THE EFFECT OF NUTRIENT LIMITATION ON THE DRUG RESISTANCE OF *PROTEUS* SPECIES

A thesis submitted by

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A typical polymyxin-resistant *Proteus mirabilis*, NCTC 5887, was compared with a polymyxin-sensitive clinical isolate, RBH. Their quantitative nutritional requirements were found to be the same. A minimal medium was formulated. For sensitivity tests, an excess of KCl was added, because these strains, and other strains tested, formed stable small-colony variants, with drug-resistance patterns different from the original organisms. Variants grew readily in the minimal medium, but relatively slowly at high KCl concentrations.

Stationary-phase cultures induced by glucose-depletion (C-dep) were compared with cultures depleted of Mg^{2+} (Mg-dep) or phosphate (P-dep) before being glucose-depleted. Mg-dep and P-dep RBH were more resistant to polymyxin than C-dep. Although the minimum inhibitory concentrations of cetrimide, chlorhexidine and phenol were almost the same for these strains, the sensitivities of cells after nutrient depletion were very different. For example, the sensitivity to cetrimide of Mg-dep RBH > C-dep > P-dep, but for 5887 C-dep > Mg-dep and P-dep. Results were also modified by the constituents of the test menstruum. Using chemostat cultures, faster-growing *Proteus* were more sensitive to polymyxin than slow-growing ones.

5887 contained twice the 2-keto-3-deoxyoctonic acid found in RBH, and its cell walls contained more phospholipid. Mg-dep RBH cell walls contained more phospholipid than C-dep, and the ratios of individual phospholipids were changed. These results support the hypothesis that polymyxin binds to lipopolysaccharide and/or phospholipid in the outer membrane, resistance depending on a reduction in the amount of polymyxin reaching the inner membrane. No evidence was found that cell wall cations were related to resistance. The variations in drug sensitivity and cell wall chemistry demonstrate the need for defined growth conditions and reproducible inocula for the microbiological assay of antibiotics and other antibacterial agents.

Key words: *Proteus*, disinfectant testing, nutrient-depletion, cell walls, polymyxin.

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ABBREVIATIONS

EDTA	ethylenediaminetetraacetic acid
KDO	2-keto-3-deoxyoctonic acid
LPS	lipopolysaccharide
PL	phospholipid
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
DPG	diphosphatidylglycerol
PC	phosphatidylcholine
QAC	quaternary ammonium compound
CTAB	cetyltrimethylammonium bromide
EGTA	ethyleneglycol-Bis-(β-aminoethylether)-N:N'-tetraacetic acid
MIC	minimum inhibitory concentration
REL	readily extractable lipid
EIF	ether insoluble fraction

ORIGIN AND SCOPE OF THE WORK

It has been shown that cation depletion of *Pseudomonas aeruginosa* in batch culture increases resistance to polymyxin (Brown & Melling, 1969b; Boggis, 1971) and affects colligative and other properties and cell wall chemistry (Eagon *et al.*, 1975; Kenward, 1975). Nutrient limitation of *Ps.aeruginosa* in continuous culture also affects the cell wall chemistry and resistance to antibacterial agents (Robinson *et al.*, 1974; Melling *et al.*, 1974; Finch & Brown, 1975). These results led to the belief that changes in the growth environment result in gross alterations in the outer membrane of an organism which are reflected in its response to the action of antibacterial agents. Variations in response due to the environment could lead to misleading results when assaying antibiotics and other antibacterial agents microbiologically. It is therefore important to define the characteristics of cells in different environments for the production of reproducible inocula for such purposes.

Proteus species are usually highly resistant to polymyxin (Sud & Feingold, 1970) unlike other enterobacteria and *Ps. aeruginosa*. This has been attributed to the cell envelope (Teuber, 1969; Sud & Feingold, 1970).

It was the objective of this study to investigate the nutritional requirements of *Proteus mirabilis* and to exploit the effect of nutrient limitation of growth in bringing about envelope changes in order to investigate the resistance of *Proteus* species to polymyxin. Two strains of *P. mirabilis* were used, polymyxin-resistant NCTC 5887 and a polymyxin-sensitive clinical isolate RBH. A chemically defined medium suitable for the growth of both

strains in batch and continuous cultures was devised. The effect of different nutrient depletions on the resistance of 5887 and RBH to polymyxin and other membrane active agents was examined. The effect of growth rate and nutrient limitation on the sensitivity of continuous cultures to the same agents has also been investigated. In addition, the whole cell and cell wall chemistry of carbon depleted and magnesium depleted cultures have been compared.

1. INTRODUCTION

1.1 The Gram-Negative Cell Envelope

1.1.1 Introduction

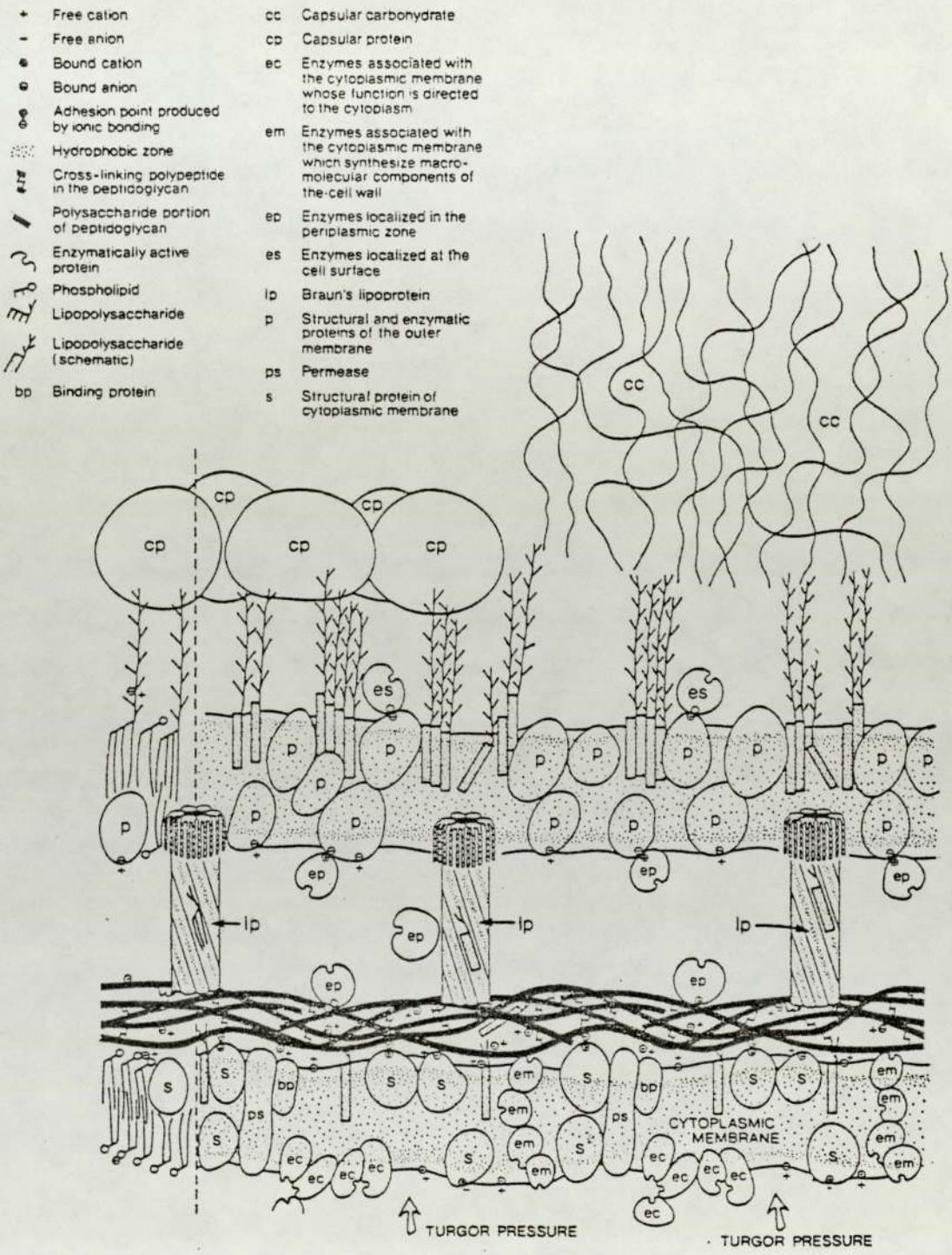
The cell envelope of gram-negative bacteria is a unique, multilayered and complex structure. It plays a vital role in regulating the internal environment of the cell and in the cell's interactions with external factors. It affords an explanation of the wide range of environments in which Gram-negative bacteria can grow.

In recent years, there have been many reviews on various aspects of the Gram-negative cell envelope. Early studies on its chemistry (Salton, 1964; Martin, 1966; Rogers & Perkins, 1968) led to models of its structure and functions (Glauert & Thornley, 1969; Martin, 1969; Reaveley & Burge, 1972; Braun, 1973; Costerton *et al.*, 1974). Figure 1 shows a model proposed by Costerton & Cheng (1975). Although some aspects of the outer membrane will have to be modified in the light of recent work (Nikaido & Nakae, 1979), it is still a useful basis for discussion. As can be seen, the cell envelope includes the cytoplasmic membrane, the rigid peptidoglycan complexed to lipoprotein, the periplasmic space, the outer membrane and other structures external to the outer membrane such as carbohydrate or protein slime layers. The basic chemical units are the peptidoglycan - lipoprotein complex (Rogers, 1970), lipopolysaccharide (LPS) (Osborn *et al.*, 1972) and phospholipid (PL) (Bell *et al.*, 1971).

1.1.2 The cytoplasmic membrane

The cytoplasmic membrane is the innermost structure of the cell envelope and separates the cytoplasm from the cell wall.

Fig. 1. The Gram-negative cell envelope (Costerton & Cheng, 1975)



Its chemical composition is similar to that of other biological membranes (Schnaitman, 1970; Martin & Macleod, 1971; Osborn et al., 1972). Singer's liquid crystal model (1972; 1974) of membrane architecture would appear to represent the arrangement of PL and proteins in the cytoplasmic membrane (Fig. 2a & b). In that model, the PL forms a liquid hydrophobic bimolecular layer which is modified by proteins "floating" in it (Jost et al., 1973). Proteins may be associated through hydrophobic interactions with either the inner or the outer aspects of the membrane (Fox, 1972). Some of these proteins appear to be permeases and involved in substrate transport through the relatively impermeable PL layer (Fox, 1972; Kaback & Hong, 1973); others are probably structural. Binding proteins have been shown to be associated with the cytoplasmic membrane (Pardee & Watanabe, 1968). These proteins are functionally important, and substrate transport may be greatly affected if specific binding proteins are released from the cells by osmotic shock (Berger & Heppel, 1972; Rosen & Vasington, 1971; Weiner & Heppel, 1971).

The cytoplasmic membrane is normally forced outward against the rigid peptidoglycan - lipoprotein complex by osmotic pressure (Costerton & Thompson, 1972), although in plasmolysed cells of Escherichia coli there appear to be regular areas of adhesion between the cytoplasmic membrane and the peptidoglycan - lipoprotein complex (Bayer, 1968), which persist even when the cytoplasm and cytoplasmic membrane shrink inwards in hypertonic conditions.

The cytoplasmic membrane is a vital interface for the cell envelope because within this layer the structural components of the cell wall are synthesized and assembled. Due to the close association between the cytoplasmic membrane and the cell wall,

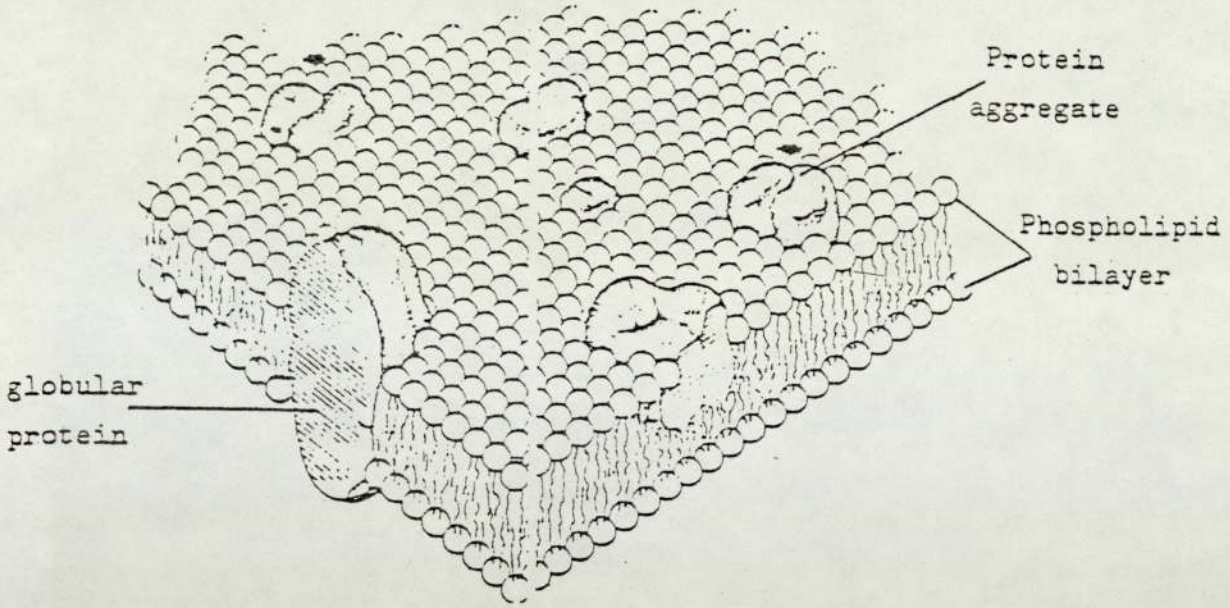


Fig 2a. A schematic representation of the three-dimensional organization of the cytoplasmic membrane (Singer, 1972).

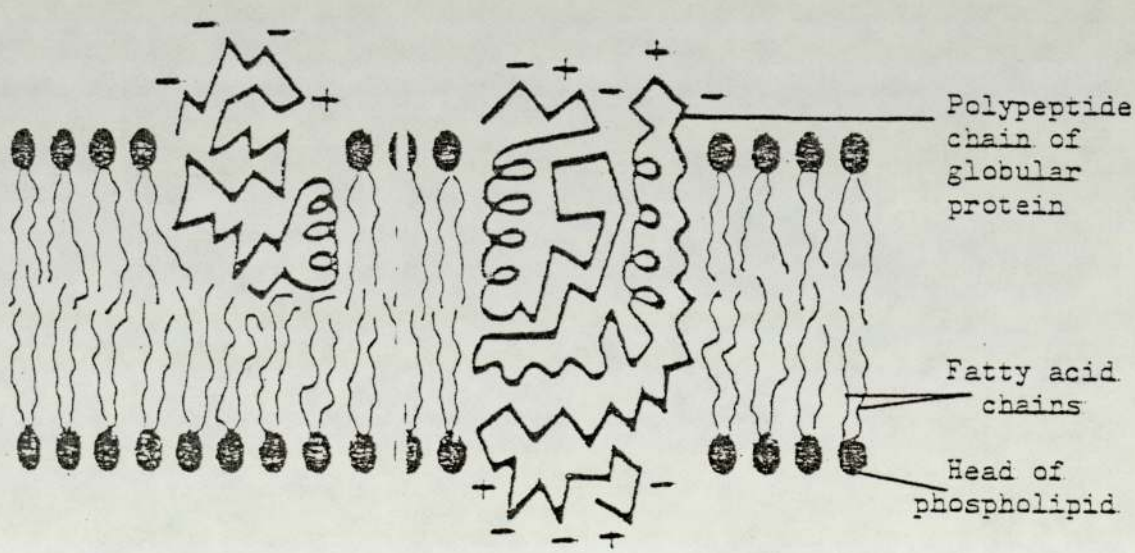


Fig 2b. A diagramatic representation of a cross section of the cytoplasmic membrane (Singer, 1972).

environmental conditions that affect the cytoplasmic membrane would reasonably be expected to produce effects in the composition of the outer membrane with resulting changes in the permeability control of the organism. Gilleland et al. (1974) using freeze-etching techniques, have shown that magnesium-depleted cultures of Pseudomonas aeruginosa are changed in both the outer and the cytoplasmic membranes. The cytoplasmic membrane of these cells appeared disorganized with the disruption of the net-like array of particles and large smooth areas. The appearance of large plaque areas was attributed to the loss of cytoplasmic membrane proteins. Corresponding changes have also been observed, under the same conditions of magnesium depletion, in cytoplasmic membranes of E. coli (Fiil & Branton, 1969).

