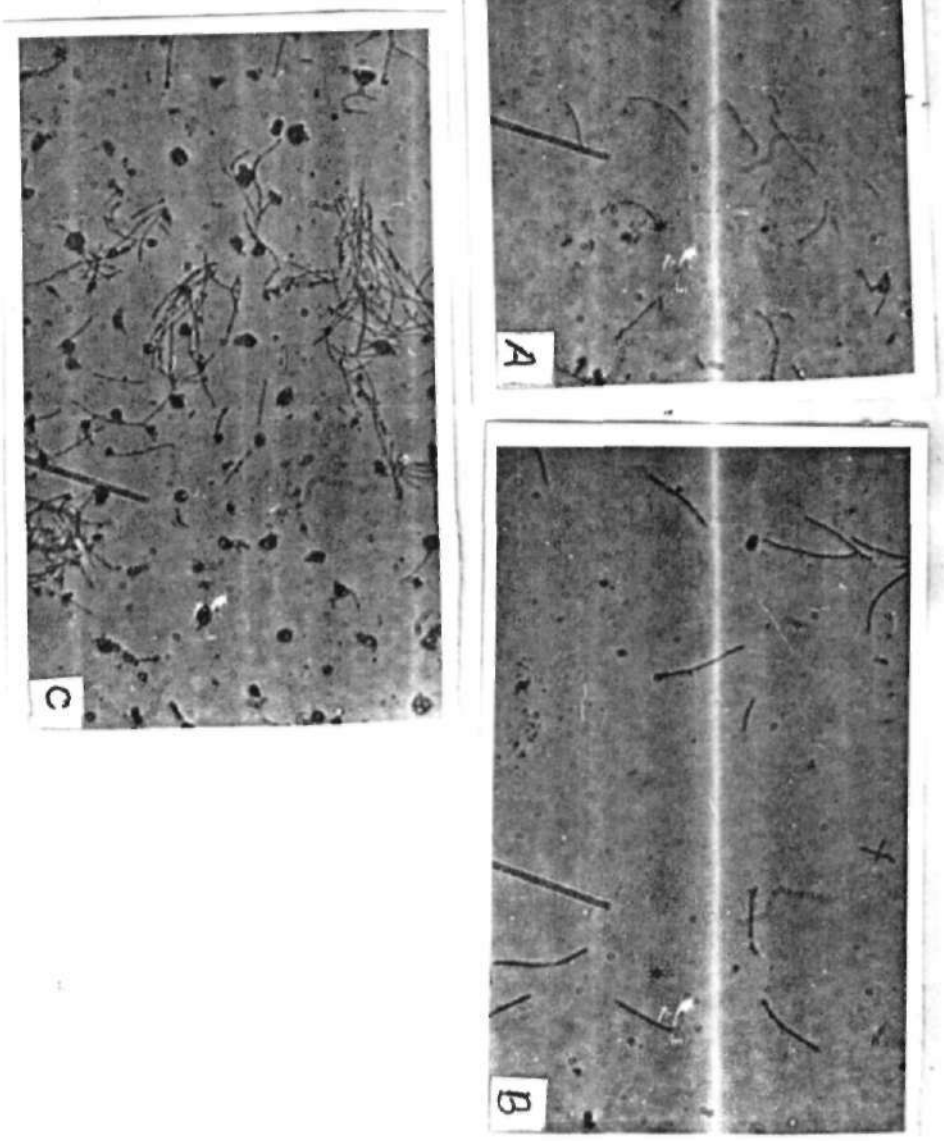


Fig 2.8a

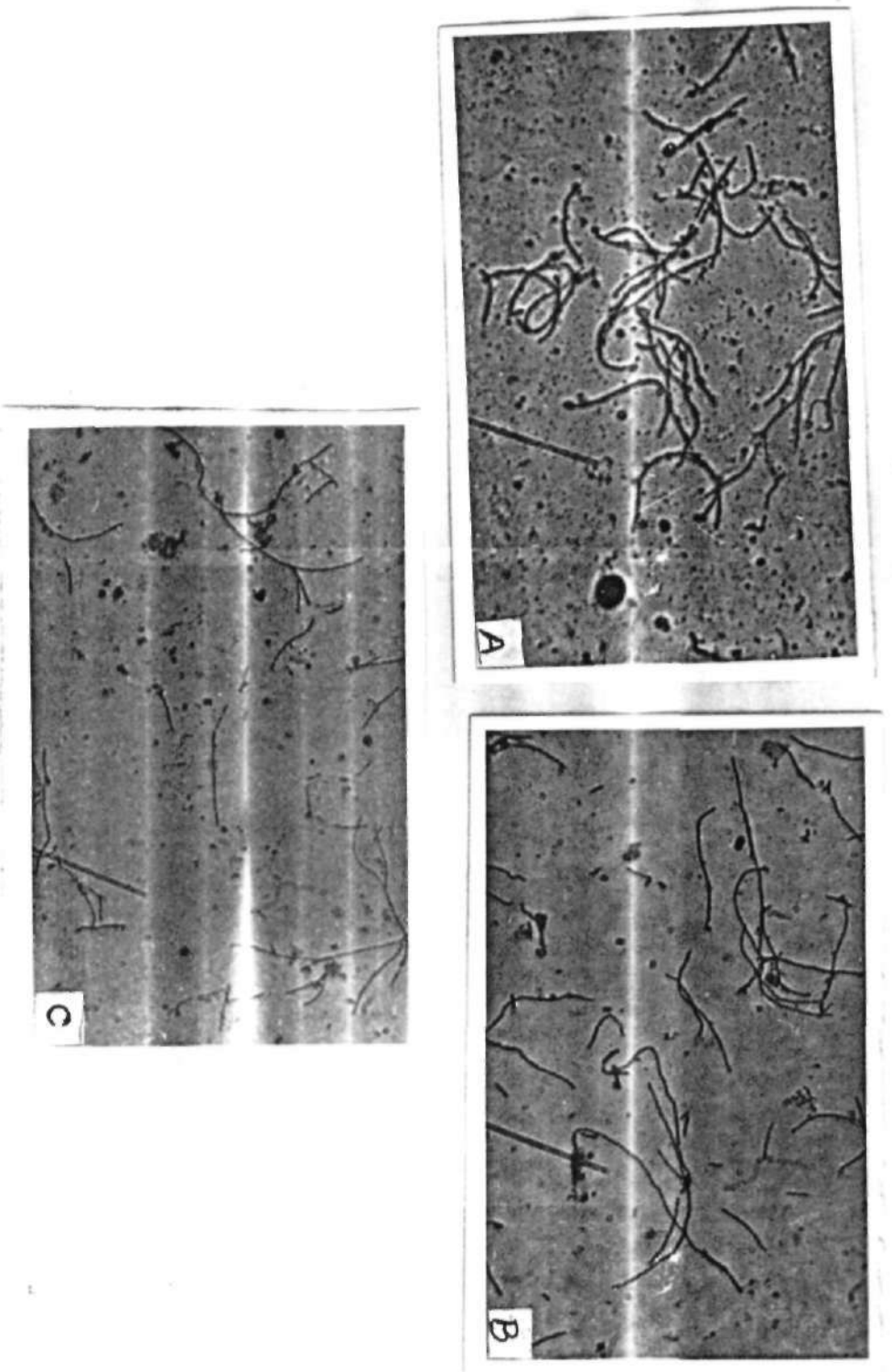
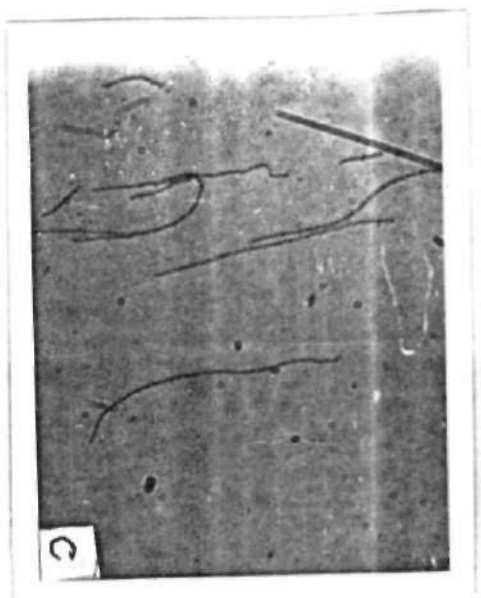
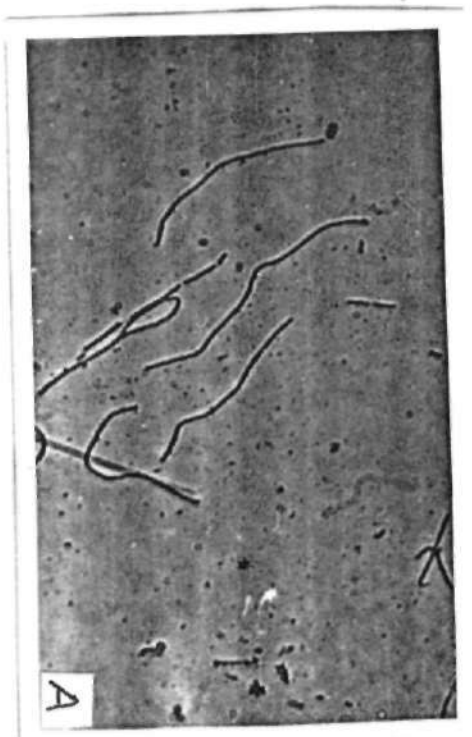
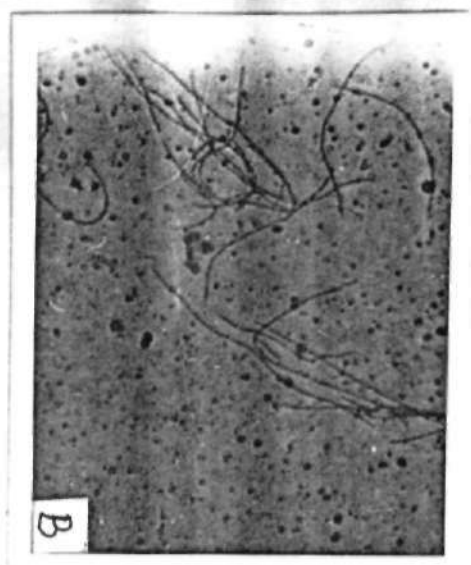


Fig 2.8b







2.5 Discussion of Results

The activity of an antibacterial agent is quite often assessed through minimum inhibitory concentration (M.I.C) determination in broth dilution or minimum bactericidal concentration (M.B.C) determination on agar gradient. From the M.I.C results, one would rather consider the five isolates (A-E) as resistant to Rocephin$^{(R)}$ (with isolate C being the most sensitive among them). This disposition might not be out of place if one considers the reported range of Rocephin$^{(R)}$ serum peak level of 40-200 ug/ml (mean of 75 ug/ml) (Eron et al, 1983). However, it is known that inoculum size affects Rocephin$^{(R)}$ M.I.C value considerably especially between 10^5-10^7 CFU/ml (Angehrn et al, 1980; Neu et al, 1981). Thus the values obtained in this work are probably higher than the actual values considering the fact that the inoculum size used (2.8×10^6 CFU/ml) falls in the upper limit of that suggested by Bristol Laboratory (1976). However, emergence of strains of Ps. aeruginosa resistant to Rocephin$^{(R)}$ has been described earlier (Angehrn et al, 1980).

