A thesis entitled

A CHEMICAL AND BIOLOGICAL STUDY OF GENTAMICIN-RESISTANCE IN *PSEUDOMONAS AERUGINOSA*

by

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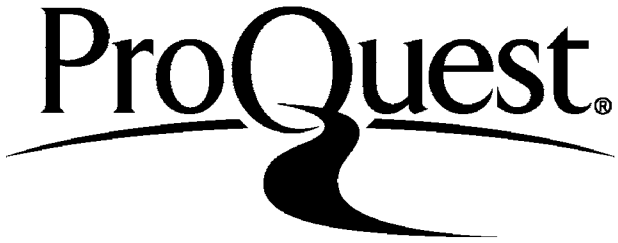

Abstract

Microelectrophoresis was used to study the surface properties of cells of strains of P. aeruginosa. Cells of gentamicin-resistant strains (not carrying R-factors) were distinguished from cells of gentamicin-sensitive strains by their characteristic shaped pH-mobility curves. The resistant strains had significant amounts of surface lipid, in contrast to negligible amounts on sensitive strains. There was no relationship between divalent cation content and gentamicin sensitivity/resistance of cells.

Cells carrying R-factors mediating gentamicin-resistance generally exhibited pH-mobility curves characteristic of gentamicin-sensitive strains; they also had negligible amounts of surface lipid. Unlike other resistant strains, these strains could be cured and could transfer resistance to acceptor strains. The transconjugant strains had identical surface properties to those of the parent acceptor strains.

The results are discussed in terms of two distinct 'natural' resistance mechanisms, (a) R-factor mediated and (b) another mechanism, which may be exclusion, a mutation at ribosome level or enzyme inactivation controlled by chromosomal genes. The resistance of one of the R-factor strains was partly due to R-factor mediation and partly to another mechanism.

Calcium and magnesium ions in the growth medium increase the resistance of cells to gentamicin. Cells acquiring resistance in this way displayed gentamicin-resistant type pH-mobility curves but had less surface lipid and higher maximum negative mobility

values than 'naturally' resistant strains. Cells becoming sensitive on growth in calcium-deficient medium displayed the properties of gentamicin-sensitive cells. Cells grown in highly calcium-supplemented medium possessed greater amounts of polysaccharide and surface divalent cations than did cells grown in calcium-deficient medium. These results are discussed in terms of a barrier mechanism of resistance. A weak 1:1 calcium-gentamicin complex, detected in microcalorimetric studies, may contribute to the antagonistic effect of calcium against gentamicin.

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CONTENTS

Summary

Gentamicin is an aminoglycoside antibiotic which has been used with considerable success against infections of Pseudomonas aeruginosa, an organism which has proved to be exceptionally resistant to other antibiotics. However, as has happened with other antibiotics, the increasing use of gentamicin has been paralleled by an increasing number of reports of gentamicin-resistant strains.

Cell microelectrophoresis was used to examine the surface properties of a series of gentamicin-sensitive and gentamicin-resistant strains of P. aeruginosa; the findings of this study confirm the observations of Pechey and James (1974) and Pechey et al (1974), relating surface properties with resistance to gentamicin. Cells of gentamicin-sensitive strains grown at 37 ^{o}C in nutrient broth exhibited a characteristic type of pH-mobility curve with a maximum mobility value in the pH range 5.5 to 6.5 and a minimum mobility value between pH 7.5 to pH 8.5. These cells also had negligible surface lipid as detected electrophoretically in the presence of SDS. Cells of gentamicin-resistant strains grown at 37 ^{o}C in nutrient broth exhibited a characteristic type pH-mobility curve with a maximum mobility value (which was generally lower than that of gentamicin-sensitive strains) in the pH range 7.5 to 8.5; no minimum mobility value was observed up to pH 10.5. Resistant strains had significant amounts of surface lipid. These pH-mobility curves showed that the surfaces of all strains are complex; amino and carboxyl are present as ionogenic groups. The variation of mobility value

with pH value is a result of some rearrangement of surface components similar to that reported by Hill and James (1972) for cell walls of Staphylococcus aureus. The characteristic surfaces associated with different MIC values and the associated high surface lipid content of resistant cells may indicate some form of barrier mechanism of resistance.

The concentration of calcium and magnesium ions in the growth medium affects the level of resistance of cells of P. aeruginosa to gentamicin, resistance increasing as the concentration of these ions increases. The effect of this acquired resistance on the surface properties of cells of P. aeruginosa was examined. It was found that gentamicin-sensitive strains which became gentamicin-resistant as the divalent cation content of the growth medium increased, acquired a gentamicin-resistant type pH-mobility curve but they did not acquire extra surface lipid. Gentamicin-resistant strains which became gentamcin-sensitive when grown in calcium-deficient medium acquired gentamicin-sensitive type pH-mobility curves and had decreased amounts of surface lipid. Cells grown in calcium-supplemented media tended to have higher negative maximum mobility values than did the same cells grown in calcium-deficient medium.

An analysis of macromolecular components of whole cells of the same strain grown in calcium-deficient and highly calcium-supplemented media indicated that cells grown in the latter medium had a higher percentage of polysaccharide whilst those grown in the calcium-deficient medium had a higher percentage of protein.

Atomic absorption spectrophotometric analysis and EDTA-lysis showed that cells grown in the calcium-supplemented medium had a higher calcium content and a higher level of surface cations whilst the cells grown in calcium-deficient medium appeared to have compensated for the low levels of calcium in the growth medium by having a higher content of magnesium.

Divalent metal ions are known to help to stabilise the Gram-negative (and especially P. aeruginosa) cell envelope. EDTA-lysis of cells extracts these metal ions and this is accompanied by a release of macromolecular components such as lipopolysaccharide and lipoprotein. The effect of calcium and magnesium ions on resistance to gentamicin may be due to the metal ions binding increased amounts of these components to the cell surface, thus forming an increased barrier to gentamicin penetration. This could be in accordance with the changes in surface properties and gross composition seen after growth in media containing differing concentrations of divalent cations. Attempts to demonstrate such a barrier experimentally by suspending cells, grown in calcium-deficient and calcium-supplemented media, in buffered gentamicin solutions proved inconclusive. There was no difference between the cells grown in the different media, both were sensitive to gentamicin. However, this may have been due to the gentamicin binding to the cells during suspension and then causing cell death when they were all growing on nutrient agar (conditions under which the cells would have their normal resistance levels, the effects of high levels of divalent cations on resistance only lasting as long as the cells are growing at that concentration).

Divalent cation analysis (of magnesium, calcium, zinc and iron(II)) and EDTA-lysis of whole cells of resistant and sensitive strains of P. aeruginosa grown in nutrient broth, showed that there was no pattern, either in total cell or surface divalent cation content that could be related to resistance to gentamicin.

It is possible that the antagonistic effect of magnesium and calcium ions towards gentamicin is caused by cation/gentamicin interactions which would inactivate the antibiotic or alter it in some way so that its ease of access into the bacterial cells was impaired. Microcalorimetry experiments indicated that there was a slight interaction between calcium and gentamicin in which a weak 1:1 calcium-gentamicin complex was formed.

A study of P. aeruginosa strains known to carry R-factors mediating gentamicin-resistance suggested that the resistant strains previously examined acheived their resistance by some mechanism other than due to R-factors. Thus the strains showing enzyme-inactivation of gentamicin generally exhibited gentamicin-sensitive type pH-mobility curves and negligible surface lipid. They also differed from other resistant strains in that the resistance could be eliminated by known curing agents (SDS and acridine dyes) and resistance could be transferred during mating experiments to gentamicin-sensitive acceptor strains. The acquisition of resistance by these strains lead to increased levels of resistance (although always lower than in the donor strain) but no detectable surface changes. One strain was examined which appeared to possess both types of resistance, being

both cured by proflavine and able to transfer resistance and yet exhibiting a gentamicin-resistant type pH-mobility curve and a high level of surface lipid.

CHAPTER 1

INTRODUCTION

1.1 Classification of Pseudomonas aeruginosa

Micro-organisms may be divided into eight major groups: algae, bacteria, moulds, pleuropneumonia-like-organisms (PPLO), protozoa, rickettsia, viruses and yeasts; inclusion in a group depends on the possession of distinct characteristics.

The bacteria, or schizomycetes, are usually divided into ten orders and the orders divided into families (Table 1.1). The different families are determined by such characteristics as cell shape, motility, the presence or absence of flagella if motile, reaction to Gram stain, growth requirements, optimum temperature of growth and the types of fermentations which the cultures are capable of carrying out under aerobic and anaerobic conditions.

The Gram stain serves as a useful preliminary characterisation technique, an organism being described as Gram-positive, Gram-negative or Gram-variable depending on its ability to retain the crystal violet dye after mordanting with iodine solution. The Gram stain also reveals cell morphology and whether or not the cells possess spores.

P. aeruginosa, the organism used in this investigation, is a Gram-negative, non-spore-forming rod (dimensions 1.5 x 0.5 μm) which is actively motile by means of a polar flagellum. It is usually non-capsulate and is sometimes fimbriated. It grows aerobically between 5 and 43 ^{o}C utilising a wide range of energy sources and producing two water soluble pigments, pyocyanine and fluorescein and a characteristic odour. The colonies are dark greyish, large, low-convex with an irregular, translucent, spreading edge. It can produce acid oxidatively from glucose, it rapidly liquefies

Table 1.1

A classification of bacteria

with particular reference to Pseudomonas

Order	Family	Genus*
Eubacteriales		
Hyphomicrobiales		
Clamydobacteriales		
Pseudomonadales	Pseudomonadaceae	Pseudomonas
	Spirillaceae	
Actinomycetales		
Caryoplanales		
Beggiatoales		
Myzobacteriales		
Spirochaetales		
Mycoplasmatales		

*Genus	Sub-generic group	Species
Pseudomonas	Acidovorans	
	Fluorescent	P. aeruginosa
		P. fluorescens
		P. putida
	Alcaligenes	

gelatin and unlike most Gram-negative rods gives a positive oxidase reaction.

As a pathogen P.aeruginosa is often implicated in urinary tract infections and infections due to catheterisation or other diagnostic or therapeutic instrumentation. It commonly infects deep wounds and burns and causes acute purulent meningitis after cranial injury or accidental introduction after lumbar puncture.The infections are normally localised but in the cases of infants or debilitated persons, invasion of the blood stream resulting in fatal generalised infection may occur, especially when radiation therapy or antineoplastic drugs are being used.

1.2 Bacterial Anatomy

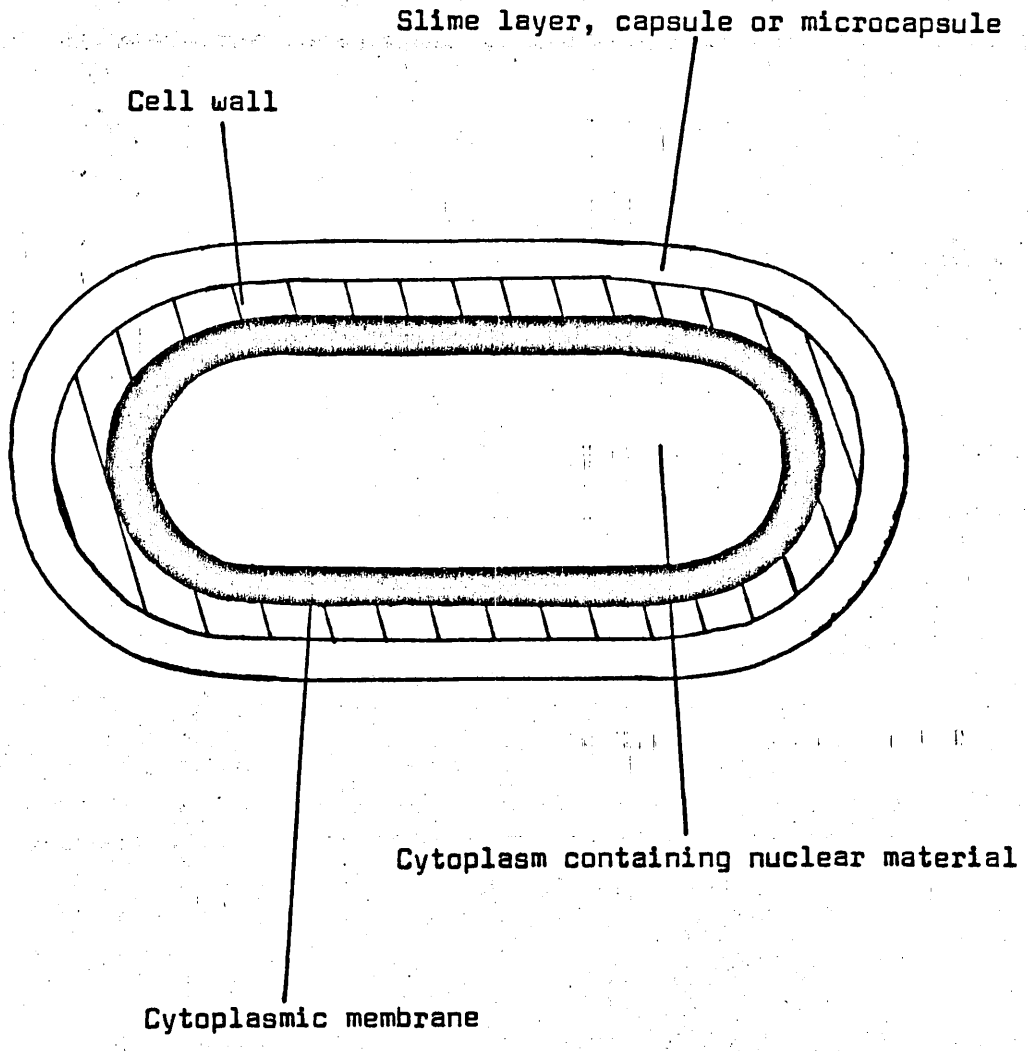
Figure 1.1 shows a cross-section through a typical bacterial cell, illustrating the basic cellular organisation. The cytoplasm is surrounded by a membrane which is in turn surrounded by a cell wall. In many organisms a capsule or slime layer envelops the cell and surface appendages may also be present.

The cytoplasm is a slightly viscous colloidal complex of water, amino acids, proteins, fats, carbohydrates and inorganic material, often with stored nutrients and oils suspended in it. Storage particles and ribosomes give the cytoplasm a granular appearance; the ribosomes are the cytoplasmic site of protein synthesis. The nuclear material, or nucleoid, lies in the cytoplasm and is not bounded by a nuclear membrane.

Figure 1.1

Diagrammatic representation of

a cross-section through a bacterial cell



There are three types of appendage on bacterial cells; flagella, cilia and fimbriae (pili). P. aeruginosa has a single, polar flagellum protruding through the cell surface, but continuous with the cell cytoplasm. It is composed almost entirely (98%) of a protein called flagellin. The flagella bring about cell motility by their movement and are also the site of the H-antigens. Cilia also impart motility to bacterial cells and also move liquid over the surface of the organism, thus facilitating the supply of nutrients and the removal of waste products. Fimbriae are very fine hair-like structures, which are known to confer haemagglutination, adhesive and antigenic properties to the cells.

Many micro-organisms have capsules or slime layers surrounding them, lying external to, but in close contact with, the cell wall. The amount produced depends on the nature of the growth medium and the production of such layers may be stimulated by unfavourable growth conditions. Capsules are often correlated with virulence in pathogenic forms.

Wilkinson (1958) classified capsules into three main types:

(a) macrocapsules, at least 0.2 μm thick, having a definite external surface;

(b) microcapsules, less than 0.2 μm thick, and usually detected immunologically;

(c) slime layers, which accumulate in the medium at the surface and have little anatomical significance.

Capsular material consists of about 98% water. Cells of Gram-negative species have chemically more complex capsular material

than have cells of Gram-positive species.

The composition of the slime layers deposited by strains of P. aeruginosa vary quite widely. Eagon (1962) estimated that mannose constituted about 50% of the dry slime material and that appreciable amounts of DNA, RNA and protein were present, whilst Bartell et al (1970) also reported that polysaccharide was the main constituent with a significant proportion of protein. Other constituents reported in the slime layer of P. aeruginosa include glucose, rhamnose, fucose, galactose, glucosamine, galactosamine and glucuronic acid (Doggett et al, 1964; Brown et al, 1969; Bartell et al, 1970).

1.3 The Gram-Negative Cell Envelope

The Gram-negative cell envelope consists of a number of layers of varying chemical and physical nature. Some layers are intimately associated with neighbouring structures and thus there are no clear boundaries between different structures. Because of this all the structures external to the cytoplasm (including the cytoplasmic membrane) are considered as a single multi-layered unit termed the cell envelope.

The cell envelope surrounds the protoplast and being rigid, gives a microbial cell its particular shape. It is capable of withstanding osmotic pressure; that across the cytoplasmic membrane in Gram-negative bacteria is generally about 12 atmospheres. The cell wall of Gram-positive bacteria e.g. Staphylococcus aureus is capable of withstanding an osmotic pressure of up to 30 atmospheres.

The cell envelope provides the permeability barrier between cytoplasm and environment and is responsible for the antigenicity and the response to pyocines and bacteriophages.

Most of the techniques used to isolate whole cell walls or envelopes are based on that of Salton and Horne (1951) in which the bacterial cells are first ruptured either by shaking cell suspensions with ballotini glass beads in a Mickle (1948) disintegrator or in the Braun homogeniser. The homogenate is then heated at 90 ^{o}C to destroy autolytic enzymes released by the cells, which would otherwise degrade the cell walls. The walls are separated from the undamaged cells by low speed centrifugation and finally a pellet of crude cell wall preparation is obtained by high speed centrifugation. Robertson and Schwab (1960) used density gradient centrifugation to obtain homogeneous preparations. The material obtained is washed repeatedly, first in either 0.1 mol dm^{-3} phosphate buffer solution or in 0.1 mol dm^{-3} sodium chloride solution and then with water. Fragments of cytoplasmic membrane adhering to the walls are removed by the action of enzymes such as trypsin, RNase or DNase (Cummins and Harris, 1956). Such isolation of whole cell envelopes has made the determination of the gross chemical composition of these structures possible. However, it is possible that the method of isolation may affect the results obtained. For example Roberts *et al* (1967) showed that washing removed some envelope components of *P. aeruginosa* and enzyme treatment may remove part of the outer envelope membrane as well as cytoplasmic membrane (Braun and Rehn, 1969).

Peptidoglycans, proteins, lipids, polysaccharides, lipopolysaccharides and lipoproteins are the main classes of chemical

constituents found in Gram-negative cell envelopes. There is a wide range of amino acids (up to 21) similar to that normally found in most proteins (Salton, 1952), compared with Gram-positive cell walls which may contain as few as three amino acids. In P. aeruginosa the envelope contains 13 amino acids, four of which, alanine, glutamic acid, lysine and diaminopimelic acid, are associated with the peptidoglycan layer (Mandelstam, 1962).

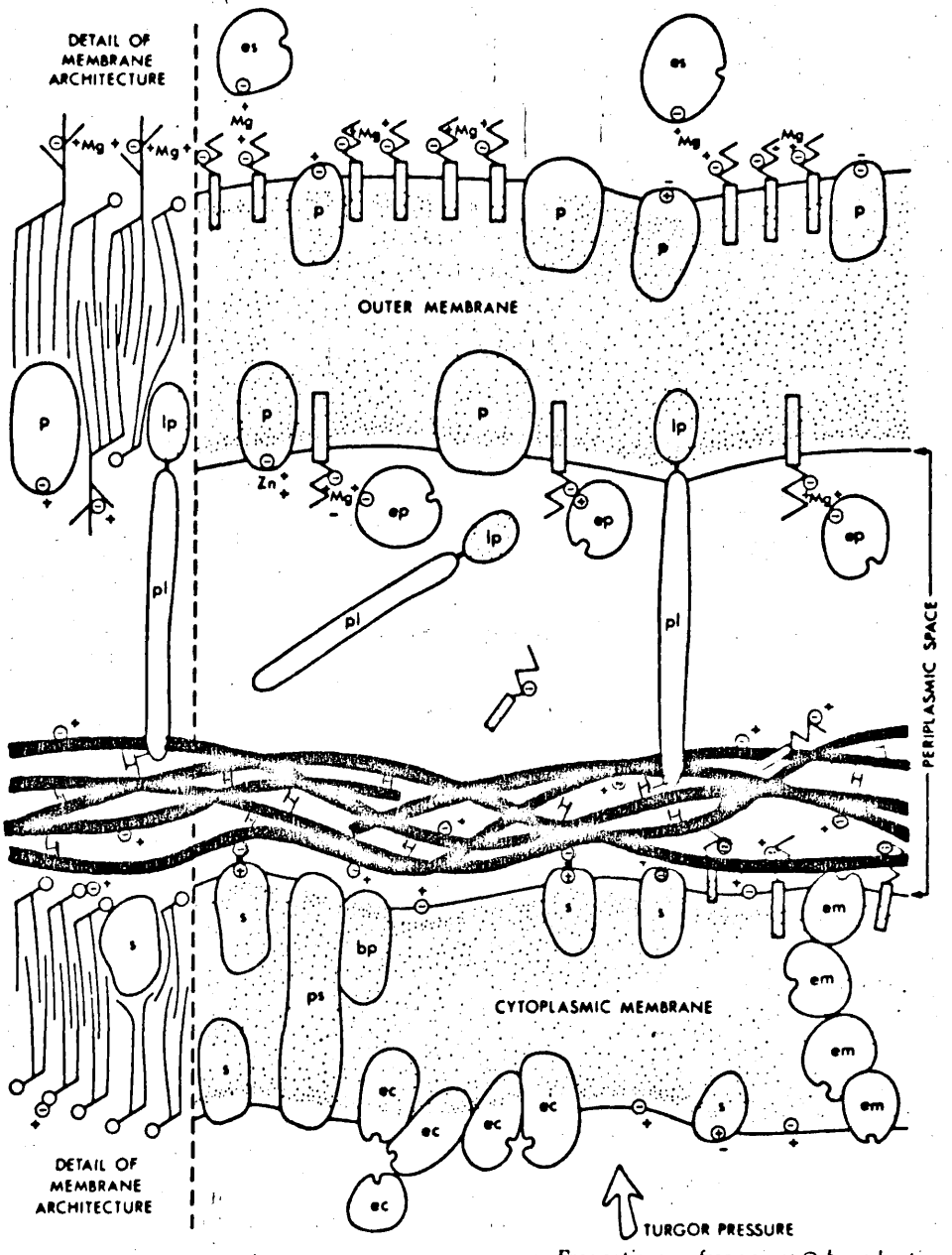
Much early information on the structure of Gram-negative cell envelopes came from thin-section electron-microscopical investigations. For example Kellenberger and Ryter (1958) using this technique established the multi-layered structure of the cell envelope of the Gram-negative bacterium Escherichia coli. Similar results by other workers (e.g. Murray, 1962) led to the suggestion by Clarke and Lilly (1962) and Brown et al (1962) that the cell envelope of most, if not all, Gram-negative bacteria consisted of two unit, or compound membranes each with the structure of protein-lipid-lipid-polysaccharide, and separated by a rigid layer of peptidoglycan. Thus the inner compound membrane would be analogous to the cytoplasmic membrane of Gram-positive bacteria and the peptidoglycan layer and the outer compound membrane would replace the peptidoglycan layer which constitutes almost the whole of the Gram-positive cell wall. A more detailed structure was proposed by De Petris (1967) from electron microscopic studies of Esch. coli.

The present state of knowledge allows the structure and composition of the Gram-negative cell envelope (Figure 1.2) to be discussed in some detail.

Figure 1.2

Schematic diagram of Gram-negative cell envelope

(Costerton et al, 1974)



+, Free cation; −, free anion; ⊕ bound cation; ⊖ bound anion; adhesion point produced by ionic bonding; hydrophobic zone; covalent bond; cross-linking polypeptide in the peptidoglycan; polysaccharide portion of peptidoglycan; enzymatically active protein; phospholipid; lipopolysaccharide; lipopolysaccharide (schematic); bp, binding protein; ec, enzymes associated with the cytoplasmic membrane whose function is directed to the cytoplasm; em, enzymes associated with the cytoplasmic membrane which synthesize macromolecular components of the cell wall; ep, enzymes localized in the periplasmic zone; es, enzymes localized at the cell surface; lp, lipid portion of Braun's lipoprotein; p, structural and enzymatic proteins of the outer membrane; pl, protein portion of Braun's lipoprotein; ps, permease; s, structural protein of cytoplasmic membrane.

1.3.(a) Cytoplasmic membrane

Examinations of isolated pure preparations of the cytoplasmic membrane of Gram-negative bacteria have shown that the chemical composition of this membrane is similar to that of other biological membranes (Schnaitman, 1970; Martin and MacLeod, 1971; White *et al*, 1972). Freeze-etching studies have shown that the frozen cytoplasmic membrane cleaves along a median hydrophobic zone (Bayer and Remsen, 1970) and that this zone is traversed by protein 'studs' (DeVoe *et al*, 1971), which Fox (1972) and Tourtellotte and Zupnik (1973) have suggested may be involved in substrate transport. Fox has also proposed that structural membrane proteins are built into the continuous phospholipid bilayer and also that other proteins may be associated with the inner or outer aspects of the membrane by hydrophobic interactions. Binding proteins such as the leucine-binding protein of *Esch. coli* (Nakane *et al*, 1968) are associated with the cytoplasmic membrane (Figure 1.2). Certain enzymes such as the adenosine-hydrolysing enzyme of *Esch. coli* are located at the outer surface of the cytoplasmic membrane (Hochstadt-Ozer, 1972).

The cytoplasmic membrane is the site where the structural components of the cell wall are synthesised and assembled.

1.3.(b) Peptidoglycan-lipoprotein complex

Peptidoglycan is present in the Gram-negative cell envelope in highly variable amounts, comprising 2.4% of the cell weight in *Spirillum serpens* (Kolenbrander and Ensign, 1968) but only 1.2% of the cell weight in the marine pseudomand B.16 (Forsberg *et al*, 1972).

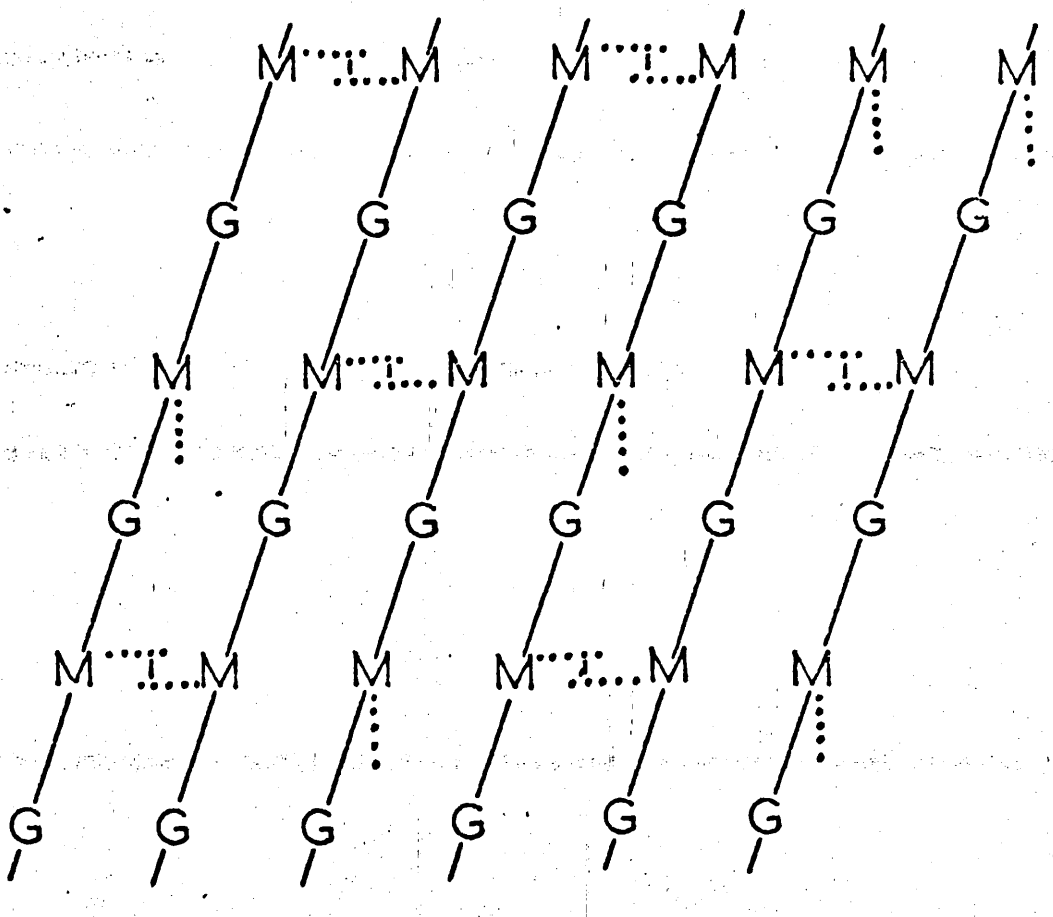
It constitutes about 10% of the Gram-negative cell envelope, compared to 40-90% of the Gram-positive wall. The peptidoglycan layer is about 2-3 nm thick in the Gram-negative envelope and 20-80 nm thick in the Gram-positive wall. Forsberg et al(1970a) have shown that cells bounded only by their peptidoglycan layer maintain their shape, even though other cell layers may contribute to cellular rigidity (Carson and Eagon, 1966; Cox and Eagon, 1968) or maintain cellular shape (Henning et al, 1973).

Electron micrographs of fixed and sectioned cells often show an electron transparent region between the peptidoglycan layer and the cytoplasmic membrane. However, it may be that this does not occur in the living cell.

Strominger et al (1959), Mandelstam and Rogers (1959), Rogers and Perkins (1959), Hancock (1960) and Mandelstam and Strominger (1961) determined the overall structure of peptidoglycan, and found that it consists of a polysaccharide backbone of alternating β-1, 4-linked N-acetyl glucosamine and N-acetyl muramic acid residues (Figures 1.3 and 1.4). The first Gram-negative peptidoglycan was isolated by Weidel et al (1960) and its structure was found to be essentially similar to that in Gram-positive cells. Attached to the N-acetyl muramic acid molecules are peptide subunits, consisting of L-alanine, D-glutamic acid, diaminopimelic acid (or its decarboxylation product L-lysine) and D-alanine. These peptide chains are often linked together by peptide bridges but in the case of Esch. coli and probably of P. aeruginosa the bridging is via direct bonding from the D-alanine of one subunit to the (D)-asymmetric carbon atom of the meso-diaminopimelic acid of the neighbouring

Figure 1.3

A schematic representation of the peptidoglycan of the cell envelope of Escherichia coli



Key

Glycan chains composed of:

G N-acetylglucosamine

M N-acetylmuramic acid

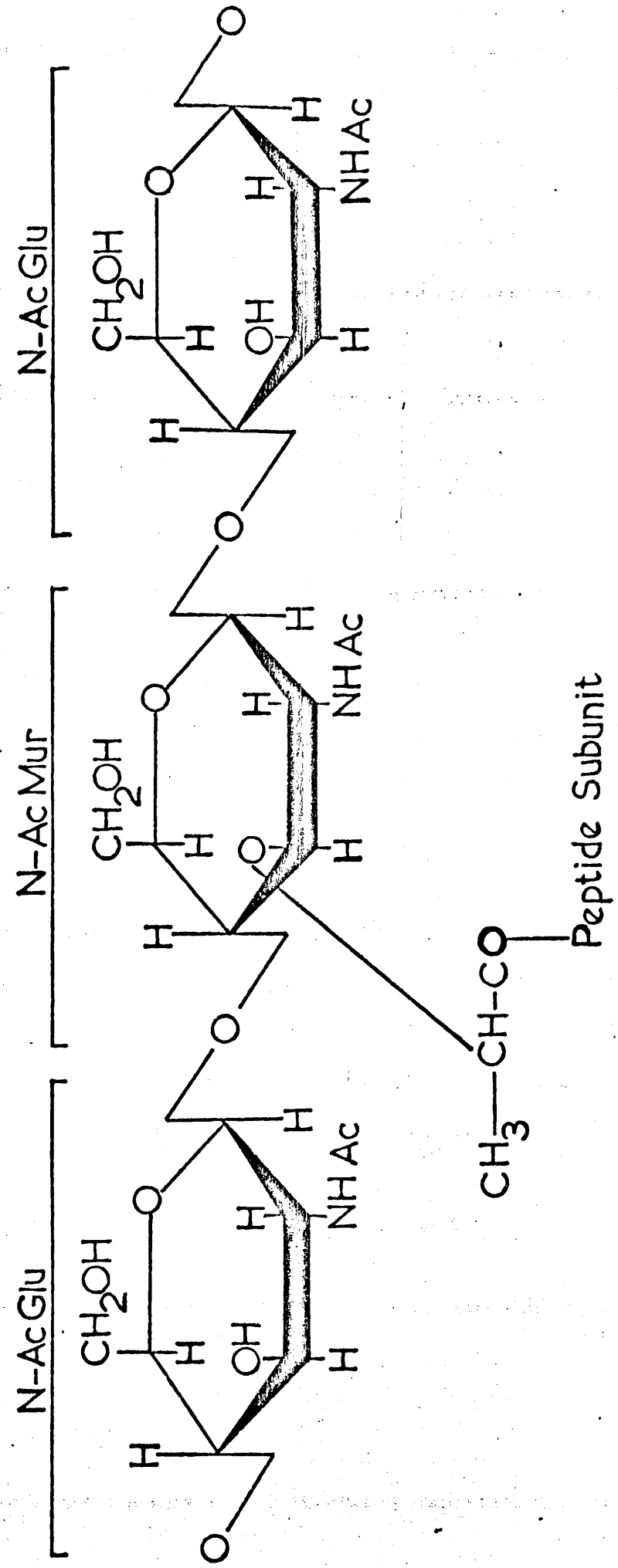
Vertical dots represent peptide subunits attached to N-acetylmuramic acid. Horizontal dots represent peptide subunits cross-linking between adjacent glycan strands.

Figure 1.4

A portion of the glycan strand of peptidoglycan showing the site of attachment of the peptide subunit

Key

N - Ac Glu	=	N-acetylglucosamine
N - Ac Mur	=	N-acetylmuramic acid


N-Ac Glu
N-Ac Mur
N-Ac Glu
NH Ac
CH2OH
OH
CH3—CH—CO
Peptide Subunit

subunit (Figure 1.5). The greater thickness of the peptidoglycan layer in the Gram-positive cell wall suggests a three dimensional arrangement compared to the thinner peptidoglycan layer in the Gram-negative envelope, indicative of a two dimensional monolayer structure (Ghuysen,1968; Keleman and Rogers, 1972). The arrangement of cross-linked peptide subunits is probably random, resulting in a loose network, unlike some Gram-positive peptidoglycans where all the peptide subunits are cross-linked, thus giving a more rigid, tightly knit structure.