1.1.3 The peptidoglycan-lipoprotein complex

In this rigid layer, the structural polymer peptidoglycan (sometimes referred to as murein) is associated covalently in many Gram-negatives with a substantial amount of a specific lipoprotein (Braun, 1973). The peptidoglycan is composed of polysaccharide chains that consist of alternating molecules of N-acetyl glucosamine and N-acetylmuramic acid linked by 1,4 glycosidic bonds. These polysaccharide chains are cross-linked by a peptide bond between the meso-diaminopimelic acid and the D-alanine of the neighbouring peptide side-chains (Fig. 3).

The major role of peptidoglycan is to maintain the shape and the rigidity of the cells. Forsberg et al. (1970) have shown that cells bounded only by their peptidoglycan layer maintain their shape. Isolated peptidoglycan "sacculi" also retained the shape of the cell from which they were derived (Forsberg et al.,

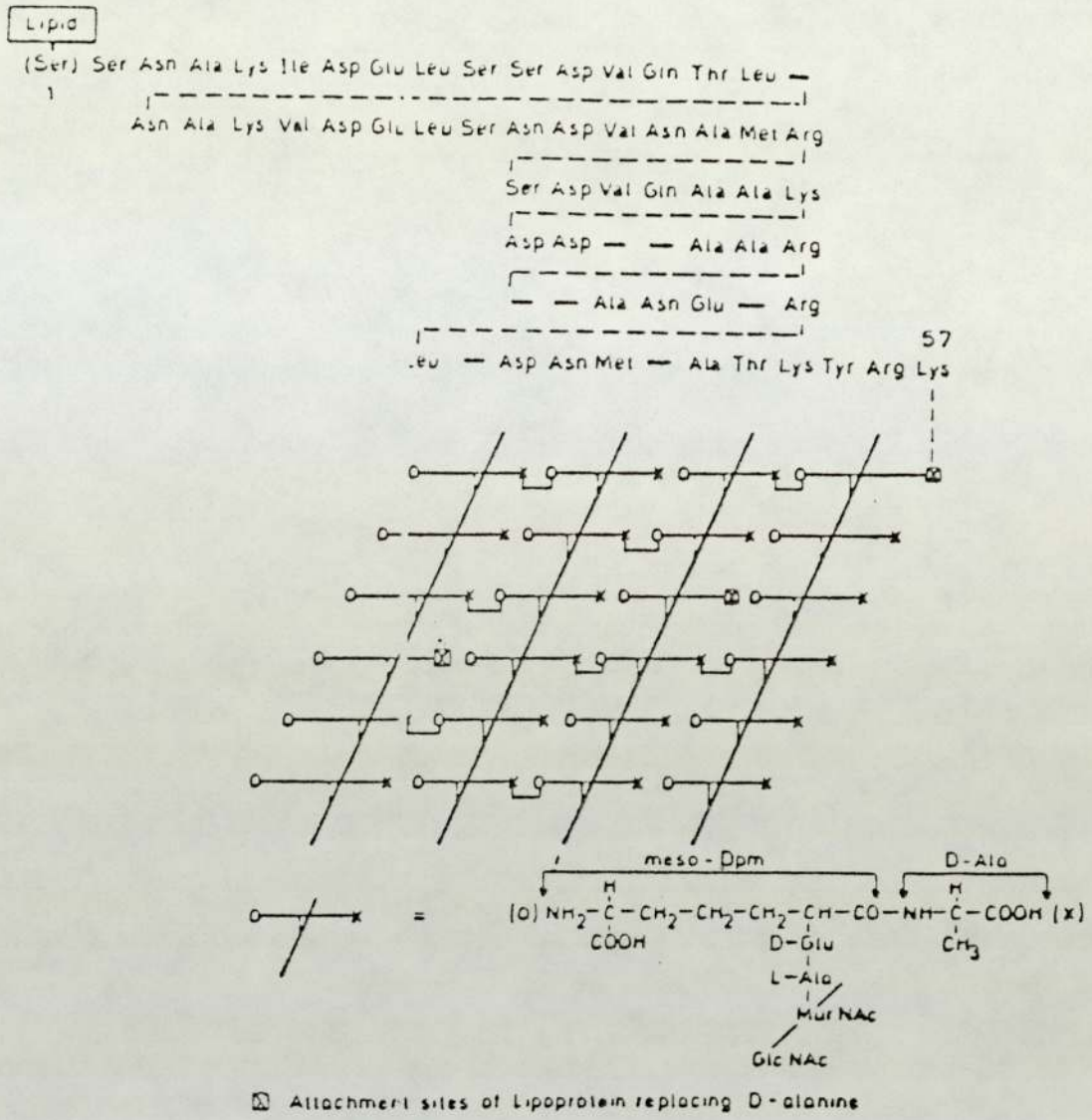


Fig 3. The lipoprotein-murein complex of E. coli (Braun, 1973).

The parallel heavy lines symbolize the polysaccharide chains. They are cross-linked by the T-like peptide side chains which are drawn to allow a long-range covalent fixation of the murein, which is a necessity for E. coli and other Gram-negative bacteria. Since E. coli is cross-linked only to the variable extent of 15 - 30% some links between meso-Dpm (O) and D-Ala (X) were left open. But despite the lack of some cross-linking peptide bonds, the conformation of the peptide side chain is considered to be the same for all. On average, one lipoprotein is covalently linked to every 10 - 12th disaccharide unit of the murein. three attachment sites are indicated in the murein net. The sequence of only one lipoprotein molecule is drawn and presented in a way that emphasizes its repetitive design.

1972). There is some evidence that the peptidoglycan layer may also act as a barrier to certain drugs (Burman et al., 1972; Tseng & Bryan, 1974).

Some of the peptidoglycan peptide side chains act as sites for the covalent bonding of the lipoprotein molecules (Fig. 3). It has been suggested that this covalently-linked lipid component serves to anchor the outer membrane by hydrophobic interactions with phospholipids in the latter (Schnaitman, 1971).

Proteus mirabilis was initially reported to lack the covalently-linked lipoprotein (Braun et al., 1970). However, its presence has since been firmly established (Gmeiner et al., 1978). These authors reported that there was a very significant difference in the quantity of lipoprotein in E. coli [1 lipoprotein per 10-12 subunits of peptidoglycan (Braun, 1975)] and in early stationary phase P. mirabilis, in which lipoprotein is linked to only about 1 peptidoglycan subunit in 80. They also noted a great variation in the amount of covalent lipoprotein in Proteus depending on the conditions of growth, with much higher quantities of lipoprotein being present at certain states of growth.

Metal cations are assumed to play no structural role in the peptidoglycan-lipoprotein complex, which was unaffected by ethylenediaminetetraacetic acid (EDTA) (Heilman, 1972).

1.1.4 The periplasmic space

The periplasmic space, which is unique to Gram-negative bacteria, is the area bounded on the inside by the cytoplasmic membrane and on the outside by the outer membrane of the cell wall. This implies that the periplasmic space and the peptidoglycan-lipoprotein complex occupy the same zone of the cell wall. This

area contains several exclusively periplasmic enzymes, such as guanosine 5'-triphosphate (Brockman & Heppel, 1968). Several other enzymes present are not exclusively periplasmic, but a large proportion of their molecules are found in this area (Dvorak et al., 1970). The penicillin degrading enzymes (β-lactamases) seem to be located in the periplasmic space of Ps. aeruginosa and other Gram-negative bacteria (Brown, 1975; Richmond, 1975).

There is much evidence which suggests that periplasmic enzymes are associated with LPS and other structural components of the cell wall and that they are distributed throughout the periplasmic space (Cheng et al., 1971; Garrard, 1972; Ingraham et al., 1973; Lindsay et al., 1973).

1.1.5 The outer membrane

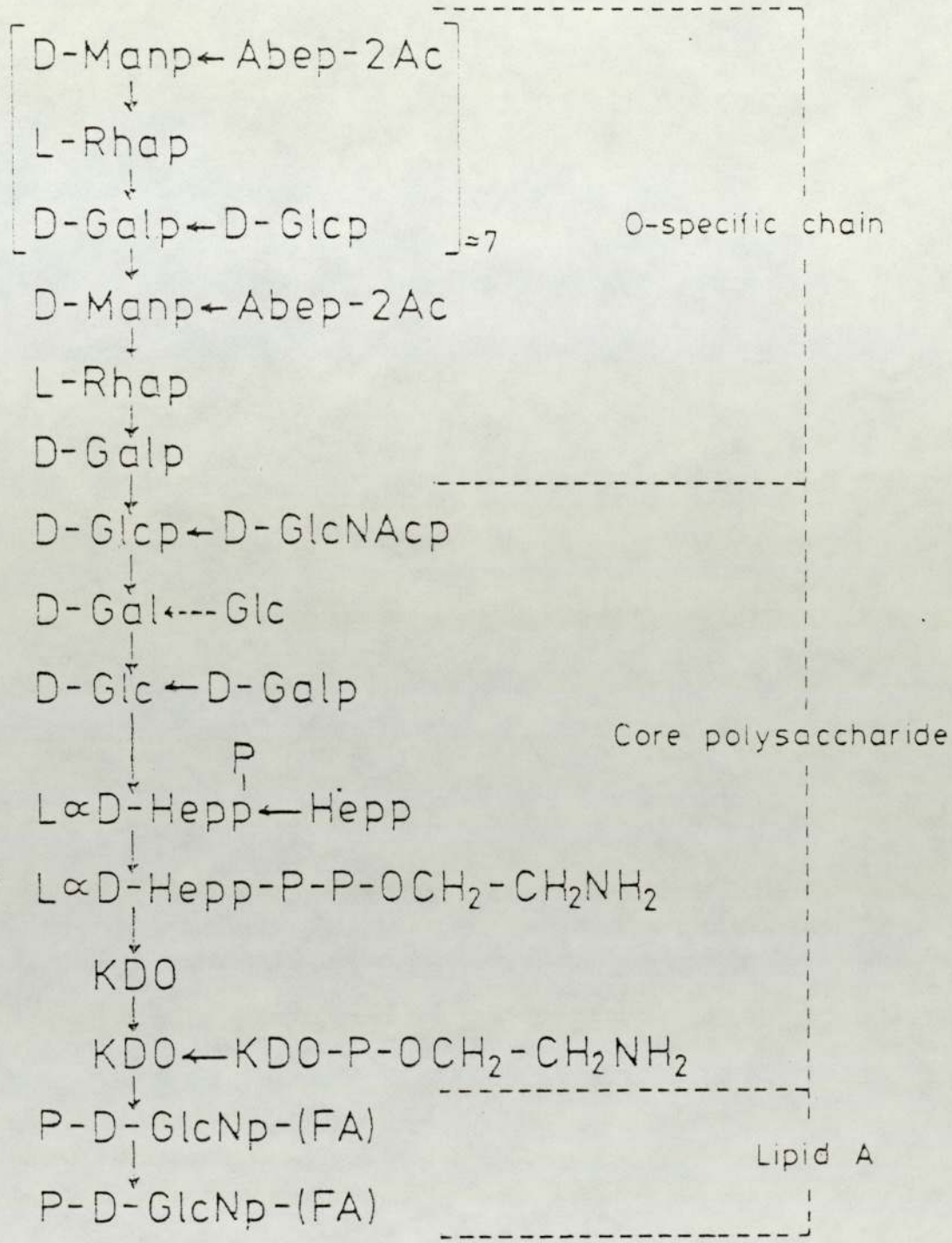
A recent review of the outer membrane of Gram-negative bacteria by Nikaido & Nakae (1979) has covered in detail the composition, structure, function, assembly and growth of this layer. The major components of the outer membrane are proteins, LPS and PL. The PL are hexagonally arranged in a closely-packed bilayer containing protein molecules (Costerton et al., 1974). In Enterobacteriaceae, the PL composition of the outer membrane is very similar to that of the cytoplasmic membrane; thus it contains mostly phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and very small amounts of diphosphatidylglycerol (DPG) (Osborn et al., 1972a). This supports the recent finding that there is a rapid exchange of PL between the two membranes (Jones & Osborn, 1977a, b).

Unlike the cytoplasmic membrane, the outer membrane is

asymmetrical. It contains LPS, which, it has been suggested, covers 41% of the outer surface of "smooth" cells, the remainder being covered by protein (Nikaido & Nakae, 1979). Fig. 4 shows the chemical structure of the LPS of Salmonella typhimurium (Luderitz et al., 1974). LPS is an amphipathic molecule with a hydrophilic, polysaccharide portion and a hydrophobic portion called lipid A. Glauert & Thornley (1969) suggested that the hydrophobic lipid A portion of the LPS is located within the hydrophobic core of the outer membrane, while the hydrophilic sugar part of the molecule protrudes into the surrounding environment. The hydrophilic portion of LPS usually consists of two parts. The peripheral portion called the O-antigen, consists of oligosaccharide repeating units and carries the serological specificity. In Salmonella, these oligosaccharide repeating units are mainly composed of neutral sugars, sometimes in combination with N-acetylated amino sugars. However, in P. mirabilis this portion of LPS contains, in addition, charged constituents such as uronic acids or amino acids (Gmeiner, 1975a).

P. mirabilis strain D52 seems to be unusual in that its O-specific polysaccharide contains ribitol phosphate and ethanolamine phosphate as charged constituents (Gmeiner, 1975b). Except for E. coli O100 (Jann et al., 1970), phosphate esters have not been reported as O-antigen constituents for other Gram-negative bacteria. The proximal portion of polysaccharide, called the "R-core", usually contains the sugar acid 2-keto-3-deoxyoctonic acid (KDO). This portion contains a very high density of charged groups. The absence of unsaturated fatty acids has been correlated with the low fluidity exhibited by the hydrophobic portion (endotoxic region, lipid A) (Nikaido et al., 1977).

Fig. 4. Structure of S. typhimurium lipopolysaccharide (Luderitz et al., 1974).



Man-Mannose; Abe-Abequose; Rha-Rhamnose; Glc-Glucose; Gal=Galactose; GlcN=Glucosamine; Hep=L-glycero-D-mannose; KDO=2-keto-3-deoxy-octonate; Ac=Acetate; p=Phosphate; P=esterified phosphate; FA=fatty acid

Gmeiner (1975a) suggested that P. mirabilis produces two types of LPS, which appear to differ in their amount of strain-specific O-antigen linked to the R-core - lipid A. These were LPS I and II. From amounts of KDO recovered with the two types of LPS, they calculated that only $^1/_4$ to $^1/_3$ of the R-core - lipid A moiety carried long chains of the O-antigen strain-specific polysaccharide (LPS II).