In the assessment of antibacterial activity of agents, a lot of focus has been on their bactericidal

effects and elucidation of their molecular targets (Kadurugamuwa et al, 1985b). Since host defence mechanisms and antibacterial agents may act in synergy, it has been suggested (Gemmell, 1984a) that concentrations of the latter below those that will kill in vitro (i.e. sub-MIC) should be considered in the evaluation of any effect that might be achieved in-vivo. Such effects may be due in part to other changes associated with the cell surface and to reduction in the synthesis of virulence factors and are of potential clinical implications (Lorian, 1980; Zak & Kradolfer, 1979).

Sub MICs of antibiotics, that does not affect growth rate has also been used to study virulence determinants in some gram-negative organisms (Kadurugamuwa et al, 1985a, 1985b & 1985c). Biochemical and ultrastructural studies were not done in this work to show the salient but perhaps important effects of subMIC of antibiotics.

The results reported above have shown that subMIC of Rocephin$^{(R)}$ which did not have any significant effect on growth rate had pronounced effect on the morphological state of Ps. aeruginosa, particularly filamentation. This was shown by a strain-dependent optimum antibiotic concentration below the M.I.C for maximal filamentation. For instance, the clinical

isolate showed greater filamentation with increasing antibiotic concentration up to a point (150ug/ml) whereas the wild type showed increase in length of filament with lowering antibiotic concentration with an optimum at about 40ug/ml while being at the same time more resistant than the clinical isolate.
A lot of works have been reported on morphological effect of antibiotics involving both high and low concentrations. For instance, Greenwood & Eley (1982) while comparing the antipseudomonal effect of some newer beta-lactam antibiotics using high concentrations, found that the various cephalosporins tested could not prevent growth of some Ps. aeruginosa strains in the first few hours of exposure, during which time the bacteria elongated to form long filaments. It was also noted that the various agents differed in the length of time that elapsed before growth was completely stopped. In a similar report, Elliot & Greenwood (1984) working with concentrations of azlocillin, ticarcillin and cefsulodin, showed that the primary response of Ps. aeruginosa was failure of the division process resulting into filamentation. Ultrastructural studies further revealed that the filaments subsequently underwent further morphological changes, the nature of which varied with the antibiotic tested.

Actually the morphological response expressed in the presence of antibiotics depends on the concentration used and it varies between the two extremes of filamentation and spheroplast formation which lyse if environment is isotonic. For example, Lorian & Sabath (1972) compared the morphological effects of penicillins and cephalosporins on Proteus mirabilis. They found that at inhibitory and subinhibitory concentrations, penicillins produced filaments of up to 93u length without any globular outpounchings whereas none of the cephalosporins produced filament longer than 14u at any concentration tested. They also observed that the predominant morphological response to cephalosporins especially at and above inhibitory concentrations is globular outpounchings from short bacilli and this was presumed to be an early stage of spheroplast formation. At concentrations from 4-32 times MIC, penicillins also produce similar globules.

The minimum concentration of an antibiotic that could produce a structural change seen either by light or electron microscope is termed the minimum antibiotic concentration (MAC) of the agent and was first proposed by Lorian & de Freitas (1979). However, Lorian (1975) had in an earlier report used the MAC/MIC ratio to describe sub MIC which he found

to have similar effects on both resistant and sensitive strains of E. coli exposed to sub MICs of ampicillin when they were examined microscopically for structural changes. This is at variance with the observations reported in this work. Thus there seems to be a constitutional difference in the envelope material responsible for filamentation between isolates C&E and this envelope material is probably not involved in determining the susceptibility of the isolates to the killing effect of Rocephin(R). This latter suggestion agrees with an earlier reported view that filamentation is not a determining factor in therapeutic efficacy (Ryan & Monsey, 1981). Also the fine details of morphological effects of antibiotics appear to be dependent on the antibiotic, the concentration used and the species and sometimes strain of bacteria challenged.

Chapter Three

3. CELL SURFACE HYDROPHOBICITY DETERMINATION

3.0 Introduction

Hydrophobicity is an index (or a reflection) of the hydrophobic nature of bacterial cell surface. Hydrophobic interactions have been implicated in adherence of bacteria to phagocytes (Van Oss, 1978), host tissues (Rosenberg et al, 1981), partitioning of bacteria at liquid : liquid and liquid : air interfaces etc.

A number of methods have been employed in studying the hydrophobic nature of cells. These include binding of hydrocarbon and fatty acids to cells and cell components, measurement of the force required to remove hydrocarbon-bound cells, partitioning of bacteria in aqueous polymer two-phase systems, hydrophobic interaction chromatography and contact angle measurements of dried cell layers. It is said that no single method adequately describes cell surface hydrophobicity (Rosenberg et al, 1980a) because the experimental conditions employed influence the observed hydrophobic interaction to some degree and also various non-hydrophobic effects often interfere.

Bacterial adherence to hydrocarbon method (Rosenberg, 1984) was used in this work. It is a simple and rapid technique for determining surface hydrophobicity. Its advantages include : (i) elaborate equipment not required, (ii) quantitative results are simple and rapidly obtained and, (iii) also the fact that since hydrophobic interactions have been implicated in the ability of certain bacteria to adhere to mammalian cells (Stendahl et al, 1973), adherence to liquid hydrocarbons provides a defined surface for adherence comparable with attachment to the less-defined biological surface (Rosenberg et al, 1982).

3.1 Materials and Method

The isolates (C & E) with and without 1/24 of M.I.C of Rocephin$^{(R)}$ were grown in 100ml of MH broth in a shaker bath at 37°C and 80 opm as described earlier. The final concentrations of Rocephin$^{(R)}$ added were 7.5 ug/ml for isolate C (C_T) and 12.5 ug/ml for E (E_T).

After 180 minutes and 240 mins. of growth enough volume of each culture was harvested and washed once in Phosphate Urea Magnesium (PUM) buffer. The washed cells were resuspended to OD_{470nm} 1.0.

Tubes, previously washed, soaked in dilute acid solution (to remove soap) and later rinsed with distilled water and dried were filled with 0.2, 0.4 and 0.6 ml of n-Hexane, each volume in duplicate and in two sets. The same was done for Xylene.

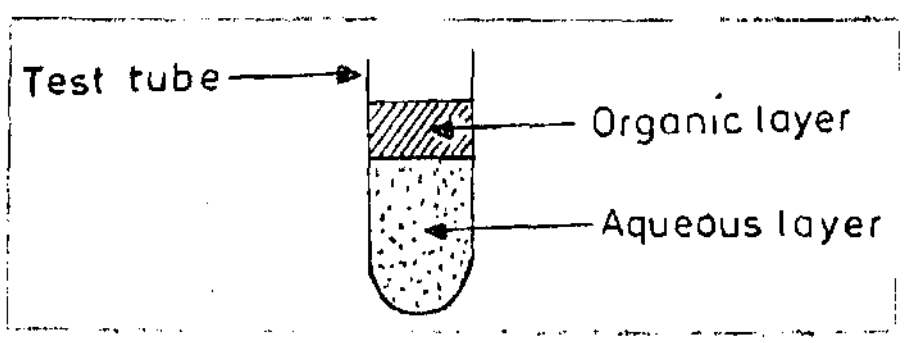
The resuspended cultures were each divided into two halves and to one half of each culture, 10%v/v serum was added in a 100ml conical flask, mixed well and incubated in a water bath at 37°C for 15 minutes. The opsonised cells were then harvested, washed once with P.U.M. buffer and resuspended in same P.U.M. buffer to OD_{470nm} 1.0.

1ml of unopsonised suspension of each culture was added to their respective tubes in one set of tubes for the two organic solvents while the opsonised cells suspensions were similarly added to their respective tubes in the second set of tubes.

After addition of culture to hydrocarbon, 15 minutes was allowed for equilibrium to be reached, then the two phases were mixed using Whirl's mixer for 90 seconds. The tubes were left for 15 minutes to allow for separation of the two phases, thereafter the aqueous phase was withdrawn and the OD_{470nm} taken.

The OD_{470nm} of the aqueous layer was compared with the initial OD_{470nm} of the cell suspension to determine the fraction of the initial population left in the aqueous phase after mixing with the hydrocarbon.

Fig. 3.1 - The Bi-phase partitioning system.



3.2 Results

The cell surface hydrophobicity measurements of the two isolates when treated with Rocephin(R), serum separately and Rocephin(R) with serum together are as presented in figures 3.2, 3.3 and 3.4. The percent hydrophobicity index indicates what fraction of the cells in the bulk aqueous phase is left after interaction with the hydrocarbon.

From the results obtained, the isolates were moderately hydrophobic, with the clinical isolate being more hydrophobic than the wild type (see Fig. 3.2).

Treatment with 1/24 of M.I.C of Rocephin(R) increased hydrophobicity, more in the exponential phase than in the stationary phase. The effect of Rocephin(R) on the wild type was so much pronounced as to reverse the trend of relative hydrophobicity between the two isolates at both phases of growth (Fig. 3.2).

