A thesis titled

"A PHYSICAL CHEMICAL STUDY OF CELLS OF STRAINS OF PSEUDOMONAS AERUGINOSA"

by

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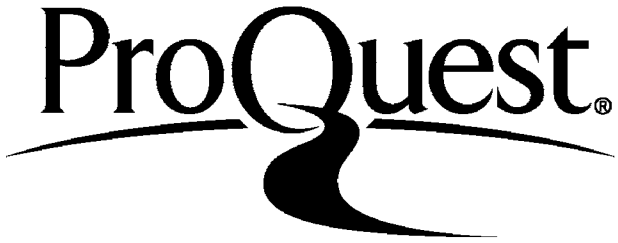

ABSTRACT

Electrokinetic studies were used to investigate differences in surface components associated with cells of Pseudomonas aeruginosa which were sensitive, naturally resistant or had an induced in vitro ("trained") resistance to the antibiotic gentamicin. The standard method of electrophoretic mobility measurement was modified for measurements at 10°C, at which temperature mobility values were not influenced by cell motility.

The pH-mobility curves of cells grown at 37°C on solid medium revealed that cells of resistant strains could be distinguished from cells of sensitive strains. The surfaces of all cells probably carry charged carboxyl and amino groups and the differences between the surfaces of sensitive and resistant cells are due, in part, to differing ratios of these two components. In addition, cells with medium-level and high-level natural resistance to gentamicin are distinguished by their pH-mobility curves after treatment with 1-fluoro-2,4-dinitrobenzene. Cells with induced resistance form a third, distinct, category of resistant cells for which the surface properties revert back to those of the parent, sensitive, cells after initial disturbance at the onset of "training"; this induced resistance is unstable.

Although cells of all strains contain the same proportions of total solvent-extractable lipid, this was composed of different types of lipid and was distributed

in different ways. There was little, if any, detectable surface lipid on cells of sensitive strains, while cells of naturally resistant and induced resistant strains possessed significant amounts of neutral lipid at the surface; there was an apparent relationship between the level of resistance and the amount of surface lipid possessed by the cells.

This thesis comprises a report of full-time research undertaken by the author in the Physical Chemistry Laboratories of Bedford College, University of London, from September 1970 to July 1973.

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CONTENTS

SUMMARY

Gentamicin is an aminoglycoside-type antibiotic first isolated by Weinstein et al in 1963. Its main advantage in therapeutic use has been its high level of activity against a broad spectrum of bacterial species, but most especially against strains of Pseudomonas aeruginosa which have proved resistant to other antibiotics (Barber and Waterworth, 1966). However, in common with most other antibiotics its increasing use has been accompanied by an increasing proportion of the species isolated from infected patients being resistant; in some cases of intensive gentamicin therapy very high levels of resistance of cells of P.aeruginosa have resulted (Yourassowsky et al, 1971). This investigation reports an electrokinetic study of cells of P.aeruginosa with special reference to their resistance to gentamicin.

There is no precedent for such a study of cells of P.aeruginosa and it was, therefore, necessary to derive standard growth and preparation procedures and to select optimum experimental conditions whereby a reproducible cell surface was obtained and cell suspensions were electrophoretically homogeneous. Cells of P.aeruginosa are flagellated, and in suspension they are motile, and so an important condition was the measurement of electrophoretic mobility values at the reduced temperature of $10^{o}C$, under which condition motility is suppressed and measured mobility values are unaffected by random cell mobility. Observing this condition, and the other optimum procedures

and conditions of length of incubation, washing of cells and the ionic strength of the buffer solution, the cell surface components were stable and reproducible within the pH range 2 to 11.

The pH-mobility curves of gentamicin sensitive, naturally resistant and in vitro "trained" resistant cells of P.aeruginosa grown at 37°C on nutrient agar were investigated. It was possible to distinguish between sensitive and naturally resistant cells by the shape and position of their pH-mobility curves. This is the first report of any difference, Weinstein et al (1971) having reported that gentamicin sensitive and naturally resistant cells of P.aeruginosa are microbiologically indistinguishable, apart from their different responses to the antibiotic. There was no correlation between the different surface properties of the cells and their phage type or their spectrum of resistance to other antibiotics.

Cells of sensitive strains had a maximum mobility value in the pH range 5.5 to 6.5, a minimum mobility value between pH 7.5 and 8.5 and the isoelectric point, extrapolated from the pH-mobility curves, was in the pH range 1.5 to 2.0. Cells of resistant strains also exhibited a maximum in the pH-mobility curve, but the maximum value was significantly lower than that for sensitive cells and was in the pH range 7.5 to 8.5; no minimum mobility value was observed at higher pH values and the extrapolated isoelectric point was in the pH range 2.5 to 3.3. These pH-mobility curves showed the surfaces of cells of all strains to be complex, unlike those of

Klebsiella aerogenes which consist only of acidic polysaccharide carboxyl groups (Lowick and James, 1957). Nor were the pH-mobility curves typical of a pH-titration curve for a surface possessing more than one ionogenic species as demonstrated by Plummer et al (1962). The decreases of negative mobility at pH values higher than that at which the maximum mobility value occurs suggest that some rearrangement of surface components is occurring, similar to that involving the ribitol teichoic acid in cell walls of Staphylococcus aureus (Hill and James, 1972a).

The pH-mobility curves for cells with "trained" resistance to gentamicin were moved to higher mobility values immediately after growth in the presence of a new, increased, antibiotic concentration, and in one case the sinusoidal-shape of the pH-mobility curve was lost. However, prolonged subculturing in the presence of the same concentration was accompanied by a reversion of the pH-mobility curve back to that of the parent, sensitive, cells. This, and the fact that the "trained" resistance was unstable, showed that in vitro induced resistance to gentamicin is not the same as naturally-occurring resistance.

The naturally resistant cells were separated into those with medium-level and those with high-level resistance to gentamicin. Whilst the pH-mobility curves for cells of both types were similar, treatment of the cells with FDNB yielded very different curves, thus the difference in response to gentamicin is reflected in the surface properties. However, the blocking of surface amino groups with FDNB produced very similar surfaces for sensitive

and medium-level resistant cells which suggests that the differences between the pH-mobility curves for normal cells of the two types of strain are due in part, to the surface amino groups. The curves for cells of sensitive and medium-level resistant strains treated with FDNB were typical of a surface possessing only one anionic species; the surface pK value suggested that this species was possibly the γ-carboxyl group of glutamic acid.

The ratios of carboxyl to amino groups calculated from mobility values of normal cells and cells treated with FDNB showed that sensitive cells have a greater proportion of surface carboxyl groups than resistant cells. It is these differences in carboxyl/amino ratio that are responsible for the observed differences in the extrapolated isoelectric points for normal resistant and sensitive cells.

Despite the evidence for the presence of surface amino groups, treatment of cells with aldehydes did not result in the change of surface properties predicted by Heard and Seaman (1961). These authors claimed that the reaction between cell surface amino groups and aldehydes would increase the net negative surface charge. However, cells of P.aeruginosa so treated showed little or no change in mobility, any change being an increase of net positive surface charge, suggesting that the aldehydes were either not reacting with surface amino groups, or that other, unspecific, reactions were occurring.

The spectrum of activity of EDTA against cells of

P.aeruginosa was unrelated to the spectrum of activity of gentamicin, but against cells of 70% of the strains the actions of gentamicin and EDTA were synergistic. EDTA removes divalent metal ions from the cell envelope of P.aeruginosa, and thus impairs envelope integrity (Wilkinson, 1970). Therefore the mode of synergism between the actions of gentamicin and EDTA may be that the latter assists the penetration of the former to its ribosomal target; this in turn suggests that there is a barrier within the cell envelope to the penetration of gentamicin.

Other workers have demonstrated correlations between the surface lipid of bacterial cells and their antibiotic resistance (Lowick and James, 1957; Hill et al, 1963; Hugo and Stretton, 1966). The electrophoretic measurement of surface lipid on cells of P.aeruginosa similarly showed a correlation with the resistance of the cells to gentamicin. Gentamicin-sensitive cells possessed little, or no, surface lipid whereas resistant cells possessed significantly greater amounts, and there appeared to be a direct relationship between the amount of surface lipid and the level of resistance to gentamicin. Furthermore, cells with "trained" resistance to gentamicin possessed increased amounts of surface lipid compared with their sensitive parent cells. The shape and position of the pH-mobility curves for cells with "trained" resistance were eventually similar to that for the parent cells which suggests that the increased surface lipid is neutral lipid and not phospholipid. Other studies have shown that cells with the greatest amounts of surface lipid and the

highest resistance to gentamicin have a greater proportion of neutral lipid and free fatty acids at the expense of phospholipid, whilst retaining the same proportion of total lipid as gentamicin-sensitive cells.

These results do not prove conclusively that neutral surface lipid is a cause of gentamicin resistance in cells of P.aeruginosa, but together with the other results presented in this investigation they do clearly demonstrate a link between cell envelope structure, and in particular cell surface structure, and resistance to gentamicin.

C H A P T E R I

INTRODUCTION

I.1. Classification of Pseudomonas aeruginosa

All microorganisms may be divided into eight major groups: algae, protozoa, yeasts, moulds, bacteria, pleuropneumonia-like-organisms (PPLO), rickettsia and viruses. Members of each group possess distinct characteristics essential for inclusion in the group. The borders, however, are diffuse, and are the subject of much discussion.

The bacteria, or schizomycetes, are usually divided into ten orders (Table I.1(a)). The families in each order are determined by cell shape; motility; the presence or absence of polar or peritrichous flagella, if motile; reaction to the Gram stain; growth requirements; optimum temperature of growth; types of fermentations which the cultures are capable of carrying out aerobically or anaerobically; and other characters found to be of use in differentiation.

The response to the Gram stain can prove very useful as a preliminary characterisation technique. The organisms are described as Gram-positive, Gram-negative or Gram-variable, according to their ability to retain crystal violet dye after mordanting with iodine solution. The colour of the stained cells is not only a determinative character in itself, but allows gross morphology to be observed, i.e. whether the cells are spherical cocci, cylindrical or rod-like, spiral or helicoidal. The Gram stain also indicates whether the cells are spore forming, and these morphological considerations lead to a classification of bacteria as shown in Table I.2.

The genus Pseudomonas of the family Pseudomonadaceae,

which contains all the aerobic pseudomonads, is defined by Stanier et al (1966) as containing unicellular organisms which are Gram-negative rods; whose major axis is straight or curved but never helical; they are motile by means of one or more polar flagella; they do not form spores, stalks or sheaths; their energy-yielding metabolism is respiratory and never fermentative or photosynthetic; they use molecular oxygen as the terminal oxidant although some may use denitrification as an alternative anaerobic respiratory mechanism; and they are mainly chemo-organotrophs but some are chemo-lithotrophs utilising H_2 as the energy source. The only groups of organisms not excluded by this definition are small-celled parasitic vibroid bacteria of the genera Campylobacter and Bdellovibrio, although it is now possible to exclude Campylobacter by measurement of the guanine and cytosine content of the DNA.

The genus Pseudomonas is subdivided into three sub-generic groups according to characters such as the ability to fluoresce, type of flagellation, ability to form pigment and biochemical reactions (Stanier et al, 1966). Pseudomonas aeruginosa is a member of the fluorescent Pseudomonads sub-generic group (Table I.1(b)) and is distinct from other members of this group in that it only shows monotrichous flagellation, produces pyocyanine pigment, is the only fluorescent pseudomonad pathogenic to man and has the highest maximum growth temperature.

Thus Pseudomonas aeruginosa, the organism used in this investigation, may be described as a Gram-negative

TABLE I.1

(a) A classification of bacteria with particular reference to Pseudomonas.

Order	Family	Genus
Eubacteriales		
Hyphomicrobiales		
Clamydobacteriales		
Pseudomonadales	Pseudomonadaceae	Pseudomonas
	Spirillaceae	Vibrio
		Spirillum
Actinomycetales		
Caryoplanales		
Beggiatoales		
Myzobacterales		
Spirochaetales		
Mycoplasmatales		

(b) A classification of Pseudomonas aeruginosa

Genus	Sub-generic group	Species
Pseudomonas	Fluorescent	P.aeruginosa;
		P.fluorescens;
		P.putida
	Acidovorans	P.acidovorans;
		P.testosteroni
	Alcaligenes	P.alcaligenes;
		P.pseudoalcaligenes;
		P.multivorans;
		P.stutzeri;
		P.maltophilia

TABLE I.2

A classification of some heterotrophic bacteria according to Gram-stain response and morphology

Gram-positive

(a) Spheres

(i) Chain cocci
- Streptococci
- Diplococci
- Leuconostoc

(ii) Cluster cocci
- Micrococci
- Gaffyka
- Sarcina
- Staphylococci

(b) Rods

(i) Spore-forming
- Lactobacilli
- Cornybacteria
- Mycobacteria
- Norcardia
- Streptomyces
- Propionibacteria

(ii) Non-spore-forming
- Bacilli
- Chlostridia

Gram-negative

(a) Spheres
- Neisseria
- Veillonella

(b) Spirals
- Vibrio
- Spirillum

(c) Cylinders

(i) Oxidative
- Pseudomonas
- Xanthomonas
- Acetobacter
- Azotobacter
- Rhizobium

(ii) Fermentative
- Escherichia
- Erwinia
- Seratia
- Klebsiella

non-spore-forming rod of dimensions 1.5 x 0.5 μm, actively motile by virtue of a polar flagellum, usually non-capsulate and sometimes fimbriated. It grows aerobically between 5 and 43°C utilising a wide range of energy sources and producing a musty smell, a diffusable green-brown pigment, and a fluorescent pigment. Colonies are dark-greyish, large, low-convex with an irregular spreading edge which is translucent. It can produce acid oxidatively from glucose, it rapidly liquefies gelatin and unlike most Gram-negative rods gives a positive oxidase reaction.

P.aeruginosa is sometimes present on healthy human skin and in small numbers in the intestinal flora of man and animals and is thus isolated from sewage. As a pathogen it is usually associated with pyogenic cocci or members of the Enterobacteriaceae. It is often implicated in urinary tract infections and infections due to catheterisation or other diagnostic and therapeutic instrumentation. It commonly infects deep wounds and burns and causes acute purulent meningitis following cranial injury or accidental introduction after lumbar puncture. Infections are usually localised but in infants and debilitated persons it may invade the blood stream resulting in fatal generalised infection, especially when the patient is receiving antineoplastic drugs or radiation therapy.

I.2. Typing of strains

Since bacteria readily adapt to different environmental conditions, a crude form of strain differentiation is to type according to the source. However, one strain may spread through various environments without noticeable change, as in cross-infection in hospital wards, and to follow such strain migration and to discover its source a more precise typing method, making fine subdivisions over the whole species, is required.

There are two widely used methods of strain-typing; serological typing and phage typing. A third method, peculiar to P.aeruginosa, is pyocine typing.

Serological typing depends on an antigen-antibody response and is performed using O-antisera usually obtained from rabbit serum. Habs (1958) described this method, and showed that it is easy to perform; although some 'types' are rather common, some of these may be further subdivided by the recognition of heat-labile antigens (Lányi, 1970). Phage typing depends on the susceptibility of a strain to the lytic action of a series of bacteriophages (Gould and McLeod, 1960). The reproducibility of this method is less good than in, say, staphylococcal phage typing, and more than two differences in response are required as evidence of different strains. The third method of typing depends on characterising the pyocine produced by the strains which has a lethal effect on other strains. This involves either determining the spectrum of inhibition of the pyocine (Darrell and Wahba, 1964; Gillies and Govan, 1966; Govan and Gillies, 1969), or by treating the test strain

with a variety of cell-free pyocine preparations (Osman, 1965).

Singly, none of these methods serves to differentiate all strains which may occur in one cross-infection outbreak. However, combination of two or more of these methods has shown an increase in the number of recognisable types (Wahba, 1965; Csiszar and Lányi, 1970), and the most widely used system, and the one used in the present investigation is serological typing and phage typing as a hierarchical system in which phage typing patterns are used to make subdivisions within serotypes.

I.3. Bacterial Anatomy

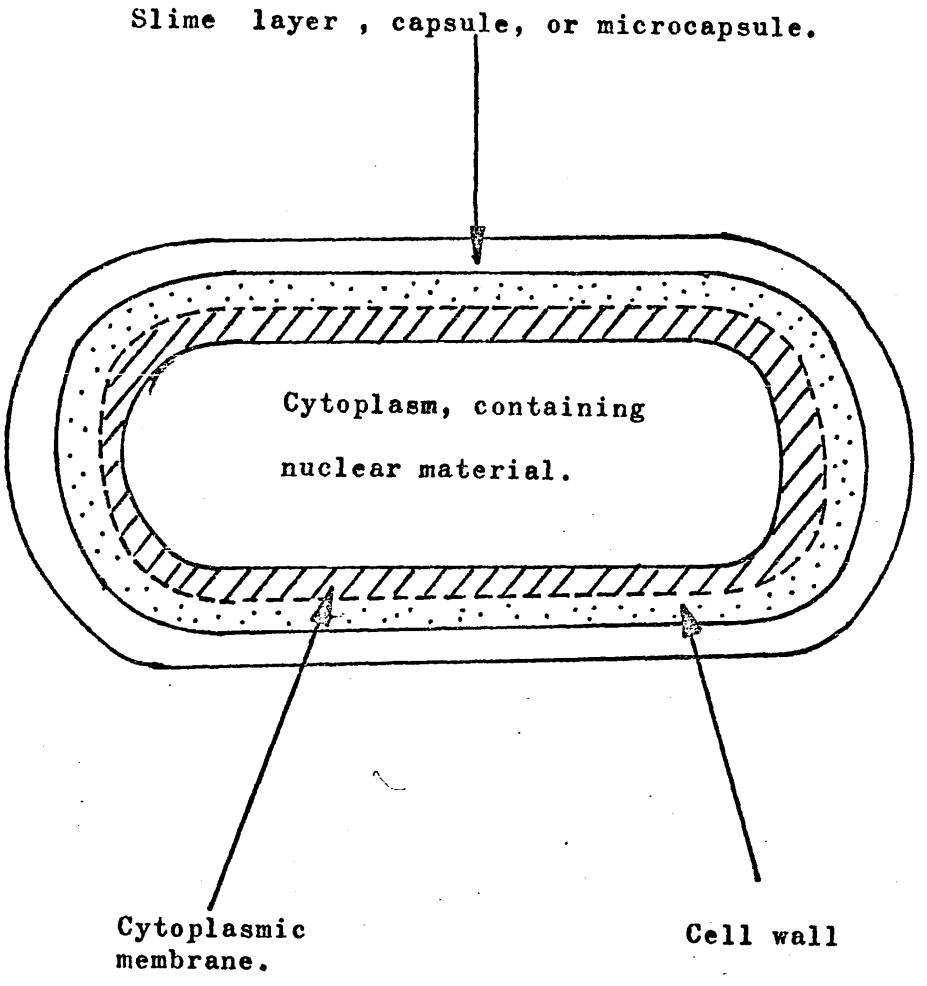
This section consists of a detailed consideration of the physical and chemical structure of the bacterial cell wall, and a brief survey of the other bacterial components.

Figure I.1, a cross-section of a typical cell, shows the basic cellular organisation. The cytoplasm is surrounded by a membrane, which in turn is enveloped by a cell wall, and possibly a capsule or slime layer. Surface appendages may 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. The granular appearance of the cytoplasm is due to the storage particles and the ribosomes. The ribosomes are the cytoplasmic site of protein synthesis, and many ribosomes are often attached to one molecule of

FIGURE I.1

A diagrammatic representation of the anatomical relationship between the outer layers of a typical bacterial cell.



ribonucleic acid, RNA, in the form of a polysome (Schlessinger, 1964). The ribosomes of these procaryotic cells are not associated with membranes or endoplasmic reticulum. Elaborate vesicular and lamellar invaginations of the protoplasmic membrane called mesosomes are concerned with the electron transport systems of bacterial cells. Electron transport is always associated with the membrane fraction of cell homogenates. In nitrogen-fixing and photosynthetic bacteria where high-energy coupling reactions take place, mesosomes can reach extreme proportions of size and shape.

The nuclear material, or nucleoid, lies in the cytoplasm without a nuclear membrane. After the nuclear material has divided, the daughter nucleoids are thought to be drawn apart by mesosomes. The mesosomes also appear to be closely associated with septum formation after nuclear division (Imaeda and Ogura, 1963). Free structures in the cytoplasm resembling mitrochondria have never been detected in procaryotic cells.

Surface appendages on bacterial cells are of three types; flagella, cilia and fimbriae (or pili), each of which can be readily distinguished from the surface layers. Several different arrangements of flagella are observed ranging from the single polar flagellum of P.aeruginosa, (monotrichous), through the polar tuft of flagella of Spirillum serpens, (multitrichous), to the distribution of flagella around the surface as in the Eubacteriales (peritrichous). The diversity of flagellation has been used as a criterion for the classification of bacteria

(Rhodes, 1965). The single polar flagellum of P.aeruginosa is a thread-like structure protruding from the cell surface, but continuous with the cell cytoplasm, (Murray and Birch-Anderson, 1963), with a diameter of 15-20 nm. It consists of 98% of a protein called flagellin, which has a low relative molecular mass, (40,000) and whose globular molecules contain only 14 or 15 amino-acids, which are arranged in a spiral pattern with a periodicity of 25 nm (Lowy and Hanson, 1965). The flagella impart motility to the cell by virtue of their movement and are the site of the H-antigens.

Many strains of the Enterobacteriaceae are non-motile but possess very fine hair-like structures called fimbriae, which are known to confer haemagglutination, adhesive and antigenic properties to the cells. Cilia, like flagella, impart motility to bacterial cells and also move liquid over the surface of the organism, aiding removal of waste products and supply of nutrients. The three types of surface appendages are easily removed by vigorous agitation of the cells in suspension; the loss may occur naturally without affecting cell viability.

Capsules and slime layers surround many microorganisms, lying external to, but in close contact with the outer cell wall. The amount produced depends on the nature of the growth medium, and the production of such layers may be stimulated by unfavourable conditions. Capsules are often correlated with virulence in pathogenic forms. The capsulate form of the Smith strain of Staphylococcus aureus is able

to resist phagocytosis whereas the non-capsulate variant is readily ingested (Koenig, 1962). The slime layer of P.aeruginosa has been implicated in resistance to chemotherapy (Brown and Richards, 1964), in toxicity and pathogenicity (Liu et al, 1961) and as a protective antigen in experimental infection (Alexander et al, 1966; Alms and Bass, 1965; and Liu et al, 1961).

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 but the macromolecular components of some species have been well studied. The type specific heteropolysaccharides of pneumococci are well known, and are used in the fine classification of the species. Similar series of polysaccharides are produced by group B streptococci, but little is known of their structure. Streptococci in groups A and C have capsules containing hyaluronic acid, a polymer of N-acetyl-glucosamine and glucuronic acid. Certain species of bacilli produce capsules consisting of polyglutamic acid either alone or in association with polysaccharides.

The capsular material of cells of Gram-negative species is chemically more complex than that of Gram-positive. In certain Gram-negative organisms,

extracellular polysaccharides often occur as microcapsules in association with lipoprotein. These complexes contain the O-somatic antigens, which are important in relation to the classification and pathogenicity of the organisms.

The slime layer deposited by some strains of P. aeruginosa is mainly polysaccharide with a significant proportion of protein (Bartell et al, 1970). Although the composition varies from strain to strain quite widely, the components of the slime layer include glucose, rhamnose, fucose, mannose, galactose, glucosamine, galactosamine, glucuronic acid, RNA, and deoxyribonucleic acid, DNA (Bartell et al, 1970; Doggett et al, 1964; and Brown et al, 1969).

I.4. The Bacterial Cell Envelope

There are marked differences between the cell walls of Gram-positive and Gram-negative bacteria (Table I.3). This investigation is concerned with the Gram-negative rod, Pseudomonas aeruginosa, and so more detailed consideration will be given to the structure and composition of the Gram-negative cell envelope, with particular reference to strains of this organism.

The Gram-negative cell envelope. Because the surface layers of Gram-negative bacteria are more complex chemically and structurally than those of Gram-positive bacteria (Table I.3), it is more usual to consider what is known as

TABLE I.3

A comparison of the cell envelopes of Gram-negative and Gram-positive bacteria

Gram-positive (e.g. *Staphylococcus aureus*)	Gram-negative (e.g. *P.aeruginosa*)
1) The cell wall presents a unified, well defined, separate structure, distinct from the cytoplasmic membrane.	1) An exceedingly complex organisation of the outer cell layers. No clear boundary between different structures. A multi-layered envelope arrangement including the cytoplasmic membrane.
2) Hydrolysis yields only a limited range of amino acids. Little protein and enzymic activity.	2) Hydrolysis yields up to 21 amino acids from protein units present. Much enzymic activity.
3) Mucopeptide forms 40-90% of cell wall material. Probably a multi-layer network.	3) Mucopeptide only accounts for 5-10% of envelope material. Probably a monolayer.
4) Contains 1-5% wall lipid.	4) Contains up to 20% envelope lipid.
5) If present, a capsule consists of simple polysaccharides and occasionally polypeptide.	5) Capsules consist of complex polysaccharide, protein-polysaccharide and lipo-polysaccharide complexes.
6) Capable of withstanding an osmotic pressure of up to 30 atmospheres.	6) Osmotic pressure across cytoplasmic membrane generally of the order of 12 atmospheres.

the cell envelope, which includes all the structures external to the cytoplasm as a single, but multi-layered unit (Salton, 1967).

This cell envelope surrounds the protoplast, and because of its rigidity gives a particular microbial cell its characteristic shape. It provides the permeability barrier between the cytoplasm and the environment and is responsible for the antigenicity and response to pyocines, to bacteriophages and to many antibiotics and other chemical reagents. In addition, many metabolic capabilities of the cell are dependent, at least in part, on the structure and organisation of these surface layers.

Many techniques have been employed for the isolation of whole cell walls or envelopes; most are based on that of Salton and Horne (1951), in which the bacterial cells are first ruptured by shaking cell suspensions with ballotini glass beads in a Mickle (1948) disintegrator, or in its high energy form, the Braun homogeniser. The homogenate is then heated at $90^{o}C$ for 10 minutes to destroy autolytic enzymes released from cells, which degrade cell walls. The walls are then separated from the undamaged cells by low speed centrifugation, and finally a pellet of crude wall preparation is obtained by high speed centrifugation. Robertson and Schwab (1960) used density gradient centrifugation to obtain homogeneous preparations. The material obtained is repeatedly washed first either in 0.1 mol dm^{-3} phosphate buffer solution, or in 1.0 mol dm^{-3} sodium chloride solution and then with water. The adhering fragments of the cytoplasmic membrane are removed from the walls by the action of enzymes such as trypsin, RNase or DNase (Cummins and Harris, 1956). Isolation of whole cell

envelopes in this way has enabled the gross compositions of the structures to be determined although the method of isolation may have affected the results of the analysis: Roberts et al (1967) showed that some envelope components of P.aeruginosa are removed simply by washing, and enzyme treatment may remove part of the outer envelope membrane as well as the cytoplasmic membrane (Braun and Rehn, 1969).

The principal classes of chemical constituents found in Gram-negative cell envelopes are glycosamino-peptides (mucopeptide), proteins, lipids, polysaccharides, lipopolysaccharides and lipoproteins. Further analysis of these constituents has shown that Gram-negative cell envelopes contain a wide range of amino acids (up to 21) similar to that normally encountered in most proteins (Salton, 1952), unlike Gram-positive cell walls which may contain as few as three amino acids. The envelope of P.aeruginosa contains 13 amino acids, four of which, alanine, glutamic acid, lysine and diaminopimelic acid, are associated with the mucopeptide layer (Mandelstam, 1962). Because of the lower mucopeptide content the amino sugar composition of Gram-negative envelopes is less than that of Gram-positive walls, and a further marked difference between the two types of cell envelope is that lipid constitutes up to 20% by weight of the Gram-negative envelope, as opposed to less than 5% for Gram-positive (Salton, 1953).

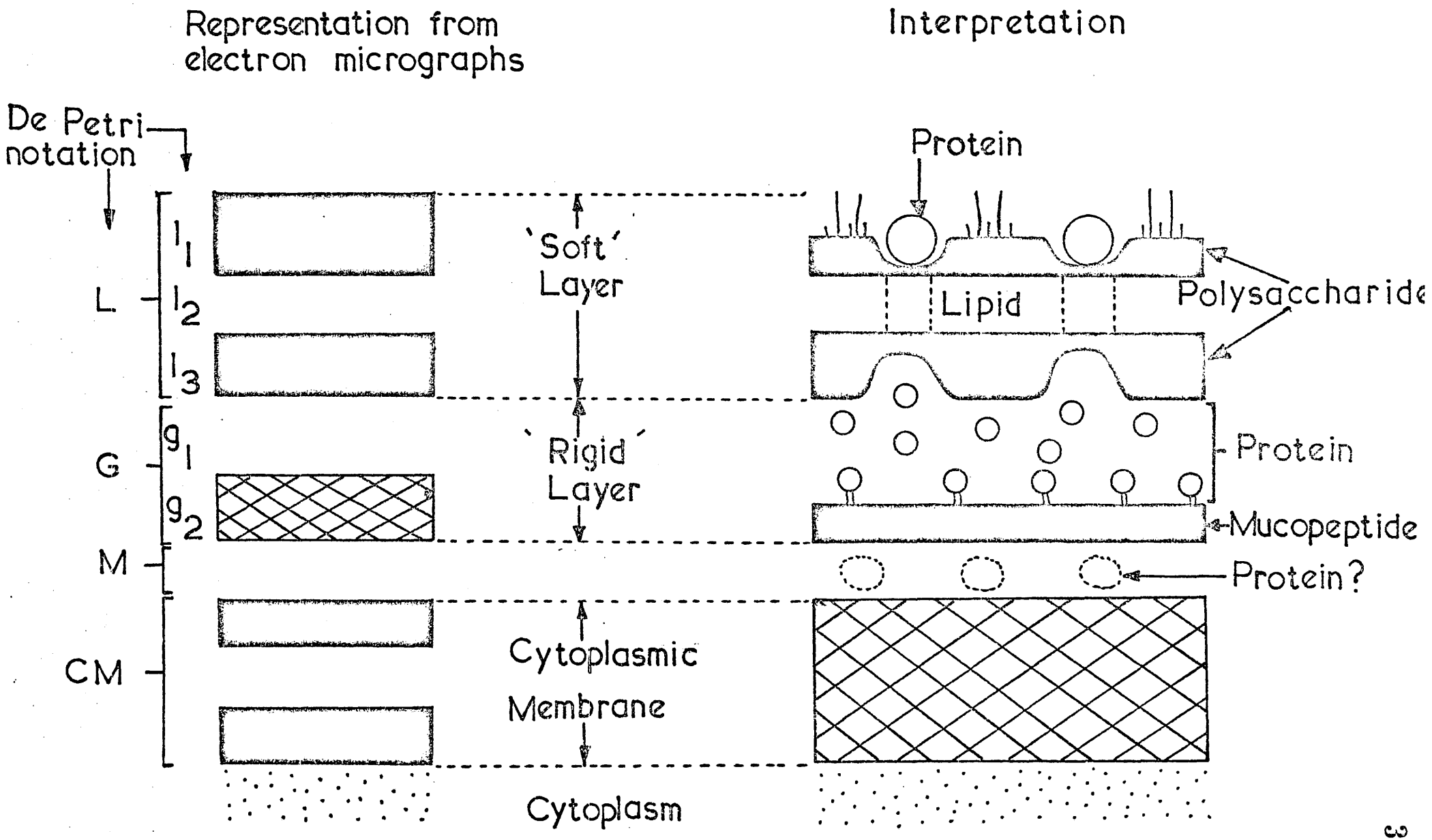
However, whilst there exists detailed knowledge about the specific macromolecular components of the Gram-negative envelope, and whilst the functions of the envelope are well-defined, there is little knowledge of the organisation of these components to form functional structures. Only

recently has it been possible to isolate selected parts of the envelope of P.aeruginosa (Martin and McLeod, 1971), and work is now beginning on their detailed characterisation. Much of the earlier information on Gram-negative envelope structure came from thin-section electron-microscopical investigations and by this method Kellenberger and Ryter (1958) were first able to establish the multi-layered envelope structure of the Gram-negative bacterium Escherichia coli. Similar results were obtained for other Gram-negative bacteria by Murray (1962) and Giesbrecht and Drews (1962), and for a marine pseudomonad by Brown et al (1962). This 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 mucopeptide. On this model the inner compound membrane would be analogous to the cytoplasmic membrane of Gram-positive bacteria, and the rigid mucopeptide layer and the outer compound membrane together would replace the mucopeptide layer which constitutes almost the whole of the Gram-positive cell wall.

Confirmation of this general structure came from thin-section electron microscopic studies of E.coli and other Gram-negative bacteria by Murray et al (1965). The most detailed model of the structure of the Gram-negative cell envelope comes from the electron microscopic study of E.coli by De Petri (1967) whose proposed structure is shown in Figure I.2. This shows a mosaic of proteins and polysaccharides on the outer surface (l_1) linked by a lipid

FIGURE I.2

Model of the structure of the Gram-negative cell envelope of Escherichia coli as suggested by electron microscopy (De Petri, 1967).


Representation from electron micrographs
Interpretation
De Petri notation
L
l1
l2
l3
G
g1
g2
M
CM
'Soft' Layer
'Rigid' Layer
Cytoplasmic Membrane
Cytoplasm
Protein
Lipid
Polysaccharide
Protein
Mucopeptide
Protein?

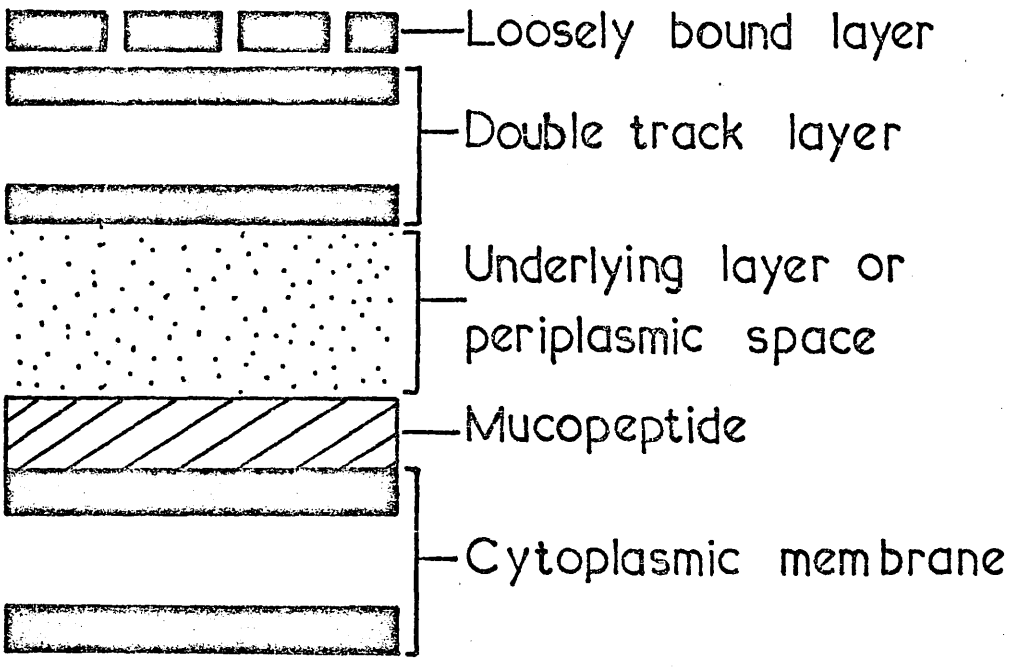
layer (l_2), consisting of two types of lipid, to a layer of polysaccharide (l_3). Layer G consists of two layers of protein globular elements, one covalently linked to the mucopeptide layer and one unlinked: the existence of proteins in the space M between layer g_2 and the lower membrane has been postulated. This lower membrane is analogous in structure and composition to the Gram-positive cytoplasmic membrane.

Forsberg et al (1970a; 1970b) were able to remove the outer layers of a marine pseudomonad by saline washing, analyse them for their components and examine them under the electron-microscope. They discovered three outer layers; a loosely bound layer, a double track layer and an underlying layer which was electron-transparent (Figure I.3). All three layers had essentially the same composition of lipid, which was mainly phospholipid, protein and carbohydrate containing both amino- and non-amino-sugars. 18 different amino-acids were isolated from all three layers, and each layer constituted 5-8% of the dry cell weight. The loosely bound layer was held together, and held to the double track layer, by divalent magnesium cation (Mg^{2+}) bridges,and these three layers were observed to be external to the mucopeptide layer, whilst the mucopeptide appeared to be associated with the cytoplasmic membrane. Thus five distinct layers were observed.

Hoffschneider and Martin (1968) analysed an isolated double track layer and separated it into two distinct fractions, one a phenol-soluble lipoprotein and the other a water phase containing lipopolysaccharide. This, using the model of De Petri (1967), (Figure I.2) tentatively

FIGURE I.3

Cross-sectional model of the structure of the cell envelope of a marine pseudomonad (Forsberg et al, 1970a)


Loosely bound layer
Double track layer
Underlying layer or
periplasmic space
Mucopeptide
Cytoplasmic membrane

designates the outer part of the double track layer observed by Forsberg et al (1970a) as a lipoprotein layer, and the inner part as a lipopolysaccharide layer. The underlying layer of Forsberg et al (1970a), known as the periplasmic space because of its electron-transparency, was said by Heppel (1967) to contain hydrolytic enzymes, but De Petri (1967) associated it with proteic, or protein-like, particles whilst Hoffschneider and Martin (1968) and Martin (1963) describe it as a lipopolysaccharide layer.

Evidence for the view of De Petri (1967) that the periplasmic space contains proteic particles comes from the work of Braun and Rehn (1969) who isolated a lipoprotein containing 12 amino-acids from layer g_1 on the De Petri model, which was firmly and covalently bound to the mucopeptide by a terminal lysine residue. By digesting the isolated lipoprotein-mucopeptide complex with trypsin, they showed that there remained one lysine residue for every ten of the repeating units (N-acetyl glucosamine, N-acetyl muramic acid, L-alanine, D-glutamic acid, meso-diaminopimelic acid, D-alanine) of the mucopeptide. It was observed that the lipoprotein-mucopeptide complex interacts strongly both with lipids and with protein and so it is probably linked <u>in vivo</u> to the double track layer and thus extends the rigidity of the mucopeptide into the outer envelope regions, acting as a true structural protein.