The role of the outer membrane in Gram-negative bacteria as a penetration barrier has been associated with that of LPS structure. It has been shown that "deep rough" mutants of S. typhimurium, whose LPS lack most of the saccharide chains are much more sensitive than the wild-type strain to certain antibiotics and dyes, mainly hydrophobic ones (Roantree et al., 1969; Nikaido, 1976).

Rapid progress has been made recently in the identification and quantitation of the protein components of the outer membrane, using the technique of sodium dodecylsulfate - polyacrylamide gel electrophoresis (Schnaitman, 1970; Bragg and Hou, 1972; Uemura and Mizushima, 1975). Braun (1975) and Schnaitman (1973a, b; 1974a, b) have identified and described the properties of the major proteins of the outer membrane.

Braun lipoprotein, a small protein, and often covalently linked to the underlying peptidoglycan, has already been discussed. "Heat-modifiable" proteins with molecular weights in the range of 32-37000, and often called major proteins, are present in E. coli and S. typhimurium. The heat-modifiable protein, 3b, with a molecular weight of 40,000 is synthesized in E. coli K12 only when the culture is grown at 37° or at higher temperatures (Lugtenberg et al., 1976).

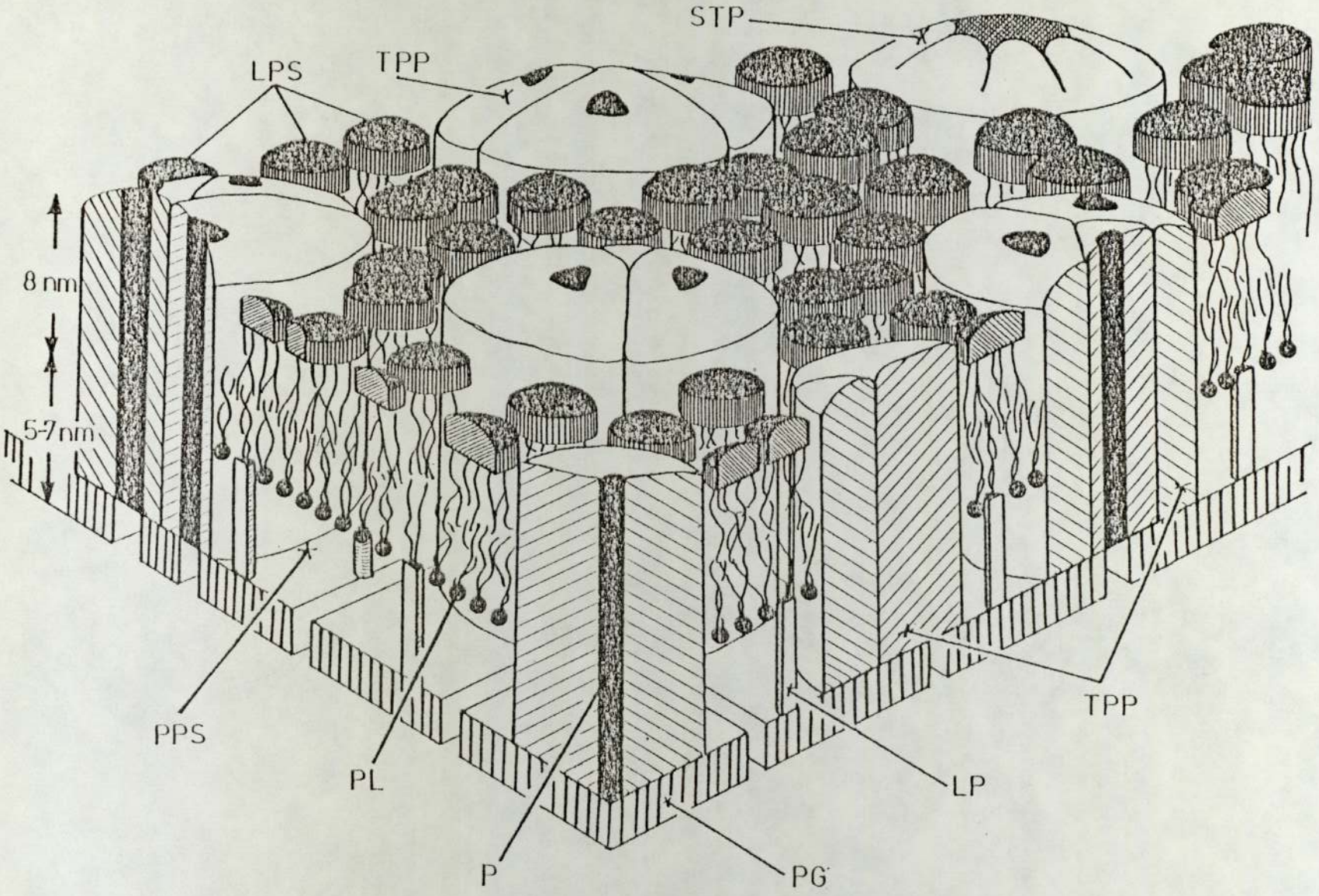
"Peptidoglycan-associated" proteins are also among the major outer membrane proteins of 32-37,000 dalton range. They have the unusual property of producing transmembrane diffusion pores when added to a PL - LPS mixture (Nakae, 1976a, b) and therefore are called "porins". Since diffusion channels are probably always necessary, porins may be expected to be a major part of the protein in the outer membrane of any Gram-negative bacterium (Nikaido & Nakae, 1979). Three species of porins were found in S. typhimurium (Ames, 1974; Ames et al., 1974; Nakae, 1976a). These were called "36K", "35K" and "34K" on the basis of their apparent molecular weights. E. coli B produces a porin similar to protein 1a of E. coli K12 (Rosenbusch, 1974). E. coli K12 produces two species of porins, 1a and 1b (Uemura & Mizushima, 1975; Lugtenberg et al., 1975; Schmitges & Henning, 1976; Bassford et al., 1977). E. coli O111 B4 and E. coli K12 strains lysogenic for phage PA-2 produce another porin, protein 2 (Schnaitman 1974a, b; Schnaitman et al., 1975; Diedrich et al., 1977). The total number of porin molecules found in a cell (E. coli) was 10^5 (Rosenbusch, 1974). It has been shown that mutation at the genetic loci involved in porin synthesis could cause the loss of any of these porins in E. coli and S. typhimurium (Foulds, 1976; Bassford et al., 1977; Nikaido & Nakae, 1979).

Three major outer membrane proteins of apparent molecular weight 39,000, 36,000 and 17,000 have been isolated from P. mirabilis (Nixdorff et al., 1977). These proteins were able to form hydrophilic pores by combining with outer membrane PL in a model membrane system, mediating the penetration of small hydrophilic molecules through the vesicle membrane in a manner similar to that observed with the native outer membrane (Nakae & Nikaido, 1975).

The trypsin-sensitive 39,000 protein and the peptidoglycan-associated 36,000 protein were equally effective in this function, whereas the 17,000 protein mediated little penetration of low molecular weight solutes. Unlike studies on other Gram-negative bacteria (Nakae, 1975; Nakae, 1976a, b), the reconstitution of Proteus model membranes did not require the presence of LPS, and divalent cations were not essential for the establishment of their permeability (Nixdorff, et al., 1977). Electron paramagnetic resonance (Nixdorff et al., 1978) provided evidence for the actual incorporation of outer membrane protein from P. mirabilis into the PL bilayer (Jost et al., 1973; Dehlinger et al., 1974; Bogges et al., 1976). These results were also consistent with the previous findings of Nixdorff et al. (1977) concerning the permeability properties of reconstituted model membranes.

Other proteins in the outer membrane are enzymes. In Enterobacteriaceae, phospholipase A, with a molecular weight of 28,000 dalton, was found to require Ca^{2+} for activity (Scandella & Konberg, 1971; Nishijima et al., 1977). Very recently, protease activity was detected in the outer membrane of E. coli K12 (Nikaido & Nakae, 1979). In Neisseria meningitidis, tetramethyl p-phenylenediamine oxidase activity was found in the outer membrane (De voe & Gilchrist, 1976). Outer membrane proteins appear to vary without affecting the viability of the cell (E. coli K12, 3b; E. coli K12 lysogenic for PA-2; mutants of E. coli and S. typhimurium (see p. 12).

Fig. 5 shows suggested structure of the outer membrane (Brown et al., 1979).


STP
LPS
TPP
8 nm
5-7 nm
PPS
PL
P
PG
LP
TPP

1.1.6 External layers and structures

Several Gram-negative bacteria have a regular patterned protein layer external to the LPS (Thornley et al., 1974). Other Gram-negatives also have a loosely-associated capsule or slime layer composed mainly of LPS. Other structures which extend out of the bacterial cell envelope are the flagella and pili. Koffler & Smith (1972) have reported on the structure and biochemistry of flagella. Pili are long thin appendages that arise from the cell surface and may serve for the transfer of genetic material and as receptors for RNA and DNA phages (Bradley, 1966, 1973), and be involved in pathogenicity (Brinton, 1967; Weiss & Raj, 1972).

1.1.7 The role of divalent cations in the Gram-negative cell envelope

Rouf (1964) has shown that magnesium, calcium, manganese, iron and zinc are present in significant quantities in Gram-negative bacteria (E. coli, Sphaerotilus natans). Eagon (1969) also reported mangesium and calcium as major elements, iron as a minor element, and zinc, lead, cobalt, copper, manganese and strontium as trace elements in the cell walls of Ps. aeruginosa. The whole cells of four strains of Proteus vulgaris contained comparable amounts of magnesium but much less calcium than the cells of Ps. aeruginosa growing under the same conditions (Brown & Wood, 1972). However, the P. vulgaris cell walls contained much less magnesium as well as less calcium in comparison with Ps. aeruginosa. Magnesium is considered to be a structural compoent of the outer membrane of Gram-negative bacteria (Costerton et al., 1974). Calcium has also been implicated in the structural organization

of the walls of Ps. aeruginosa (Asbell & Eagon, 1966) and E. coli (Leive, 1968).

Magnesium is an essential element for the growth of several Gram-negative bacteria in defined media (Webb, 1949). It is essential for ribosomal integrity and activity (Tissieres et al., 1959; McCarthy, 1962), the activity of many enzymes, including those involved in the synthesis of cell wall components (fatty acids: Knivett & Cullen, 1967; peptidoglycan: Garrett, 1969; LPS: Edstrom & Heath, 1967; PL: White et al., 1971), stability and permeability control in membranes (Laderberg, 1956; Brock, 1962) and ribosomal and ribonucleic acid (RNA) synthesis (Cohn & Ennis, 1967).

1.1.7.1 The structural role of metal cations in the Gram-negative cell envelope. Divalent cations, especially magnesium, play a very important role in the envelope of Gram-negative bacteria. This role is associated with the structure and stability of the envelope and with the activity of membrane-bound enzymes.

Laderberg (1956) demonstrated that magnesium is required for cytoplasmic membrane stability, since spheroplasts of E. coli tend to lyse when magnesium is not included in the medium together with sucrose 20% and penicillin 10^3 u ml^{-1}. Repaske (1958) found that the sensitivity of E. coli and Ps.aeruginosa to lysozyme was increased after pretreatment with an ion-exchange resin. He suggested that the metal cations present in the walls of untreated cells prevented the lysozyme action by steric hindrance.

It has been shown that E. coli treated with EDTA is more permeable to actinomycin (Leive, 1968). This treatment had not damaged the cytoplasmic membrane, since two transport systems tested were unaffected. The addition of metal cations could not

restore the impermeability to actinomycin without the occurrence of an energy-requiring process. It was concluded that EDTA removed magnesium from the outer membrane and caused steric or chemical changes which require energy metabolism for repair. The time and energy requirement for the repair of EDTA-damaged outer membrane observed by Leive (1968) was possibly for LPS synthesis and for binding and stabilizing of the new LPS layer. These two steps required energy and the presence of magnesium. This explanation is consistent with the finding of Leive (1965) that EDTA released LPS from the outer membrane and that the LPS layer is stabilized by cationic bridges (Costerton et al., 1974). It is also compatible with the observation of Sanderson et al. (1974) that mutant strains of S. typhimurium possessing defective LPS were more sensitive to actinomycin and to other drugs attacking targets inside the outer membrane than were strains with wild-type LPS.

The activity of penicillinase in cells of Aerobacter aerogenes and Aerobacter ozoenae was increased in the presence of EDTA (Hamilton-Miller, 1965). The author concluded that the EDTA altered the permeability of the cells to benzylpenicillin. This change of permeability was later found to be blocked by magnesium or calcium, but not by magnesium or calcium salts of EDTA (Hamilton-Miller, 1966).

Asbell & Eagon (1966) found that EDTA-treated cells of Ps. aeruginosa did not lyse when they were suspended in a hypertonic solution of sucrose. However, these cells were lysed on suspension in water and were termed osmoplasts. They were made stable by the addition of most divalent and trivalent cations. It was only the addition of cations normally present in the cell walls (Ca^{2+},

Mg^{2+} and Zn^{2+}) that permitted these osmoplasts to multiply. Monovalent cations did not restore the stability and viability of osmoplasts. This suggests that cross-linking was involved in restoring osmotic stability.

The cells of Rhizobium trifolii growing under conditions of calcium-depletion developed fragile walls even in the presence of a high magnesium concentration (Humphrey & Vincent, 1962). From this observation and from electrophoretic mobility studies (Humphrey et al., 1968) the authors suggested that calcium binding was below the surface of the outer membrane and might be involved in the packing of the peptidoglycan layer or in cross-linking between the peptidoglycan and LPS. This conclusion was supported by evidence presented by Boggis (1971). Using Ps. aeruginosa, he found that in certain conditions ethylene glycol-Bis-(β-aminoethylether)-N:N'-tetra-acetic acid (EGTA, a specific Ca^{2+} chelator) could not remove magnesium and disrupt the outer membrane and consequently could not penetrate to deeper structures which were stabilized by calcium. EDTA, because of its similar affinities for both cations, was able to remove magnesium and calcium and cause cell lysis.

Brown (1964, 1965) suggested that magnesium and other divalent cations might be involved in providing a stiffening mechanism for the lipoprotein in the membranes of Sarcina lutea by forming salt bridges between adjacent carboxyl groups. Shah & Schulum (1965) have reported the presence of similar salt bridges for PL.

Ps. aeruginosa and other pseudomonads with a high sensitivity to EDTA have an unusually high phosphate concentration in their cell walls (Wilkinson, 1975). These appear to be polyphosphate

residues substituted on the R-core polysaccharide and have high metal-binding capacity. It has been suggested that the outer membrane of Ps.aeruginosa depends upon cationic cross-linking between LPS anionic residues and other components for structural stability and integrity to a greater extent than other Gram-negative bacteria (Wilkinson & Galbraith, 1975). In P. mirabilis D52, the O-antigen specific chain of LPS II contains phosphate residues as ribitol phosphate and ethanolamine phosphate (Gmeiner, 1975a). If this is true with P. mirabilis it would imply more binding capacity for cations by the cell surface than other Gram-negatives. However, Proteus species are resistant to the action of EDTA (Chapman & Russell, 1978).