Treatment of the two isolates with serum also increased hydrophobicity and also much more in the exponential phase than in the stationary phase but without altering the order of relative hydrophobicity between the two isolates (see Fig. 3.3).

Treatment of the two isolates with antibiotic and serum together also increased hydrophobicity more in the exponential phase than in the stationary phase (Fig. 3.4). Also the increase was generally more than was observed with either Rocephin(R) or serum alone in C. Also the order of relative hydrophobicity between the isolates was not altered (see Fig. 3.4 and figures 3.2 and 3.3).

Results similar to that of n-Hexane was obtained for Xylene.

3.3 Discussion of Results

From the results, both Rocephin(R) and serum individually have hydrophobicity increasing effect on

Fig.3·2

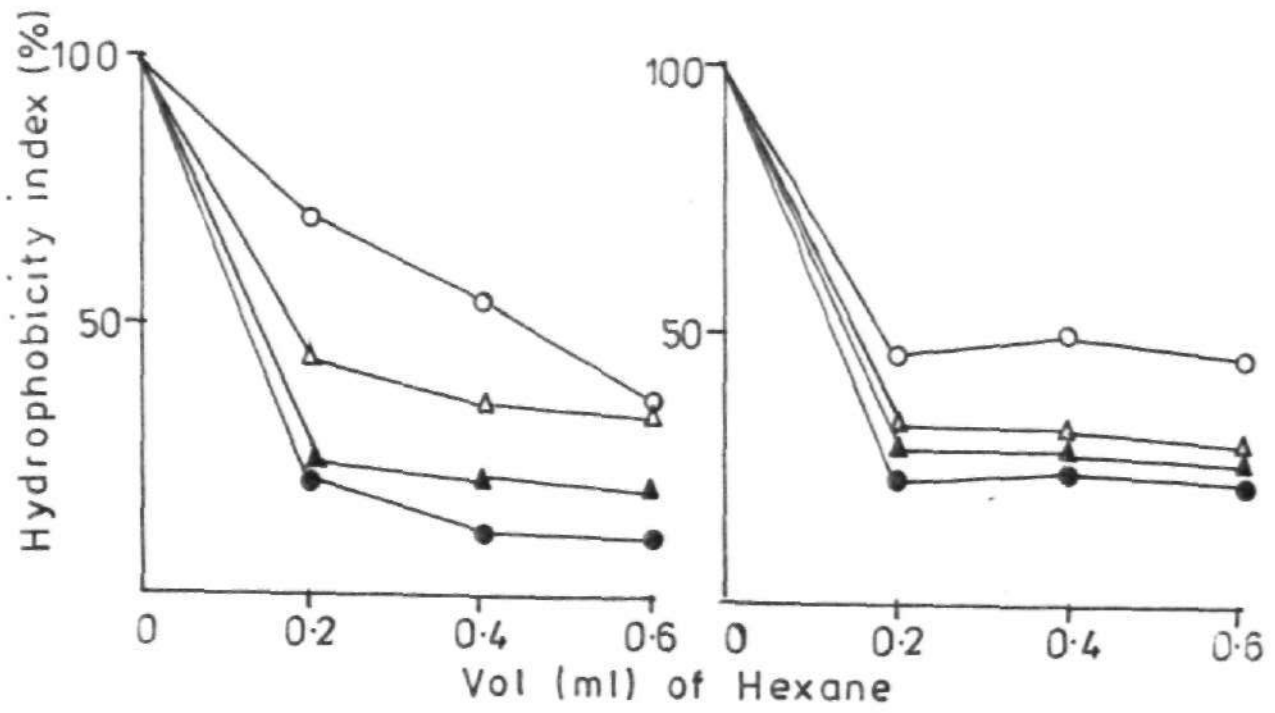

Log Phase
Stationary Phase
Hydrophobicity index (%)
100
50
0
0·2
0·4
0·6
Vol (ml) of Hexane

Fig.3·3

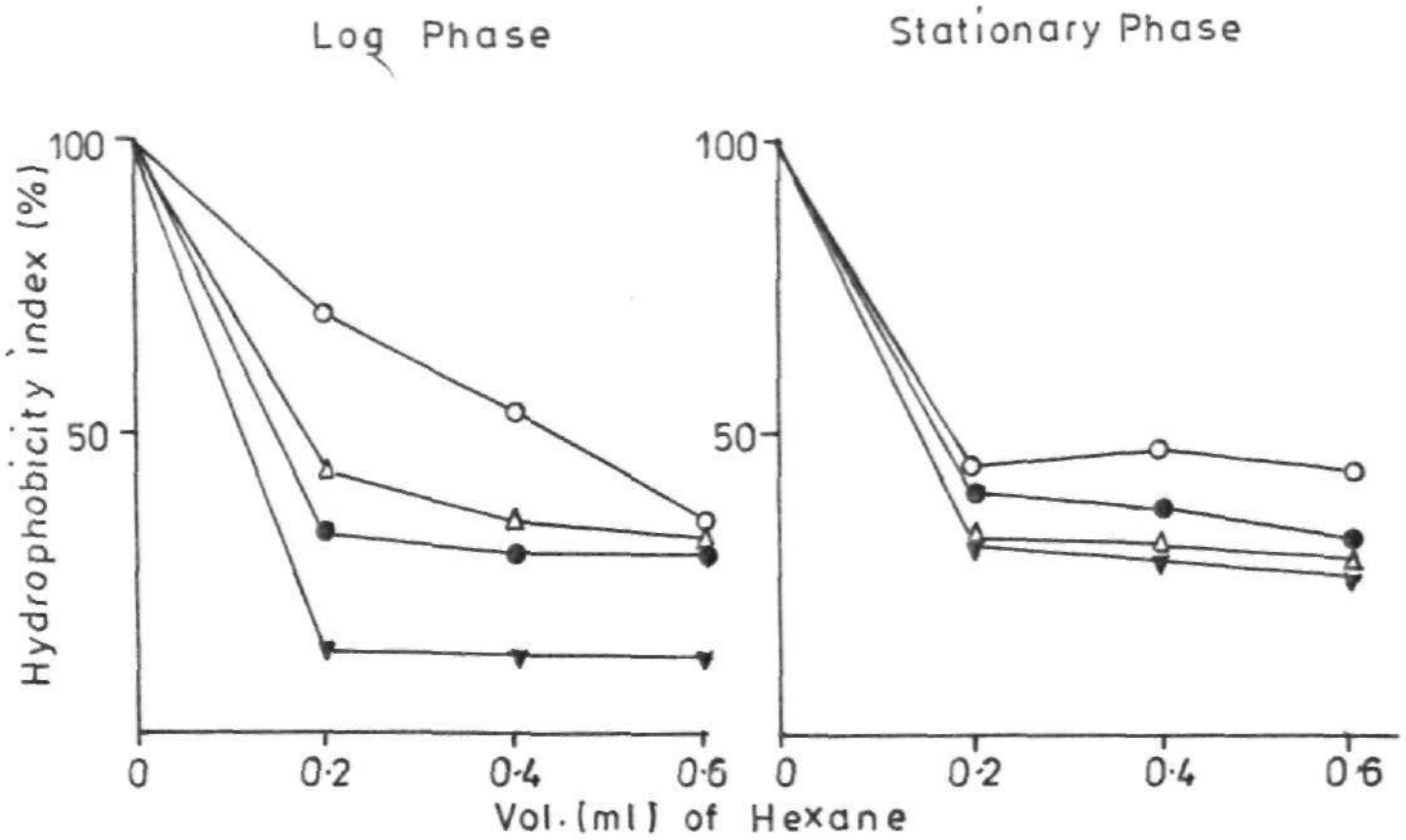
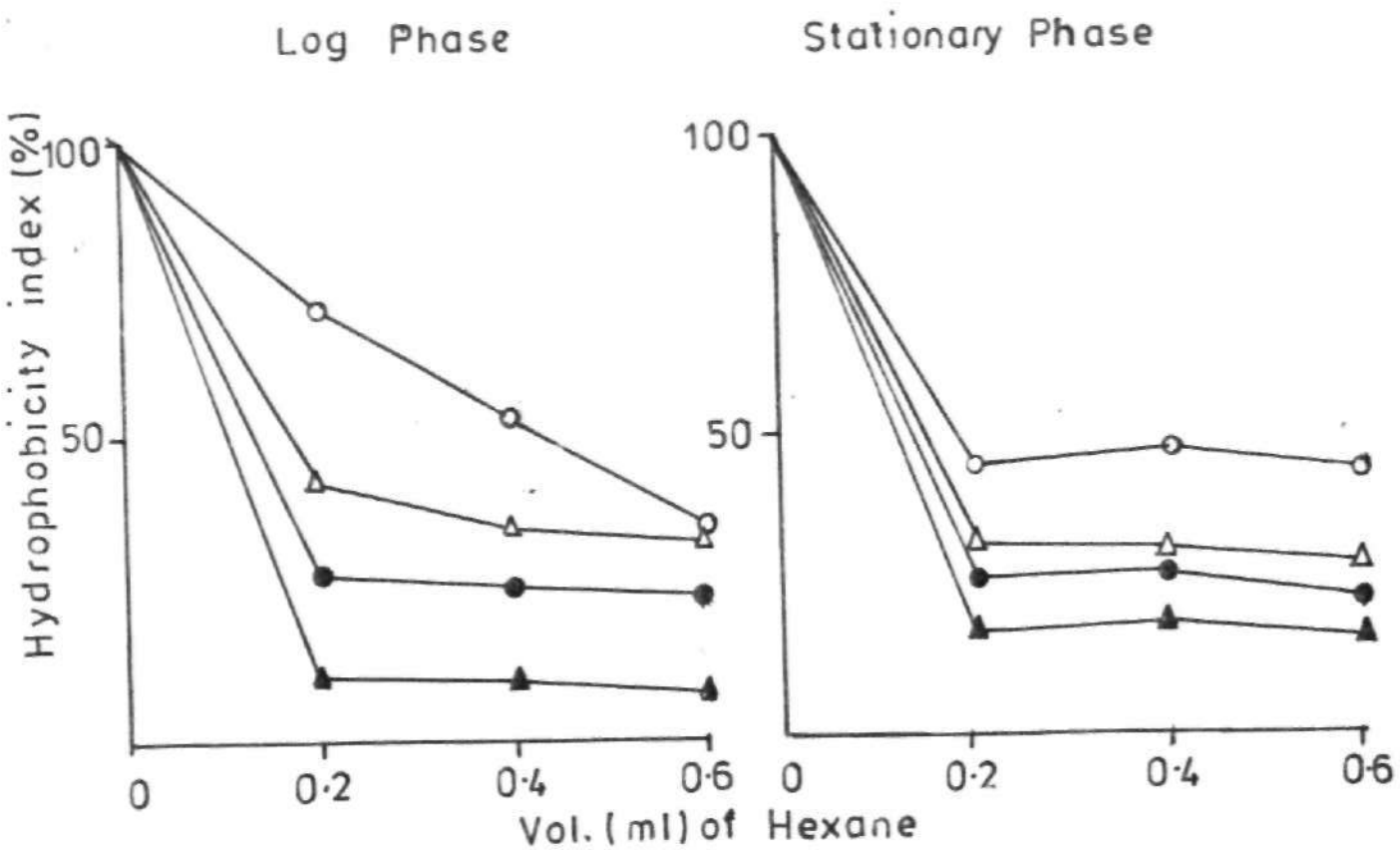