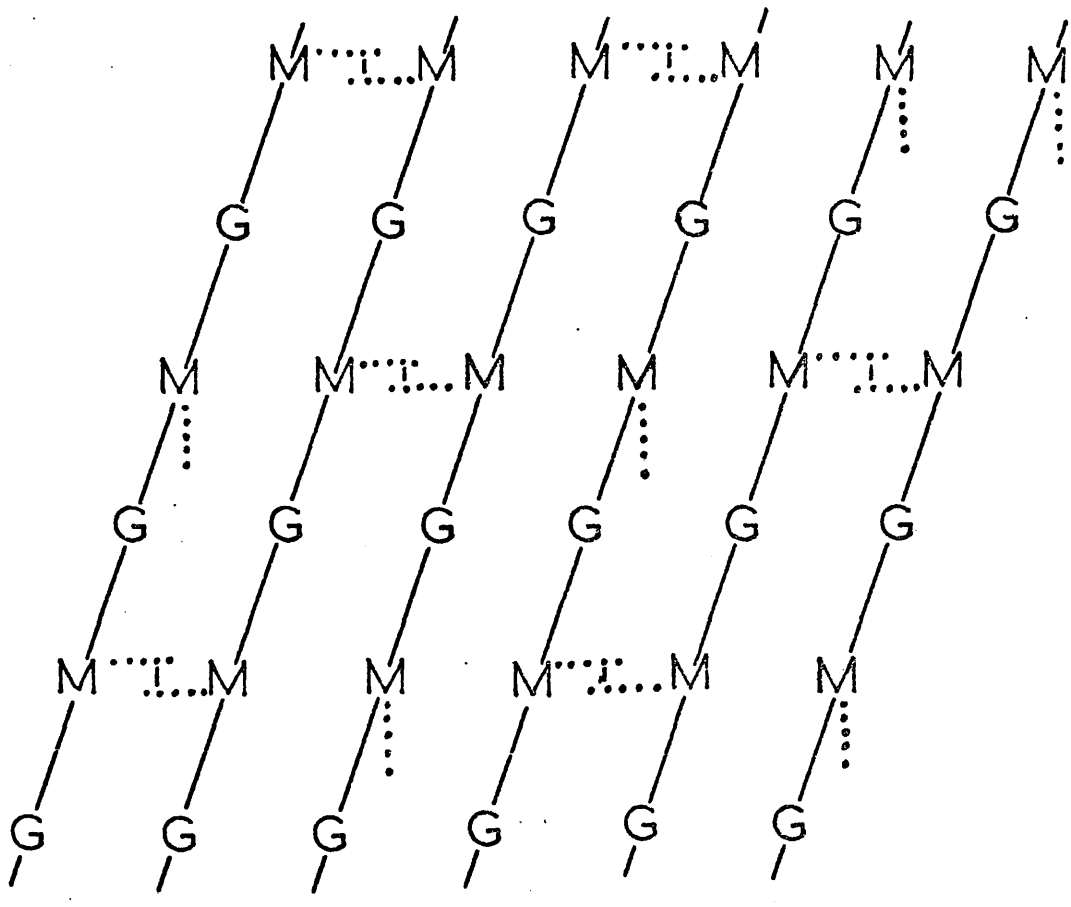
Recently freeze-etching electron-microscopic investigations have helped to elucidate Gram-negative cell envelope structure further. Weiss and Fraser (1973) demonstrated particulate members of the cytoplasmic membrane

in P.aeruginosa similar to those observed in E.coli. They suggested that these members were functional membrane components such as multi-enzyme complexes, or that they were associated with membrane transport functions. They also observed hemispherical structures, which had not been observed in E.coli, which were thought either to be sites where cell envelope subunits are sequestered during growth or to be lipid micelles. Van Gool and Nanninga (1971) confirmed the model proposed by Forsberg et al (1970a) by observing 4 fracture faces, and hence 5 discreet layers, in the envelope of E.coli. Three of these layers were associated with the outer membrane, and two with the cytoplasmic membrane. However Lickfield et al (1972), in a similar study of P.aeruginosa, observed three cytoplasmic membrane layers and five outer membrane layers. This may be due to greater fracturing of the envelope, and it must be remembered that such freeze etching studies give no direct information on the constitution of the structures exposed.

The fundamental building block upon which all these structures are based is the mucopeptide layer, otherwise known more specifically as peptidoglycan, glycosaminopeptide, glycopeptide or murein. It constitutes only about 10% of the Gram-negative envelope and is 2-3 nm thick, whereas it constitutes 40-90% of the Gram-positive wall and is 20-80 nm thick. Strominger et al (1959), Mandelstam and Rogers (1959), Rogers and Perkins (1959), Hancock (1960, and Mandelstam and Strominger (1961) elucidated its overall structure (Figure I.4), and suggested that it is a polysaccharide backbone of alternating β-1, 4-linked N-acetyl

FIGURE I.4

A schematic representation of the mucopeptide 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.

glucosamine and N-acetyl muramic acid residues (Figure I.5). The polysaccharide, or glycan strands have peptide subunits attached to the N-acetyl-muramic acid residue consisting of L-alanine, D-glutamic acid, (L)-meso-diaminopimelic acid and D-alanine. These peptide subunits are linked together often by peptide bridges, but in the case of E.coli and probably 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 subunit (Figure I.6). The arrangement of cross-linked peptide subunits is probably random, giving a loose network, unlike some Gram-positive mucopeptides where all the peptide subunite are cross-linked giving a very tightly knit and more rigid structure. The first Gram-negative mucopeptide was isolated by Weidel et al (1960) and its structure was essentially similar to that in Gram-positive cells, although the greater thickness of the mucopeptide in the latter case suggests a three dimensional arrangement whereas the narrower mucopeptide layer in the Gram-negative envelope suggests a two-dimensional monolayer structure (Ghuysen, 1968; Keleman and Rogers, 1972).

Weidel and Peltzer (1964) suggest that the mucopeptide layer alone provides rigidity in the Gram-negative cell wall as in the Gram-positive case, but Voss (1964), Fleck (1965) and Shafa and Salton (1960) have all presented evidence to suggest that there is another component which contributes perhaps equally to the rigidity of the Gram-negative cell envelope; this component could be the lipoprotein isolated by Braun and Rehn (1969).

FIGURE I.5

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

Key

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

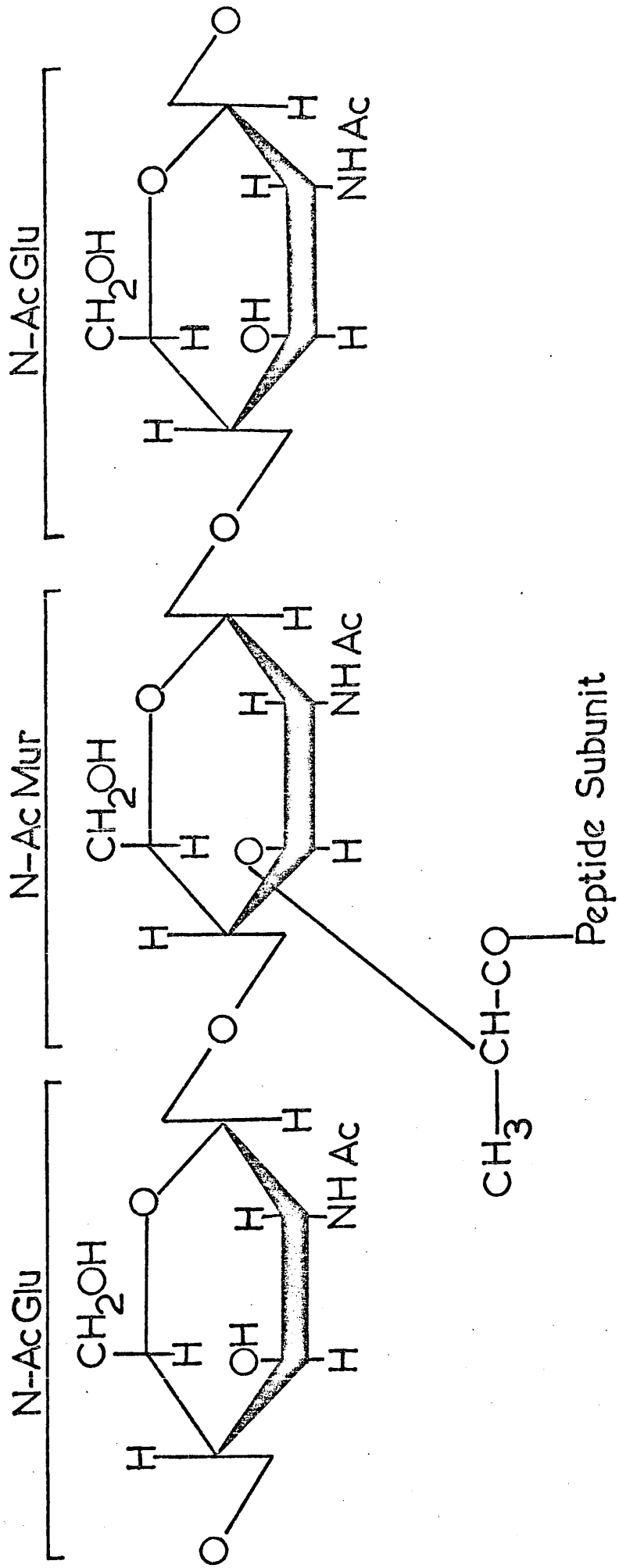
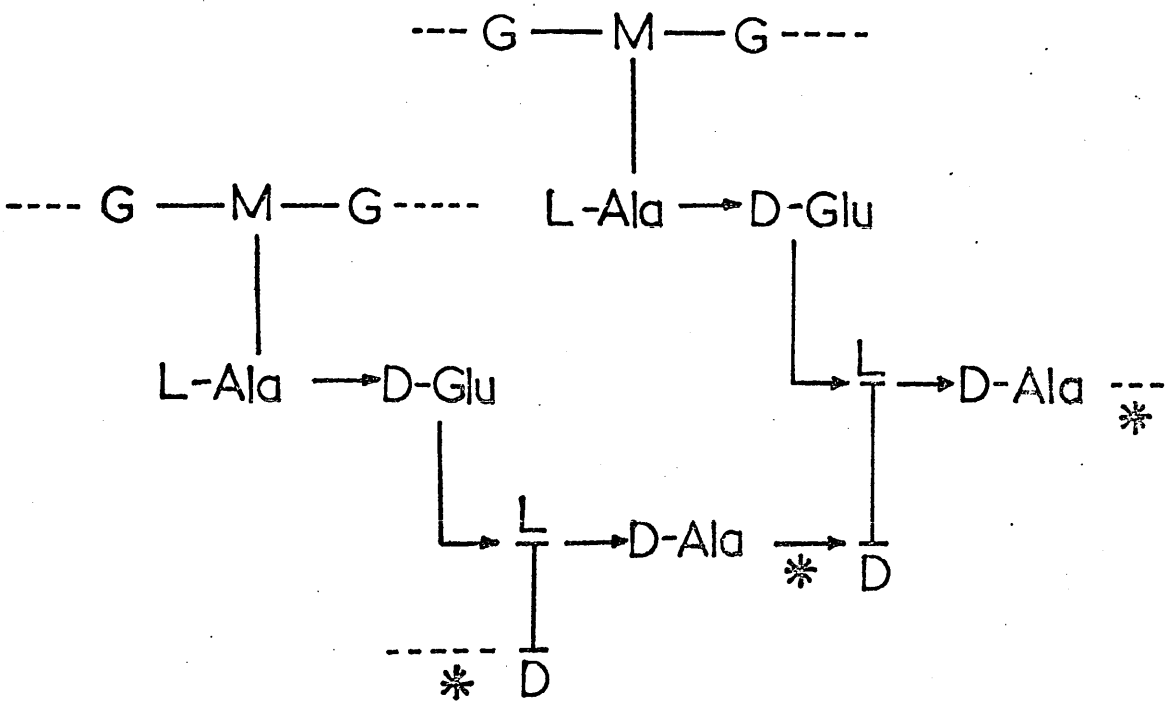

N-Ac Glu
N-Ac Mur
N-Ac Glu
NH Ac
CH2OH
OH
CH3—CH-CO
Peptide Subunit

FIGURE I.6

The structure of the peptide subunits of mucopeptide 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.

Eagon et al (1965a; 1965b) first illustrated the importance of divalent metal ions in isolated envelopes of P.aeruginosa and since then small, but fixed, amounts of Zn^{2+}, Mg^{2+} and Ca^{2+} have been shown to be essential for cell envelope integrity (Eagon and Carson, 1965; Absell and Eagon, 1966; Roberts et al, 1970; and Wilkinson, 1970). Forsberg et al (1970a) found that an outer loosely bound layer of a marine pseudomonad was held together and held to the envelope by divalent metal ion bridges; and 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. Absell and Eagon (1966) believe that during the biosynthesis of the lipopolysaccharide sacculus, negatively charged subunits are "trapped" by forming ionic and covalent bonds intermediated by divalent metals. Certainly the site of the divalent ion is stereospecific as Mn^{2+}, which is not found in isolated envelopes, cannot replace the naturally occurring divalent ions.

Gilleland et al (1973) were able to observe in a freeze-etching electron-microscopic study of P.aeruginosa that the removal of envelope divalent metal ions caused the loss of spherical protein units from the inner part of the outer membrane envelope (i.e. adjacent to the muco-peptide, see Figure I.2). The addition of Mg^{2+} to the cell

suspension medium precipitated the reaggregation of these protein units back into the cell wall. Loss of these protein units caused osmotic fragility of the cells, but stability returned with the addition of Mg^{2+}. This suggests the role of the lipoprotein layer is that of a permeability barrier external to the cytoplasmic membrane and protecting the mucopeptide, a feature observed by Burman et al (1972).

Readily extractable lipid in the cell envelope of P.aeruginosa accounts for about 16% of the total cell weight, and almost half of this is phospholipid (Bobo and Eagon, 1968). Firmly bound lipid accounted for about 9% of the cell weight much of which is incorporated into the lipopolysaccharide layer. Forge et al (1973) showed that the hydrocarbon tails of the phospholipids are packed side by side and suggests that these regular arrays of phospholipids are important in the structural integrity of the outer envelope membrane. These phospholipids are centrally placed in this membrane whilst the proteins are superficially placed and less important to the integrity of the outer membrane. Other studies of the lipopolysaccharide of the P.aeruginosa envelope by Chester et al (1972) have shown that the alanine is probably not part of a chain of alternating alanine and galactosamine residues but is present as a side chain on the galactosamine residues of the main polysaccharide chain.

The lipopolysaccharide of the P.aeruginosa envelope is important in that it constitutes, in part, the O-somatic

antigens which are associated with the cell surface and are important in the serotyping of strains. These antigens contain specific polysaccharides, lipids and proteins (Homma and Suzuki, 1964) and whilst the polysaccharide alone is not antigenic, it is variations in its structure which bring about changes in antigenicity. The polysaccharides of the O-antigens contain between 5 and 8 different sugars, but these always include heptose, D-glucose, D-galactosamine and D-glucosamine.

I.5. Antibiotics

Antibiotics were originally defined as compounds produced by microorganisms, including fungi, which at low concentrations inhibited growth and other activities of other microorganisms. As early as 1889, Doehle was recommending the use of pyocyanase, produced by Pseudomonas pyocyaneus, against the anthrax bacillus, and in 1896, Gosio obtained a crystalline substance from the penicillin mould, mycophenolic acid, which was also antagonistic to Bacillus anthraxis. However, these compounds were too unspecific in their toxic action to be clinically useful and it was in 1913 that Ehrlich set out the theory of selective drug action to aid the search for a "magic bullet". This "bullet" would be either toxic only to its target, the parasite, or be preferentially bound only to its target. His search led to the discovery of the arsenical agents (Voegtlin and Smith, 1920), and the sulphonamides (Domagk, 1935). However, the discovery

of penicillin by Fleming (1929) and its development for specific use against bacteria by Florey et al (1940) truly heralded the "antibiotic age".

The subsequent search for antibiotics has yielded hundreds of new toxic substances most of which have been naturally-occurring products. Gale (1963) divided antibiotics into five groups according to the general biochemical function upon which they acted:

(i) energy metabolism;

(ii) the function of bacterial membranes;

(iii) the synthesis of protein;

(iv) the metabolism of nucleic acid;

(v) the synthesis of peptidoglycan (mucopeptide).

Penicillin falls into group (v), inhibiting the cross-linking of the cell wall mucopeptide (Tipper and Strominger, 1965), whereas gentamicin falls into group (iii).

Gentamicin is a member of the aminoglycoside "family" of antibiotics, which also includes streptomycin, kanamycin, and neomycin. It is effective against a broad spectrum of Gram-negative and Gram-positive bacteria (Barber and Waterworth, 1966; Weinstein et al, 1963) and is most often used clinically in cases of infection due to P.aeruginosa which proves to be resistant to other antibiotics. Commercial preparations of gentamicin contain a mixture of four components (Figure I.7) isolated from submerged cultures of Micromonospora purpurea (Weinstein et al, 1963) of which gentamicins C_1, C_{1a} and C_2 are the most abundant and the most active.

Gentamicin, and the other aminoglycoside antibiotics, act upon the smaller (30 S) ribosomal subunit of the

FIGURE I.7

The structures of the gentamicins

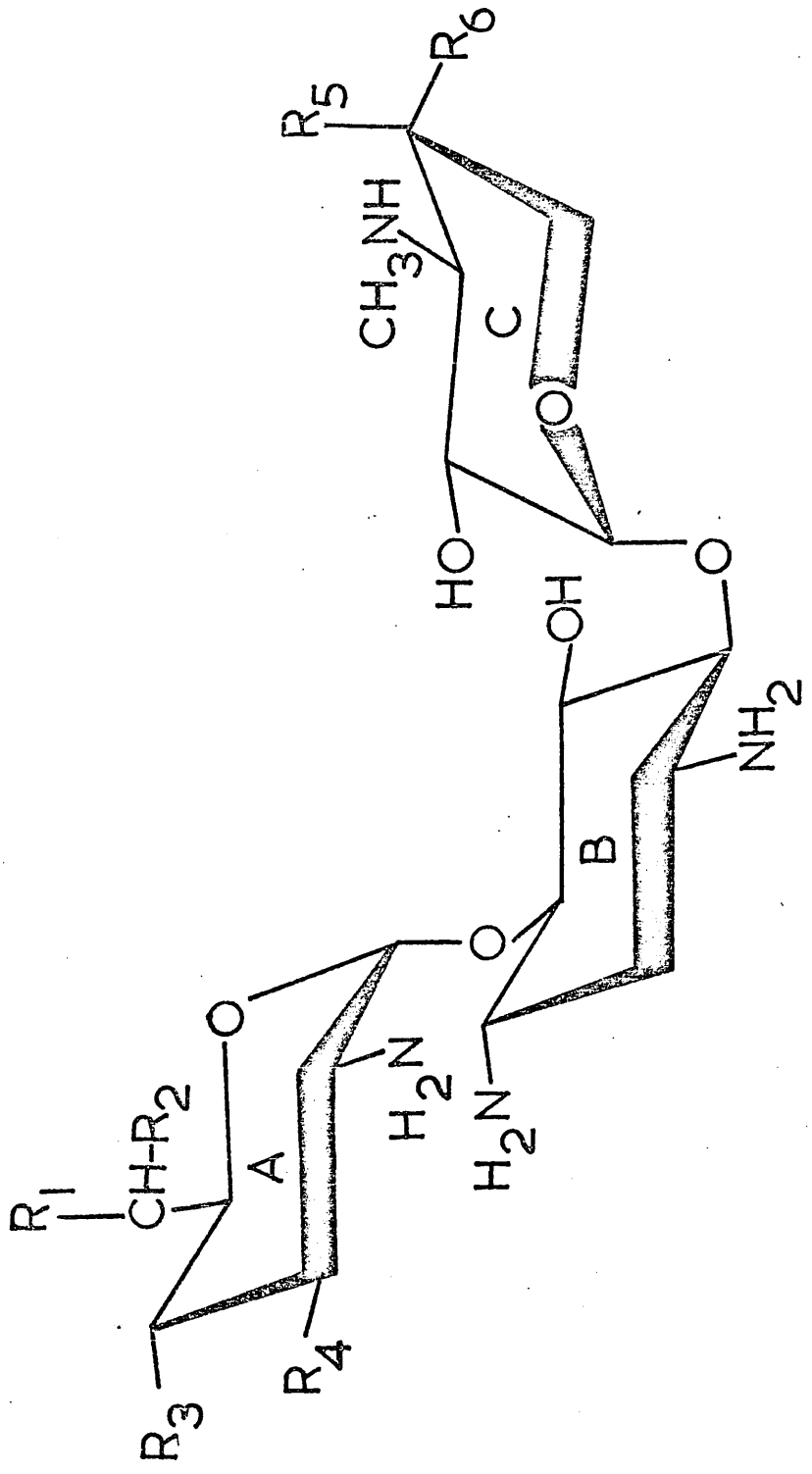
Key

	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
OH
NH2
HO
O
CH3NH
C
O
R5
R6

bacterial cell (Hahn and Sarre, 1969). The ribosome takes part in protein synthesis, a process which involves the assembling of polypeptides from L-amino acids. The amino acids are attached to a specific transfer RNA (tRNA) by enzymes and are carried to the ribosome. There, specificity in the protein synthesis is achieved by matching the incoming tRNA with a messenger RNA (mRNA) on the ribosome, whose nucleotide sequence is governed by the DNA. Once bound on or in the ribosome, the amino acid is attached to the adjacent nascent peptide and a translocation reaction shifts the elongated peptide along to a neighbouring ribosomal binding site, allowing another amino acid molecule to be brought to the ribosome and another peptide bond to be formed.

The effect of the aminoglycoside antibiotics is to bind preferentially to the ribosome and thus to disturb the protein synthesis. This binding has been shown to cause misreading or miscoding in vitro where anomalous amino acids are incorporated into the polypeptides to form proteins that are useless to the cell (Davies et al, 1964). This miscoding in itself is not lethal to the cell (Gorini and Kataja, 1965) 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 kills the cell (Gale et al, 1972). The differences in the spectrum and intensity of activity of the aminoglycoside antibiotics is probably due to the differences in ribosomal binding site. The binding site of streptomycin is probably the P10 protein (using the Nomura notation in Ozaki et al, 1969) and although the

binding site for gentamicin is unknown it is almost certainly a collection of several sites on the 30 S ribosomal subunit.

I.6. Antibiotic resistance

In 1887, Kosaihof noted that "bacteria tend to adapt to poisonous agents", and in the early days of chemotherapy, Ehrlich, discussing the treatment of syphilis, recommended the use of "massive doses" of Salvarsan to guard against any resistant spirochaetes which might have developed during prolonged treatment with smaller amounts. The problem became of major importance in the 1930s when the use of sulphonamides was severely limited by the development of resistant organisms, and soon after the introduction of penicillin as a therapeutic agent in the 1940s, there was evidence of the presence of penicillin-resistant organisms (Barber, 1947). In fact a pattern has built up whereby the introduction of a new antibiotic is followed by a "honeymoon period" when it is widely effective, but subsequently a higher and higher proportion of resistant organisms are isolated until the antibiotic is undermined as a therapeutic agent. The resistance of Staphylococcus aureus to benzyl-penicillin provides an example, for in 1943 17% of these organisms were resistant, but by 1961 nearly 70% were (Munch-Peterson and Boundy, 1962).

In biochemical terms, Gale et al (1972) consider resistance to antibiotics to be due to four mechanisms:

(a) modification of the target in the cell;

(b) reduction of the physiological importance of the target;

(c) prevention of access to the target;

(d) synthesis by the bacteria of an enzyme capable of inactivating the inhibitor.

In the case of resistance of P.aeruginosa to gentamicin, one of these mechanisms has been shown to be present, and at least one of the others is implicated. These resistance mechanisms will now be discussed:

(a) Modification of the target. In such cases the target is most often an enzyme and modification occurs so that, for example, the preferential binding to the enzyme of the inhibitor rather than the substrate, is reversed, inactivating the inhibitor but retaining enzyme activity. This mechanism has been observed in the binding of sulphonamides to the enzyme tetrahydropteroic acid synthetase (Hotchkiss and Evans, 1960; Ortiz, 1970). An example of this mechanism may also be found in the aminoglycoside antibiotics, where resistant mutant cells show no binding of streptomycin to the P10 protein of the 30S ribosomal subunit unlike its sensitive parent. This may be due to an altered primary amino acid sequence of the protein (Nomura, 1970). Cases are known where the modification of the target site to inactivate the inhibitor also inactivates the enzyme (Adelberg, 1958) and in this case survival of the cell is ensured at the cost of metabolic efficiency.

(b) Reduction in the physiological importance of the target
This mechanism of resistance reduces the dependence of the

cell upon the inhibited process by adaptation. Such adaptation was invoked by Barber (1962) to explain resistance to penicillin not involving β-lactamase production (part (d)), and was termed "intrinsic resistance". In the case of penicillins, this "intrinsic resistance" may be due to two factors, the inaccessibility of the target and a reduced dependence of the cell upon the target. Gram-negative organisms have a higher "intrinsic resistance" to penicillins than Gram-positive, because the mucopeptide target is more deeply buried in the envelope structure and so less accessible to the antibiotic. Also, in pseudomonads the mucopeptide is only a small part of the envelope structure and is not solely responsible for envelope rigidity and integrity unlike most Gram-positive organisms (I.4) and this may be why the pseudomonads in general have a higher "intrinsic resistance" to penicillins.

Dean (1971) provides evidence for a selective adaptation process which suggests that resistance of Klebsiella aerogenes to streptomycin can be due either to a build up of enzymes prior to the inhibited process which eventually swamp the antibiotic action, or a change of enzyme balance which allows the cell to continue metabolising by the greater operation of a minor pathway which is less susceptible to inhibition. This latter mechanism is an example of the reduction of importance of the target site.

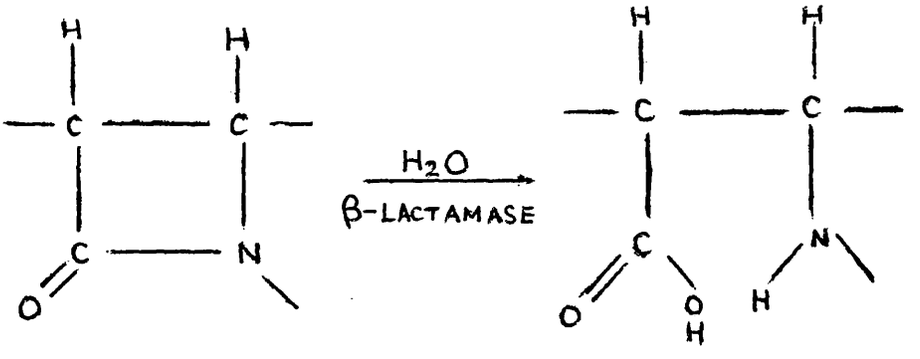
(c) Prevention of access. The resistance of organisms due to a failure to achieve sufficient antibiotic concentrations within the cell is an important mechanism and a complex one. It seems unlikely that it is caused

simply by the placing of an extra molecular barrier around the cell as this would probably equally inhibit the passage of molecules needed by the cell. However cases where the failure to build up a sufficient internal antibiotic concentration have caused resistance are known. For example tetracycline initially passes freely into and out of bacterial cells, but its low concentration inside the cell induces a change in permeability, preventing outflow of tetracycline. The antibiotic concentration thus builds up and kills the cell by preventing protein synthesis (Franklin, 1966, 1967). Resistant cells, however, fail to provide the permeability barrier in the outward direction and so a lethal antibiotic concentration is never achieved (Franklin, 1967; Franklin and Higginson, 1969).

Several workers have shown that the structure of the envelope P.aeruginosa, and especially the outer double track membrane, influences its resistance to antibiotics, probably by an exclusion mechanism (for review see Brown, (1971). Hamilton (1970) showed that P.aeruginosa became resistant to membrane active antibiotics by preventing their penetration, and polymyxin, one such antibiotic, has been shown to be inactive in some cases because of lack of uptake by the cells (Few and Schulman, 1953; Newton, 1956). It is also possible that an exclusion mechanism may be operating in conjunction with other antibiotic resistance mechanisms, such as the production of inactivating enzymes; this has been postulated by Smith et al (1969) and Roe et al (1971).

(d) Synthesis of inactivating enzymes. Resistance by this method is achieved in two ways; by the production of enzymes which destroy the antibiotic by opening one or

more covalent bonds in the antibiotic molecule, or by the production of enzymes which chemically substitute key antibiotic residues and thus make them inactive. An example of the former is the production of β-lactamase by S.aureus which opens the β-lactam peptide bond in penicillin (Saudi and Woodruff, 1949):



Examples of the second type of enzymes are found in the resistance of bacteria, including P.aeruginosa, to aminoglycoside antibiotics. There are three types of these substituting enzymes: (i) adenylylation enzymes using ATP as a source of the adenylyl residue; (ii) phosphorylating enzymes, also using ATP, but as a source of a phosphate group; and (iii) acetylating enzymes, using acetyl-CoA as the source of an acetyl group. These enzymes are probably located in the periplasmic space (Davies et al, 1969). Brzezinska et al (1972) suggested that P.aeruginosa can produce an enzyme which adenylylates the garosamine ring of gentamicin and Mitsuhashi et al (1971) isolated an enzyme from P.aeruginosa which acetylates the purpurosamine and deoxystreptamine rings of gentamicin C components.

P.aeruginosa produces a phosphorylating enzyme (Kobayashi et al, 1971a; 1971b; 1972; and Davies et al, 1969) but this only inactivates gentamicin A, streptomycin, kanamycin, paramomycin and neomycin, leaving the gentamicin C components fully active. Results such as this have enabled the speficicity of the enzyme substrates to be determined. It is known that the adenylylating enzyme shows a preference for hydroxyl groups in the D-threo configuration, as in streptomycin; the acetylating enzyme attacks free primary 6-amino groups on six-membered sugar or deoxysugar rings glycosidically linked to deoxystreptamine moieties; and the phosphorylating enzyme attacks 3′-hydroxyl groups on six-membered sugar or deoxysugar rings linked to deoxystreptamine moieties (Davies et al, 1971). Because of these substrate specificities gentamicin C_1, C_2 and C_{1a} are poor substrates for the phosphorylating enzyme whilst gentamicin A is a good substrate, and gentamicins C_1, C_2 and A are poor substrates for the acetylating enzyme whilst gentamicin C_{1a} is a good substrate. Acetylation of an antibiotic does not necessarily lead to inactivation, but phosphorylation causes complete loss of activity, implicating the 3′-hydroxyl groups and not the 6-amino groups of the gentamicin molecule in its binding to the ribosome.

All four of these types of resistance to antibiotics require extra genetic material within the cell, either as part of the chromosome, or extra-chromosomally on a plasmid (or R-factor). The inactivating enzymes (type (d)) are known to be mediated by plasmids as shown by the ability to transfer them from cells of one strain to those of another both in vitro (Sykes and Richmond, 1970) and in

vivo (Roe et al, 1971). The transfer of these plasmids may assist the spread of antibiotic resistance but it is probably less important than the selective pressures of widespread antibiotic therapy resulting in the presence of genes coding only for antibiotic resistance being present at the onset of infection. The other three types of resistance mechanism, (a) to (c), only require the modification of chromosomal genes already existing and may be more readily acquired than those requiring extra-chromosomal genes, and may be responsible for levels of resistance less than the characteristically high-level resistance caused by inactivating enzymes.

I.7. Electrophoresis

At any solid-liquid interface there exists a potential difference due to the asymmetrical distribution of ions. This leads to the four electrokinetic phenomena: electrophoresis, electroosmosis, sedimentation potential and streaming potential. The first two refer to the movement of the phases relative to each other under the influence of an applied electric field; electrophoresis is the movement of the solid phase relative to the liquid and electro-osmosis is the movement of the liquid relative to a stationary solid. The present discussion is largely concerned with the phenomenon of electrophoresis.

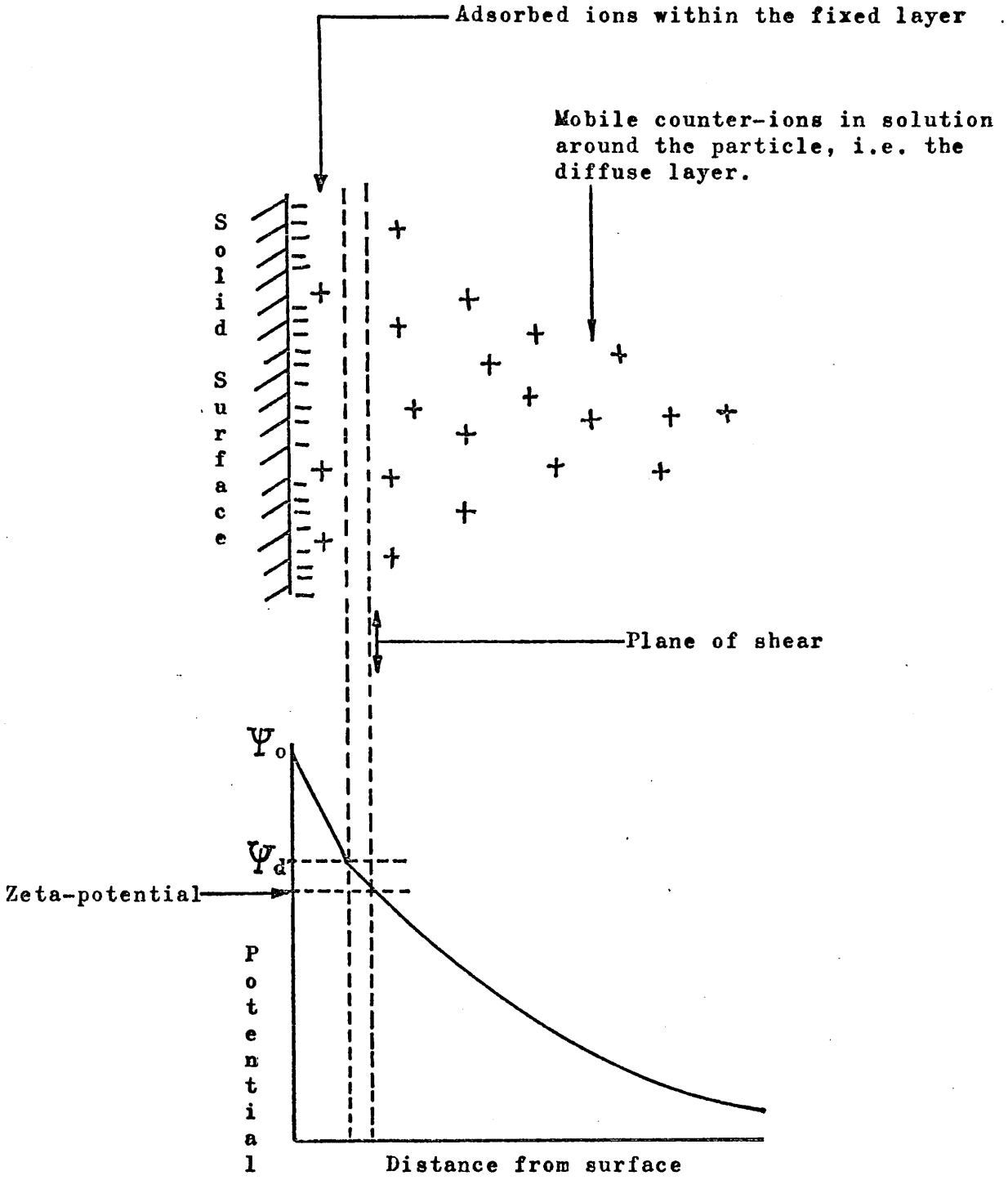
Early observations on electrokinetic phenomena date from Reuss (1809), but it was Quincke (1861) who first

put forward the concept of the electrical double layer, visualising two parallel layers of charges or ions, of uniform charge density but opposite sign, held at a small distance apart, one firmly attached to the solid surface, and the other in the liquid. Helmholtz (1879) discussed the theory mathematically and defined the zeta potential. Perrin's idea of a parallel plate condenser form of a double layer was succeeded by theories of Gouy (1910) and Chapman (1913). These introduced the idea of a diffuse double layer, in which the potential decreased exponentially to zero over the distance $^{1}/_{K}$, the statistical thickness of the double layer, an equilibrium in the diffuse layer being maintained between the opposing forces of the potential field, tending to order the ions, and the forces of thermal motion tending to redistribute them randomly. Stern (1924) recognised the possibility of specific ion adsorption and also the finite size of the ions and utilised both these hypotheses and suggested that 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, followed by an exponential decrease over the diffuse outer atmosphere of the Gouy-Chapmen theory (Figure I.8). A condition of electrical neutrality is maintained, since the surface charge is equal in magnitude, but of opposite sign, to the total charge in the fixed and diffuse parts of the double layer.

The phenomenon of electrophoresis arises when an electric field is applied, and particle movement relative to a suspension of liquid results. This causes the

FIGURE I.8

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



development of a shear plane within the double layer, and the particle velocity is governed by the magnitude of the potential at this plane, known as the zeta-potential.

The zeta-potential, Z, is related to the electrophoretic mobility, $\bar{v}$, the velocity per unit potential gradient, by the general equation:

$$\bar{v} = \text{constant.} \frac{\epsilon_r Z}{\pi n}$$

where ϵ_r is the relative permittivity and n the coefficient of viscosity within the double layer.

Smoluchowski (1921) evaluated the constant as $^1/_4$, independent of shape, size and orientation of the particle, whereas Debye and Huckel (1924) derived an equation where the constant depended on the particle shape and its orientation in the field. Henry (1931) accounted for the differences in the theories of Smoluchowski and Debye and Huckel, and derived a value for the constant of $^1/_4 f(Kr)$ where f(Kr) is a power series in Kr, where r is the radius of curvature. This theory, for a sphere of any size, took full account of the effect of distortion of the external field and furthermore showed that the Smoluchowski equation is valid if $Kr > 100$. Working with bacterial cells of size 10^{-4} cm, the Smoluchowski equation is valid if the ionic strength exceeds 10^{-3} mol dm^{-3} (James, 1957).

The Smoluchowski equation assumes that the particle is non-conducting, that the applied field may be simply added to the field of the double layer, and that the conductance, viscosity and relative permittivity are the same in the double layer as in the bulk medium. Gittens

and James (1963) showed that the first of these assumptions is untrue when dealing with ionogenic material such as bacterial surfaces where the surface charge arises from groups such as amino, carboxyl and phosphate located in the cell surface structures, and where there is no charge due to ion adsorption (Gittens, 1962). This raises serious objections against discussing particulate electrophoresis observations in terms of the zeta-potentials or the surface charges but these objections are not tenable if all observations of electrophoretic mobility are discussed as such. Furthermore, in this investigation, conditions of constant ionic strength, viscosity and relative permittivity were used to enable the direct comparison of results.

I.8. Particulate Microelectrophoresis

The method used throughout this investigation for determining the electrophoretic mobility of a bacterial cell is based upon that of Ellis (1911). A bacterial suspension is contained in a closed glass chamber, and a potential difference is applied across the suspension between two electrodes. Using a microscope, the migration of the suspended bacteria is observed, and individual cells are timed moving across a graticule in the eyepiece. However, when an electric field is applied across a system, not only will the bacteria move relative to the suspension liquid, but due to electroosmosis the

suspension liquid will move relative to the glass surface of the observation chamber. 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 wall, and v_P the velocity of the particles relative to the liquid. In dealing with a closed system, the liquid will flow along the top and bottom faces of the chamber, and return through the centre, resulting in a variation of v_L, and hence v_o, with depth. As the liquid is being continuously deformed within a closed system, it follows that there must be a plane at which the liquid is stationary; at this level $v_o = v_P$. In the flat cell of rectangular cross-section, 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 whose width/thickness ratio is K, such that

$$a_o = \tfrac{1}{2} \pm \sqrt{\frac{1}{12} + \frac{32}{\pi^5 K}}$$

where a_o is the fractional depth measured from the top inside surface. For a cell with K greater than 20, 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_0 the velocity of the particle observed at depth x. For a symmetrical cell the curve of v_0 against x should be a parabola symmetrical about the centre.