1.1.7.2 Role in enzyme-catalyzed reactions. The divalent cations (Mg^{2+} & Ca^{2+}) are also involved in activating specific enzymes associated with the synthesis of the cell wall. When the magnesium concentration decreased, the proportion of unsaturated fatty acids (C_{16} and C_{18}) in E. coli was increased with a corresponding decrease in the level of saturated cyclopropane fatty acids (C_{17} and C_{19}) (Knivett & Cullen, 1965). In vivo, magnesium stimulated the synthesis of PG but not PE or DPG in cell-free extracts of E. coli (Benns & Proulx, 1974). It was found that magnesium was the most effective activator of the synthetase enzymes which are located at the cytoplasmic membrane, whereas calcium was effective for lipases which are present in the outer membrane (E. coli: White et al., 1971; Bell et al., 1971; S. typhimurium: Bell et al., 1971; Osborn et al., 1972). These observations are compatible with the general view that in cellular systems, most of the magnesium is intracellular while calcium is extracellular or on the cell surface. The enzymes involved in LPS synthesis and for

peptidoglycan precursors in E. coli, both of which being located at the cytoplasmic membrane, have been shown to require magnesium for their activities (Ito & Strominger, 1964; Ghalambor & Heath, 1966; Edstrom & Heath, 1967).

1.1.8 Effect of nutritional deficiencies on Gram-negative bacteria

1.1.8.1 Magnesium. Lack of magnesium affects different metabolic, structural and morphological aspects of the bacterial cell.

A major effect due to magnesium deficiency is on ribosomal structure and function. Kennell & Magasanik (1962) observed changes in the ribosomal content of various bacteria when incubated in magnesium-depleted media. In a magnesium-limited chemostat culture of A. aerogenes, it was found that, on increasing the growth rate, the ribosome, RNA and magnesium concentrations in the cell were increased (Tempest et al., 1965). Tempest & Strange (1966) found that RNA : Mg ratio is constant in Mg^{2+}-limited A.aerogenes at several dilution rates, and the similar finding for Pseudomonas putida (Sykes & Tempest, 1965) supports the hypothesis that the ribosome and RNA content and hence rate of protein synthesis may well be controlled by the amount of magnesium available in the cells.

The magnesium concentration also effects the size of bacterial cells. Tempest & Ellwood (1969) reported that the size of magnesium-limited A. aerogenes cells grown in a chemostat increased with increases in the growth rate. This increase was accompanied by a decrease in the cell wall content of the cells. However, when the degree of magnesium-limitation increased by lowering the dilution rate, these cells synthesised cell walls which contained less total carbohydrate (Tempest & Ellwood, 1969),

but more LPS. The structure of the LPS was also altered. Washed magnesium-limited cells of Bacillus subtilis and A. aerogenes adsorb more magnesium than similarly treated phosphate-limited cells, when suspended in 0.017% NaCl containing different $MgCl_2$ concentrations (Meers & Tempest, 1970). This supports a previous observation of Tempest & Strange (1966) that magnesium is loosely adsorbed to the cell surface of A. aerogenes, when harvested from media containing excess magnesium, and can be removed by washing in normal saline. These two observations imply that magnesium-limited cells have little or no surface-bound magnesium.

The effect of magnesium-depletion on the chemistry of Ps. aeruginosa cell envelope was described by Gilleland et al. (1974). These authors found that the envelope of magnesium-depleted cultures contained more carbohydrate, KDO and protein, but less phosphorous, than of magnesium-adequate cultures. These changes were associated with the presence of an increased number of highly compact spherical units in the middle layer of the outer membrane, which are thought to comprise a protein - LPS complex (Rogers et al., 1969).

The magnesium content of magnesium-limited chemostat-grown A. aerogenes increased on raising the growth rate (Tempest & Strange, 1966), and was half that of the equivalent carbon-limited cells (Tempest et al., 1965). Fiil & Branton (1969) reported a 2-fold difference in magnesium content between magnesium-limited and carbon-limited E. coli cells, although no difference was detected in the magnesium content of the respective cell envelopes. Brown & Watkins (1970) found that a polymyxin- resistant strain of Ps. aeruginosa contained much less PL and magnesium compared with the sensitive strain. It was shown that the same strain

becomes resistant to polymyxin under magnesium-depleted conditions (Brown & Melling, 1969b). Günther et al. (1975) found an increase in the total phospholipid of Mg^{2+}-depleted E. coli and also a change in the pattern of PL with an increase only in DPG and PE. Relative to total extracted phospholipid, there was more DPG and less PG and PE. The increase in phospholipid content seems to correspond to the increase in membrane and infolding of the cytoplasmic membrane which was observed in magnesium-depleted E. coli by electron microscopy (Fiil & Branton, 1969). The same authors also reported an increase in the number of spherical subunits arranged in paracrystalline fashion in the middle layer of the cytoplasmic membrane of magnesium-depleted cells, similar to those found by Gilleland et al. (1974). Gilleland et al (1974) observed that these subunits were disorganised and were not removed by EDTA. They suggested that resistance to EDTA induced by magnesium-depletion was associated with an increase in the protein - LPS complex of the cell wall.

Significant changes in the wall cations and PL content after magnesium-depletion of Ps. aeruginosa have been reported by Kenward (1975). He showed that magnesium-depleted cells resistant to EDTA and polymyxin had a different PL pattern from the sensitive magnesium-adequate cells, and that magnesium-depleted cells grown in excess calcium or manganese had a similar phospholipid composition to that of magnesium-depleted cells but were sensitive to EDTA and polymyxin. Accordingly the author concluded that not only could PL composition be related to resistance to polymyxin as might be implied by several other workers (Brown & Watkins, 1970; Pechey et al., 1974; Feingold et al., 1974) but cation content might assume a role in this respect. Brown & Melling (1969b) and

Boggis (1971) also reported that magnesium-depleted Ps. aeruginosa loaded with calcium was almost as sensitive to polymyxin and EDTA as the control cells. Finch (1976) obtained similar results using the same strain of Ps. aeruginosa grown in batch and in continuous culture. These observations suggest that calcium may replace magnesium in a structural role in the cell wall and are compatible with the view that calcium is mainly involved extracellularly (William & Wacker, 1967).

Most of the evidence reviewed so far has shown that magnesium deficiency results in chemical and morphological alterations in the outer membrane. It may also affect the functioning of the cytoplasmic membrane. Brock (1962) found that magnesium-depleted E. coli showed an increased permeability to o-nitrophenyl β-D-galactoside, mimicking the action of novobiocin which binds magnesium. However, Sykes & Tempest (1965) found that the rate of oxidation of exogenous glucose was reduced by magnesium-limitation of Ps. putida in a chemostat and concluded that permeability to glucose was reduced and that the main effect of magnesium-limitation upon this process was at the site of glucose access into the cells. This conclusion is consistent with the evidence of Kunding & Roseman (1971) that the transport of sugars in E. coli is governed by magnesium-dependent enzymes at the cytoplasmic membrane.

Using a cell-free extract of E. coli, the addition of magnesium with adenosine triphosphate (ATP) was found to affect the distribution of ^{14}C-labelled glycero-3-phosphate in different PL. Most of the label was found in the glycerophosphate moeity of the phospholipid (Benns & Proulx, 1974). These authors suggested that the addition of magnesium and ATP stimulated the

formation of a larger pool of endogenous phosphatidyl group precursors, and it is possible that magnesium deficiency may result in a decrease of these precurosrs and consequently an alteration in the PL composition of the cell wall.

In conclusion, it seems that the main effect of magnesium deficiency, in addition to its effect on ribosomes, is on the structure of the cell envelope. This may be a reflection of changes in the synthesis of the components such as LPS, PL and protein. These chemical and morphological changes of the envelope may well affect its permeability to different substrates and antibacterial agents.

1.1.8.2 Phosphate. The role of phosphorus is mainly associated with the ribosomes and membranes. Tempest et al. (1966) reported that, regardless of growth rate, the molar ratio of cell-bound Mg : K : RNA : P remained constant in K-limited A. aerogenes grown in a chemostat. This indicated a functional relation between these substances. It also implied that the bulk of K and P is located at the ribosome together with the bulk of RNA and Mg. Changes in K, Mg and P content of A.aerogenes were also measured as functions of the bacterial RNA content (Dicks & Tempest, 1966). The alterations of RNA content were caused by changing the incubation temperature at a fixed dilution rate (under K-limitation) or by changing the growth rate at a fixed temperature (under Mg- or P-limitation). In all cases the alterations in RNA content were accompanied by corresponding changes in the bacterial Mg, K and P contents, and the same molar ratio of these substances was maintained. It was suggested that an intensive uptake system is present for phosphate in

phosphate-depleted cells of Micrococcus lysodeikticus (Friedberg, 1977), and the rate of transport increases in presence of K^+ and Mg^{2+}.

Horiuchi et al. (1959) studied the effect of phosphate depletion on the RNA content of E. coli. They found that the amount of labelled ^{32}P in RNA reached its maximum when the inorganic phosphate was exhausted and it then decreased by 30% over the next 3 - 4 h, to reach a plateau. It seems likely that a large portion of ^{32}P from the degraded RNA was utilized for DNA synthesis, because the growth of the bacteria (measured as absorbance at 650 mμ) continued until 3 - 4 h after the apparent exhaustion of inorganic phosphate. During this time, the amount of DNA and protein doubled and bacterial counts increased 5- to 10-fold, indicating a reduction in the content of nucleic acid and protein per bacterium. Finally, the cells assume a smaller, spherical form. Similar observations were also reported for Ps. aeruginosa (Hou et al., 1966).

Phosphorus is present in the cell envelope of Gram-negative bacteria as a constituent of PL and LPS. The high P content in LPS of several species of Pseudomonas has been correlated with their high sensitivity to EDTA (Wilkinson, 1968; Key et al., 1970; Wilkinson, 1975; Wilkinson et al., 1973). This suggests that divalent cations in the cell walls are associated with highly metal-binding phosphate groups forming inter- and intramolecular cross-linkings of cell wall components. This seems compatible with the report that phosphate-depleted Ps. aeruginosa was insensitive to lysis by EDTA and polymyxin and increasing sensitivity was linearly dependent upon phosphate concentration in the medium (Boggis, 1971).

Phosphate-depletion also induces the formation of intracytoplasmic membranes (Lutsch & Venker, 1969). Dorrer & Teuber (1977) demonstrated that a phosphate-depleted culture of Pseudomonas fluorescens produced less membrane PE, PG and DPG and concomitantly a positively-charged ornithine amide lipid was synthesized.

It has been shown that in E. coli, as the specific growth rate (governed by phosphate concentration) increases, the mean cell volume increases to a maximal value and then decreases. This is unlike when the growth rate is increased by increasing the concentration of glucose; the mean cell volume increased. The mean cell volume is not an exponential function of the growth rate (Shehata & Marr, 1971). These authors reported that the mean cell volume of phosphate-limited E. coli has a maximal value at a specific growth rate of 0.68 h^{-1}.

The phosphate concentration in the medium has also been involved in culture longevity. Gentry et al. (1971) demonstrated that phosphate concentrations high enough to distort secondary metabolism caused glycerol-grown cells of Ps.aeruginosa to die early in the stationary phase, unlike cells exposed to low phosphate concentrations or removed from the presence of lethal phosphate concentrations; these remain viable for many months. The same authors suggested that in studies involving post-vegetative growth phase cultures, the amount of phosphate available should be carefully controlled and buffer systems other than phosphate be employed.

1.2 Mode of Action of Polymyxin and Other Membrane-Active Antibacterial Agents

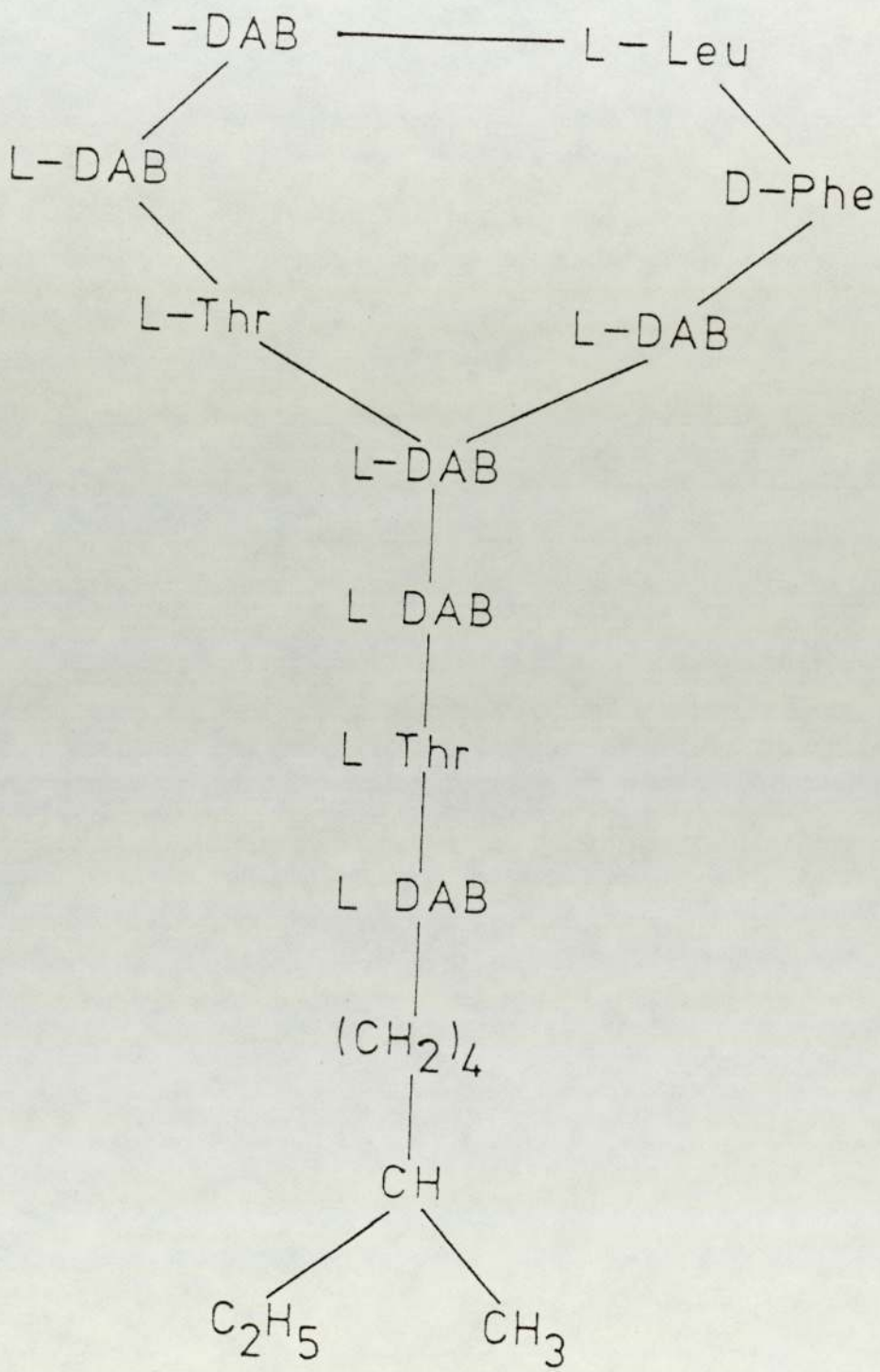
1.2.1 Introduction

A common feature of the antibacterial action of phenol, the cationic surface-active agents, cetrimide and chlorhexidine and the polymyxins,which are polypeptide antibiotics, is their ability to disrupt membrane permeability barriers (Newton, 1958; Russell, 1969; Longworth, 1971; Hamilton, 1971). For this reason they were termed "membrane-active" agents (Hamilton, 1971). These agents have similar effects on bacterial cells (Salton, 1968): absorption to the cell surface, membrane disorganization, leakage of low molecular weight compounds and finally gross lysis. High concentrations of such agents cause the release of low molecular weight compounds without gross lysis, possibly due to inhibition of autolytic enzymes (Newton, 1953; Rye & Wiseman, 1964) or the precipitation of cytoplasmic components (Hugo & Longworth, 1966). The polymyxins are surface-active and resemble the cationic detergents in their properties (Newton, 1953). However, cationic detergents are more active against Gram-positive, while in general polymyxins are more active against Gram-negative bacteria.