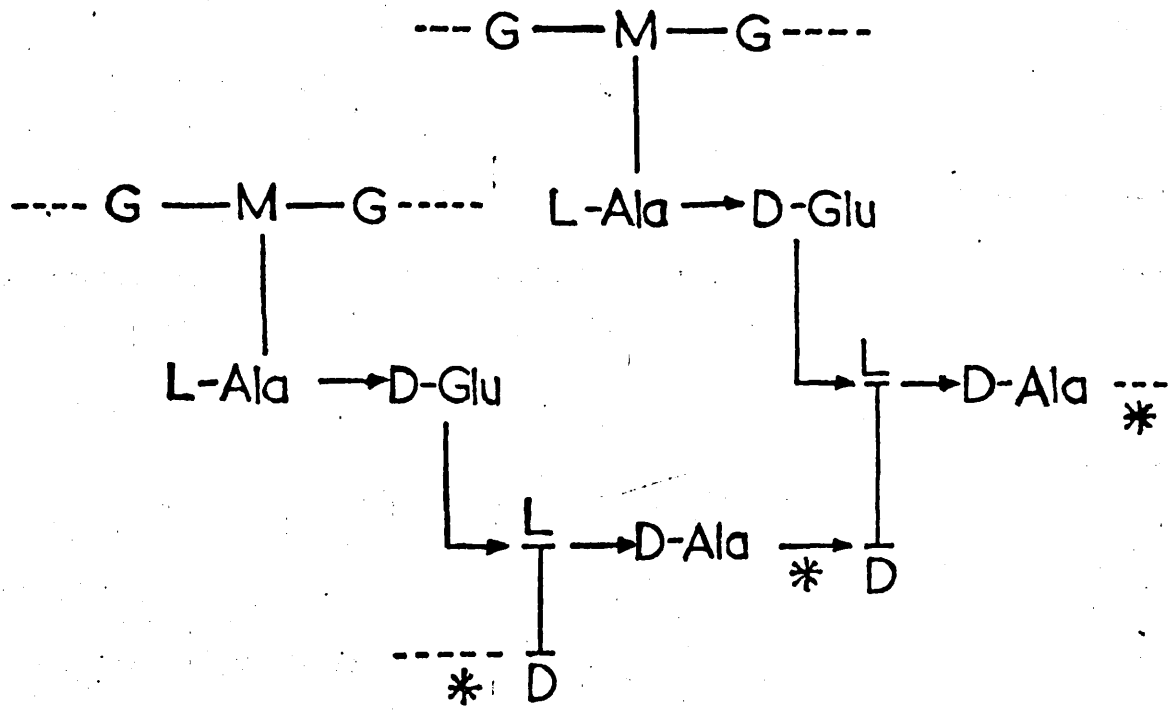
A lipoprotein (Figure 1.2), 12 to 14 nm long and composed of 57 amino acids, has been shown to be covalently linked to the peptidoglycan and to extend outwards towards the outer membrane in several enteric bacteria (Braun and Rehn, 1969; Braun et al, 1970; Braun and Sieglin, 1970; Braun and Wolff, 1970; Braun and Bosch, 1972). It has been suggested (Schnaitman,1971) that the covalently linked lipid component of this lipoprotein anchors the outer membrane by hydrophobic interactions with outer membrane phospholipids. Consistent with this suggestion is the fact that proteolytic digestion causes a separation of the outer membrane from the peptidoglycan layer (Braun and Sieglin, 1970). The covalently linked peptidoglycan-lipoprotein complex of the Gram-negative cell wall may thus serve as a rigid foundation for the whole cell envelope.

1.3.(c) Periplasmic zone

The periplasmic area is that between the cytoplasmic membrane and the cell wall outer membrane; thus it contains the

Figure 1.5

The structure of the peptide subunits of peptidoglycan showing the site of cross-linking between adjacent glycan strands of N-acetylglucosamine and N-acetylmuramic acid



Key

G	N-acetylglucosamine	M	N-acetylmuramic acid
L-Ala	L-alanine	D-Ala	D-alanine
D-Glu	D-glutamic acid	*	Site of cross-linking
L–D	Meso-diaminopimelic acid, with asymmetric carbon atoms designated L and D.		

peptidoglycan layer (Figure 1.2).

Periplasmic enzymes, binding proteins and pigments are released from cells in which the barrier layer of the outer cell wall has been disturbed by such techniques as exposing cells of *P. aeruginosa* to 0.2 mol dm^{-3} Mg^{2+} or growing cells at an elevated pH (Cheng *et al*, 1970).

The evidence currently available suggests that periplasmic enzymes are associated with structural components of the cell wall including lipopolysaccharide and that they are distributed throughout the periplasmic zone without notable concentration.

1.3.(d) Outer membrane layer

Electron micrographs of embedded and sectioned cells reveal the profile of this layer to be the same as other biological membranes, suggesting that the phospholipids and proteins form a bilayer. Studies of the isolated outer membrane show that it contains phospholipids and proteins (Forsberg *et al*, 1970a,b; Schnaitman, 1970a,b; Osborn *et al*, 1972a,b; White *et al*, 1972) and also variable amounts of polysaccharide (DePamphilis and Adler, 1971; Osborn, 1971; Rothfield and Romeo, 1971; Schnaitman, 1971; Osborn *et al*, 1972a,b). The main body of the membrane seems to be formed by proteins and phospholipids (Forge and Costerton, 1973; Forge *et al*, 1973) whilst the oligosaccharide portion of the lipopolysaccharide appears to be associated with the inner and outer surfaces (Cheng *et al*, 1971; Schnaitman, 1971).

The phospholipids of the outer membrane are quantitatively similar to those of the cytoplasmic membrane in *Esch. coli*, but are

qualitatively different (Osborn et al, 1972b; White et al, 1972). Also the phospholipids of the outer membrane are arranged in an hexagonally close-packed bilayer with a d-spacing of 0.44 nm (Rothfield et al, 1966; Burge and Draper, 1967; Forge and Costerton, 1973; Forge et al, 1973), again indicating similarities with a typical membrane. A cleavage plane in the outer cell wall membrane has been observed in freeze-etching studies (Dvorak et al, 1970; DeVoe et al, 1971; Forge et al, 1973; Gilleland et al, 1973), indicating that the hydrophobic parts of the phospholipid and lipopolysaccharide molecules form a zone in the centre of the outer cell wall membrane similar to that found in typical membranes. Using freeze-etching, Lickfield et al (1972) and Gilleland et al (1973) have shown that the outer membrane of the cell wall of P. aeruginosa contains a granular and predominantly proteinaceous subunit. Furthermore, Gilleland et al (1973) have shown that exposure to tris(hydroxymethyl)aminomethane-ethylenediaminetetraacetate (Tris-EDTA) removes these granules and that the addition of Mg^{2+} to the cell suspension causes these protein units to be reaggregated back into the cell wall. Schnaitman (1970b) has shown that outer membrane proteins differ from cytoplasmic membrane proteins and that one type of protein may have a structural function in the outer membrane; Osborn et al (1972b) have reported phospholipase activity in another outer membrane protein.

The hydrophobic lipid A portions of the lipopolysaccharide molecules are beleived to associate with the hydrophobic zone of the outer membrane, leaving the polysaccharide portions of the molecules protruding from the inner and outer surfaces of the membrane

(Cheng et al, 1971; Lindsay et al, 1973).

Divalent metal ions are known to be important in maintaining cell envelope integrity and in retaining lipopolysaccharide in the outer cell membrane (Eagon et al, 1965; Wilkinson, 1967; Leive,1968; Wilkinson, 1970; O'Leary et al, 1972). Roberts et al (1970) and Wilkinson (1970) suggest that the divalent metal ions are involved with phosphate groups in the cross linking of lipopolysaccharide and in this way are involved in the integrity of the envelope. Asbell and Eagon (1966) have suggested that during the biosynthesis of the lipopolysaccharide sacculus, negatively charged subunits are 'trapped' by forming ionic bonds intermediated by divalent metals.

Costerton et al (1974) have proposed a model of the outer membrane involving a protruding 'picket-fence' pattern on both surfaces consisting of lipopolysaccharide oligosaccharides carrying the 'O' antigen at their distal tips,the polysaccharide chains forming an ordered and cross-linked mass (Figure 1.2). The interaction of the protruding oligosaccharides helps to strengthen the cell wall. The proteins and phospholipids which constitute the basic outer membrane are exposed between this oligosaccharide 'mat' to a greater or lesser degree, possibly acting as specific receptor sites. These receptor sites could consist of glycoprotein (colicin receptors, Sabet and Schnaitman,1973), specialised lipopolysaccharide (phage receptors, Lindberg and Hellerquist, 1971) and phospholipid (Glauert and Thornley, 1969) with the polar heads at the surface. In the case of P. aeruginosa the polar heads will characteristically be mainly of phosphatidylethanolamine (Meadow, 1975).

The outer membrane constitutes a barrier, susceptible to

damage by a number of agents, which excludes a wide variety of molecules and also retains enzymes and structural components within the periplasmic space (Costerton et al, 1974).

1.4 Antibiotics

Antibiotics were originally defined as compounds produced by micro-organisms, which at low concentrations inhibit growth and other activities of other micro-organisms.

Gentamicin is an aminoglycoside antibiotic, a group which also includes kanamycin, neomycin, tobramycin and streptomycin. Gentamicin is obtained from submerged cultures of Micromonospora purpurea; commercial preparations contain a mixture of three components, gentamicins C_1, C_{1a} and C_2, in approximately equal proportions. The structures of the gentamicins are shown in Figure 1.6.

Gentamicin is effective against a large number of Gram-negative and Gram-positive bacteria (Weinstein et al, 1963; Barber and Waterworth, 1966) but is most frequently used in cases of infection due to P. aeruginosa, an organism which has proved resistant to many other antibiotics.

Gentamicin inhibits bacterial growth by inhibiting protein biosynthesis (Hahn and Sarre, 1969). Along with the other aminoglycoside antibiotics it acts upon the smaller (30 S) ribosomal subunit of the bacterial cell. The ribosomes are the cytoplasmic site where protein synthesis occurs, a process involving the assembling of polypeptides from amino acids. The individual amino acids are attached to a specific transfer RNA (tRNA) by enzymes and

Figure 1.6

The structures of the gentamicins

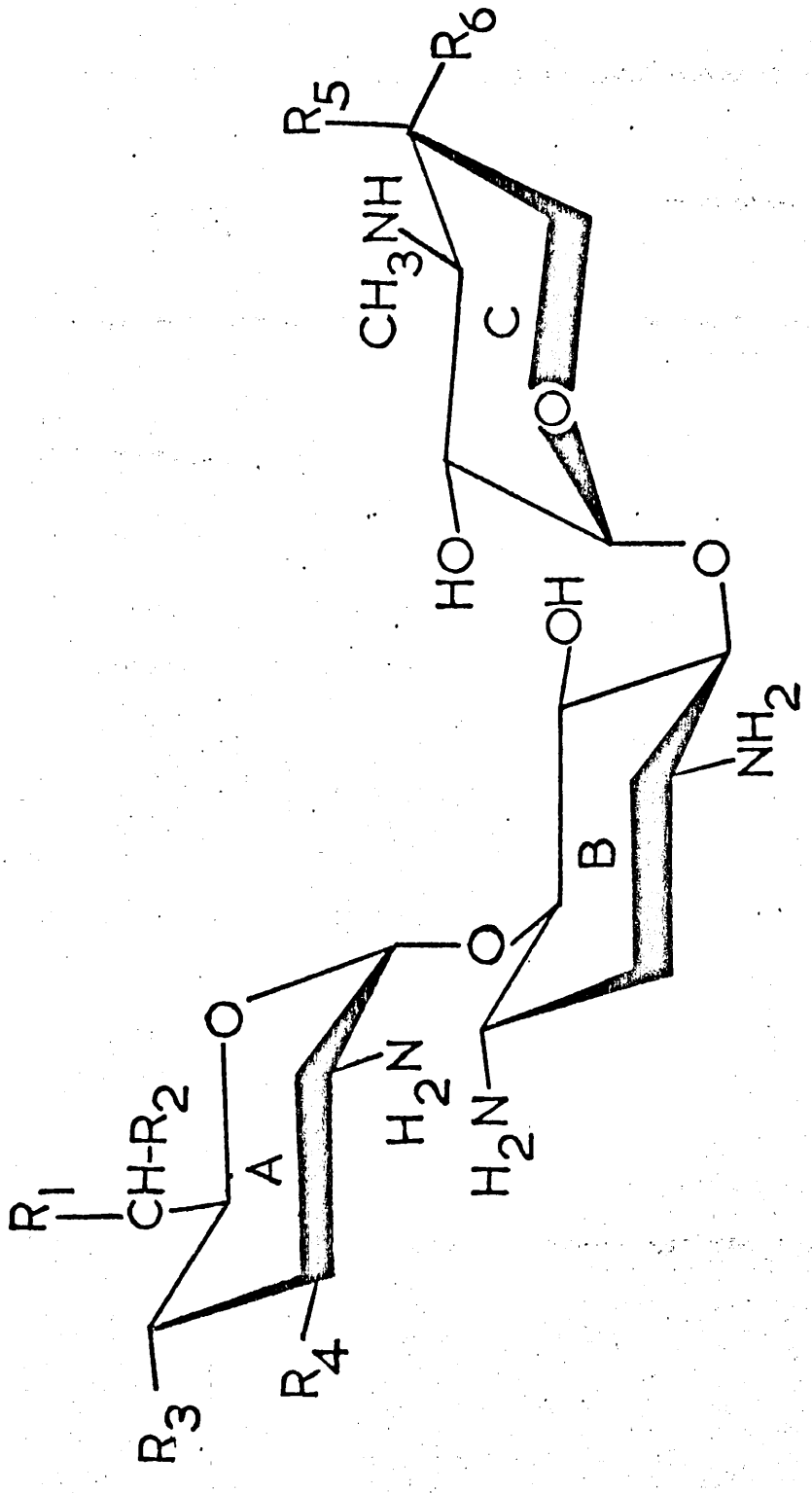
Key

	R_1	R_2	R_3	R_4	R_5	R_6
Gentamicin A	H	OH	OH	OH	H	OH
C_{1a}	H	NH_2	H	H	OH	CH_3
C_2	CH_3	NH_2	H	H	OH	CH_3
C_1	CH_3	$NHCH_3$	H	H	OH	CH_3

A = purpurosamine ring

B = deoxystreptamine ring

C = garosamine ring


R1
CH-R2
O
A
R3
R4
H2N
O
B
H2N
NH2
OH
HO
O
CH3NH
C
O
R5
R6

are carried to the ribosomes. There the incoming tRNA is matched with a messenger RNA (mRNA) on the ribosome. The nucleotide sequence of the mRNA is governed by the DNA, this allowing specific proteins to be synthesised. Once bound on or in the ribosome, the amino acid is attached to the adjacent nascent peptide and a translocation reaction moves the elongated peptide along to a neighbouring ribosomal binding site, thus allowing another amino acid molecule to be brought to the ribosome and consequently another peptide bond to be formed.

Aminoglycoside antibiotics bind preferentially to the ribosome and thus interfere with protein synthesis. This binding causes *in vitro* misreading or miscoding, anomalous amino acids being incorporated into the polypeptide chains, thus forming proteins that are useless to the cell (Davies *et al*, 1964). Gorini and Kataja (1965) have reported that this miscoding is not in itself lethal to the bacterial cell, but it is thought that the bound antibiotic causes the ribosome to attach itself to the mRNA in an aberrant fashion which irreversibly inhibits protein synthesis and thus kills the cell (Gale *et al*, 1972). Differences in ribosomal binding sites may be the reason for differences in the spectrum and intensity of the aminoglycoside antibiotics.

1.5 Antibiotic Resistance

As with other antibiotics, the widespread use of gentamicin for the treatment of bacterial and especially *P. aeruginosa* infections, has been matched by an increasing percentage of resistant organisms.

Generally speaking, drug resistance is achieved by two main methods; either the bacterial cell changes in some way and thus becomes less susceptible to the antibiotic, or the cell changes the antibiotic, thus rendering it inactive. Gale et al (1972) have listed four mechanisms of antibiotic resistance:

(a) the target in the cell is altered;

(b) the physiological importance of the target is reduced;

(c) access by the drug to the target is reduced, resulting in exclusion;

(d) the production of drug degrading enzymes.

(a) Modification of the target

An example of this type is resistance to the aminoglycoside antibiotic streptomycin, in which a mutation changes the amino acid sequence of the 30 S ribosome subunit, resulting in the antibiotic not being bound to the ribosomes of resistant mutant cells (Nomura, 1970).

(b) Reduction in the physiological importance of the target

The Gram-negative wall may perhaps be considered as a modified Gram-positive wall, the former having less peptidoglycan than the latter with the addition of the outer cell wall membrane. Also evidence suggests that other macromolecular components, as well as the peptidoglycan layer, contribute towards cell rigidity in Gram-negative bacteria (Section 1.3). Thus it may be that the reduced importance of the peptidoglycan in Gram-negative bacteria contributes to an intrinsic resistance to anti-peptidoglycan agents.

Another example of this type of resistance occurs when P. aeruginosa is grown in magnesium-depleted medium (Brown and

Melling, 1969a,b; Gilleland et al, 1974), when the cells become resistant to both EDTA and polymyxin B. These agents cause cell lysis and normally cells grown in magnesium-sufficient media would be susceptible to them.

(c) Exclusion

Considerable evidence is now available pointing towards the envelope of Gram-negative bacteria and of P. aeruginosa in particular, having a significant role in antibiotic resistance by an exclusion mechanism. In such a mechanism an otherwise active agent would fail to achieve an effective concentration at its site or sites of action. In many cases much lower concentrations of antibiotics are required for enzyme inhibition in cell free systems than for inhibition of cell growth. For example, Hurwitz et al (1962) found that much less actinomycin D was needed for inhibition of the DNA-dependent RNA polymerase in Esch. coli, than was needed for inhibition of growth. Crypticity studies have also been used to demonstrate the relative ease of penetration of antibiotics through the envelope, crypticity values being obtained by comparing the rate of enzymatic destruction of an antibiotic using broken cell preparations, with that obtained using equal numbers of whole cells. Richmond and Sykes (1973) have reported comparatively high crypticity values with P. aeruginosa for cephaloridine.

EDTA-treatment sensitises P.aeruginosa to the action of lysozyme (Gray and Wilkinson, 1965a,b) and also potentiates the action of several, unrelated antibacterial substances. This potentiating action of EDTA can be blocked or reversed by adding divalent cations, especially magnesium and calcium (Brown and

Richards,1965). It has been suggested that EDTA increases cell permeability by removing divalent cations and thus disrupting the wall structure; EDTA removes lipoprotein, lipopolysaccharide and lipid from the cell wall (Colobert, 1958; Gray and Wilkinson, 1965b; Leive, 1965).

Studies using whole cells, protoplasts and spheroplasts of P.aeruginosa and other species have shown that resistance in many cases was due to non-penetration. Hamilton (1970) showed that in P.aeruginosa among other strains, the wall acted as a barrier to membrane active agents.

Surface lipid changes have been implicated in antibiotic resistance. Pechey et al (1974) found a relationship between surface lipid and gentamicin MIC in P.aeruginosa using electrophoretic mobility studies, whilst Ivanov et al (1964) found that by extracting P.aeruginosa with petroleum ether (without altering viability), the sensitivity to a number of antibiotic agents became enhanced.

A correlation between uptake and sensitivity to polymyxin has been reported for several species including P.aeruginosa (Few and Schulman, 1953; Newton, 1956).

(d) Drug degrading enzymes

Three different mechanisms are at present known by which aminoglycoside antibiotics can be enzymatically modified and thus inactivated:

(i) acetylation of amino groups;

(ii) adenylation of hydroxyl groups;

(iii) phosphorylation of hydroxyl groups.

These enzymes render antibiotics inactive by substituting key residues. The adenylating and phosphorylating enzymes both use

ATP, the former as a source of the adenyl residue and the latter as a source of a phosphate group. The acetylating enzymes use acetyl CoA as the source of an acetyl group.

Reports of P.aeruginosa producing a phosphorylating enzyme (Davies et al, 1969; Kobayashi et al, 1971a,b; 1972) have appeared. These enzymes only inactivate gentamicin A. Gentamicin (Figure 1.6) contains a purpurosamine ring, a 2,3,4,6-tetradeoxyamino sugar which lacks hydroxyl groups (except in gentamicin A, which is not present in commercial preparations) and thus makes the molecule resistant to attack by phosphorylating enzymes. Bryan et al (1974) and Kabins et al (1974) have reported gentamicin adenylating enzymes in P.aeruginosa and Mitsuhashi et al (1971), Bryan et al (1974) and Jacoby (1974) have reported gentamicin acetylating enzymes in P.aeruginosa. Such studies have made the determination of the specificity of the enzyme substrates possible. Brzezinska et al (1972) have described gentamicin inactivation by acetylation at the 3-amino group of the 2-deoxystreptamine ring, the gentamicin C antibiotics all being excellent substrates, unlike gentamicin A. The adenylating enzymes show a preference for hydroxyl groups in the D-threo configuration.

Davies et al (1969) have suggested that these enzymes are probably located in the periplasmic space. If this is the case it would confer an advantage on the cell compared to the extracellular enzymes of Gram-positive cells (Percival et al, 1963). Thus enzymes located in the periplasmic space are less liable to dilution than extracellular enzymes and in many cases are acting on a substrate with restricted access, thus less enzyme is necessary. Also, the population effect involved with inactivation by extracellular

enzymes is not apparent.

Enzymatic resistance to gentamicin in strains of P.aeruginosa has been shown to be extrachromosomally (plasmid) mediated and the transfer of both gentamicin adenylating and gentamicin acetylating resistance has been reported (Bryan et al, 1974; Jacoby, 1974; Korfhagen and Loper, 1975). The transfer of such plasmids or R-factors may well assist the spread of antibiotic resistance, resistant strains passing the plasmids on to sensitive strains, thus causing these to become resistant in turn.

The first three types of resistance described, (a) to (c) do not require such an acquisition of extrachromosomal genes, only the modification of genes already existing.

1.6 Electrophoresis

At any solid-liquid interface there is an electrical double layer caused by an excess of ions on the solid phase and an equal amount of ionic charge of opposite sign distributed in the liquid phase near the interface.

One of the first theories of the double layer was proposed by Helmholtz (1879) in which two parallel layers of charges, of uniform charge density but of opposite sign, are held a short distance apart, one layer firmly attached to the surface and the other in the liquid. Gouy (1910) modified this theory, proposing the idea of a diffuse double layer, in which the potential decreases exponentially to zero over the distance $1/\kappa$, the statistical

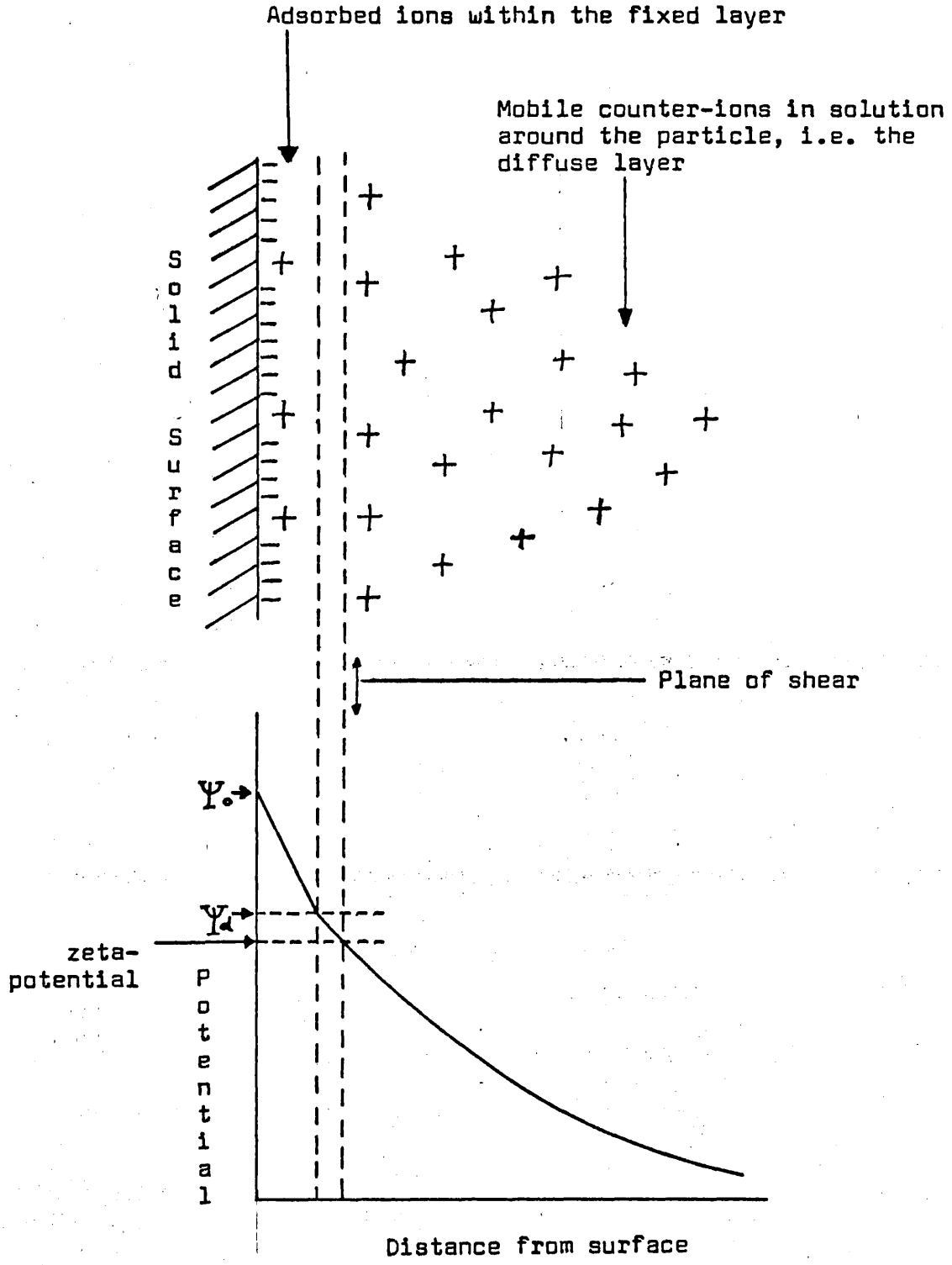
thickness of the double layer, an equilibrium in the diffuse layer being maintained between the opposing forces of the potential field which tends to order the ions and the forces of thermal motion which tend to redistribute them randomly. Stern (1924) produced a model for the double layer which allows for the finite size of ions. Thus the fall in potential at the interface can be divided into two parts, a sharp fall over the molecular condenser in contact with the surface where ions are fixed to the solid surface and then an exponential decrease over the diffuse outer atmosphere of the Gouy-Chapman theory (Figure 1.7). Electrical neutrality is maintained as the surface charge is of equal magnitude, although opposite in sign, to the total charge of the fixed and diffuse regions of the double layer.

Electrophoresis occurs when, under the influence of an applied electric field, the charged solid phase and the liquid phase move relative to one another. This causes the development of a shear plane within the double layer. The potential at this plane is known as the zeta potential and the magnitude of the zeta potential determines particle velocity under an applied electric field.

In biological cells the charge originates from ionogenic groups such as carboxyl, phosphate and amino, located in the surface structures. For such surfaces there is no charge due to adsorption (Gittens, 1962).

Figure 1.7

The structure of, and the electrical potentials associated with, the double layer at a solid-electrolyte interface



1.7 Particulate Microelectrophoresis

In this investigation a method based on that of Ellis (1911) was used to determine the electrophoretic mobility of bacterial cells. A potential difference is applied across a bacterial suspension contained in a closed glass chamber. The migration of the suspended bacteria is observed with a microscope and individual cells are timed moving across a graticule in the eyepiece.

When an electric field is applied across a system, not only do the bacteria move relative to the suspension medium, but the suspension medium also moves relative to the glass surface of the observation chamber as a result of electroosmosis of the suspension medium. Thus the observed particle velocity, v_o, is given by the expression:

$$v_o = v_L + v_P$$

where v_L is the velocity of the suspension medium relative to the glass surface, and v_P the velocity of the bacteria relative to the liquid, this being constant at all depths within the chamber. In dealing with a closed system, the liquid flows along the two inside faces of the chamber, towards the negative electrode and returns through the centre, causing a variation of v_L and hence of v_o with depth. As the liquid is being continuously deformed within a closed system, there must be a plane at which the liquid is stationary. Thus $v_L = 0$ and $v_o = v_P$ at this level. For the flat cell of rectangular cross section used, this plane is observed at two levels equidistant from the cell centre. Komagata (1933) derived an expression for the position of the stationary levels

in a cell with a width/thickness ratio, K, such that:

$$\frac{s}{d} = 0.500 \pm (0.0833 + \frac{32}{\pi^5 K})^{\frac{1}{2}}$$

where s/d is the fractional depth measured from the inside surface. For a cell with a K value greater than 20, the stationary levels are at 0.21 and 0.79 of the total depth from an inside face (Abramson, 1934), and the mean particle velocity, v, may be determined by the method of Ellis, whereby

$$v = \frac{1}{x_1} \int_0^{x_1} v_o \, dx$$

where x_1 is the cell depth, and v_o the velocity of the particle observed at depth x. For a symmetrical cell the curve of v_o against x should be a parabola symmetrical about the centre.

Cell and electrode design has been extensively reviewed by James (1957) and Seaman (1965). With the apparatus used in this investigation (Gittens and James, 1960) the applied field strength (X) is best calculated from conductance and current data, using the equation:

$$X = \frac{I}{q\,k}$$

where I/A is the current, k the conductivity of the suspension medium, and q/m^2 the cross sectional area of the cell. Moyer (1936) showed that the use of the applied voltage to measure the field strength may lead to large errors (up to 50%), as slight changes

in the electrodes may result in large changes in the field strength, without affecting the applied voltage.

1.8 Application of Microelectrophoresis to the Study of the Bacterial Cell Surface

It is essential that electrophoretic measurements be made on cells suspended in a medium of known chemical composition, pH and ionic strength.

By varying the pH of the suspension medium whilst maintaining the ionic strength at a constant value, valuable information about the nature of surface ionogenic groups on cells of fixed age may be obtained. For example the ionogenic groups of a carboxyl-amino type surface will be titrated according to the equations:

$$RCOO^- + H^+ \rightleftharpoons RCOOH$$

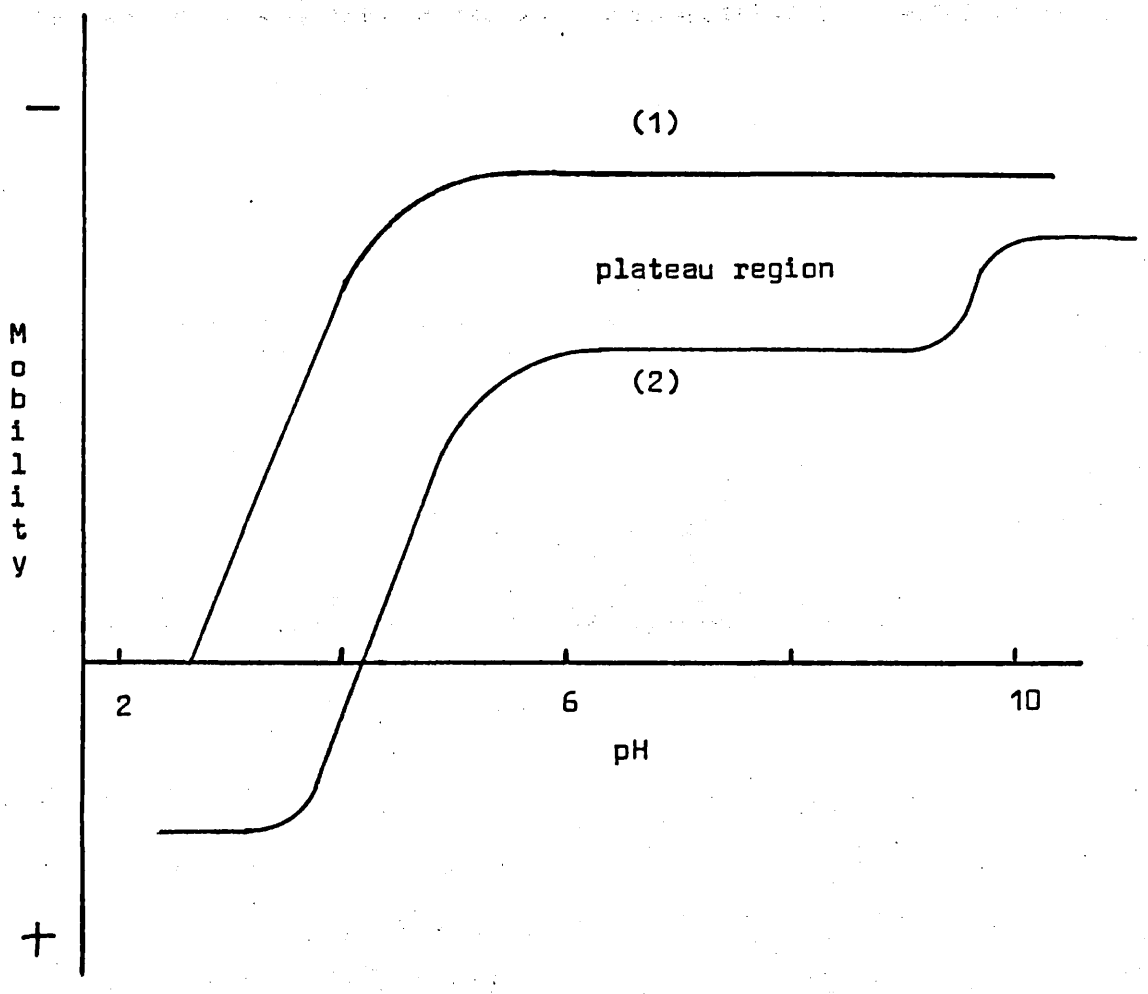
$$RNH_2 + H^+ \rightleftharpoons RNH_3^+$$

The sigmoid type pH-mobility curve typical of a carboxyl-amino type surface is shown in Figure 1.8. At low pH values the surface charge is due to the positively charged amino groups but as the pH increases (hydrogen ion concentration decreases), the positive charge becomes reduced and the negative charge increases as the carboxyl groups ionise. An isopotential point occurs between pH 4 and 5, the exact value being determined by the relative numbers of carboxyl and amino groups present and their respective pK values. Between pH values 5 and 9, a plateau region occurs

Figure 1.8

(1) The pH-mobility curve characteristic of a surface with ionogenic carboxyl groups only.

(2) The pH-mobility curve characteristic of a surface with both ionogenic amino and carboxyl groups.



where both the carboxyl and amino groups are fully ionised, the mobility then depends on the relative numbers of carboxyl and amino groups present. Beyond pH 9 the effective negative charge increases due to the suppression of the amino group ionisation. Such curves have been obtained experimentally by Plummer et al (1962) and by Hill et al (1963) for the carboxyl-amino surface of cells of Streptococcus pyogenes.

Curve 1 (Figure 1.8) is a typical titration curve for a surface with carboxyl groups only. At low pH values the carboxyl groups are undissociated and as the pH of the suspension medium is increased, the carboxyl groups become ionised, causing the negative charge to rise. This characteristically simple type of pH-mobility curve was obtained for the polysaccharide surface of Klebsiella aerogenes by Lowick and James (1957) and for human erythrocytes (Seaman, 1965).