Cell and electrode design has been extensively reviewed by James (1957) and Seaman (1965). The cell used in this work was that described by Gittens and James (1961). With this apparatus the applied field strength (X) is best calculated from conductance and current data, using the equation:

$$X = \frac{I}{qk}$$

where I is the current (amp), k is the conductivity of the suspension medium, and q is the cross-sectional area of the cell (m^2). Mayer (1936) showed that the use of the applied voltage to measure the field strength may lead to errors of up to 50%, since slight changes in the electrodes may result in large changes in the field strength, without affecting the applied voltage.

I.9. Application of Microelectrophoresis to the Study of the Bacterial Cell Surface

Electrophoretic measurements must be made on cells suspended in a medium of known chemical composition, pH and ionic strength.

Valuable information about the nature of surface

ionogenic groups on cells of fixed age may be gained by varying the pH of the suspension medium, whilst the ionic strength is maintained at a constant value, e.g. with a carboxyl-amino type surface, the ionogenic groups will be titrated according to the equations:

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

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

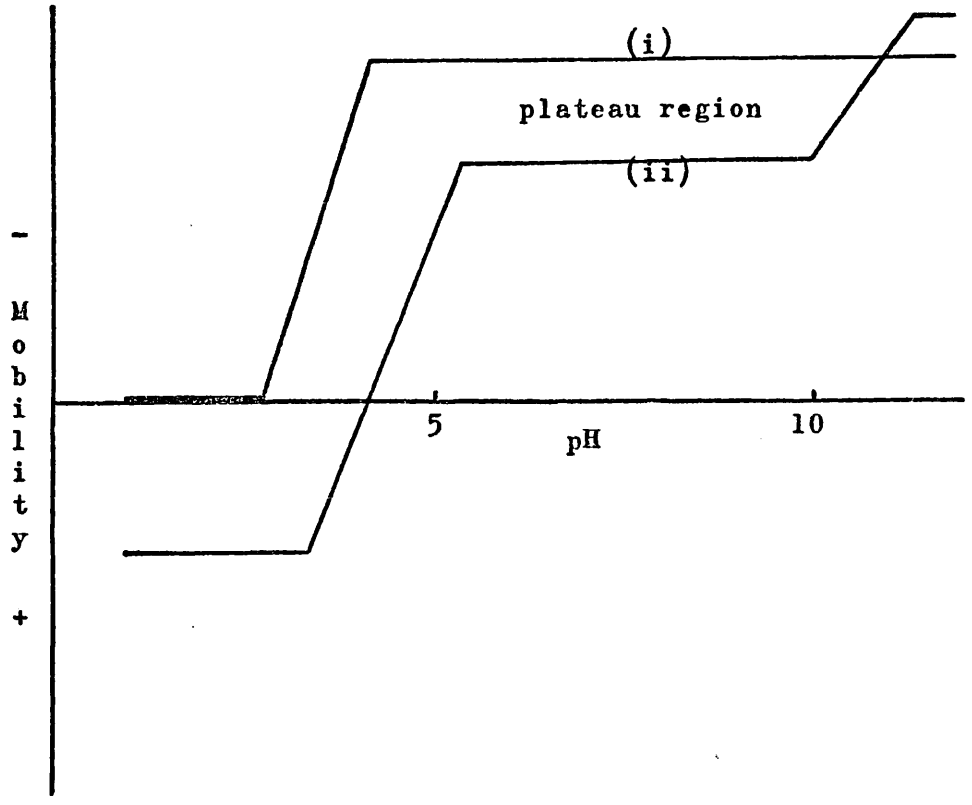
At a low pH, the surface charge will be due to the positively charged amino groups, but on decreasing the hydrogen ion concentration (increasing the pH) not only is the positive charge reduced, but the carboxyl groups ionise, giving rise to a negative charge. The sigmoid type pH-mobility curve, typical of a carboxyl-amino surface, is shown in Figure I.9. An isopotential point occurs between pH 4 and 5, the exact value being determined by the relative numbers of amino and carboxyl groups present and their pK values. Between pH 5 and 9, a plateau region occurs where both amino and carboxyl groups are fully ionised, the mobility then depending on the relative numbers of carboxyl and amino groups present. The effective negative charge increases beyond pH 9, as shown, due to suppression of the amino group ionisation. Such curves have been obtained experimentally (Douglas, 1959; Plummer et al, 1962).

The pH-mobility curves characteristic of a surface with ionogenic carboxyl groups only is shown in Figure I.9, and was obtained for the polysaccharide surface of Klebsiella aerogenes by Lowick and James (1957).

FIGURE I.9

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

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

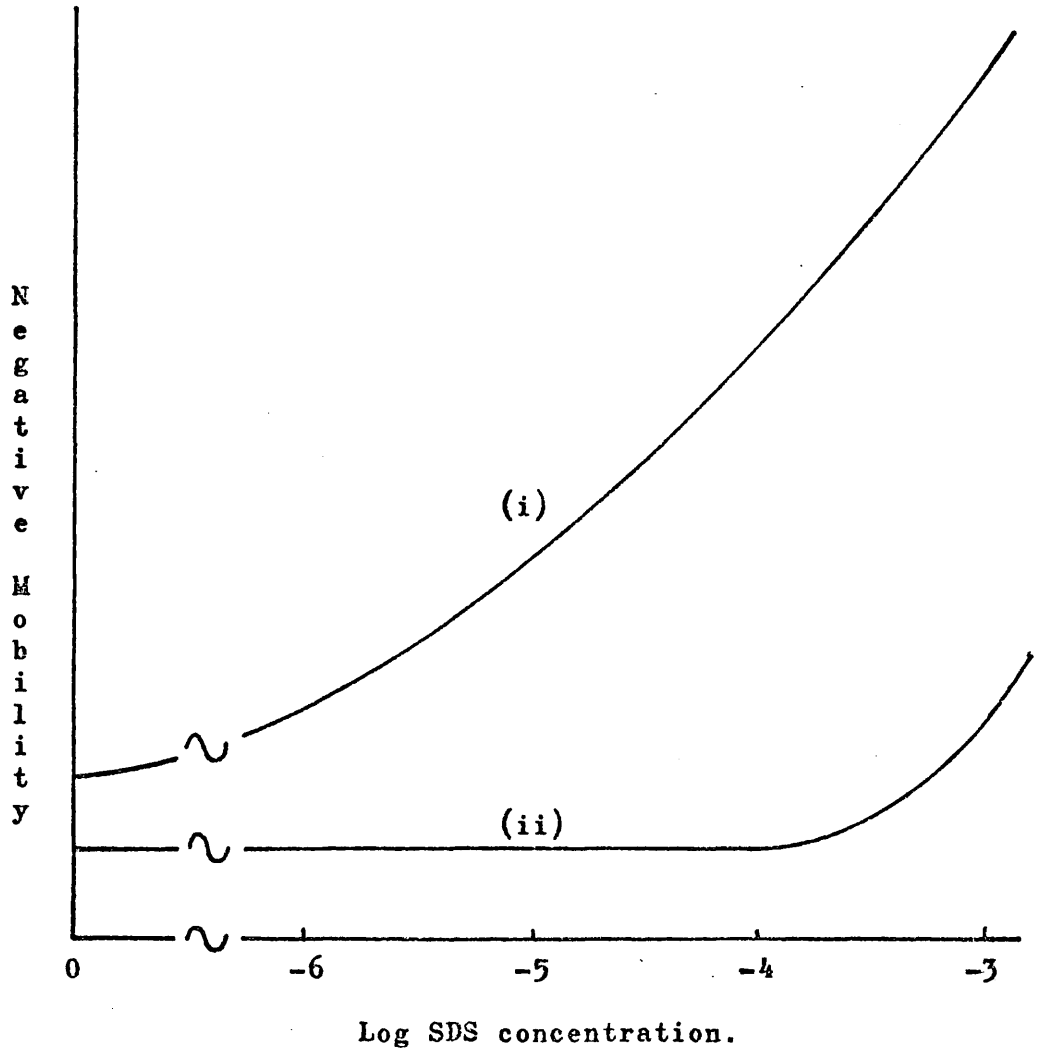


When varying the pH of the suspension medium, care must be taken not to cause irreversible changes in the surface by using extreme pH values. After suspending cells at a high, or low, pH the reversibility must be checked by rewashing and measuring the mobility at pH 7 The mobility value should not differ significantly from that of control cells at pH 7. An irreversible change indicates cell surface damage, making useful interpretation difficult. This variation of mobility with pH will only give information on the nature and quantity of surface ionogenic groups.

Powney and Wood (1940) observed that the negative mobility of oil droplets increased when measured in the presence of the detergent sodium dodecyl sulphate (SDS). This was due to the solution of the hydrophobic hydrocarbon chain of the surface active agent in the oil droplet with the negatively charged sulphate group orientated outwards into the medium and contributing to the mobility of the droplet. Dyar (1948) observed the same increase in negative mobility when mobility values of bacterial cells having large amounts of lipid on the surface were determined in the presence of SDS, and this is shown in Figure I.10. The method of measuring cell mobilities in the presence and absence of 10^{-4} mol dm^{-3} SDS has been used to detect lipid located on the cell surface of strains of Staphylococcus aureus (Hugo and Stretton, 1966) and K.aerogenes after repeated growth in crystal violet (Lowick and James, 1957). An SDS concentration above 10^{-4} mol dm^{-3} causes increased and variable mobility values for surfaces both with and without surface lipid, due to non-specific

FIGURE I.10

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



adsorption and cell lysis.

I.10. Objects of this Investigation

Previous workers have shown that the surface structures of bacterial cells, as revealed by pH-mobility studies, can be correlated with factors such as origin and antibiotic resistance (for example see Hill and James, 1972a, 1972b). No such studies have been performed on cells of Pseudomonas aeruginosa and so the objects of this investigation are:

(i) to standardise the application of particulate microelectrophoresis to the study of P.aeruginosa,

(ii) to use this method to investigate P.aeruginosa cell surface structure,

(iii) to study the correlation between cell surface structure and antibiotic resistance of P.aeruginosa with particular reference to gentamicin.

C H A P T E R I I

TECHNIQUES

II.1. Bacteriological Techniques

(a) Media and growth conditions

Nutrient agar was prepared by adding 25 g of powdered Oxoid Nutrient Broth No.2 to 1 dm^3 distilled water, and then adding 1% w/v of Oxoid Agar No.1 to this solution. The solution was distributed into 100 cm^3 lots in 3 oz medical bottles and immediately sterilised by autoclaving at 15 lb in^{-2} for 20 minutes. Agar plates were prepared by melting a sufficient amount of this stock agar in a steam bath and aseptically pipetting 10 cm^3 amounts into sterile plastic petri dishes and allowing the agar to cool.

Nutrient broth was prepared by adding 13 g of powdered Oxoid Nutrient Broth (Code CM1) to 1 dm^3 distilled water, distributing into 50 cm^3 lots in 8 oz medical bottles and sterilising by autoclaving as before.

Agar plates containing any required concentration of gentamicin were prepared by adding a given volume of a stock aqueous gentamicin solution (2000 $\mu g\ cm^{-3}$) to the solid agar and then re-sterilising by autoclaving, before pipetting into petri dishes as before.

Eosin methylene blue, (EMB), agar was prepared by adding 37.5 g of powdered Oxoid Eosin Methylene Blue Agar to 1 dm^3 distilled water and boiling the solution to dissolve the solid before autoclaving at 15 lb in^{-2} for 20 minutes. The liquid agar was cooled to 60°C and vigorously shaken to re-oxidise the indicator and finally aseptically pippetted in 10 cm^3 lots into sterile plastic petri dishes.

Unless otherwise stated all agar plates were inoculated

from the parent culture with a sterile platinum loop and incubated for 18 hr at 37°C. Nutrient broth cultures were inoculated from parent broth cultures with one drop from a sterile 1 cm^3 pipette, and incubated for 18 hr at 37°C with the bottle sloped and loosely capped.

(b) Strains

The strains of Pseudomonas aeruginosa (Table II.1) used in this investigation were obtained from the following sources:

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

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

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

When received, all the strains were growing on nutrient agar slopes and were stored in the laboratory on similar slopes at 5°C. The strains were maintained by subculturing onto fresh slopes bi-monthly.

The strain of Klebsiella aerogenes used for calibration of the electrophoresis apparatus (Table II.1) was resuscitated from a freeze-dried ampoule by aseptically opening the ampoule, taking up the sample in 0.5 cm^3 nutrient broth and then inoculating 50 cm^3 nutrient broth with one drop of this suspension. The broth was incubated and the resulting culture kept at 5°C. The strain was maintained

TABLE II.1.

The strains used in this investigation , with the code numbers used in the text and associated characteristics.

Key:

Source: Nic. = Nicholas Research Institute, Slough.
MRC = MRC Industrial Injuries and Burns Unit,Birmingham.
B = Brugmann Hospital , Belgium.
M = Dr.M.McDonough, Bedford College,London.

MIC : Gen. = Gentamicin
Neo. = Neomycin
Kan. = Kanamycin
Str. = Streptomycin
Car. = Carbenicillin

Serological typing : NT. = Not typable

Phage Reactions : NT. = Not typable. Figures in brackets show weak reactions.

TABLE II.1.

Strain Reference Numbers (*P.aeruginosa*) Text	Laboratory	Source	M.I.C./ ug cm^{-3} Gen.	Neo.	Kan.	Str.	Car.	Serological Group	Phage Reactions
1	1 (NCTC 8058)	Nic	1.25	6.3	50	3.0	400	6	7 16 (31) 44 F8 109 (352) 1214 (M4) (Col.11)
2	2	Nic.	5	312	78	39	250	9	7 21 (24) 31 44 68 F8 109 (119x) 352 1214 (M4) (Col.11)
3	3 (NCTC 6749)	Nic.	≤0.16	3.2	50	12.5	100	6	7 21 44 (F8) (M4)
4	4	Nic.	1.5	12.5	100	>100	400	11	F7
5	72	Nic.	12	>100	100	>100	50	4	NT.
6	96	Nic.	25	156	78	39	>1000	6	(68) 73
7	B32	MRC	25	625	156	312	250	4	(24) 68 119x 352 (M4) Col.11
8	100	B	8000	>1000	>5000	156	390	11	44 109 119x 352 1214 M6 Col.11
9	101	B	8000	5000	>5000	1250	3100	11	44 352 1214 Col.11
10	102	B	25	1250	78	312	250	11	NT.
1A/20	1A/20	1	25	312	>5000	625	250	NT.	44 (F8) 109 352 (1214) (M4) Col.11
1A/50	1A/50	1	55	312	>5000	312	500	NT.	(44) (F8) 109 352 (M4) Col.11
1A/100	1A/100	1	120	312	>5000	625	250	NT.	(24) (109) Col.11
1B	1B	1	3.1	39	78	39	500	6	(7) 44 F8 109 352 (1214) (M4) Col.11
Escherichia coli.K 12		M	0.61	≤9.7	24	9.7	31		
Klebsiella aerogenes (NCTC 418)									

by subculturing into fresh nutrient broth bi-monthly.

The strain of Escherichia coli K12 (Table II.1) was received on a nutrient agar plate from Dr. M. McDonough, (Department of Botany, Bedford College, London N.W.1) and was maintained by daily subculturing onto a fresh agar plate whilst in constant use, and at other times by bi-monthly subculturing on agar slopes kept at $5^{o}C$.

(c) Growth of strains for experimental purposes

All strains of P.aeruginosa, after transfer from the storage slope, were grown once on nutrient agar plates in the quantities required for further examination immediately before being harvested. In some preliminary experiments nutrient broth cultures were used, these were inoculated with a sterile loop from the storage slope into the broth sample.

For experimental purposes cells of K.aerogenes were inoculated from the storage culture into 50 cm^3 nutrient broth and then incubated before harvesting.

(d) Growth of cells during training to gentamicin

P.aeruginosa, strain 1, was trained, using the method described by Rolinson et al (1960), to develop a resistance to gentamicin, enabling it to grow in the presence of 20 $\mu g\ cm^{-3}$ of the antibiotic in nutrient agar initially, and subsequently, by continued training, to grow in the

presence of 50 and 100 μg cm^{-3} of the antibiotic.

The sensitive strain 1 was initially grown at 37°C on nutrient agar containing a concentration of gentamicin of half the minimum inhibitory concentration (MIC). Sparse, slow-growing cultures were obtained at first, but after several more passages at the same concentration of the antibiotic, faster growing, larger colonies were obtained. This resulting culture was then inoculated onto a plate containing double the previous concentration of antibiotic. This stepwise increase in tolerance to the antibiotic was repeated until luxuriant growth was obtained on nutrient agar plates containing 20 μg cm^{-3} of gentamicin; at this point the strain was designated strain 1A/20. This strain was split, one half was repeatedly grown at this same concentration of antibiotic whilst the other half was subjected to further training until it acquired tolerance to a gentamicin concentration of 50 μg cm^{-3}, and was designated strain 1A/50. This strain was again split, one half being grown repeatedly at the same antibiotic concentration, the other trained until it acquired tolerance to a gentamicin concentration of 100 μg cm^{-3}, and was designated strain 1A/100.

(e) Cleaning and sterilisation of apparatus

All glassware was washed and scrubbed in tap water and then rinsed once in tap water and once in distilled water. It was finally dried and sterilised in an oven at 150°C overnight. Pipettes and Pasteur pipettes were

plugged with cotton wool, put into metal containers and re-sterilised.

All solutions required in a sterile condition were autoclaved at a pressure of 15 lb in^{-2} for 20 minutes.

Contaminated disposable apparatus was immersed in a 1% lysol solution, and glass pipettes in a 2% Milton solution. Other contaminated glassware was autoclaved before washing.

II.2. The Microelectrophoresis Apparatus

(a) Description

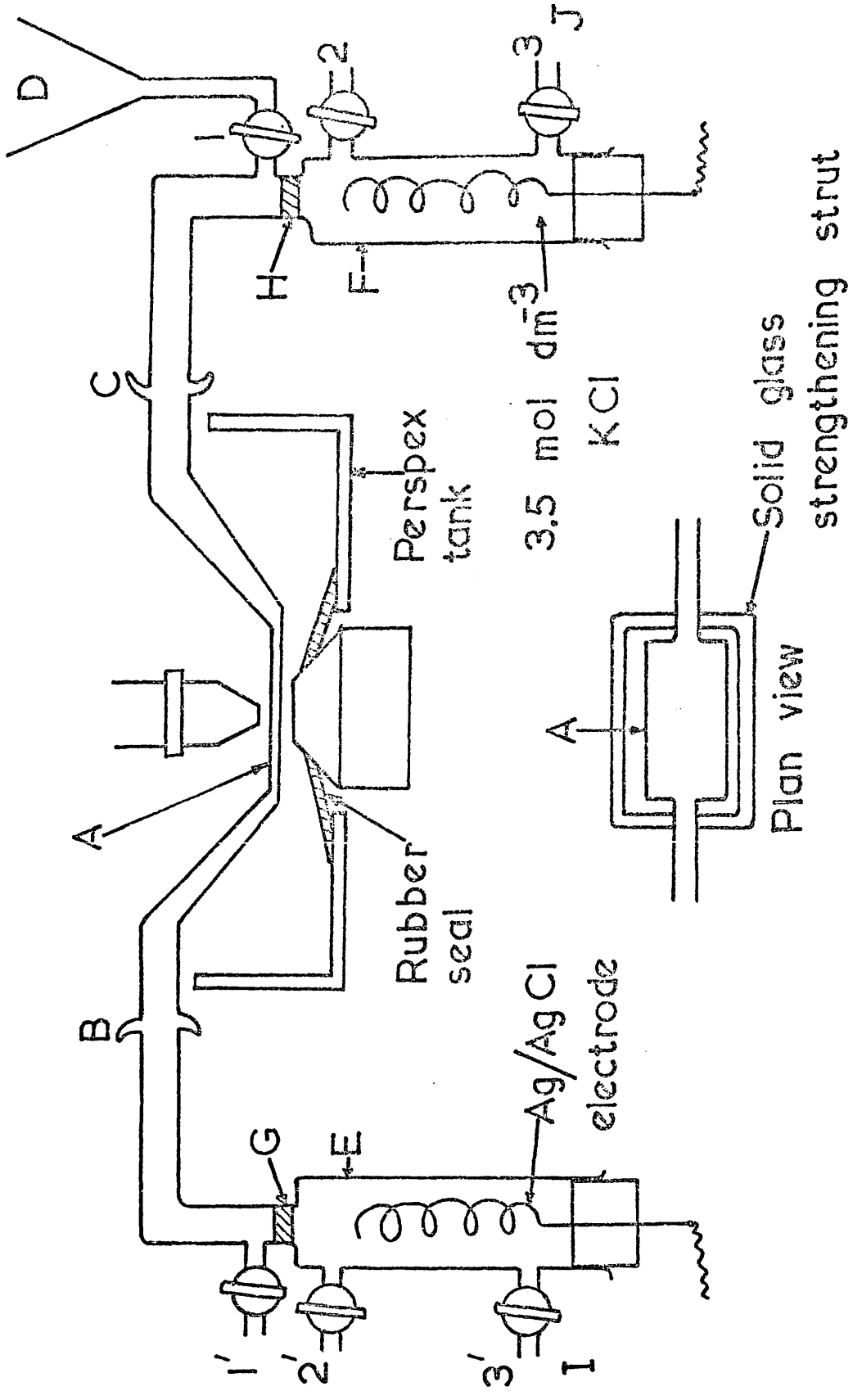
The apparatus used was that developed by Gittens and James (1961); this is shown in Figure II.1.

The electric field was applied across a suspension of bacterial cells contained in the glass observation chamber A. Observing the cells with a microscope, the velocity due to a known applied field was determined by timing individual cells 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. This was attached to side arms fitted with hemispherical ground glass joints (B and C) and filled with suspension from the reservoir (D) by opening taps 1 and 1'.

A constant electric field was applied between the Ag.AgCl/KCl electrode systems in the compartments (E and F). The electrodes consisted of spirals of silver wire

FIGURE II.1

A diagrammatic representation of the microelectrophoresis apparatus


D
1
2
3
J
H
F
C
A
B
G
E
Perspex tank
Rubber seal
3.5 mol dm^{-3} KCl
Ag/AgCl electrode
1'
2'
3'
I
Solid glass strengthening strut
Plan view

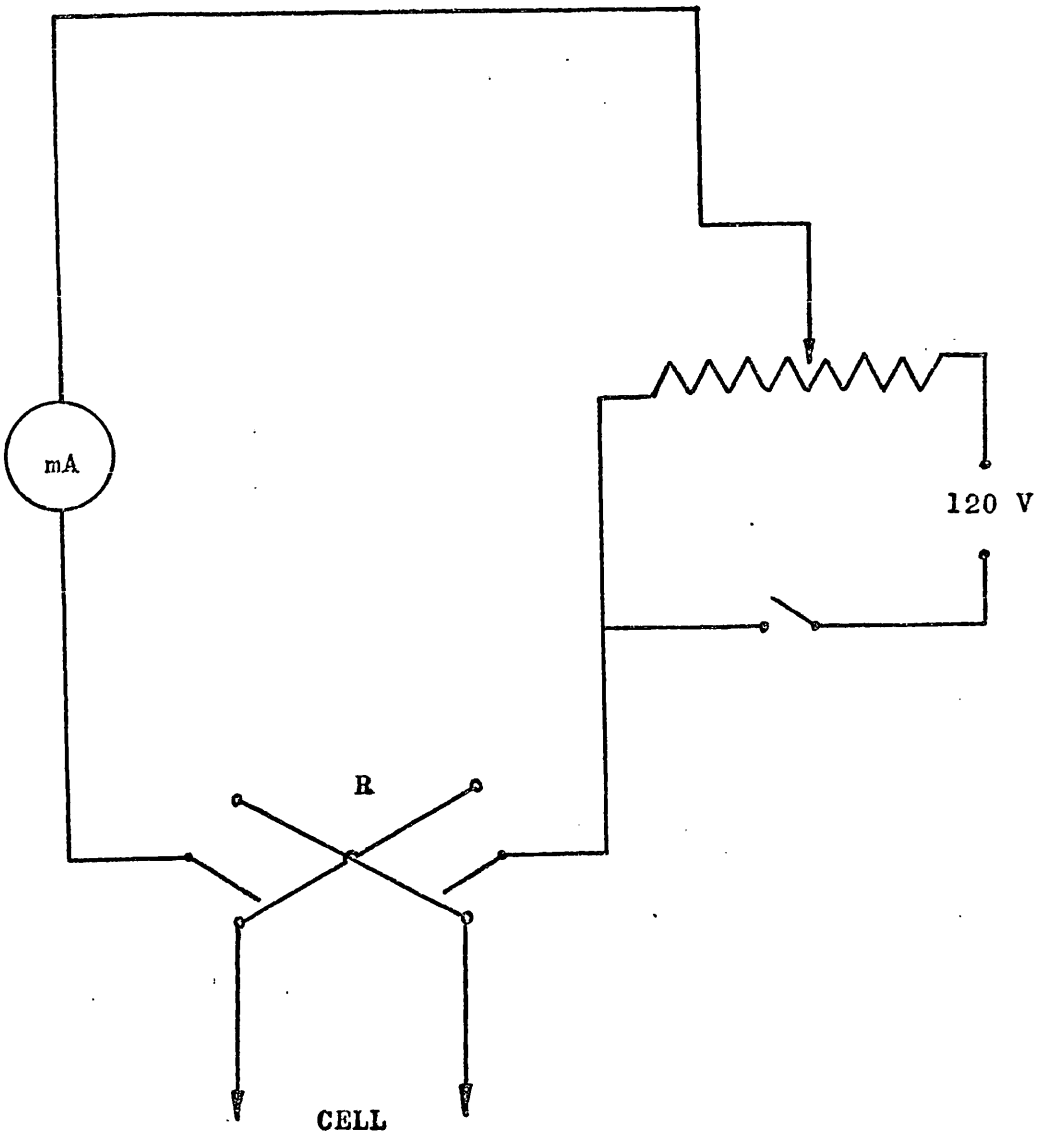
(diameter 1.5 mm, length 16 cm) mounted in the compartments by means of rubber bungs, through which the wire passed forming a water-tight seal. The silver wire was first cleaned by abrasion with emery cloth and then by immersion in dilute nitric acid. It was then anodised in 0.1 mol dm^{-3} HCl using a platinum cathode at a current density of 25 A m^{-2} for 60 minutes, to give a grey-purple deposit of AgCl on the electrode. The electrodes were then placed in the compartments which were 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). The electrode compartments were refilled at regular intervals with the electrolyte solution from reservoirs using taps 2 and 3,, 2′ and 3′.

The electrical circuit used (Figure II.2) was based on that of Abramson et al (1942). The voltage was supplied by two 60 v batteries connected in series and controlled by a variable resistance of maximum resistance 10 000 ohm. The current flowing was measured by a Sangamo-Western multi-range milliammeter connected in series with the cell. The applied potential could be reversed by using the switch R, which in its "off" position shorted the electrodes to prevent electrode polarisation.

A Beck (London model) microscope was used to observe the cells in the chamber. This was fitted with an annulus phase contrast condenser and objective (x 40) and angled stem (x 1.5) and a focusing eyepiece (x 10), with a cross-hatched graticule. Illumination came from a 30 watt projector lamp; the light was reflected from a substage concave mirror focused onto the phase plate.

FIGURE II.2

The electrical circuit used in the microelectrophoresis apparatus.



Since the viscosity and conductivity of the buffer solution, and hence the cell electrophoretic mobility were temperature dependent, it was essential to maintain the bacterial suspension at a constant temperature. This was made possible by immersing the observation chamber in a small waterbath adapted to fit on the stage of the microscope. The water in the bath was maintained at any required temperature in the range 5°C to 30°C to within 0.05°C by a Julabo Paratherm II heating and stirring unit and a Grant cooling unit working in opposition. The waterbath system was constructed of Perspex sheeting, the small tank was cut away beneath the observation chamber to allow the condenser lens of the microscope system to pass through. A flexible, water-tight fitting, joining the condenser to the Perspex sheeting, was provided by a rubber diaphragm. Both the condenser and objective lenses were waterproofed with lacquer.

An additive stop watch reading to 0.01 s was used to record the time taken by a bacterial cell to cross a given number of squares on the graticule.

(b) Mode of operation

To ensure good and reproducible electrical connections through the sintered glass plates, the following procedure was carried out before use of the apparatus.

50 cm^3 of KCl solution were flushed through each electrode compartment by opening taps 3 and 2, and 3′ and 2′; then taps 2 and 2′ were closed and tap 1′ opened,

forcing solution through the sintered glass plates. The observation chamber was then flushed through with a large volume of distilled water to remove the electrolyte which had been forced through the sintered plates. The chamber was finally filled with buffer solution at the temperature, pH and ionic strength of the suspension to be examined. In all operations great care was taken to ensure that no air bubbles were trapped in the closed system.

Cells of Klebsiella aerogenes were dried onto the inner surface of the observation chamber before assembly. By focusing on these reference particles the depth of the chamber was found in arbitrary units on the microscope micrometer scale. In routine use, the stationary levels were located at fractional depths of 0.21 and 0.79 from the top inside surface. All mobility measurements were made at the upper stationary level.

The velocity of a cell in focus at the stationary level, selected at random, was measured by recording the time taken for the cell to cross a given number of graticule squares under the influence of an applied electric field. A time of between 1.5 and 4 s was considered suitable, and the number of squares across which it was timed and the applied electric field were adjusted accordingly. For each suspension at least 40 cells were timed, and by reversing the current with switch R the cells were timed in both directions. The potential was never applied for long periods of time in either direction to minimise electrode polarisation during operation.

The mobility values obtained for suspensions of cells of the same strain grown on different occasions were

reproducible to within ± 3%. Cultures with mobility values differing by 10% or more were considered significantly different.

(c) Calibration of apparatus

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:

$$\bar{v} = \frac{V}{X} = \frac{nL}{t}.\frac{qk}{I} = \frac{Lq}{tI}.J\,G \qquad II.1$$

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 cell, and I/A is the current flowing. $k/ohm^{-1}m^{-1}$ is the conductivity of the buffer solution, which was obtained from the measured conductance (G/ohm^{-1}) and the cell constant J/cm^{-1} of the conductance cell.

The values of G, I and t were obtained experimentally. It is not possible, however, to determine accurately the cross-sectional area (q) of a rectangular observation chamber. This difficulty was overcome by using a standard particle, which had a known absolute mobility ($\bar{u}_s$), when suspended in a buffer solution of a known pH and ionic strength. By timing this under the conditions previously described, a "cell-constant" for the chamber, K, was determined, where K was given by:

$$K = Lq\,J = \frac{\bar{u}_s\,t\,I}{nG} \qquad II.2$$

The value of K also includes the cell constant of the conductance cell. The subsequent timings (t′) on bacterial cells under examination were converted to mobility values using the relationship:

$$\bar{u} = \frac{K n G'}{t' I} \qquad \text{II.3}$$

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

The standard particles were bacterial cells harvested from 18 hr cultures of K.aerogenes grown in nutrient broth at 37°C (II.1(c)). The cells were sedimented out of broth by centrifugation, washed twice and resuspended in acetate-veronal buffer solution. The standard conditions for determining the mobility values of these particles are at an ionic strength of 0.02 mol dm^{-3}, at a pH of 7.0 and at 25°C; under these conditions the absolute mobility is $-1.67 \times 10^{-8} m^2 s^{-1} V^{-1}$. This value was obtained as a result of extensive calibration studies of suspensions of K.aerogenes against human erythrocytes as standard (Gittens 1962). K was determined before each set of electrophoretic measurements were made.

All mobility values are quoted without sign or units; a value of 1.14 means that the particle is negatively charged with an electrophoretic mobility (towards the positive electrode) of $1.14 \times 10^{-8} m^2 s^{-1} V^{-1}$.

Before a new observation chamber was commissioned, the symmetry of the cell was checked by the method of Hartman Bateman and Lauffer (1952). A velocity-depth curve (III.1) was plotted by timing cells of K.aerogenes at various depths throughout the cell at constant field strength.

The fractional cell depth from the centre of the cell, x, was plotted against the reciprocal of the time taken by the bacterium to traverse two squares of the eyepiece graticule (u). Typical results for the cell used gave a parabolic curve at 25°C described by the equation:-

$$u = -1.835\ x^2 - 0.0408\ x + 0.481 \qquad \text{II.4}$$

Integration of this equation over the complete depth gives the mean electrophoretic velocity:

$$\bar{u} = 2 \int_0^{0.5} (-1.835\ x^2 - 0.0408\ x + 0.481)\ dx$$

from which $\bar{u}$ was calculated to be 0.317. Substituting this value back in equation II.4 gave the two positions of the stationary level as +0.288 and -0.310 from the centre. The stationary levels were therefore at fractional depths of 0.212 and 0.81 from the top of the cell.

II.3. Buffer Solutions

All solutions were prepared from Analar grade chemicals and dissolved in glass distilled water.

(a) Veronal-acetate buffer solution

In all mobility determinations veronal-acetate buffer solutions with a pH range of 3.0 to 9.5 were used as a suspending electrolyte (Michaelis 1931). 5 dm^{-3} of stock solution (I = 0.5 mol dm^{-1}) contained:

0.15 mol dm^{-3} sodium barbiturate	154.635 g
0.15 mol dm^{-3} hydrated sodium acetate	102.0675 g
0.20 mol dm^{-3} sodium chloride	58.450 g

This stock solution was stored at 5°C. The buffer solutions were prepared from the stock solution by diluting with distilled water to give the required ionic strength, I, and adding HCl (1 mol dm^{-3}) to give the required pH. The pH of each solution was measured using an E.I.L. (Model 23A) pH meter. The conductance of each buffer solution was measured with a Wayne-Kerr (B221) Universal Bridge in a bottle type cell at temperature equilibrium in the thermostat water bath at the same temperature as that used for measuring the electrophoretic mobility.

(b) Sørensens phosphate buffer solution

This was used as a standard buffer solution of known pH for calibration of the pH meter.

Solution A - 0.667 mol dm^{-3} disodium hydrogen orthophosphate dodecahydrate 23.88 g dm^{-3}

Solution B - 0.667 mol dm^{-3} potassium dihydrogen orthophosphate 9.074 g dm^{-3}

A solution containing equal volumes of these solutions on mixing has a pH = 6.81.

II.4. Preparation of Cell Suspensions for Electrophoresis

Cells of P.aeruginosa grown on nutrient agar were washed off the solid surface with glass distilled water and divided into the required number of aliquots. The cells were centrifuged out of suspension, and then washed twice with, and finally resuspended in the required buffer solution of known pH and ionic strength.

The cells of K.aerogenes were harvested by centrifuging the nutrient broth suspensions, and washing the cells twice in the appropriate buffer solution before resuspension. The concentration of the cells in suspension for mobility measurements was about 2×10^8 cells per cm^3. Mobility measurements were made on the cell suspensions as soon as possible after preparation.

II.5. Measurement of Minimum Inhibitory Concentrations of Antibiotics and Antibacterial Agents

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

A series of metal capped tubes containing a range of concentrations of the antibacterial agent in 5 cm^3 nutrient broth solution were prepared such that each tube contained half the concentration of antibacterial agent of its predecessor, by means of a two times serial dilution from one tube to the next. For heat-stable antibiotics these tubes were then autoclaved. For heat-labile antibiotics

the first solution was made up by aseptically weighing the antibiotic into sterile nutrient broth; subsequent dilutions into sterile nutrient broth were performed aseptically.

Each tube was inoculated with approximately 10^5 bacteria from an 18-hr agar plate culture. The tubes were then incubated for 48 hr at 37°C and the lowest concentration of antibacterial agent in which there was no turbidity, and hence no growth, was recorded as the MIC.

II.6. Detection of Surface Lipid

Bacterial surface lipid was detected (I.9) by observing the change in the electrophoretic mobility of the cells in the presence of a low concentration of an anionic surface active agent, sodium dodecyl sulphate (SDS).

The cells were harvested and then washed (II.4) in barbiturate buffer solution ($I = 0.005$ mol dm^{-3}, pH 7.0) which contained SDS at a concentration of 1×10^{-4} mol dm^{-3}. The mobility obtained for these cells was compared with that for cells in buffer solution at the same ionic strength and pH in the absence of SDS. An increase of the negative value of mobility greater than 10% for the cells in the presence of SDS was statistically significant and indicative of the presence of surface lipid; the size of the increase gave an indication of the amount of surface lipid.

II.7. Treatment of Cells with Chemical Agents

(a) Treatment of Cells with Sodium Metaperiodate

The cell surfaces were modified by the mild oxidative action of sodium metaperiodate by a method modified after Garrett (1965) and Brewer (1966) who used it to destroy and remove teichoic acid from the surface of Gram-positive bacteria.

Cells of 18 hr cultures grown on nutrient agar plates were harvested and washed once in distilled water, and then once in physiological saline (0.85%, w/v, NaCl). The cells were suspended in 0.1 mol dm^{-3} aqueous ammonia to remove any ester-linked alanine and then washed in distilled water to remove any alanine and aqueous ammonia. The material was then suspensed in barbiturate buffer solution pH 6.0 (I = 0.02 mol dm^{-3}) containing sodium metaperiodate (0.05 mol dm^{-3}) for 30 minutes in a water bath at 37°C.

The oxidised cells were divided into the required number of aliquots, centrifuged from the sodium metaperiodate solution and washed twice in barbiturate buffer solutions (I = 0.005 mol dm^{-3}) at suitable pH values. Mobility determinations were then made on cells in these suspensions.

(b) Treatment of Cells with 1-fluoro-2,4-dinitrobenzene

To block free amino groups on the cell surface a

method modified after Ingram and Salton (1957) and Hill (1963) was used.

Cells of 18 hr cultures grown on nutrient agar plates were harvested in distilled water and centrifuged out of suspension. They were then resuspended in an aqueous solution of sodium bicarbonate (3.3 mg cm^{-3}) containing 1-fluoro-2,4-dinitrobenzene (FDNB) at one third saturation concentration. This suspension was shaken vigorously for five hours in the dark, and all excess FDNB was removed by four washings in distilled water. The cells were prepared for mobility measurements by washing twice in the appropriate barbiturate buffer solution.

(c) Treatment of cells with aldehydes

Cells from an 18 hr culture of P.aeruginosa were harvested and resuspended in an aqueous solution of the aldehyde at the required concentration. This suspension was kept at 37°C for one hour before the cells were centrifuged out of suspension, washed twice and finally resuspended in barbiturate buffer solutions of the required pH and ionic strength prior to mobility measurements.

In some experiments alkaline aqueous solutions of the aldehyde were used. These solutions were buffered by the addition of 5 g of sodium bicarbonate to 1 dm^3 of the aqueous aldehyde solution; this increased the pH from 4 to 7.6.

(d) Treatment of cells with ethylenediaminetetraacetic acid (EDTA)

Cells from an 18 hr culture of P.aeruginosa were harvested and resuspended in barbiturate buffer solution containing a sub-lethal concentration of the di-sodium salt of ethylenediaminetetraacetic acid, EDTA, (93 $\mu g\ cm^{-3}$ where the MIC of EDTA was 388 $\mu g\ cm^{-3}$, and 931 $\mu g\ cm^{-3}$ where the MIC of EDTA was 3201 $\mu g\ cm^{-3}$). This suspension was kept at 37°C for 30 minutes, and the cells were then centrifuged out of suspension, washed once in distilled water and then twice, before being finally resuspended in, barbiturate buffer solutions of the required pH and ionic strength prior to mobility measurements.

C H A P T E R I I I

DETERMINATION OF THE OPTIMUM EXPERIMENTAL CONDITIONS

Throughout this, and subsequent, chapters only typical curves are plotted. These are representative of replicate experiments performed under identical conditions.