1.2.2 Polymyxin

The polymyxins (Fig. 6) are surface-active cationic cyclic polypeptides with both lipophilic (6-methyl-octanoic acid) and lipophobic groups (positively-charged amino acids in the cyclic peptide chain) (Few & Schulman, 1953; Newton, 1956). They exhibit a wide spectrum of antibacterial activity against Gram-negative bacteria and lesser activity against Gram-positive bacteria and fungi (Storm *et al.*, 1977). Among the

Fig. 6. Structure of Polymyxin B



DAB-diaminobutyric acid; Leu-leucine; Thr-threonine; Phe-phenylalanine

Enterobacteriaceae, only Proteus species and Serratia marcescens are naturally resistant to polymyxins (Russell, 1963; Sud & Feingold, 1972). These antibiotics are bacteriostatic at low concentrations and bactericidal at higher concentrations (Storm et al., 1977).

It appears that the primary site of action of polymyxins is at the bacterial cytoplasmic membrane resulting in rapid permeability changes (Few, 1955; Newton, 1956; Chen & Feingold, 1972; Teuber, 1974). Other effects of polymyxins on cellular functions, which include inhibition of active transport, respiration, RNA and DNA synthesis, LPS, peptidoglycan and protein synthesis, are likely to be secondary effects of the primary attack (Teuber, 1974). After treatment with a minimum bactericidal concentration of polymyxin, the permeability of sensitive bacteria for small molecular weight compounds is increased causing the release of materials absorbing at 260 nm (Newton, 1953). These compounds include adenine, adenosine, guanine and guanosine (Storm et al., 1977). The rate of their release is enhanced by increasing the polymyxin concentration. However, maximum leakage is observed in the presence of a polymyxin concentration which kills 99% of Ps. aeruginosa; higher concentrations inhibit leakage (Newton, 1953). The leakage of 260 nm-absorbing materials was also inhibited by divalent cations (Nakajima & Kawamata, 1965). This is consistent with other reports that Mg^{2+} and Ca^{2+} antagonize the antimicrobial activity of polymyxin B and its binding to bacteria (Newton, 1953; Chen & Feingold, 1972; Storm et al., 1977).

Teuber (1974) has examined the kinetics of the breakdown

of the membrane permeability of S. typhimurium by polymyxin B, using cells preloaded with ^{14}C-methyl α-D-glucopyranoside, and followed the efflux of the label following the addition of the antibiotic. He found that the effect of polymyxin B on the permeability of the cells was very rapid and could be detected within 15 - 30 sec contact with the antibiotic. The permeability changes either preceded or occurred simultaneously with cell death. Nucleic acid and protein synthesis were also inhibited. The latter effects are now thought to be secondary to membrane damage because polymyxin B can inhibit the growth and respiration of Gram-negative bacteria without entering the cells (Laporte et al., 1977). The rapid effects of polymyxins on bacterial membrane permeability would lead to an effect on respiration and oxidative phosphorylation (Storm et al., 1977). Wahn et al. (1968) reported the immediate decrease in oxygen uptake by E. coli following the addition of polymyxin B. The extent of inhibition was proportional to the antibiotic concentration. Teuber (1974) reported similar observations with S. typhimurium, although the response of inhibition was slower. A comparison between the kinetics for changes in membrane permeability and inhibition of respiration indicated that the effect on respiration occurred after changes in membrane permeability (Storm et al., 1977). Inhibition of bacterial respiration by polymyxin B has been shown to be accompanied by a depressed level of intracellular ATP (Storm et al., 1977). The rate of this decrease in ATP concentration was dependent upon antibiotic concentration.

For polymyxin to exert its primary action at the cytoplasmic membrane, it must first overcome the permeability barrier of the outer membrane. This in turn implies that polymyxin must

be able to interact with the LPS or protein of the external surface of the outer membrane. There have been several reports of the interaction of polymyxin with lipopolysaccharide. Lopes & Innis (1969), using electron microscopy, showed that when the LPS prepared from E. coli was exposed to polymyxin B, the typical ribbon-like structure broke down, with only short sections or completely disaggregated materials remaining. They concluded that polymyxin B causes loss of structural integrity of LPS. Bader & Teuber (1973) reported that polymyxin B binding to O-antigenic LPS of S. typhimurium involved electrostatic and possibly hydrophobic interactions. Using LPS isolated from mutants of S. typhimurium lacking various regions of the LPS side-chain, they were able to show that the lipid A region was the binding site. The more exposed the lipid A the greater was the capacity to bind polymyxin. All the lipid A fractions isolated from these mutants had identical polymyxin binding capacity. Earlier evidence that the presence of the O-antigen side chain influenced the binding of polymyxin had come from electrophoretic studies. McQuillen (1956) showed that the binding site of polymyxin was some way below the cell surface, but in a rough mutant (with exposed lipid A region) the presence of bound polymyxin at the cell surface could be detected. Lipid A is the source of the endotoxic activity of Gram-negative bacteria. The neutralization of endotoxin by polymyxins is further indirect evidence for polymyxin binding at the outer membrane. The lethality of E. coli endotoxin for chick embryo, rabbits and adrenalectomized mice was neutralized by polymyxin B and E (Rifkind & Palmer, 1966; Rifkind, 1967). Similar observations were made with the endotoxins of Ps. aeruginosa (Rifkind, 1967).

The same author reported that the antibiotic activity of polymyxin B was inhibited by endotoxin preparations and that endotoxins derived from polymyxin B-sensitive strains were more inhibitory than were those obtained from polymyxin-resistant strains. Corrigan & Bell (1971) and Cooperstock (1974) also reported inactivation of endotoxins by polymyxin. However, in contrast to Rifkind's results, Cooperstock (1974) found that the bactericidal and detoxifying properties of polymyxin B were not directly related, since polymyxin-resistant P. mirabilis produced highly sensitive endotoxin.

Changes in the morphology of the outer membrane as a result of polymyxin action have been revealed by electron microscopy. Wahn et al. (1968) observed protrusions or bleb formation at the cell surface of E. coli at polymyxin B concentrations up to 10 $\mu g\ ml^{-1}$. Higher concentrations caused rapid lysis and destruction of cytoplasm in addition to bleb formation. Koike et al. (1969) have also observed blebs on the cell surface of E. coli and Ps. aeruginosa upon treatment with polymyxin B or E. They also noticed that the number of blebs produced by polymyxin was increased with increasing antibiotic concentration and was inhibited by Mg^{2+}. Freeze-etching studies of polymyxin B-treated S. typhimurium revealed the presence of numerous blebs only on the outermost surface which corresponds to the external monolayer of the outer membrane (Schindler & Teuber, 1975).

Changes in the outer membrane permeability due to polymyxin B have been detected by the selective release of periplasmic enzymes (Cerny & Teuber, 1971, 1972; Teuber & Cerny, 1973). Page & Tsang (1975) have also reported the release of periplasmic enzymes from six strains of Ser. marcescens upon treatment with

polymyxin B . Other evidence for the destruction of the permeability barrier of the outer membrane arises from studies on synergism between polymyxin and other antibacterial agents. Sud & Feingold (1972; 1975) reported that pretreatment of P. mirabilis with polymyxin B renders the cells osmotically fragile and sensitive to some surface active agents and to the bactericidal action of normal serum. Polymyxin B has been used with lysozyme to produce spheroplasts (Warren et al., 1957; Teuber, 1970). This indicates that polymyxin causes the destruction of the outer membrane barrier, allowing lysozyme to reach the peptidoglycan layer.

The resistance of P. mirabilis to polymyxin was attributed to its outer membrane by Teuber (1969), who demonstrated that conversion of P. mirabilis to L-forms or spheroplasts increased its sensitivity to polymyxin B 400-fold. The sensitivity was lost after reconversion to the bacillary form. Sud & Feingold (1970) came to the same conclusion on the basis of liposome studies that showed equal polymyxin sensitivities for liposomes prepared from lipids extracted from several P. mirabilis strains with different polymyxin B sensitivity. This conclusion was also supported by the studies of Suling & O'Leary (1975) which showed that P. mirabilis was made sensitive to polymyxin B by several detergents. Similarly, Brown & Richard (1965) found that the activity of polymyxin B against Ps.aeruginosa was potentiated by the sodium salt of EDTA. Ps.aeruginosa becomes resistant to polymyxin B when Mg-depleted (Brown & Melling, 1969b). This suggests that Mg^{2+}-starvation changes the chemical nature of the outer membrane masking the initial binding site of polymyxin, or that the replacement of Mg^{2+} by organic cations

(Wilkinson, 1975) may prevent polymyxin from reaching its site of action at the cytoplasmic membrane.

The ability of LPS to complex with polymyxins in resistant cells may well be the basis of their resistance to these antibiotics (Tsang et al., 1976).

The effects of polymyxins on the cytoplasmic membrane are due either to direct interaction with the antibiotics or are indirect effects resulting from damage to the outer membrane (Storm et al., 1977). Newton (1956), using fluorescent-labelled polymyxin, showed that it was equally bound to the outer membrane and cytoplasmic membrane of Ps.aeruginosa. Binding was completely inhibited by added divalent cations (Newton, 1953), and he proposed that polymyxin and divalent cations were competing for the same negatively-charged phosphate groups on the cell surface. Teuber & Bader (1976a, b) reported that the two membranes (outer and inner) prepared from S. typhimurium bound equivalent amounts of polymyxin per mole of lipid phosphate. They suggested that polymyxins bind preferentially to negatively-charged phospholipids or lipopolysaccharide in the membranes.

The perturbation of cytoplasmic membrane structures after disrupting the outer membrane could result from the association of polymyxins with membrane lipids or protein or both. However, there is no evidence for polymyxin - protein association, and no indication of specific protein receptors in the membrane have been found (Storm et al., 1977). Therefore it can be concluded that the polymyxins bind and disrupt the structure of membranes by interaction with membrane lipids.

It has been shown that polymyxins have strong affinities for bacterial phospholipids and LPS and that the structure of

lipid aggregates, monolayers and liposomes are affected by polymyxins (Few, 1955). Bliss et al. (1949) showed that polymyxin activity was inhibited by exogenously added PL. Few (1955) found that the activity of polymyxin E against Pseudomonas denitrificans was inhibited by bacterial phospholipids and he proposed that the binding of polymyxins to the bacterial membranes involves electrostatic interactions with the negatively-charged phospholipids.

An additional mechanism of polymyxin action, involving proton exchange between polymyxin and the amino group of PE, leading to irreversible damage in membranes containing PE as the dominant phospholipid has been proposed (Hsu Chen & Feingold, 1973; Feingold et al., 1974). They found that the permeability of lipid bilayers for polar molecules was rapidly increased upon treatment with physiologically significant levels of polymyxin B. They concluded that polymyxin susceptibility of biological membranes requires the presence of a threshold density of PE, since liposomes containing PE were sensitive to polymyxin B, whereas those prepared from N-methyl, dimethyl or trimethyl substituted PE were insensitive. However, Teuber & Bader (1976a), on the basis of studies on the normally polymyxin-resistant Acholeplasma laidlawii fused with phospholipid vesicles, concluded that the presence of PE was not a crucial determinant for polymyxin susceptibility. Furthermore, Imai et al. (1975) reported that phosphatidyl choline (PC) (N-trimethy substituted PE) liposomes were sensitive to polymyxin B as long as they contained negatively-charged amphipathic molecules such as DPG. The low affinity of the polymyxins for PC is the most consistent observation that has been reported in various studies of polymyxin - PL association (Storm et al., 1977).

It seems clear that polymyxin-induced permeability changes are not dependent on the presence of PE, but do require the presence of negatively charged amphipathic molecules (Storm et al., 1977).

In conclusion, the sequence of events of polymyxin action can be simply summarized as follows. Initially polymyxin binds to the LPS of the outer membrane disrupting its permeability barrier to reach its primary site of action at the cytoplasmic membrane; at this site the antibiotic binds to the phospholipids causing permeability changes in the cytoplasmic membrane followed by inhibition of the biochemical processes of the cells. Cell lysis and death is either simultaneous with or after membrane damage.

1.2.3 Cetrimide

Cetrimide, a cationic surfactant with antimicrobial properties, is a mixture of quaternary ammonium compounds (QACs), composed mainly of tetradecyltrimethyl ammonium bromide together with smaller quantities of dodecyl and hexadecyl derivatives (Smith et al., 1975).

Several mechanisms for the antibacterial activity of QACs have been suggested. These include enzyme inactivation, denaturation of essential cell protein and disorganization and destruction of the cytoplasmic membrane (Sykes, 1965). Bacterial cells have a high affinity for QACs. McQuillen (1950) reported that excess QACs reduced or reversed the negative charge on bacteria. Salton (1951) studied the adsorption of cetyltrimethyl-ammonium bromide (CTAB) by six different organisms including P. vulgaris and found that the amount of CTAB adsorbed per cell at saturation level was more than the amount corresponding to a

closely packed monolayer of the detergent. This suggests additional binding sites for CTAB other than those on the cell surface. Further evidence for this was supplied by Salt & Wiseman (1968) who reported a diphasic uptake curve of CTAB by E. coli. He suggested that after the initial adsorption on the cell surface, the individual cells build up a critical amount of CTAB before the second phase of uptake and penetration into the cells commences. This conclusion agrees with that of Salton (1968), who postulated that the initial effect of a charged surfactant appears to be ionic adsorption at the bacterial surface until a sufficient concentration has accumulated to inflict damage on the cytoplasmic membrane, which then becomes permeable to small molecules. Leakage of 260 nm-absorbing materials takes place 5 min after exposure to CTAB and this is proportional to the percentage of cells killed by CTAB, using concentrations below that required for 99.99% killing (Salton, 1951). A rapid release of cellular constituents was observed using a concentration sufficient for maximum killing. Salton (1951) reported that leakage of cell constituents, inhibition of sensitive enzymes and cytological damage can all be observed simultaneously with cell death. He suggested that the interaction between detergent molecules and cell components such as proteins, enzymes and lipoprotein could account for all these events including cell death. Surfactants usually interact with these components, but the most likely mechanism of cytolysis appears to involve an interaction with the permeability barrier of the cell, specifically the anionic lipids of the membrane (Newton, 1958).

Cetrimide, like chlorhexidine, can precipitate the cell contents at concentrations greater than those which disorganize the

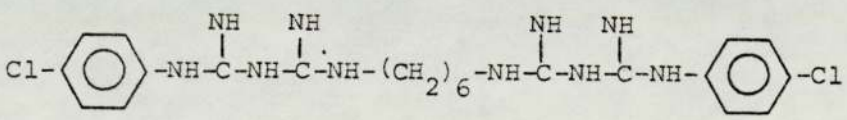
cytoplasmic membrane and cause lysis (Davies et al., 1968). The formation of spheroplast-like forms was concomitant with lysis in Vibrio cholerae (Parton & Jeynes, 1975).

As the concentration of Mg^{2+} in the suspending medium is increased, the maximum amount of CTAB taken up by the cells of E. coli is reduced by an amount that is a function of the Mg^{2+} concentration (Salt & Wiseman, 1970). These results suggest that the appearance and shape of the first phase of CTAB uptake is due to competition with such ions as Mg^{2+} for sites at the cell surface and that CTAB uptake is an ion-exchange process.

It has been shown that CTAB and cetrimide do not act in a similar fashion on growing cells of E. coli; CTAB tends to cause cell aggregation whereas cetrimide causes cell lysis (Smith et al., 1975). These authors suggested that the lytic effect is related to the presence of other QACs in cetrimide. This may be evidence indicating a relationship between alkyl chain length in cationic detergents and lysis.