The pH-mobility curves of cells of P. aeruginosa are unlike those of model surfaces and are dependent on the gentamicin resistance of the cells. Typically, cells of gentamicin-sensitive strains of P. aeruginosa are characterised by a steep increase in the negative mobility between pH 2 and 5 and a maximum negative mobility at around pH 5.5 to 6.5. Each curve also shows a minimum negative value between pH 7.5 and 8.5. The pH-mobility curves of cells of gentamicin-resistant strains of P. aeruginosa are characterised by an increased negative mobility over the pH range 3 - 7.5 and a maximum mobility value at pH 7.5 - 8.5 with decreasing mobility values at higher pH values; no minimum value has been observed (Pechey and James, 1974). Clearly such pH-mobility curves

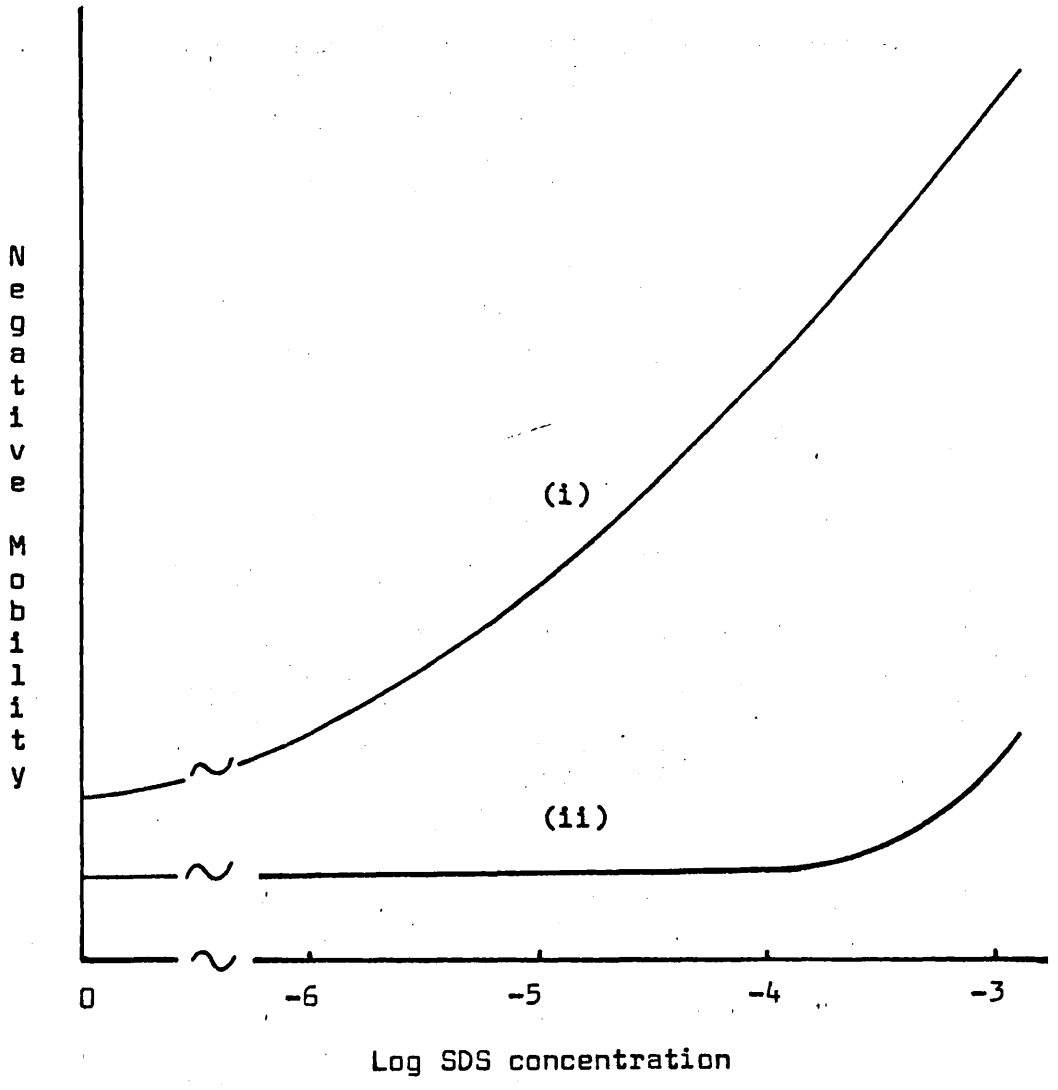
cannot be explained in terms of the simple titration of the surface ionogenic groups as no plateau mobility values were observed.

When the pH of the suspension medium is varied, care must be taken not to cause irreversible changes in the surface by using extreme pH values. After suspending cells at a high or low pH, the reversibility must be checked by rewashing and measuring the mobility of the cells at pH 7. The mobility value should not differ significantly from that of control cells at pH 7. An irreversible change indicates cell surface and maybe cell wall damage, making useful interpretation difficult. In all the work reported, pH-mobility curves were plotted over a range where no irreversible surface changes occurred. The variation of mobility with pH will only give information on the nature and quantity of surface ionogenic groups.

Powney and Wood (1940) observed an increase in the negative mobility of oil droplets when measured in the presence of the detergent sodium dodecyl sulphate (SDS). This was caused by the solution of the hydrophobic hydrocarbon chains of the surface active agent (SDS) in the oil droplets, with the sulphate groups orientated outwards into the medium, thus contributing to the negative mobility of the droplets. Dyar (1948) also observed a similar increase in negative mobility in the presence of SDS for bacterial cells which had large amounts of surface lipid (Figure 1.9). The measurement of mobility values in the presence and absence of 10^{-4} mol dm^{-3} SDS has been used to detect surface lipid on the cell surface of strains of Staph. aureus (Hugo and Stretton, 1966), K. aerogenes (Lowick and James, 1957) and P. aeruginosa (Pechey

Figure 1.9

The variation of electrophoretic mobility with the concentration of SDS in the buffer solution for cells (i) with, and (ii) without surface lipid (diagrammatic)



et al, 1974). At SDS concentrations above 10^{-3} mol dm^{-3}, increased and variable mobility values occur both for surfaces with and without lipid; this is caused by non-specific adsorption and/or cell lysis.

1.9 Objects of the Present Investigation

In a previous study (Pechey and James, 1974; Pechey et al, 1974) it was shown that changes in the surface structure of cells of P. aeruginosa as revealed by pH-mobility studies, can be correlated with differing levels of gentamicin-sensitivity/resistance in these cells.

The objects of this investigation are:

(a) to confirm the previous results using a larger number of gentamicin-resistant and gentamicin-sensitive strains of P. aeruginosa;

(b) to investigate aspects of the effects of divalent metal ions on cells of P. aeruginosa; previous reports (Zimelis and Jackson, 1973) indicate that the presence of magnesium and calcium ions in the growth medium has an enhancing effect on gentamicin-resistance in this organism;

(c) to study a series of strains of P. aeruginosa which possess R-factor mediated gentamicin-resistance, with particular reference to any characteristic surface properties that these strains may display.

Most emphasis has been placed on the use of particulate electrophoresis to detect changes in bacterial surface structures

but other techniques such as atomic absorption spectrophotometry, microcalorimetry and various microbiological techniques have also been used.

CHAPTER 2

EXPERIMENTAL TECHNIQUES

2.1 BACTERIOLOGICAL TECHNIQUES

2.1.(a) Strains

The bacterial strains used in this work are listed in tables 2.1, 2.2 and 2.3. They were obtained from the following sources:

(i) Mr. J. T. Magee, Nicholas Research Institute, 225 Bath Rd, Slough, Buckinghamshire;

(ii) Dr. E. Schoutens, Brugmann Hospital, Institut Pasteur du Brabant, Bruxelles, Belgium;

(iii) Dr. E. J. L. Lowbury, M.R.C. Industrial Injuries and Burns Unit, Birmingham Accident Hospital, Birmingham, Warwickshire;

(iv) Mr. Slocombe, Beecham Research Laboratories, Brockham Park, Betchworth, Surrey;

(v) Dr. A. R. Ronald, Health Sciences Centre, University of Manitoba, Winnipeg, Manitoba R3E 0Z3, Canada;

(vi) Dr. J. C. Loper, Department of Microbiology, University of Cincinnati Medical Center, Cincinnati, Ohio 45267;

(vii) Dr. G. A. Jacoby, Massachusetts General Hospital, Boston, Massachusetts 02114;

(viii) Dr. L. E. Byran, Department of Medical Bacteriology, University of Alberta, Edmonton, Canada;

(ix) Dr. M. McDonough, Department of Botany, Bedford College, Regents Park, London NW1.

Table 2.1

Bacterial strains

Strain	Gentamicin resistance/sensitivity	Source
P.aeruginosa		
1	S	(i)
2	S	(i)
3	S	(i)
72	S	(i)
75	S	(i)
96	S	(i)
100	R	(ii)
101	R	(ii)
102	S	(ii)
B.32	S	(iii)
103	R	(iv)
104	R	(iv)
105	R	(iv)
106	S	(i)
107	R	(i)
Smith 2234	R	(v)
Lesko 5681	S	(v)
Lesko 5892	S	(v)
Tait 6767	S	(v)
Tait 6903	S	(v)
Cheekie 6428	S	(v)
Cheekie 7247	S	(v)
Kuch 6925	S	(v)
Kuch 7051	S	(v)

Table 2.2

Abbreviations :-

Gm - gentamicin

Rm - rifampicin

Cb - carbenicillin

Sm - streptomycin

Tc - tetracycline

Su - sulphonamide

Cm - chloramphenicol

Km - kanamycin

Table 2.2

P.aeruginosa strains used for R-factor studies

Strain	Antibiotic characteristics	Source
PL1	Mutant isolate of 280, Gm sensitive, Rm resistant	(vi)
PL1 (RPL11)	Transconjugant selected using PL11 as donor	(vi)
PL11	RPL11 (Gm, Cb, Sm, Tc, Su, Cm)	(vi)
PU21	Gm sensitive, Rm resistant	(vi) & (vii)
PU21 (pMG1)	Transconjugant selected using Capetown no.18 as donor	(vii)
Capetown no.18	pMG1 (Gm, Sm, Su)	(vii)
PU21 (pMG2)	Transconjugant selected using Stone no.130 as donor	(vii)
Stone no.130	pMG2 (Gm, Sm, Su)	(vii)
PU21 (R64)	Transconjugant containing R64 (Cb, Cm, Gm, Km, Su) R64 originally from Esch. coli K-12 J5(R64)	(vii)
280	Gm sensitive, Rm resistant	(viii)
280 (R130)	Transconjugant selected using 130	(viii)
1310	Gm sensitive, Rm resistant	(viii)
1310 (R130)	Transconjugant selected using 130	(viii)
130	R130 (Sm, Su, Gm)	(viii)
280 (R151)	Transconjugant selected using POW 151	(viii)
POW 151	R151 (Sm, Su, Cb, Gm)	(viii)
1/Cb	Cb resistant	Strain 1
3/Cb	Cb resistant	Strain 3

Table 2.3

Other Bacterial Strains

Strain	Source
Escherichia coli	
K 12	(ix)
B. CL 224	(ix)
K. CL 265	(ix)
NCIB 86	(ix)
Klebsiella aerogenes	
NCTC 418	NCTC

The strains of Pseudomonas aeruginosa were grown overnight on nutrient agar slopes in loosely capped universal bottles at 37°C and then stored at 5°C.

The strain of Klebsiella aerogenes used for calibration of the electrophoresis apparatus was stored at 5°C as a 50 cm^3 nutrient broth culture in a tightly capped 100 cm^3 medical bottle.

The strains of Escherichia coli were received on nutrient agar plates and were subsequently stored on nutrient agar slopes at 5°C.

All strains were routinely maintained by bimonthly subculturing onto fresh agar slopes or into fresh nutrient broth for K. aerogenes. However, when strains were in continual use they were subcultured more frequently.

2.1.(b) Media

Nutrient agar was prepared by adding 25 g of powdered Oxoid Nutrient Broth No. 2 to 1 dm^3 distilled water, and then adding Oxoid Agar No. 1 to give 1% w/v agar. The solution was distributed in 350 cm^3 lots into 500 cm^3 screw top bottles and sterilised by autoclaving. Agar plates and agar slopes were prepared by melting the stock agar in an autoclave and aseptically pipetting 10 cm^3 portions into sterile plastic petri dishes or into sterile 25 cm^3 universal bottles which were then sloped before the agar solidified.

Nutrient broth was prepared by adding 13 g of Oxoid Nutrient Broth (code CM 1) powder to 1 dm^3 distilled water. This was then

distributed into bottles in appropriate volumes (usually 50 cm^3 in 100 cm^3 medical bottles) and sterilised by autoclaving.

Agar plates containing gentamicin were prepared by adding the appropriate weight of gentamicin to a given volume of melted agar. The agar was then re-sterilised by autoclaving, before pouring into petri dishes.

Agar plates containing carbenicillin or rifampicin, which are both heat labile antibiotics, were prepared by autoclaving the required volume of agar and then cooling this to between 40-50°C before adding the weighed antibiotic aseptically.

Eosin methylene blue,(EMB),agar was prepared by adding 37.5 g of Oxoid Eosin methylene blue agar powder to 1 dm^3 distilled water, the solution was boiled to dissolve the solid and then autoclaved. The liquid agar was cooled to 60°C and vigorously shaken to re-oxidise the indicator and finally aseptically pipetted into sterile plastic petri dishes.

The synthetic medium was made up using Analar grade chemicals as follows :-

Sodium barbitone	7.0 g
Potassium chloride	3.0 g
Sodium citrate ($2H_2O$)	0.5 g
Ammonium sulphate	1.0 g
Magnesium sulphate ($7H_2O$)	0.0862 g
Glucose	2.0 g

These were dissolved in glass distilled water and made up to 970 cm^3, the pH being adjusted to 7.0 by the inclusion of HCl. This basal medium was supplemented when required with calcium ions in the form of calcium chloride, magnesium ions as magnesium

sulphate (in addition to the magnesium ions in the basal medium), zinc ions as zinc sulphate, iron(II) ions as iron(II) sulphate and potassium ions as potassium chloride (again, in addition to the potassium ions in the basal medium). This solution was then autoclaved, and after cooling 30 cm^3 of sterile 10% sodium glycerophosphate solution was added to give a final volume of 1 dm^3. The finished medium was then distributed aseptically as required into sterile glassware, usually in 50 cm^3 batches in 100 cm^3 medical bottles.

2.1.(c) Growth of strains for experimental purposes.

Unless otherwise stated all agar plates were inoculated from an initial culture or storage culture with a sterile platinum loop and incubated for 18 h at 37°C.

Similarly, unless otherwise stated nutrient broth cultures were inoculated from the parent culture using a sterile platinum loop and incubated for 18 h at 37°C with the bottles sloped and loosely capped.

Synthetic media cultures were grown (again unless otherwise stated) by inoculating 50 cm^3 starter cultures from the storage culture with a sterile platinum loop and incubating for 18 h at 37°C with the bottle sloped and loosely capped. The experimental cultures (again 50 cm^3) were then grown from the starter by the same procedure, except that the period of incubation was 24 h.

When larger volumes were required, 300 cm^3 volume cultures of nutrient broth or synthetic medium were used. An overnight starter

culture was grown in a 50 cm^3 volume of the appropriate medium, using the technique described above. 2.5 cm^3 of this culture was then used to inoculate 300 cm^3 of medium, contained in a 1 dm^3 flask. The culture was then stirred with a magnetic stirrer and incubated at 37 °C in a water bath for 18 h for nutrient broth, or until the late exponential phase for synthetic media.

2.1.(d) Determination of growth curves for growth in synthetic medium.

A 300 cm^3 sample of synthetic medium (either calcium-deficient or calcium sufficient, 22.5 mmol dm^{-3} Ca^{2+}) was inoculated with P. aeruginosa (strain B32 or 107) (see 2.1.(c)). The culture was incubated at 37 °C with vigorous stirring and small samples were taken aseptically at about 1 h intervals and the absorbance of each suspension read at 625 nm against a blank of distilled water, using an S.P.600 spectrophotometer. A growth curve was plotted for each strain in each medium of absorbance against time.

2.1.(e) Growth of cells during training to carbenicillin.

P. aeruginosa strains 1 and 3 were trained, using the method described by Rolinson et al (1960), to develop a resistance to carbenicillin, thus enabling them to grow in the presence of 800 μg cm^{-3} of carbenicillin in nutrient agar.

Initially the strains were grown at 37 °C on nutrient agar containing carbenicillin at half the minimum inhibitory concentration (MIC). At first, sparse and slow growing cultures were obtained,

but by using these to inoculate the next generation, at the same concentration, larger and faster growing colonies were produced. This procedure was then repeated in stepwise fashion, each time doubling the concentration of antibiotic, until the strains grew vigorously on nutrient agar containing 800 $\mu g\ cm^{-3}$ of carbenicillin. The strains were then designated 1/Cb and 3/Cb. These were maintained on nutrient agar slopes in the presence of carbenicillin.

2.1.(f) Measurement of MIC of antibiotics

The minimum inhibitory concentration, MIC, of antibacterial agents (i.e. the lowest concentration of antibiotics required to prevent growth) was measured by the method of Gould (1960)

A series of metal capped test tubes containing a range of concentrations of antibiotic in 5 cm^3 of nutrient broth or synthetic medium were prepared by two times serial dilution from one tube to the next. Pure antibiotic free medium was used as control. When gentamicin in nutrient broth was used the tubes were autoclaved. When synthetic medium or heat-labile rifampicin or carbenicillin were used, a stock solution of antibiotic was prepared by aseptically weighing the antibiotic into sterile medium for subsequent, aseptic dilutions.

Each tube was inoculated with approximately 10^5 bacteria from an 18 h culture (grown in the appropriate medium). The tubes were then incubated at 37 ^{o}C for 48 h and the lowest concentration

of antibiotic in which no growth (i.e. no turbidity) was observed, was recorded as the MIC.

2.1.(g) Transfer of R-factors between strains of P. aeruginosa.

Gentamicin-resistant strains of P. aeruginosa were used as R-factor donors and strains sensitive to gentamicin but resistant to another antibiotic were used as acceptors. These latter were strain PU21 which is rifampicin resistant (rifampicin M I C $>1600\ \mu g\ cm^{-3}$) and strains 1/Cb and 3/Cb which were trained from strains 1 and 3 respectively to be carbenicillin resistant (carbenicillin MIC $>800\ \mu g\ cm^{-3}$).

Exponentially growing nutrient broth cultures of a donor and an acceptor strain were mixed and incubated together in nutrient broth in a 100 cm^3 medical bottle lying flat at 37°C and then spread onto agar plates containing gentamicin and also rifampicin or carbenicillin for counterselection of the donor. Thus only transconjugants grew on these plates. The conditions of the experiments varied slightly but usually 15-16 h cultures were used for matings although in one experiment 9 h cultures were used. Similarly 0.2 cm^3 of donor culture and 0.2 cm^3 of acceptor culture were usually incubated with 1.6 cm^3 of nutrient broth, but in one experiment the donor to acceptor ratio was 1:3 (i.e. 0.1 : 0.3 cm^3). For controls 0.4 cm^3 donor or acceptor culture was incubated with 1.6 cm^3 nutrient broth. The mating mixture was incubated for 5 h or 24 h. After mating (in some cases also after 5 h of a 24 h mating) 0.1 cm^3 of culture was inoculated onto selection plates.

The gentamicin concentration in these plates was either 5 or 8 $\mu g\ cm^{-3}$ and the carbenicillin concentration 800 $\mu g\ cm^{-3}$ or the rifampicin concentration 300 $\mu g\ cm^{-3}$.

2.1.(h) Transfer of R-factors from P. aeruginosa to Esch. coli

Four gentamicin-sensitive strains of Esch. coli were used as potential acceptors of R-factors and gentamicin-resistant strains of P. aeruginosa were used as possible donors.

0.5 cm^3 of an overnight nutrient broth culture of a P. aeruginosa donor strain was mixed with 0.5 cm^3 of an overnight nutrient broth culture of an Esch. coli acceptor strain in a volume of 4.0 cm^3 nutrient broth in a 100 cm^3 medical bottle. This mating mixture was then incubated in one of several ways in an attempt to effect R-factor transfer. The methods used were :

(i) the mixed culture was incubated at 37°C overnight, with the bottle lying flat;

(ii) the mixed culture was incubated at 37°C for 2 h and then shaken at room temperature for 16 h;

(iii) the mixed culture was shaken at room temperature for 2 h and then incubated at 37°C for 16 h;

(iv) the mixed culture was shaken at 37°C for 16 h.

In one series of experiments the donor cells were ruptured with a French Press before mixing with the acceptor cells, in an attempt to release R-factors and thus facilitate their uptake by the acceptor cells.

After mating, 0.1 cm^3 of each mixed culture was spread onto several plates containing EMB agar (section 2.1.(b)) and also gentamicin at a concentration of 10 $\mu g\ cm^{-3}$. For controls, cultures of Esch. coli or P. aeruginosa were spread onto similar plates. P. aeruginosa cells grew on all plates but only Esch. coli cells which had attained gentamicin resistance would grow on the plates containing gentamicin. P. aeruginosa cells can be distinguished from Esch. coli cells growing on EMB agar as they do not ferment lactose and thus appear translucent. Esch. coli cells on the other hand do ferment lactose and appear deep blue with a green metallic sheen in reflected light.

2.1.(i) Cleaning and sterilizing of apparatus.

All glassware was washed and scrubbed in tap water and then rinsed twice in distilled water. It was then dried overnight in an oven at 100°C.

Glassware, solutions and growth media required in a sterile condition were autoclaved at 15 lb in^{-2} for 30 minutes. Pipettes and Pasteur pipettes were plugged with cotton wool and put into metal canisters prior to autoclaving.

Contaminated glassware was autoclaved before washing. Contaminated disposable apparatus was first immersed in a 1% lysol solution.

2.2 Microelectrophoresis Techniques.

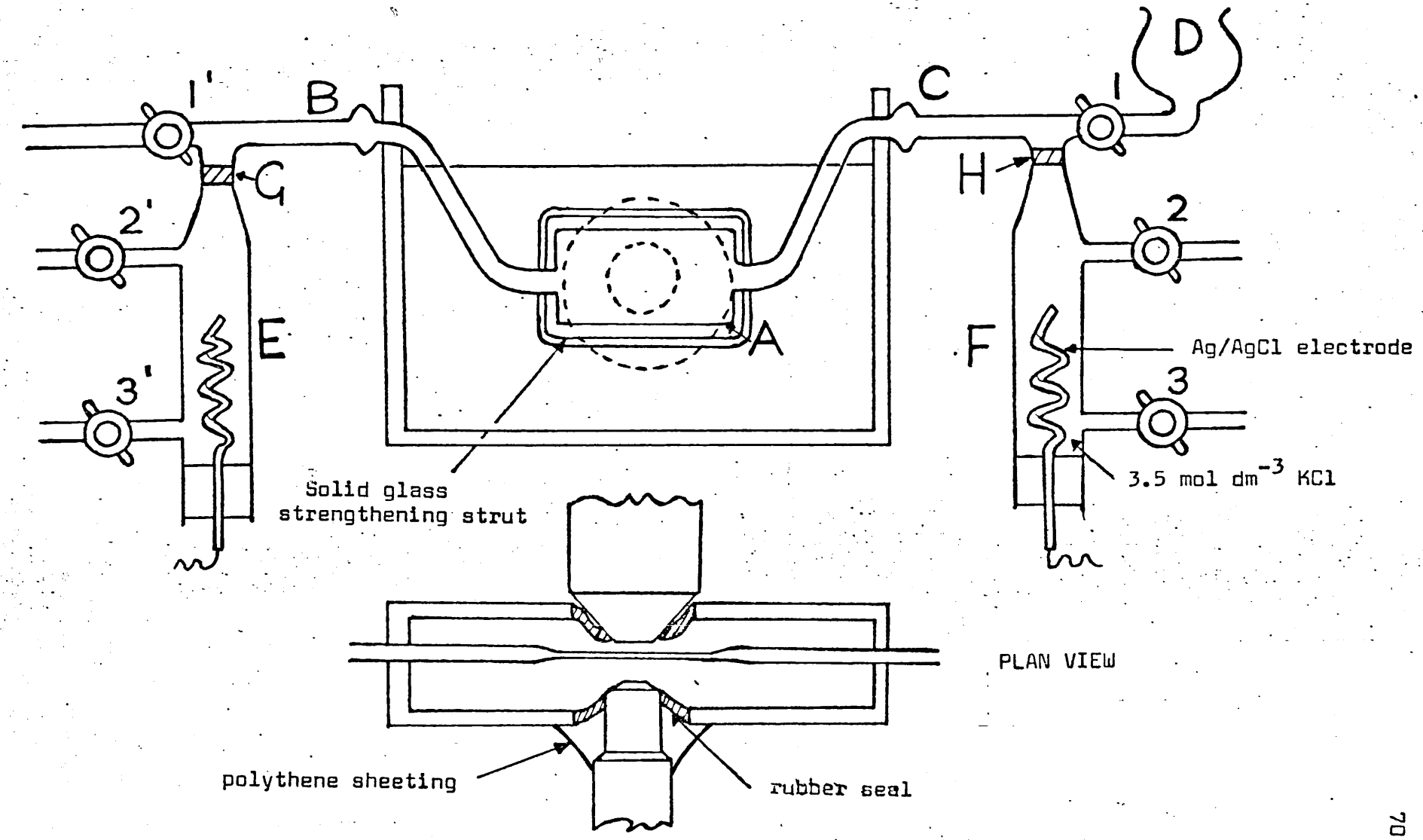
2.2.(a) General description

The apparatus is shown in Figure 2.1. The cell and electrode compartments used were those developed by Gittens and James (1960).

The electric field was applied across a suspension of bacterial cells contained in the glass observation chamber (A). The cells were observed under a microscope and the velocity of a cell due to a known applied electric field was determined by timing an individual cell across a given number of squares in an eyepiece graticule.

The rectangular chamber (A) was made from two optically flat Hysil plates (40 x 25 x 0.5 mm), fused to give a separation of 0.5 mm. Glass rods sealed around the outside of the cell afforded protection and helped strenghten the structure. The side arms were constructed by sealing 10 mm bore Pyrex tubing directly onto the chamber (A). These were bent in the plane of the cell so that the chamber could be completely immersed in a water bath. The side arms were connected to the electrode compartments via hemispherical Quickfit joints (B and C). These were greased and metal clips attached to ensure leak proof seals. Suspensions were introduced into the cell from the reservoir (D) by opening taps 1 and 1'. A constant electric field was applied between the Ag. AgCl/ KCl electrode systems in compartments (E) and (F). The electrodes consisted of 25 cm lengths of 2 mm diameter pure silver wire in

Figure 2.1 Microelectrophoresis cell and electrode compartments



the form of a coil mounted on rubber bungs with some wire protruding for electrical connections. Initially the wire was cleaned with 50% nitric acid, then after coiling the electrodes were anodised in series in 0.1 mol dm^{-3} hydrochloric acid using a platinum cathode, until a purple-grey coating of AgCl was deposited on each electrode. The electrodes in their rubber bungs, were then inserted in the electrode compartments which had been filled with potassium chloride solution (3.5 mol dm^{-3}). Electrical contact with the bacterial suspension was made through the sintered glass discs (G and H).

Since the viscosity and conductivity of the buffer solution, and hence cell electrophoretic mobility are temperature-dependent, it is essential to maintain the bacterial suspension at a constant temperature. For this purpose the cell was immersed in a small water bath which was maintained at 10 $\pm$ 0.5^{o}C by water circulated from a large water bath. The water in this latter was maintained at constant temperature by a Grant cooling unit and a Shandon heating and stirring unit, working in opposition.

A Rank Bros. Particle Micro-electrophoresis apparatus Mark II was used and this included the small water bath and circulating pump, electrical circuit, microscope unit and timing device. The small perspex water bath had a circular well at the front to accommodate the objective lens of the microscope. To prevent condensation in this well and on the objective lens, due to the low temperature of the circulating water, this cavity was covered over with polythene sheeting and a gentle current of warm air blown in from a hot air blower. The objective lens penetrated the sheeting and could be moved freely. The microscope used gave

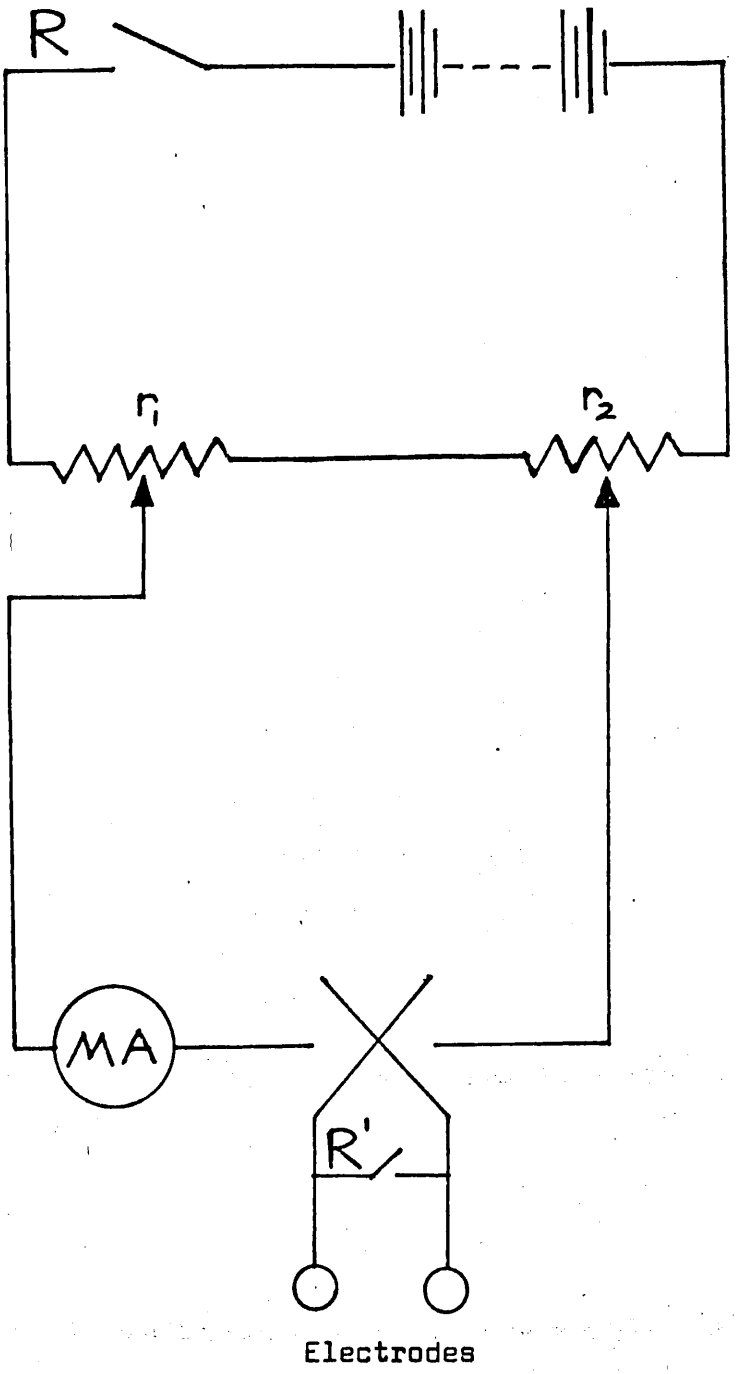
an overall magnification of x 600 and a low power dark ground illuminating condenser was employed. The light source was a 12 V, 100 watt lamp; to prevent the intense light source from causing temperature rises and convection currents in the observation chamber, a quartz disc and glass heat filter were placed in front of the lamp.

The electrical circuit is shown diagrammatically in Fig. 2.2. The mains voltage was rectified and the DC voltage controlled by a variable resistance. The applied potential could be reversed using the switch k', which also allowed the electrodes to be shorted when not in use, thus preventing polarisation. A milliameter was used to measure the current flowing through the electrophoresis chamber. The time taken by a bacterium crossing a given number of squares on the eyepiece graticule was recorded using an electromagnetic timer, operating from the mains frequency.

Before assembly of the electrophoresis chamber between the electrodes, cells of K. aerogenes were dried onto the inner surfaces of the observation chamber. The chamber was mounted in the water bath in a lateral position between the condenser and objective lens of the microscope. Bolts, attached to perspex bars, held the chamber firmly in the water bath; protective sponge foam was wrapped around the arms before tightening the bolts. Care was taken to ensure that the cell was horizontal when viewed from the front, vertical when viewed from the side and at right angles to the optical axis of the microscope.

Figure 2.2

The electrical circuit for the Microelectrophoresis apparatus



2.2.(b) Mode of operation

The following procedure was carried out each time before use of the apparatus, to ensure good and reproducible electrical connections through the sintered glass discs.

About 50 cm^3 of KCl solution from the reservoir were flushed through each electrode compartment by opening taps 3 and 2, and 3' and 2'; taps 2 and 2' were then closed and tap 1' opened, thus forcing the solution through the sintered glass discs. A large volume (50 cm^3) of distilled water was then flushed through the observation chamber to remove electrolyte which had been forced through the sintered discs. Finally the chamber was filled with buffer solution at the temperature, pH and ionic strength of the suspension to be examined. Movement of the objective by coarse and fine micrometer screws enabled the microscope to be focused on the bacteria on the front and back surfaces of the chamber and the depth of the chamber was recorded from the calibrations on the fine adjustment screw. In practice there was found to be little variation of depth from day to day. This scale was also used to focus the microscope on the required calculated position of the stationary level within the cell; all mobility measurements were made at the front stationary level.

The cell suspension to be investigated was introduced into the observation chamber and allowed to come to thermal equilibrium. The current was then adjusted to give a time of 2-4 s for the bacterial cells to cross a given number of squares of the graticule. At least 20 cells were timed for each suspension, timing the cells in each direction by reversing switch R' (i.e. at least

40 timings were made). The suspension was then flushed out through the rubber tubing into a reservoir below the bench containing lysol and the chamber left filled with distilled water. The chamber was fed by gravity, but to remove air bubbles a partial vacuum was also applied. Great care was taken at all times to exclude air bubbles from the closed system and to prevent the introduction of grease into the observation chamber.

2.2.(c) Calibration of the apparatus

The symmetry of the observation chamber was checked by determining the velocity-depth curve using cells of K. aerogenes. The bacteria were grown in nutrient broth at 37 ^{o}C for 18 h, harvested, washed twice with barbiturate-acetate buffer solution (pH = 7.0, I = 5 x 10^{-3} mol dm^{-3}) and resuspended in the buffer solution. At various known cell depths bacteria in focus were timed across a fixed distance in the eyepiece graticule at constant field strength. The reciprocal of the excursion time was plotted against the fractional cell depth from the centre of the cell. Typical results for the cell used are shown in Figure 2.3.