Log Phase
Stationary Phase
Hydrophobicity index (%)
100
50
0
0·2
0·4
0·6
Vol. (ml) of Hexane

Fig. 3·4


Log Phase
Stationary Phase
Hydrophobicity index (%)
100
50
0
0·2
0·4
0·6
Vol. (ml) of Hexane

the two isolates. These increasing effects of the two when combined was cummulative on the clinical isolate whereas on the wild type they appeared to be antagonistic. In fact, it seems the whole effect of serum is nullified by the effect of the antibiotic while the observed increase in hydrophobicity when the two effects were combined is due to the difference in the two effects on the wild type.

Various cell surface components are known to affect cell surface hydrophobicity. For example proteins and other molecular components of the bacterial envelope e.g. glycolipids, phospholipids, lipoteichoic acid (LTA) (Ofek et al, 1983; McBride et al, 1984; Parker & Munn,1984). For instance McBride et al (1984) compared hydrophobic strains of Streptococcus mutans with variant strains that were less hydrophobic, they found that hydrophobic cells contained high molecular weight cell surface proteins that were absent in cells with reduced hydrophobicity. In another report, Tylewska et al (1980) suggested that M-protein is involved in determining the hydrophobic property of group A streptococci.

Apart from the major proteins, it is known however, that the bacterial OM contains some minor

proteins which are inducible or derepressible and would under appropriate environmental conditions reach levels in the OM comparable with those of the major proteins (Hammond et al, 1984), a situation that may also be reflected by an increase in hydrophobicity.

A group of workers, Miorner et al (1983) worked with group A streptococci and they believed that LTA is the major cell wall component responsible for surface hydrophobicity of these organisms.

Differences in localisation of phospholipids in the outer and inner leaflets of the OM may account for differences in surface hydrophobicity between two isolates C & E as suggested by Nikaido & Nakae (1979). If however, the distribution of phospholipids is similar in two isolates, other differences like ability to synthesize complete LPS structure will determine relative hydrophobicity due to the shielding effect of the long hydrophilic carbonhydrate chains of the LPS (Rosenberg et al, 1982).

Russell & Furr (1986) reported that removal of part or much of the LPS and the absence of one or more porins would result in phospholipids appearing in the outer leaflet of the OM in the form of a classical membrane structure and this will cause a

resultant increase in hydrophobicity. Also Kadurugamuwa et al (1985a) observed that sub-M.I.C of some cephalosporins increased cell surface hydrophobicity by exposure of otherwise masked components.

A lot of antibacterial agents are known to inhibit synthesis of, or inactivate the LPS structure e.g. *inhibition of surface polysaccharide synthesis* by Diphenylamine (Feingold, 1969), LPS as a polyphosphate compound being inactivated by interaction with Polymyxin B (Rifkind, 1967) and Polymyxin B alteration of antigenic protein and LPS component effected by the complexing as well as the degradative effects of the antibiotic on the LPS component of the OM (Tsang & Brown, 1980). This inhibition of LPS polysaccharide synthesis or its degradation will result in increase in surface hydrophobicity due to reduced shield from the deminuted LPS according to Rosenberg et al (1982).

When bacteria are exposed to serum, they become opsonised with such serum factors as specific antibodies e.g. IgG (heat stable), and complement (heat labile) serving as opsonins (Davies et al, 1968). Van Oss (1973) have reported that the Fc fragment of IgG is strongly opsonic and hydrophobic in nature while the Fab fragment has no opsonic activity and is quite hydrophilic. Also, Hahn (1983) believed that immunoglobulin of the IgG

class react via their Fab pieces with antigen on the bacterial outer wall leaving the Fc part of the antibody molecule at the surface for attachment to any Fc receptors. It is further believed that the antibodies provide the bacteria with a more hydrophobic surface via the Fc fragment (Van Oss, 1973). Complement has also been reported to improve cell surface hydrophobicity through the activation of the third component of complement C3 to yield C3b which is deposited on the cell surface (Van Oss, 1973; Verhoef, 1982 & Hahn, 1983).

The observed differences in the degree of hydrophobicity between isolates C & E may be attributed to one or more of many factors e.g.

(a) absence of one or more surface protein(s) in the wild type using the explanations of McBride et al (1980), Tylewska et al (1980) and Parker & Munn (1984).

(b) differences in the localisation of membrane structures like phospholipid between the two isolates (Nikaido & Nakae 1979) and,

(c) there may be differences in the degree of shield provided by the LPS structure on the cell surface (Rosenberg et al, 1982).

The observed increase in hydrophobicity due to serum in this work is in agreement with earlier reported cases and is probably complement-mediated or due to antibodies or a combination of the two. Although the antibiotic also improved hydrophobicity, however, this might involve more than a straight forward exposition of hitherto masked components because judging by the magnitude of increase in hydrophobicity in the wild type, the antibiotic is probably acting on more than one system as might have been the case in the clinical isolate. This probable other system(s) probably unique for the wild type might as well explain why the effects of antibiotic and serum combined were not cummulative. It thus appear that the antibiotic acts on an envelope component in the wild type by exposing it such that in the presence of serum part of the complement-antibody system is used to mask it in such a way that the masking opsonin does not contribute to hydrophobicity. This proposition seem likely if after exposition, the envelope component in question also contributes to hydrophobicity, hence the high magnitude of Rocephin$^{(R)}$ influence on the wild type relative to the clinical isolate. As regards the non-contribution of masking opsonins to hydrophobicity, the reports of

Hahn (1983) and Van Oss (1973) should be considered and also assuming that the exposed envelope component (which is hydrophobic in nature) serve as Fc receptors then the Fc fragment of antibody will attach to it leaving the rather hydrophilic Fab fragment to the exterior. If this happens, then the influence of the antibiotic-sensitive and rather potentially very hydrophobic envelope component in the wild type would have been eliminated, leaving the two isolates with their common features in their relative relationship as evidence by figures 3.2, 3.3 & 3.4 responding to antibiotic and serum.

Chapter Four

4. INVESTIGATION OF ADHESIVE PROPERTIES OF ISOLATES C & E

4.0 Introduction

Bacterial adhesiveness is the ability of a bacterium to attach to host cells and tissues and it is a necessary step before the bacterium can invade or penetrate the host tissue. The consequences of attachment vary depending on the organism involved. For instance, in enteric infection due to E. coli, the toxin producing ability of the organism is of no effect if it cannot attach itself to the epithelium and become established in the intestine. Same is true for Vibrio cholerae (Fuller, 1972). Indeed, bacterial adhesiveness is a critical step in the pathogenesis of an infection and is either advantageous or disadvantageous to the host depending on the specificity of the surface-surface interaction between the bacterium, host tissue and host's PMN. A bacterium attached to host tissue may not be easily phagocytosed whereas a good PMN-bacterium contact will result in the bacterium being strongly phagocytosed since adherence has also been implicated in the process of phagocytosis.

Bacterial adhesion as a cell surface phenomenon has been shown to be influenced by several substances

including antibiotics. For instance, while investigating parasitism by Mycoplasma pneumoniae on respiratory epithelium, Hu et al (1977) found that an OM protein P, was important in attachment capabilities to ensure a successful membrane - membrane interaction between host and parasite. Also, Klein et al (1985) observed that sub M.I.C of some beta-lactam antibiotics especially those penicillins and cephalosporins that cause filamentation increased the adhesion of E. coli with mannose sensitive (MS) type of adhesin, while those antibiotics that cause ovoid cell formation (e.g. Mecillinam) did not change or only slightly reduce MS adhesion. However, all these antibiotics had no effect on strains with mannose resistant (MR) adhesins.

Azthreonam increased adherence of both MS and MR strains while vancomycin and fosfomycin reduced the adherence of both strains. Similarly, sub M.I.Cs of ampicillin, cefoxitin, penicillin G, erythromycin all decreased the adherence of gonococci to mammalian cells (Jacques et al, 1985). Hence it seems that the various components of the cell envelope on which the various antibiotics excert their effects are either primarily or secondarily influencing adhesion. In fact, it has been said that the mechanism of adhesion

is implicated in determining whether adhesiveness will favour pathogenicity or susceptibility to host defence by PMN phagocytic action (Eden et al, 1984).

4.1 Materials and Method

The isolates (C & E) with and without 1/24 of respective M.I.C of Rocephin$^{(R)}$ were grown in 30ml of MH broth in a shaker bath at 37^{o}C and 80 opm as described earlier.

Glass slide cut into pieces 1cm x 2cm, plastic petri dish in 12mm x 22mm and a prothesis catheter in pieces of 1cm length. These were washed thoroughly with distilled water and sterilized. They were added in two pieces each into each culture flask 45 minutes after addition of antibiotic and removed 45 minutes later, rinsed in distilled water to remove loosely attached cells. The pieces were then put one each in universal bottle containing 9.9ml sterile distilled water. This is for exponential phase studies. For the stationary phase studies, the sterile biomaterial pieces were added 105 minutes after addition of antibiotic and removed 45 minutes later.