III.1. Determination of the optimum experimental conditions for mobility measurements

(a) The effect of washing on the surface properties of the cells

18-Hour cells of *P.aeruginosa* strain 1 were harvested and the suspension divided into seven aliquots. Cells of the first aliquot were centrifuged out of suspension and suspended in buffer solution (pH = 7.8, I = 5×10^{-3} mol dm^{-3}) i.e. zero washing. Cells of the other six aliquots were washed in the same buffer solution from one to six times and the velocity of cells under a fixed applied field strength determined at $10^{o}C$ for each suspension. The number of washings had no significant effect on the reciprocal mean timings, $^{1}/_{t}$, proportional to the mobility (Table III.1) which shows that no cell surface components were removed by the washing procedure in a way that affected the mobility. In future experiments the cells were washed twice in the required buffer solution before measuring their mobility values.

TABLE III.1

The effect of repeated washing on the electrophoretic velocity of cells of P.aeruginosa

No.of washings	0	1	2	3	4	5	6
Velocity/S^{-1}	0.351	0.350	0.347	0.346	0.351	0.348	0.350

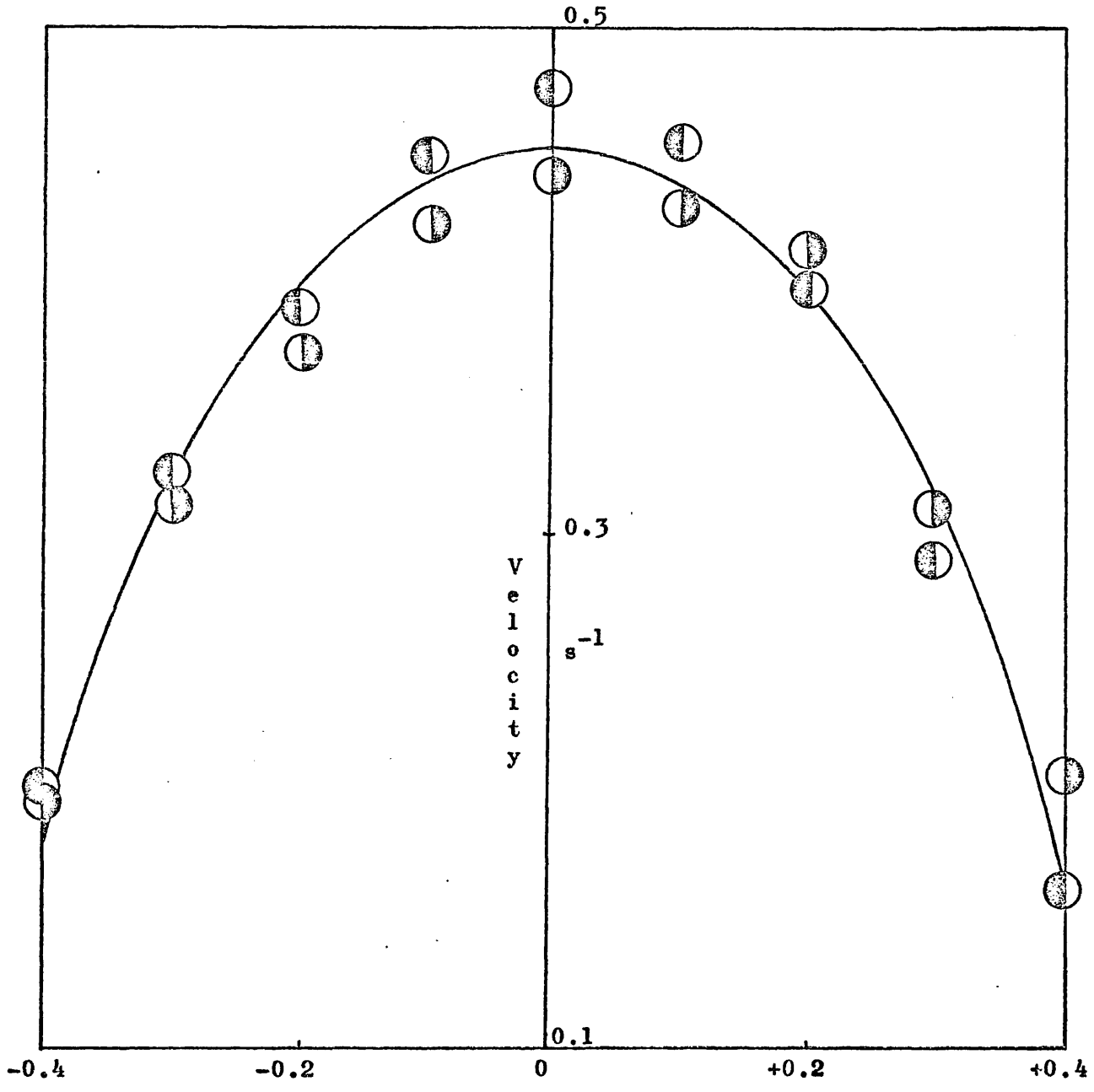
(b) The effect of the temperature of measurement on the mobility of the cells

The velocity-depth curves (Figure III.1) were obtained for 18-hour cells of Klebsiella aerogenes suspended in veronal-acetate buffer solution (pH = 7.4, I = 0.02 mol dm^{-3}). Measurements were made with a current of 1mA and at two different temperatures, 15 and 25°C. The conductivities of the buffer solutions were measured at the same temperature as that at which mobility measurements were made.

The close agreement between the curves shows that the symmetry of the cell remained unaltered with the variation in temperature. Thus variation of the cell symmetry was without effect on subsequent mobility measurements. The positions of the stationary levels calculated from these curves (II.2) were 0.212 and 0.810 of the cell depth at 25°C, and 0.204 and 0.781 at 15°C. This good correlation between the values and with the calculated value allowed the use of the mean values 0.21 and 0.79 of the cell depth from the top inside surface in all subsequent measurements.

FIGURE III.1

Velocity-depth curves for cells of Klebsiella aerogenes measured at 15 and 25°C in veronal-acetate buffer solution (pH = 7.4, I = 5 x 10^{-3} mol dm^{-3}).



Key

Measured at 15°C.

Measured at 25°C.

Figure III.2 shows the pH-mobility curves obtained for cells of K.aerogenes suspended in veronal-acetate buffer solution ($I = 0.02$ mol dm^{-3}) measured at 10, 15 and 25°C. The mobility values of the cells suspended at pH 7, which were used for calibration at the different temperatures, were obtained from the expression:

$$\bar{v}_T = \frac{\bar{v}_{25} \cdot n_{25}}{n_T} \qquad \text{III.1}$$

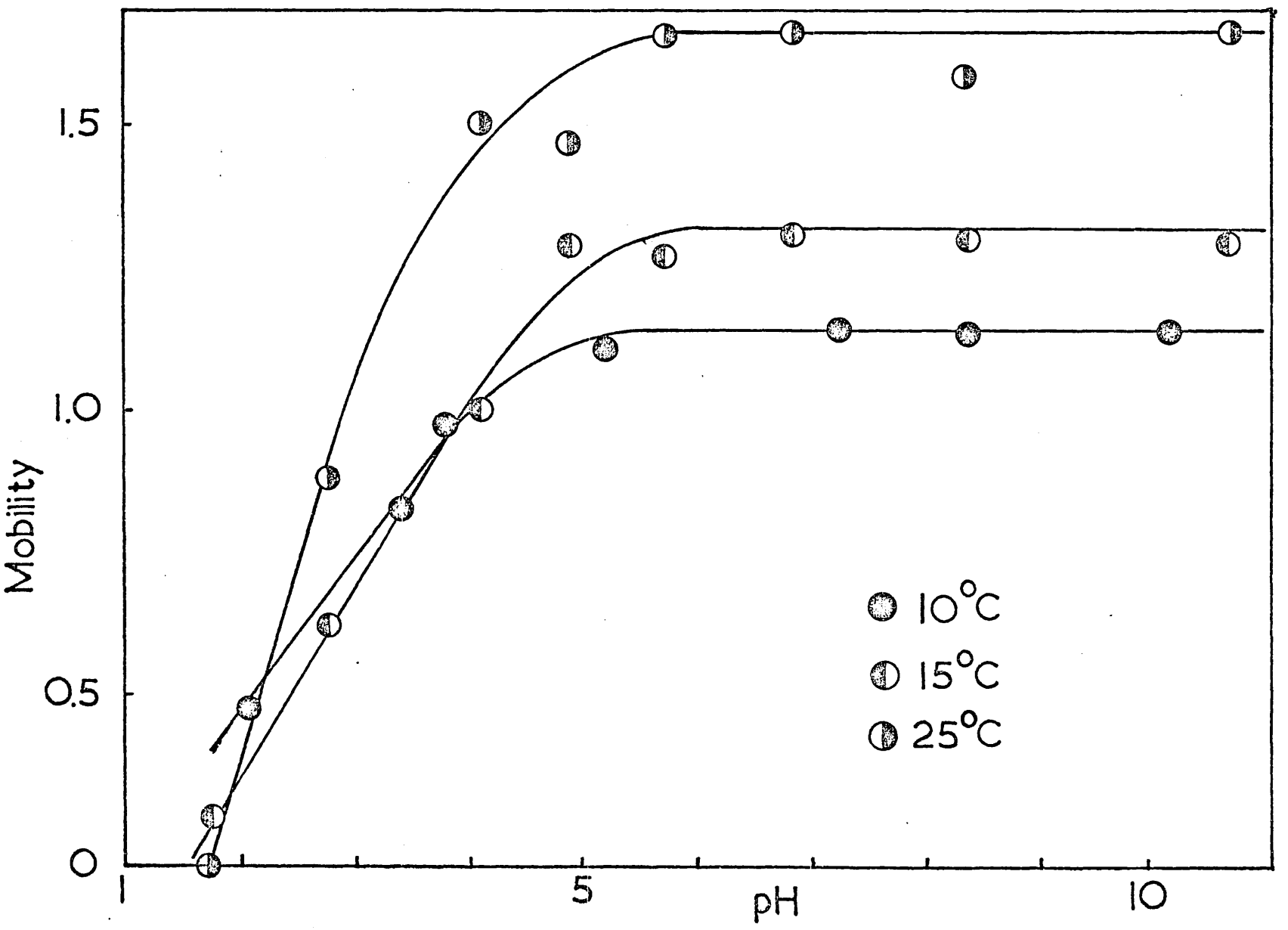
where $\bar{v}_T$ and $\bar{v}_{25}$ are the mobility values at temperatures T, and 25°C respectively (Gittens, 1962), and n_T and n_{25} are the viscosity coefficients of water at temperatures T and 25°C respectively.

The curves are typical of a carboxyl surface with pK between 2.5 and 3.0 and a plateau mobility value at pH values greater than 5. The only differences between the curves are the absolute mobility values of the plateaux which are a direct consequence of the change in the viscosity of the suspending medium with change in temperature. Thus the nature of the pH-mobility response was unaltered by a change in temperature between 10 and 25°C, only the absolute value of the mobility was affected by the change.

Cells of P.aeruginosa are flagellated, and in suspension they are motile. In order that this motility,

FIGURE III.2

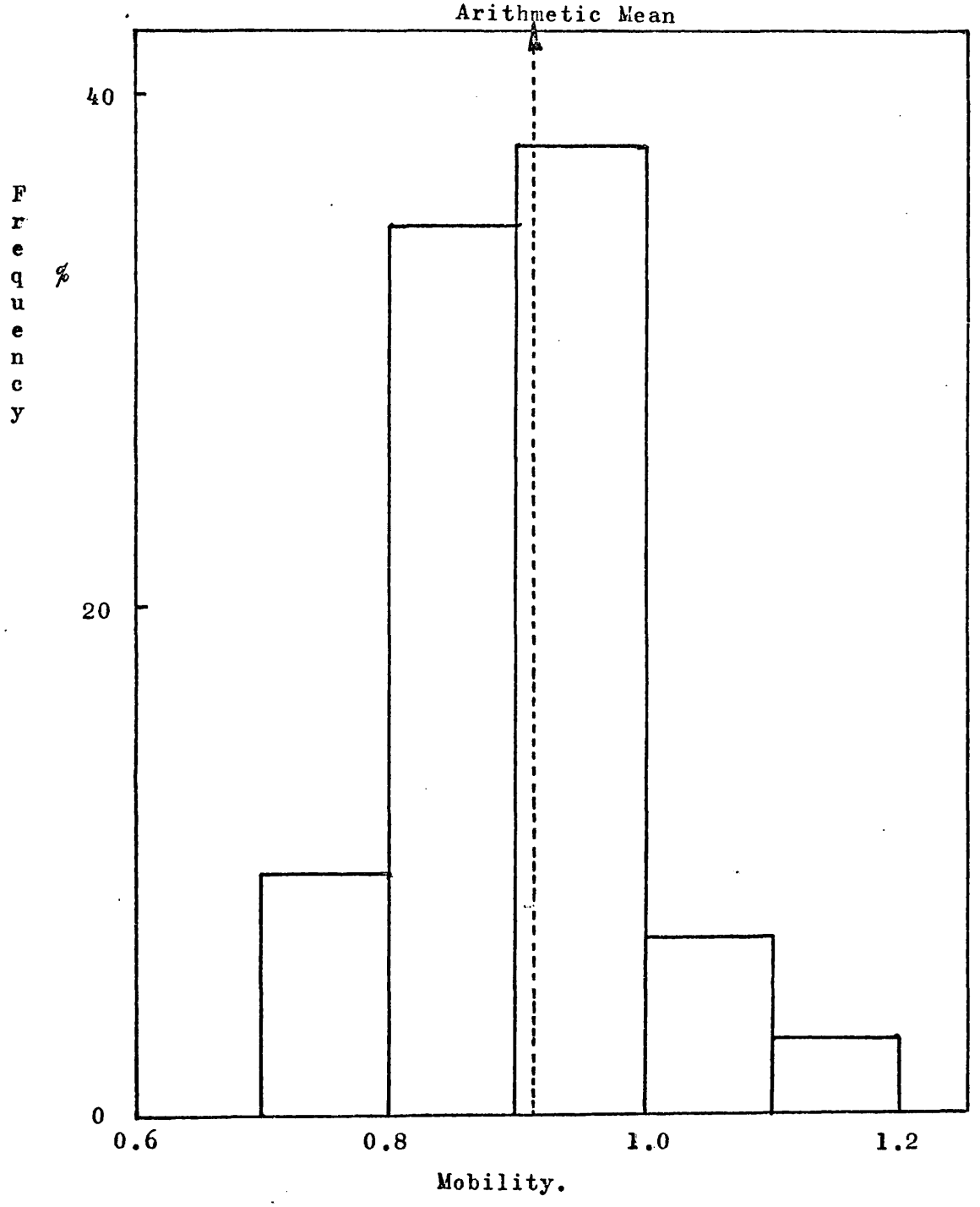
pH-mobility curves for 18-hr cells of *Klebsiella aerogenes* suspended in veronal-acetate buffer solution ($I = 2 \times 10^{-2}$ mol dm^{-3}) measured at 10, 15 and 25°C


1.5
1.0
0.5
0
Mobility
1
5
10
pH
10°C
15°C
25°C

i.e. intrinsic mobility, of the cells should not affect measurements of the electrophoretic mobility, conditions whereby this motility was suppressed were required. A sample of cells of strain 1 was suspended at pH 8.5 and at ionic strength 5×10^{-3} mol dm^{-3} and washed into the observation chamber of the electrophoresis apparatus. Mobility measurements were attempted at 25°C, but even on the application of a high field strength the movement of the cells was random. As the temperature was gradually reduced, so the movement of the cells in the absence of an electric field lessened, and the movement under an applied field became more homogeneous. At 15°C no cells were able to defy an applied current of 2mA or more by moving towards the cathode under their own motility. It appeared that no further improvement in the homogeneity of the cell suspension was obtained as the temperature was lowered from 13°C to 5°C and so a series of 100 individual measurements of electrophoretic mobility was made on a fresh suspension at 10°C. A histogram showing the range of mobility values for individual cells in this suspension was constructed (Figure III.3). The mobility values ranged from 0.7 to 1.2, with 73% of all values lying between 0.8 and 1.0. The arithmetic mean was 0.912 and the limit of accuracy at a 95% probability limit was 0.912 ± 0.018; thus a difference in mobility value of 10% was considered significant. This compared favourably with the accuracy of other workers using suspensions of cells of Staphylococcus aureus, (Brewer, 1966), and Streptococcus pyogenes (Hill, 1963) and showed the suspension of P.aeruginosa to be electrophoretically homogeneous.

FIGURE III.3

Histogram showing the range of mobility values of cells of Pseudomonas aeruginosa strain 1 measured at 10 °C, pH = 8.5. and I = 5×10^{-3}.



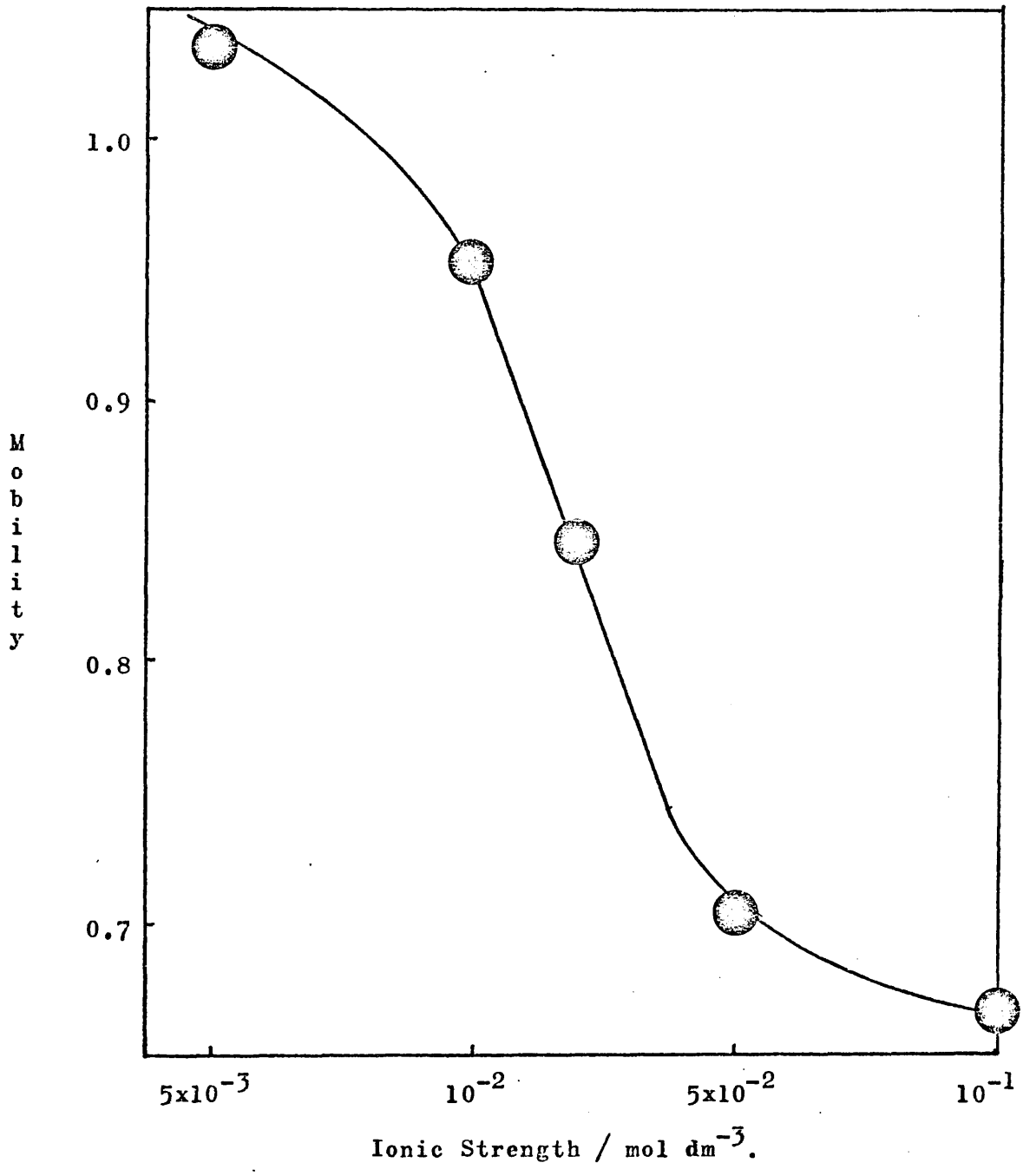
(c) The effect of the ionic strength of the buffer solution on the mobility of the cells

The ionic strength of the buffer solution in which the cells are suspended affects their electrophoretic mobility values by altering the dimensions of the electrical double layer at the cell surface and hence altering the zeta-potential. It is desirable to use a buffer solution of ionic strength which gives the highest mobility value for a given cell suspension, under fixed conditions of pH and temperature. In this way the greatest possible difference is obtained between cells of the same strain suspended at different pH values.

The variation of the mobility values of cells P.aeruginosa strain 1 suspended at different ionic strengths but the same pH (7.8), and measured at $10^{o}C$ is shown in Figure III.4. The curve shows a marked increase in the mean mobility value of cells as the ionic strength decreases from 1×10^{-1} to 5×10^{-3} mol dm^{-3}. At ionic strengths less than 5×10^{-3} mol dm^{-3} the buffer capacity of the veronal-acetate buffer solution, and hence the accuracy with which its ionic strength is known, is impaired. Thus it was decided to use an ionic strength of 5×10^{-3} mol dm^{-3} in almost all experiments in order to obtain the greatest differentiation between different suspensions and strains.

FIGURE III.4

The variation of the mobility values of cells Pseudomonas aeruginosa strain 1 with the ionic strength of the veronal-acetate buffer solution at constant pH (7.8), and temperature (10 °C).



(d) The effect of the growth medium on the surface properties of the cells

pH-mobility curves for 18-hour cells of P.aeruginosa strain 1 simultaneously harvested from agar plate cultures and nutrient broth cultures are shown in Figure III.5. The measurements, made at the same temperature ($10^{o}C$) and ionic strength (5×10^{-3} mol dm^{-3}), give superimposable pH-mobility curves and showed that nutrient broth cultures were comparable with agar plate cultures, and therefore the difference between these two growth media had little or no effect on the surface properties of the cells.

(e) The effect of the length of incubation on the surface properties of the cells

Cultures of strain 1 grown on nutrient agar were harvested 18 and 42 hours after incubation. The mobility values of suspensions were determined at $10^{o}C$, an ionic strength of 2×10^{-2} mol dm^{-3}, and over a range of pH values. The results (Figure III.6) showed that the length of incubation had no effect upon the surface properties of the cells between 18 and 42 hours.

(f) The deterioration of suspensions on storing

It was envisaged that during the course of some experiments it might prove necessary to delay the measurement of mobility for up to 24 hours after harvesting the bacteria. Therefore cells of P.aeruginosa strain 1

FIGURE III.5

pH-mobility curves for 18-hr cells of P.aeruginosa (strain 1) harvested from nutrient broth and nutrient agar cultures grown at $37^{\circ}C$. Measurements made at $10^{\circ}C$, $I = 5 \times 10^{-3}$ mol dm^{-3}

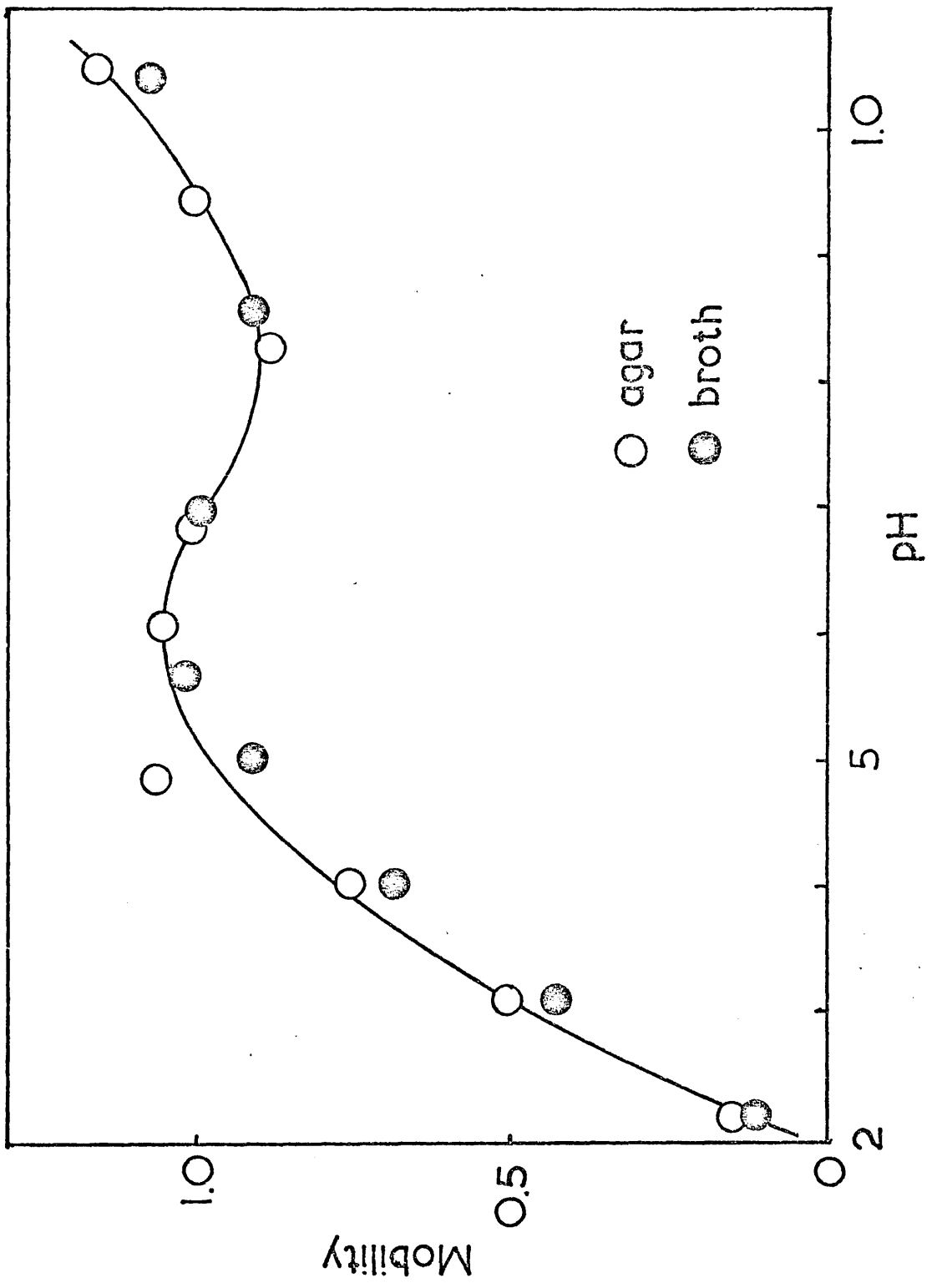
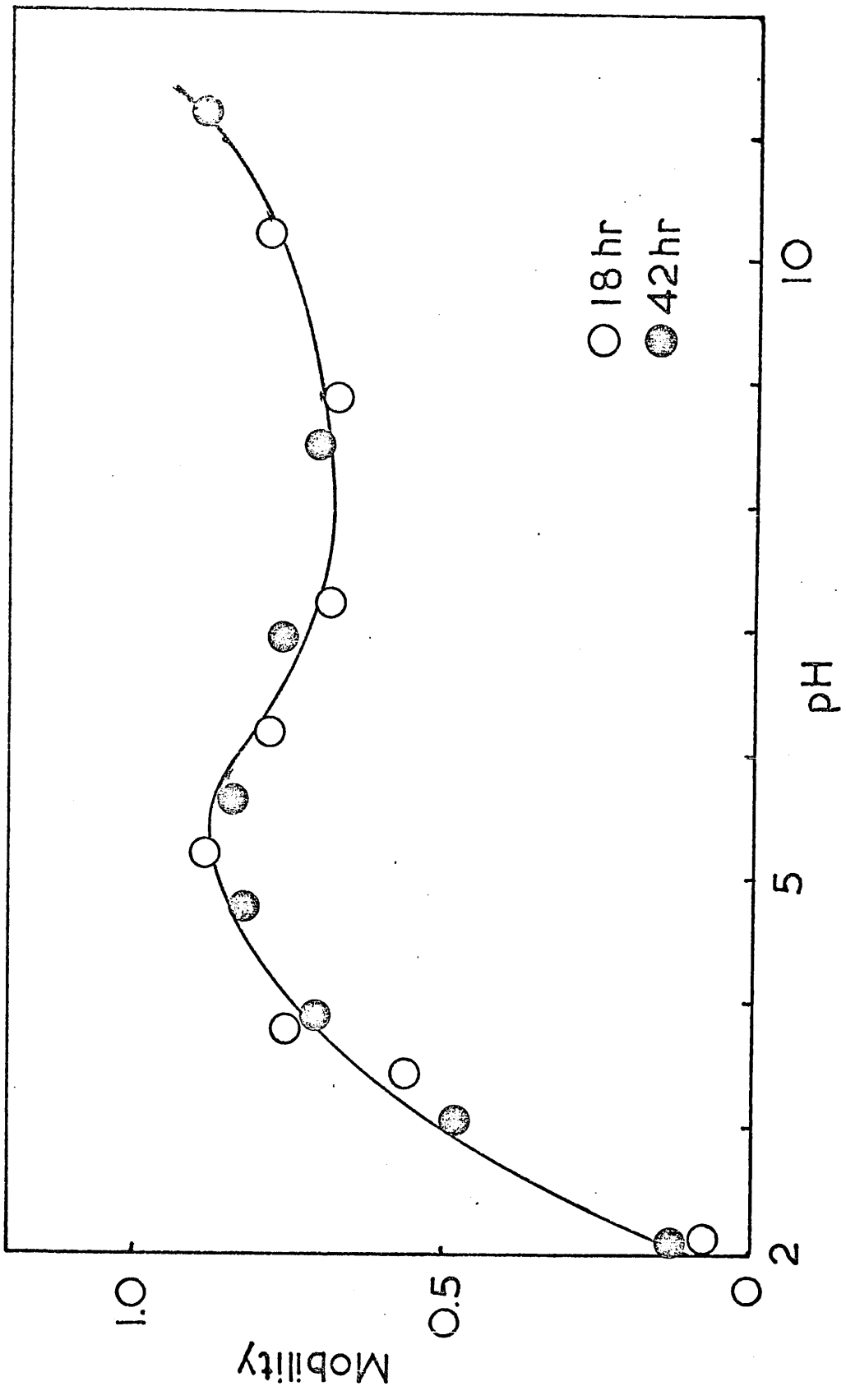

Mobility
0.1
0.5
1.0
2
5
10
pH
agar
broth

FIGURE III.6

pH-mobility curves for cells of P.aeruginosa (strain 1) incubated for 18 and 42 hr at 37°C on nutrient agar plates. Measurements made at 10°C, $I = 2 \times 10^{-2}$ mol dm^{-3}


Mobility
1.0
0.5
0
2
5
10
pH
18 hr
42 hr

were prepared in the usual way for mobility measurements at three different pH values and then each suspension split into two samples. For the cells of one sample of each suspension, the mobility was measured immediately, whilst the cells of the other sample were stored for 24 hours at 5°C before their mobility values were determined without further washing (Table III.2).

TABLE III.2

The effect of storage on the mobility of cells of P.aeruginosa

	Mobility	
pH	Time 0	24 hr
8.50	0.787	0.802
5.64	0.959	0.923
4.77	0.887	0.862

Thus storing suspensions of cells in buffer solutions over a range of pH values for up to 24 hours at 5°C has no significant effect upon the measured mobility of the cells.

(g) The effect on the mobility of cells of the presence of gentamicin in the buffer solution

Cells of P.aeruginosa strains 1 and 7, grown on nutrient agar in the absence of gentamicin were harvested,

washed and finally resuspended at a concentration of approximately 10^6 bacteria cm^{-3} in veronal-acetate buffer solution (pH = 7.2, I = 5 x 10^{-3} mol dm^{-3}) containing gentamicin in the range 0 - 50 μg cm^{-3}. The mobility values were determined for the cells of each strain in each suspension. Cells originally suspended in buffer solution containing the highest gentamicin concentration of 50 μg cm^{-3} were washed twice, and finally resuspended in the same buffer solution but in the absence of gentamicin and their mobility values re-determined. A limited experiment was performed with cells of strain 8 but using a buffer solution containing only the highest gentamicin concentration.

The presence of the gentamicin lowered the negative mobility values of cells in all strains in direct proportion to the antibiotic concentration (Figure III.7). Further, the decrease of mobility for a given gentamicin concentration was similar for strains 7 and 8 for which resistance to gentamicin was high but less for strain 1 which was sensitive to gentamicin. The effect of the gentamicin was reversible, since the mobility of cells after suspension in gentamicin solution, followed by washing and resuspension in standard buffer solution, was the same as that of the control cells, within the limits of experimental error. The effect of the gentamicin on the mobility values was not due to any alteration of the pH, ionic strength or conductivity of the buffer solution. Instead its effect was probably due to one or both of the following:

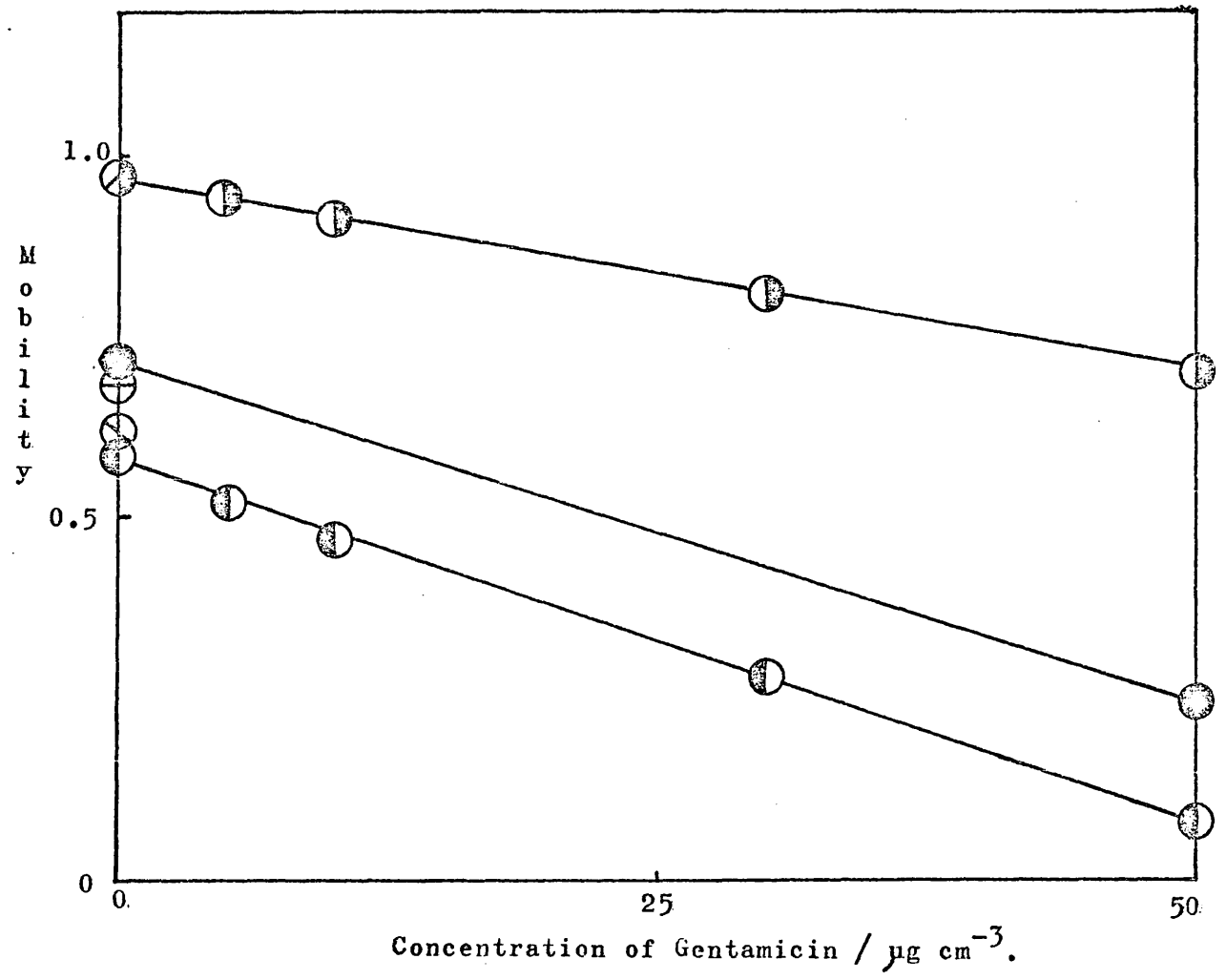
(i) the reversible adsorption of gentamicin onto the surface of the cells, leaving one or more of its amino

groups protruding into the medium, and hence contributing a positive charge to the cells because of its protonation; (ii) specific ion-pairing of the protonated amino groups of the gentamicin molecule with the negatively charged surface carboxyl groups which would prevent these carboxyl groups from contributing to the negative mobility of the cells. According to this latter suggestion, the cells of the more highly gentamicin-resistant strains 7 and 8 should have a greater density of surface carboxyl groups which would produce the greater decrease of negative mobility in the presence of gentamicin. This, however, is contrary to the fact that the observed mobility values of cells of the resistant strains, measured in the absence of gentamicin, are less than the mobility values of cells of the sensitive strain. The greater lowering of the mobility of cells of resistant strains by gentamicin is most probably due to a more preferable orientation of the surface carboxyl groups.

These results show that gentamicin in the buffer solution has a significant, but reversible effect on the surface charge but any gentamicin which may be present in the growth medium and which is carried over will be removed by washing during preparation. Measurements in the presence of gentamicin will be significantly affected and were subsequently avoided.

FIGURE III.7

The effect of the presence of gentamicin in the buffer solution on the mobility of cells of Pseudomonas aeruginosa strains 1, 7, and 8. Measurements made at 10 °C, constant pH (7.2), and I = 5 x 10^{-3} mol dm^{-3}.



Key.

- Cells of strain 1.
- Cells of strain 1, resuspended from 50 back to 0 μg cm^{-3}.
- Cells of strain 7.
- Cells of strain 7, resuspended from 50 back to 0 μg cm^{-3}.
- Cells of strain 8.
- Cells of strain 8, resuspended from 50 back to 0 μg cm^{-3}.

III.2. The effect of temperature, and time, of incubation on gentamicin MIC measurement

Three identical sets of media containing twofold dilutions of gentamicin in nutrient broth were prepared (II.5) for each of strains 1 and 7 of P.aeruginosa. For strain 1, ten tubes covered the concentration range 0 - 10 $\mu g\ cm^{-3}$, and for strain 7 ten tubes covered the concentration range 0 - 200 $\mu g\ cm^{-3}$. Each solution was inoculated and one series of solutions for each strain was incubated at 25°C, a second at 37°C and the third at 43°C. The tubes were examined 16, 24, 48 and 72 hours after inoculation (Table III.3).