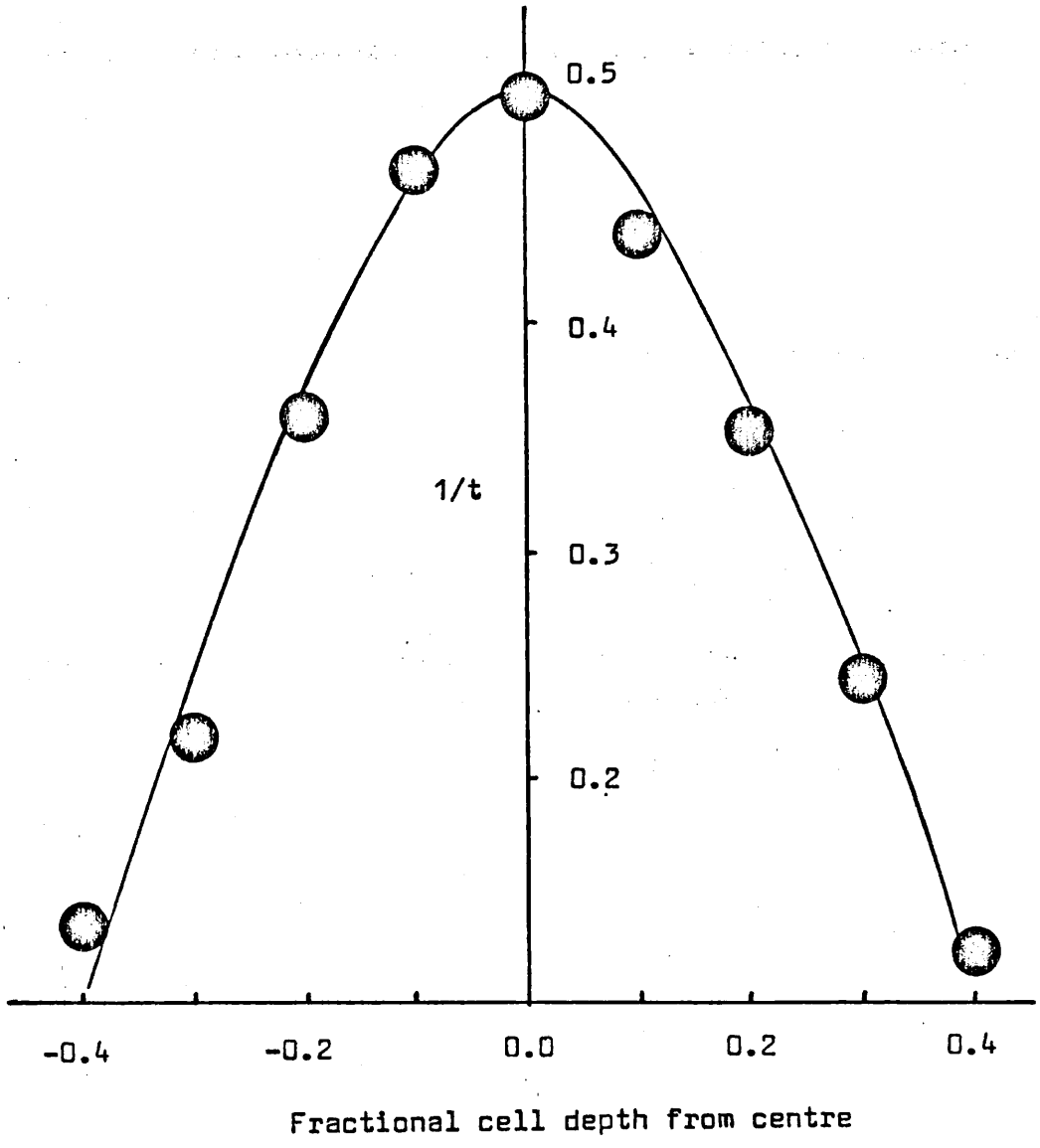
The equation of the velocity-depth parabola is of the form:

$$\frac{1}{t} = a + bx + cx^2 \qquad (2.1)$$

Where x denotes the fractional depth from the centre of the cell and t the excursion time measured at this depth.

Figure 2.3

Velocity-depth curve for cells of K. aerogenes measured at 25 ^{o}C in barbiturate-acetate buffer solution (pH = 7.0, I = 5 x 10^{-3}mol dm^{-3})



The equation obtained for the cell in use was:

$$\frac{1}{t} = 0.468 + 0.0033x - 2.266x^2 \qquad (2.2)$$

The small value of "b" indicates that the calculated and geometrical centres are very close and therefore the chamber can be accepted as symmetrical. Integration of equation 2.2 over the complete depth gives the mean reciprocal time as 0.279 s^{-1}. Substitution of this value back into equation 2.2 gives values of x corresponding to the positions of the two stationary levels of +0.289 and -0.288 from the centre of the chamber. Therefore, the stationary levels are at fractional depths of 0.211 and 0.788 from the front inside surface of the cell, these positions being in close agreement with those predicted theoretically for a cell of the same size (Komagata, 1933).

The electrophoretic mobility of a particle, $\bar{v}/m^2s^{-1}V^{-1}$, is defined as the particle velocity, v/ms^{-1}, per unit potential gradient, X/Vm^{-1}. It is given by the expression:

$$\bar{v} = \frac{v}{X} = \frac{nL}{t} \cdot \frac{qk}{I} = \frac{nL}{t} \cdot \frac{qJG}{I} \qquad (2.3)$$

Where nL/m is the distance travelled (n is the number of squares of side L/m) in time t/s; q/m^2 is the cross sectional area of the chamber, and I/A is the current flowing. $k/ohm^{-1}\ m^{-1}$ is the conductivity of the buffer solution obtained from the measured conductance, G/ohm^{-1} and the cell constant, J/m^{-1} of the conductance cell.

The values of G, I and t are obtained experimentally. However, since it is not possible to determine accurately the cross sectional area (q) of a rectangular observation chamber, a standard particle of known absolute mobility ($\bar{u}_s$) was used. Standard particles in suspension were timed and an apparatus constant K, which included the cell constant J of the conductance cell, was obtained; K is given by:

$$K = L\,q\,J = \frac{\bar{u}_s\ t\ I}{n\ G} \qquad (2.4)$$

Subsequent timings (t') on bacterial cells under examination were converted to mobility values using the relationship:

$$\bar{v} = \frac{K\ n\ G'}{t'I'} \qquad (2.5)$$

where the primed values are those obtained for cells in that particular suspension.

The standard particles used were cells of K. aerogenes grown for 18 h at 37 ^{o}C in nutrient broth. These were centrifuged, washed twice and resuspended in 5 x 10^{-3} mol dm^{-3} barbiturate-acetate buffer at pH 7.0. Under these standard buffer conditions the cells have an absolute mobility of -1.45 x 10^{-8} m^2 s^{-1} V^{-1} at 10 ^{o}C. This value was obtained from extensive calibration studies of suspensions of K. aerogenes against human erythrocytes as standard (Gittens, 1962 and Pechey, 1973). K was determined before each set of electrophoretic measurements was made, to ensure that there were no changes in mobility which could be attributed to changes of the apparatus constant.

All mobility values will be quoted without units and are negative values except where prefixed by +. A value of 1.2 for example means that the particle is negatively charged with an electrophoretic mobility (towards the positive electrode) of 1.2 x 10^{-8} m^2 s^{-1} V^{-1}.

2.2.(d) Buffer solutions

Barbiturate-acetate buffer solutions were used as the suspending electrolyte (Michaelis, 1931) for mobility determinations. Analar grade chemicals were used and dissolved in glass distilled water. 5 dm^3 of stock solution (I = 5 x 10^{-1} mol dm^{-3}) contained:

0.15 mol dm^{-3} sodium barbitone	154.6350 g
0.15 mol dm^{-3} hydrated sodium acetate	102.0675 g
0.20 mol dm^{-3} sodium chloride	58.4500 g

This stock solution was stored at 5 ^{o}C. The buffer solutions were prepared by diluting with distilled water to give the required ionic strength, I. The pH was adjusted using either HCl (1 mol dm^{-3}) or NaOH (1 mol dm^{-3}). An E.I.L. (Model 23A) pH meter was used to measure pH. The conductance of each buffer solution was measured in a bottle-type conductivity cell at 10 ^{o}C using a Wayne-Kerr (B221) Universal Bridge.

2.2.(e) Preparation of cell suspensions for electrophoresis

Cells of P. aeruginosa grown on nutrient agar were washed from the surface with glass distilled water, divided as required

into aliquots and centrifuged out of suspension. The cells were then washed twice in and finally suspended in buffer solutions of ionic strength 5×10^{-3} mol dm^{-3} and appropriate pH.

Cells P. aeruginosa grown in nutrient broth or synthetic medium and cells of K. aerogenes grown in nutrient broth were divided into aliquots, sedimented and then washed twice and resuspended as before.

The suspensions for mobility measurements contained about 2×10^{8} cells per cm^{3}. Mobility measurements were made as soon as possible after preparation of the suspensions.

2.2.(f) Detection of surface lipid

The surface lipid of bacteria was detected by comparing the mobility values of cells of the same culture suspended in buffer and in buffer containing a low concentration of sodium dodecyl sulphate (SDS), an anionic surface active agent.

Cells were harvested and washed in buffer solution ($I = 5 \times 10^{-3}$ mol dm^{-3}, pH 7.0) containing SDS at a concentration of 1×10^{-4} mol dm^{-3}. The mobility values of these cells were compared with those of cells suspended in buffer of the same ionic strength and pH but in the absence of SDS. An increase of negative mobility greater than 10% in the presence of SDS was considered significant; the size of the increase was taken to be an indication of the amount of surface lipid.

2.3 Chemical Analysis of Whole Bacterial Cells

2.3.(a) Growth and preparation of cells for analysis

P. aeruginosa, strain 832, was grown to the late logarithmic phase in synthetic medium. The cultures were centrifuged, and the cells washed four times with saline (1.5×10^{-1} mol dm^{-3}) and finally suspended in about 50 cm^3 of saline.

2.3.(b) Determination of bacterial dry weight

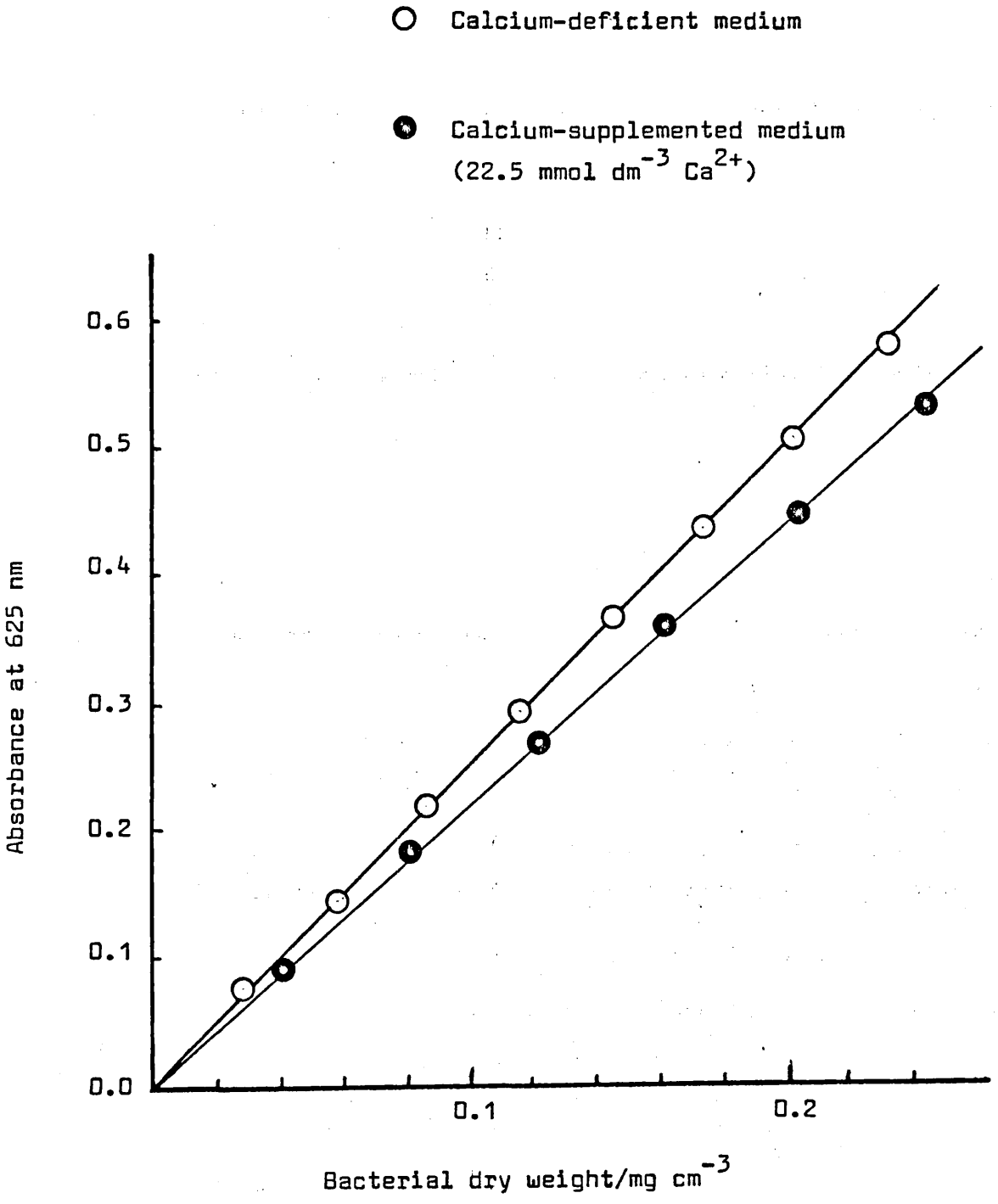
The dry weight of bacteria/mg cm^{-3} of a particular suspension was determined by one of two methods:

(i) absorbance against dry weight:

standard curves were produced for cells grown in both calcium-deficient and calcium-sufficient media. The cells were centrifuged out from the supernatant growth medium, washed four times with saline and finally resuspended in saline to give an absorbance of about 0.5 at 625 nm against a blank of saline. Samples were then diluted with saline to give a series of concentrations down to 1/8 th of the original. The absorbance of each suspension was measured. Finally 15 cm^3 samples of stock suspension and saline (for NaCl correction) were pipetted into weighed 25 cm^3 beakers, and dried to constant weight at 100 - 105 ^{o}C. Standard curves were then plotted of absorbance against dry weight (Figure 2.4) from which the dry weight/mg cm^{-3} of any suspension could be determined from its absorbance.

Figure 2.4

Dry weight v absorbance standard curves for P.aeruginosa strain 832 grown in calcium-deficient and calcium-supplemented media



(ii) direct weighing:

duplicate 15 cm^3 samples of suspension and of saline were pipetted into weighed 25 cm^3 beakers and dried to constant weight at 100 - 105 ^oC. The bacterial weight/mg cm^{-3} was thus determined directly.

2.3.(c) Chemical analysis of cell suspensions

After dry weight determination the remainder of the suspension was analysed as soon as possible. Standard curves were produced for each analytical procedure.

(i) Protein was estimated by the Biuret method. 3 cm^3 samples of suspensions (in duplicate), standard solutions (bovine serum albumin) or saline for the blank were pipetted into boiling tubes in an ice bath. 1.5 cm^3 of 3.0 mol dm^{-3} sodium hydroxide solution was then added to each tube and the tubes placed in boiling water for 10 minutes. They were then cooled in ice and 1.5 cm^3 of 5% copper sulphate solution was added to each. Each tube was sealed with parafilm and thoroughly shaken. After 15 minutes at room temperature the solutions were transferred to 25 cm^3 universal bottles and the precipitated $Cu(OH)_2$ removed by centrifugation at 6000 rpm for 15 minutes. The absorbances of the violet supernatants were measured at 550 nm against the saline blank (P.E. 124 spectrophotometer) and the protein contents read off the standard curve.

(ii) Polysaccharide was assayed by the anthrone method. Anthrone reagent was prepared fresh each day by adding 30 cm^3 of distilled water to 0.2 g anthrone in a 250 cm^3 flask and then slowly adding 100 cm^3 of concentrated sulphuric acid, cooling the flask under a running tap. Care was taken to ensure that no anthrone remained undissolved. When the solution was cool 8 cm^3 absolute ethanol was added and the reagent again cooled. 1 cm^3 samples of test suspensions (in duplicate), standard glucose solutions or saline blank, were pipetted into boiling tubes standing in an ice bath and 10 cm^3 of anthrone reagent added to each. The tubes were transferred to a boiling water bath for 10 minutes and finally cooled in ice water again. The absorbances of the green solutions were measured at 625 nm and the polysaccharide contents of the test suspensions obtained from the standard curve.

(iii) For DNA and RNA analysis, 9 cm^3 suspensions (in duplicate) were treated with perchloric acid to extract the nucleic acids. Each suspension was centrifuged and the cells suspended in 5 cm^3 0.2 mol dm^{-3} perchloric acid for 20 minutes to remove free nucleic acid. After centrifuging again the cells were transferred in 15 cm^3 of 0.5 mol dm^{-3} perchloric acid to 50 cm^3 glass stoppered flasks. These were heated at 70 ^{o}C for 30 minutes during which time they were shaken. The temperature was then slowly raised to 85 ^{o}C with the flasks being occasionally shaken. On cooling, the contents were transferred to 25 cm^3 universal bottles and the flasks rinsed

out with 2 cm^3 of 0.5 mol dm^{-3} perchloric acid; this was also added to the contents of the universal bottles. After centrifuging, the supernatants were removed from the cell debris and the DNA and RNA contents determined.

DNA was estimated by the diphenylamine method (Burton, 1956); the reagent consisted of 1 g of diphenylamine in 100 cm^3 of glacial acetic acid plus 2.75 cm^3 of concentrated sulphuric acid. This solution was stored in the dark. When it was to be used 0.1 cm^3 of a 16 mg cm^{-3} aqueous acetaldehyde solution was added to 20 cm^3 of the reagent. 3 cm^3 of this final diphenylamine solution was then added to 1.5 cm^3 of supernatant, standard DNA solutions (calf thymus DNA) or perchloric acid (for blank) in boiling tubes. The tubes were then capped with glass marbles and left overnight at 30 ^oC in a water bath. The absorbances of the blue solutions were measured at 595 nm and the DNA contents read from the calibration curve.

RNA was assayed by the orcinol method. The reagents were: orcinol (1g) in absolute alcohol (10 cm^3) and iron(III) chloride (33 mg) in concentrated hydrochloric acid (100 cm^3). 0.3 cm^3 of orcinol reagent and 3 cm^3 iron(III) chloride solution were added to 3 cm^3 of extract, standard solutions (yeast RNA) or perchloric acid in boiling tubes. These were capped with glass marbles, heated for 45 minutes in a boiling water bath and then cooled to room temperature. The absorbances of the green solutions were measured at 667 nm and the RNA contents read from the calibration curve.

CHAPTER 3

THE GENTAMICIN RESISTANCE AND SURFACE PROPERTIES OF CELLS OF PSEUDOMONAS AERUGINOSA GROWN AT 37 ^{o}C

The experiments described in this chapter were undertaken to confirm and extend the findings of Pechey (1973), Pechey et al (1974) and Pechey and James (1974), relating gentamicin-resistance or gentamicin-sensitivity in P. aeruginosa, with cell surface electrophoretic properties and surface lipid content. The bacterial strains were either gentamicin-sensitive, or gentamicin-resistant in which the resistance is probably not due to the presence in the cells of R-factors. Similar studies on strains of P. aeruginosa which were known to possess R-factor mediated gentamicin-resistance are reported in Chapter 6.

Serum levels of gentamicin greater than 12 to 15 μg cm^{-3} are known to be seriously ototoxic (Jao and Jackson, 1963; Wërsall et al, 1969) and thus clinically not acceptable. This provides a useful and practical method of defining gentamicin-resistance or gentamicin-sensitivity; a gentamicin-resistant strain of P. aeruginosa is one for which the MIC of gentamicin is greater than 12 μg cm^{-3} and a gentamicin-sensitive strain one for which the MIC of gentamicin is less than, or equal to, 12 μg cm^{-3}. This definition will be used throughout, the terms gentamicin-resistant and gentamicin-sensitive being used for growth in nutrient broth or on nutrient agar, unless otherwise stated.

3.1 MIC of Gentamicin for P.aeruginosa Strains

The results of the determination of the MIC of gentamicin, and, where applicable, the results of Pechey (1973) are shown in Table 3.1.

Table 3.1

MIC of gentamicin for strains of P.aeruginosa grown at 37 °C in nutrient broth

Strain	MIC/μg cm^{-3}	
	Current	Pechey
1	0.1	1.25
2	1.5	5
3	0.2	⩽ 0.16
3/Cb	0.2	—
72	6.2	12
75	0.8	—
96	3.1	25
100	8000	8000
101	8000	8000
102	6.2	25
B32	3.1	25
103	1250	—
104	1250	—
105	1250	—
106	1.5	—
107	25	—
Smith 2234	25	—
Lesko 5681	< 1.5	—
Lesko 5892	6.2	—
Tait 6767	< 1.5	—
Tait 6903	1.5	—
Cheekie 6428	< 1.5	—
Cheekie 7247	3.1	—
Kuch 6925	< 1.5	—
Kuch 7051	3.1	—
PU21	< 0.75	—
PL1	0.25	—
280	0.075	—
1310	0.075	—

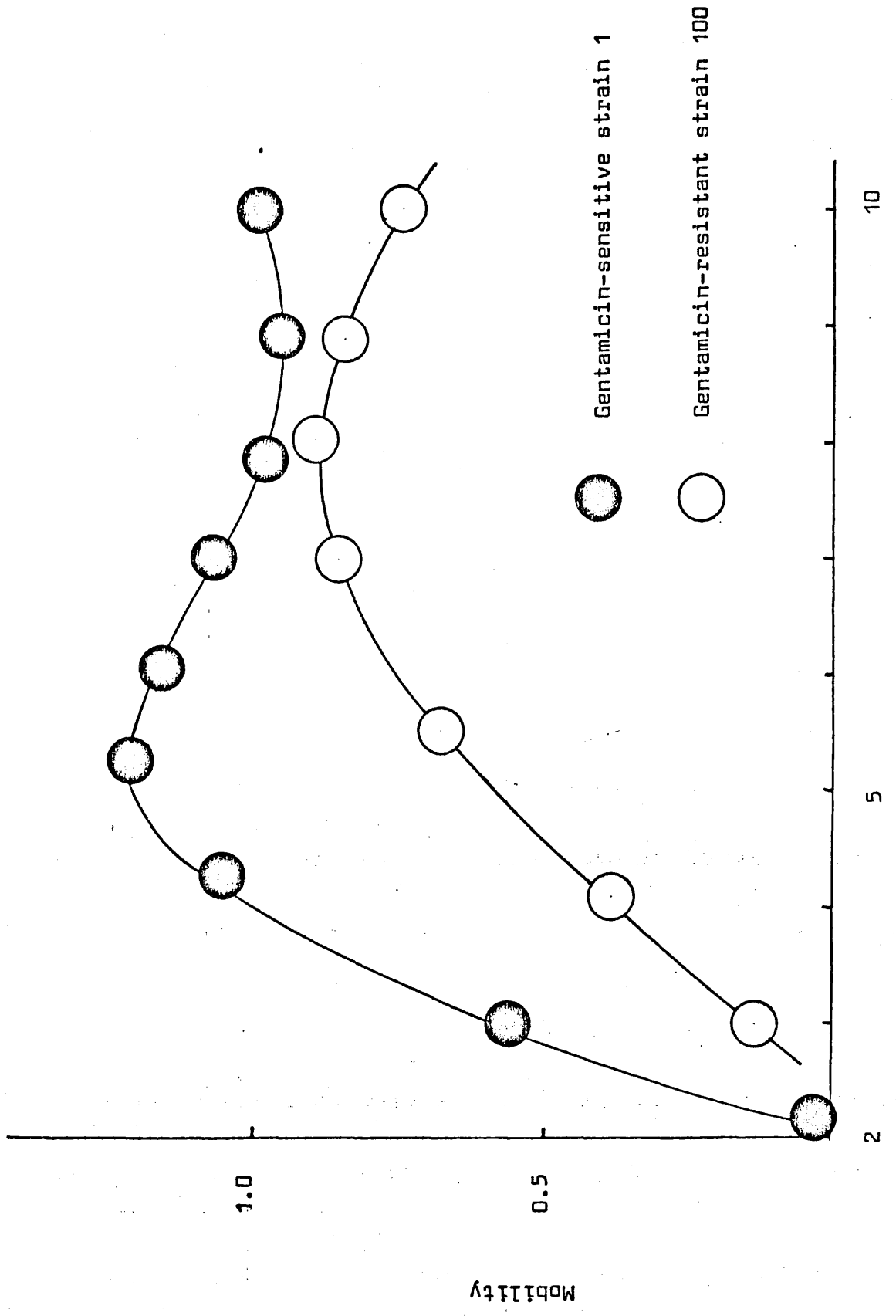
A marked decrease in the MIC of gentamicin values of both sensitive and medium-level resistant strains (except strain 3) has occurred over a period of some 4 - 5 years. During this period the strains have been subcultured onto fresh nutrient agar slopes in the absence of gentamicin at regular intervals. As a result of this, strains 96, 102 and 832, which had previously been clssified as medium-level resistant strains ($12 > MIC < 100\ \mu g\ cm^{-3}$), have now become sensitive strains. This decrease in the MIC of gentamicin has not happened with strains 100 and 101 which show high-level resistance to gentamicin, even though they have been maintained under the same conditions as the other strains. Also, over the period of this work (2 - 3 years), the MIC of gentamicin for the high-level resistant strains 103, 104 and 105, has not changed.

3.2 pH-Mobility Curves for Cells of P. aeruginosa Grown at 37 °C for 18 h in Nutrient Broth or on Nutrient Agar

In the previous study, Pechey (1973) found that cells of gentamicin-sensitive strains of P. aeruginosa grown at 37 °C on nutrient agar for 18 h, had a characteristically shaped pH-mobility curve which was different from the type observed for cells of gentamicin-resistant strains. Typical gentamicin-sensitive and gentamicin-resistant type pH-mobility curves are shown in Figure 3.1. Gentamicin-sensitive cells show a steep increase in negative mobility between pH 2 and 5 with a maximum negative

Figure 3.1

Typical pH-mobility curves for cells of P.aeruginosa grown at 37 ^{o}C for 18 h in nutrient broth or on nutrient agar


Gentamicin-sensitive strain 1
Gentamicin-resistant strain 100
Mobility
1.0
0.5
2
5
10
pH

mobility between pH 5 and 7. Each curve also passes through a minimum value between pH 7.5 and 9, the difference between maximum and minimum being 10-40% of the maximum mobility value. Positive mobility values were occasionally recorded in solutions of low pH, although this was not observed in the strains studied by Pechey. Suspension in buffer solutions of pH less than 2 or greater than 11 was avoided as this caused irreversible changes at the cell surface. The sinusoidal shape of the gentamicin-sensitive pH-mobility curves is not explicable in terms of a simple pH-titration of surface ionogenic groups. In such a titration, positive or negative surface groups, acting either singly or in combination, would result in one or more plateau mobility values at increasing negative values as the pH increased. It may be that a rearrangement of cell surface components causes the observed decline in negative mobility between pH 5 and 9. This would occur if amino groups, previously hidden, became exposed or if carboxyl groups, which were contributing to the mobility of the cell at pH 5.5 becme concealed. James and Brewer (1968) suggested that such a re-orientation (in this case of charged groups of the cell surface teichoic acid, thus exposing previously hidden phosphate groups) could account for the increased negative mobility values of cells of Staphylococcus aureus at pH values from 3-4, causing a maximum mobility value at pH 3.5 in the mobility curve.

The pH- mobility curves of gentamicin-resistant cells are unlike those of gentamicin-sensitive cells and show a characteristic increase in negative mobility over the pH range 3 - 7.5 and a maximum value at pH 7.5 - 8.5, with lower mobility

values at higher pH values. No minimum value was observed for gentamicin-resistant cells at values less than 10.5. Generally the maximum mobility values for resistant cells are lower than the maximum mobility values for gentamicin-sensitive cells (usually 0.8-1.2 as opposed to 1.1-1.7); the maxima also occur at higher pH values (1-3 pH units). The rate of increase in mobility values over the pH range 3-7.5 is less than that for gentamicin-sensitive cells over the range 2-5.

A summary of the results of pH-mobility curves for the various strains is shown in Table 3.2. The pattern observed by Pechey is maintained, in that cells of sensitive strains exhibit a sensitive-type pH-mobility curve while cells of resistant strains have a resistant-type pH-mobility curve. Strains 102 and 832 which were medium-level resistant strains when examined previously, have over the years become sensitive to gentamicin with normal sensitive-type pH-mobility curves (Figures 3.2 and 3.3). Strains 1, 100 and 101 have pH-mobility curves which are essentially unchanged from those recorded by Pechey.

All of these strains were examined after growth in nutrient broth and several of them were also examined after growth on nutrient agar; the change of media had no significant effect on surface properties, i.e. the shape and position of the pH-mobility curves.

Table 3.2 Summary of characteristic shape of pH-mobility curves and S-values for strains of P.aeruginosa grown at 37 ^{o}C in nutrient broth

Strain	Gentamicin-sensitive/ -resistant	pH-mobility curve*	S-value
1	S	S	0
3	S	S	0
3/Cb	S	S	0
100	R	R	17
101	R	R	18
102	S	S	0
832	S	S	0
103	R	R	14
104	R	R	25
105	R	R	14
106	S	S	0
107	R	R	10
Smith 2234	R	R	12
Lesko 5681	S	S	6
Lesko 5892	S	S	12
Tait 6767	S	S	4
Tait 6903	S	S	5
Cheekie 6428	S	S	6
Cheekie 7247	S	S	8
Kuch 6925	S	S	5
Kuch 7051	S	S	5
PU21	S	S	7
PL1	S	S	0
280	S	S	0
1310	S	S	0

* characteristic sensitive (S) or resistant (R) type

Figure 3.2

pH-mobility curves for strain 102 grown at 37 °C in nutrient broth or on nutrient agar

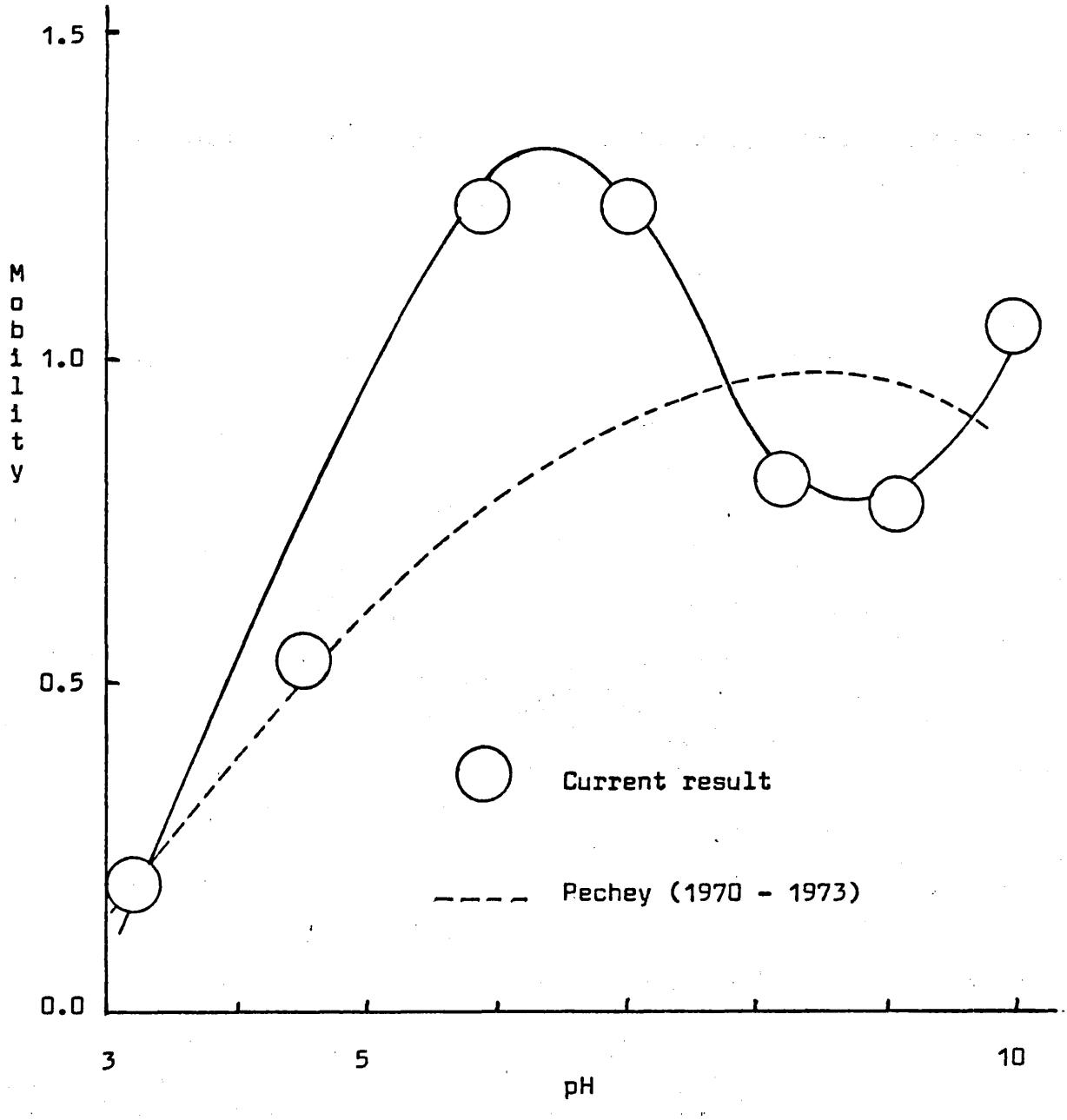
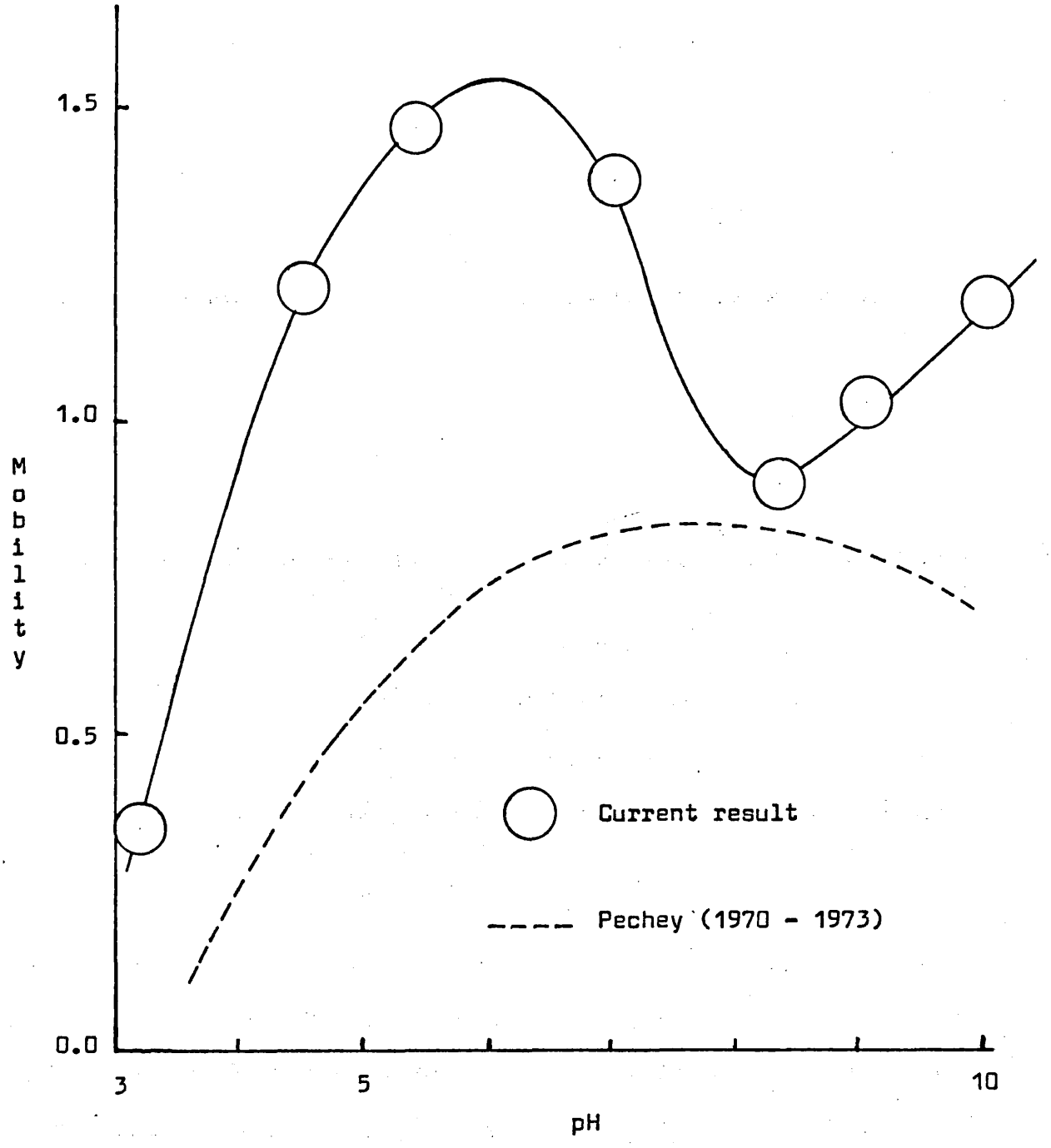


Figure 3.3

pH-mobility curves for strain 832 grown at 37 ^{o}C in nutrient broth or on nutrient agar



3.3 Surface Lipid on Cells of P. aeruginosa

The S-value of cells of Staph. aureus was defined by Marshall (1969) as the increase in the mobility of cells suspended in barbiturate-acetate buffer solution at pH 7 containing sodium dodecyl sulphate, SDS, (10^{-4} mol dm^{-3}), expressed as a percentage of the mobility of the cells in buffer solution alone. The S-value gives a quantitative measure of the amount of surface lipid possessed by the cells as measured by the method of Dyar (1948) (Section 1.8). In this investigation the same definition of S-value is used, except that the mobility values have been measured in barbiturate-acetate buffer solution of ionic strength 5×10^{-3} mol dm^{-3}.
S is given by the expression:

$$S = \frac{(\bar{v}_{SDS} - \bar{v}) \times 100}{\bar{v}}$$

where $\bar{v}_{SDS}$ is the mobility value in the presence of SDS (10^{-4} mol dm^{-3}) and $\bar{v}$ is the mobility value in buffer solution alone.