Each piece in sterile distilled water was then subjected to vigorous mixing using Whirl's mixer for 2 minutes to dislodge cells that have adhered to the piece. The dislodged cells culture (assumed to be a

1:100 dilution) was further diluted, plated out and then incubated at 37°C for 18 hours. The viable cells were then counted.

All the above procedures were repeated using sterile biomaterial pieces that had earlier been coated with serum and dried.

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4.2 Results

The adhesive properties of the isolates both antibiotic treated and untreated, at both phases of growth, on the biomaterials before and after coating with serum are as presented in figures: 4.1, 4.2 and 4.3. The percent adhesion shown is the fraction of the bacterial population on the surfaces compared to the population in the medium.

In the untreated cells, the clinical isolate adhered more in the stationary phase than in the exponential phase whereas the wild type adhered more in the exponential phase. This pattern was the same for all the substrates.

Also, among the untreated cells, the clinical isolate adhered less than the wild type in the exponential phase but adhered more in the stationary phase.

On treatment with Rocephin$^{(R)}$ the clinical isolate adhered less than the wild type at both

phases of growth on catheter whereas on plastic and glass the clinical isolate adhered more at both phases of growth. Also antibiotic treated cells of the clinical isolate adhered more than the control in the exponential phase on catheter and plastic and less on glass, but adhered less than the control at the stationary phase. For the wild type, the antibiotic treated cells adhered less than the control in the exponential phase but adhered more in the stationary phase on catheter and plastic but less on glass.

Serum improved the adhesion of the antibiotic untreated cells of the clinical isolate whereas it reduced that of the wild type on all surfaces tested in the exponential phase. Serum also generally decreased the adhesion of antibiotic treated cells of the two isolates in the exponential phase. In the stationary phase serum increased adhesion of antibiotic untreated cells of the wild type on all test surfaces whereas for the clinical isolate it increased adhesion on catheter and decreased it on plastic and glass. Serum also increased the adhesion of antibiotic treated cells of the wild type in the stationary phase, whereas it increased that of clinical isolate on catheter and plastic but decreased it on glass.

Fig.4·1

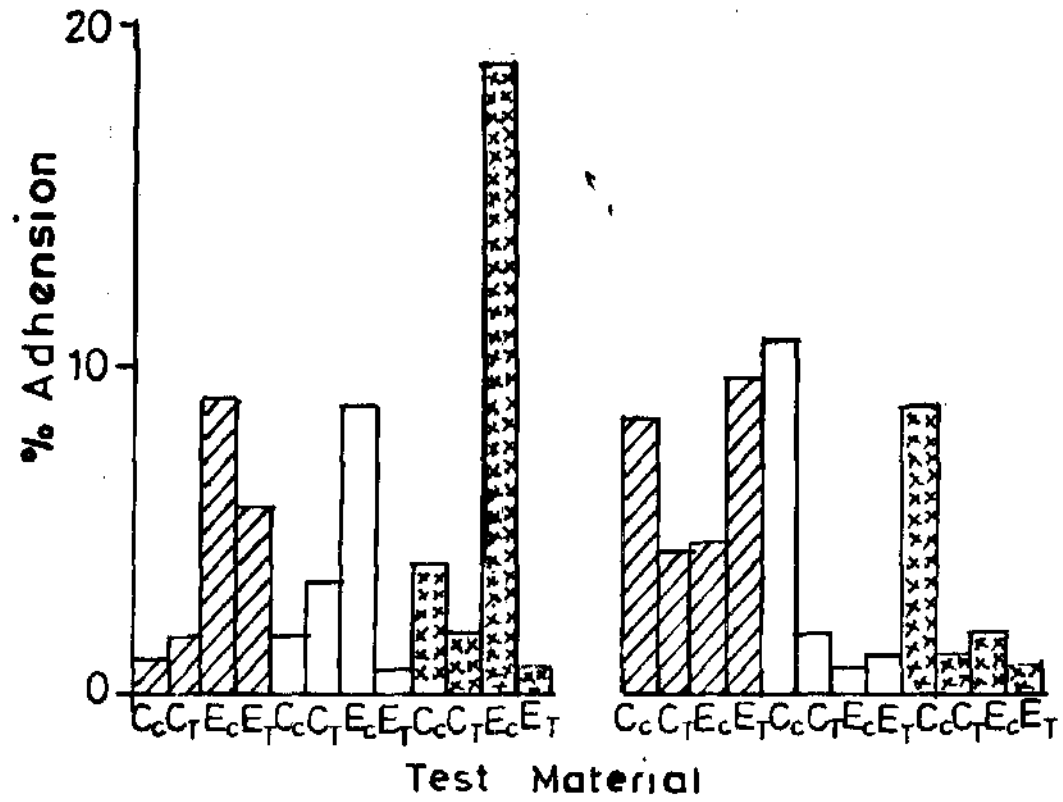

Log Phase
Stationary Phase
20
10
0
% Adhension
CcCTEcETCcCTEcETCcCTEcETCcCTEcET
CcCTEcETCcCTEcETCcCTEcETCcCTEcET
Test Material

Fig.4.2 Log Phase

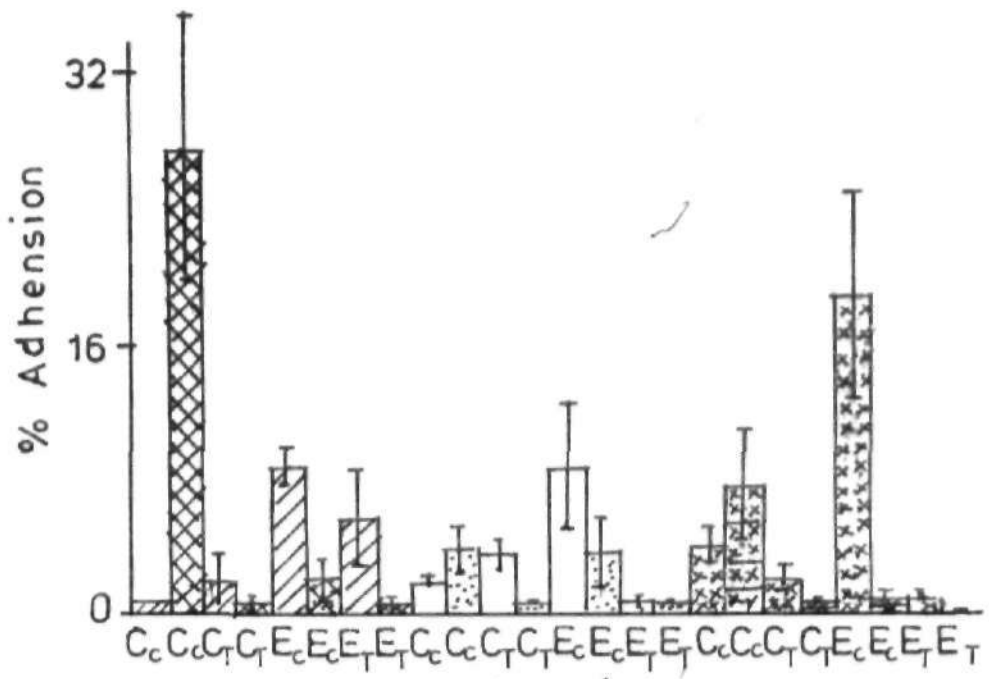
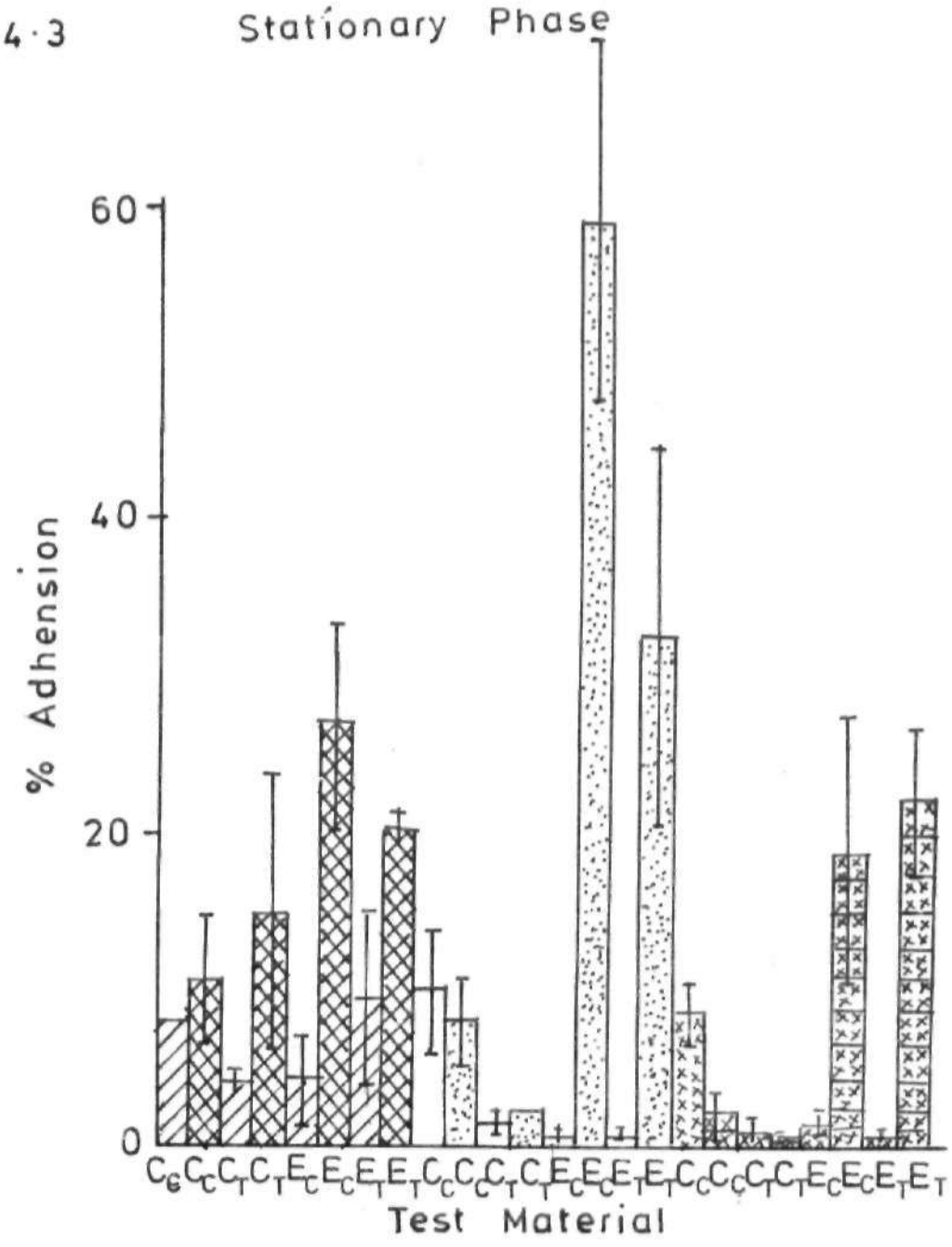