The cells incubated at 25°C took longer to grow than those incubated at 37°C for both strains, due to the slower growth rate at the lower temperature. At both 25 and 37°C the cells were fully grown after 24 hours' incubation. The cells in the tubes incubated at 43°C were not fully grown for strain 1 until 48 hours had elapsed. For cells of both strains the measured minimum inhibitory concentration (MIC) of gentamicin was lower than that measured at the lower temperatures. This is probably due to the higher temperature, the maximum for growth of P.aeruginosa, potentiating the effect of the antibiotic and causing an apparent increase in antibacterial effect. 37°C corresponds most nearly to the in vivo environment and in vitro this temperature shows the quickest development of growth, therefore in all subsequent measurements of the MIC of antibiotics and antibacterial agents the growing cultures were incubated at this temperature; the MIC was read after 48 hours to ensure full growth.

TABLE III.3

The effect of temperature and length of incubation on the measured MIC of gentamicin for cells of (a) strain 1, and (b) strain 7 of P.aeruginosa

(a) P.aeruginosa, strain 1

Temp. of incubation /°C.	MIC/μg cm^{-3}			
	16 hr	24 hr.	48 hr	72 hr
25	0.625	1.25	1.25	1.25
37	1.25	1.25	1.25	1.25
43	0	≤0.039	0.078	0.078

(b) P.aeruginosa, strain 7

Temp. of incubation /°C	MIC/μg cm^{-3}			
	16 hr	24 hr	48 hr	72 hr
25	12.5	25	25	25
37	25	25	25	25
43	0	≤ 0.78	≤0.78	≤0.78

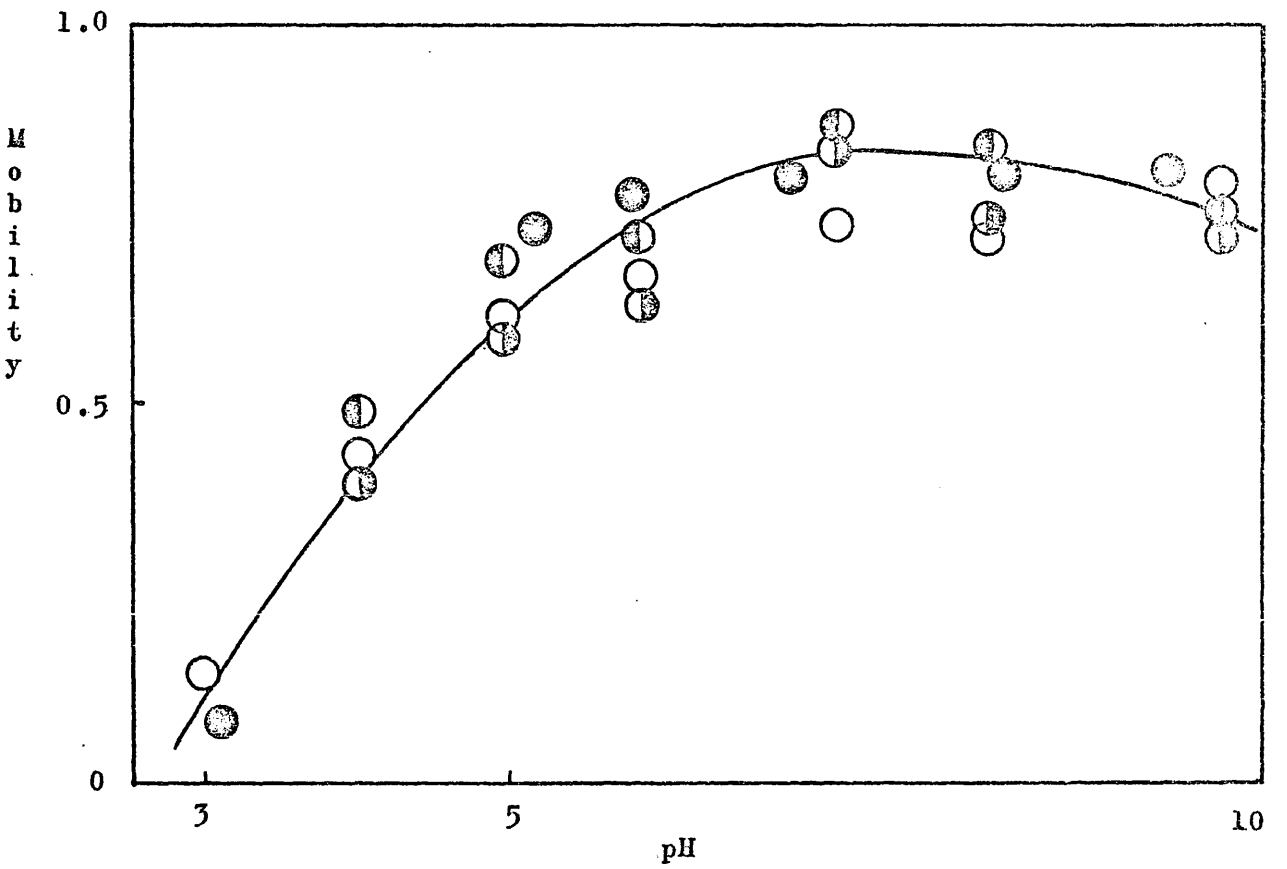
III.3. The effect of growth of cells on gentamicin-agar

Yourassowsky et al (1971) showed that gentamicin resistance in P.aeruginosa can be unstable and can be unaccountably lost. To overcome this, the possibility of stabilising resistant strains by growing in the presence of gentamicin was investigated.

Cells of a recent hospital isolate of P.aeruginosa (strain 7), resistant to gentamicin (MIC = 25 $\mu g\ cm^{-3}$) were subcultured daily at 37°C for two weeks in three parallel series; one on nutrient agar (antibiotic-free), one on agar containing a gentamicin concentration of 1 $\mu g\ cm^{-3}$, and the third on agar containing a gentamicin concentration of 10 $\mu g\ cm^{-3}$ (i.e. 0, 4 and 40% respectively of the MIC of gentamicin for the strain). The pH-mobility curves for cells of this strain before the experiment, and after 12 subcultures, are shown in Figure III.8. They show that subculturing in the presence of a concentration of gentamicin up to 40% of its MIC had no effect on the shape or position of the pH-mobility curve. The MIC of gentamicin before the experiment and after 12 subcultures in the absence of, and in the presence of 1 $\mu g\ cm^{-3}$ of the antibiotic, was 25 $\mu g\ cm^{-3}$. The MIC after 12 subcultures in the presence of 10 $\mu g\ cm^{-3}$ of the antibiotic was 100 $\mu g\ cm^{-3}$. Thus the presence of a low gentamicin concentration affected neither the surface properties nor the level of resistance, but a higher antibiotic concentration (40% of the MIC) produced a considerable increase in tolerance of the bacteria to the antibiotic without affecting the surface properties. During the limited duration of this experiment no loss of resistance

FIGURE III.8

The effect of repeated subculture of cells of Pseudomonas aeruginosa strain 7 onto nutrient agar plates containing 0, 1 and 10 $\mu g\ cm^{-3}$ of gentamicin. Measurements made at 10 ^{o}C and $I = 5 \times 10^{-3}$ mol dm^{-3} in veronal-acetate buffer solution.



Key.

- Before experiment.
- After 12 subcultures in the absence of gentamicin.
- After 12 subcultures at a gentamicin concentration of 1 $\mu g\ cm^{-3}$.
- After 12 subcultures at a gentamicin concentration of 10 $\mu g\ cm^{-3}$.

to gentamicin was observed in cells grown repeatedly in the absence of the antibiotic; further, no loss of resistance was observed in any of the strains used in this investigation as a result of the bi-monthly subculturing of cells onto fresh nutrient agar, for routine maintenance of the strains, over a period of three years. Thus there seems little danger that experimental results will be affected by a loss of gentamicin-resistance.

III.4. Summary

(a) Mobility measurements on cells of P.aeruginosa are best made at 10°C in veronal-acetate buffer solution of ionic strength 5×10^{-3} mol dm^{-3} after twice washing the cells in the required buffer solution. Under these conditions the cells are non-motile and the suspensions are electrophoretically homogeneous. The mobility value of cells of K.aerogenes suspended in 5×10^{-3} mol dm^{-3} buffer solution at 10°C used to calibrate the apparatus daily was 1.45, as calculated from equation III.1 and the data of Gittens (1962).

(b) Cells of P.aeruginosa grown on nutrient agar or in nutrient broth have identical surface properties.

(c) Cells of P.aeruginosa incubated for periods between 18 and 42 hours exhibited coincident pH-mobility curves.

(d) Prepared suspensions of cells of P.aeruginosa may be stored for up to 24 hours at 5°C without deterioration, as revealed by the consistency of the surface properties.

(e) Gentamicin contamination from the growth medium does not affect the mobility of washed cells, but gentamicin in the suspending medium during measurement lowered the negative mobility of the cells.

(f) Measurements of the MIC of antibiotics and antibacterial agents for strains of P.aeruginosa are best made after 48 hours incubation at 37°C.

(g) Low concentrations of gentamicin in the growth medium do not affect the pH-mobility response or the MIC of gentamicin for resistant strains of P.aeruginosa.

These experimental conditions were adhered to throughout this study, unless stated otherwise, and in all subsequent experiments the cells of P.aeruginosa were grown on nutrient agar.

C H A P T E R I V

pH--MOBILITY CURVES FOR CELLS OF PSEUDOMONAS AERUGINOSA GROWN AT 37°C ON NUTRIENT AGAR

Serum levels of gentamicin in excess of 12 to 15 $\mu g\ cm^{-3}$ are seriously ototoxic (Jao and Jackson, 1963; Wërsall et al, 1969) and hence not clinically acceptable. Thus a useful practical definition of a gentamicin-resistant strain of P.aeruginosa is one for which the MIC of gentamicin is greater than 12 $\mu g\ cm^{-3}$. Conversely those strains for which the MIC of gentamicin is less than, or equal to, 12 $\mu g\ cm^{-3}$ are described as gentamicin-sensitive. This definition will be used throughout. In addition, it is apparent from Table II.1 that the strains of P.aeruginosa resistant to gentamicin may be subdivided into those for which the MIC lies between 12 and 100 $\mu g\ cm^{-3}$, i.e. with medium-level resistance, and those for which the MIC is in excess of $10^3\ \mu g\ cm^{-3}$, i.e. showing high-level resistance. Similarly, these definitions will be used throughout.

IV.1. Cells of gentamicin-sensitive strains

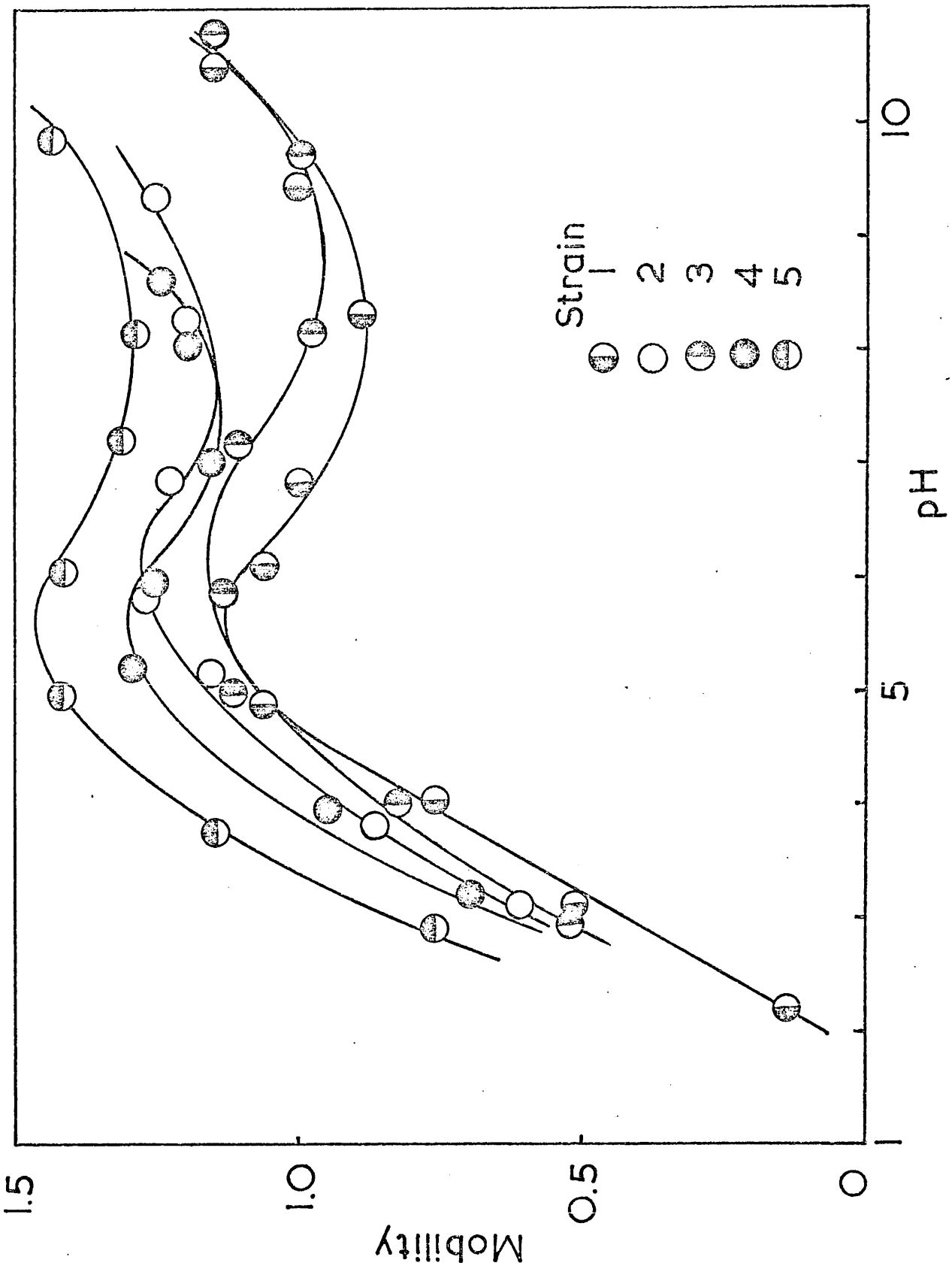
The pH-mobility curves for 18-hour cells of strains 1, 2, 3, 4 and 5 all of which are sensitive to gentamicin are shown in Figure IV.1. Each curve is characterised by a steep increase in mobility between pH 2 and 5 and a maximum negative mobility value between pH 5.5 and 6.5. Each curve also shows a minimum mobility value between pH 7.5 and 8.5; the difference between the maximum and minimum being 9 - 18% of the maximum mobility value. For none of the strains was a positive mobility value

obtained at low pH values. pH values less than 2 and greater than 11 were not used as under these conditions denaturation or irreversible changes occurred at the surface such that cells first suspended in a buffer solution at pH 1.5 or 11.5 and then resuspended in a buffer solution at pH 7.0 did not have the same mobility value as that of cells which were immediately suspended in buffer solution at pH 7.0 after harvesting and washing.

The sinusoidal shape of the pH-mobility curves (Figure IV.1) cannot be explained as simply a pH-titration of the surface ionogenic groups. In such a titration, positive or negative surface groups, either singly or in combination, would result in a plateau mobility value, or a series of plateau mobility values at increasing negative values as the pH increased. James and Brewer (1968) suggested that the maximum mobility value in the pH-mobility curve for cells of Staphylococcus aureus was caused by a re-orientation of charged groups of the cell surface teichoic acid, exposing previously hidden phosphate groups which increased the negative mobility value of the cells at pH 3.5. Teichoic acid has never been reported to be a component of the P.aeruginosa cell envelope but this does not exclude the rearrangement of other cell surface components causing the observed decrease in negative mobility between pH values 5 and 8 by exposing previously hidden amino groups, or by concealing carboxyl groups which at pH 5.5 were contributing to the mobility of the cells.

FIGURE IV.1

pH-mobility curves for 18 hr cells of P.aeruginosa strains 1, 2, 3, 4 and 5, all of which are sensitive to gentamicin. Cells grown at 37°C on nutrient agar plates.


Mobility
1.5
1.0
0.5
0
1
5
10
pH
Strain
1
2
3
4
5

IV.2. Cells of gentamicin-resistant strains

The pH-mobility curves for cells of strains 6, 7 and 10 of P.aeruginosa, which show medium-level resistance to gentamicin are shown in Figure IV.2. The curves, which are quite unlike those for cells of sensitive strains, are characterised by an 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 ever observed for cells of resistant strains at pH > 10. The maximum mobility vslues are all lower than those for gentamicin-sensitive strains (0.7 - 1.0 for medium-level resistant strains as opposed to 1.1 - 1.5 for sensitive strains) and occur at higher pH values by between 1 and 3 pH units. The rate of increase in mobility values over the pH range 3 - 7.5 is less than that for sensitive strains over the pH range 2 - 5.

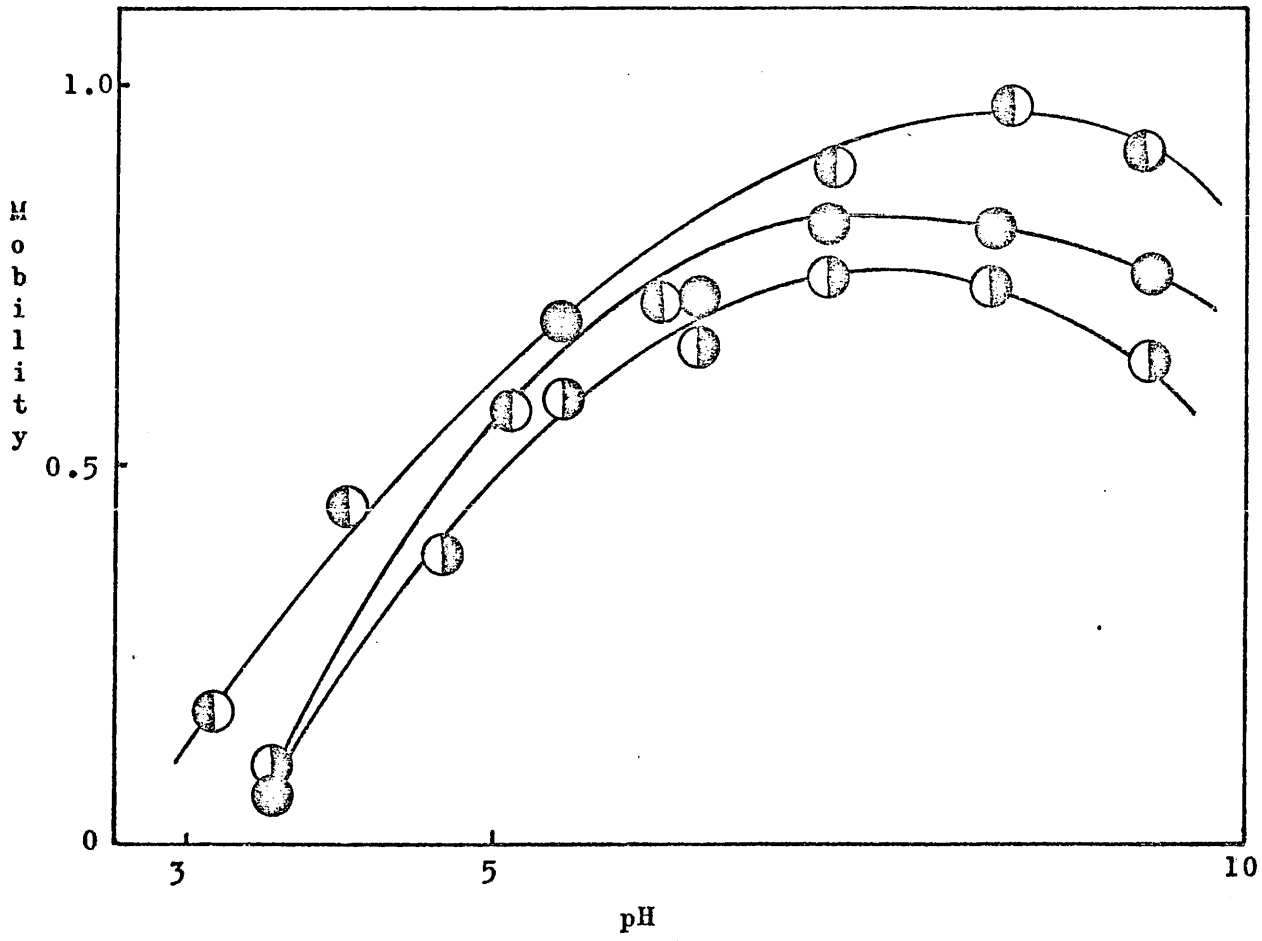
The pH-mobility curves for strains 8 and 9 of P.aeruginosa, which show high-level resistance to gentamicin (Figure IV.3) are of the same general appearance as those of medium-level resistant strains. A maximum mobility value occurs between pH values 7.5 and 8.5, and lies within the range 0.7 and 1.0 for strain 8 (i.e. the same as for the medium-level resistant strains) but within the range 1.1 - 1.5 for strain 9.

IV.3. Cells trained to grow in the presence of gentamicin

Cells of the gentamicin-sensitive strain 1 were grown on nutrient agar plates containing successively

FIGURE IV.2

pH-mobility curves for cells of Pseudomonas aeruginosa strains 6,7 and 10 which possess medium-level resistance to gentamicin, ($12\ \mu g\ cm^{-3} < MIC < 100\ \mu g\ cm^{-3}$).



Key.

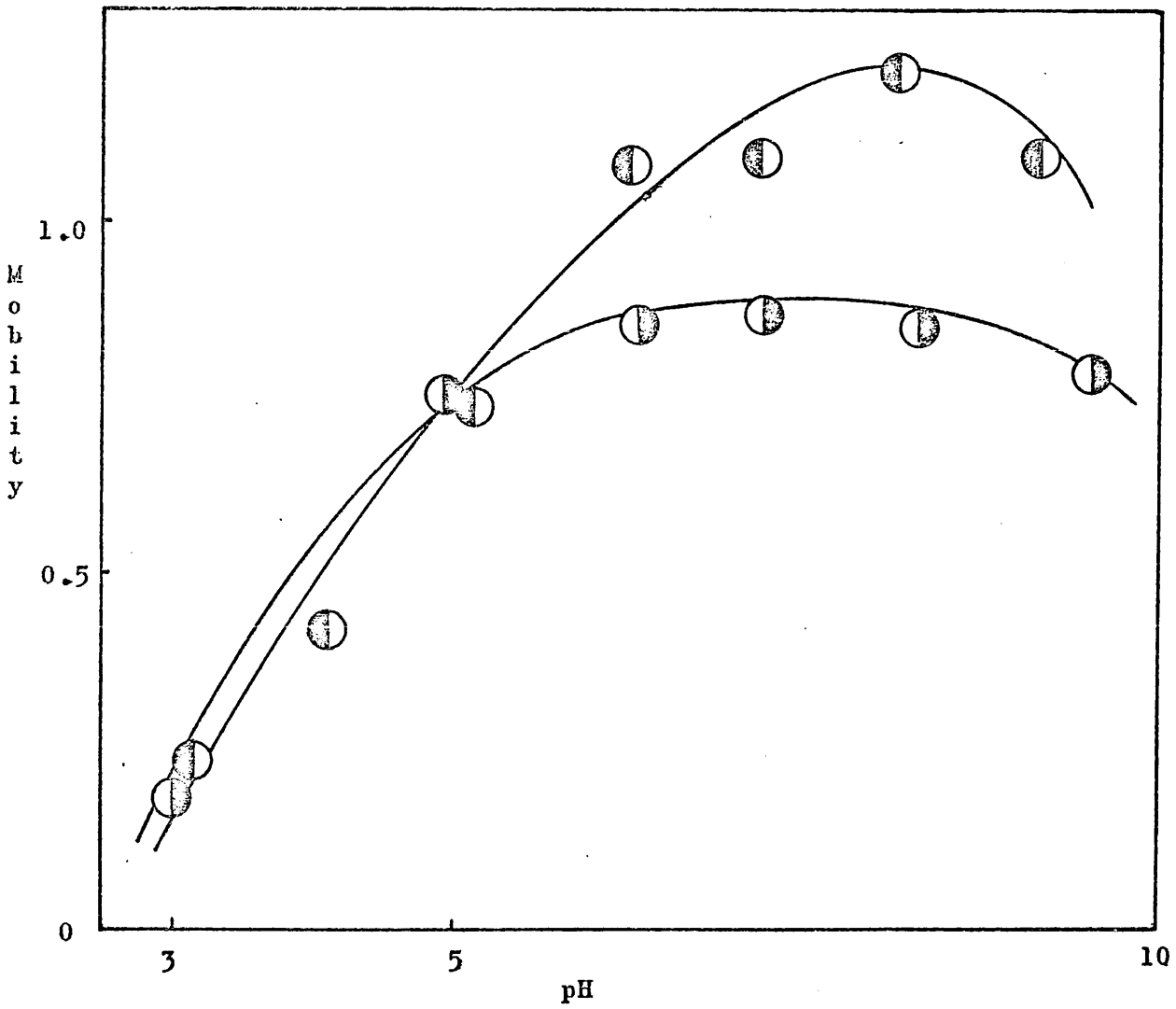
Cells of strain 6.

Cells of strain 7.

Cells of strain 10.

FIGURE IV. 3

pH-mobility curves for cells of Pseudomonas aeruginosa strains 8 and 9 which possess high-level resistance to gentamicin (MIC $> 10^3$ µg cm^{-3}).



Key.

Cells of strain 8.

Cells of strain 9.

higher concentrations of gentamicin (II.1d) until three parallel strains were obtained; these were subcultured daily in the presence of gentamicin concentrations of 20, 50 and 100 μg cm^{-3} in nutrient agar, (strains 1A/20, 1A/50 and 1A/100 respectively). In addition, after 63 subcultures of strain 1A/20 in the presence of 20 μg cm^{-3} of gentamicin in nutrient agar, the strain was split, one half maintained as before and the other subcultured daily on nutrient agar in the absence of gentamicin (strain 1B). All mobility determinations on cells of strains regularly subcultured in the presence of gentamicin were performed on cells that had been grown once in the absence of the antibiotic before harvesting and washing; this ensured that the organisms were not suffering from any damage caused to the cell surface as a result of growth in the presence of the antibiotic.

The pH-mobility curve for strain 1A/20 showed a marked variation with the number of subcultures in the presence of gentamicin (20 μg cm^{-3})(Figure IV.4). Whilst retaining the sinusoidal shape of its sensitive parent, strain 1, the value of its maximum mobility increased markedly at first from 1.1 for strain 1 to 2.0 after 83 subcultures. After 55 further subcultures at the same gentamicin concentration the peak mobility value decreased to 1.3, and after 177 subcultures it was found to be closest to that of strain 1, at 0.98. The pH-mobility curve for strain 1A/50 (Figure IV.5) showed a similar increase in the maximum mobility value soon after being subcultured in the presence of 50 μg cm^{-3} of gentamicin. After only 9 subcultures the pH-mobility curve showed a maximum

FIGURE IV.4

pH-mobility curves for cells of P.aeruginosa (strain 1A/20) after various numbers of subcultures on nutrient agar containing gentamicin (20 $\mu g\ cm^{-3}$). Cells grown once in the absence of gentamicin on nutrient agar plates at $37^{\circ}C$ immediately before harvesting.

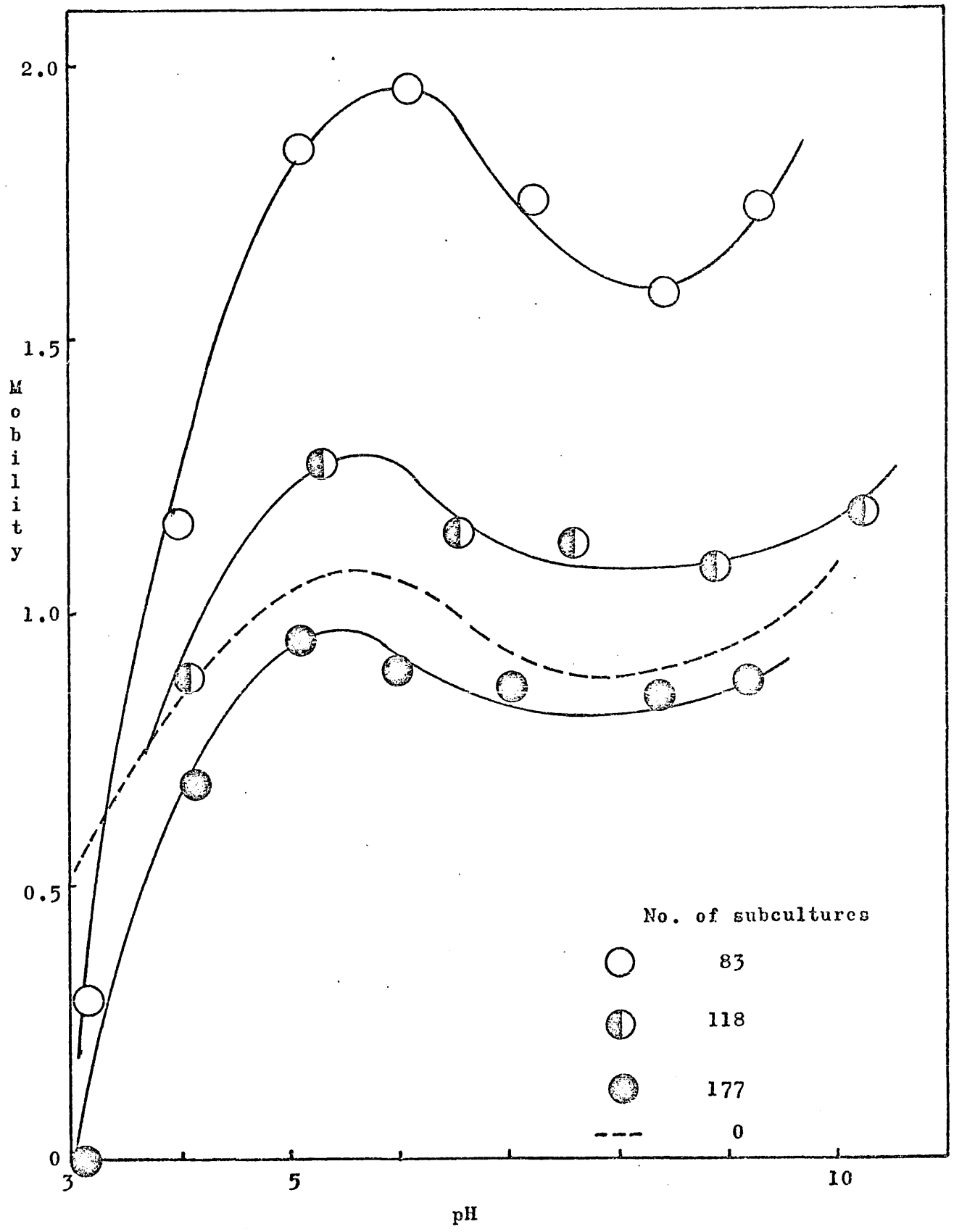
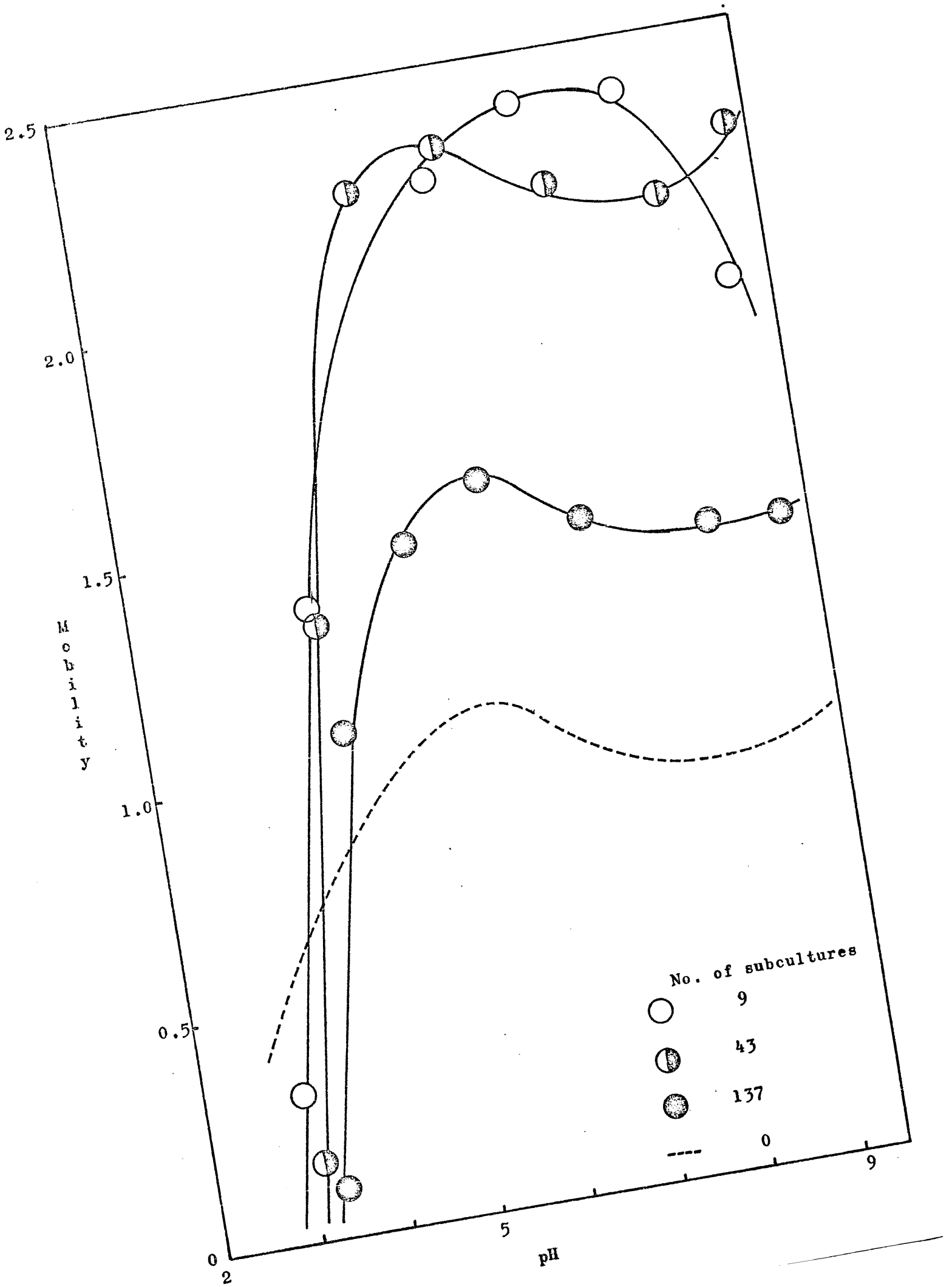

2.0
1.5
1.0
0.5
0
Mobility
3
5
10
pH
No. of subcultures
83
118
177
0

FIGURE IV.5

pH-mobility curves for cells of P.aeruginosa (strain 1A/50) after various numbers of subcultures on nutrient agar containing gentamicin (50 $\mu g\ cm^{-3}$). Cells grown once in the absence of gentamicin on nutrient agar plates at $37^{o}C$, immediately before harvesting.


2.5
2.0
1.5
1.0
0.5
0
Mobility
2
5
9
pH
No. of subcultures
9
43
137
0

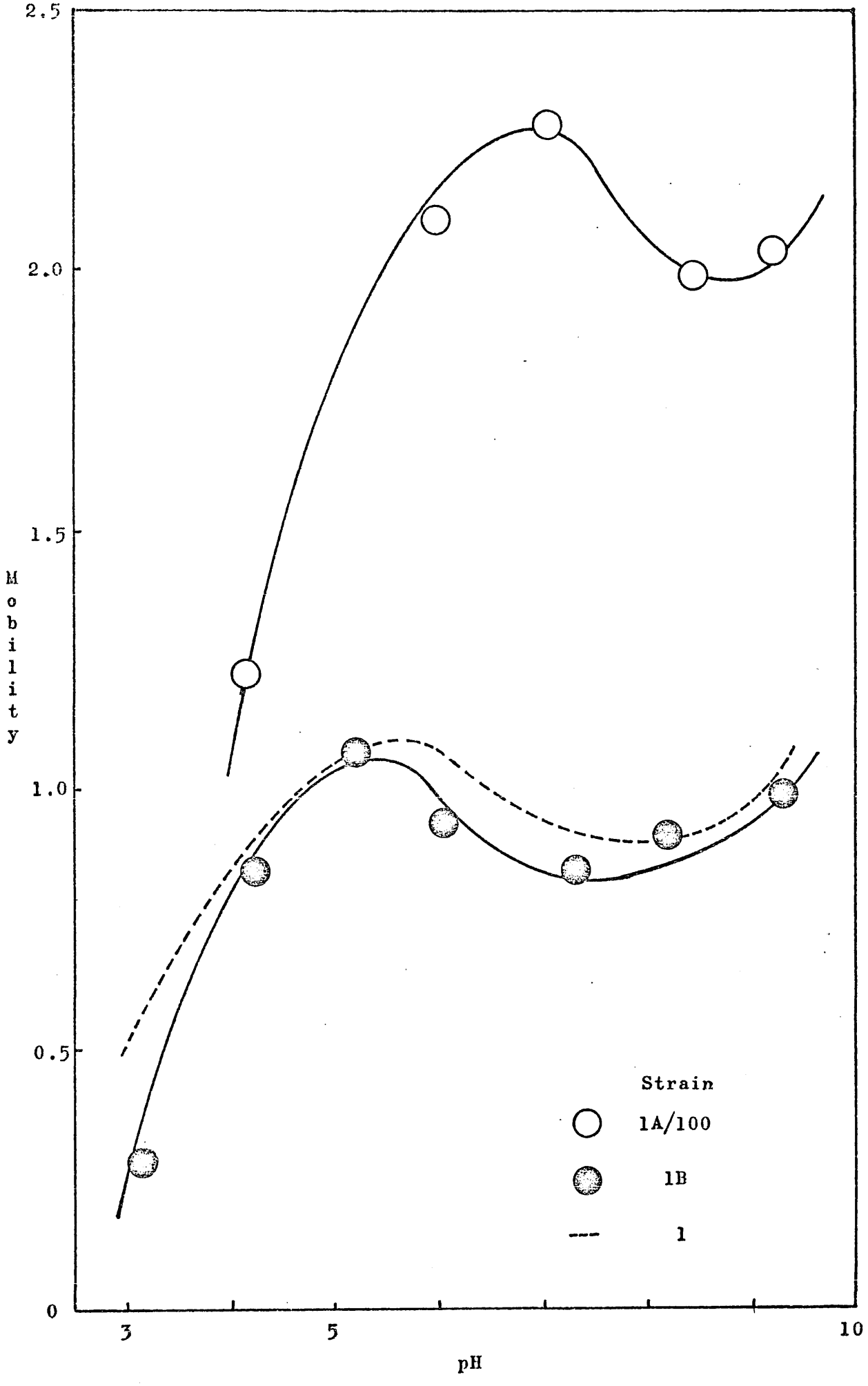
mobility value at pH 7.5 as did the naturally-occurring resistant strains. However after 34 further subcultures the sinusoidal shape, characteristic of the sensitive strains, had returned, although the maximum was still displaced to a high mobility value (2.3). After 137 subcultures the pH-mobility curve for strain 1A/50 retained its sinusoidal shape but the maximum mobility value had decreased to 1.6. Comparison with the behaviour of the pH-mobility curve for strain 1A/20 suggests that further subculturing in the presence of the same gentamicin concentration will bring the pH-mobility curve for strain 1A/50 to approximately that of the sensitive parent strain 1.