1.2.4 Chlorhexidine

Chlorhexidine is another cationic surface active agent with a potent antimicrobial activity (Davies et al., 1954).



1,6-di-(4-chlorophenyldiguanido)hexane

The mode of action of chlorhexidine is in many ways similar to that of QACs and polymyxins (Hugo & Longworth, 1964; Davies et al., 1968). Adsorption isotherms for the uptake of chlorhexidine by the cells of E. coli and Staph. aureus is of an L-type diphasic curve (Hugo & Longworth, 1964). The failure of chlorhexidine to

neutralize the negatively-charged surface of E. coli, even in a concentration producing a level of drug adsorption much greater than the amount required to form a monolayer around the cell, suggests that chlorhexidine is not adsorbed in a monolayer form, but rather in aggregate accumulation (Hugo & Longworth, 1966). The uptake of chlorhexidine was increased with increasing pH and this was shown to be due to a change in the ionization of the cell surface (Hugo & Longworth, 1964). The leakage of cellular constituents on treatment with a low concentration of chlorhexidine suggests that membrane disruption is a direct and primary effect of chlorhexidine action (Hugo & Longworth, 1964), while at high concentrations, chlorhexidine inhibits leakage by precipitation of the cytoplasmic constituents, although such high doses are more rapidly bactericidal. Inhibition of autolytic enzymes or congealing of the cytoplasmic membrane was suggested as an explanation for such effects (Hugo & Longworth, 1964). The cytological observations reported by Hugo & Longworth (1965) agree with the biochemical results; a chlorhexidine concentration which causes maximum leakage ruptures the cells leaving empty ghosts, and higher concentrations cause the appearance of a coagulated cytoplasm but without loss of cytoplasmic materials. Newton (1953), using polymyxin E, showed similar biochemical and cytological effects with Ps. aeruginosa. High chlorhexidine concentrations which cause maximum leakage also inhibit dehydrogenase activity in E. coli (Hugo & Longworth, 1966). The same authors reported that chlorhexidine was unable to cause cell wall lysis or impair cell wall synthesis.

The effects of chlorhexidine on Clostridium perfringens are similar to its effects on aerobes (Hugo & Daltrey, 1974).

Chlorhexidine inhibits RNA, glycolysis and protein synthesis at concentrations below the minimum inhibitory concentration (Daltrey & Hugo, 1974). At high concentrations, RNA and protein were precipitated; also chlorhexidine did not facilitate ion passage or proton translocation across the cytoplasmic membrane. Membrane ATPase and the active transport of amino acids were also inhibited.

In conclusion, it seems that chlorhexidine exerts a range of effects on the cytoplasmic membrane, from inhibition of biochemical functions to structural damage and leakage. High concentrations cause gross damage resulting in lysis (in the case of C. perfringens), killing and precipitation of the cell contents.

1.2.5 Phenol

Phenol is an anionic antiseptic. Its bactericidal activity is related to the degree of uptake by the cells (Bean & Das, 1966). These authors have shown that the adsorption isotherms for phenol and other phenolic derivatives are bilinear and resemble Giles isotherm type "S" (Giles et al., 1960). Gilbert et al. (1978) reported "C" type adsorption isotherms for another group of uncoupling phenolics, with the exception of two compounds which exhibited "Z" curve isotherms (Giles & Toliu, 1964; Giles et al., 1974). They demonstrated a correlation between drug concentration at the inflection point of the isotherm and the marked structural disorganization of the cytoplasmic membrane and outer layer of the cell envelope of the tested organisms; gross cytoplasmic membrane damage was indicated by the release of cellular constituents. This suggested that increased phenol uptake resulted from an increase in the available interactive surface for adsorption (Gilbert et al., 1978). Similar findings were reported earlier

by Bean & Das (1966), but protein precipitation instead of leakage was observed. This may well be due to different effective concentrations for different phenolic compounds used in the two studies, since high phenol concentration reduced the leakage after coagulating the cell contents (Pullman & Reynolds, 1965). Measurements of electrophoretic mobility have also shown a correlation between the uptake and toxicity of phenols (Loveday & James, 1957).

Phenol itself has been shown to cause damage to the permeability barrier of E. coli, permitting the release of low molecular weight substances (Pullman & Reynolds, 1965). This suggests that cytoplasmic membrane damage is the primary effect of phenol, with the lipids and protein as the target sites (Judis, 1966; Starr & Judis, 1968). The addition of a bacteriostatic concentration of phenol was shown to inhibit immediately the synthesis of protein, RNA and DNA in growing cells of E. coli, although respiration and synthesis of ATP continued for some little time (Pullman & Reynolds, 1965). After the removal of phenol, the cells rapidly recovered. This suggests that apart from the membrane damage and consequent leakage of cellular materials, no other significant damage has been observed. The action of phenol on the cytoplasmic membrane of the same organism has also been shown to affect its permeability to sugars and access of organic acids to oxidizing enzymes (Hugo & Street, 1952). Judis (1966) found that binding of 2,4-dichlorophenol was affected by changes in pH of the reaction mixture, while the other compounds including phenol itself were bound to the same extent between pH 4.9 and 9.6. These results suggest that the ionized form of a phenolic compound is bound considerably less than the

unionized form. This implies that unionized phenol is the active form; consequently phenols are more active at acid pH values. Organic materials, including proteins like serum albumin, were shown to interfere with the action of phenols (Judis, 1966), possibly by competing for phenols with the bacterial cell or preventing access of phenols by coating the cell.

1.3 Bacterial Growth in Continuous Culture

1.3.1 Introduction

In batch culture, the growth rate is related to the limiting nutrient at low concentrations, but not at high concentrations which allow maximum growth rate. Microbial growth in continuous culture takes place under steady state conditions; that is, growth occurs at a constant rate and in a constant environment. The rate of growth in continuous culture can be maintained at submaximal rates by controlling a steady state concentration of the growth limiting nutrient in the culture below that required for maximum growth rate. Unlike batch culture, in continuous culture parameters such as pH, oxygen, metabolic products and population densities are kept constant and can be easily controlled. The most commonly used continuous culture is a single-stage flow-controlled system. This was first described by Monod (1950), and independently by Novick & Szilard (1950) who named it the "chemostat".

1.3.2 Theory

Exponential growth of bacteria in batch culture is represented by the equation:

$$\frac{dx}{dt} = \mu x \qquad (1)$$

where x is the concentration of organisms (dry weight/unit volume) at time t and dx/dt is the growth rate (actual rate of increase of concentration of organisms). Equation (1) can be expanded to define the specific growth rate (μ) (rate of increase/unit of organism concentration) of bacterial culture in relation to the doubling time (t_d):

$$\mu = \frac{1}{x} \cdot \frac{dx}{dt} = \frac{d\ln x}{dt} = \frac{\ln 2}{t_d} \qquad (2)$$

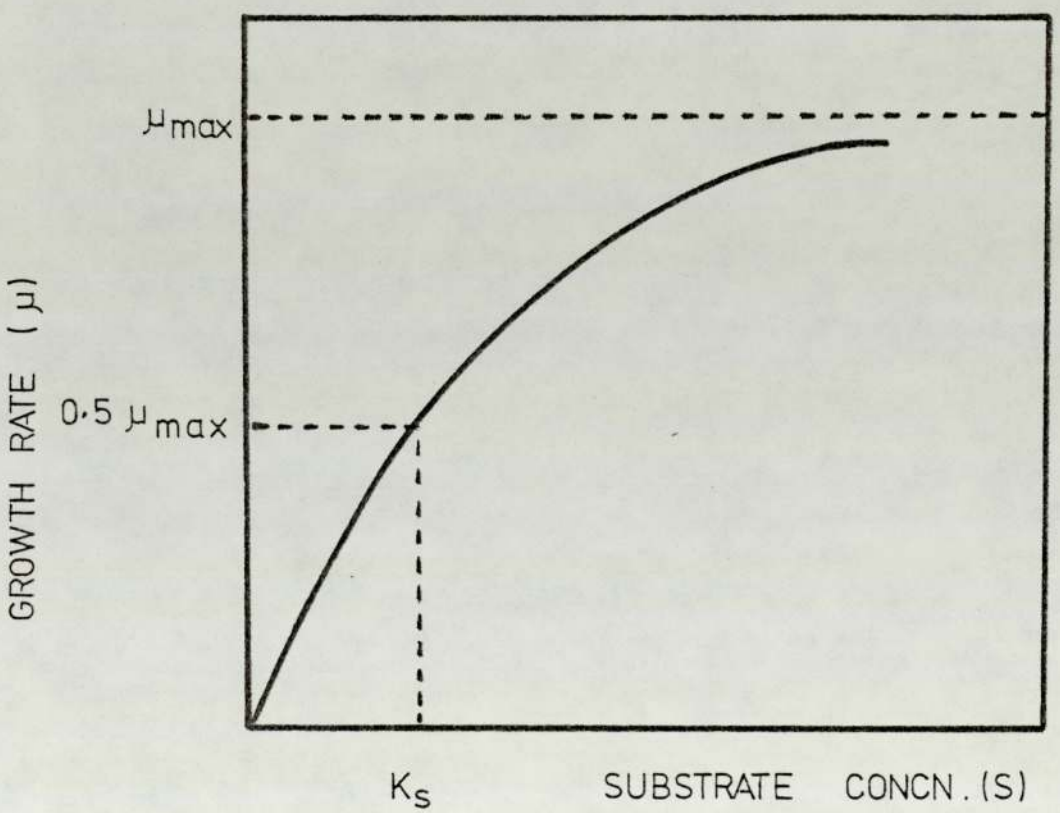
In the above equation, μ and t_d are usually assumed to be constants; this is only correct when all substrates necessary for growth are present in excess (Herbert et al., 1956).

When bacteria are inoculated into a suitable growth medium, growth will take place and continue at the possible maximum rate under the given conditions until one of the essential nutrients in the medium is depleted. If all other nutrients are in excess, this nutrient is called the growth-limiting nutrient. Monod (1942, 1950) found the relation between the specific growth rate and the concentration of the growth-limiting nutrient:

$$\mu = \mu_m \left(\frac{S}{K_s + S}\right) \qquad (3)$$

where μ_m is the maximum growth rate, S is the concentration of the growth-limiting substrate and K_s is the saturation constant, numerically equal to the nutrient concentration at which $\mu = \frac{\mu_m}{2}$. According to this equation, the specific growth rate varies with the concentration of the growth-limiting nutrient. A diagrammatic representation of equation (3) shows a Michaelis-Menten type of saturation curve (Fig. 7). This shows that μ is proportional to the growth-limiting substrate at low concentrations but it approaches a maximum value μ_m as the substrate concentration increases. Monod (1942) also found that in batch culture there is a simple relationship between growth and utilization of substrate. The growth rate is a constant fraction, Y, of the substrate utilization rate:

Fig. 7. Relation between the specific growth rate (μ) and the growth limiting substrate concentration (S). μ_{max} is the maximum specific growth rate and K_s, a constant (Harder et al., 1977).



$$\frac{dx}{dt} = -Y \frac{ds}{dt} \qquad (4)$$

where Y is the yield constant = $\frac{\text{weight of bacteria formed}}{\text{weight of substrate used}}$

In a continuous culture vessel, the growth rate of bacteria is governed by the dilution rate (D) of the culture, where $D = f/v$, f = flow rate of fresh medium added to the culture vessel/unit time and v is the volume of the culture. Therefore the dilution rate is the number of volume changes per unit time and its dimension is reciprocal time, usually h^{-1}. The residence time of non-dividing particles is determined from D. Thus the rate at which particles are lost in the effluent is proportional both to the number remaining, x, and to D.

$$-\frac{dx}{dt} = Dx \qquad (5)$$

and $\frac{1}{D}$ is the mean residence time of a particle (Powell, 1965).

The increase in the concentration of bacteria in the continuous culture vessel is shown by the balance equation (Herbert et al., 1956):

increase = growth rate - rate of output

and since growth rate is μx (equation 1) and rate of output is Dx (equation 5)

$$\frac{dx}{dt} = \mu x - Dx \qquad (6)$$

$$= x(\mu - D)$$

From the balance equation, it can be seen that when $\mu = D$, $\frac{dx}{dt} = 0$, x is constant and the culture will be in a steady state. However, this equation does not define the dilution rates at which the steady state is possible. These can be determined from the effect of dilution rate on the concentration of substrate in

the culture vessel, since μ_m depends on S (equation 3).

In a steady state

$$x(\mu - D) = 0$$

$$\text{and } \mu = D$$

therefore from equation 3

$$D = \mu_m\left(\frac{\bar{S}}{K_s + \bar{S}}\right) \qquad (7)$$

($\bar{S}$ is the concentration of growth-limiting substrate in a steady state).

If the growth-limiting substrate is entering the culture vessel at a concentration S_r (reservoir concentration) and flowing out at a concentration S, the net rate of change of substrate concentration can be obtained by another balance equation (Herbert et al., 1956):

$$\text{Increase} = \text{input} - \text{output} - \text{consumption}$$

$$= \text{input} - \text{output} - \frac{\text{growth}}{\text{yield constant}} \quad \text{(from eq. 4)}$$

$$\text{therefore } \frac{ds}{dt} = DS_r - DS - \frac{\mu x}{Y} \qquad (8)$$

Substituting μ from equation (3) in equations (6) and (8):

$$\frac{dx}{dt} = x\left[\mu_m\left(\frac{S}{K_s + S}\right) - D\right] \qquad (9)$$

$$\frac{ds}{dt} = D(Sr - S) - \frac{\mu_m x}{Y}\left(\frac{S}{K_s + S}\right) \qquad (10)$$

These two fundamental equations (9 and 10) define the behaviour of a continuous culture in which the basic growth relations have been described previously (equations 1 to 4). In a steady state, when Sr and D are held constant and D does not exceed a critical value (see Fig. 8), then unique values of $\bar{x}$ (bacterial concentration in a steady-state) and $\bar{S}$ exist for which both $\frac{dx}{dt}$ and $\frac{ds}{dt}$ are zero (Herbert et al., 1956). Therefore by solving equations 9 and 10,

the steady state values of $\bar{x}$ and $\bar{S}$ can be determined:

$$\bar{S} = K_s \left(\frac{D}{\mu_m - D}\right) \qquad (11)$$

$$\bar{x} = Y (Sr - \bar{S}) = Y \left[Sr - K_s \left(\frac{D}{\mu_m - D}\right)\right] \qquad (12)$$

Thus, if the values of the growth constants μ_m, K_s and Y are known, the steady state concentrations of bacteria and substrate in the culture vessel can be predicted for any value of D and concentration of the inflowing substrate (Sr) by using equations 11 and 12. Equation (11) shows that $\bar{S}$ is independent not only of $\bar{x}$ but also of Y and Sr. Thus it is possible to grow bacteria in continuous culture at high densities at growth-limiting substrate concentrations.