Fig.4·3



4.3 Discussion of Results

Bacterial adhesion to surfaces is a complex phenomenon as evidenced by the various forces and explanations associated with it by many workers. For instance, Hogt et al (1985) associated the attachment of coagulase-negative staphylococci to FEP (a polymeric biomaterial) with hydrophobic bonding, just as Marshall (1972) believed that the secondary, firm irreversible process in adhesion is mediated by polymeric bridges. Other workers also supported the idea of involvement of polymers in adhesion e.g. LPS (Kamberger, 1979a, b), capsule and slime layer, (Dazzo, 1979; Leban & Whitmoyer, 1979), Pili (Ofek et al, 1977). However, Criado et al (1985) believed that the first step in bacterial adhesion to host cells or different polymeric substrates is directly related to the surface tension of bacteria. This is an idea strongly supported by Absolom et al (1983) who went further to include the surface tension of the substrate and the suspending medium. Also separate mechanisms had been implicated in the adhesion of Vibrio proteolytica to hydrophilic (e.g. glass) and hydrophobic substrates (Paul & Jeffrey, 1985). The involvement of protein in the hydrophobic adhesion was also suggested. The multiplicity of the mechanism of adhesion is further supported by Jann et al (1981) who worked on E. coli

and described pili as the mechanism of adhesion in strains with MS^+ and MR^+ adhesins. In fact bacterial adhesion is generally considered to involve both specific (polymers) and non-specific (charged groups, extracellular bacterial products etc) processes (Ward, 1980; ØGAARD et al, 1985).

The observation in this work that the clinical isolate adhered more in the stationary phase than in the exponential phase while the reverse was the case for the wild type might be emphasising the involvement of hydrophobicity in adhesion. The clinical isolate's adhesion might be mediated by polymeric bonding while that of the wild type is not. However, if one considers the fact that, that adhesion pattern cut across all the biomaterials (which includes glass, a hydrophilic substrate), this may suggest the involvement of other non-specific mechanism(s) in addition to the polymeric bridging and that probably the degree of contribution by polymer and non-specific forces varies between the two isolates. Also the observation that the clinical isolate adhered less than the wild type in the exponential phase and adhered more than the wild in the stationary phase is still amenable to the above explanations.

That antibiotic treated cells of the clinical isolate adhered less than the wild type on catheter

at both phases of growth while adhering more than the wild type on plastic and glass also at both phases of growth can probably be best explained in terms of surface tension. It then means that the antibiotic lowered the surface tension of the wild type relative to the clinical isolate to a level lower than that of the medium, hence the greater attachment of the wild type to the low surface tension polymeric catheter while the clinical isolate showed greater attachment to the more hydrophilic and hence higher surface tension plastic and glass according to Absolom et al (1983). This might be the case considering the rather high sensitivity of an envelope component in the wild type to Rocephin$^{(R)}$ (see fig. 3.2 on hydrophobicity).

It was also observed that antibiotic treated cells of the clinical isolate adhere more than the control on catheter and plastic (hydrophobic substrates) but adhered less than the control on glass in the exponential phase. This is further testimony that adhesion in the clinical isolate is mediated by polymers (hydrophobic) since the antibiotic was also found to increase hydrophobicity and this will lower the surface tension of the treated cells relative to the control. However, that antibiotic treated clinical isolate cells adhered

generally less than the control at stationary phase tend to introduce the influence of phase of growth. This is because the treated cells were still more hydrophobic (and hence lower surface tension) than the control. This is probably an indication for an extracellular adhesive factor according to ØGAARD et al (1985), produced in the stationary phase and whose production is probably inhibited by the antibiotic.

The observed general greater adherence of control cells of the wild type relative to the antibiotic treated cells of the same strain in the exponential phase further supports that fact that adhesion in the wild type is not mediated by polymeric or hydrophobic bridges and at the same time it underplays the role of surface tension in adhesion since treated cells were more hydrophobic and hence with lower surface tension. However, that treated cells of the wild type adhered more than control in the stationary phase on catheter and plastic but less on glass, while conforming with surface tension reasoning, probably shows how the main mechanism of adhesion in this strain is influenced by phase of growth.

When the biomaterials were coated with serum the control cells of the clinical isolate adhered

more in exponential phase than when biomaterials were not coated while in the case of the wild type control cells adhered less when biomaterials were coated. This is still in support of the earlier suggested mechanisms of adhesion since proteins adsorbed on the biomaterials will improve their surface hydrophobicity and will therefore favour polymeric bonding.

That the wild type control cells adhered generally more in the stationary phase when the substrates were serum coated tend to confirm the effect of phase of growth on its main mechanism which yields to surface tension considerations and the extracellular adhesive factor. However, the improved adhesion of the clinical isolate on catheter and its reduced adhesion on plastic and glass in the stationary phase when the substrates were serum coated would probably be explained by polymeric bridging, interaction of charged groups and repulsive electrostatic forces according to Heckels et al (1976) and Ward (1980).

The adhesion patterns of the antibiotic treated cells of the two isolates at both phases of growth when biomaterials were serum coated, present a very complex picture and are probably due to interaction and balance of forces between the various factors earlier mentioned.

It is thus noteworthy that while hydrophobicity correlates with adherence in the clinical isolate, there was no such correlation in the wild type. Adherence also appeared to involve both specific mechanism (clinical) and non-specific (both isolates) mechanism involving extracellular adhesive factor, charged groups and surface tension. Also adhesion is influenced by the strain of the organism, phase of growth, antibiotic, presence of serum and type of material presenting the adhesion surface.

Chapter Five

5. DETERMINATION OF SUSCEPTIBILITY TO PHAGOCYTIC UPTAKE BY HUMAN POLYMORPHONUCLEAR LEUCOCYTES

5.0 Introduction

Phagocytosis is the ingestion, killing and digestion of bacteria and foreign particles by single white blood cells e.g. polymorphonuclear (PMN) leucocytes. It is a major defence mechanism by which the immune system protects the body against bacterial infection. The process comprises of a number of separate steps and activities, these include : (1) the initial event which may be described as the signal; followed by (2) the pursuit i.e. the directional locomotion (chemotaxis) of phagocytes towards the foreign particle, which in the case of a traumatic injury may involve the emigration of phagocytes through the endothelial lining of the blood vessels; (3) the surface recognition of the particle or bacterium and (4) the engulfment and destruction of the invader (Van Oss, 1973).

For many types of bacteria to be ingested, they must first be coated with one or more of the serum proteins IgG, IgM or complement(C3). This process is called opsonisation and it enables the white blood cells to recognise the bacteria, it enhances the

attachment of the bacteria to and its subsequent engulfment by the PMN.

Bacterium attachment to PMN induces pseudopod formation to surround it and when completely engulfed forms a phagocytic vacuole or phagosome (Verhoef, 1982). The bacterium is then killed and digested when lysosomes present in the white blood cells fuse with the phagosome and discharge their contents such as Lysozyme, peroxidase, lactoferrin and cationic proteins into the vacuole. Such phagocytic PMNs later die and forms pus (Hahn, 1983).

Phagocytosis is one of the potential sites where antibiotics act on host defences (Peterson, 1982). Antibacterial agents are known to affect the phagocytic function of phagocytes. Their effect on chemotaxis, phagocytic uptake and killing has been examined using a variety of experimental methods and was recently reviewed by Mandell (1982). Van Oss (1975) reported the observation that Gentamycin increased the hydrophilicity of phagocytes while at the same time made bacteria more hydrophobic with a resultant enhancement of phagocytosis. Kadurugamuwa et al (1985a) also found that some cephalosporins decreased the amount of capsule on Klebsiella pneumonia 327 which led to an increase in contact angle (and hence increase in hydrophobicity) and phagocytosis.