Cells of 18 h cultures (usually grown in nutrient broth but occasionally on nutrient agar with no apparent effect on results) were harvested and washed in buffer solution. A portion of the cells was suspended in buffer solution and a portion in buffer solution containing SDS. The mean mobility value of the cells in each suspension was determined. At the SDS concentration used no denaturation or irreversible changes in cell surface was observed (Pechey 1973).

The results (Table 3.2) show that there is a correlation between MIC of gentamicin and the S-value of the strain. All strains

sensitive to gentamicin have S-values less than 10 (except Lesko 5892) and all gentamicin-resistant strains have S-values greater than 10, suggesting a direct connection between the amount of surface lipid and the degree of gentamicin-resistance. In this context it is interesting to note the results for the paired Lesko, Tait, Cheekie and Kuch strains. These are clinical isolates; the second strain of each pair (i.e. the higher code number) with higher gentamicin-resistance, is beleived to have evolved from the less resistant strain (isolated earlier) during *in vivo* treatment with gentamicin. From the results there appears to have been an increase in surface lipid to match the increased resistance which has occurred with each pair.

The change from gentamicin-resistance to gentamicin-sensitivity in strains 102 and 832 already noted, is also correlated with a change in S-value. In the current study the S-values are consistent with those shown by gentamicin-sensitive type strains, whereas previous values (16.4 for strain 102 and 16.1 for strain 832) were characteristic of resistant strains.

There has also been a dramatic fall in the S-value for strains 100 and 101. Pechey *et al* (1974) record these strains as having S-values of 67 and 63.9 respectively as opposed to the current values of 17 and 18. However, if there has been a fall of detectable surface lipid in these two strains it is not matched by a corresponding fall in MIC of gentamicin or any observable change in their pH-mobility curves.

3.4 Summary

(a) Over a period of about four years the MIC of gentamicin in all but one of a series of gentamicin-sensitive and and medium-level gentamicin-resistant strains of *P. aeruginosa* has fallen appreciably. The MIC values of two highly resistant strains remained unaltered.

(b) Cells of gentamicin-sensitive strains of *P. aeruginosa* showed characteristic pH-mobility curves which exhibited a maximum negative mobility value in the pH range 5 to 7, and a minimum mobility value between pH 7.5 to 9.

(c) Cells of gentamicin-resistant strains showed characteristic pH-mobility curves which exhibited maximum mobility values at pH 7.5 to 8.5.

(d) Gentamicin-resistant cells had S-values in excess of 10 indicating significant surface lipid. Gentamicin-sensitive cells had S-values normally lower than 10 indicating low or negligible quantities of surface lipid.

(e) Two previously resistant strains which had become gentamicin-sensitive now exhibited sensitive-type pH-mobility curves and low S-values.

CHAPTER 4

THE DIVALENT METAL ION CONTENT OF CELLS OF P.AERUGINOSA GROWN AT 37 ^{o}C IN NUTRIENT BROTH

There have been numerous reports of the enhancing effect of magnesium and calcium ions on gentamicin-resistance in P.aeruginosa (see Chapter 5). Therefore, as gentamicin resistance is increased by the presence of these divalent metal ions, it seemed possible that the converse may be true; i.e. that naturally occurring gentamicin-resistant strains of P.aeruginosa may contain higher levels of divalent metal ions than gentamicin-sensitive strains. In order to investigate this possibility, atomic absorption spectrophotometry was used to determine the divalent cation content of whole cells of gentamicin-resistant (excluding strains displaying R-factor mediated gentamicin resistance) and gentamicin-sensitive strains of P.aeruginosa. Ethylenediaminetetraacetic acid (EDTA) lysis was used to examine cell surface cation content.

4.1 Atomic absorption spectrophotometric analysis of whole cells of P.aeruginosa grown at 37°C in nutrient broth.

Cells of P.aeruginosa (strains 1, B 32, 100 and 101) were grown for 18 h in 1 dm^3 batches of nutrient broth. The cultures were centrifuged and the cells washed three times with distilled water and finally suspended in water to give a volume of about 20 cm^3. The suspensions were then poured into clean beakers and the water evaporated off and the cells dried to constant weight by heating at 100-105°C. Duplicate suspensions were thus treated for each strain examined. The dried bacteria were scraped from the beakers, pewdered as much as possible and 0.25 g of each sample accurately

weighed out. These bacterial samples and also the weighed chemicals for standard curves were then wet ashed using the technique of Eagon (1969). 10 cm^3 of an acid mixture, composed of conc. HNO_3, conc. H_2SO_4 and 60-62 % $HClO_4$ in a 5:1:2 (v/v) ratio was added to each weighed sample. The beakers were then heated on a hot plate at low temperature for several minutes. The temperature was then increased and the mixtures heated until fumes of H_2SO_4 were evolved. Heating was then continued until the volume of each was reduced to 2-3 cm^3. After cooling, each digested mixture was transferred to a 10 cm^3 volumetric flask and made up to volume using distilled water. The same method was used to produce a reagent blank.

The duplicated extract solutions were analysed for magnesium, calcium, iron(II) and zinc ions, using a Southern Analytical A300 atomic absorption spectrophotometer. These particular metals were examined because they were reported by Eagon (1969), to be the most abundant divalent cations in the cell wall of P.aeruginosa. For calcium analysis, strontium chloride was added to test samples, standards and blank solutions, to give a strontium concentration of 500 $\mu g\ cm^{-3}$ in each. The strontium was used to minimise interference with calcium absorption by such ions as aluminium and phosphate. Similarly, for magnesium analysis, strontium chloride was added to give a strontium concentration of 1000 $\mu g\ cm^{-3}$.

The concentrations of the same four divalent cations in Oxoid nutrient broth was also examined using the same technique.

The results for the bacterial analysis are shown in Table 4.1. The 'total' divalent cation content of the cells, expressed as the sum of the contents of the four divalent cations analysed, is also shown. The duplicate results for each cation, for each strain, showed good agreement with each other.

There is no apparent relationship (Table 4.1) between the cellular content of divalent metal ions and the MIC of gentamicin of the strains examined. The results for each individual cation and for 'total' divalent cation content, are in close agreement between the four strains; the strains actually having the highest and lowest 'total' cellular percentage of divalent cations were the two highly gentamicin-resistant strains, 100 and 101 respectively.

The results of this study show broad agreement with those reported by Eagon (1969) for P.aeruginosa strain OSU 64 cell walls (Table 4.1). However, he observed that the calcium content was higher and exceeded that of iron(II). This difference can probably be attributed to differences in growth media (See Table 4.2), rather than to differences between the divalent cation content of whole cells and cell walls. According to Eagon (1969) the cation content of whole cells and cell walls is essentially similar.

The results of the metal ion analysis of nutrient broth are shown in Table 4.2. These concentrations are for nutrient broth as used for bacterial growth. All the divalent cations examined were present in the nutrient broth in the same order of abundance as they appeared in the whole cells. It thus appears that there could

Table 4.1

Divalent cation content of whole cells of P. aeruginosa grown at 37 ^{o}C in nutrient broth

Strain	MIC of gentamicin /μg cm^{-3}	Magnesium*	Calcium*	Iron(II)*	Zinc*	Total*
1	< 1.0	0.27	0.029	0.060	0.006	0.365
B32	3.0	0.27	0.028	0.057	0.005	0.360
100	8000.0	0.29	0.026	0.062	0.006	0.384
101	8000.0	0.22	0.025	0.064	0.006	0.315
OSU 64**	not known	0.20	0.105	0.012	0.008	0.325

* expressed as % dry cell weight.

** Eagon (1969).

Table 4.2

Divalent cation content of Oxoid nutrient broth

Divalent cation	Concentration /mmol dm^{-3}
Magnesium	6.1×10^{-2}
Calcium	2.0×10^{-2}
Iron (II)	2.25×10^{-2}
Zinc	2.14×10^{-3}

be a direct relationship between availability and uptake of these divalent cations by cells of P.aeruginosa at the concentration levels used.

The cells of all the strains, including strain OSU 64, had a 'total' divalent cation content of between 0.31 and 0.38 %. It may be that this represents the required level of these cations, a level which is achieved with varying concentrations of the cations according to availability.

4.2 EDTA-Lysis of Cells of P.aeruginosa Grown at 37 °C in Nutrient Broth

P.aeruginosa is particularly sensitive to EDTA-lysis (Gray and Wilkinson, 1965; Eagon and Carson, 1965; Haque and Russell, 1974). It is beleived that the main action of EDTA is the extraction of

divalent cations from the cell envelope (Roberts et al, 1970); this causes the loss of complex cell components which in turn leads to weakening of the cell envelope and consequent cell lysis.

Cells of three highly gentamicin-resistant strains (100, 101 and 105) and three gentamicin-sensitive strains (1, 3 and 832) of P.aeruginosa were grown overnight in nutrient broth. Duplicate 100 cm^3 batches of each culture were centrifuged and the cells washed aseptically three times with 0.05 mol dm^{-3} barbiturate-acetate buffer solution. The control batch of cells for each culture was then suspended in barbiturate-acetate buffer solution (pH=8.6) to give an initial absorbance reading of about 0.7 at 660 nm against a blank of buffer solution. The corresponding test batch of cells was suspended in barbiturate-acetate buffer containing 1×10^{-2} mol dm^{-3} EDTA (pH = 8.6) to give an initial absorbance of about 0.7 at 660 nm. The suspensions were then left stationary at 30 ^{o}C and their absorbances measured at approximately hourly intervals over a period of about five hours. Graphs (Figures 4.1 and 4.2) were plotted of absorbance against time for both control and test suspensions for each strain.

The rate and extent of lysis of each suspension was determined by the rate and extent of the decrease of absorbance with time. All the strains examined showed such evidence of lysis when treated with EDTA. The rate of lysis was greatest in the first 1 - 2 h thereafter becoming less rapid. For all the strains tested, lysis was still incomplete after 5 h. Over the test period the absorbances of the control suspensions also decreased but at a considerably slower rate than for the test suspensions.

Figure 4.1

EDTA-lysis of cells of P.aeruginosa grown at 37 °C in nutrient broth

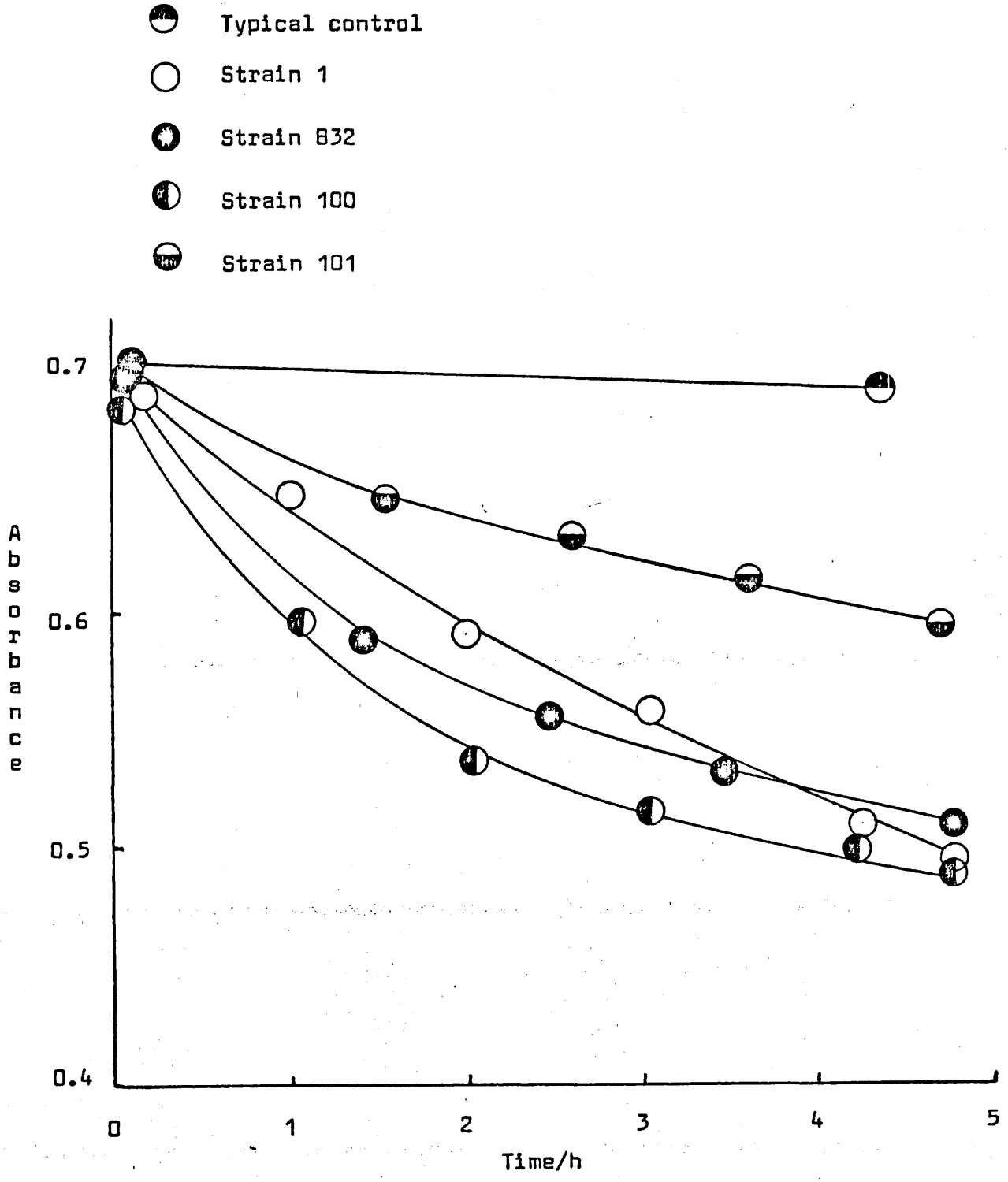
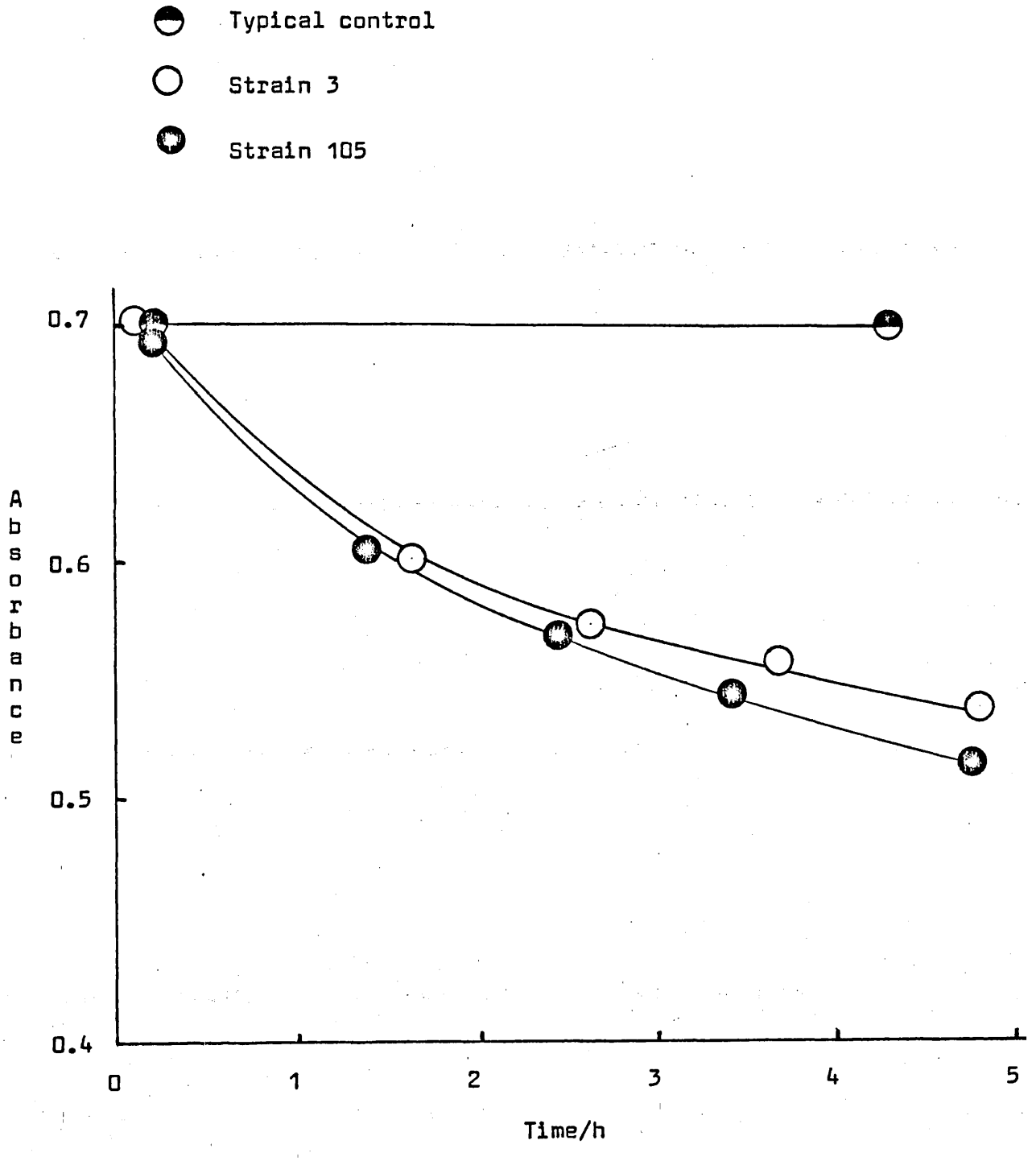


Figure 4.2

EDTA-lysis of cells of P.aeruginosa grown at 37 °C in nutrient broth



There was no correlation between the MIC of gentamicin of the strains and the rate or extent of their lysis by EDTA over the test period. This confirms the results of Pechey (1973) relating MIC of EDTA to MIC of gentamicin for strains of P. aeruginosa. However, there was good correlation between EDTA lysis and divalent cation content determined by atomic absorption spectrophotometry for strains 100, 101, 1 and 832 (Section 4.1). Thus strain 100, which had the highest divalent cation content, was the most effectively lysed strain. Strains 1 and 832 were intermediate for both lysis and divalent cation content and strain 101, which had the lowest divalent cation content, was the least effectively lysed. Strains 3 and 105 (gentamicin-sensitive and gentamicin-resistant respectively) both showed lysis comparable to strains 1 and 832.

4.3 Summary

(a) There was no relationship between divalent cation content (Ca, Mg, iron(II) and Zn) and the gentamicin -resistance or sensitivity of the strains.

(b) The relative abundances of the four divalent cations in whole cells and in nutrient broth were similar.

(c) There was a direct relationship between the amount of divalent cation in the cells and the effectiveness

of their lysis by EDTA. The effectiveness of lysis of cells of P. aeruginosa by EDTA was not related to the gentamicin-resistance or gentamicin-sensitivity of the strains.

CHAPTER 5

THE EFFECT OF DIVALENT METAL IONS ON GENTAMICIN-RESISTANCE IN CELLS OF P.AERUGINOSA

Calcium and magnesium ions in growth media have an antagonistic effect against the action of gentamicin on P. aeruginosa; increasing levels of these ions causing the susceptibility of the bacteria to decrease (Gilbert et al, 1971; Zimelis and Jackson,1973). It is possible that the divalent metal ions complex with the gentamicin molecules, thus inactivating them or impairing their progress into the bacterial cells in some way or that the metal ions interact with the bacterial cells and thus increase the intrinsic resistance of the cells, The experiments described in this chapter were performed to examine any changes in the properties of the cells which could be attributed to the presence of increased levels of magnesium and calcium in the growth medium and which could possibly give rise to increased levels of resistance.

5.1 Effect of Magnesium and Calcium Ions on the MIC of Gentamicin of Cells of P.aeruginosa

The calcium and magnesium levels in the basal medium (Section 2.1.(b)) were determined using atomic absorption spectrophotometry (Section 4.1). The results are shown in Table 5.1. Calcium was added to the basal medium as calcium chloride and magnesium as magnesium sulphate to give the required levels (5 or 22.5 mmol dm^{-3}). The final concentrations were checked using atomic absorption spectrophotometry.

The MIC of gentamicin of eight strains of P. aeruginosa (six sensitive and two highly resistant to gentamicin when grown in nutrient broth) were determined for growth at 37 ^{o}C in the different media (Table 5.1).

Table 5.1 Effect of addition of calcium and magnesium ions on the MIC of gentamicin of strains of P.aeruginosa

MIC of gentamicin/μg cm^{-3} for growth at 37 °C in:

Strain		Nutrient broth	Basal medium	BM(Ca1)	BM(Ca2)	BM(Mg1)	BM(Mg2)
	Ca conc^{n}/ mmol dm^{-3}	2×10^{-2}	2.5×10^{-3}	2.5×10^{-3} + 5	2.5×10^{-3} + 22.5	2.5×10^{-3}	2.5×10^{-3}
	Mg conc^{n}/ mmol dm^{-3}	6.1×10^{-2}	0.35	0.35	0.35	0.35 + 5	0.35 + 22.5
1		0.1	0.02	—	0.4	—	—
B32		3	1.5	100	200	25	200
102		6	3	100	200	50	200
106		1.5	0.75	—	50	—	—
107		25	6	—	400	—	—
Smith		25	6	—	400	—	—
100		8000	500	—	> 16000	—	—
101		8000	500	—	> 16000	—	—

The results are consistent in showing an increased MIC value for each strain, for each increase in calcium or magnesium concentration. Thus the MIC values were lowest for growth in basal synthetic medium and highest for growth in basal medium plus 22.5 mmol dm^{-3} calcium or magnesium. Strains 100 and 101 remained gentamicin-resistant (MIC $>$ 12 $\mu g\ cm^{-3}$) even in the calcium-deficient medium, whilst strain 1 remained gentamicin-sensitive even at very high levels of calcium ions. However, borderline sensitive/resistant strains changed their resistance-type with the nature of the calcium or magnesium levels in the growth medium. Thus strains Smith and 107 which are gentamicin-resistant when growing in nutrient broth or calcium-supplemented media became gentamicin-sensitive when grown in calcium-deficient medium i.e. basal medium. Conversely strains 102, 106 and B32 which are gentamicin-sensitive when tested in nutrient broth or calcium-deficient media became gentamicin-resistant when grown in calcium-supplemented media.

Calcium had a greater effect in increasing the MIC of gentamicin than did magnesium when 5 mmol dm^{-3} of either was added to basal medium. Strain B32 had an MIC value two tubes higher (i.e. factor of 4) with the added calcium than with the magnesium and strain 102 had an MIC value one tube higher (i.e. factor of 2). These results may be due to the magnesium being used in part for metabolism other than cell wall synthesis, thus less is available to enhance gentamicin-resistance than for calcium. At a higher concentration of magnesium this effect may be masked and so when the calcium and magnesium concentrations were increased by 22.5 mmol dm^{-3} the MIC values were the same for each strain. When

equal concentrations (11.25 mmol dm^{-3}) of both calcium and magnesium were used, the MIC of gentamicin values of strains 832 and 102 were the same as when only one cation was added at this concentration. Thus there appeared to be no competitive or synergistic effect between calcium and magnesium ions.

All the bacterial strains tested gave a higher MIC of gentamicin when grown in nutrient broth than when grown in basal medium.

5.2 Effect of Repeated Growth of Cells of P.aeruginosa in Calcium-Deficient and Calcium-Sufficient Media

The MIC of gentamicin of P. aeruginosa strains 832 and 102 were determined for growth in nutrient broth. The cells were then grown ten times successively at 37 ^{o}C in calcium-deficient and calcium-sufficient (8M(Ca 2)) media. They were then grown overnight at 37 ^{o}C in nutrient broth and their MIC of gentamicin in nutrient broth again determined. These MIC results were the same as initially, showing that growth at the different calcium levels had not caused any permanent cell changes or selected out mutant cells, with regard to resistance.

5.3 Effect on MIC of Gentamicin of Other Cations

Zinc and iron(II), along with calcium and magnesium constitute the four most abundant divalent cations in the cell envelope of P. aeruginosa (Eagon, 1969). Basic synthetic medium

was supplemented with various levels of zinc as zinc sulphate or iron(II) as iron(II) sulphate. Complete inhibition of growth at 37 ^{o}C was observed when the concentration of zinc exceeded 0.75 mmol dm^{-3}; and of iron(II) exceeded 1.5 mmol dm^{-3}. In contrast, no inhibition of growth was observed with calcium and magnesium supplemented media at any concentration used. Zinc and iron(II) ions present at lower concentrations did not enhance the MIC of gentamicin of strains 102 or B32.

Potassium, added as potassium chloride, had no enhancing effect on the MIC of gentamicin of strains 102 and B32; at high potassium concentrations growth tended to be inhibited.

5.4 Growth Curves of Cells of P.aeruginosa Strains Grown at 37 ^{o}C in Calcium-Deficient and Calcium-Sufficient Synthetic Media

Growth curves were plotted (Section 2.1.(d)) for cells of strains 107 and B32 growing in calcium-deficient basal medium and calcium-sufficient medium (8M (Ca 2)) at 37 ^{o}C (Figures 5.1 and 5.2). For both strains, growth as determined from absorbance readings was much more prolific in calcium-sufficient medium than in calcium-deficient medium, the absorbance readings being about four times higher at maximum growth for the former for strain 107 and about two times higher for strain B32. The length of the lag-phase was not altered by growth in the different media. Strain 107 reached the stationary phase of growth very much more quickly in calcium

Figure 5.1 Growth curves for cells of P.aeruginosa strain 107 growing at 37°C in calcium-deficient and highly calcium-sufficient media

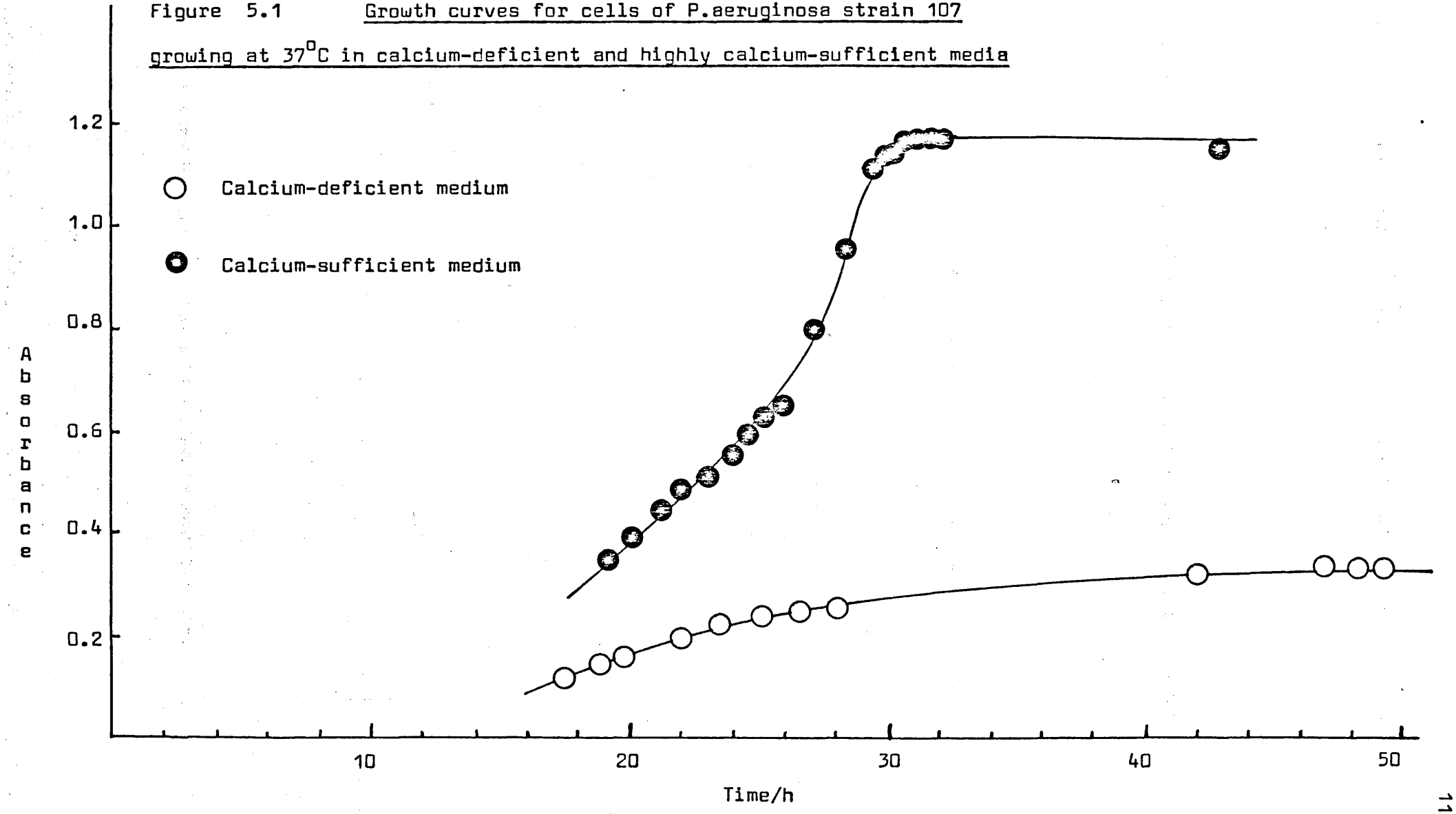
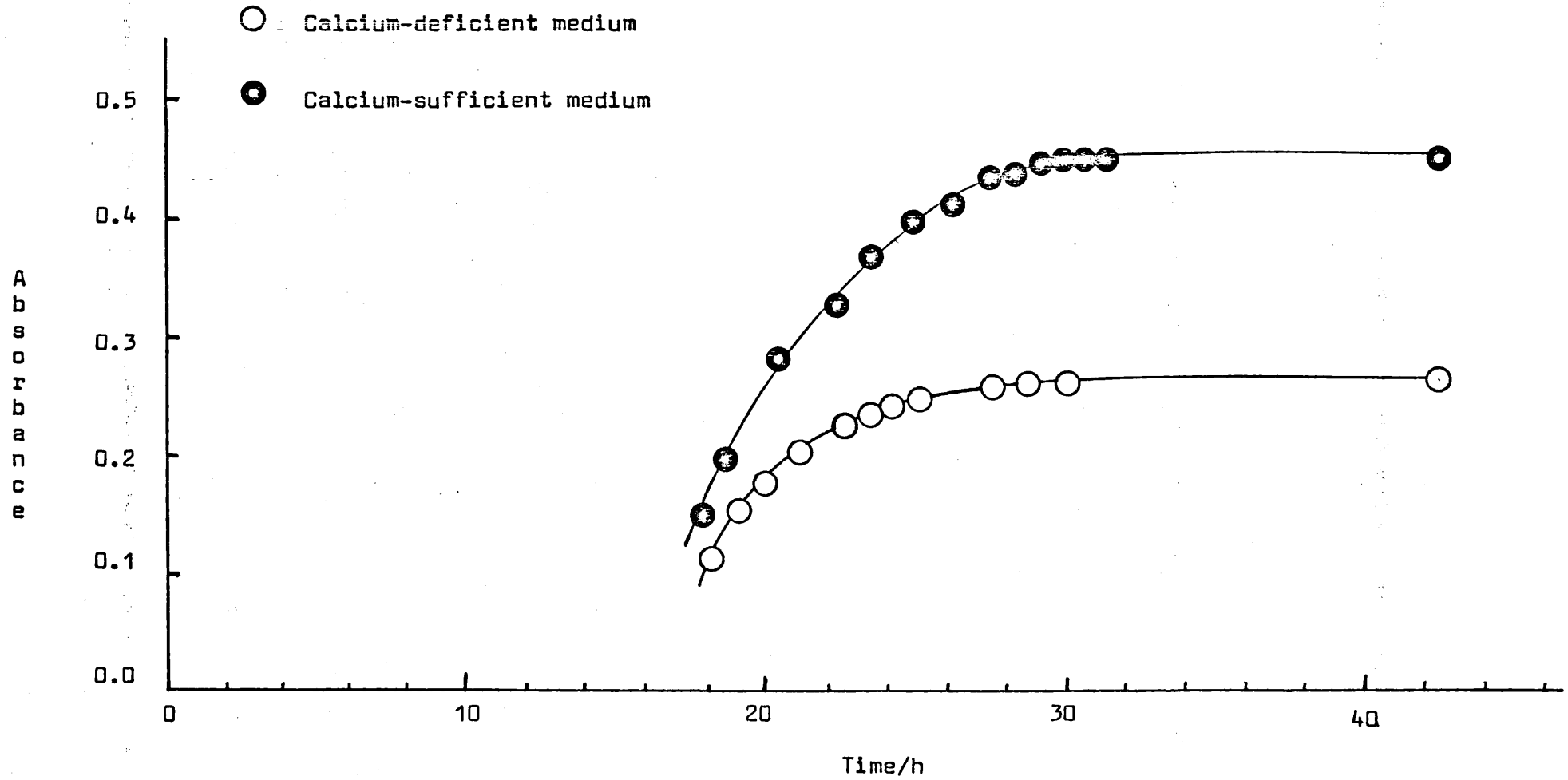


Figure 5.2 Growth curves for cells of P.aeruginosa strain B32 growing at 37 ^{o}C in calcium-deficient and highly calcium-sufficient media



-sufficient medium than in calcium-deficient medium. The stationary phase was reached at approximately the same time for both media with strain B32.