Figure IV.6 shows the similar behaviour of strain 1A/100 in that after 60 subcultures in the presence of gentamicin (100 $\mu g\ cm^{-3}$), its pH-mobility curve retains the sinusoidal shape of strain 1, characteristic of a sensitive strain, but its maximum mobility value has increased to 2.3. Further subculturing may bring about the decrease of this mobility value. The pH-mobility curve for strain 1B (Figure IV.6) is almost identical with its parent strain 1 and suggests that the reverse process of allowing the cells to grow in the absence of gentamicin which results in the loss of gentamicin-resistance (Table II.1) is not associated with large disturbances of the pH-mobility curve. (MIC of gentamicin for strain 1B = 3.1 $\mu g\ cm^{-3}$.)

A possible explanation for the observed behaviour of the pH-mobility curves of these strains during training to gentamicin is that when the metabolism of the cells is disturbed by growth in a sub-lethal, or increased antibiotic

FIGURE IV.6

pH-mobility curves for cells of P.aeruginosa strain 1A/100 after 60 subcultures on nutrient agar containing gentamicin (100 $\mu g\ cm^{-3}$) and cells of strain 1B after 25 subcultures on nutrient agar in the abssnce of gentamicin following 63 subcultures in the presence of gentamicin (20 $\mu g\ cm^{-3}$). All cells grown once in the absence of gentamicin on nutrient agar plates at $37^{o}C$ immediately before harvesting.


2.5
2.0
1.5
1.0
0.5
0
Mobility
3
5
10
pH
Strain
1A/100
1B
1

concentration, the number and relative proportion of positively and negatively charged surface groups is affected by the altered metabolism. As the cells become more able to utilise the alternative metabolic routes so the cell surface structures return, progressively, back to normal. This would probably be the case in an adaptation mechanism of attaining resistance. If the mechanism was a process of selection of resistant mutants, the explanation of the behaviour of the pH-mobility curves would be that these mutants have different surface properties from the less resistant cells, but that after a prolonged period of growth in the presence of the antibiotic, these surface properties revert to those of the less resistant cells.

IV.4. Summary

(a) Mobility measurements may be made on cells of P.aeruginosa in the pH range 2 - 11 without causing denaturation or irreversible changes to the cell surface components.

(b) Cells of gentamicin-sensitive strains of P.aeruginosa showed characteristic pH-mobility curves which exhibited maximum negative mobility values of 1.1 to 1.5 at pH 5.5 to 6.5, and minimum mobility values at pH 7.5 to 8.5; the difference between the maximum and the minimum mobility values was 9 - 18% of the maximum value.

(c) Cells of medium- and high-level resistant strains of P.aeruginosa showed characteristic pH-mobility curves which exhibited maximum mobility values at pH 7.5 to 8.5, and

for medium-level resistant strains the maximum mobility value was 0.7 to 1.0.

(d) pH-mobility curves of sensitive cells trained to acquire gentamicin resistance showed a prolonged period of perturbation before achieving a curve similar in shape and position to that of the sensitive parent cells; during this period the pH-mobility curves were displaced to higher mobility values and initially the sinusoidal shape of the curve was lost.

C H A P T E R V

SURFACE LIPID

Marshall (1969) defined the S-value for cells of Staphylococcus aureus as the increase in the mobility of the cells suspended in 2 x 10^{-2} mol dm^{-3} veronal-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 suspended in buffer solution alone. This S-value gave a quantitative measure of the amount of surface lipid possessed by the cells as measured by the method of Dyar (1948)(see I.9). The same definition of the S-value will be used in this investigation but referring to the mobility values determined in veronal-acetate buffer solution of ionic strength 5 x 10^{-3} mol dm^{-3}. Thus S is given by the expression:

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

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-hour cultures were harvested and washed in water. A portion of the cells was resuspended in buffer solution and a portion in buffer solution containing SDS. The mobility values of the cells in both suspensions were determined. As a control experiment, cells suspended in buffer solution containing SDS were washed twice and finally resuspended in buffer solution alone and their mobility values re-determined. In all cases the re-determined mobility values were the same, within the limits of experimental error, as those of cells immediately

suspended in buffer solution alone. It is thus apparent that the SDS caused no denaturation or irreversible changes in cell surface structures and thus the experimental S-values have significance.

The results of this study are shown in Table V .1, and the results for gentamicin-sensitive and medium-level gentamicin-resistant strains are displayed in Figure V.1 as a function of the MIC of gentamicin for the strains, including the trained strain 1A/20. The degree of correlation between the S-value and the MIC of gentamicin for the strains shown in Figure V.1 is expressed by the correlation coefficient for the calculated best straight line shown of 0.96. All the strains sensitive to gentamicin (MIC $\leqslant$ 12 $\mu g\ cm^{-3}$) have S-values less than 10 and all the medium-level gentamicin-resistant strains (12 $\mu g\ cm^{-3} <$ MIC $<$ 100 $\mu g\ cm^{-3}$) have S-values greater than 10, that is, greater than the 10% limit above which the presence of surface lipid is confirmed (II.6) This suggests a connection between the degree of gentamicin resistance and the amount of surface lipid. Figure V.1 also shows that during the process of <u>in vitro</u> training to acquire gentamicin resistance strain 1, in becoming the more resistant strain 1A/20, has acquired more surface lipid and follows the general pattern displayed by the sensitive and medium-level resistant strains.

The high-level gentamicin-resistant strains would not fit on the straight line shown in Figure V.1 possessing MICs of gentamicin 300 times greater than any of the medium-level resistant strains. However, the higher level of resistance shown by strains 8 and 9 is associated with

TABLE V.1

The relationship between the S--value of cells of P.aeruginosa and the MIC of gentamicin

Strain	Mobility		S-value	MIC/μg cm^{-3}
	Control	In SDS		
1	1.18	1.23	4.5	1.25
2	1.16	1.20	2.9	5
3	1.19	1.20	0.1	$\leq$0.16
4	1.18	1.21	1.9	1.5
5	1.36	1.48	8.7	12
6	0.73	0.89	22.8	25
7	0.72	0.83	16.1	25
8	0.81	1.35	67.0	8000
9	1.04	1.70	63.9	8000
10	0.94	1.10	16.4	25
1A/20 (86)*	1.67	2.12	26.6	25
1A/20 (183)*	1.17	1.38	17.5	25
1A/50 (12)*	2.29	2.49	8.6	55
1A/50 (47)*	2.09	2.58	23.4	55
1A/50(142)*	1.33	1.54	15.7	55
1A/100(61)*	2.16	2.57	18.7	120
1B (125)*	0.79	0.98	23.4	3.1

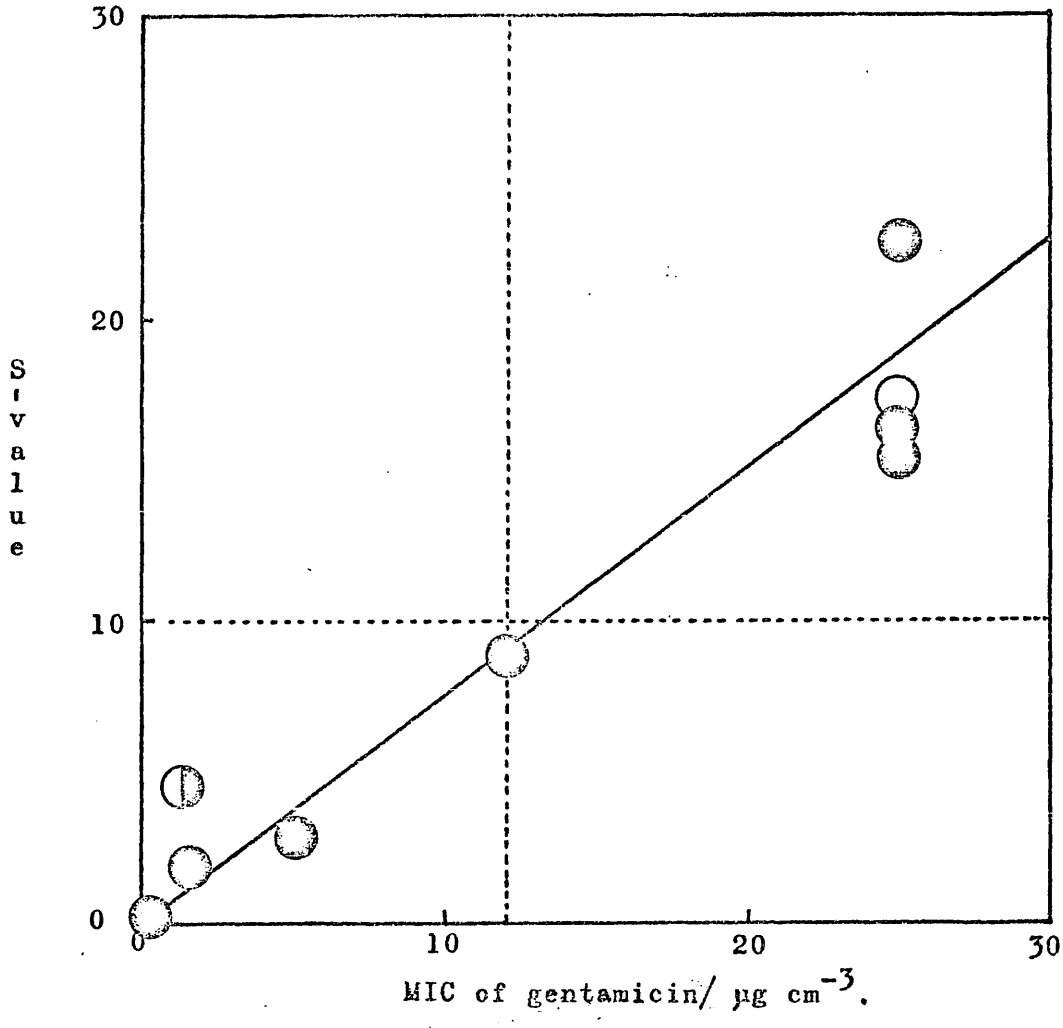
* Figures in brackets refer to the number of subcultures received by the cells in the presence of the given concentration of gentamicin.

a higher S-value than any of the medium-level resistant strains. This suggests that the adsorption of SDS, onto the cell surface, follows a Langmuir-type adsorption isotherm culminating in a plateau S-value of about 65, the value obtained for strains 8 and 9. The lack of strains of P.aeruginosa with MICs of gentamicin between 25 and 8000 $\mu g\ cm^{-3}$ prevents testing this hypothesis.

The S-values for the other trained strains 1A/50 and 1A/100 are comparable with that of strain 1A/20 and suggest that further increase in resistance beyond that of strain 1A/20 is not reflected in increased surface lipid. However the absolute differences in mobility for the trained cells measured in the presence and absence of SDS does show an increase with increasing MIC of gentamicin; the increase in mobility for cells of strain 1A/100 was twice that for cells of strain 1A/20, after 183 subcultures in the presence of gentamicin (20 $\mu g\ cm^{-3}$), the comparability of their S-values being a result of the higher mobility in the absence of SDS for cells of strain 1A/100. The S-value for strain 1B (i.e. after repeated growth in the absence of gentamicin) remained at the higher level of its immediate parent strain 1A/20 rather than falling to that of its ultimate sensitive parent strain 1. This suggests that although strain 1B has lost its acquired resistance to gentamicin (Table II.1) during growth in the absence of the antibiotic, the altered metabolic pathways whereby strains 1A/20, 1A/50 and 1A/100 acquired resistance to gentamicin, and which resulted in their increased surface lipid content are still operating to a certain extent. The absolute difference in mobility values measured in

FIGURE V.1

The variation of the S-value for cells of P.aeruginosa with the MIC of gentamicin.



Key.

Cells of gentamicin-sensitive and medium-level gentamicin-resistant strains of P.aeruginosa.

Cells of P.aeruginosa strain 1.

Cells of P.aeruginosa strain 1A/20.

the presence and absence of SDS is in fact less than that for strain 1A/20. The S-value for cells of strain 1A/20 was higher after 86 subcultures in the presence of gentamicin than it was after 183 subcultures. This suggests that the S-value passed through a maximum after increasing from the value for cells of the parent strain 1. This behaviour is probably related to the perturbation observed with the pH-mobility curve for cells of this strain during which the maximum mobility value passed through a maximum and then decreased as the number of subcultures in the presence of gentamicin increased (IV.3). A maximum was also observed in the S-value for cells of strain 1A/50 and this is probabl also related to the perturbation observed with the pH-mobility curve. However, although the pH-mobility curve for cells of strain 1A/20 had returned to that of cells of its parent strain 1 after 177 subcultures, the S-value was still higher than that for cells of the parent strain. Whether the S-value and hence the amount of surface lipid for cells of the trained strain remains greater than that for cells of the parent strain will be revealed by further subculturing.

C H A P T E R V I

THE INVESTIGATION OF CELL-SURFACE AMINO GROUPS

VI.1. The effect of FDNB on the mobility of cells

1-Fluoro-2,4-dinitrobenzene (FDNB) irreversibly reacts, under alkaline conditions, with amino groups according to the reaction:

$$C_6H_3F(NO_2)_2 + RNH_2 \longrightarrow RNH[C_6H_3(NO_2)_2] + HF$$

Hill (1963) showed that this reaction with bacterial cell-surface amino groups caused an increase in the negative mobility value of the cells by preventing the protonation of the substituted amino groups which prevented these groups from contributing positive charges to the cells.

Treatment of cells of P.aeruginosa strains 1, 7 and 8 with FDNB (II.7b) caused an increase in the negative mobility values of the cells at pH values higher than 5, compared with control cells which had undergone the same treatment, but in the absence of FDNB (Figures VI.1 and VI.2); this suggests that the FDNB has blocked the surface amino groups in the manner shown previously. The shapes of the pH-mobility curves for the treated cells of the gentamicin-sensitive strain 1 and medium-level resistant strain 7 are similar in shape and position, showing a plateau mobility value at pH $>$ 7.5. The different shapes of the curves for untreated cells of the two strains are therefore possibly due to the cell-surface amino groups. The increase of the maximum mobility value for cells of strain 1 is less than that for cells of strain 7 suggesting that more surface amino groups have reacted with the FDNB on cells of the latter strain, which in turn leads to the

FIGURE VI.1

pH--mobility curves for cells of P.aeruginosa strains 1 and 7, treated with FDNB.

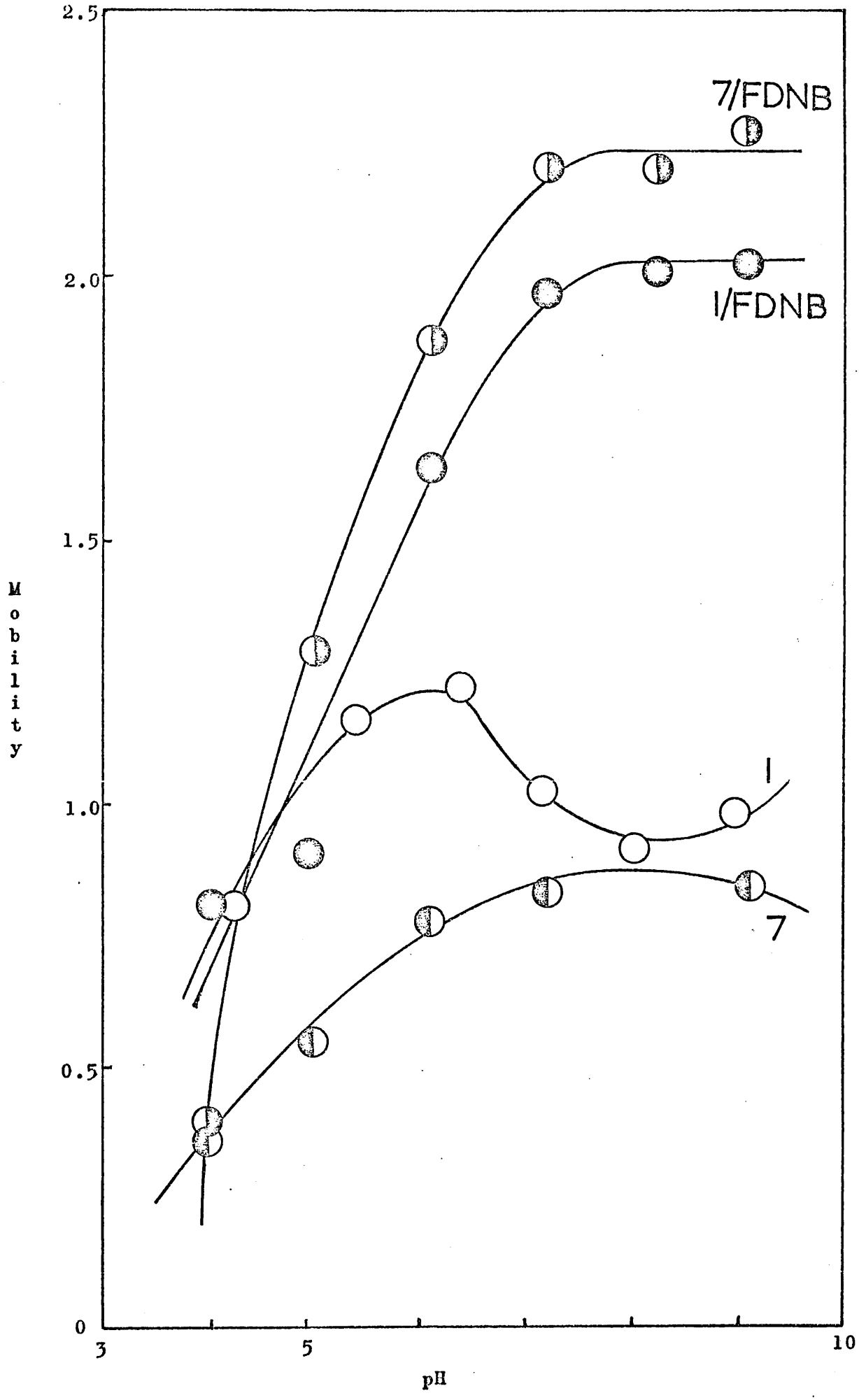
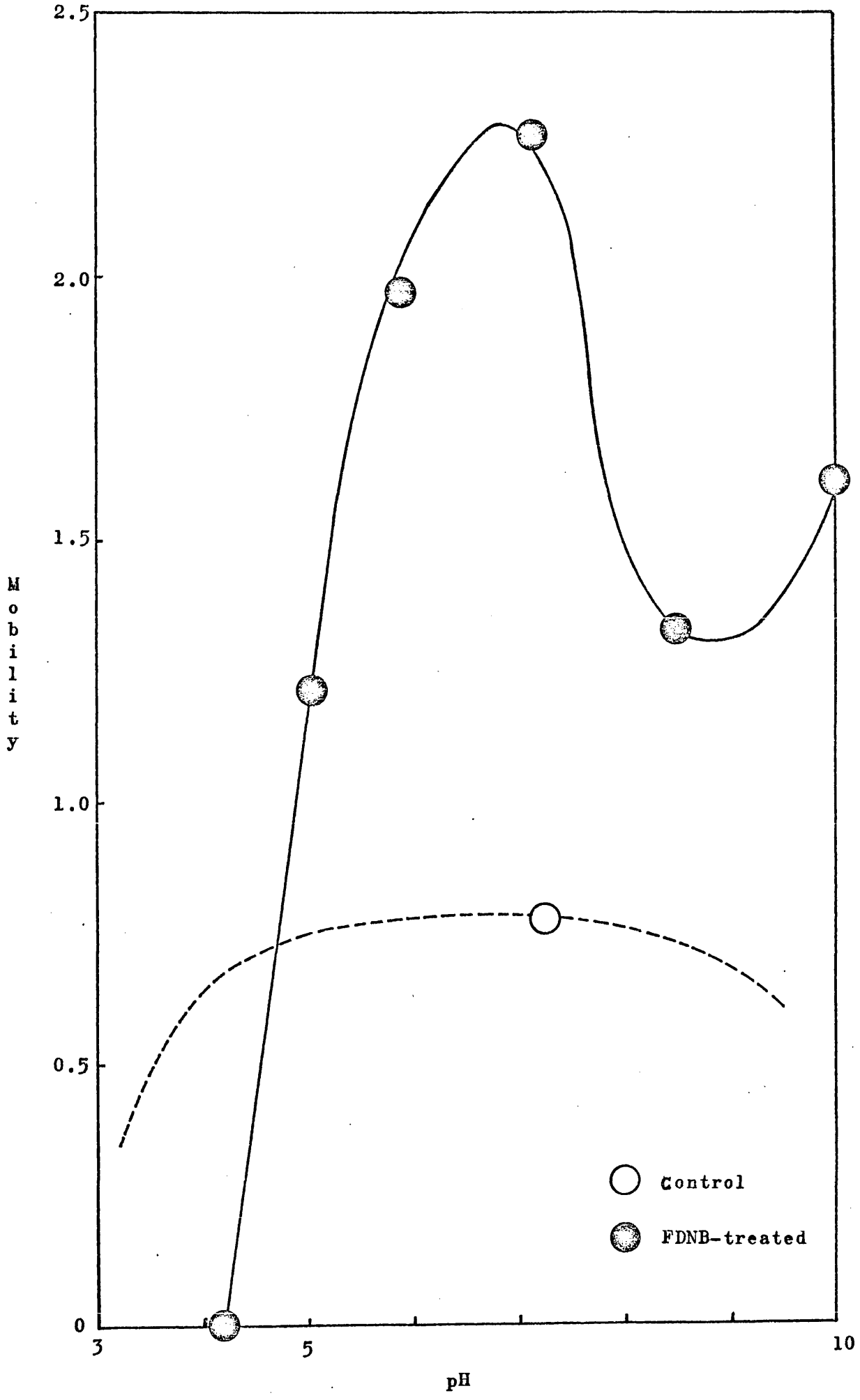

2.5
2.0
1.5
1.0
0.5
0
Mobility
3
5
10
pH
7/FDNB
I/FDNB
I
7

FIGURE VI.2

pH-mobility curve for cells of P.aeruginosa (strain 8) after treatment with FDNB.


2.5
2.0
1.5
1.0
0.5
0
Mobility
3
5
10
pH
Control
FDNB-treated

possibility that the lower general mobility values for cells of the medium-level resistant strain are due to a greater density of surface amino groups on cells of this strain.

The pH-mobility curve for FDNB-treated cells of the high-level gentamicin-resistant strain 8 is different from those for cells of strains 1 and 7. The curve for treated cells of strain 8 whilst exhibiting a general increase in mobility values, as did those for treated cells of strains 1 and 7, does not possess a plateau mobility value at pH $>$ 7.5, but has a sinusoidal shape similar to that for untreated cells of sensitive strains, having a maximum mobility value at pH 7 and a minimum at pH 9. This marked difference in electrophoretic response to FDNB treatment suggests that the surface properties of normal cells of the high-level resistant strain 8 are totally different from normal cells of the medium-level resistant strain 7, even although the general shapes of the pH-mobility curves for cells of the two strains are similar.

VI.2. The effect of aldehydes on the mobility of the cells

Aldehydes, in general, react with cationogenic groups, that with amino groups being according to the equation:

$$RNH_2 + R'CHO \longrightarrow RN : CHR' + H_2O$$

The lower aldehydes have a stabilizing effect on red blood cells in suspension without drastic effect upon the

cell mobility as the surface is polyanionic (Heard and Seaman, 1961). Where the surface is also cationic, aldehydes would be expected to cause an increase of negative mobility value by preventing, say, amino groups from contributing a positive charge by the above reaction.

Treatment of cells of strain 1 with a 2% aqueous solution of formaldehyde, HCHO, (II.7c) had no effect on their mobility values compared with those of control cells treated in the same way but in the absence of formaldehyde (Figure VI.3). This lack of effect was not due to a reversal of the action of formaldehyde by washing the cells in buffer solution prior to mobility measurement, as the same result was obtained when the mobility values of the cells were measured in buffer solutions containing 2% formaldehyde.

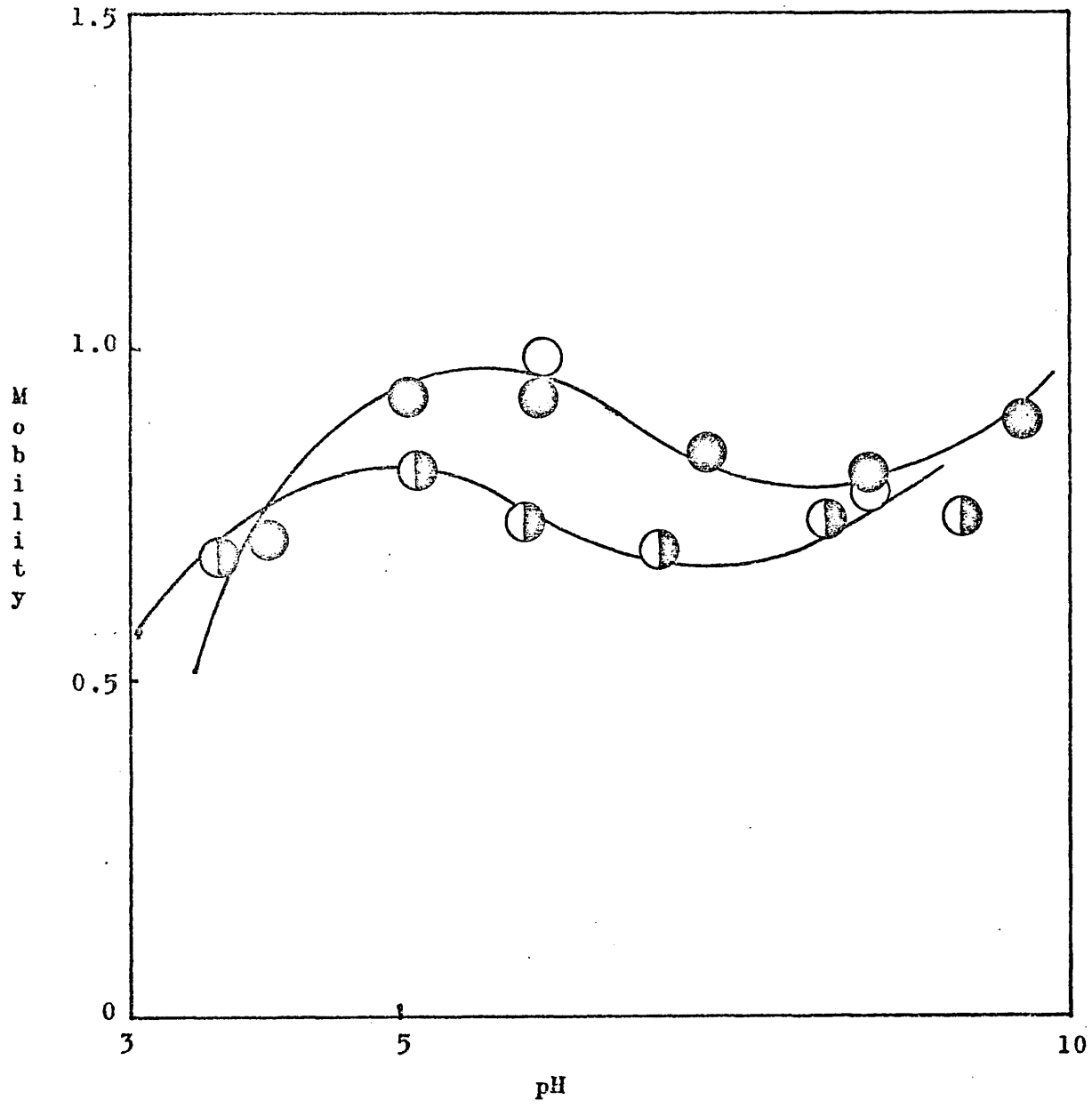
Treatment of cells of strain 1 with a 2% aqueous solution of acetaldehyde, CH_3CHO, however, did have an effect upon the pH-mobility curve (Figure VI.3). This effect was opposite to that which would result from the aldehyde-amino interaction described previously, in that the mobility values of the treated cells were less negative than those of control cells. The cause of this is unknown, as is the reason why acetaldehyde has an effect upon the surface properties of the cells which formaldehyde does not. A reaction between acetaldehyde and surface carboxyl groups is possible:

$$RCOOH + CH_3CHO \rightleftharpoons RCOOCCH_3(H)OH \xrightarrow{R'COOH} RCOOCCH_3(H)OCOR' + H_2O$$

where R'COOH may be an impurity in the acetaldehyde or an

FIGURE VI.3

The effect of acetaldehyde and formaldehyde on the surface properties of cells of *P.aeruginosa* strain 1.



Key

Control cells.

Cells treated with formaldehyde.

Cells treated with acetaldehyde.

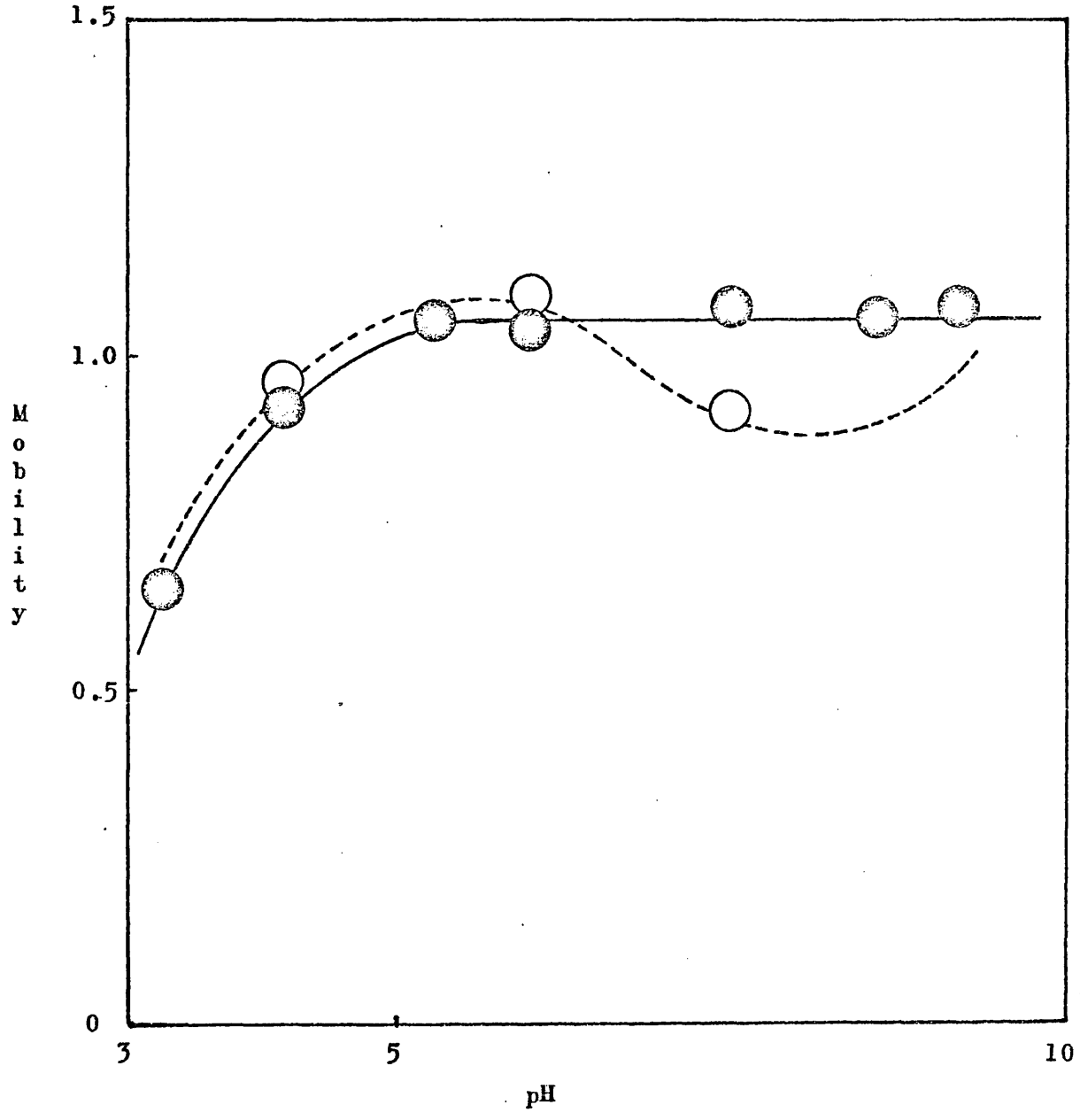
adjacent carboxyl group. This reaction would prevent the surface carboxyl groups from contributing a negative charge, and thus lower the negative mobility of the cells. However, such a reaction is improbable under the experimental conditions used and as long as surface amino groups are present to compete for the aldehyde.

Glutaraldehyde, or glutaric dialdehyde, $CHO(CH_2)_3CHO$, is used as a fixative for bacterial cells, especially for electron microscopy, because it possesses two aldehyde groups. These are thought to hold firmly together adjacent protein chains in the cell envelope structure by forming a bridge between amino groups on the protein molecules.

The effect on the pH-mobility curve of cells of P.aeruginosa strain 1 of treatment with a 1% aqueous solution of glutaraldehyde was to transform the sinusoidal shape of the curve to that of a plateau at pH > 5.5, the mobility value of which was approximately the same as the maximum mobility value for untreated control cells (Figure VI.4). Thus, as with formaldehyde and acetaldehyde, the expected increase in negative mobility value for the cells due to the blocking of the surface amino groups was not observed. The same results were obtained when the cells were treated under alkaline conditions by buffering the aqueous glutaraldehyde solution with sodium bicarbonate. Thus the effect of the glutaraldehyde on the mobility of cells of strain 1 was not pH dependent, unlike its effect upon the mobility of cells of Escherichia coli and Bacillus subtilis (Munton and Russell, 1972). Further, the effect of the glutaraldehyde was irreversible as the cells

FIGURE VI.4

The effect of glutaraldehyde on the surface properties of cells of P.aeruginosa strain 1.



Key

○ Control cells.

● Cells treated with glutaraldehyde.

were washed, following treatment, from 0 - 6 times without significant effect on the mobility value of the cells.

The fact that the sinusoidal-shaped curve for untreated cells is transformed into a plateau at pH > 5.5 after glutaraldehyde treatment suggests that the dialdehyde, by bridging between adjacent envelope structures, is able to prevent a re-orientation of cell surface components which is responsible for the sinusoidal shape of the pH-mobility curve for untreated cells. If such bridging between adjacent amino groups is occurring then these amino groups are unlikely to be able to contribute to the surface charge of untreated cells, since their blocking by glutaraldehyde does not cause an increase in the negative mobility value of the treated cells.

VI.3. The effect of Glutaraldehyde and FDNB, in combination on the surface of cells

Since glutaraldehyde and FDNB, whilst nominally reacting with the same surface amino groups, show widely differing effects upon the mobility of the bacteria, the effect of treating the cells with both, consecutively, was investigated.

18-Hour cells of strain 1 were harvested and the suspension divided into 4 aliquots. The cells in the first aliquot were prepared in the usual way for the measurement of mobility. Cells in the second aliquot were treated with a 1% aqueous solution of glutaraldehyde

(II.7c); those in the third were treated with FDNB (II.7d), and the cells in both prepared for the measurement of the mobility in the usual way. The cells in the fourth aliquot were first treated with a 1% aqueous glutaraldehyde solution, washed twice in water and then treated with FDNB before final suspension for the measurement of mobility by washing twice in water and twice in the required buffer solution. The pH-mobility curves for the cells of all four aliquots confirm the effects of FDNB and glutaraldehyde singly and show that the effect of treating the cells first with glutaraldehyde and secondly with FDNB lowers the mobility value of the plateau caused by the glutaraldehyde treatment alone (Figure VI.5). This suggests that there are no free surface amino groups remaining to react with FDNB after the glutaraldehyde treatment as there is no increase in negative mobility value of the cells treated with both reagents over those treated only with glutaraldehyde. The reason for the further reduction in negative mobility value of the cells treated with both reagents below that for cells in the other three aliquots is unknown.

The way in which the pre-treatment of cells of strain 1 with FDNB affects the mobility of those cells when subsequently treated with glutaraldehyde (i.e. the reverse of the previous experiment) was also investigated. The cell suspension was again split into 4 aliquots; cells were (1) prepared normally, (2) treated with FDNB, washed twice in water and then treated with glutaraldehyde, (3) treated with glutaraldehyde only, and (4) treated with FDNB only. After the normal washing and suspension in the required buffer solutions the mobility values were

FIGURE VI.5

pH-mobility curve for cells of P.aeruginosa (strain 1) after treatment with glutaraldehyde and subsequently FDNB.