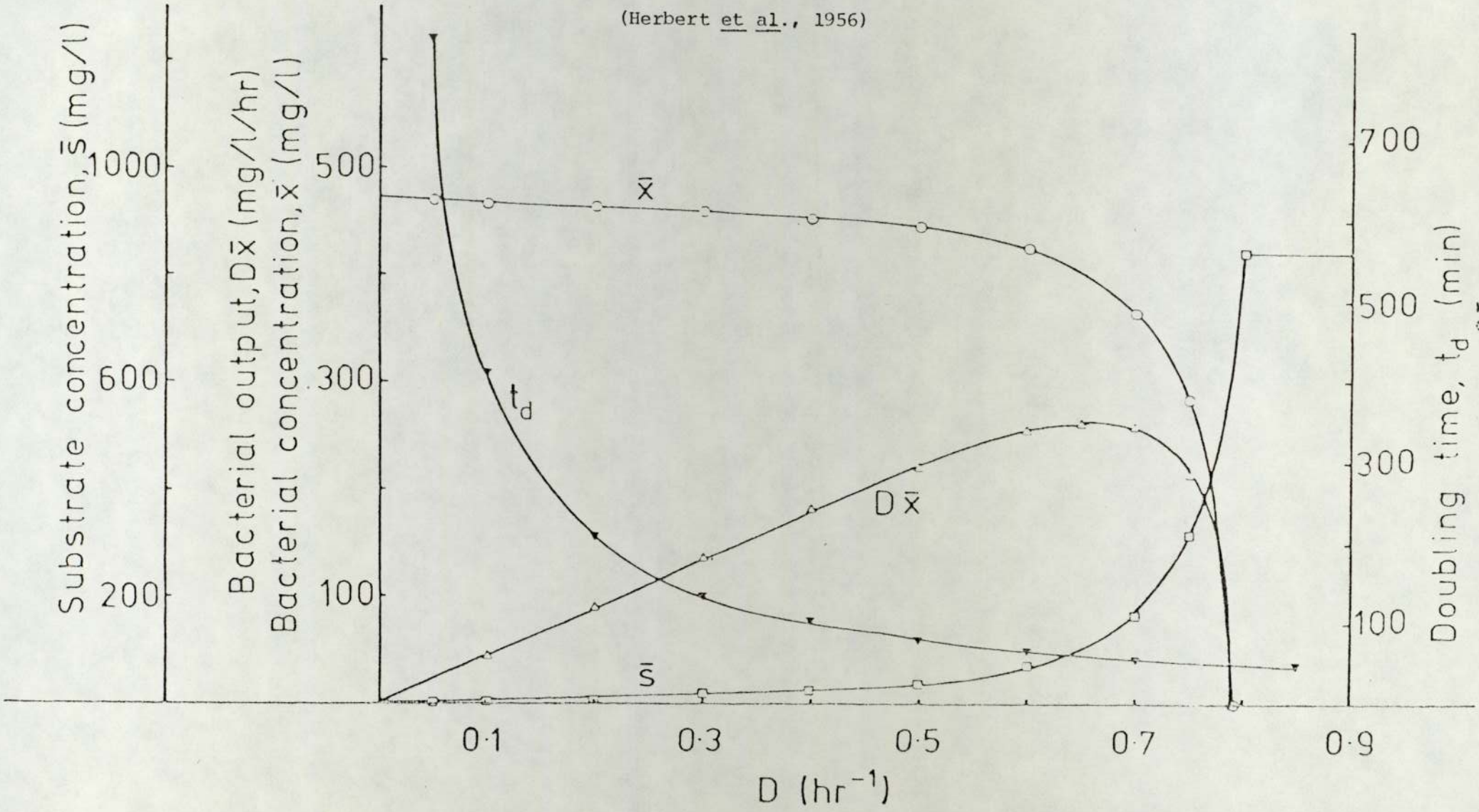
Fig. 8 shows the steady-state relationships in continuous culture between dilution rate, $\bar{x}$ and $\bar{S}$ at a chosen S_r for some experimental values of K_s, Y and μ_m determined from batch culture. A critical value of the dilution rate designated as D_c can be seen in Fig. 8. Above this value a complete washout of bacteria occurs; it is equal to the highest value of μ which is attained when $\bar{S}$ has its highest value S_r. The value of D_c was shown by Herbert et al. (1956) to be given by:

$$D_c = \mu_m \left(\frac{S_r}{K_s + S_r}\right) \qquad (13)$$

When $S_r >> K_s$, which is true in most cases, then $D_c \approx \mu_m$. Furthermore, from Equation (6), at all dilution rates greater than D_c, $\frac{dx}{dt}$ is negative and bacteria will be washed out of the culture vessel faster than they can grow.

The output of bacteria in unit time from continuous culture in steady-state is equal to the product of flow rate and

Fig. 8. Theoretical steady-state relationships in a continuous culture (Herbert et al., 1956)



concentration of organisms in the vessel; the output/unit volume of culture is therefore $D\bar{x}$ and from equation (12) Herbert *et al.* (1956) derived the following equation:

$$\text{output} = D\bar{x} = DY \left[Sr - K_s \left(\frac{D}{\mu_m - D} \right) \right] \qquad (14)$$

From Fig. 8 it can be seen that there is a value of D for which $D\bar{x}$ is a maximum. This implies that for any system there is a particular dilution rate, D_m, which gives the maximum output of bacteria in unit time. This D_m value was found by Herbert *et al.* (1956) to be given by:

$$D_m = \mu_m - \left(1 - \sqrt{\frac{K_s}{K_s + S_r}}\right) \qquad (15)$$

From these equations, it is possible to determine the value of S_r for any required bacterial concentration at $D = D_m$. Substituting $\mu_m \left(1 - \sqrt{\frac{K_s}{K_s + S_r}} \right.$ for D in equation (11) and solving for S_r gives

$$S_r = \frac{2\bar{x}Y - Y^2K_s \pm \sqrt{Y^4K_s^2 + 4\bar{x}Y^3K_s}}{2Y^2} \qquad (16) \text{ (Finch, 1976)}$$

Therefore, when Y and K_s are known, an S_r value which gives the concentration of growth-limiting nutrient for a desired bacterial concentration ($\bar{x}$) can be calculated.

1.4 The Effect of Inoculum History Upon the Sensitivity of Bacteria to Antimicrobial Agents

1.4.1 Introduction

Variations in the effects of antimicrobial agents or any other form of stress applied to a bacterial population are due to the physical and biochemical properties of the individual bacteria. The history of the population may greatly influence these properties, and consequently affect the response of bacteria (Farewell & Brown, 1971).

Environmental factors which affect bacterial sensitivity may be conveniently divided into two group, those which cause phenotypic changes in the bacterial cell and those which may modify the response of bacteria to antimicrobial agents by interacting with the cell or the agent, but do not necessarily induce a phenotypic change. Such effects may occur during treatment of cultures before testing (Melling & Brown, 1975). To obtain reproducible results, it is therefore very important to define and control the population history as well as the conditions involved subsequent to the culture stage. This chapter will discuss the effects of preinoculum history on sensitivity testing of antibacterial agents. It will include growth conditions of the culture and post growth pretreatment conditions of the inoculum.

1.4.2 Effects of growth conditions

1.4.2.1 Nutrient-depletion in batch culture

Brown & Melling (1969a, b) studied the effect of Mg^{2+} concentration in a chemically-defined growth medium on the sensitivity of Ps. aeruginosa to antibacterial agents. Mg^{2+}-depleted stationary-phase cultures were more resistant to polymyxin and

EDTA than glucose-depleted cultures, and showed increased sensitivity as the Mg^{2+} concentration was raised. Other cations, particularly Ca^{2+} were able to restore the sensitivity of Mg-depleted cultures. In contrast to these results, Mg-depleted cultures were more sensitive to silver ions than glucose-depleted ones (Brown & Anderson, 1968). The results of Brown & Melling (1969a, b) have been confirmed by several other workers (Boggis, 1971; Kenward, 1975; Finch, 1976). Boggis (1971) found that the order of effectiveness of cations in the growth medium in inducing sensitivity to EDTA was $Mg^{2+} > Mn^{2+} > Ca^{2+} > Ba^{2+} > Sr^{2+}$ with Zn^{2+} having no effect and Fe^{2+} having a protective effect. It was suggested that Mg^{2+} and possibly other cations are involved in one of the sites of action of EDTA; these sites are susceptible to phenotypic variation (Melling & Brown, 1975). The addition of more Mg^{2+} to Ca^{2+}-plentiful, Mg-depleted cultures depressed the lysis caused by the chelating agent EGTA (Boggis, 1971). These results suggested that the added Mg^{2+} almost entirely replaced the Ca^{2+} associated with the surface anionic groups, which represent the target site for EGTA. The same author found that the order of effectiveness of divalent cations in the growth medium in inducing sensitivity to polymyxin was $Ca^{2+} > Mg^{2+} > Ba^{2+} > Mn^{2+} > Sr^{2+}$.

Depletion of anions may also affect resistance. Boggis (1971) reported that batch cultures of Ps. aeruginosa were more resistant to either EDTA and polymyxin as the phosphate concentration of the medium was reduced. Similarly, Dorrer & Teuber (1977) found that phosphate-depleted Ps. fluorescens was resistant to polymyxin. Sulphate-depletion also leads to an increase in resistance of Ps. aeruginosa to polymyxin, provided an energy-source is present (Klemperer, 1976).

1.4.2.2 Nutrient-limitation and growth rate in continuous culture. Nutrient limitation and growth rate have been shown to affect the sensitivity of bacteria growing in continuous culture. Melling et al. (1974) found that Mg-limited Ps.aeruginosa grown in continuous culture was less sensitive than the carbon-limited and phosphate-limited cultures to polymyxin, EDTA, gentamicin, streptomycin and tetracycline. Furthermore, for both carbon-limited and Mg^{2+}-limited cultures, the slower the growth rate, the greater was the resistance. Finch & Brown (1975) using a different test system, also reported that carbon-limited continuous cultures of Ps.aeruginosa became progressively more resistant to lysis by EDTA as the growth rate was decreased; the addition of Ba^{2+} to the medium had no effect whereas the addition of Ca^{2+} resulted in cells which were sensitive to EDTA at all growth rates. However, unlike Melling et al. (1974) they found that Mg^{2+}-limited cells were very resistant to lysis by EDTA at all growth rates; the addition of Ba^{2+} again had no effect, whereas the addition of Ca^{2+} rendered the slow-growing cells sensitive to EDTA. They also found that the sensitivity of both Mg^{2+}-limited and carbon-limited cultueres to lysis by polymyxin did not change with growth rate although the carbon-limited were the most sensitive. The addition of Ba^{2+} to the media was again without effect; addition of Ca^{2+} increased the sensitivity of the Mg^{2+}-limited cells.

Gilbert & Brown (1978b) found that fast-growing cultures of Ps.aeruginosa were more sensitive to 3-chlorophenol and 4-chlorophenol than slower growing ones, and glucose-limited cells were generally more sensitive than Mg-limited ones. They also noted that 3-chlorophenol had greater activity than 4-chlorophenol at slow growth rates, but at faster rates of growth their activity

was similar. Melling et al. (1974) observed that phosphate-limited cultures of Ps. aeruginosa had a higher sensitivity to polymyxin B compared with Mg^{2+}-limited cultures. No significant effect was reported due to changing the growth rate in phosphate-limited conditions; in carbon-limited conditions a 30-fold difference in sensitivity existed between the slowest (0.05) and the fastest (0.7) growth rates.

1.4.2.3 Growth in complex media. Complex media are routinely used for minimum inhibitory concentration (MIC) determination. Variations in the media, particularly of cations, affect the results. As the same culture is used for growth and testing, one cannot distinguish phenotypic characters in the cells due to growth in a particular medium from effects of the medium on the cell-drug interaction.

The presence of excess cations in complex media may well be sufficient to render otherwise sensitive bacteria resistant to some antibiotics, possibly by exclusion mechanisms (Brown, 1975). Tseng et al. (1972) reported that the Mg^{2+} content of the growth medium affected the sensitivity of Ps. aeruginosa to streptomycin. Inclusion of 0.1 M $MgCl_2$ into a low-phosphate medium gave a 3-fold increase in the MIC and reduced the uptake of streptomycin 8-fold. Similarly, Dienstag & Neu (1972) reported an increase in the MIC of tobramycin against Ps. aeruginosa on addition of Ca^{2+} to the medium. They found Ca^{2+} more effective than Mg^{2+} in this respect. There have also been several reports of antagonistic effects of Ca^{2+} and Mg^{2+} on the activity of gentamicin against Ps. aeruginosa. Waitz & Weinstein (1969) observed that disc sensitivity testing of Ps. aeruginosa produced variable zone diameters depending upon the medium used. Garrod & Waterworth (1969) reported a 32-fold

variation in the sensitivity of Ps. aeruginosa to gentamicin depending upon the medium constituents and the type of agar used for producing solid media. In general, an increase in the MIC of the antibiotic has resulted from increasing the Mg^{2+} content of the medium. For example, a high Mg^{2+} and Ca^{2+} content on trypticase soy agar caused a 4-fold increase in the MIC of gentamicin against 39 Ps. aeruginosa strains in comparison with results on Mueller-Hinton agar with less Mg^{2+} and Ca^{2+} (Washington et al., 1970). Similar results were obtained by Gilbert et al. (1971). However, such variations in sensitivity were small in similar tests using E. coli and other Gram-negative organisms (Garrod & Waterworth, 1969; Washington et al., 1970). Brown (1975) related these results to the type of cation effects described by Newton (1954), which involved competition between the antibiotic and cations for receptor sites on the cell surface.

Sands et al. (1963) noted that the presence of agar in the test medium reduced the activity of eight out of 19 phenolics tested against Staph aureus. In a subsequent report, Sands & Bennett (1964) observed that washing of agar with water prior to use removed the materials responsible for the inhibition of phenol activity. Similarly, the washing of agar by water reduced the MIC of polymyxin, neomycin, kanamycin and streptomycin (Hanus et al., 1967). This effect was attributed to the removal of Ca^{2+} and Mg^{2+}. Variations in agar quality have also been correlated with the size of inhibition zones. Using ion agars rather than conventional agar, larger zones were obtained with polymyxin and aminoglycosides (Bechtle & Scherr, 1958). Such variations were expected since agar components are agarose and agaropectin. Agaropectin is sulphated and contains cation-binding acid residues

(Araki, 1959). Furthermore, agar may react with protein molecules (Brishammar et al., 1961) and basic compounds such as polymyxin and neomycin (Ford et al., 1955) making true evaluation difficult.

The content of NaCl in the growth medium has also been found to affect the sensitivity of bacteria to antibiotics. Wick & Welles (1967) observed a 10-fold increase in the MIC of nembramycin for both Staph. aureus and Salmonella typhosa on increasing the added NaCl concentration from 0% to 5%. The addition of NaCl to nutrient broth was found to increase the MIC of gentamicin for Ps.aeruginosa, Ser. marcescens and E. coli (Medeiros, 1971). This effect was subsequently attributed to the competitive displacement of loosely-bound Mg^{2+} at the cell surface by Na^{+} which would increase changes due to depletion of Mg^{2+} (Melling & Brown, 1975).

1.4.2.4 Temperature of the growth medium. Growth temperature also seems to affect the sensitivity of bacteria to antimicrobial agents. In general, the higher the temperature, the greater the sensitivity. This effect may well involve changes in the cell wall fatty acids and lipid composition which cause the exclusion of the agents (Melling & Brown, 1975). The resistance of Ps. aeruginosa grown on trypticase soy agar to acetic acid, chlorine dioxide, glutaraldehyde and QACs was increased by growth at 25° rather than 37° (Carson et al., 1972). The sensitivity of several strains of Ps. aeruginosa to serum and antibiotics was affected by growth temperature (Muschel et al., 1969). The sensitivity to serum, polymyxin E, mitomycin C, erythromycin and actinomycin D was increased at 41° instead of 37°. Annear (1968) reported a 30-fold reduction in the MIC of methicillin for R-factor-mediated methicillin-resistant Staph. aureus when grown at 43° rather than

31°. No such change occurred with methicillin-sensitive bacteria. This effect was attributed to the failure of plasmid replication to keep pace with cell division (Ashevov, 1966).