5.5 pH-Mobility Curves and Surface Lipid of Cells of P.aeruginosa Grown in Synthetic Media

pH-mobility curves were obtained for cells of P. aeruginosa strains grown at 37 ^{o}C in calcium-deficient and highly calcium-sufficient (BM (Ca 2)) media for 24 h. The results are summarised in Table 5.2.

The pH-mobility curves for cells of strain 1 are shown in Figure 5.3. Both curves are of a gentamicin-sensitive type but the cells grown in calcium-sufficient medium consistently tended to give slightly higher mobility values than did the cells grown in the calcium-deficient medium.

The pH-mobility curves of gentamicin-sensitive strains 102, B32 and 106 (i.e. when grown in nutrient broth, see Section 3.1) were all of normal gentamicin-sensitive type for growth in calcium-deficient medium. However, after growth in calcium-sufficient medium (which causes the cells to become gentamicin-resistant) the cells all displayed gentamicin-resistant type pH-mobility curves. The pH-mobility curves of strain B32 are shown in Figure 5.4. Thus the shape of the pH-mobility curves matches the MIC of gentamicin for these strains in the different media and so parallel the results previously described (Section 3.2) for gentamicin-sensitive and

Table 5.2 pH-mobility curve and surface lipid results for cells of P. aeruginosa grown in synthetic medium

Strain	Growth medium					
	Basal medium		BM(Ca 2)		BM(Mg 2)	
Strain	pH-mobility curve	S-value	pH-mobility curve	S-value	pH-mobility curve	S-value
1	S	Negligible	S	Negligible	—	—
B32	S	Negligible	R	Negligible	R	Negligible
102	S	Negligible	R	Negligible	R	Negligible
Smith	S	9	R	15	—	—
107	S	Negligible	R	9	—	—
106	S	Negligible	R	Negligible	—	—

S and R refer to characteristic shape of pH-mobility curves (i.e. sensitive or resistant type respectively)

Figure 5.3

pH-mobility curves for cells of P.aeruginosa strain 1 grown at 37 ^{o}C in calcium-deficient and highly calcium-sufficient media

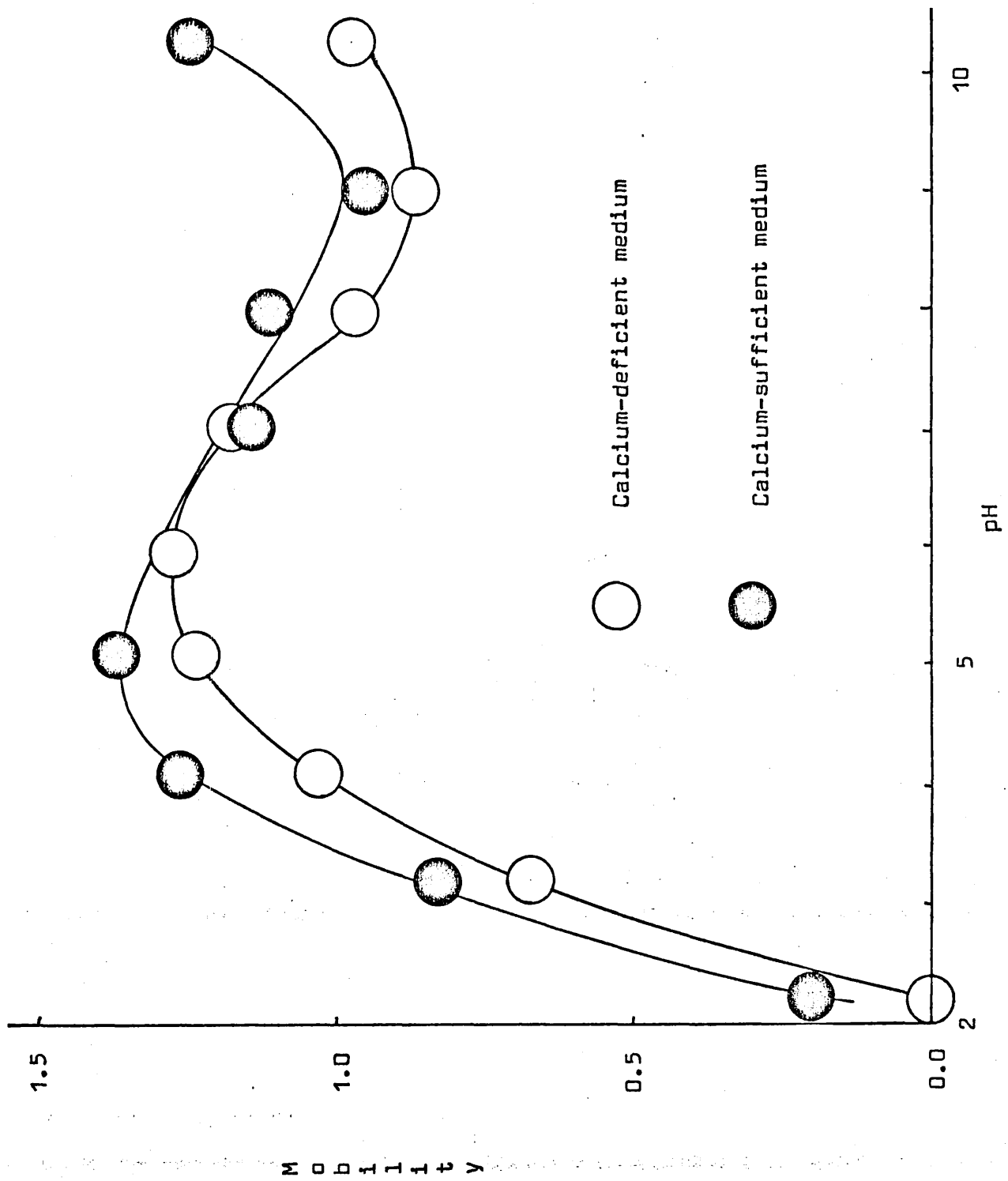
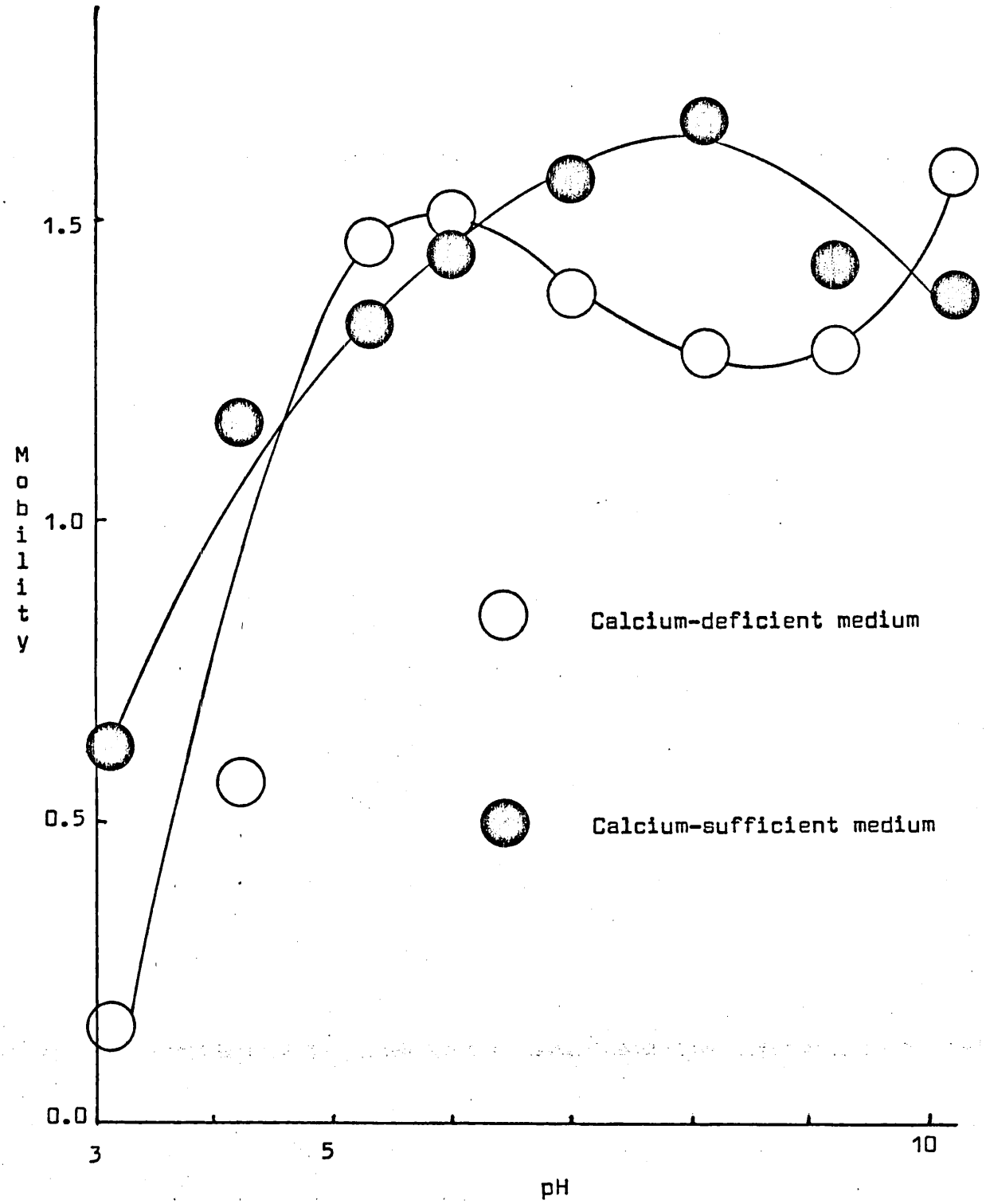

Mobility
1.5
1.0
0.5
0.0
2
5
10
pH
Calcium-deficient medium
Calcium-sufficient medium

Figure 5.4

pH-mobility curves for cells of P.aeruginosa strain 832 grown at 37 °C in calcium-deficient and highly calcium-sufficient media



gentamicin-resistant cells. However, as with the cells of strain 1, the pH-mobility curves for these strains showed a tendency to have a higher maximum mobility value when grown in calcium-sufficient medium, although it is more difficult to compare in this case because of the different shapes of the resistant and sensitive type curves. This tendency was seen with both strains 832 and 102 but not with strain 106 which had an unaltered maximum mobility value. The pH-mobility curves of cells of these strains after growth in calcium-deficient medium and nutrient broth were indistinguishable.

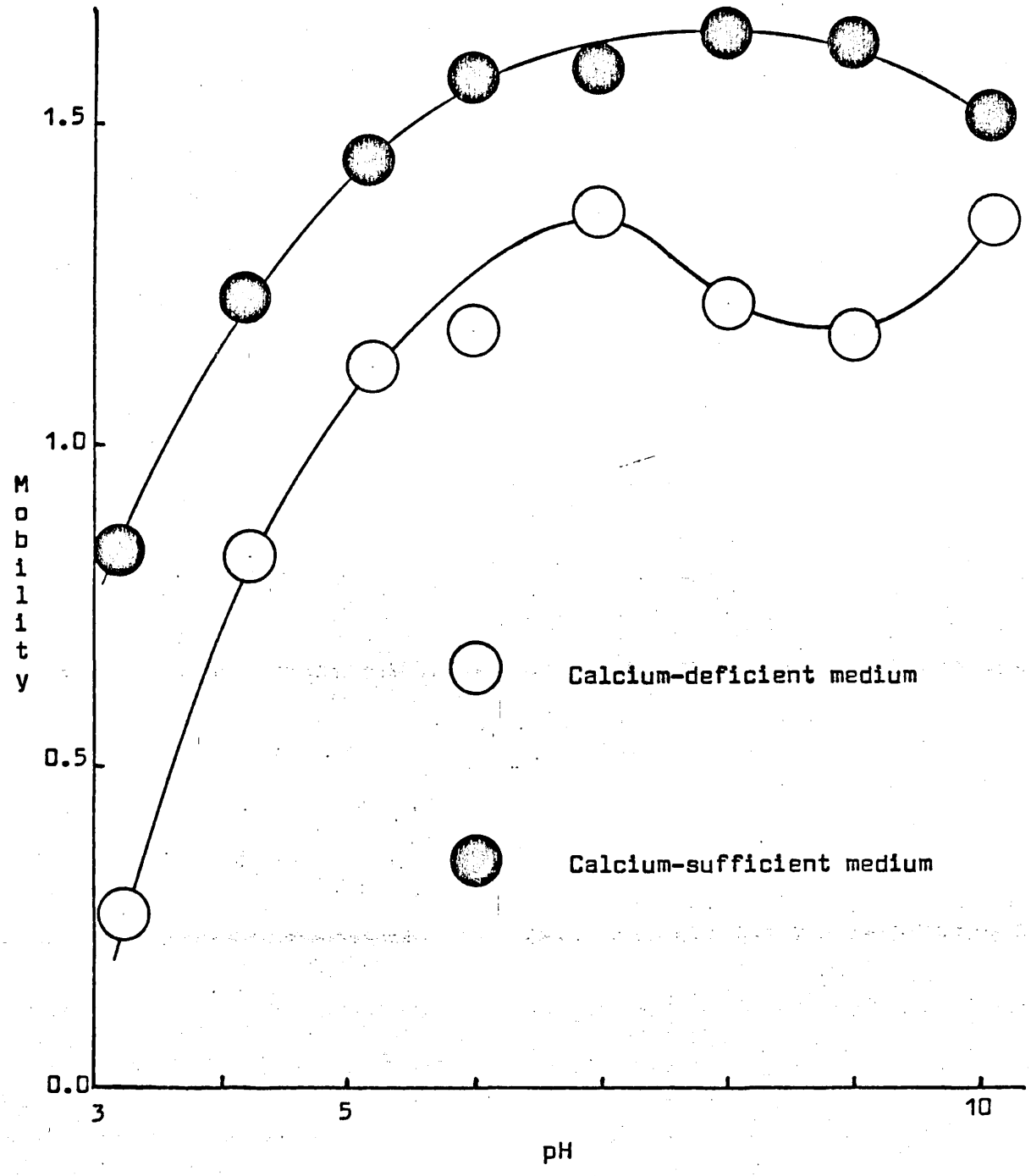
The pH-mobility curves of cells of gentamicin-resistant strains 107 and Smith showed a similar pattern. When grown in calcium-sufficient medium (or nutrient broth) these strains displayed gentamicin-resistant type pH-mobility curves whilst they had gentamicin-sensitive type pH-mobility curves when grown in calcium-deficient medium. The results for strain Smith are shown in Figure 5.5.

The S-values of cells of the strains examined are also listed in Table 5.2. No appreciable surface lipid was detected on cells of any of the gentamicin-sensitive strains in calcium-deficient or calcium-sufficient media. However, the gentamicin-resistant strains 107 and Smith, which show significant surface lipid when grown in nutrient broth had comparable surface lipid after growth in calcium-sufficient medium but reduced surface lipid after growth in calcium-deficient medium.

Since there is a difference in the growth characteristics of cells grown in calcium-deficient and calcium-sufficient media,

Figure 5.5

pH-mobility curves for cells of P.aeruginosa strain Smith grown at 37 °C in calcium-deficient and highly calcium-sufficient media



a comparison of the surface properties after 24 h growth may not be valid on account of differences of age. Cells of strains 107 and 832 were therefore grown to the late exponential phase of growth in calcium-deficient basal medium and calcium-sufficient medium (BM(Ca 2)). The surface properties of these cells were identical with those for cells grown for 24 h.

Cells of strains 832 and 102 were also grown in magnesium -supplemented medium (BM(Mg 2)) and cells of strain 832 grown in calcium-supplemented medium (BM(Ca 1)) and the surface properties of the cells examined. The results were comparable to those obtained for these strains in highly calcium-supplemented medium (BM(Ca 2)).

There is thus a clear correlation between the concentration of calcium and magnesium ions in the growth medium, the MIC of gentamicin and changes in the surface properties of cells of P. aeruginosa.

5.6 Effect of Suspension of the Cells in Calcium/Buffer Solution on the Surface Properties of Cells of P.aeruginosa

One possible explanation for the enhancing effect of calcium and magnesium ions on gentamicin-resistance in P.aeruginosa is that the ions bind irreversibly onto the surface of the cells and thus perhaps act as a barrier to gentamicin entering the bacterial cell. To examine this possibility, cells of strain 832 were grown in nutrient broth at 37 ^{o}C for 18 h. The culture was divided into two portions and the cells in each portion centrifuged and washed twice

with barbiturate-acetate buffer ($I = 5 \times 10^{-3}$ mol dm^{-3}, pH = 7.0). The cells in one portion were suspended in barbiturate-acetate buffer containing calcium chloride (Ca^{2+} = 22.5 mmol dm^{-3}) for 24 h at 37 ^{o}C while the cells in the other were suspended in barbiturate-acetate buffer as a control. The cells in both the test and control suspensions were then washed twice and finally suspended in barbiturate-acetate buffer and the electrophoretic mobilities of the cells determined at pH 7.0. There were no observable differences between the mobility values of cells in the two suspensions and thus no binding of calcium ions was detected by this method.

5.7 Calcium and Magnesium Content of Whole Cells of P.aeruginosa Grown in Synthetic Media

Whole cells of P.aeruginosa strain 832 grown at 37 ^{o}C in nutrient broth, calcium-deficient or calcium-sufficient medium (BM(Ca 2)) to the late logarithmic phase, were analysed for calcium and magnesium content (Section 4.1). The results are listed (as percentage dry weight) in Table 5.3; duplicate results showed good agreement.

The cells grown in highly calcium-sufficient medium had about ten times the calcium content of cells grown in calcium-deficient medium and about three times that of cells grown in nutrient broth. The calcium content of the nutrient broth was higher than that of calcium-deficient medium but was lower than that of calcium-sufficient

Table 5.3

Calcium and magnesium content of cells of P.aeruginosa strain 832 grown at 37 ^{o}C in synthetic media and nutrient broth

Growth medium	Calcium*	Magnesium*
Calcium-deficient (basal medium)	0.008	0.32
Calcium-sufficient (BM(Ca 2))	0.090	0.25
Nutrient broth	0.028	0.27

* expressed as % dry cell weight.

(BM(Ca 2)) medium. This was mirrored in the calcium content of cells grown in these three media; those grown in basal medium having the lowest calcium content and those grown in calcium-sufficient (BM(Ca 2)) medium having the highest calcium content. Although the magnesium content of the basal medium and the calcium-sufficient medium were the same, the magnesium content of the cells grown in basal medium was higher than of cells grown in calcium-sufficient medium. It thus appears that cells grown in calcium-sufficient medium need less magnesium than cells grown in basal medium. This could probably be due to the use of calcium ions to perform some of the same functions. Nutrient broth had a magnesium content lower than the synthetic media

and cells grown in nutrient broth had a magnesium content lower than those grown in calcium-deficient medium but higher than those grown in calcium-sufficient medium. The zinc and iron(II) contents of cells grown in synthetic media were not determined; since these ions were not added to the medium and Analar grade chemicals were used the contents of these cations in the cells were probably small.

5.8 Analysis of Macromolecular Components of Cells of P.aeruginosa Grown in Synthetic Media

Cells of strain B32 were grown at 37 ^{o}C to the late logarithmic phase in calcium-deficient or calcium-sufficient (BM(Ca 2)) medium, and the resulting whole cells analysed for protein, polysaccharide, DNA and RNA content (Section 2.3). The results (as percentage dry weight of cell) are listed in Table 5.4.

Table 5.4

Macromolecular analysis of whole cells of P.aeruginosa strain B32 grown at 37 ^{o}C in synthetic media

Medium	Protein*	Polysaccharide*	DNA*	RNA*
Basal medium	71.0	5.1	6.0	11.9
BM(Ca 2)	68.5	6.7	5.9	11.2

*expressed as % dry cell weight.

Good agreement was obtained between duplicate results for each component for each medium; the determination of protein content was the least accurate technique.

The DNA content and the RNA content of the cells were the same for cells grown in calcium-deficient or in calcium-sufficient medium. However there was a significantly higher percentage of polysaccharide in the cells grown in the highly calcium-sufficient medium compared with that of cells grown in calcium-deficient medium. There was also a marginally, possibly not significantly lower protein content in the cells grown in the highly calcium-sufficient medium than in those grown in the calcium-deficient medium. The same trends were obtained when the dry weights of bacteria were determined by direct weighing or from absorbance standard curves. One possible interpretation of these results is that the calcium ions are used by the cells in binding polysaccharide units, probably in the cell envelope.

5.9 EDTA Lysis of Cells of P.aeruginosa Grown at 37 ^{o}C in Synthetic Media

Cells of P. aeruginosa strains 832 and 102 were grown in basal medium, calcium-sufficient medium (BM(Ca 2)) or magnesium-sufficient medium (BM(Mg 2)). These were suspended in barbiturate-acetate buffer solution containing EDTA as lytic agent (Section 4.2) and lysis was followed by plotting the absorbance at different times (Figures 5.6 and 5.7).

Figure 5.6 EDTA-lysis of cells of P.aeruginosa grown at 37 °C in basal medium and highly calcium-sufficient medium

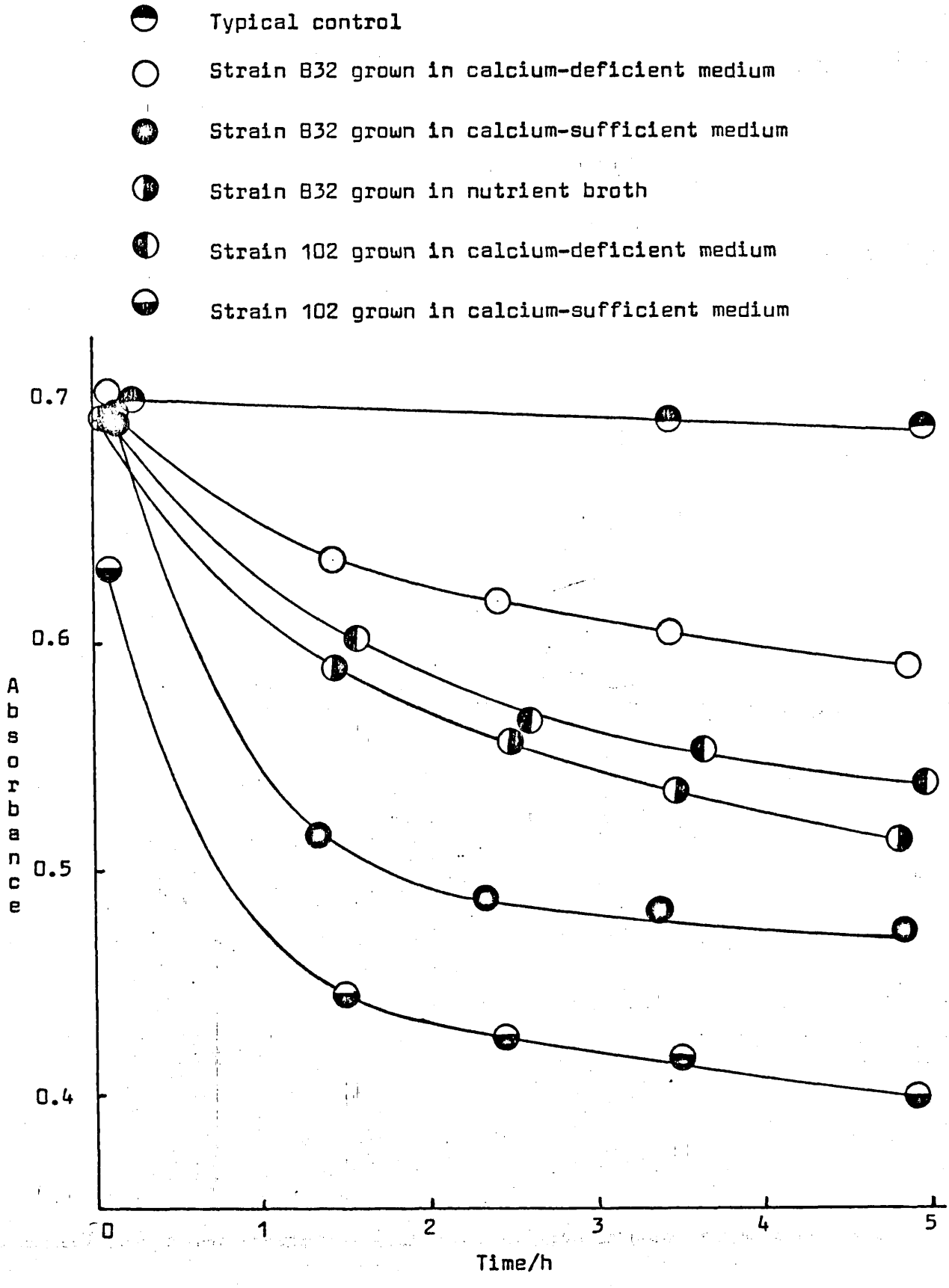
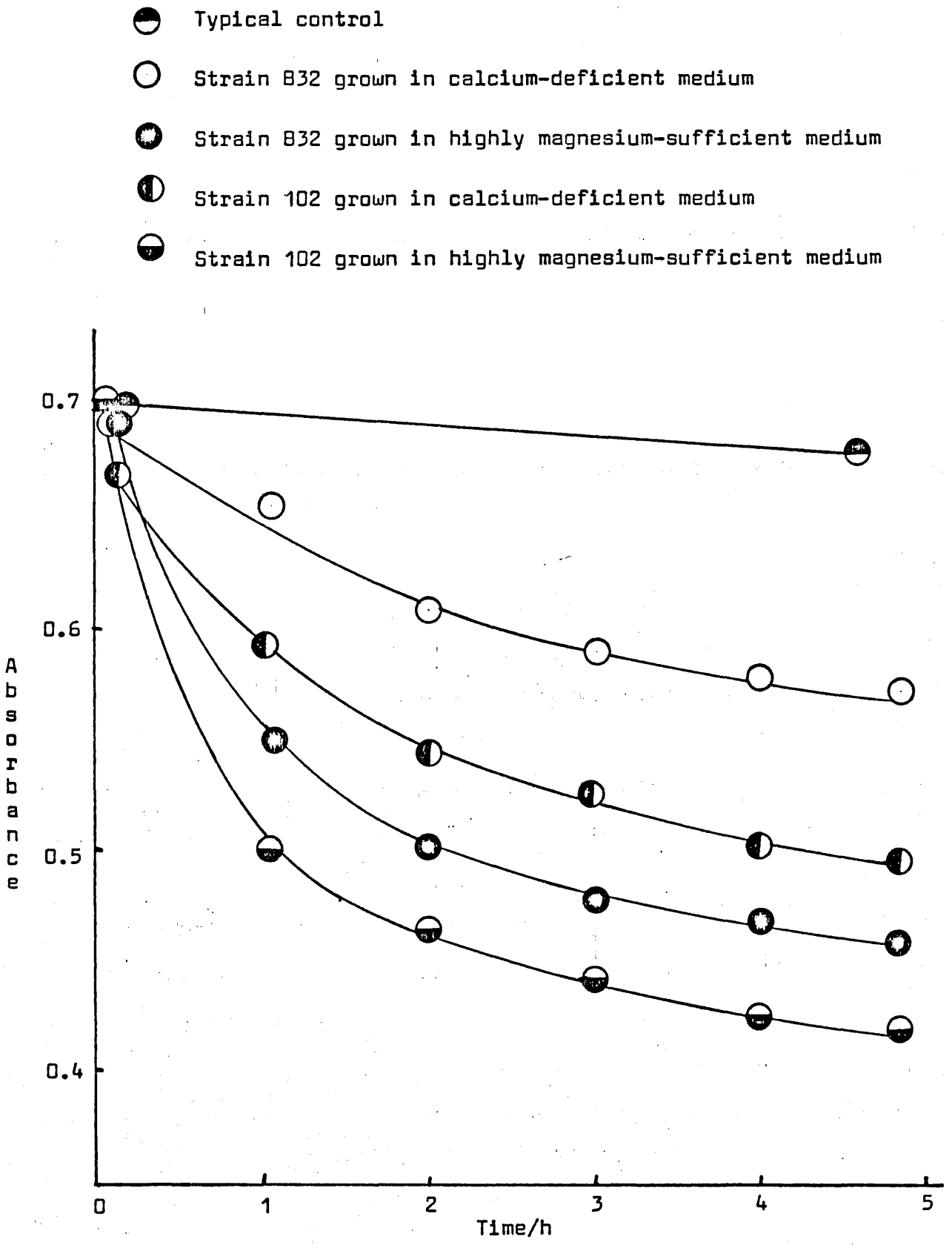


Figure 5.7 EDTA-lysis of cells of P.aeruginosa grown at 37 °C in basal medium and highly magnesium-sufficient medium



The cells of both strains lysed much less readily over the test period of about 5 h when they were grown in calcium-deficient basal medium than when they were grown in highly calcium or magnesium-sufficient media. The results for the lysis of cells grown in highly calcium or magnesium-sufficient media were indistinguishable.

These results indicate that a detectable proportion of the extra magnesium and calcium ions provided in the magnesium and calcium-supplemented media may reside in the outer cell envelope of the bacteria, where they are susceptible to chelation by EDTA. Thus if these cations are involved in maintaining cell envelope integrity this could lead to the increased rate of lysis compared to cells grown in basal medium. These extra cations may thus contribute to gentamicin-resistance. The results for growth in basal medium suggest that the increased magnesium uptake detected by atomic absorption spectrophotometry (Section 5.7) in cells of strain 832, does not lead to a high level of magnesium in the cell envelope.

Cells of strain 102 appear to concentrate greater levels of divalent metal ions in the outer cell envelope than strain 832 for growth in any given medium, leading to more efficient lysis by EDTA.

Cells of strain 832 grown in nutrient broth lyse more rapidly than when grown in basal medium, but less rapidly than when grown in the calcium or magnesium-supplemented media.

The absorbances of the control suspensions decreased over the experimental period but to a much lesser extent than did the test suspensions.

5.10 Effect of Suspension in Gentamicin/Buffer Solutions on Cells of P.aeruginosa

It is possible that cells of P. aeruginosa grown in calcium-sufficient medium acquire a barrier to gentamicin molecules entering the cell. A series of experiments was performed to compare the effects of different calcium concentrations in the growth medium with gentamicin uptake (if any) during suspension of cells in buffered gentamicin solutions.

Cells of strains 832 and 107 were grown in basal medium or calcium-sufficient medium (BM(Ca 2)) and cells of the resistant strains 100 and 101 were grown in nutrient broth. The cultures were centrifuged and the cells washed aseptically three times with barbiturate-acetate buffer ($I = 5 \times 10^{-3}$ mol dm^{-3}, pH = 7.0) and finally suspended in sterile buffer solution to give a suspension with an absorbance of about 0.1. 0.5 cm^3 of each suspension was then pipetted into both 25 cm^3 sterile buffer (as a control) and 25 cm^3 sterile buffer containing an appropriate concentration of gentamicin. These suspensions were then shaken at 37 ^{o}C for periods of up to 24 h. Equal portions of each suspension were then centrifuged and the cells again washed aseptically three times to remove any gentamicin, and finally the cells were suspended in about 20 cm^3 of buffer solution. Samples from each suspension were plated onto nutrient agar.

Cells of both strains 100 and 101 grew profusely on nutrient agar after suspension in gentamicin concentrations of 1000 $\mu g\ cm^{-3}$. Thus cells of these strains (MIC = 8000 $\mu g\ cm^{-3}$) were still highly

resistant to gentamicin during suspension in buffered gentamicin solution. The controls for these and other strains grew well on nutrient agar after suspension in buffer solution. For strains 832 and 107 there was no detectable difference by this technique, between cells grown in basal medium and cells grown in calcium-sufficient medium. Cells grown in both media grew on nutrient agar after suspension in gentamicin concentrations lower than their MIC of gentamicin (determined in nutrient broth), but failed to grow after suspension in gentamicin levels equal to or greater than their MIC. Thus the higher levels of resistance induced by growth in calcium-supplemented medium compared with growth in basal medium appeared to be lost during suspension in buffered gentamicin solutions.

Possible explanations for these results are that cellular changes caused by different levels of calcium ions in the growth medium do not alter resistance but that resistance is due to some other mechanism, such as gentamicin/calcium interaction; or that during suspension the gentamicin binds firmly to the bacterial surfaces (Bryan and Van Den Elzen, 1975), and thus when the cells originally grown in calcium-supplemented medium are grown on nutrient agar they revert to having lower levels of calcium and thus become susceptible to the bound gentamicin.

5.11 Lytic Effect of Gentamicin on Cells of P.aeruginosa

Cells of strains 832 and 107 grown for 18 h in basal medium or in calcium-sufficient medium (BM(Ca 2)), were centrifuged and washed three times with barbiturate-acetate buffer ($I = 5 \times 10^{-3}$

mol dm^{-3}, pH = 7.0) and suspended in buffer solutions containing various concentrations of gentamicin. The absorbance of each suspension was measured at intervals over a period of 24 h at 660 nm.

No lytic effects were caused by either lethal or sub-lethal concentrations of gentamicin; any lysis in the test solutions was matched by that occurring in the control suspensions.

5.12 Summary

(a) Both magnesium and calcium divalent cations in the growth medium enhanced gentamicin-resistance in strains of *P. aeruginosa*.

(b) Higher densities of growth were observed for given strains when grown in calcium-sufficient media rather than calcium-deficient media.

(c) Cells of *P. aeruginosa* grown in media containing different concentrations of calcium and magnesium exhibited different surface properties. On acquiring gentamicin-resistance due to growth in divalent cation rich media, cells of sensitive strains exhibited gentamicin-resistant type pH-mobility curves. Cells of gentamicin-resistant strains on becoming gentamicin-sensitive due to growth in calcium-depleted medium exhibited gentamicin-sensitive type pH-mobility curves and reduced surface lipid.

(d) Cells of P. aeruginosa grown in calcium-supplemented medium had enhanced levels of cell calcium and higher levels of polysaccharide. Cells grown in calcium-deficient medium appeared to contain slightly higher levels of protein.

(e) Cells of P. aeruginosa grown in calcium or magnesium-supplemented media had higher levels of surface divalent cations as detected by EDTA-lysis of cells, than did cells grown in basal medium.

CHAPTER 6

STUDIES OF R-FACTOR MEDIATED GENTAMICIN-RESISTANCE IN CELLS OF P. AERUGINOSA

The following experiments were undertaken to determine (a) whether any surface changes, detectable by electrophoretic techniques, occurred in cells of P. aeruginosa when they acquired R-factor mediated gentamicin-resistance, and (b) to compare the surface properties of R-factor containing gentamicin-resistant strains with those of gentamicin-resistant strains of P.aeruginosa, already examined, which do not contain R-factors. Experiments to transfer gentamicin-resistance to gentamicin-sensitive strains of P.aeruginosa and to eliminate gentamicin-resistance were also performed. The antibiotic characteristics of R-factor containing strains discussed in this chapter are listed in Table 2.2.