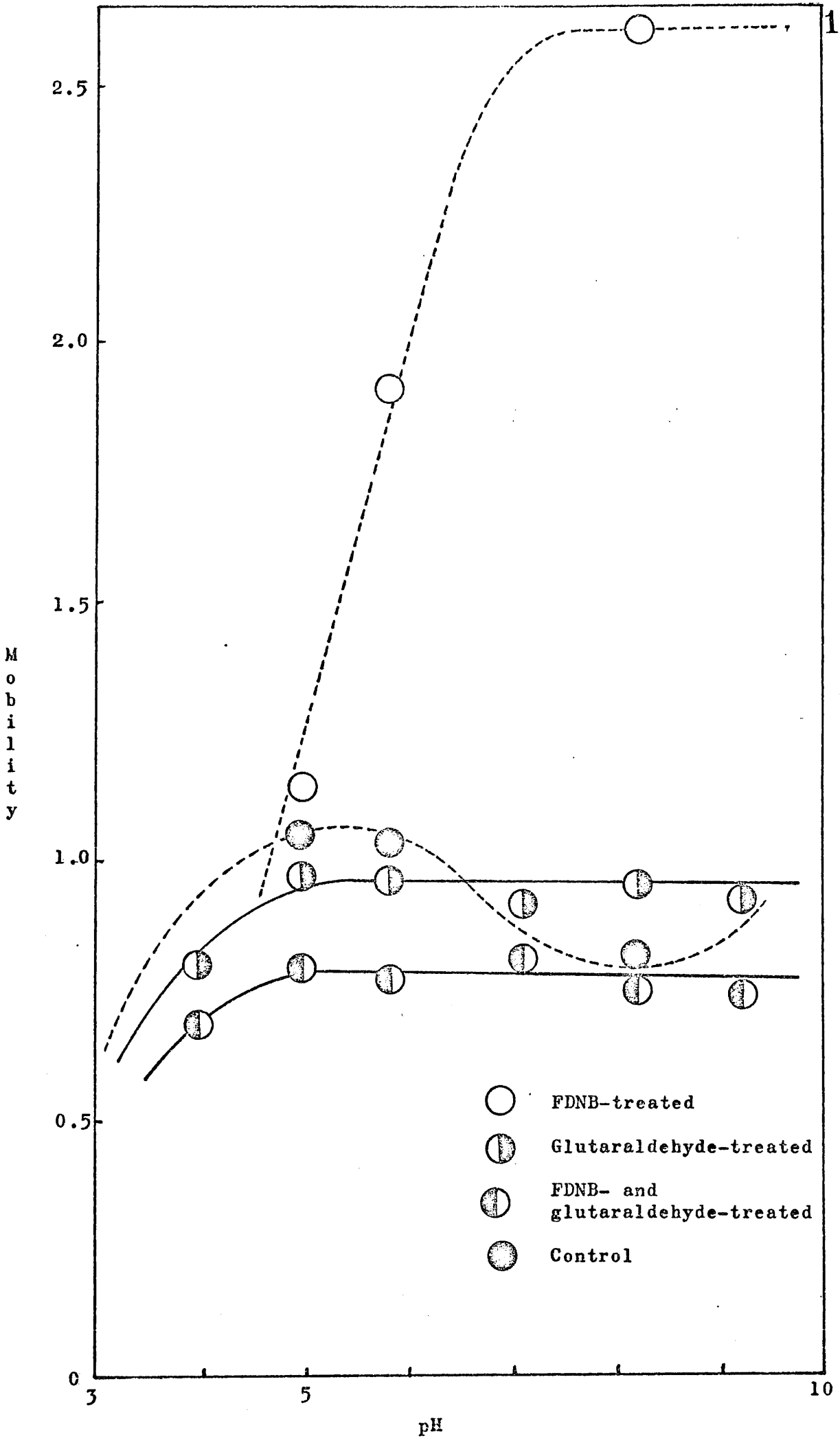
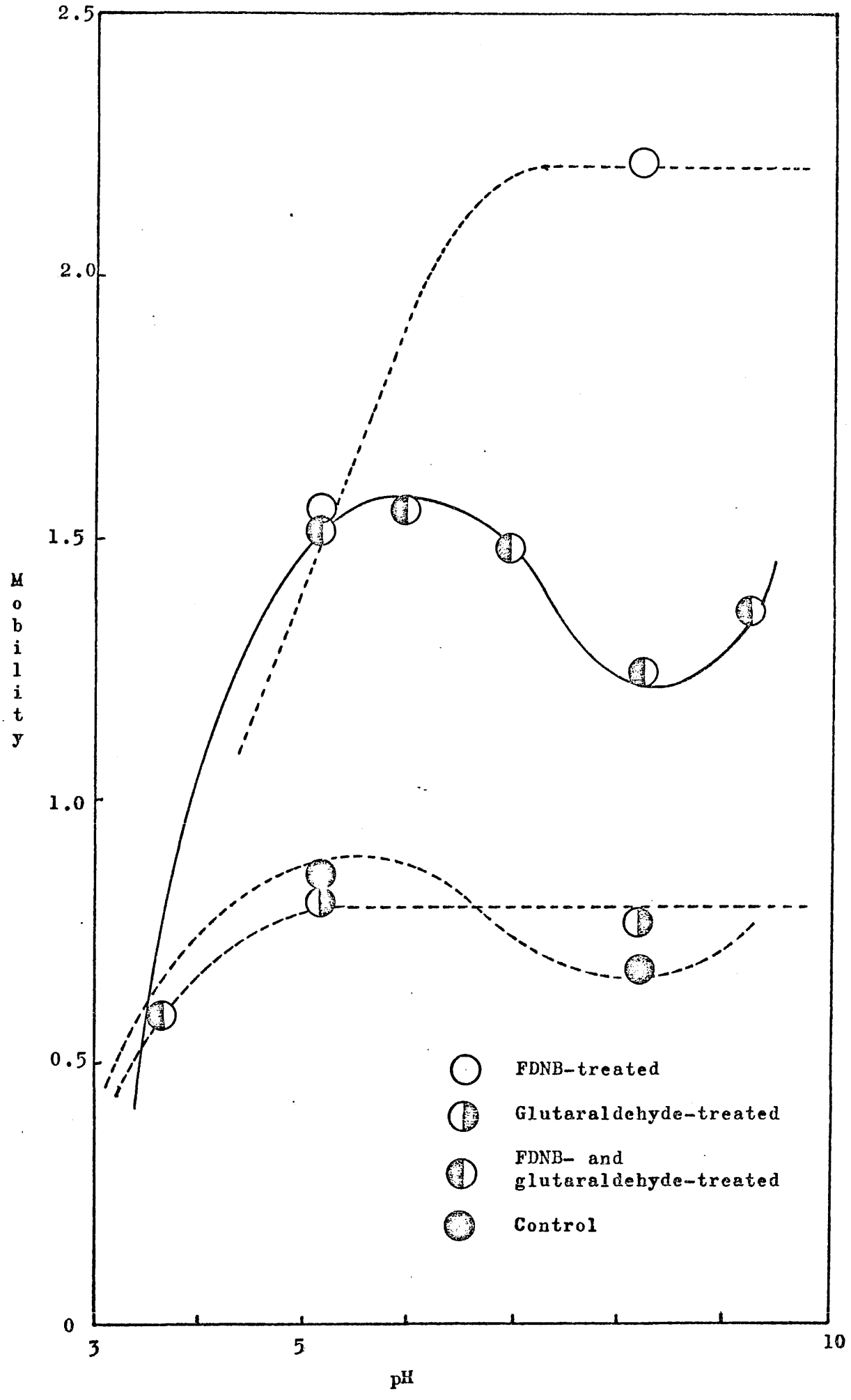

2.5
2.0
1.5
1.0
0.5
0
Mobility
3
5
10
pH
FDNB-treated
Glutaraldehyde-treated
FDNB- and glutaraldehyde-treated
Control

FIGURE VI.6.

pH-mobility curves for cells of P.aeruginosa (strain 1) after treatment with FDNB and subsequently glutaraldehyde.


2.5
2.0
1.5
1.0
0.5
0
Mobility
3
5
10
pH
FDNB-treated
Glutaraldehyde-treated
FDNB- and glutaraldehyde-treated
Control

determined. The pH-mobility curves for cells of the four aliquots (Figure VI.6) once again confirm the effects of glutaraldehyde and FDNB singly and show that treatment with glutaraldehyde following treatment with FDNB lowers the high mobility values caused by the latter to a level intermediate between that for untreated and FDNB-treated cells. This shows that the FDNB does not exclude the glutaraldehyde and hence either it does not react with all the available surface amino groups or FDNB and glutaraldehyde do not react with the same surface groups. The reason why glutaraldehyde, which alone transforms the sinusoidal-shaped curve for normal cells of strain 1 into a plateau, should transform the plateau resulting from FDNB treatment back into a sinusoidal-shaped curve is unknown. It is possible that glutaraldehyde causes the observed decrease in negative mobility value compared with the FDNB treated cells by reacting with the surface carboxyl groups, FDNB having blocked all the available surface amino groups.

VI.4. Summary

(a) FDNB-treatment of cells of strains 1 (gentamicin-sensitive), 7 (medium-level gentamicin-resistant) and 8 (high-level gentamicin-resistant) caused an increase in the negative mobility values of the cells.

(b) FDNB-treatment of cells of strains 1 and 7 transformed the pH-mobility curves for cells of both strains into plateaux at pH > 7.5, but treatment of cells of strain 8

transformed the pH-mobility curve into a sinusoidal-shaped curve.

(c) Formaldehyde-treatment of cells of strain 1 had no effect on the pH-mobility curve.

(d) Acetaldehyde-treatment of cells of strain 1 caused a decrease in negative mobility values at all pH values, but the shape of the curve was unchanged.

(e) Glutaraldehyde-treatment of cells of strain 1 transformed the pH-mobility curve from a sinusoidal-shaped curve into a plateau at pH $>$ 5.5, the mobility value was approximately the same as the maximum mobility value for untreated cells.

(f) Pre-treatment of cells of strain 1 with glutaraldehyde prevented the increase in negative mobility value due to FDNB-treatment observed for cells not so pre-treated.

(g) Pre-treatment of cells of strain 1 with FDNB did not prevent glutaraldehyde from reacting at the cell surface, and this reaction lowered the negative mobility values due to the pre-treatment and resulted in a sinusoidal-shaped pH-mobility curve like that for normal cells, but at higher mobility values.

C H A P T E R V I I

THE EFFECT OF EDTA ON CELLS OF PSEUDOMONAS AERUGINOSA

The divalent metal ions, Mg^{2+}, Ca^{2+} and Zn^{2+} are essential for the integrity of the cell envelope of P.aeruginosa (I.4). Cell envelope disruption has been caused by treating cells of P.aeruginosa with ethylenediaminetetraacetic acid (EDTA) which, because it readily forms stable chelating complexes with divalent metal ions, is believed to extract these ions from the cell envelope (Eagon and Carson, 1965; Roberts et al, 1970). The following experiments were performed to investigate (a) the bacteriostatic effect of EDTA, (b) the effect of EDTA on the surface properties of cells of P.aeruginosa.

VII.1. The bacteriostatic effect of EDTA, alone and in combination with gentamicin

The MIC of EDTA, in the form of its disodium salt, was measured for cells of strains of P.aeruginosa (II.5). The results (Table VII.1) showed no obvious correlation between gentamicin resistance and resistance to EDTA (i.e. MIC > 1000 $\mu g\ cm^{-3}$). Despite this lack of correlation between the effects of the two reagents, cells of strain 1A/20, having acquired greater resistance to gentamicin, had also become resistant to EDTA. However, cells of strain 1A/50 showed the same MIC of EDTA as cells of strain 1 whilst the MIC of EDTA for cells of strain 1A/100 was much lower. This may be due to the perturbation of the cell surface structures, as shown by the pH-mobility curves during

training to gentamicin (IV.3). This could render the cell envelope more susceptible to EDTA attack, but as the training proceeds and the cell surface structures revert back to those of the sensitive parent strain, greater resistance to EDTA is attained. The result for strain 1B shows that as the unstable acquired resistance to gentamicin was lost by growth in the absence of the antibiotic, so the acquired resistance to EDTA was also lost.

Synergism between the effects of two antibacterial agents is shown by a four-fold, or greater, decrease in the MIC of both or a two-fold decrease in the MIC of one and an eight-fold decrease for the other when used in combination; smaller decreases in the MIC than these show that the combined effect of the two reagents is only additive. Accordingly to investigate the possibility of synergism between the effects of gentamicin and EDTA against P.aeruginosa, for each strain three series of liquid media were prepared containing two-fold dilutions of gentamicin and EDTA, as its disodium salt, each in 5 cm^3 nutrient broth. One series of six solutions covered the concentration range from the MIC of both reagents for the strain to zero, and the other two series' six solutions covered the concentration ranges of twice the MIC of gentamicin and half the MIC of EDTA to zero, and twice the MIC of EDTA and half the MIC of gentamicin to zero. All these solutions were sterilised, inoculated with cells of the test strain and incubated as described for normal MIC determinations.

The results are shown in Table VII.1, where a four-fold reduction in the MICs of gentamicin and EDTA means the

TABLE VII.1

The bacteriostatic effect on cells of P.aeruginosa of EDTA, both alone and in combination with gentamicin.

Strain	MIC of gentamicin /μg cm^{-3}	MIC of EDTA /μg cm^{-3}	Effect of EDTA and gentamicin in combination
1	1.25	388	++
2	5	3102	++
3	≤0.16	3102	0
4	1.5	3102	++
5	12	97	0
6	25	3102	++
7	25	3102	0
8	8000	3102	+
9	8000	3102	++
10	25	3102	+++
1A/20 (230)*	25	1551	+
1A/50 (191)*	55	388	0
1A/100(109)*	120	97	0
1B (171)*	3.1	194	0

Key

0 = 2 fold reduction in MICs of gentamicin and EDTA, i.e. an additive response and hence no synergism.

+ = 4 fold
++ = 8 fold
+++ = 16 fold

reduction in MICs of gentamicin and EDTA, i.e. synergism exists.

* Figures in brackets represent number of subcultures in presence of the appropriate gentamicin concentrations.

average reduction for both reagents, i.e. either a four-fold decrease in MIC for both or a two-fold decrease in the MIC for one and an eight-fold decrease for the other; similarly for two-, eight- and sixteen-fold reductions. For cells of seven of the ten strains (No.1 to 10), gentamicin and EDTA showed synergism in their effect, but only an additive effect against cells of the other three strains. There is no obvious correlation between the effects of gentamicin and EDTA singly and their effect in combination. The trained strains showed a greater resistance to the combined action of gentamicin and EDTA than did the parent strain 1, for which an eight-fold reduction in the MICs was observed; a four-fold reduction was observed for strain 1A/20 and only a two-fold reduction was observed for strains 1A/50, 1A/100 and 1B.

VII.2. The effect of EDTA on the surface properties of the cells

Washed cells of strain 1 (sensitive to gentamicin and EDTA) and 8 (resistant to gentamicin and EDTA) were suspended in aqueous EDTA solution at a concentration approximately ½ of the MIC and at a bacterial concentration of approximately 5×10^8 cells cm^{-3} for 30 minutes at 37°C. They were then prepared for the determination of mobility values (II.7d). Treated cells of these strains were subcultured onto nutrient agar plates where growth showed that cell viability had not been lost during treatment. There was no change in

the absorbance of suspensions of cells (a) in the absence, and (b) in the presence of EDTA, over a period of three hours indicating that no cell lysis had occurred.

The pH-mobility curve for EDTA-treated cells of strain 1 (Figure VII.1) shows a similar shape between pH 3 and 6 to that of normal cells. At pH values greater than 6, however, the mobility values for treated cells are always greater than those for normal cells, and a rapid increase in negative mobility value between pH 6 and 7.5 gives a maximum value of 2.4 with a subsequent minimum value of 1.3 at pH 8. This increase in negative mobility value may be due to the negatively charged surface groups no longer being associated with the divalent metal cations which have been removed by the EDTA. The shape of the curve between pH 6 and 8 may be the result of configurational changes at the cell surface first revealing and then concealing these negatively charged groups. This configurational change at the surface could be that responsible for the sinusoidal shape of the pH-mobility curve observed for normal cells of a sensitive strain.

The pH-mobility curve for EDTA-treated cells of strain 8 (Figure VII.2) is different from that of treated cells of strain 1 in that the increase in negative mobility between pH 6 and 7.5, beyond that for normal cells is not followed by a minimum mobility value at pH 8. Again the increased negative mobility values of treated cells may be due to the negatively charged surface groups deprived of the metal counter-ions by EDTA. The lack of a minimum

FIGURE VII.1

pH-mobility curve for cells of P.aeruginosa (strain 1) after treatment with EDTA (93 $\mu g\ cm^{-3}$).

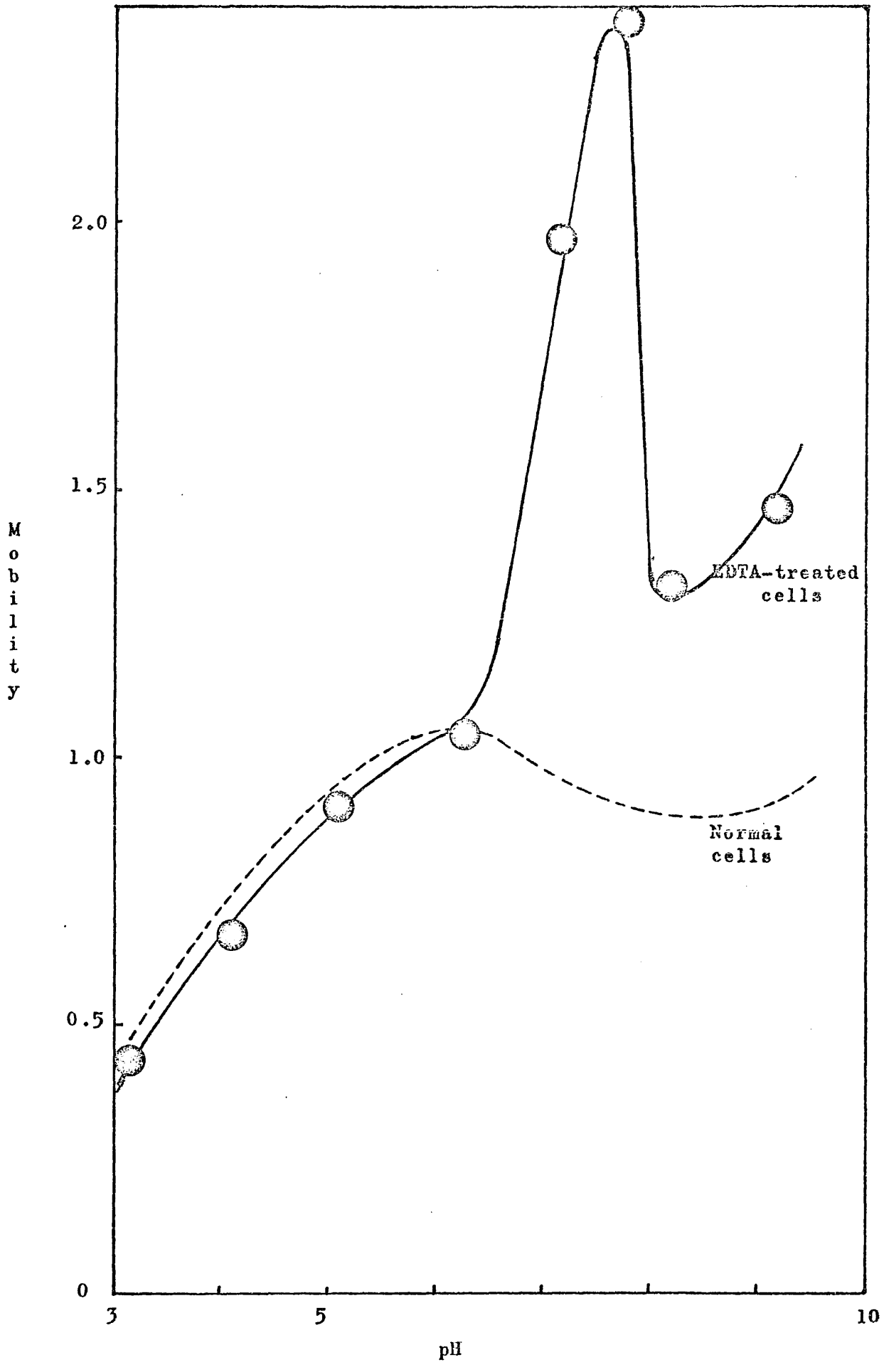
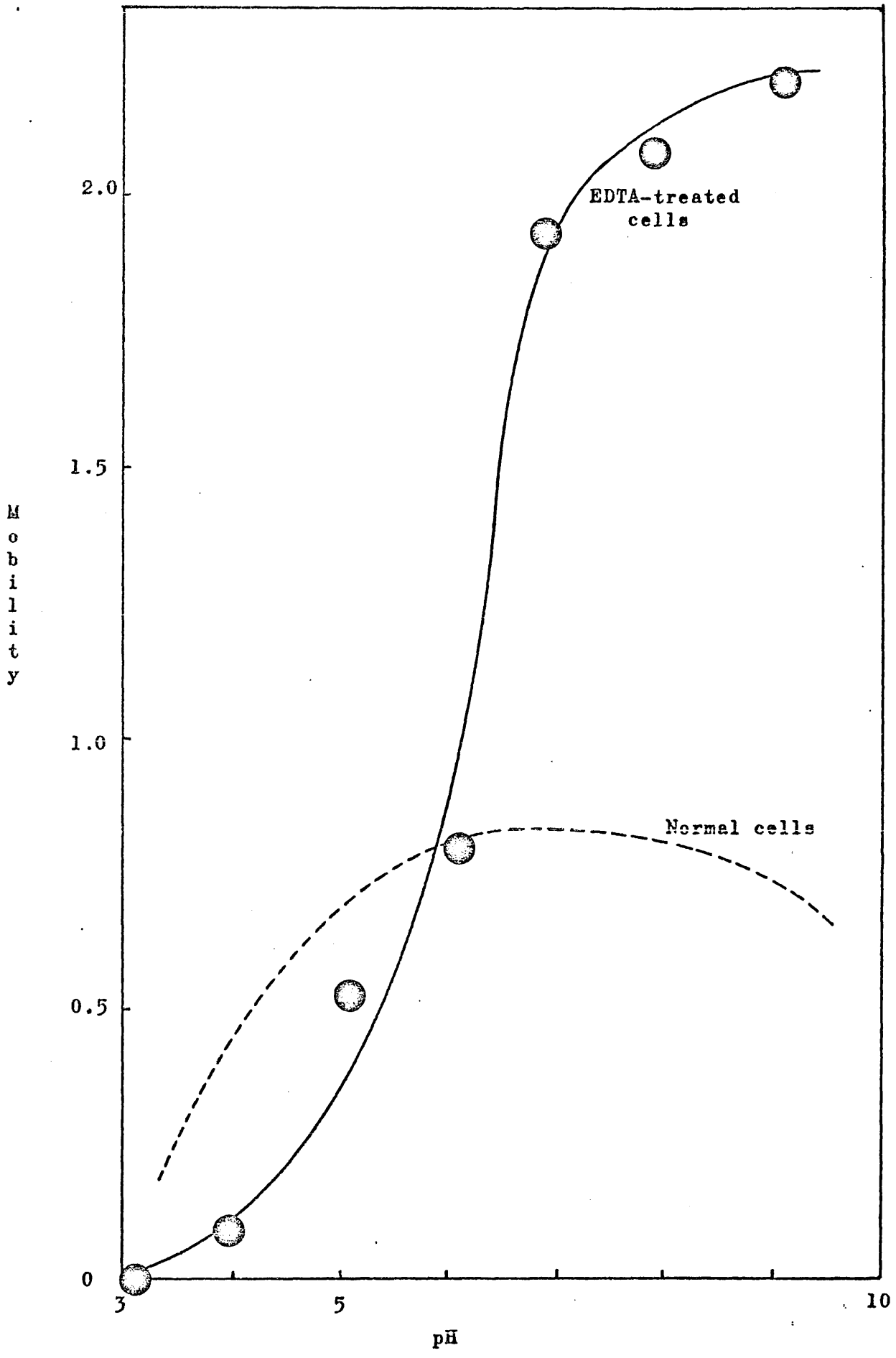

2.0
1.5
1.0
0.5
0
Mobility
EDTA-treated cells
Normal cells
3
5
10
pH

FIGURE VII.2

pH-mobility curve for cells of P.aeruginosa (strain 8) after treatment with EDTA (930 $\mu g\ cm^{-3}$).


2.0
1.5
1.0
0.5
0
Mobility
EDTA-treated cells
Normal cells
3
5
10
pH

mobility value at pH 8 is possibly due to the lack of the configurational change at the surface which may be responsible for the minima observed for treated and untreated cells of the gentamicin-sensitive strain 1. However, an effect due to the difference in EDTA concentration used in the two experiments is not ruled out.

The effect of EDTA on the surface lipid possessed by the cells of strain 8 was investigated by incubating the cells for 30 minutes at $37^{o}C$ in an aqueous solution containing EDTA at concentrations 0, 37 and 930 $\mu g\ cm^{-3}$ (i.e. approximately 0, 1 and 25% respectively of the MIC of EDTA for the cells). The cells were then washed twice in water and once in either buffer solution containing SDS (10^{-4} mol dm^{-3}) or buffer solution alone. The S-values for the variously treated cells were calculated from their measured mobility values (Table VII.2). The S-values were not significantly altered by the EDTA-treatment; they were slightly lower for treated cells than untreated cells but this may be due to the increased mobility values of the treated cells measured in the absence of SDS. Whatever the exact effect of EDTA on the cell surface, the surface lipid had not been disrupted.

The S-value for cells treated in the absence of EDTA was lower than that measured in the normal way both before and after this experiment (V). This difference is probably a result of the extended treatment and washing procedure

TABLE VII.2

The effect on the surface lipid of treating cells of P.aeruginosa (strain 8) with sub-lethal concentrations of EDTA (i and ii are duplicate determinations)

	EDTA concentration /μg cm^{-3}	Mobility		S-value
		Control	In SDS	
(i)	0	0.81	1.08	34
	37	0.98	1.19	22
	930	1.84	2.27	23
(ii)	0	0.76	0.96	26
	37	0.82	1.28	57
	930	1.55	2.01	30

undergone by the cells.

VII.3. Summary

(a) EDTA exhibited a bacteriostatic effect of cells of all strains of P.aeruginosa used in this investigation, although to widely different extents.

(b) Gentamicin and EDTA showed synergism in their combined effect against 7 out of 10 naturally occurring strains of P.aeruginosa.

(c) There was no obvious correlation between the bacteriostatic effects of gentamicin and EDTA singly or between their effects singly and in combination. Training to gentamicin caused an increased resistance to the combined action of the two reagents and after a long period resulted in increased resistance to EDTA singly. This acquired resistance to EDTA singly was lost as gentamicin-resistance was lost during subculturing in the absence of the antibiotic.

(d) Pre-treatment of cells with EDTA at sub-lethal concentrations caused an increase in the negative mobility values; gentamicin-sensitive and -resistant cells showed different responses.

(e) Cell-surface lipid was not removed by sub-lethal EDTA concentrations.

CHAPTER VIII

THE EFFECT OF SODIUM METAPERIODATE ON THE SURFACE PROPERTIES OF CELLS OF PSEUDOMONAS AERUGINOSA

Teichoic acid may be removed from bacterial cell envelopes by oxidation with sodium metaperiodate (Brewer, 1966), a process which also removes other readily oxidisable envelope components.

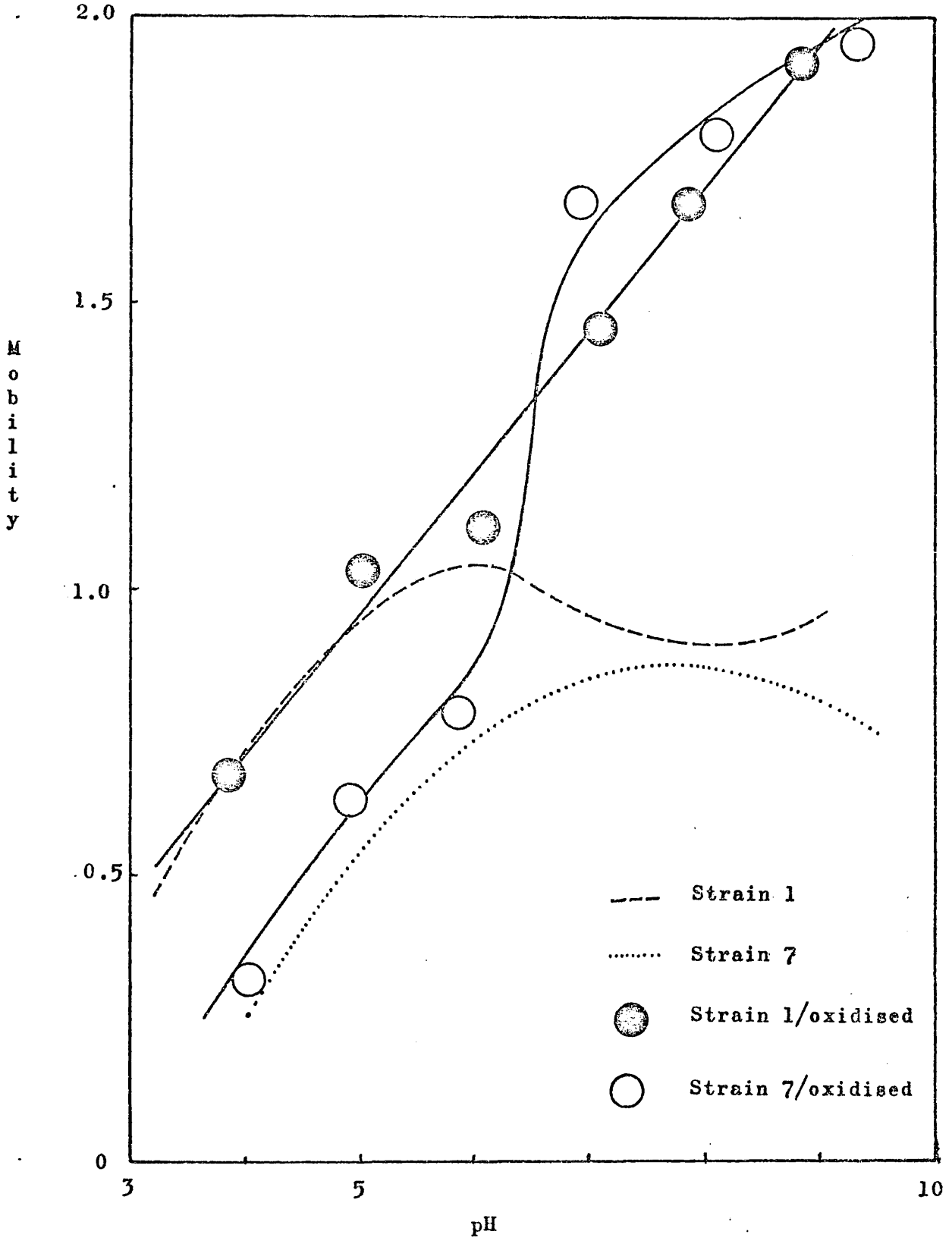
Cells of 18-hour cultures of strains 1 and 7 were harvested and treated with sodium metaperiodate ($NaIO_4$) before measuring their mobility values (II.7a). The negative mobility values for cells of both strains were increased at all pH values (Figure VIII.1). The increase was slight between pH 3.5 and 6 for cells of both strains but at pH $>$ 6 the values for treated cells were as much as double the values for normal cells. The pH-mobility curve for cells of the gentamicin-sensitive strain 1 was almost linear, which could be characteristic of a non-ionogenic surface where the charge was due only to adsorbed ions; the pH-mobility curve for cells of the medium-level gentamicin-resistant strain 7 was almost discontinuous between pH 6 and 7. It is interesting that the mobility values of cells of both strains at pH $>$ 6 are similar, suggesting that the treated cell surfaces may be more alike than those of normal cells.

The removal of surface teichoic acid from cells of Staphylococcus aureus by this method resulted in a lowering of negative mobility values between pH 3 and 5 as the phosphate groups of the acid molecule were no longer able to contribute a negative charge (Hill and James, 1972a). Similar behaviour was not observed here where the increase in negative mobility occurred at higher pH values. Teichoic

acid has never been reported in the cell envelope of P.aeruginosa, but these results show that the sodium metaperiodate has removed a readily oxidisable cell envelope component which has either revealed previously concealed negative groups or has removed positively charged surface groups.

FIGURE VIII.1

pH-mobility curves for cells of P.aeruginosa (strains 1 and 7) after oxidation with sodium metaperiodate (5×10^{-2} mol dm^{-3}).


2.0
1.5
1.0
0.5
0
Mobility
3
5
10
pH
Strain 1
Strain 7
Strain 1/oxidised
Strain 7/oxidised

C H A P T E R I X

THE TRANSFER OF R-FACTOR MEDIATED GENTAMICIN RESISTANCE IN PSEUDOMONAS AERUGINOSA

The resistance to gentamicin of the strains of P.aeruginosa used in this investigation was at two distinct levels; those possessing medium-level resistance where the MIC of gentamicin was intermediate between 12 and 100 μg cm^{-3} and those with high-level resistance for which the MIC was greatly in excess of 1000 μg cm^{-3} (Table II.1). The latter type of resistance is characteristic of enzymic inactivation or destruction of the antibiotic, whereas the former is characteristic of the levels of resistance achieved by other mechanisms such as modification or adaptation within the cell to overcome the effect of the antibiotic (I.6). The inactivating and destructive enzymes are often mediated by extra-chromosomal DNA which in some cases has been successfully transferred from resistant cells of one organism to sensitive cells of another with corresponding transfer of antibiotic resistance (Benveniste and Davies, 1971; Roe et al, 1971). To investigate the possibility of R-factor mediated gentamicin-resistance in cells of the different types of strains of P.aeruginosa used in this investigation, a transfer was attempted between cells of P.aeruginosa strain 7 (medium-level gentamicin-resistant), 8 (high-level resistant) and 1A/50 (trained resistant) and cells of Escherichia coli K12 (gentamicin-sensitive), using a method modified after Benveniste and Davies (1971).

The MICs of gentamicin for all three donor strains of P.aeruginosa and the recipient strain of E.coli were determined immediately prior to the transfer. 0.5 cm^3 of an 18-hour nutrient broth culture of the donor strain was

mixed with 0.5 cm^3 of an 18-hour nutrient broth culture of the recipient strain (both approximately 5 x 10^8 bacteria cm^{-3}) in 4 cm^3 nutrient broth. The mixed culture was incubated at 37°C for 2 hours and then, with agitation, for 16 hours. The recipient cells were separated from donor cells by spreading 0.1 cm^3 of the mixed culture onto each of 10 plates (approximately 10^7 bacteria per plate) containing eosin-methylene-blue (EMB) agar. After incubation for 24 hours at 37°C colonies resulting from donor cells of P.aeruginosa appeared translucent on the agar surface because they do not ferment lactose; colonies resulting from recipient cells of E.coli appeared deep blue with a green metallic sheen in reflected light because they do ferment lactose. In control experiments with mixed cultures of E.coli and P.aeruginosa, pure cultures of E.coli were successfully obtained by picking E.coli colonies off the EMB agar surface and plating them out onto EMB agar plates. A further plating out of individual colonies on EMB agar plates confirmed the purity of the cultures. To isolate only those recipient cells resistant to gentamicin (the frequency of transfer is approximately 1 in 10^5), the antibiotic was included in the EMB agar at a concentration of 10 μg cm^{-3}.

After replicate experiments no colonies of E.coli were observed on the EMB-gentamicin agar, indicating that there had been no transfer of an R-factor mediating gentamicin-resistance between the cells of P.aeruginosa strains 7, 8 and 1A/50, and the cells of E.coli. Further experiments were performed in which the mixed donor and recipient

culture was incubated for two hours statically, then two hours with agitation and finally for 16 hours statically, but again no transfer was observed. It is possible that none of the three donor strains used was resistant to gentamicin by production of an inactivating enzyme coded for by a transferable extra-chromosomal gene. This does not, however, exclude the possibility of inactivating enzyme production coded for by a non-transferable chromosomal gene. The failure to obtain transfer could also be the result of incompatibility between donor and recipient, and a different strain of E.coli might prove compatible, as might a gentamicin-sensitive strain of P.aeruginosa as used recently by Knothe et al (1973), although the latter presents greater problems of satisfactory donor-recipient separation.

CHAPTER X

DISCUSSION

The zeta-potential at the cell-electrolyte interface is determined partly by the nature and quantity of the ionogenic groups at the surface and partly by the pH and ionic strength of the suspending medium. Although the coefficient of viscosity, η , and relative permittivity, ϵ_r, within the electrical double layer are generally assumed to have the value of bulk water, recent work indicates that this may not be so. Thus any conversion of mobility values to zeta-potentials using the Smoluchowski equation:

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

(where ϵ_o is the permittivity of free space) and the subsequent discussion of the variation of the zeta-potential is open to criticism. However, if the pH and ionic strength of the suspending electrolyte are kept constant, then it is permissible, for cells of fixed age, to discuss changes of the experimentally determined mobility in terms of changes in the nature and quantity of surface charged groups. This is the experimental situation obtaining in this work.

To interpret the results obtained in particulate electrophoresis it is further necessary to derive experimental conditions and procedures whereby the surface under study is reproducible and free from adsorbed material e.g. growth material, toxins or antibiotics. It was initially established that the

motility of cells of P.aeruginosa, evident at room temperature, is completely suppressed at 10°C. From the experimental physical standpoint it was shown that the measurement of mobility values at this low temperature did not give rise to experimental difficulties such as changes in the symmetry of the observation chamber, nor was the shape of the pH-mobility curve affected by measurement at this lower temperature. Accordingly, 10°C was adopted as the temperature of measurement throughout this study to ensure that reported changes in mobility were independent of cell motility. It was not possible to remove material from the cell surface by repeated washing, and therefore reported changes in the mobility of cells, which have been washed twice in buffer solution, represent true changes in the surface properties of the cells. Conditions of ionic strength were selected whereby the maximum difference between different suspensions and strains was obtained. The nature of the pH-mobility response of cells of P.aeruginosa was independent of the length of time of incubation between 18 and 42 hours, independent of the physical state of the growth medium and unaffected by the overnight storage of prepared suspensions at reduced temperature. These conditions and procedures (III.4) are therefore presented as a standard method for the reproducible measurement of electrophoretic mobility values of cells of P.aeruginosa.

It was necessary to calibrate the apparatus daily before use with a particle of known mobility. The accepted primary standard reference particle is the human

erythrocyte suspended in 0.667 mol dm^{-3} phosphate buffer solution at pH 7.35. The secondary standard reference, 18-hr cells of K.aerogenes suspended in veronal-acetate buffer solution at pH 7.0, was used in this investigation. It was soon established that cells of a given strain of P.aeruginosa, after 18-hr growth on nutrient agar, had a reproducible surface when prepared in the defined manner and when the mobility values were measured under the stated conditions. Analysis of the mobility values obtained on different occasions, for cells of the same strain, showed that the confidence limit of a single mean was 3% at $p = 0.05$. With this degree of reproducibility, cells with mobility values differing by 10% or more were considered to be significantly different, and hence to have different surface components or different proportions of the same surface components.

Cells of all strains were electrokinetically homogeneous with an unimodal population irrespective of their phage type or spectrum of antibiotic resistance and irrespective of the pH of suspension medium or the effect of chemical treatments. Care was taken to ensure that denaturation of the cell surface did not occur at the pH values used to determine the mobility. The influence of extreme pH conditions was estimated by the reversibility of the surface after exposure. After cells had been suspended in a buffer solution of extreme pH, they were centrifuged out of that solution and resuspended in buffer solution at pH 7; if these cells did not have a mobility value to within 3% of that of control cells

suspended immediately in buffer solution at pH 7, the surface was considered to be denatured and the results discarded. The cells were reversible when suspended in buffer solutions in the pH range 2 to 11, without loss of ~~reversibility~~ viability and denaturation; most work, however, was limited to the pH range 3 to 10.