5.1 Materials and Method

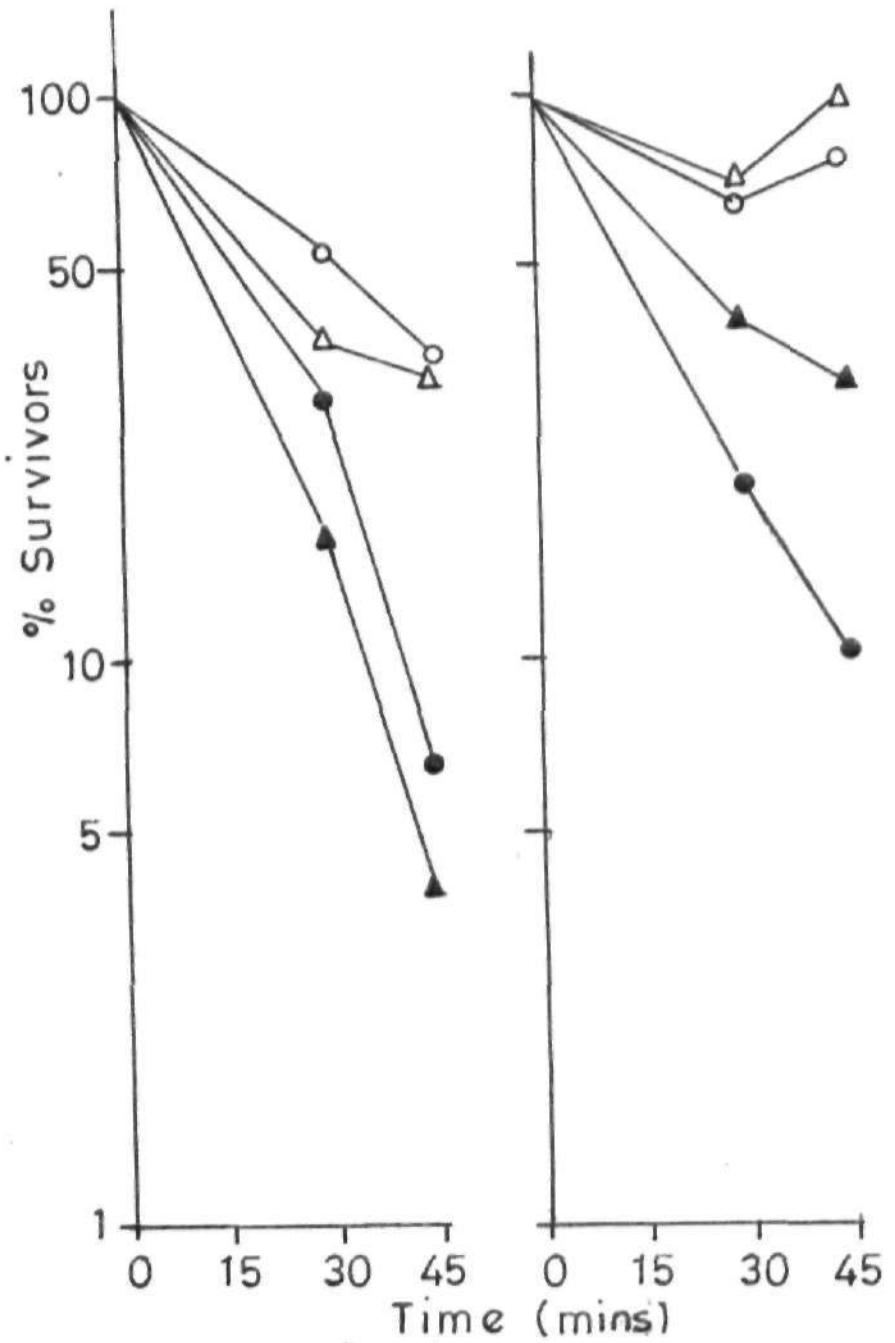
The isolates (C & E) with and without 1/24 of their respective M.I.C of Rocephin$^{(R)}$ were grown in 100ml of MH broth in a shaker bath at 37^{o}C and 80 opm as described earlier. After 180 minutes and 240 minutes of growth 10mls of each culture was harvested, washed once with sterile MH broth and resuspended to O.D$_{470nm}$ of 0.2.

For each culture at each time of harvest, 0.5ml of the resuspended culture was put inside a sterile tube and 0.5ml of fresh heparinised whole human blood was added. The tubes were incubated at 37^{o}C and shaken at 80 opm. At timed interval 0.1ml from the blood-bacteria mixture was withdrawn and added to 0.9ml of ice-cold sterile distilled water and left for 30 minutes. The withdrawn samples were further diluted, plated out and incubated at 37^{o}C for 18 hours. Control culture at O.D$_{470nm}$ 0.2 was similarly diluted and plated out. The viable cells were then counted.

5.2 Results

The susceptibility of the two isolates with and without antibiotic treatment, to phagocytic uptake by human PMN is as presented in Fig. 5.1. The percent survivor shows the fraction of the initial

Fig. 5.1 Log Phase Stationary Phase



bacterial population that remained viable after the various exposure times.

The two isolates were fairly sensitive to phagocytosis in the exponential phase but highly resistant in the stationary phase. Also the wild type was more resistant to phagocytic uptake than the clinical isolate in the exponential phase but less resistant in the stationary phase.

Treatment with subMIC of Rocephin$^{(R)}$ drastically increased the sensitivity of the two isolates to phagocytosis at both phases of growth but the increase in sensitivity at the exponential phase was more than that in the stationary phase. Also the observed change in order of relative susceptibility to phagocytosis between the two isolates (untreated) with phase of growth was maintained in the antibiotic treated cells.

5.3 Discussion of Result

Phagocytosis as a phenomenon occurs in the body after the particles or bacteria to be ingested might have been opsonised. It is partly a surface phenomenon because there has to be contact between phagocytic cells and the bacteria. PMN are known to have receptor sites for opsonin components like Fc and C3b (Gemmell, 1984b). However, the extent

of opsonisation and hence the degree of phagocytosis, depends on the surface properties of the bacterium. Since whole blood was used in studying phagocytosis in this work, it follows therefore that isolates C & E were exposed to a complete complement of opsonin content of serum.

The observed difference in sensitivity to phagocytic uptake between the two isolates may be due to a difference in extent of opsonisation which may be attributable to : presence or absence or a difference in length, of the LPS O-chain. This explanation is according to Engels et al (1985), who found that the O-chain deficient mutant of a strain of Ps. aeruginosa was opsonised and phagocytosed to a greater extent than the parent strain. Also that isolates C & E were more phagocytosed in the exponential phase than the stationary phase may be an indication of changes in surface properties with phase of growth. For instance, bacterial resistance to phagocytosis may be mediated by other cell surface structures apart from polysaccharide capsule and O-antigen as suggested by Robins et al (1980). Such surface structures may be a protein e.g. 20K protein in gonococci (Parsons et al, 1985) and an unspecified OM protein in Treponema pallidium (Cockayne et al, 1986).

Other such antiphagocytic OM proteins have also been reported e.g. OM protein Tra^{t} 5 in E. coli (Aguero et al, 1984), M-protein in group A streptococci (Jack-Weis et al, 1982). Hence if isolates C & E elaborated such proteins at stationary phase or the growth conditions become favourable for synthesis of antiphagocytic minor OM protein to a level comparable with those of the major proteins (considering the suggestion of Hammond et al, 1984), then the observed relative resistance at stationary phase should be expected. The reversed relative susceptibility with phase of growth could be due to loss or elaboration of a particular envelope component in one of the isolates at the stationary phase.

The increase in susceptibility when the isolates were treated with Rocephin$^{(R)}$ could be due to exposure of envelope components that were previously masked, resulting in increase in cell surface hydrophobicity. The increased susceptibility could also be due to loss of envelope components that antagonise opsonisation. For example, using sub MIC of lincomycin and clindamycin, Gemmell & Amir (1979) showed that production of M and T proteins in Streptococcus pyrogenes was impaired and later correlated the reduction in the M protein content of

the treated cells with their susceptibility to phagocytosis by PMN. Also Gemmell et al (1981) showed that the absence of M protein potentiated opsonisation by normal human serum lacking specific antibodies to the surface antigens.

The increased susceptibility with antibiotic treatment could also be a result of inhibition of synthesis of or inactivation of the LPS structure. The result of these effects will be better opsonisation and greater phagocytic uptake by PMN.

The observed improvement of phagocytic uptake due to antibiotic treatment in this work is in agreement with some earlier published reports. For instance, Lorian & Atkinson (1984) reported that antibiotic-induced filaments of gram negative bacilli were much more susceptible to bactericidal action of PMN than were the bacilli (sub MIC of Rocephin$^{(R)}$ treated cells of isolates C & E were morphologically filamentous).

Also Kadurugamuwa et al (1985b) were inclined in their conclusion towards the believe that reduced synthesis of enterochelin and capsules can significantly reduce the capacity of the bacteria to survive in the host. This is because they observed that more

surface-exposed antigens were accessible to antibodies in the presence of subMICs of antibiotics compared with only three surface-exposed antigens in cells grown in the absence of cephalosporins. By implication, greater antibody fixation means greater tagging and hence better opsonic recognition and subsequent phagocytosis by PMN.

This report is also in partial agreement with that of Cates & Caparas (1981). They exposed Haemophilus influenza type b to 1/8 M.I.C of ampicillin and chloramphenicol and observed that after one hour, sub MIC ampicillin improved PMN killing action while sub MIC chloramphenicol had no effect on PMN killing action. This difference in effect by the two antibiotics might not be unconnected with their site of action. However, this report contradicts an earlier one by Bar-Shavit et al (1980). These workers studied the uptake of E. coli cells by mouse peritoneal macrophages. They showed that the mannose-binding activity of E. coli determined its recognition by the phagocytic cells. They also found that non-septate filamentous form that resulted from growth of the organism in the presence of cephalexin was less internalised (uptake only 20%) by peritoneal macrophages than the normal rod-shaped single cells (90% uptake) after 30 minutes.

Chapter Six

6. GENERAL DISCUSSION AND CONCLUSION

Quite often the antibacterial activity of antibiotics is assessed through minimum inhibitory concentration (MIC) or minimum bactericidal concentration (MBC) determination. However, antibiotic resistance on the other hand has been attributed to various mechanisms such as : (a) production of antibiotic degrading enzymes by microorganisms, (b) drug exclusion due to permeability barrier and (c) a change in the bacterium itself that will render it less vulnerable (Brown, 1975). Drug exclusion involves the bacterial cell envelope which serves as a permeability barrier especially in Gram-negative organisms, thereby preventing the accumulation of such agents up to their lethal concentration. Then the question is, has the low concentrations attained by such agents any effect on the viability of such bacteria in an infected host? This question plus other reports e.g. (a) the association of widespread use of antibiotics with increase in the incidence of antibiotic resistance among Gram-negative organisms both in hospitals and the general environment by Al-Jebouri & Al-Meshhadani (1985), (b) that antibiotic therapy promotes transfer of R-factors in the gut with the transfer probability being highest sometime after the withdrawal of

therapy (Anderson et al, 1973), coupled with the common problems of non-compliance to dosage regimen and large scale antibiotic abuse in the society generated interest in investigating the effect of low level of antibiotics on some pathogenicity determinants. Hence the use of subminimum inhibitory concentration (subMIC) of Rocephin$^{(R)}$ on two isolates (C & E) of Ps. aeruginosa in this work.