6.1 R-Factor Transfer Experiments

In these experiments attempts to transfer resistance from R-factor carrying strains Stone no. 130 and Capetown no. 18 were compared with attempts to transfer resistance from gentamicin-resistant strains of P.aeruginosa which did not contain R-factors.

6.1.(a) Transfer between potential R-factor donor strains and a rifampicin-resistant acceptor strain

Only strains Capetown no.18 and Stone no.130, which were known to possess transmissible R-factors, were capable of transferring increased gentamicin-resistance to strain PU21 (Section 2.1.(g)). The transfer of resistance was consistently positive for these two strains, and was most pronounced when a 1 ; 3 donor to acceptor

ratio was used and the mating mixture was incubated at 37 °C for 24 h. Resistant strains 100, 101, 103, 104, 105, 107 and Smith all gave negative results; thus they did not carry transmissible R-factors.

6.1.(b) Transfer between potential R-factor donor strains and carbenicillin-resistant acceptor strains

R-factors from strains Capetown no 18 and Stone no 130 were transferred to carbenicillin-resistant strains 1/Cb and 3/Cb. The resulting transconjugant strains were subsequently found to transfer gentamicin-resistance to cells of strain PU21. As before, other resistant and potential R-factor donor strains were incapable of transferring gentamicin-resistance when strains 1/Cb and 3/Cb were used as acceptors.

6.1.(c) Transfer between potential R-factor donor strains and Esch. coli strains

It was not possible by using any of the techniques described in section 2.1.(h) to transfer gentamicin-resistance from gentamicin-resistant strains (even strains known to carry transmissible R-factors) of P. aeruginosa to strains of Esch. coli.

6.2 Curing of R-Factors

Acridine dyes are routinely used to cure sex (F) factors. They can also cure drug resistance (R) factors at a reportedly much

lower efficiency (Mitsuhashi et al, 1961;Watanabe and Fukasawa, 1961). Acridines are believed to induce curing by binding to DNA molecules. Sodium dodecyl sulphate (SDS), an anionic surface active agent has also been reported to be very efficient in curing R-factor mediated drug resistance (Adachi et al, 1972; Maliwan et al, 1975). The sex pili are beleived to be the site of action of SDS. In this study the acridine dye proflavine and SDS were used in curing experiments.

Gentamicin-resistant strains Capetown no.18, Stone no.130, POW 151, PL11 (all known to possess R-factor mediated gentamicin-resistance), 100, 101, 104 and 105 were grown overnight in nutrient broth at 37 ^{o}C. 100 cm^3 medical bottles with 50 cm^3 nutrient broth containing 10% w/v SDS, 50 cm^3 nutrient broth containing 50 $\mu g\ cm^{-3}$ proflavine and 50 cm^3 nutrient broth for controls were then inoculated from each culture. These were incubated at 37 ^{o}C for 72 h. A loop of each culture was then streaked (in duplicate) onto nutrient agar plates and incubated overnight to produce isolated colonies. Each of 100 such single colonies per plate was then used to inoculate individual agar plates containing gentamicin at half the MIC of gentamicin of that particular strain. The plates were incubated at 37 ^{o}C for up to 96 h. After incubation the plates were examined for growth, absence of growth being taken as evidence of curing. The results are listed in Table 6.1 as the total frequency of drug susceptible colonies among survivors, i.e. percentage curing.

All the control plates showed near 100% growth and so effective comparison with the test plates was possible.

Table 6.1 Curing of R-factors mediating gentamicin-resistance in P. aeruginosa

Strain	% curing after 72 h incubation in presence or absence of curing agents		
	Control	Proflavine treated	SDS treated
Capetown no.18	0	0	0
Stone no.130	0	72	20
PL11	10	85	12
POW 151	0	0	7
100	0	0	0
101	0	0	0
104	0	0	0
105	0	0	0

R-factor carrying strain Capetown no.18 showed no evidence of curing of gentamicin-resistance by either SDS or proflavine, whilst strain Stone no.130 was slightly cured by SDS and very efficiently cured by proflavine. Jacoby (1974) reported that curing was not observed when strains carrying R-factors pMG1 and pMG2 were treated with SDS and acriflavine. Strain POW 151 was slightly cured by SDS but not by proflavine. Strain PL11 was very efficiently cured by proflavine but was not cured by SDS.

Strains 100, 101, 104 and 105 showed no evidence of curing after treatment with SDS and proflavine, which taken with their inability to transfer gentamicin-resistance (Section 6.1) strongly suggests that these strains are resistant to gentamicin by some mechanism other than by R-factor mediation.

6.3 MIC of Gentamicin of Strains of P. aeruginosa

The values of the MIC of gentamicin are listed in Table 6.2. When an R-factor was introduced into a gentamicin-sensitive acceptor strain, there was always an increased level of gentamicin-resistance in the latter. In 8 of the 10 examples shown, the acceptor strain changed from a gentamicin-sensitive to a gentamicin-resistant strain. All of the five donor strains were gentamicin-resistant but in no case did the acquisition of R-factors give an acceptor strain a gentamicin-resistance equal to that of the parent donor strain. It is of interest that PL11, a donor strain with one of the higher levels of resistance only conferred a negligible resistance on the acceptor strain; the

Table 6.2

MIC values of gentamicin for the various strains of P. aeruginosa

Donor		Acceptor		Transconjugant		Increase in resistance
Strain	MIC/$\mu g\ cm^{-3}$	Strain	MIC/$\mu g\ cm^{-3}$	Strain	MIC/$\mu g\ cm^{-3}$	
PL11	200	PL1	0.25	PL1 (RPL11)	3.0	12
Capetown no.18	100	PU21	< 0.75	PU21 (pMG1)	25.0	>33
Stone no.130	200	PU21	< 0.75	PU21 (pMG2)	25.0	>33
130	50	280	0.075	280 (R130)	1.8	24
POW 151	100	280	0.075	280 (R151)	7.5	100
130	50	1310	0.075	1310 (R130)	30.0	400
Capetown no.18	100	1/Cb	0.10	1/Cb (pMG1)	> 8.0	> 80
Stone no.130	200	1/Cb	0.10	1/Cb (pMG2)	> 8.0	> 80
Capetown no.18	100	3/Cb	0.20	3/Cb (pMG1)	> 8.0	> 40
Stone no.130	200	3/Cb	0.20	3/Cb (pMG2)	> 8.0	> 40

increase of resistance was by a factor of 12, compared with an in excess of 33 fold increase of resistance using the comparably resistant strains Capetown no.18 and Stone no.130 as donors.

6.4 pH-Mobility Curves and Surface Lipid of Cells of P. aeruginosa Grown at 37 °C in Nutrient Broth

The results for the strains under consideration are shown in Table 6.3. As has already been reported (Section 3.2), cells of the R-factor acceptor strains PL1, PU21, 1310, 280 and 3/Cb showed normal gentamicin-sensitive type pH-mobility curves and had low S-values. In none of the strains examined did the acquisition of R-factors by the acceptors have any effect on the surface properties of the cells, in as far as they could be detected from pH-mobility curves or S-value determinations. This is seen for example in Figure 6.1, where the pH-mobility curves for cells of strain PU21 and cells of three R-factor containing derivatives of strain PU21 are superimposable; the cells of all four strains also had low S-values.

Cells of the R-factor donor strains Capetown no.18, Stone no.130, 130 and POW 151 are exceptional in that they are the first 'naturally' gentamicin-resistant strains of P. aeruginosa (i.e. excluding in vitro produced transconjugants discussed above) examined in this laboratory, the cells of which showed typical gentamicin-sensitive type pH-mobility curves with low S-values after growth at 37 °C in nutrient broth (Figures 6.2 and 6.3). In marked

Table 6.3

Summary of the MIC of gentamicin and the surface properties of R-factor donor, acceptor and transconjugant strains of P.aeruginosa

Strain	MIC of gentamicin /$\mu g\ cm^{-3}$	pH-mobility* curve	S-value
PL11**	200	R	17
PL1 (RPL11)	3	S	0
PL1***	0.25	S	5
Capetown no.18**	100	S	2
PU21 (pMG1)	25	S	3
Stone no.130**	200	S	0
PU21 (pMG2)	25	S	0
PU21 (R64)	> 200	S	0
PU21***	< 0.75	S	7
130**	50	S	0
280 (R130)	1.8	S	4
POW 151**	100	S	0
280 (R151)	7.5	S	0
280***	0.075	S	0
1310 (R130)	30	S	0
1310***	0.075	S	0
3/Cb***	0.2	S	0
3/Cb (pMG1)	> 8	S	0

* characteristic sensitive (S) or resistant (R) type

** donor

*** acceptor

Figure 6.1

pH-mobility curves for cells of strains PU21, PU21 (pMG1), PU21 (pMG2) and PU21 (R64) grown at 37 °C in nutrient broth

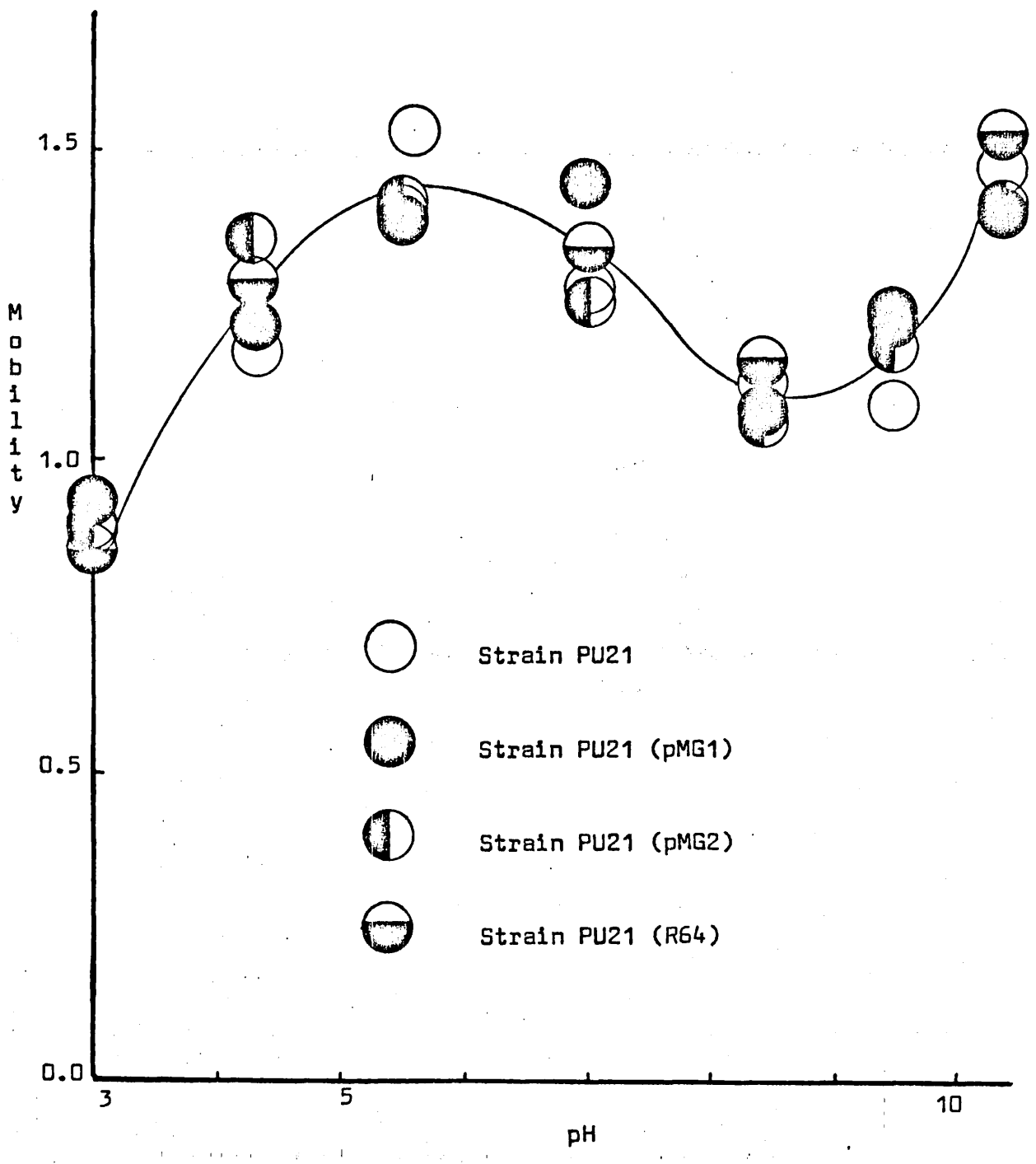


Figure 6.2

pH-mobility curves for cells of strains Capetown no.18 and Stone no.130 grown at 37 °C in nutrient broth

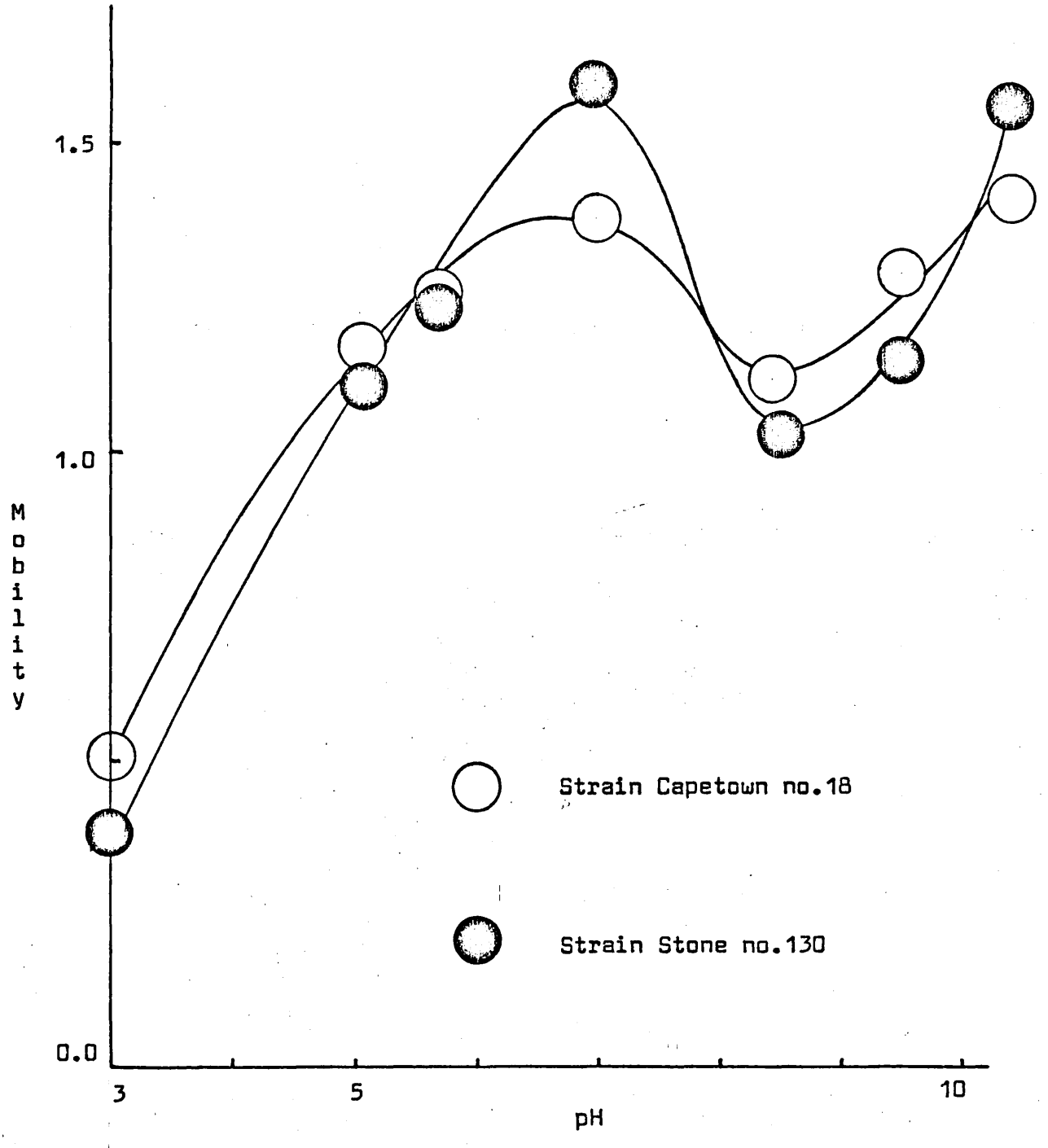
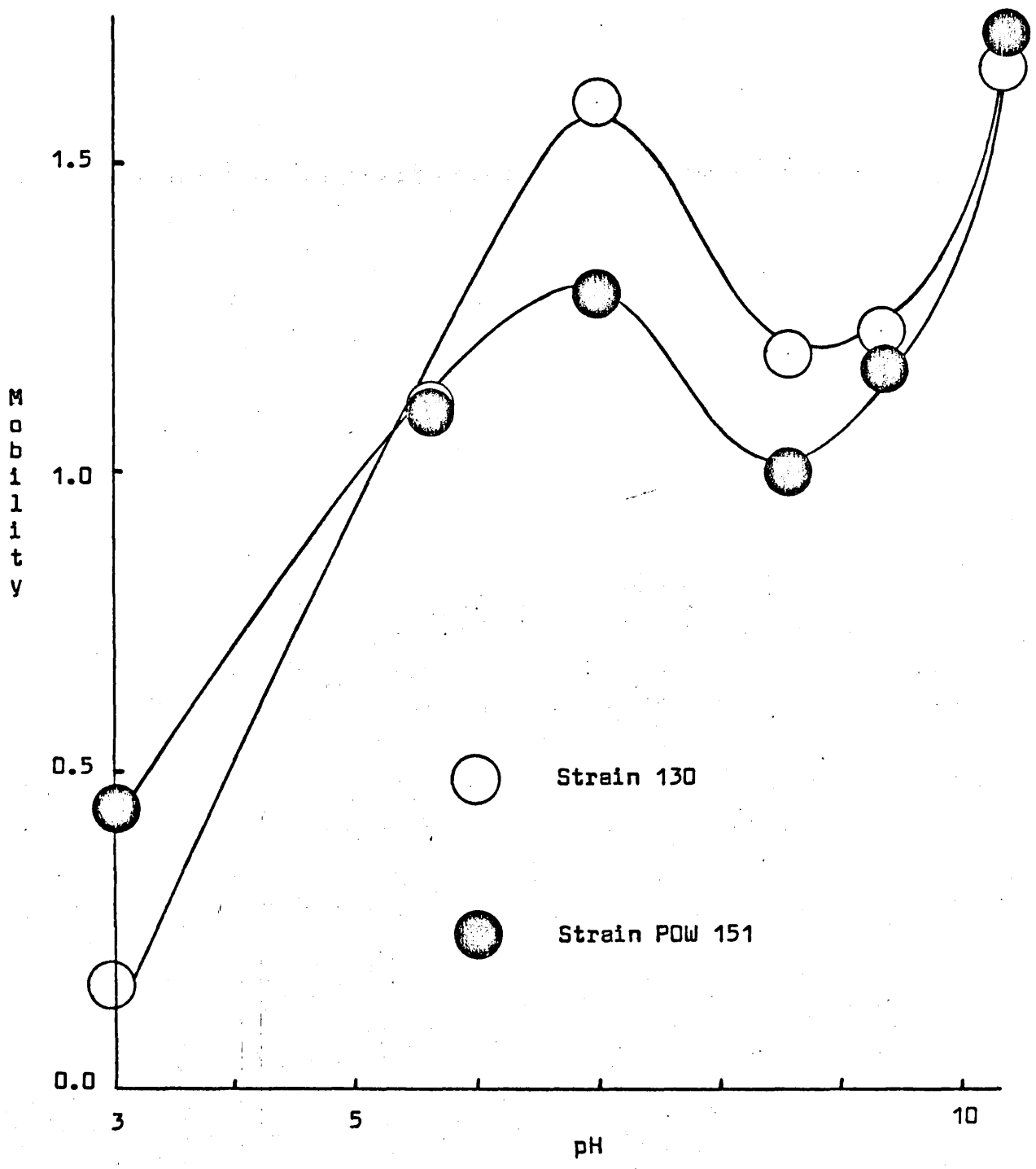


Figure 6.3

pH-mobility curves for cells of strains POW 151 and 130 grown at 37 °C in nutrient broth



contrast, cells of the gentamicin-resistant donor strain PL11 (MIC = 200 $\mu g\ cm^{-3}$) exhibited a 'normal' gentamicin-resistant type pH-mobility curve and an associated high S-value (Figure 6.4). There was no change in the shape or position of the pH-mobility curve of cells of the acceptor strain (PL1) after the acceptance of R-factor RPL11.

6.5 Growth of R-Factor Strains in Calcium-Sufficient and Calcium-Deficient Synthetic Media

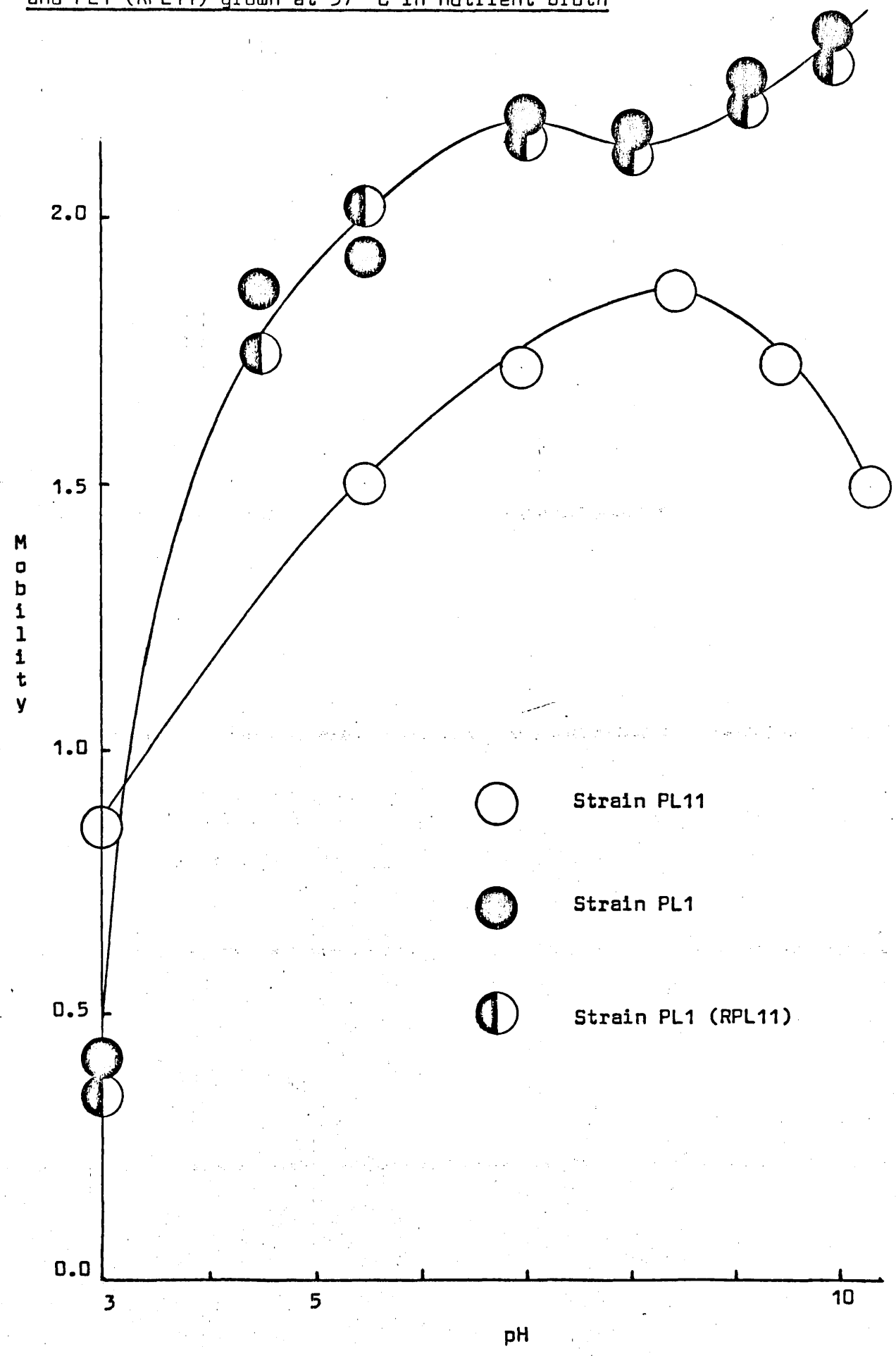
The MIC of gentamicin of R-factor strains PL11 and Stone no.130 grown in calcium-deficient (calcium concentration 2.5×10^{-3} mmol dm^{-3}) and highly calcium-sufficient (calcium concentration 22.5 mmol dm^{-3}) media were determined (Table 6.4).

Table 6.4

Effects of calcium ion concentration on the MIC of gentamicin of R-factor carrying strains of P.aeruginosa

Strain	MIC of gentamicin $/\mu g\ cm^{-3}$		
	calcium-deficient medium	calcium-sufficient medium	nutrient broth
PL11	100	> 400	200
Stone no.130	100	> 400	200

Figure 6.4 pH-mobility curves for cells of strains PL11, PL1 and PL1 (RPL11) grown at 37 °C in nutrient broth



Both strains showed a 'normal' response to variation of calcium concentration in the growth medium (Section 5.1), the MIC of gentamicin was lower in the calcium-deficient medium and higher in the highly calcium-sufficient medium, as compared to MIC determinations made in nutrient broth. These results correspond to those obtained for gentamicin-sensitive strains and gentamicin-resistant strains not carrying R-factors.

6.6 Summary

(a) P.aeruginosa strains Capetown no.18 and Stone no.130 which were known to possess R-factor mediated gentamicin-resistance, transferred gentamicin-resistance to gentamicin-sensitive acceptor strains.

(b) One strain known to carry R-factors was cured by SDS and by proflavine, one by SDS only and one strain by proflavine only. The fourth strain was not cured by either agent.

(c) The acquisition of R-factors mediating gentamicin-resistance, by gentamicin-sensitive strains of P.aeruginosa resulted in increased MIC of gentamicin values. However, the transconjugant strains always had lower MIC values than their corresponding donor strains.

(d) No changes in surface properties were observed in cells of P. aeruginosa which had acquired R-factors.

(e) Cells of four 'parent' R-factor carrying strains of P. aeruginosa had gentamicin-sensitive type pH-mobility curves and low S-values. Cells of a fifth strain had a gentamicin-resistant type pH-mobility curve and a high S-value.

(f) The MIC of gentamicin of two R-factor carrying strains of P. aeruginosa were determined in calcium-deficient and highly calcium-sufficient media. The former decreased and the latter increased the MIC, as compared to growth in nutrient broth.

CHAPTER 7

A STUDY OF THE INTERACTION OF DIVALENT METAL IONS WITH GENTAMICIN

Calcium and magnesium ions may enhance gentamicin-resistance in cells of P. aeruginosa by interacting with gentamicin molecules. Thus the gentamicin-resistance of the cells need not actually alter, but it is possible that the cells would be able to grow in higher concentrations of gentamicin because the metal ions effectively reduce the concentration of gentamicin in its active form. This interaction might have two effects; either the metal ions bind firmly to the gentamicin molecule and by altering the configuration of the antibiotic or by masking active sites on the molecule, reduce its potency; or the binding of the metal ions onto the gentamicin effectively increase the size of the antibiotic molecule, thus making entry into the bacteria more difficult.

Since gentamicin does not exhibit any strong absorption in the u.v. or visible regions of the spectrum, any method in the detection of metal ion-gentamicin complexes based on Job's plot cannot be used. The technique of microcalorimetry has been employed increasingly in recent years in the study of such complexes. The method is very sensitive in detecting the heat changes which accompany chemical reactions and is thus a method of following the extent of the reactions. An LKB-10700-1 flow microcalorimeter fitted with a mixing cell was used. A solution of calcium chloride or magnesium sulphate of known concentration was pumped into the cell at the same rate as a solution of gentamicin chloride or sulphate of fixed concentration. When equilibrium was reached the deflection of the trace of rate of heat output against time from the baseline, was taken as a measure

of the heat change for the reaction between the two solutions.

The commercial gentamicin available was in the form of of gentamicin sulphate (88 g sulphate = 50 g base) and thus when this was reacted with calcium chloride solution there was a possible problem of heat change arising from gentamicin/chloride and calcium/sulphate interactions. To overcome this, gentamicin sulphate was converted to gentamicin chloride (and also to the gentamicin base to investigate the reaction between gentamicin base and calcium chloride). The gentamicin preparation consisted of 34.9% gentamicin C_1, 32.51% gentamicin C_2 and 32.59% gentamicin C_{1a}. The relative molecular masses of these components are:

$$C_1 = C_{21}H_{44}O_7N_5 = 477$$

$$C_2 = C_{20}H_{42}O_7N_5 = 463$$

$$C_{1a} = C_{19}H_{40}O_7N_5 = 449$$

the average molecular mass of the laboratory sample was taken as 463.3 and using this value 2 dm^3 of 0.01 mol dm^{-3} gentamicin solution were made up using conductance water. A strong anion exchange resin ('Amberlite' IRA-400(Cl)) was used to convert gentamicin sulphate to gentamicin chloride and gentamicin base. Columns were approximately half filled with a slurry of the resin in the chloride form. These were then drained and back washed with an upward flow of water until the waste water at the top was clear. The flow was then stopped, excess water drained off and the resin washed with conductance water. 500 cm^3 of 3% NaOH solution was then passed through one column to regenerate the

resin in the hydroxyl (base) form and 500 cm^3 of 3% NaCl solution passed through the other to regenerate the resin in the chloride form. About 2 dm^3 of conductance water was used to wash each column and this in turn was washed away with about 200 cm^3 of gentamicin solution. About 500 cm^3 of gentamicin sulphate was then passed through each column and collected; periodically the eluent solutions were tested with barium chloride solution to ensure that no sulphate was coming down and with silver nitrate solution to ensure that gentamicin chloride was coming through one column but not the other.

The relative potency of the three gentamicin solutions was tested against P. aeruginosa strains 1 and 832. The results are shown in Table 7.1.

Table 7.1

MIC for P. aeruginosa strains 1 and 832

Strain	MIC/$\mu g\ cm^{-3}$		
	gentamicin sulphate	gentamicin chloride	gentamicin base
1	0.1	0.2	0.2
832	3.0	3.0	6.0

The gentamicin solutions were not significantly diluted during passage through the ion exchange resin; the chloride form

is slightly less potent than the sulphate form and the base slightly less than the chloride form.

To investigate heat changes evolved when gentamicin reacts with calcium or magnesium ions, the two standard solutions under consideration were pumped into the microcalorimeter (at the same rate of 20 $cm^3\ h^{-1}$). Corrections for the heat of dilution of both salt solution and gentamicin solution were applied. The procedure adopted was, firstly to pass distilled water and calcium chloride or magnesium sulphate solution into the cell to obtain a base line on the recorder; this automatically corrects for the heat of dilution of the salt solution. A gentamicin solution was then pumped in instead of the water until a constant deflection from the base line was obtained. This deflection after correction for the heat of dilution of the given gentamicin solution was then a measure of the reaction between the two solutions. Using this technique it was found by reacting gentamicin chloride with calcium chloride and magnesium sulphate with gentamicin sulphate, that both calcium and magnesium react endothermically and with approximately the same heat change with gentamicin. Calcium chloride reacted exothermally with gentamicin base but this was considered unreliable as an indication of calcium/gentamicin complex formation due to the possibility of heat changes involved in gentamicin/chloride interaction.

The experiment was repeated with varying concentrations of calcium chloride (7.5×10^{-4} to 6×10^{-1} mol dm^{-3}) but with a fixed concentration of gentamicin (6×10^{-3} mol dm^{-3}). The pH of the gentamicin chloride solution was 6.

The deflection on the thermogram (arbitrary scale) was then plotted against final (i.e. after 1:1 mixing in the cell) calcium concentration (Figure 7.1). The shape of this graph indicates that a weak complex is formed between calcium and gentamicin; the magnitude of the reaction increasing for each increase of calcium concentration, up to calcium concentrations considerably in excess of the gentamicin concentration. The turnover point of the graph is thus not readily located. If the initial and the main slope of the graph are extrapolated as shown, the lines cross at a concentration of 3×10^{-3} mol dm^{-3} calcium; this is indicative of a 1:1 calcium/gentamicin complex;

For the system,

$$Ca + Gm \rightleftharpoons CaGm$$

If α is the fraction of gentamicin which has formed a complex then,

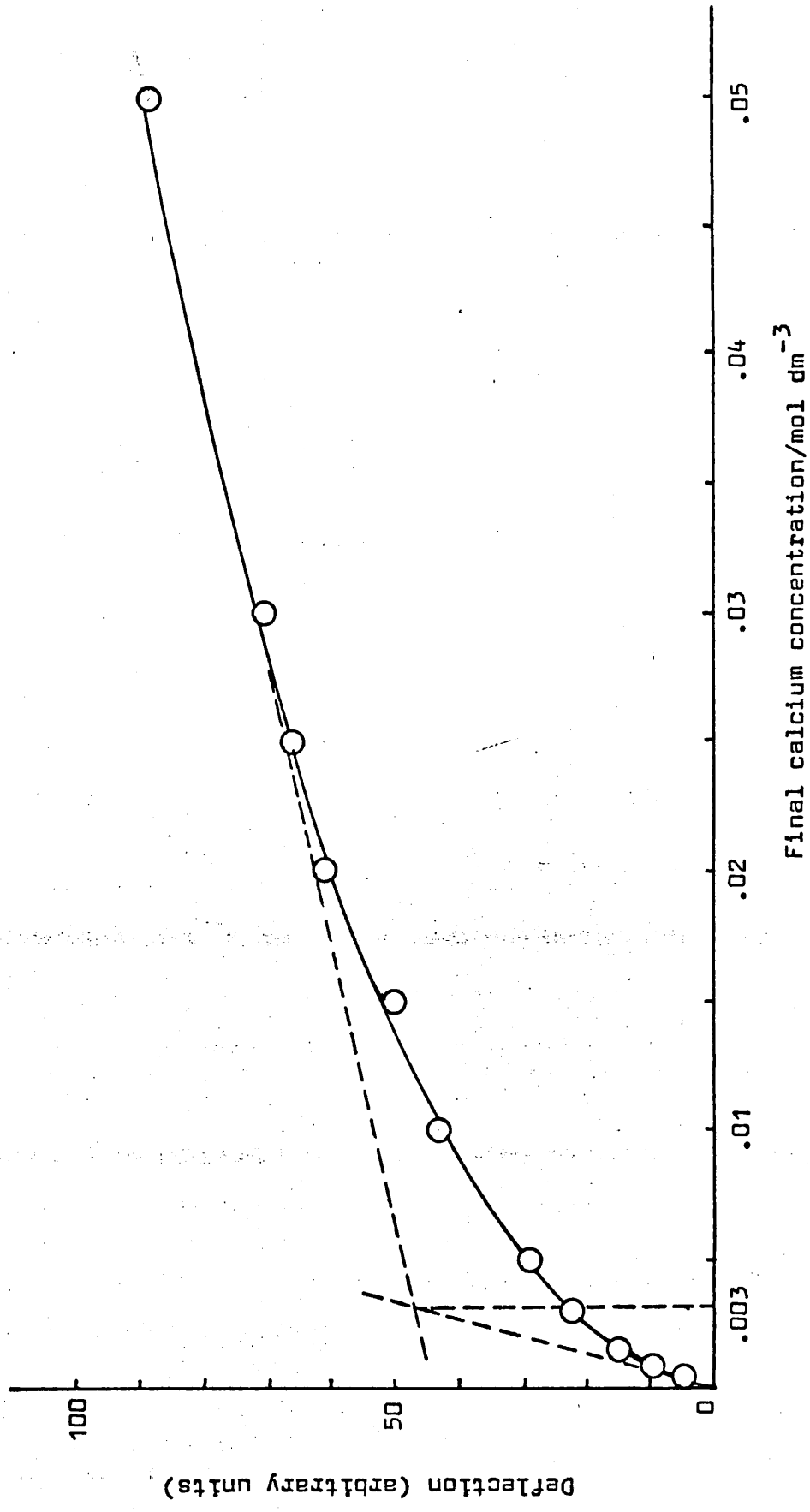
$$K = \frac{c_{(Gm)} \alpha}{c_{(Ca)} (1-\alpha) \times c_{(Gm)} (1-\alpha)}$$

$$K = \frac{\alpha}{c_{(Ca)} (1-\alpha)^2} \qquad (1)$$

α is obtained experimentally from the ratio:

$$\frac{\text{deflection at the given concentration}}{138}$$

Figure 7.1 Graph of deflection against final calcium concentration



where 138 was the maximum deflection obtainable from reacting 0.006 mol dm^{-3} GmCl solution with calcium chloride solutions.