The pH-mobility curves for cells of various strains of P.aeruginosa show at least two distinct types of response (Figures IV.1, IV.2 and IV.3). Curves for typical cells of gentamicin-sensitive (A), medium- (B) and high-level (C) resistant strains are shown in Figure X.1. The curves for cells of strains sensitive to the antibiotic action of gentamicin (i.e. MIC $\leqslant$ 12 $\mu g\ cm^{-3}$) are all characterised by a maximum negative mobility value in the pH range 5.5 to 6.5 and a subsequent minimum value in the pH range 7.5 to 8.5; the difference between the maximum and minimum mobility values is 9 to 18% of the maximum value. The curves for cells possessing medium-level resistance to gentamicin (i.e. 12 $\mu g\ cm^{-3} <$ MIC $<$ 100 $\mu g\ cm^{-3}$) are all characterised by a maximum mobility value in the pH range 7.5 to 8.5 (i.e. at higher pH values than that for sensitive cells); no minimum mobility value at higher pH values was ever observed. The rate of increase of negative mobility between pH 3 and the pH at the maximum mobility value for the medium-level resistant cells is

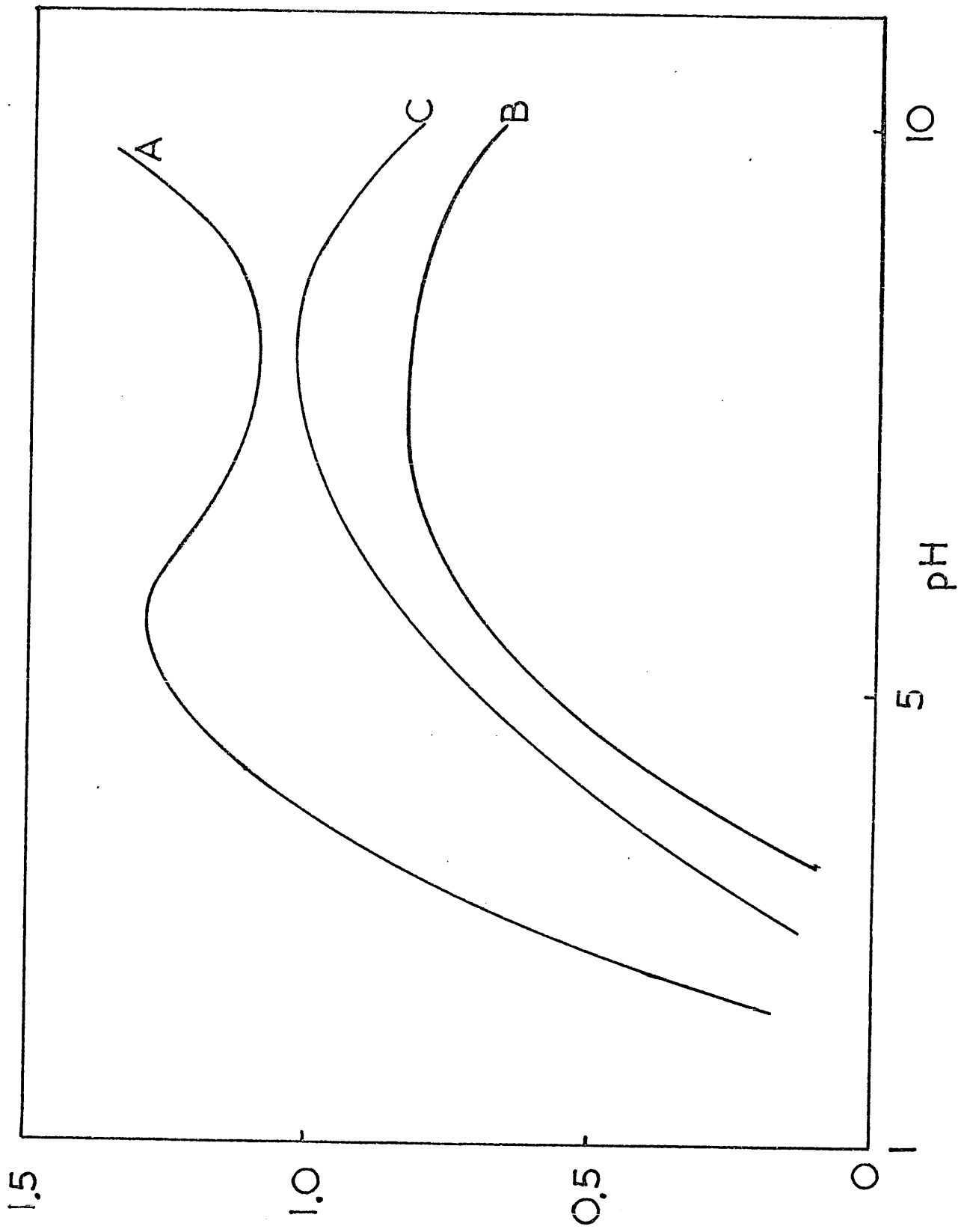
FIGURE X.1

Typical pH-mobility curves for 18 hr cells of *P.aeruginosa* possessing medium-level and high-level resistance to gentamicin and cells sensitive to gentamicin.

Curve A - typical of gentamicin-sensitive cells

Curve B - typical of medium-level gentamicin-resistant cells

Curve C - typical of high-level gentamicin-resistant cells


Mobility
1.5
1.0
0.5
0
5
10
pH
A
B
C

less than that for sensitive cells, and the maximum mobility value lies in the range 0.7 to 1.0 as opposed to 1.1 to 1.5 for sensitive cells. The pH-mobility curves for cells possessing high-level resistance to gentamicin (i.e. MIC $>$ 1000 $\mu g\ cm^{-3}$) are characterised by a maximum mobility value in the same pH range as that for medium-level resistant cells; but the range of maximum mobility values is 0.85 to 1.25. Curves A, B and C in Figure X.1 emphasise that the pH-mobility curve for typical cells of strains sensitive to gentamicin is completely non-superimposable upon the curve for typical cells of gentamicin-resistant strains with either high- or medium-level resistance. Figure X.1 also shows that the maximum mobility value for high-level resistant cells may be slightly higher than that for medium-level resistant cells.

Against the aminoglycoside antibiotics, gentamicin, neomycin, kanamycin and streptomycin, the strains of P.aeruginosa used in this investigation exhibit a one-way cross-resistance (Table II.1); cells resistant to gentamicin are always resistant to the other related antibiotics, but the reverse is not necessarily true. This is a general feature of this group of antibiotics, and suggests that gentamicin is the key member of the group, a fact which is borne out by there being no obvious correlation between resistance to the aminoglycoside antibiotics other than gentamicin listed in Table II.1 and the type of pH-mobility curve obtained for cells of P.aeruginosa. Carbenicillin, an antibiotic widely used against P.aeruginosa

is totally unrelated to the aminoglycoside antibiotics either by its structure or by its site and mode of action on the bacterial cell, and again there is no correlation between the shape of the pH-mobility curve obtained for the cells of P.aeruginosa and their resistance to this antibiotic. Furthermore, there is no correlation between the shape of the pH-mobility curve and the phage-typing pattern of the strains. This all supports the suggestion that the observed differences in the pH-mobility curves for cells of different strains of P.aeruginosa are a function of the different levels of resistance shown by the cells to gentamicin.

The pH-mobility curve characteristic of gentamicin-sensitive cells shows the surface to be complex, and not consisting of only one charged species, as does the surface of K.aerogenes which contains only polysaccharide carboxyl groups (Lowick and James, 1957). Nor can the pH-mobility curve for sensitive cells of P.aeruginosa be interpreted simply as a pH-titration curve of a surface consisting of more than one ionogenic species as obtained by Plummer et al (1962), as this would consist of a series of plateau mobility values at increasingly negative values as the pH increased, and not the sinusoidal-shaped curve which was obtained for the sensitive cells. This sinusoidal type curve suggests that there is some rearrangement or reorientation of the cell-surface components, revealing positively charged, or concealing negatively charged groups. Hill and James (1972,a) conclusively demonstrated

that the maximum value in the pH-mobility curve at low pH values for cells of Staphylococcus aureus (a more pronounced maximum than observed in the present work) was due to surface rearrangement, exposing phosphate groups of cell wall teichoic acid, which at higher pH values were concealed. A similar situation is observed here for cells of P.aeruginosa, but the extrapolated isoelectric point is too high to be due to phosphate groups. The curves characteristic of medium- and high-level gentamicin resistant cells also show a decrease of the negative mobility value at pH values greater than that at which the maximum mobility value occurs. This decrease is less pronounced and occurs at higher pH values than in the curves for sensitive cells, but still suggests a rearrangement of cell-surface components. Because of the complex nature of the surface it was not possible to obtain the pK values of any surface groups from the pH-mobility curves (cf. Lowick and James, 1957).

To carry out a more detailed investigation of the differences between the surface properties and structures of the cells of the three types of strain (gentamicin-sensitive, medium-level resistant and high-level resistant) three strains, one typical of each type, were chosen for further experiments. Strain 1 was chosen as typical of gentamicin-sensitive strains; strain 7 as typical of medium-level resistant strains; and strain 8 as typical of high-level resistant strains.

The result of treating cells of all three types with FDNB was to increase the negative mobility values

(Figures VI.1 and VI.2), showing that all three types of cell possessed surface amino groups which had been blocked by FDNB. For cells of both the sensitive and medium-level resistant strains, this treatment produced very similar results, yielding plateaux in the pH-mobility curves at greatly increased negative mobility values. The surface of these cells is now typical of a surface with a single dissociating anionic group; the pK values for the surfaces were approximately 4.7, a value which is too high for either sulphate or phosphate groups, which are both strongly acidic, and too high for an acidic polysaccharide carboxyl group. However, this value is close to the pK value of the γ-carboxyl group of glutamic acid (4.3) which is a constituent of the P.aeruginosa cell envelope. On account of its incorporation into cell wall protein the pK of this carboxyl group would not be expected to have the same value as when glutamic acid is in free solution. It is therefore possible that the single anionic species present on the surfaces of cells of both strains after treatment with FDNB is this same carboxyl group of glutamic acid. The pK value for the FDNB-treated cells of the high-level resistant strain (Figure VI.2) is approximately 5.0, which suggests that the surface of cells of this strain also possesses carboxyl groups, possibly also from the same glutamic acid; this conclusion must be regarded as tentative because of the unusual response to treatment with FDNB.

The response to treatment with FDNB is not the only similarity in behaviour between the gentamicin-sensitive

cells of strain 1 and the medium-level resistant cells of strain 7; the effect of mild oxidation, with sodium metaperiodate, on the surface structures of cells of these two types of strain was also similar. This oxidation produced, in cells of strain 1, an almost linear increase of mobility with pH. At pH $>$ 6 the mobility values for metaperiodate-oxidised cells of the medium-level resistant strain 7 were very similar to those for the oxidised cells of strain 1. The mobility values at pH $\leq$ 6, for cells of both strains, were not significantly different from the values for normal cells (Figure VIII.1). Treatment of cells of Staphylococcus aureus with sodium metaperiodate removed and destroyed cell-wall teichoic acid and this was reflected by the absence of the contribution of the teichoic acid phosphate groups to the cell surface charge which resulted in the absence of a peak mobility value at low pH values for treated cells (Hill and James, 1972a). An analogous situation is not observed with the periodate-treated cells of P.aeruginosa used in this investigation, most probably because teichoic acid is not an important envelope constituent; contrast cells of S.aureus. Nevertheless a readily oxidisable component is obviously removed from the cell surfaces of both gentamicin-sensitive and medium-level resistant cells, and this results in a surface which is similar for cells of both types, and comparison with the effects of treatment with FDNB would suggest the component removed has affected the cell-surface amino groups, as their blocking with FDNB also produces a similar surface for cells of the two types.

The isoelectric points extrapolated from the pH-mobility curves for all the gentamicin-sensitive cells lie in the pH range 1.5-2.0, for the medium-level resistant cells in the range 2.5-3.3, and for the high-level resistant cells in the range 2.5-2.7. The significant difference between the extrapolated isoelectric points for the sensitive and the two types of resistant cells is due to the different ratios of carboxyl to amino groups on the surfaces of sensitive and resistant cells (Table X.1). The charge densities (σ) of surface groups were calculated from the equation:

$$\sigma/C\ m^{-2} = 3.713 \times 10^{-3} \sqrt{c}\ \sinh\left[\frac{e\zeta}{2kT}\right]$$

where $c/mol\ m^{-3}$ is the concentration of the buffer solution used to measure electrophoretic mobility values; e is the charge on the electron; k is the Boltzmann constant; T/K is the temperature and ζ/mV the zeta-potential which is directly related to the electrophoretic mobility value. To calculate σ_{COO^-} the mobility value at pH 7.5 for cells treated with FDNB was used (Figures VII.1 and VII.2), i.e. when no protonated amino groups contributed to the surface charge. The mobility value at the same pH value for control cells was used to calculate σ_{total}, and assuming that the surface is comprised of constituents possessing only carboxyl and amino groups, algebraic subtraction gives the value of $\sigma_{NH_3^+}$. The pH-mobility curves for normal cells of all three strains and for cells of strain 8 treated with FDNB are complex (i.e. there may be a

rearrangement of surface components as the pH varies) therefore the ratios of the charge densities due to surface carboxyl and amino groups are only relevant to the state of the surfaces at pH 7.5. They do, however, provide a guideline for comparing the surfaces of the different cells under the same conditions and show that the ratio of carboxyl/amino groups for gentamicin-sensitive cells (1.91) is greater than that for medium- and high-level resistant cells (1.60 and 1.56 respectively). The greater proportion of carboxyl groups on the sensitive cell surface is responsible for the lower isoelectric point. The lower ratio of carboxyl/amino groups on the surface of the resistant cells is also reflected in the lower general level of negative mobility values for these cells, i.e. they exhibit a greater positive mobility due to the greater proportion of amino groups.

The importance of the amino groups in the surface chemistry of cells of P.aeruginosa is further indicated by the two different types of response to treatment with FDNB. It is observed that whilst the pH-mobility curves for untreated high- and medium-level resistant cells are very similar, treatment of these cells with FDNB and hence the blocking of the surface amino groups results in very different responses. The treated medium-level resistant cells have a pH-mobility curve which possesses a plateau (Figure VI.1) whilst the pH-mobility curve for FDNB-treated high-level resistant cells is transformed into a sinusoidal-shaped curve (Figure VI.2). Thus, not only are the surface amino groups responsible for the

TABLE X.1

Comparison of some properties of cells of various strains of *P.aeruginosa*

	Strain 1	Strain 7	Strain 8
MIC of gentamicin/$\mu g\ cm^{-3}$	1.25	25	8000
$^{**}\sigma_{Total} \times 10^3/C\ m^{-2}$	2.05	1.82	1.54
$^{*}\sigma_{NH_3^+} \times 10^3/C\ m^{-2}$	2.27	3.03	2.82
$^{*}\sigma_{COO^-} \times 10^3/C\ m^{-2}$	4.33	4.83	4.41
$\frac{\sigma_{COO^-}}{\sigma_{NH_3^+}}$	1.91	1.60	1.56
pK of surface carboxyl groups	4.7	4.7	5.0
Extrapolated isoelectric point	1.9	3.3	2.7

**From pH-mobility curves of control cells

*From pH-mobility curves of cells treated with FDNB

differences in shape and position of the pH-mobility curve between sensitive cells and resistant cells, but they are also responsible for differences of surface properties between cells with high-level and medium-level resistance to gentamicin.

Unfortunately the lack of detailed knowledge of the composition of the outer envelope layers of cells of P.aeruginosa precludes the assignment of the carboxyl groups, demonstrated to be constituents of the surface, to particular envelope components. However, a recent chromatographic analysis of the envelope components which contribute surface amino groups has been performed in this laboratory (Stewart, 1973). Whole cells were first treated with FDNB and then hydrolysed before the dinitrophenyl amino-acid derivatives, so liberated, were analysed. This showed that the free amino groups in the cell protein of cells of gentamicin-sensitive, medium-level and high-level resistant strains originated from L-alanine and lysine. These two amino acids, together with glutamic acid which, as discussed earlier, is the possible source of the surface carboxyl groups, are components of the cell envelope of P.aeruginosa (Mandelstam, 1962); whilst they are known to be associated with the underlying mucopeptide layer, they may also be present in the protein of the outer part of the double track layer of the cell envelope.

Further information on the surface anionic groups was obtained from the study of the effect of gentamicin in the buffer solution on the surface properties of the cells. This is a purely reversible phenomenon, since on

repeated washing the gentamicin is removed from the surface. The mobility values measured in the presence of gentamicin showed that a given concentration of the antibiotic caused a greater decrease in the mobility value of cells of medium- and high-level gentamicin resistant strains (strains 7 and 8 respectively) than in the mobility value of cells of the gentamicin-sensitive strain 1 (Figure III.7). This effect is probably due either to the reversible adsorption of gentamicin onto the cell surface or to the specific ion-pairing of cell-surface carboxyl groups with the amino groups of the gentamicin molecule. If the latter is the case then either the resistant cells possess more carboxyl groups, or the carboxyl groups on resistant cells are more favourably orientated for ion-pairing than they are on sensitive cells. The results of treatment with FDNB have already demonstrated that the resistant cells are likely to possess fewer carboxyl groups as the ratio of carboxyl/amino groups is lower than for sensitive cells, and so the latter explanation of specific ion-pairing is the more probable. Thus if gentamicin combines with the surface by specific ion-pairing, then the inference is that, although the surface pK values of FDNB-treated cells suggest that both sensitive and resistant cells possess surface carboxyl groups from the same source, the environment of those groups is different on the two types of cell; surface carboxyl groups on gentamicin-sensitive cells are probably more sterically hindered than those on both medium- and high-level resistant cells.

The reaction of the aldehydes formaldehyde, acetaldehyde

and glutaraldehyde with the charged groups on the surfaces of cells of the gentamicin-sensitive strain 1 was not as expected. Guthe (1959) considered that the interaction of formaldehyde with haemoglobin was *via* the amino groups of the protein. Furthermore, Heard and Seaman (1961) have made the statement that cationic groups, such as amino groups, present in the electrophoretic shear plane would react with aldehydes and these amino groups would then be unable to contribute a positive charge; the loss of this positive charge would be reflected, they claim, by an increase of negative mobility of the particles. Since it has been shown that erythrocytes do not possess surface amino groups, these authors were unable to confirm this theory. Treatment of cells of *P.aeruginosa* with FDNB confirmed the presence of surface amino-groups in the electrical double layer, and yet treatment with any of the three aldehydes mentioned caused no increase of negative mobility, in fact treatment with acetaldehyde produced a decrease in the negative charge. Whilst aldehydes do not react exclusively with amino groups, the fact that they cause an increase of positive mobility is indicative of a reaction with anionic groups, such as the carboxyl groups believed to be present, but as long as amino groups are present such a reaction seems very improbable. Acetaldehyde and formaldehyde may react unspecifically with other anionic groups or they may react with groups below the outer cell layers which form part of the electrical double layer, and in so doing reveal additional cationic groups in the shear plane.

Although exact reactions of these aldehydes with components on the cell surface of P.aeruginosa are not fully understood they are not simply, as predicted by Heard and Seaman (1961), reactions with cell-surface amino groups.

Munton and Russell (1972) observed that both aqueous- (i.e. acid) and alkaline-glutaraldehyde caused a decrease in the negative surface charge of both cells of Escherichia coli and spores of Bacillus subtilis, the greater decrease being observed in aqueous solution. This they attributed to the cross-linking effect of glutaraldehyde between amino groups in cell wall structures as proposed by Hughes and Thurman (1970) for B.subtilis. In accordance with the known ability of glutaraldehyde to cross-link, and so stabilise, proteins (Habeeb and Hiramoto, 1968) Munton's and Russell's evidence for the aldehyde-amino interaction is the absence of an inflection in the pH-mobility curve at pH 9-10 of glutaraldehyde treated cells. However, such an interaction is not wholly compatible with their observed decrease of negative mobility unless at the same time as glutaraldehyde may be cross-linking between amino groups, other surface changes are also occurring which cause the decrease in negative mobility.

The effect of glutaraldehyde, both under aqueous and alkaline conditions on gentamicin-sensitive cells of P.aeruginosa strain 1 was to convert the sinusoidal pH-mobility curve into a normal titration type curve with the plateau at approximately the same mobility value as the peak value (Figure VI.4). This may be attributed

to the cross-linking effect of glutaraldehyde stabilising the lower cell envelope structures and in preventing any rearrangement which was responsible for the sinusoidal-shaped curve. The fact that this cross-linking between amino-groups does not cause an increase in negative mobility value may be due to the same unspecific reactions at the cell surface that resulted in a decrease of negative mobility in the investigations of Munton and Russell (1972). Thus whilst no firm evidence is revealed as to the exact nature of the glutaraldehyde-cell surface interaction it is not simply, or wholly, an aldehyde-amino interaction as is often postulated.

The effects of the combined treatment of cells with glutaraldehyde and FDNB tend to confirm this hypothesis. The fact that pre-treatment of cells with glutaraldehyde prevents FDNB from blocking the surface amino groups, which would increase the net negative surface charge, shows that glutaraldehyde reacts with all the available surface amino groups. Furthermore the fact that the pre-treatment of cells with FDNB, and hence the blocking of the surface amino groups, does not prevent glutaraldehyde from causing a decrease in negative cell mobility values suggests that glutaraldehyde reacts with other surface components as well as with the surface-amino groups. It is these other, unspecified, reactions which are probably responsible for the glutaraldehyde treatment not causing an increase of negative mobility and these reactions must not be ignored when discussing the mechanism by which glutaraldehyde reacts when stabilising proteins and when

used for "fixing" preparations for electron-microscopical investigations

. Cells of P.aeruginosa with resistance to gentamicin, at whatever level, always exhibit a homogeneous response to the antibiotic at all temperatures. Although the MIC decreases with increase of growth temperature no heterogeneous response occurs, as was observed in the case of naturally-occurring methicillin-resistant S.aureus. Among the naturally-occurring strains of P.aeruginosa used in this investigation three types have been distinguished on grounds of the level of resistance to gentamicin, i.e. sensitive, medium-level resistant and high-level resistant, all of which also possess distinguishable surface characteristics. A fourth type of strain has also been studied; in this type, resistance was induced in the laboratory from a naturally-occurring sensitive strain and therefore possessed "trained" resistance to gentamicin. The resistance was acquired as a result of successive transfers of cells, originally sensitive to gentamicin, in media containing gradually increasing concentrations of the antibiotic. The mechanism by which the cells acquired resistance was probably either by a process of adaptation, or by a process of the selection of resistant mutant cells. In the former, the training can be envisaged as a gradual change of emphasis in the metabolic pathways

within the cells as proposed by Dean (1971). Thus as the cells experience a new or increased concentration of antibiotic, the metabolic process affected by the antibiotic is less used, and previously used minor pathways are developed and utilised which avoid the antibiotic action. In the second mechanism, only those cells which had mutated and which were therefore resistant to the increased antibiotic concentration survived and grew at the expense of the sensitive or less resistant cells. This process of mutant selection would occur at each increase of the antibiotic concentration; unless the mutation rate was exceptionally low the resistance would be acquired rapidly.

It is impossible to say with certainty which mechanism is operative during the training method used in this investigation as there is no certainty that all cells subcultured at a new antibiotic concentration formed colonies. However, the length of the growth period increased at each new antibiotic concentration and then gradually decreased during successive passages in the presence of the same concentration of antibiotic, returning almost to that observed before the concentration was increased. This suggests that the adaptive mechanism is operating by a gradual, enforced use of less favourable metabolic pathways; mutants might be expected to show no such variation in the length of the growth period. Furthermore it was only after the second increase in gentamicin concentration (i.e. the third different concentration experienced by the cells) that the

concentration of antibiotic in the growth medium exceeded the initial MIC of gentamicin for the cells.

Further evidence that the mechanism of acquiring the trained resistance was adaptive comes from the surface properties of the cells as revealed by their pH-mobility curves during training. Since cells required for electrophoretic studies were always grown in the absence of the antibiotic immediately prior to harvesting, the observed changes in the surface properties could not be attributed to gentamicin carried over from the growth medium, or to changes brought about by growth in the presence of the antibiotic. During the first few subcultures in the presence of a new concentration of gentamicin there was a large increase in the maximum mobility value, and in one case the characteristic sinusoidal shape of the curve was briefly lost (Figures IV.5 and IV.6). Further subculturing at the same antibiotic concentration, however, was accompanied by a decrease in the maximum mobility value until after a sufficient number of subcultures the pH-mobility curve was finally observed to be almost coincident with that of the sensitive parent cells. This pattern of disturbance of the cell surface properties was observed for cells trained to grow in the presence of gentamicin concentrations of 20 and 50 $\mu g\ cm^{-3}$ and also, probably, in 100 $\mu g\ cm^{-3}$. If the mechanism of acquiring resistance were that of mutant selection, the mutant cells would be expected to show consistent surface properties, i.e. not time-variable, even although they might be different from the parent cells.

The variation of the pH-mobility curve of cells during training may reflect the internal changes occurring in the cell whereby the metabolic route is changing in order to overcome the action of the antibiotic; as the cells become more able to metabolise in the presence of the given antibiotic concentration so the disturbance at the cell surface diminishes and the pH-mobility curve returns to normal. This in turn suggests that the synthesis of the cell surface structures is not directly affected by the antibiotic as increased tolerance to the antibiotic is not associated with a permanent change in surface properties. This might not be the case if gentamicin were an inhibitor of envelope synthesis and not of protein synthesis.

The surface properties of the trained cells suggest that this type of resistance is indeed a third, distinct, type of resistance, unlike the naturally-occurring resistance. Cells with natural resistance have pH-mobility curves distinct from those of sensitive cells, whereas the trained resistant cells all possess pH-mobility curves of the same sinusoidal shape as the parent, sensitive, cells, and although this is initially displaced to higher mobility values, continued subculturing in the presence of the same gentamicin concentration produces cells with pH-mobility curves very similar to that of the parent cells. Further evidence that the trained resistance is different from natural resistance lies in the lack of stability of the former. Cells trained to grow in the presence of a gentamicin concentration of 20 $\mu g\ cm^{-3}$

(MIC = 25 $\mu g\ cm^{-3}$) when repeatedly grown in the absence of the antibiotic became progressively more sensitive. The MIC of gentamicin for the cells decreased, after 130 subcultures to 3.1 $\mu g\ cm^{-3}$, i.e. almost back to that of the sensitive parent cells. Such a loss of resistance was never observed for cells of naturally resistant strains. Similar conclusions were reached by Weinstein et al (1971) who showed that laboratory-induced resistance was unstable and cells with this type of resistance were much more nutritionally demanding and less virulent than naturally resistant cells. However, these workers do not give the method of inducing the resistance, and therefore strict comparison is not possible.

It is interesting that the training of cells to be resistant to gentamicin caused, at the same time, training to the other aminoglycoside antibiotics; streptomycin, neomycin, kanamycin, but not to the unrelated antibiotic carbenicillin (Table II.1). Thus the mechanism of training overcomes the inhibition of a metabolic step which is the common site of action of at least these four aminoglycoside antibiotics, but which is not the site of action of carbenicillin. Furthermore, the growth of cells already trained to be resistant to gentamicin in the absence of the antibiotic not only causes the loss of gentamicin resistance but also the loss of resistance to neomycin, kanamycin and streptomycin, with carbenicillin resistance again unaffected. This result is in accord with the known structural and site-of-action relationships between the aminoglycoside antibiotics, and also with the

belief that gentamicin is the key member of this group of aminoglycoside antibiotics.

It is apparent from the phage and serological typing of the "trained" strains that the acquisition of resistance to gentamicin has caused profound changes in the cells (Table II.1). None of the trained strains 1A/20, 1A/50 or 1A/100 are serologically typable unlike their parent strain which belongs to type 6. Furthermore cells of these three trained strains show more than two differences in phage reactions from cells of the parent strain 1, and thus qualify as separate strains in their own right. Since phage reception is partly dependent on outer envelope structure, these differences in phage reaction suggest that training has caused changes in the envelope which may be beneath the surface, as cells of the parent strain and the trained strains eventually exhibit similar pH-mobility curves, i.e. they have the same ionogenic groups. Although the trained strains qualify as separate strains on the grounds of phage and serological typing, strain 1B which was originally trained to tolerate gentamicin in the growth medium (20 $\mu g\ cm^{-3}$) but later lost its resistance by growth in the absence of the antibiotic, is indistinguishable from the parent strain. Thus the laboratory-induced acquisition of resistance has a profound, but reversible, effect upon the cells.

The prolonged training of sensitive cells of P.aeruginosa strain 1 to grow in the presence of a gentamicin concentration of 20 $\mu g\ cm^{-3}$ was accompanied by a four-fold increase in the resistance of the cells

to the bacteriostatic effect of EDTA. This evidence of an apparent link between the effects of gentamicin and EDTA is of interest because of the different modes of action of the two compounds. EDTA is known to form stable chelating complexes with the same divalent metal ions that are essential to the integrity of the cell envelope. It is thus believed to be toxic to the bacterial cells (i) by extracting these ions from the envelope and so causing envelope disruption, (ii) by starving the nutrient medium of these ions, preventing the cell from synthesizing an envelope containing them, or (iii) by preventing the cell synthesizing those proteins for which divalent metal ions, especially Mg^{2+}, are required as cofactors at the ribosome. Mechanisms (i) and/or (ii) would suggest that the acquisition of resistance to gentamicin has produced a cell envelope more resistant to the removal of metal ions by EDTA or a cell envelope less dependent on these ions for structural integrity. Mechanism (iii) might be related to changes in ribosomal structure which allowed the cells to avoid the attack of gentamicin on protein synthesis which is occurring at the ribosome.

There is evidence for the effect of EDTA being, at least in part, at the cell surface, and hence on the cell envelope. Sub-lethal concentrations of EDTA had a profound effect upon the cell surface structures as revealed by the pH-mobility curves for cells of strains 1 and 8 pre-treated with EDTA (Figures VII.1 and VII.2). These curves showed the cells to exhibit different responses to EDTA-treatment, which are probably a result

of the different surface structures observed for normal cells; nevertheless the marked effect shows EDTA to attack the cell envelope. This attack probably removes divalent metal ions, and since the environment of these positively charged ions will be net negatively charged, it is this increase of negative charge which is responsible for the observed increase of negative mobility for cells pre-treated with EDTA.

Cells trained to grow in the presence of higher gentamicin concentrations (50 and 100 $\mu g\ cm^{-3}$) showed greater sensitivity to EDTA than did the cells trained to grow in the presence of a concentration of 20 $\mu g\ cm^{-3}$, and in one case the cells showed greater sensitivity than even the parent, gentamicin-sensitive, cells (Table VII.1). This suggests that in the initial stages of training, the disruption at the cell surface, as demonstrated by the disturbance in the pH-mobility curve, renders the envelope more susceptible to EDTA attack. However as the cells adapt to the new, higher, gentamicin concentration and the cell surface structures return to normal, (as shown by the pH-mobility curve), so resistance to EDTA builds up. This increased resistance to EDTA is unstable, as is the trained resistance to gentamicin, since growth in the absence of the latter results in the loss of resistance to both reagents, and this underlines the link between the effects of the two reagents.

Despite this link between gentamicin and EDTA, during training to the former, the spectra of activity of the two

reagents against cells of the various strains of P.aeruginosa show no obvious correlation (Table VII.1); this is probably a result of their different sites of action. The results of tests for synergism between the actions of the two reagents show that against 70% of the strains (excluding "trained" strains) used in this investigation, the combination of gentamicin and EDTA is synergistic (Table VII.1). Against the gentamicin-trained cells the combination displays a smaller degree of synergism, or none at all. This probably reflects the fact that the increased resistance to gentamicin of these cells is the governing factor despite whatever way the EDTA may assist the gentamicin in its attack. Consideration of the two possible sites of action of EDTA (metal-ion starvation of the cell envelope or the ribosome) suggests two possible modes of synergism between the two reagents; (i) EDTA disrupts the cell envelope and so assists the penetration of gentamicin to its target, the ribosome, or (ii) EDTA starves the ribosome of essential divalent metal ions which renders it more susceptible to gentamicin. Gilbert et al (1971) demonstrated that Mg^{2+} starvation of cells of P.aeruginosa by a selective choice of growth medium (analogous to Mg^{2+} starvation by EDTA) caused a decrease in the in vitro resistance of the cells to gentamicin, whilst Lieve (1965) showed that EDTA causes a non-specific increase in the permeability of cells to a wide variety of substances without necessarily causing death. Both results support mechanism (i), above, for the mode of synergism of gentamicin and EDTA, and if this is the case

it implies that normally there is a penetration barrier which hinders the arrival of gentamicin at the ribosome, and that this barrier lies in the cell envelope.

The investigation of the surface lipid possessed by cells of P.aeruginosa has demonstrated a further link between the cell envelope components and the resistance of the cells to gentamicin. Electrokinetic measurement of the cell surface lipid was effected by determining the amount of the anionic surface active agent sodium dodecyl sulphate (SDS) adsorbed at the cell surface. The lipophilic hydrocarbon chain of the SDS molecule dissolves in the surface lipid of the cells and leaves its hydrophilic polar head group (SO_4^{2-}) protruding into the medium. The effect of the added negatively-charged groups is to increase the anionic mobility of the cells in proportion to the amount of lipid; this is measured electrophoretically.

The results clearly show that cells of strains with natural resistance to gentamicin possessed significantly greater amounts of surface lipid than did cells of sensitive strains (Table V.1). Furthermore, there appeared to be a direct relationship between the MIC of gentamicin and the S-value, a measure of the amount of cell-surface lipid, for cells of the eight strains numbers 1 to 7 and 10 (Figure V.1). Cells of the high-level gentamicin-resistant strains numbers 8 and 9 do not follow this direct

relationship between MIC and S-value. The S-values for cells of these strains were not 300 times greater than those for cells of any of the medium-level resistant strains as would have been expected for a linear MIC-S-value relationship; the S-values were, nevertheless, much greater than for any other strain. This suggests that the uptake of SDS at the cell surface follows a Langmuir-type uptake isotherm, achieving a maximum S-value of approximately the same value as that obtained for cells of strains 8 and 9, and it confirms that increased levels of gentamicin-resistance are associated with greater amounts of surface lipid. Unfortunately cells with natural resistance in the range 100-1000 $\mu g\ cm^{-3}$, which would enable the turnover point to be established, seem rare and were unobtainable.

It was also observed that during the process of training, the cells acquired significantly greater amounts of measurable surface lipid. In the case of cells trained to grow at a gentamicin concentration of 20 $\mu g\ cm^{-3}$ (strain 1A/20) the increase was directly proportional to the increase in the MIC of gentamicin, in the manner observed for cells of the naturally-occurring resistant strains. Cells trained to grow in the presence of higher gentamicin concentrations (strains 1A/50 and 1A/100) also possessed S-values significantly greater than that for cells of the sensitive parent strain, although the increased levels of resistance were not connected with increases of surface lipid content beyond the value observed for cells of strain 1A/20.

The nature of the surface lipid is not revealed by this investigation, but since, after a period of initial disturbance, the pH-mobility curve for cells of the trained strain 1A/20 reverted back to that of the parent cells the inference is that the surface lipid is not phospholipid; an increase in the phospholipid content at the cell surface would be reflected by an increase of negative mobility value, if not a change in the shape of the pH-mobility curve and a lowering of the isoelectric point due to the phosphate groups. Also the proposal by Roberts et al (1970) and Wilkinson (1970) that divalent metal ions form bridges between the phosphate groups of wall components might suggest that cell envelope phospholipid would be disrupted by the EDTA-extraction of such ions. However, sub-lethal concentrations of EDTA did not cause significant changes in the S-value for cells of strain 8 (Table VII.2) providing further evidence that there is no surface phospholipid which is stabilised by divalent metal ions.

A parallel study has been carried out in this laboratory on the nature and amount of lipid in whole cells of some of the strains of P.aeruginosa used in this investigation (Yau, 1973). This showed that the total chloroform-methanol extractable lipid of cells of strains 1, 7, 8 and 1A/20 was constant for all four strains within the limits of experimental error, and that there was no qualitative difference detectable in the lipid components from cells of these four strains (Table X.2). However, whilst the amount of neutral lipid and free fatty acids was

TABLE X.2

The readily extractable lipid (chloroform-methanol) of whole cells of P.aeruginosa strains 1, 7, 8 and 1A/20 together with the electrophoretically determined S-values for the cells (a measure of the amount of cell-surface lipid)

Properties		Strain 1	Strain 7	Strain 8	Strain 1A/20
Total lipid (percentage of whole cell dry weight)		9.7	10.0	10.2	10.9
LIPID	Phospholipids	(i) phosphatidyl ethanolamine (ii) cardiolipid			
	Neutral Lipids	(i) tripalmitin (ii) 1,3-distearin (iii) unspecified monoglycerides			
	Free fatty acids	(i) palmitic acid			
Neutral lipids and free fatty acids (percentage of total lipid)		38	35	48	35
Phospholipid (percentage of total lipid)		62	65	52	65
S-value		4.5	16.1	67.0	26.6

approximately constant for cells of strains 1, 7 and 1A/20, that for cells of the high-level gentamicin-resistant strain 8 was significantly greater, at the expense of the phospholipid. These results suggest that the increase of the surface lipid content for cells of strain 1, when trained to grow in the presence of gentamicin (strain 1A/20), is the result of a redistribution of the lipid within the cell, as there is no change in total lipid content. Similarly, electrophoretic results have shown that the lipid appearing at the surface during training is neutral lipid, and the fact that cells possessing very high surface lipid contents show a significant increase in the proportion of neutral lipid suggests that the surface lipid detected for all gentamicin-resistant strains is neutral lipid.

There are precedents for correlations between the amount of lipid possessed by bacterial cells and their resistance to antibiotics and antibacterial agents. Hill et al (1963) showed that naturally-occurring cells of Streptococcus pyogenes with resistance to tetracycline possessed surface lipid and that the growth of tetracycline-sensitive cells in the presence of lipid-inducing agents resulted in the appearance of surface lipid. Similarly Hugo and Stretton (1966) showed that the appearance of surface lipid on cells of S.aureus was accompanied by increased resistance to penicillin-type antibiotics. Lowick and James (1957) trained K.aerogenes to grow in the presence of crystal violet and observed that this induced the appearance of surface lipid.

Furthermore, several workers (Hugo, 1967; Brown and Watkins, 1970; Brown and Wood, 1972) have proposed that the amount and type of lipid in whole cells, and especially in the cell envelope, plays a role in determining the drug sensitivity and resistance of cells of many organisms, including P.aeruginosa, by an exclusion mechanism. Such an exclusion mechanism would come under the heading of "prevention of access" amongst the general mechanisms of bacterial resistance to antibiotics discussed by Gale et al (1972); the other mechanisms they propose are the synthesis of enzymes capable of inactivating the antibiotic, the reduction of the physiological importance of the target and the modification of the target (I.6). In cells of many strains of P.aeruginosa resistance to aminoglycoside antibiotics is due to the synthesis of inactivating enzymes which are mediated by R-factors which can be transferred from one species to another. Transferable R-factor mediated enzymatic resistance could not be demonstrated for the cells of P.aeruginosa used in this investigation; whilst this does not exclude it as a possible mechanism which was not observed due to experimental reasons, it suggests that one or more of the other resistance mechanisms is operative, at least in conjunction with enzyme production.

The conclusion from the surface lipid and whole cell lipid analyses that the cells of P.aeruginosa used in this investigation are resistant to gentamicin, at least in part, by the prevention of access of gentamicin, in which cell lipid plays a role, is attractive. However, the

result of surface lipid measurements on cells originally trained to grow in the presence of gentamicin (20 $\mu g\ cm^{-3}$) but subsequently grown in the absence of the antibiotic show that although resistance to gentamicin is lost, the increased surface lipid content is not. This, in turn, suggests that the altered metabolic pathways whereby the cells became resistant to gentamicin stimulated the redistribution of neutral lipid to the cell surface, but when the pressure of the antibiotic was removed, the pathways responsible for resistance reverted back to normal. The cells were still, however, able to maintain an increased level of surface lipid suggesting that alternative pathways are still operative to some extent. This implies that the presence of surface lipid on cells resistant to gentamicin is a result of the resistance and not a cause of it. However, as has been discussed, trained resistance to gentamicin is a wholly different mechanism from naturally-occurring resistance, and therefore the information currently available permits no definitive conclusion as to whether the presence of surface lipid on cells of P.aeruginosa naturally resistant to gentamicin is a cause of, or a result of, that resistance, or whether they are associated properties originating from a common cause.

In conclusion, this thesis contains the first report of detectable differences between cells of P.aeruginosa which are sensitive or resistant to gentamicin. This difference is especially important when considering the conclusion of Weinstein et al (1971) that despite a

detailed microbiological investigation of several strains of P.aeruginosa, strains which were resistant to gentamicin were indistinguishable from strains which were sensitive, apart from their response to the antibiotic. The surfaces of all cells probably carry charged carboxyl and amino groups, the differences between the surfaces of the resistant and sensitive cells is due, in part, to the differing ratios of these two components. In addition it has been possible to distinguish cells with three types of resistance, i.e. medium-level, high-level and "trained". Although cells of all strains are known to contain the same proportions of total solvent-extractable lipid, this was composed of different types of lipid and it was distributed in different ways. There was little, if any, detectable surface lipid on cells of sensitive strains, while cells of resistant strains possessed significant amounts of neutral lipid at the surface.

Thus although the exact nature of the surface components of cells of P.aeruginosa have not been unequivocally identified, the general anionic and cationic groups have been characterised. Differences in the relative proportions of these and neutral surface lipid have been detected and related to gentamicin resistance. As yet it has not been established whether changes in these surface components are the result of the cells becoming resistant to gentamicin or whether the changes and the level of resistance have a common origin within the cell.

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