The significance of low antibiotic concentrations in host-parasite interaction becomes more glaring if one considers the report of Sud & Feingold (1972) that polymyxin resistant bacteria undergo some changes on exposure to the antibiotic thereby becoming susceptible to killing by desoxycholate to which they were previously resistant as well as showing higher susceptibility to serum-killing. Actually low concentrations of antibiotics has been shown to effect subtle ultrastructural changes in bacterial cell wall and in biosynthesis of various virulence factors (Gemmell et al, 1982; Gemmel & O'Dowd, 1983). However, the mechanism of action of antibiotics seem to determine whether such changes will be of advantage to the host. For instance, Gemmell (1984a) reported that drugs that affect cell wall biosynthesis

may or may not potentiate phagocytosis depending on whether filamentation does occur or not at subMIC level. Also Taylor et al (1981) reported that conversion of E. coli from serum-resistant to serum -susceptible by subMIC of Mecillinam is dependent on formation of ovoid or round cells. In fact, there has been a lot of reports on the morphological effect of antibiotics involving both high and low concentrations (Elliot & Greenwood, 1984; Greenwood & Eley, 1982; Lorian & Sabath, 1972) and the response expressed ranges between two extremes of filamentation and spheroplast formation depending on the concentration used. In agreement with an earlier published report (Lorian & Atkinson, 1984) that antibiotic-induced filaments of gram-negative bacilli were more susceptible to phagocytosis than the original bacilli, the filaments produced by treating Ps. aeruginosa isolates C & E with sub MIC Rocephin(R) were more phagocytosed than the untreated cells (see figs. 2.7, 2.8, & 5.1). Although the ability of an antibiotic to cause filamentation is hereby seen to potentiate the efficacy of the host defence system, it has been noted not to determine the therapeutic efficacy of the antibiotic itself (Ryan & Monsey, 1981). This is in conformity with the observation in this work that there is a strain-dependent optimum antibiotic concentration below the MIC for maximal

filamentation. This is because while the wild isolate is more resistant to Rocephin(R) than the clinical isolate judging by their MIC values, it had a much lower concentration as its optimum concentration for maximal filamentation compared to the optimum concentration for the clinical isolate

Apart from influencing synthetic processes for cell envelope components, antibiotics have also been found to inactivate cell envelope components e.g.LPS by Polymyxin B (Rifkind, 1967); alter antigenic protein and LPS component e.g. Polymyxin B (Tsang & Brown, 1980). All these result in increased cell surface hydrophobicity. Thus the observed increase in cell surface hydrophobicity in isolates C & E on exposure to sub MIC Rocephin(R) could be due to exposure of otherwise masked envelope components and/or reduction in antigenic content at the cell surface. Also the observed higher sensitivity of the wild type (isolate E) to the hydrophobicity increasing effect of the antibiotic could be suggestive of the fact that the antibiotic is either acting on different envelope components to produce this effect in the two isolates or is acting on an additional component that is probably unique to the wild type.

As is natural during infection and antibiotic chemotherapy, serum is encountered and it influences

the complex interaction between the bacteria, antibiotic and the host both as an organic matter as well as through the antibacterial properties of its components such as immunoglobulins and complement. Ordinarily in the presence of serum bacteria and any foreign particle become coated with serum components in a process called opsonisation prior to engulfment by phagocytic cells. During opsonisation bacterial cells become coated with immunoglobulins and/or complement, both of which have been reported to increase cell surface hydrophobicity (Hahn, 1983; Van Oss, 1973). Thus the observed increase in cell surface hydrophobicity in both isolates C & E in this work on exposure to serum in the absence of Rocephin$^{(R)}$ (*see fig. 3.2*) *agrees with earlier published reports.* It is however noteworthy that serum did not alter the order of relative hydrophobicity between the isolates unlike what obtained with Rocephin$^{(R)}$. This probably suggests that while the effect of serum is purely a surface effect, the antibiotic probably acted on an additional component in the wild type which is not normally exposed at its cell surface.

When Rocephin$^{(R)}$ treated cells of the two isolates were opsonised, the hydrophobicity increasing effects of Rocephin and serum were cummulative in the clinical isolate (C) whereas in the wild type it

was not, though there was a net increase in cell surface hydrophobicity. This observation is somehow surprising since serum had hydrophobicity increasing effect and Rocephin(R) also exerted a very high hydrophobicity increasing effect in the wild type. The possibility of serum as an organic matter inactivating Rocephin(R) is however ruled out considering the fact that : (1) it does not have similar effect in the clinical isolate, (2) the cells were exposed to serum after treatment with Rocephin(R) and not concurrently and (3) it has been reported that serum does not affect the activity of Rocephin(R) (Wise et al, 1980). Also that the combined effect of Rocephin(R) and serum were cummulative in the clinical isolate tend to suggest that the sites of action of Rocephin(R) and serum differ i.e. while serum opsonins attach to their normally available receptor sites, Rocephin(R) exposes other hydrophobic components of the cell envelope by inhibition of synthetic activity or by degradation of components like the LPS and this will result in additional accessibility of opsonins to their receptors that were normally masked. However, the observed lack of synergism by Rocephin(R) and serum in the wild type portrays the antibiotic-exposed additional

cell envelope component as serving as Fc receptor which takes up the hydrophobic Fc fragment of antibody molecule, leaving the hydrophilic Fab fragment to the exterior. This would have nullified the extra increase in hydrophobicity due to that particular envelope component, thus leaving the two isolates in their relative relationship with regards to their common features responding to both Rocephin$^{(R)}$ and serum.

Another pathogenicity determining property of bacteria is adherence and is also influenced by antibiotics. Recent reports show that sub MIC of many antibiotics can alter (either increase or decrease) the ability of certain bacteria to adhere to epithelial cells (Atkinson & Amaral, 1982; Beachey et al, 1981; Lorian, 1980; Salit, 1983; Klein et al, 1985).

Adherence of Neisseria meningitidis to buccal cells was reduced by treatment with subMICs of antibiotics (Salit, 1983). Tetracycline and penicillin also markedly reduced the number of pili per meningococcus and gonococcus and the percentage of meningococci and gonococci with pili (Stephens et al, 1984). Also E. coli adherence to human buccal cells was reduced by a sub MIC of

streptomycin (Ofek et al, 1979). Similar results were reported by Eden et al (1978 & 1979) when they used sub MICs of ampicillin and amoxycillin. The reduction in adhesion may be due to a decrease in bacterial fimbriation (Vosbeck et al, 1980, 1982; Beachey et al, 1981; Eden et al, 1978) or to synthesis of non-functional fimbriae (Eisenstein et al, 1979).

Some antibiotics have however, been found to increase the adherence of some bacteria. For example, 1/4 MIC of those penicillins and cephalosporins which led to filamentation were found to increase MS adhesion (Klein et al, 1985). It was also found that azthreonam increased the adherence of MS^+ & MR^+ cells.

The observation in this work that the clinical isolate adhered more at the stationary phase than the exponential phase while the reverse was the case for the wild type might be an indication of the involvement of hydrophobicity in adhesion. The clinical isolate's adhesion might be mediated by polymeric bonding while that of the wild type is not. Also the observation that antibiotic treated cells of the clinical isolate adhered more than the control on catheter and plastic (hydrophobic substrates) but adhered less than the control on glass in the exponential phase might be a further testimony that adhesion in the clinical isolate is mediated

by polymers (hydrophobic) since the antibiotic was also found to increase hydrophobicity and this will lower the surface tension of the treated cells relative to the control. However, the general lower adhesion of treated cells of clinical isolate relative to the control at stationary phase is probably indicative of the influence of phase of growth since the treated cells were still more hydrophobic. This might be a pointer to an extracellular adhesive factor according to ØGAARD et al (1985), produced in the stationary phase but whose production is probably inhibited by the antibiotic. Hence while there is correlation between hydrophobicity and adherence in the clinical isolate, there is no such correlation in the wild type.

Another vital pathogenicity determinant is ability to resist phagocytic uptake. This has severally been reported to be influenced by cell surface hydrophobicity and bacterial adherence and would as such be influenced by treatment with subMIC of antibiotics. Phagocytosis as a phenomenon occurs in the body after the particles or bacteria to be ingested might have been opsonised with antibody components like IgG & IgM or complement. Binding of IgG to the surface of certain bacteria also alters their general surface properties.

For example, when smooth S. typhimurium cells were opsonised with IgG antibodies, their surface properties changed in a way that mimics an S-R mutation (Stendahl et al, 1974; Stjernstrom et al, 1977). However, the extent of opsonisation and hence the degree of phagocytosis depends on the surface properties of the bacterium.

Several surface components of the bacterial cell envelope have been found to affect engulfment by phagocytic cells in human infections e.g. OM proteins in gonococci (Hildebrandt et al, 1978; Parsons et al, 1985).

The observed difference in sensitivity to phagocytic uptake between the two isolates may be due to a difference in extent of opsonisation which may be attributable to : presence or absence or a difference in length, of the LPS O-chain according to Engels et al (1985).

The observed increase in susceptibility when the isolates were treated with Rocephin$^{(R)}$ could be due to exposure of envelope components that were previously masked (Kadurugamuwa et al, 1985a) or due to loss of envelope components that antagonise opsonisation (Gemmell & Amir, 1979). It could also

be a result of inhibition of synthesis or inactivation of the LPS structure (Rifkind, 1967; Tsang & Brown, 1980).

The observed increase in phagocytic uptake by PMN after antibiotic treatment in this work agrees fully with Lorian & Atkinson (1984) earlier report and partially with the report of Cates & Caparas (1981) while it contradicts an earlier one by Bar-Shavit et al (1980).

The various pathogenicity determinants are considered to be interdependent to some extent. The observed hydrophobicity measurement correlates with phagocytosis, in the exponential phase but the lack of such correlation in the stationary phase could be an indication of the effect of phase of growth and a reflection of the complexity of the host-parasite interaction. There was no clear-cut correlation between adherence and phagocytosis in the two isolates.

On the basis of morphological effect of sub MIC of Rocephin$^{(R)}$ in the two isolates, a constitutional difference in the envelope material responsible for filamentation between the two isolates is proposed. Also there was a strain dependent optimum antibiotic concentration below the MIC for maximal filamentation.

Generally, treatment of the isolates with 1/24 MIC of Rocephin$^{(R)}$ increased hydrophobicity and potentiated phagocytosis, thereby rendering the organisms less virulent. If these results are taken for their surface value, one might be tempted to encourage the haphazard use of antibiotics as is common in the society. However, considering the issues of resistance training and R-plasmid transfer in which low antibiotic concentrations have been implicated coupled with the fact that there are a lot of dynamics involved in the interaction between antibiotic, bacteria and host defence system, extreme caution is still recommended in the use of antibiotics.

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