The stability constant was thus calculated for each reaction using equation (1). The results are listed in Table 7.2. These stability constants of about 100 or less indicate a very weak complex.

Table 7.2

Final concentration of calcium/mol dm^{-3}	Deflection	α	$K_{stab.}$
3.75×10^{-4}	4.5	0.032	91.07
0.75×10^{-3}	9	0.065	99.47
1.5×10^{-3}	15	0.1087	91.22
3.0×10^{-3}	23	0.1704	82.52
0.5×10^{-2}	29	0.2101	95.21
1.0×10^{-2}	43	0.3116	65.75
1.5×10^{-2}	49	0.3550	56.9

The final gentamicin concentration was 3×10^{-3} mol dm^{-3}.

The thermodynamic functions, ΔG, ΔH and ΔS for the reaction were calculated. ΔG was determined using the equation,

$$\Delta G = - R T \ln K$$

and was found to be -11.41 kJ mol^{-1}.

ΔH was found to be 1.31 kJ mol^{-1}. The entropy change, ΔS, was then calculated using the relation,

$$\Delta G = \Delta H - T \Delta S,$$

and was found to be 42.7 J K^{-1} mol^{-1}.

CHAPTER 8

DISCUSSION

In studying antibiotic resistance in bacteria it is important to examine the surface of cell walls both because the surface is the first (and often the final) site of attack by the antibiotic and also because cell wall changes are often implicated in antibiotic-resistance mechanisms. When analysis is made after the cell walls have been treated chemically e.g. after acid hydrolysis, there is no guarantee that the molecular structure of wall components is the same in the free state as in the intact cell. The main technique employed in this investigation was particulate electrophoresis, which was used to detect any surface changes due to growth in different media or to the bacteria becoming resistant to antibiotics. The main advantage of this technique is that complete, undamaged cells suspended in buffer solution, can be studied.

The zeta-potential at the cell-electrolyte interface is determined in part by the nature and quantity of the ionogenic groups at the surface and in part by the ionic strength and pH of the suspending medium. The viscosity and relative permittivity within the electric double layer have generally been assumed to have the value of bulk water, but recent work indicates that this may not be so. This means that the calculation of zeta-potential (5) from mobility values using the Smoluchowski equation:

$$\bar{v} = \frac{\epsilon \zeta}{4\pi\eta} = \frac{\epsilon_0 \epsilon_r \zeta}{\eta}$$

(where η is the coefficient of viscosity, ϵ_r the relative permittivity of the medium and ϵ_0 the permittivity of free space)

and the subsequent discussion of the variation of the zeta-potential is open to doubt. However, if the ionic strength and pH of the suspending electrolyte are kept constant, then for cells of fixed age, changes in the experimentally determined mobility values can be interpreted in terms of changes in the nature and quantity of surface charged groups. These conditions were maintained for the present study.

In a previous investigation Pechey (1973) determined the experimental conditions and procedures necessary to give a reproducible surface free from adsorbed materials such as toxins or growth material. This was essential in order to make valid interpretations of the results obtained. Thus the measurement of mobility values was made at 10 ^{o}C, a temperature at which the motility of cells of P. aeruginosa is completely suppressed. It was found that two washings of the cells in buffer solution were sufficient to remove any adsorbed material. The ionic strength of the buffer solutions used to suspend cells for mobility measurements was 5×10^{-3} mol dm^{-3}; this was the ionic strength at which the mobility of the cells was at a maximum. The shape and position of the pH-mobility curves of cells of P. aeruginosa was independent of the time of incubation between 18 and 24 h and of the physical nature of the growth medium; it was also unaffected by overnight storage of the prepared suspensions at 5 ^{o}C.

A daily calibration of the apparatus was performed using a particle of known mobility. The accepted standard reference particles are human erythrocytes suspended in 0.667 mol dm^{-3}

phosphate buffer solution at pH 7.35. In this study a secondary standard reference (Gittens and James, 1960), 18 h cells of K. aerogenes suspended in barbiturate-acetate buffer solution at pH 7.0 was used. When these conditions for the preparation of cells and the determination of mobility values were strictly observed, the cells of a given strain of P. aeruginosa had a reproducible surface. The confidence limit for a single mean at $p = 0.05$ was 3% and thus cells with mobility values differing by 10% or more were considered to have significantly different surfaces; this may be due to the cells having different surface components or different proportions of the same components. The cells of individual strains in suspension were electrokinetically homogeneous at all pH values. Mobility determinations were restricted to pH range 2 to 11. Within this range the cell surfaces did not suffer irreversible denaturation.

The previous findings of Pechey relating characteristic surface properties to gentamicin sensitivity/resistance in strains of P. aeruginosa, were confirmed by electrophoretic studies with a larger group of gentamicin-sensitive and gentamicin-resistant strains (in which resistance was not due to the presence of R-factors - see page 182). Thus cells of gentamicin-sensitive strains (MIC $\leqslant$ 12 $\mu g\ cm^{-3}$), grown in nutrient broth displayed a pH-mobility curve with a maximum negative mobility value in the pH range 5.5 to 6.5 and a minimum value in the pH range 7.5 to 8.5; the difference between the maximum and minimum values was about 10-40% of the maximum value. The pH-mobility curves for gentamicin-resistant strains grown in nutrient broth

(MIC $>$ 12 μg cm^{-3}), are characterised by a maximum negative mobility value in the pH range 7.5 to 8.5. No minimum mobility value at higher pH values (up to pH 10.5) was observed. Typical pH-mobility curves are shown in Figure 8.1. The rate of increase of mobility between pH 3 and the pH of maximum mobility was steeper for sensitive cells than for resistant cells. In general the maximum mobility values for gentamicin-sensitive cells were higher than those for gentamicin-resistant cells. Thus cells of gentamicin-sensitive and gentamicin-resistant strains exhibit quite distinct, non-superimposable pH-mobility curves. Occasionally positive mobility values were observed in suspensions at very low pH values, a phenomenon not reported in the previous study but one which appeared to bear no relationship to gentamicin-resistance or sensitivity.

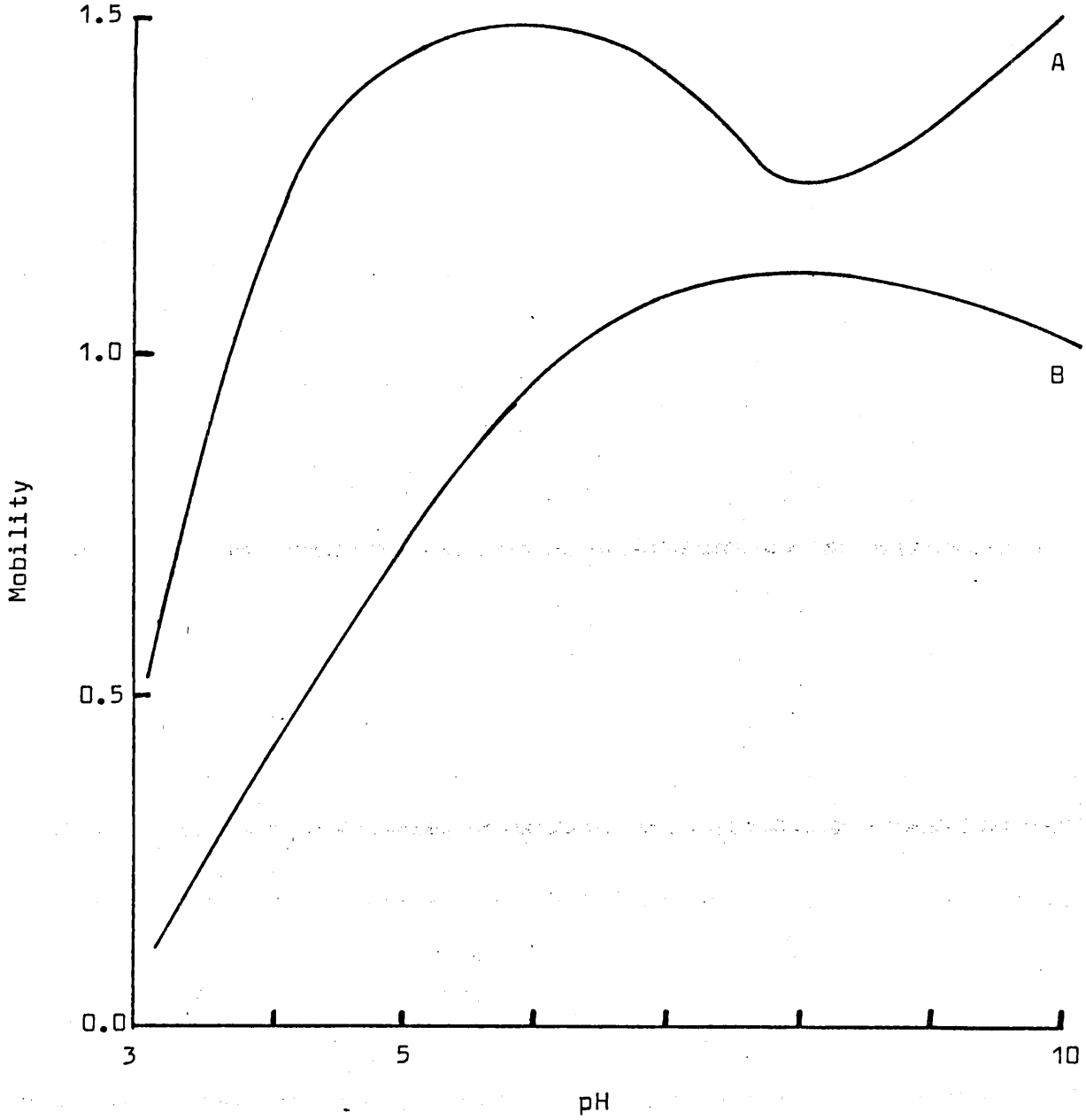
The MIC of gentamicin of all the strains except the sensitive strain 3 and the two very highly resistant strains 100 and 101 have decreased since they were first obtained (4-5 years previously). These strains have been maintained by regular subculturing onto fresh nutrient agar and stored at 5 ^{o}C. For strains 832, 96 and 102 this decrease in resistance has resulted in the strains changing from gentamicin-resistant to gentamicin-sensitive. This has been matched for cells of strains 832 and 102 by a change from a gentamicin-resistant to a gentamicin-sensitive type pH-mobility curve; this is further evidence of a link between characteristic surface properties and gentamicin-sensitivity or resistance. There is no obvious pattern between resistance to other aminoglycoside antibiotics, carbenicillin or phage-typing

Figure 8.1

Typical pH-mobility curves for gentamicin-sensitive and gentamicin-resistant strains of P.aeruginosa grown at 37 ^{o}C in nutrient broth or on nutrient agar

Curve A - Typical of gentamicin-sensitive cells

Curve B - Typical of gentamicin-resistant cells



characteristics and the type of pH-mobility curve obtained for cells of P. aeruginosa (Pechey and James, 1974). This again supports the suggestion of a link between surface properties and resistance to gentamicin.

The typical gentamicin-sensitive pH-mobility curve shows the cell surface to be complex, not consisting of a single charged species (cf. Lowick and James, 1957). Nor can the shape of the pH-mobility curve be simply explained as a pH-titration curve of a surface composed of more than one ionogenic species (cf. Plummer et al, 1962), as this would take the form of a series of plateau mobility values of increasing negative value as the pH increased. Instead the sinusoidal-type curve is indicative of some reorientation or rearrangement of the cell surface components, either revealing positively charged groups or concealing negatively charged groups as the pH increases. Hill and James (1972) suggested that such a rearrangement occurred at low pH values for cells of Staph. aureus, whereby the phosphate groups of cell wall teichoic acid become exposed, thereby giving a maximum mobility value in the pH-mobility curve. A similar pattern of change is seen for cells of P. aeruginosa but at higher pH values; the extrapolated isoelectric point is too high to be attributed to phosphate groups. The decrease in negative mobility at high pH values for cells of gentamicin-resistant strains suggested that some rearrangement of cell surface components may be occurring here also. It is impossible to obtain pK values of any surface groups from the pH-mobility curves owing to the complex nature of the surface (cf. Lowick and James,1957).

Nevertheless the presence of surface amino and carboxyl groups has been established (Pechey and James,1974). Attempts to establish the presence of teichoic acid by treatment of cells with sodium metaperiodate were unsuccessful.

The relative amount of surface lipid possessed by cells of P. aeruginosa was obtained from the increase in negative mobility resulting from the presence of a low concentration of sodium dodecyl sulphate (SDS), an anionic surface active agent in the suspension medium. The hydrocarbon chain of the SDS molecule becomes aligned with the surface lipid by hydrophobic interaction, leaving the hydrophilic (sulphate) polar head group protruding into the medium, thus increasing the negative mobility of the cells. The amount of increase of negative mobility is in proportion to the amount of surface lipid. Again, using a larger number of strains, the results confirmed those of Pechey, linking surface lipid with gentamicin-resistance; gentamicin-resistant cells (in which resistance was not due to the presence of R-factors) having significantly more surface lipid than do gentamicin-sensitive cells. An examination of the surface lipid of cells of strains B32 and 102, which over the years have changed from gentamicin-resistant to gentamicin-sensitive, showed that whereas previously they had high S-values (a measure of the amount of cell surface lipid), they now had negligible S-values, characteristic of gentamicin-sensitive type strains.

There is no quantitative difference between the total chloroform-methanol extractable lipid of gentamicin-resistant and gentamicin-sensitive cells (Pechey et al, 1974). However,

there was a qualitative difference; the highly gentamicin-resistant strain 100 had a significantly higher content of neutral lipid and free fatty acid than did the gentamicin-sensitive strain 1. An increased amount of neutral (e.g. triglycerides) surface lipid in gentamicin-resistant strains would account for the relatively lower maximum negative mobility values of these strains compared to that of sensitive strains, if the neutral lipid replaces components contributing significant negative charge to the surface. If the lipid was negatively charged then resistant cells would become more negatively charged than sensitive cells.

There thus appears to be strong evidence that for the large number of strains of P. aeruginosa examined, there is a correlation between gentamicin-resistance and a change in surface components of cells as compared with gentamicin-sensitive strains and that this change involves an increase in neutral surface lipid. These resistant strains do not carry R-factors mediating gentamicin-resistance and it may be that the different surface properties are indicative of a barrier mechanism of resistance, restricting the entry of gentamicin into the cell. Such barriers involving increased levels of lipid have been reported previously (Lowick and James, 1957; Hill et al, 1963; Hugo and Stretton, 1966); the evidence for barriers in P. aeruginosa has been discussed in Section 1.5.(c), the outer membrane layer being the most likely site of this barrier.

A barrier mechanism would have to operate in such a way that whilst the entry of gentamicin was restricted, the passage of nutrients and waste material across the outer membrane

was largely unimpeded. If the resistant cells do possess increased levels of surface neutral lipid this might act as a barrier to the large polar gentamicin molecule.

However, the wall can act as an adsorbing as well as a non-adsorbing barrier. Thus a strong interaction between phospholipid and drug has been used to explain drug resistance for penicillin (Padfield and Kellaway, 1973) and viomycin (MacKenzie and Jordan, 1970), it being proposed that the antibiotic interacts strongly at the surface, and thus remains at the surface and does not penetrate into the cell. Similarly, specific outer membrane components having a high affinity for specific drugs have been suggested as the sites of drug uptake. Thus a depletion or absence of these sites would contribute a penetration barrier to antibiotics. Any of these barrier-type resistance mechanisms might result in detectable changes in surface properties between sensitive and resistant cells.

The decrease in surface lipid of the highly gentamicin-resistant strains 100 and 101 over a period of 4 - 5 years (S-values have decreased from$\sim$60 to$\sim$20) argues against a barrier mechanism of resistance involving lipid, since the resistance of these strains has not changed. However, it may be that the lipid has become slightly more 'buried' in the cell surface, thus not altering the pH mobility curves but becoming less accessible to the SDS molecules used for detection.

Another possibility which must be considered is that the altered pH-mobility curves and increased surface lipid are a side-effect of another mechanism of resistance and not a cause

of resistance in themselves. Such a mechanism might be an altered ribosomal structure. The site of action of gentamicin is the ribosomes and thus a mutational alteration of an amino acid sequence might inhibit or even prevent the binding of gentamicin onto the ribosomes in resistant organisms; such a mechanism has been proposed to account for streptomycin resistance (Nomura, 1970).

Exclusion mechanisms have also been advanced as contributing factors in antibiotic-resistance when operating in conjunction with other mechanisms such as the production of antibiotic-inactivating enzymes (Smith *et al*, 1969; Roe *et al*, 1971). If antibiotic-inactivating enzymes are produced by the cells and the genes coding for them are located on the chromosomes they will not be detected in resistance transfer experiments. In such a situation, resistance will be passed from one generation to the next.

The effects of calcium and magnesium cations in the growth medium on the resistance of cells of *P. aeruginosa* to gentamicin are well documented and previous reports were verified in the current investigation. Thus each increase in concentration up to 22.5 mmol dm^{-3} of calcium or magnesium lead to an increased MIC of gentamicin for all strains examined. These results once again illustrate the need for an agreed and universal

standardisation of the technique for determining the MIC of gentamicin in P. aeruginosa. An increase of 22.5 mmol dm^{-3} in either the calcium or the magnesium concentration of the basal synthetic medium produced equal changes in the MIC for each strain; at lower concentrations (5 mmol dm^{-3} calcium or magnesium added) however, calcium was slightly more effective in antagonising gentamicin. A reason for this may be that at this concentration a significant proportion of the magnesium ions are required for metabolic processes other than those leading to resistance to antibiotics; this effect would be masked at higher concentrations.

The MIC values measured in the basal medium were consistently lower than those measured in nutrient broth; the former medium had less calcium but considerably more magnesium than the latter, as detected by atomic absorption spectrophotometry. Again, it may be that the use of magnesium in general metabolism means that at low concentrations of magnesium and calcium, it is mainly the latter which affects gentamicin resistance. Alternatively, the difference between the MIC values measured in the two media could be because nutrient broth is a richer medium, giving more vigorous growth and the bacteria may thus be better able to overcome the presence of gentamicin. Also calcium ions may actually be slightly more effective than magnesium ions in increasing resistance against gentamicin and this may only show up at low concentrations of these ions.

There was no competitive or synergistic effect between calcium and magnesium ions and growth in calcium-deficient and calcium-supplemented media had no permanent effect on gentamicin-

were
resistance (i.e. when cells subsequently grown in nutrient broth). There was denser growth in calcium and magnesium-supplemented media than in calcium-deficient basal medium. Potassium, zinc and iron(II) ions did not affect the MIC of gentamicin at the concentrations used; zinc and iron(II) were inhibitory at concentrations of 0.75 mmol dm^{-3} or above and 1.5 mmol dm^{-3} or above respectively.

Cells of borderline sensitive/resistant strains (i.e. sensitive or resistant when grown in nutrient broth) changed their resistance type with varying levels of calcium and magnesium ions. Thus gentamicin-sensitive strains 102, B32 and 106 became more sensitive when grown in basal medium, but became resistant when grown in highly calcium or magnesium-supplemented media. The pH-mobility curves of these strains were of normal gentamicin-sensitive type after growth in nutrient broth or basal medium, but changed to a gentamicin-resistant type with a maximum but no minimum negative mobility value after growth in highly calcium or magnesium-supplemented media. Gentamicin-resistant strains 107 and Smith became gentamicin-sensitive when grown in basal medium and the pH-mobility curves changed from characteristic gentamicin-resistant to gentamicin-sensitive type. Strain 1 was gentamicin-sensitive after growth at all calcium concentrations and gave sensitive type pH-mobility curves.

Cells of strains grown in highly calcium or magnesium-supplemented media consistently had a slightly higher maximum negative mobility value than did cells grown in basal medium or

nutrient broth (except strain 106 which had an unaltered maximum mobility value). Growth in calcium or magnesium-supplemented media had no detectable effect on surface lipid, but a decrease in surface lipid occurred when resistant strains 107 and Smith were grown in calcium-deficient basal medium.

These results provide evidence for a barrier type mechanism of gentamicin-resistance caused by the divalent metal ions. Resistance increased with increasing calcium or magnesium concentrations in the growth medium and at the same time the cells showed an associated altering of surface properties. There was thus a correlation between MIC of gentamicin and characteristic pH-mobility curves for strains grown in synthetic media, as there was for 'naturally' sensitive or resistant strains grown in nutrient broth, indicating similarities between the two types of resistance mechanism. However, there was a difference in that, unlike 'naturally' resistant strains, cells resistant due to increased levels of cation in the growth medium did not show any increase in detectable surface lipid. Also a slightly higher maximum negative mobility value was exhibited by the cells grown in calcium or magnesium-supplemented media (Figure 5.3) than those grown in calcium-deficient basal medium, whereas cells of 'naturally' resistant strains grown in nutrient broth normally had a lower maximum negative mobility value than cells of 'naturally' sensitive strains grown in nutrient broth.

A reduction of the maximum negative mobility value following growth in calcium or magnesium-supplemented media could be explained in terms of the extra metal ions binding to

negatively charged surface groups. If a binding process does in fact occur, then to obtain a net increase in negative charge there must be a change in the configuration of surface components, perhaps in such a way that negative groups, previously hidden, become exposed at the cell surface. Results indicating that cells grown in cation-supplemented media had a higher polysaccharide content and higher levels of surface cations than those grown in calcium-deficient basal medium provided evidence for such a binding process, this being consistent with the theory that the Gram-negative cell envelope is a highly variable structure, showing marked differences with changes of growth conditions.

Thus cells of strain 832 grown in highly calcium-supplemented medium had approximately ten times as much calcium as those grown in calcium-deficient basal medium. Therefore, the more calcium there was in the growth medium the more calcium the cells took up. However, although magnesium ions were present in the basal medium and in the calcium-supplemented medium in equal concentrations, cells grown in the former had a markedly higher magnesium content. It may be that cells grown in the calcium-supplemented medium use calcium to perform some functions otherwise performed by magnesium in cells grown in calcium-deficient basal medium, thus reducing the levels of magnesium in the former cells.

The results of a study of EDTA-lysis of whole cells of strains of P. aeruginosa grown in basal medium and in calcium or magnesium-supplemented media indicated that the latter cells had higher surface levels of divalent cations. It is beleived that EDTA causes the lysis of whole cells of P. aeruginosa, by removal

of the divalent cations which help to stabilise the surface layers and that this removal leads to the release of macromolecular components such as lipopolysaccharide and lipoprotein. In the current study the extent of lysis was measured by the rate of decrease in absorbance of the buffered EDTA/cell suspension; the cells grown in the highly calcium or magnesium-sufficient media were lysed more rapidly and more completely than were those grown in calcium-deficient basal medium. This is in accord with the hypothesis that the extra metal ions are used to bind components such as lipopolysaccharide at the surface, thus increasing resistance to gentamicin. Further evidence for this was obtained from an analysis of macromolecular components of whole cells of strain B32, those grown in highly calcium-supplemented medium having a higher proportion of polysaccharide and a slightly lower proportion of protein than those grown in basal medium.

It has been suggested that divalent metal ions link lipopolysaccharide and lipoprotein molecules via phosphate groups and thus help to stabilise the outer cell membrane. The Costerton (Costerton et al, 1974) model of the Gram-negative cell envelope envisages an outer network of protruding lipopolysaccharide oligosaccharide chains, whilst the hydrophobic lipid A portions of the lipopolysaccharide molecules are located in the hydrophobic region of the outer cell membrane. Thus an increase in surface lipopolysaccharide would lead to an increase in cell wall lipid, which might not be detected electrophoretically. This would correspond to increased levels of detectable surface lipid on naturally resistant cells previously reported, and could be the

reason why metal ions cause increased resistance to gentamicin.

Sanderson et al (1973) reported that a rough strain of Salmonella typhimurium with shortened oligosaccharide chains distal to the lipid A gave relatively easy penetration to antibiotics compared with the smooth strain. It may thus be that if the extra metal ions do bind increased levels of lipopolysaccharide, it is the oligosaccharide network which is at least in part responsible for increased resistance.

A study of gentamicin-sensitive and naturally gentamicin-resistant strains grown in nutrient broth, failed to reveal a similar pattern of divalent cation content or rate of cell lysis which could be related to gentamicin-sensitivity or resistance. This again implies that these two mechanisms of natural and metal-ion-induced resistance are somewhat different. It may be that naturally resistant organisms, rather than sequestering more divalent metal ions, are better able to utilise the metal ions available to them in becoming resistant to gentamicin than are sensitive strains. In this context it was observed that cells of highly resistant strains suffered a comparatively greater decrease (16-fold) in MIC value when grown in basal medium than did other strains (2 or 4-fold) and as has already been discussed, borderline resistant strains show sensitive type surface properties when induced to become sensitive by growth in basal medium. Thus divalent metal ions do have an effect on the gentamicin-resistance and surface properties of naturally resistant strains.

The metal ions, magnesium, calcium, iron(II) and zinc occurred in the same order of abundance in the whole cells as they

did in the nutrient broth growth medium. Thus cells tend to utilise these ions in relation to their availability, at least at the low concentrations used. Cells of strain 832 had a higher percentage of calcium and a lower percentage of magnesium when grown in nutrient broth than when they were grown in basal medium. The cells grown in nutrient broth however, lysed more rapidly and completely than those grown in basal medium, indicating higher levels of surface divalent cations, this being consistent with the higher MIC of gentamicin for growth in nutrient broth.

Further evidence that calcium and magnesium ions do not simply bind onto negative charges on the cell surface when cells are grown in calcium or magnesium-supplemented media was obtained by suspending cells (grown in nutrient broth) in buffer solutions containing 22.5 mmol dm^{-3} of calcium, i.e. the calcium concentration of the highly supplemented medium. The cells were then washed and were found to have unaltered electrophoretic properties. Thus calcium was not irreversibly bound to the surface, suggesting that the association of the cations with the cell wall is a more complex process occurring during growth and perhaps involving the binding of other components as previously discussed. However, these results do not eliminate the possibility that the metal ions bind at deeper levels in the cell envelope, where they might affect resistance but not be detected electrophoretically.

It is possible that the calcium or magnesium ions may antagonise gentamicin by being weakly adsorbed onto the cell surface, thus blocking negative groups, or they could be located in the double layer. When the cells are prepared for electrophoresis

these ions would be washed off. Although mobility values can be measured in the presence of divalent metal ions comparison and interpretation of the experimental results are difficult on account of changes in the ionic strength of the suspending medium.

Attempts were made to detect any barrier to entry of antibiotic molecules which resulted from growth of cells in calcium-supplemented media. This involved suspending cells grown in different concentrations of calcium, in buffered gentamicin solutions and comparing the uptake of antibiotic in terms of the concentration of gentamicin in the suspension medium required to prevent subsequent growth of the cells. However, the results proved to be inconclusive; no calcium-induced barrier could be demonstrated by this technique, but the results did not conclusively prove that such a barrier was absent in cells grown in calcium-supplemented media.

It may be that, at least in part, calcium and magnesium ions have an antagonistic effect against gentamicin, not by reacting with the bacterial cells, but by interacting with the gentamicin molecules. Such an interaction might result in (a) the blocking of active groups on the antibiotic molecule, (b) in a change in the configuration of the antibiotic molecule, (c) in an increase in the size of the molecule or (d) in a change in the surface charge of the gentamicin molecule; any of these could result in a decrease in the ease of passage of gentamicin into the bacterial cell.

The technique of microcalorimetry was used to investigate any interaction between divalent metal ions and gentamicin. Thus

by reacting solutions of gentamicin chloride with calcium chloride and gentamicin sulphate with magnesium sulphate, heat changes due solely to gentamicin/calcium and gentamicin/magnesium interactions were determined. The results obtained indicate the formation of a weak complex between calcium or magnesium and gentamicin. Any such interaction could be responsible for the alteration of the ionic nature of the antibiotic or a configurational change in the shape of the molecule, either of which would reduce the concentration of gentamicin in its active form.

A number of strains known to possess R-factors mediating gentamicin-resistance was studied (a) to investigate any changes (especially of surface properties) which may occur when cells of P. aeruginosa acquire R-factors and (b) to compare the properties of these strains with properties of resistant strains already examined which did not contain R-factors mediating gentamicin-resistance.

SDS is effective in curing R-factors by acting it is believed, on the surface pili and thus removing them and the attached plasmids from the cells. Proflavine, an acridine dye, intercalates with DNA molecules. The curing experiments using high concentrations of these agents proved generally positive with the strains known to carry R-factors; of the four strains tested one strain was cured by both agents, one strain by SDS, one strain by

proflavine while the fourth strain was not cured by either agent. None of the other gentamicin-resistant strains previously discussed (p. 166) showed any sign of curing, suggesting the absence of R-factor mediated resistance.

Plasmids conferring resistance to antibiotics are extrachromosomal elements which may divide and be passed from the parent to an acceptor cell during conjugation, thus effecting a potentially rapid spread of resistance from one strain to another. Known R-factor carrying strains readily transferred resistance to acceptor strains, while the other resistant strains never transferred resistance. These results are in complete accord with those obtained from curing experiments.

Thus the results of the curing and transfer experiments indicated that at least two separate mechanisms of resistance are in operation in these strains. Further evidence in support of this came from electrophoretic study of the surface properties of cells of R-factor donor, transconjugant and acceptor strains. Four of the five donor strains proved to be the only strains ever examined in this laboratory which although naturally resistant to gentamicin (i.e. excluding transconjugant and other strains trained to resistance) when grown in nutrient broth, exhibited gentamicin-sensitive type pH-mobility curves with a low surface lipid content. The fifth strain (PL11) exhibited a gentamicin-resistant type pH-mobility curve with an associated high level of surface lipid.

The acceptor strains (all of which had pH-mobility curves and S-values characteristic of sensitive cells) were unchanged electrophoretically when they acquired R-factors, the pH-mobility

curves of transconjugant and acceptor cells were thus superimposable (Figure 6.1). The donor strains used, which display either gentamicin-acetylating or gentamicin-adenylating activity, have been variously reported to confer multiple drug resistance and changes in pyocine and phage type onto the acceptor strains (Byran et al, 1974; Jacoby, 1974; Korfhagen and Loper, 1975). None of these have caused any changes in the surface properties of the acceptor strains. This was also true of transconjugant strain PL1(RPL11) which was indistinguishable from the acceptor strain PL1 (Figure 6.4), showing that the R-factor RPL11 was not in itself responsible for the gentamicin-resistant type pH-mobility curve and high S-value of strain PL11.

The level of resistance conferred onto the transconjugant strains during mating was always lower than the original resistance of the donor (Table 6.2). Only eight of the eleven transconjugant strains could be classified as gentamicin-resistant even though all the donor strains had MIC values in excess of 12 $\mu g\ cm^{-3}$. Donor strain PL11 only conferred a twelve-fold increase of resistance on the acceptor strain PL1, the lowest such increase observed. This can be compared, for example, with donor strain Stone no.130 which has the same MIC of gentamicin (200 $\mu g\ cm^{-3}$) and yet which increased the MIC of the acceptor strain PU21 by more than thirty-three-fold.

These results demonstrate the presence in the available resistant strains of P. aeruginosa of at least two mechanisms of resistance to gentamicin. The first type (possibly a barrier mechanism) is characterised by (a) the inability of the resistant cells to transfer resistance to cells of other strains of

P. aeruginosa (none of the strains examined was able to transfer resistance to Esch. coli strains), (b) the insusceptibility of the resistant cells to curing and (c) the cells exhibited typical gentamicin-resistant type pH-mobility curves and high S-values. The second type is R-factor mediated resistance in which the resistant cells can transfer increased resistance to other sensitive strains of P. aeruginosa without altering their detectable surface properties. This resistance can be eliminated by curing agents and the cells generally display gentamicin-sensitive type pH-mobility curves with low S-values. It may be that lack of an external resistance barrier would be of advantage to R-factor carrying strains as there would be less possibility of restriction of passage of nutrient material and wastes to and from the cells, whilst the periplasmic space could allow an effective concentration of antibiotic-inactivating enzyme to build up to counteract incoming antibiotic molecules.

It appears likely that cells of strain PL11 possess both types of resistance, since (a) they exhibit gentamicin-resistant type pH-mobility curve with a high S-value, (b) they are cured by proflavine and (c) they transfer gentamicin-resistance to acceptor strains, albeit at a much lower level than is possessed by cells of the parent strain , the inference being that the R-factor mediated resistance in the parent strains is also of a low-level.

Like all other strains of P. aeruginosa examined, strains carrying R-factors became more or less resistant to gentamicin as the calcium content of the growth medium was varied. This of itself might suggest the greater importance of interaction between

calcium and gentamicin rather than the interaction of calcium and surface components in determining calcium antagonism.

In conclusion, there appear to be at least two mechanisms of natural resistance to gentamicin in cells of P. aeruginosa. The first type is characterised by changes in cell surface properties and an increase in the amount of surface neutral lipid with increasing levels of resistance. This may be indicative of some form of barrier mechanism of resistance and very high levels of resistance (MIC = 8000 $\mu g\ cm^{-3}$) may be acheived. The second type is R-factor mediated resistance, the resistant cells producing gentamicin-inactivating enzymes which are located extrachromosomally. Typically these strains have surface properties indistinguishable from gentamicin-sensitive strains.

In addition there is a third type of facultative resistance. All strains of P. aeruginosa may be classified as gentamicin-sensitive or gentamicin-resistant relative to each other for growth in a particular medium. However, the sensitivity/resistance level of any strain may be changed by altering the concentration of calcium and magnesium ions in the growth medium, increased concentrations leading to higher resistance. This type of acquired resistance leads to altered cell surface properties and this may again indicate a barrier-type resistance, albeit achieved in a slightly different manner to the naturally resistant strains.

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