1.4.2.5 Growth phase. Most of the work on antibiotic sensitivity testing of bacteria is usually done using batch cultures. However, in such cases the term growth phase may not define completely the actual conditions of the bacterial population. The resistance of log phase cultures is usually assessed either directly, by addition of the agent to a growing culture, or by an MIC determination in which a non-replicating inoculum is often used. In complex media such as nutrient broth, even log phase growth may be a series of diauxies and the particular nutrient being consumed will vary as growth proceeds (Melling & Brown, 1975). Thus, the standardization of the culture and density at which an agent is added may be very critical. The "stationary phase" term is also imprecise, since after cessation of the growth, the metabolic state of a culture will change with time and therefore variations in resistance in relation to growth phase may be difficult to interpret. It is generally found that stationary phase cultures are less sensitive to lethal agents. For example, stationary-phase Ps. aeruginosa was reported to be more resistant to acetic acid than log phase cultures (Carson et al., 1972).

1.4.2.6 pH of the growth medium. Melling & Brown (1975) noted that the observed effects of growth medium pH on the resistance of bacteria are more closely related to the degree of ionization of some antibacterial agents or cell components than to any phenotypic variation. The effects of pH on antibiotic activity were observed soon after the introduction of antibiotic chemotherapy. The basic antibiotics were found to be more active at pH 8 than at pH 6

(Gardner & Chain, 1942; Waksman et al., 1944), while acidic antibiotics such as benzyl penicillin were more active at acid pH (Florey et al., 1946). Similarly, Abraham & Duthie (1946) found that the MIC of streptomycin for Ps. aeruginosa increased 2.5-fold with an increase in medium pH from 6 to 8. Eagle et al. (1952) investigated the effect of pH on the activity of penicillin, streptomycin, chloramphenicol and tetracycline. The activity of penicillin against Micrococcus pyogenes was decreased with increasing pH, but little change in activity occurred against E. coli. Streptomycin showed more activity with increasing pH against both organisms. Tetracycline had an optimum pH of 7.7 for activity against E. coli, but showed little change in activity against M. pyogenes. Using an antibiotic disc method, Rosenblatt & Schoenknecht (1972) confirmed that the sensitivity of E. coli to the aminoglycosides as well as to tetracycline and chloramphenicol increased with a pH change from 7.4 to 8.4. Inclusion of 5% CO_2 in the atmosphere caused a reduction in the size of the zone of inhibition due to a fall in pH. Eagle et al. (1952) also found that the activity of chloramphenicol changed with pH, showing a minimum at pH 6.6. Since chloramphenicol is not ionizable this may indicate changes in the bacteria, which may also provide an explanation for the differences in response to pH changes shown by E. coli and M. pyogenes.

Zager (1965) reported an increase in erythromycin activity at an alkaline pH against the Gram-negative bacteria E. coli, Proteus species and Ps. aeruginosa. The increased activity was attributed to an increasing proportion of unprotonated erythromycin available (Garrett et al., 1970). A combination of other factors such as ionization of cell receptors, active transport mechanisms

and competition between ionized antibiotic and H^+ or OH^- ions for cellular receptors was suggested as a possible explanation for the activity of the ionized form (Albert, 1965).

Bassett (1971) observed a decrease in the MIC of a combination of chlorhexidine and cetrimide against P. vulgaris and Pseudomonas multivorans following a pH reduction from 7.2 to 6, although their activity against Ps. aeruginosa was unchanged.

Miller & Perkins (1973) assessed different methods used to determine the effect of pH on the activity of carbenicillin on P. mirabilis. Cylinder plate assays, growth curve studies and scanning electron microscopy of the treated bacteria all indicated that its greatest activity was at pH 7.2 and the least at pH 5. However, the standard tube dilution method for MIC determinations showed no significant difference at pH 5, 6, 7 and 8.

The activities of a number of antibiotic inactivating enzymes from different bacteria have been shown to be pH-dependent. Therefore, variation in pH affecting these enzymes would affect resistance to these antibiotics (Melling & Brown, 1975).

1.4.3 Effect of post-growth conditions

The environmental factors involved in the growth medium which affect bacterial resistance also apply to situations where the growth and test conditions are so similar that an agent is added to an otherwise unaltered culture. This has been achieved by the addition of a small volume of an agent relative to the culture volume. Such an addition would cause no change in temperature, pH or the composition of the culture. In this section will be considered the effects of harvesting and handling procedures where a change in the bacterial environment takes place. It includes both the test situation and subsequent recovery situations.

1.4.3.1 Composition of the suspending medium. Bacteria transferred from one solution to another of different solute concentration may undergo changes of size and shape (Mohr & Larsen, 1963) which may lead to lysis (Christian & Ingram, 1959) and loss of viability (Carlucci & Pramer, 1960). This was attributed to a change in tonicity by Christian & Ingram (1959). Gosseling (1958) also found that reducing the difference in ionic strengths between various suspending media decreased lethal effects when bacteria were transferred from one to another. The solute concentration per se was shown to be a crucial factor by Brown & Winsley (1969), who found that an increase in the NaCl concentration of a suspending medium from 0 to 2 M reduced the leakage of 260 nm-absorbing materials from Ps. aeruginosa by about 4-fold.

The nature of the solutes in the suspending media, in addition to their concentrations, also affect bacterial survival to a great extent. Newton (1954) observed that addition of divalent cations (in order of effectivenss; $Mg^{2+} > Sr^{2+} > Ca^{2+} > Ba^{2+}$) protected washed Ps. aeruginosa against polymyxin B. Trivalent cations were even more effective and uranyl ions gave still more protection. This effect involves competition for sites on the cell surface with the antibiotic. Direct interaction, not necessarily chelation, may also take place between the cations and the agent. Tetracyclines lost their activity after neutralization by cations (Weinberg, 1957). Other compounds such as izoniazid and α-picolinic acid act by depriving the cells of essential cations. The presence of an increased concentration of the necessary cations in both cases will reduce the effectiveness of these agents.

The presence of monovalent cations in suspending media also

affects the survival of bacteria, although a number of the reported protective effects may well be the results of non-specific maintenance of tonicity (Melling & Brown, 1975). Shively & Hartsell (1964) found that the lysis of Ps. aeruginosa by EDTA-lysozyme was inhibited by NaCl (0.1-2%). Melling & Brown (1975) suggested that this effect of monovalent cations is due at least in part to the desorbtion of Mg^{2+} and other divalent cations from the cell surface.

The presence of various metabolites in the suspending medium also affects bacterial viability. Cysteine increased the rate of Ps. aeruginosa lysis in phosphate buffer (Bronheim, 1966). Peptone has been shown to exert a protective action for a number of bacteria (Curlacci & Pramer, 1960; King & Hurst, 1963; Strak & Stokes, 1957; Weiler & Hartsell, 1969). The effect of various carbohydrates added to suspending media has been investigated by Gossling (1958) who found that glucose reduced the lethal effects of changes from one suspending medium to another and by Christian (1958), who found that the use of sucrose solutions for washing Salmonella caused less change in the intracellular levels of Na^{+}, K^{+} and 260 nm-absorbing materials than salt solutions of the same water activity. The inclusion of various other organic compounds may also affect the response to antibacterial agents. Brown & Winsley (1969) reported that the presence of polysorbate 80 in suspending media for Ps. aeruginosa resulted in an immediate increase in the susceptibility of this organism to changes in pH, temperature or medium tonicity, although polysorbate 80 itself was not toxic. This agent also enhanced polymyxin B activity against Ps. aeruginosa.

1.4.3.2 pH of the suspending medium. The effect of pH on the activity of antibacterial agents has been discussed in a previous section (1.4.2.6). In this section, the effect of pH is to be considered in relations to the viability per se of the bacterial suspension after the culture stage. Brown & Winsley (1969) reported that the pH of the suspending medium for Ps. aeruginosa over the range 5.7 - 9.5 had little effect on leakage of 260 nm-absorbing materials, but the magnitude of a sudden pH change did correlate with loss of viability. Other workers have reported particular pH values as being optimal for the maintenance of viability, such as pH 8.5 for Streptococcus lactis and Streptococcus cremoris (Cowell et al., 1966) and pH 7 for chilled A. aerogenes (Strange & Postgate, 1964). However, the pH of suspending media allowing survival may be affected by other environmental conditions (Melling & Brown, 1975).

1.4.3.3 Temperature of the suspending medium. A reduction in the temperature of a bacterial suspension can result in either enhanced stability or loss of viability depending upon the rate of temperature change as well as on the composition of the suspending medium (Melling & Brown, 1975). With some exceptions, there is evidence that the viability of bacterial suspensions is maintained better at lower temperatures provided that the temperature reduction is gradual. Cook & Willis (1956) have found that Ps. aeruginosa and E. coli showed a slight increase in viability when stored at 10° rather than room temperature for a month. Storage at 37° significantly effected the viability, while the susceptibility to phenol was increased as the temperature increased.

The survival of nutrient broth grown Ps. aeruginosa was unaffected by dilution in water at temperatures over the range

6 - 37°, but lost viability on storage at -10° (Emmanouilidou-Arseni & Koumentakou, 1964), whereas Ps. aeruginosa grown in a salts-glucose medium and stored in either water, fresh medium, saline or magnesium solution at 37°, 20° or 4° retained viability best at 20°.

Chilling a Gram-negative bacteria results in a lethal effect called cold shock (Sherman & Albus, 1923; Gorrill & McNeil, 1960). A rapid temperature reduction is the characteristic feature of cold shock. Various factors have been shown to modify the bacterial response to this stress. Brown & Winsley (1969) showed that rapidly chilled Ps. aeruginosa lost viability and released 260 nm-absorbing materials, but the magnitude of this effect could be correlated with the suspending medium composition and pH. Inclusion of polysorbate 80 enhanced susceptibility to cold shock.

1.4.4 Conclusion

The data reviewed here clearly indicate that environmental circumstances profoundly influence the sensitivity of bacteria to antimicrobial agents. This variation in bacterial resistance emphasizes the problems of *in vitro* assessment of antibiotics. Consequently a considerable variation may exist in the measure of agreement between such tests and the final *in vivo* performance. Brown (1977) proposed the use of nutrient-depleted chemically-defined inocula for antibiotic assay or disinfectant testing or any pharmacopoeial tests for preservative efficacy. The physiological state of bacteria in certain *in vivo* situations may be affected by nutrient-depletion and thus a major component of biological variation might be reduced significantly. When more data about the influence of growth rate and specific nutrient-

depletion on bacterial resistance to drugs and body defence mechanisms is available, it would be possible to produce physiologically-defined and more relevant inocula to mimic the *in vivo* situation (Brown, 1977).

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Bacteria

The two strains of *Proteus mirabilis* used for most of this study were NCTC 5887 and a polymyxin-sensitive clinical isolate supplied by Dr. Martin, The Royal Berkshire Hospital, Reading, Berks, and designated RBH by us (hereafter referred to as 5887 and RBH). Five other strains of *Proteus* were also used for the investigation of small colony (Sc) variants. These were NCTC 7827, 8309 and 60, NCIB 2100 and *Proteus morgani* NCIB 10466.

All strains were maintained on nutrient agar slopes (Oxoid or Gibco) stored at 4°. Subculturing on fresh nutrient agar slopes, and streaking on to nutrient agar (Oxoid) plates to check the homogeneity of the cultures was repeated at approximately three-monthly intervals. For some purposes, 5887 and RBH were also stored in liquid nitrogen, as nutrient broth cultures, prepared from clones of typical large colony-forming organisms (Lc) isolated by terminal dilution (Meynell & Meynell, 1970) to obtain pure cultures with no small colony variants (Sc) present. 5887 and RBH were also stored on chemically-defined medium (CDM) slopes (CDM table 1 + Lab M agar 1.2%) at 4°. To prepare the latter, an overnight nutrient broth culture was serially subcultured twice in CDM, from which a CDM slope was inoculated. Fresh CDM slopes were prepared at four-weekly intervals.

2.1.2 Media

Nutrient broth (NB): Oxoid nutrient broth, C.M.1: Nutrient agar (NA): Oxoid nutrient agar, C.M.3: Oxoid Ltd., London SE1 9HF.

Gibco diagnostic nutrient agar (GNA) Dri-form: Gibco Diagnostic, Glasgow, Scotland.

Table 1

Basic chemically-defined medium

Ingredient	Final concentration Molar
Glucose	5×10^{-3}
Nicotinic acid	6×10^{-5}
NaCl	5×10^{-5}
KCl	5×10^{-5}
$(NH_4)_2SO_4$	1×10^{-3}
$MgSO_4.7H_2O$	4×10^{-5}
$(NH_4)_2HPO_4$ + $NH_4H_2PO_4$ pH 7.0	1×10^{-1}

Medium pH 7.0

Lab M agar: M.C.2. London Analytical and Bacteriological Media Ltd., London EC3R 7QJ.

MOPS (3-(N-morpholino)propanesulphonic acid): Hopkins & Williams Ltd., Essex or Sigma London, Poole, Dorset. It is an organic buffering agent with pK_a of 7.2 (range 6.5 - 7.9). Its pH was adjusted with NaOH and it was used at a concentration of 0.025 M (containing 0.0125 M-Na^+) to buffer media containing low concentrations of phosphate.

All the medium constituents except for glucose and MOPS were separately sterilised by autoclaving at 121° for 20 min. Glucose and MOPS were sterilised by filtration through a 0.45 µm membrane filter for clarification before autoclaving.

2.1.3 Chemicals

All chemicals used in the preparation of CDM and the organic

solvents used in chemical analysis were of Analar grade, and were obtained from British Drug Houses Chemicals Ltd., Poole, Dorset or Fison Scientific Apparatus Ltd., Loughborough, Leics.

Other chemicals were obtained as follows:-

Chlorhexidine gluconate 5% w/v (Hibitane); Imperial Chemical Industries Ltd., Macclesfield, Cheshire.

Cetrimide: British Drug Houses Chemical Ltd., Poole, Dorset.

Phenol: Fison Scientific Apparatus Ltd., Loughborough, Leics.

Antibiotic Multodiscs: Oxoid Ltd., London SE1 9HF.

Phospholipid standards, 2-keto-3-deoxyoctonate (KDO) as ammonium salt, Deoxyribonuclease 1(crude activity 410 Kunitz units/mg protein), Ribonuclease-A (crystallized, activity 76 Kunitz units/mg protein), and Trypsin (crystallized, activity 12000 BAEE units/mg protein): Sigma London, Poole, Dorset.

Lecithin: British Drug Houses Chemical Ltd., Poole, Dorset.

Tween 80: Koch-Light Laboratories Ltd., Colnbrook, Bucks.

Glycerol B.P and Liquid Paraffin B.P: Macarthys, Romford, Essex.

Polymyxin B sulphate was kindly given by Burroughs Wellcome & Co., London.

Water was deionised before distillation in a glass still.

2.1.4 Apparatus

Spectrophotometers: optical density and absorbance measurements were carried out in 1 cm matched glass cuvettes using a Unicam S.P.600 (Pye Unicam Instruments Ltd., Cambridge). Atomic absorption spectroscopy was carried out using a Unicam S.P.90 (Pye Unicam Instruments Ltd., Cambridge).

Mickle reciprocating shaker bath: Camlab Ltd., Nuffield Road, Cambridge.

I.E.C. centrifuge: International Equipment Company, 300 Second Avenue, Needham Heights, Massachusetts, U.S.A.

Millipore membrane filtration apparatus: Millipore U.K. Ltd., Wembley, Middlesex.

Membrane filters: Sartorious, Göttingen, West Germany. Before use, these were boiled in three changes of distilled water before sterilisation by autoclaving. This treatment was to remove wetting agents and other chemicals (Brown, Farwell & Rosenbluth, 1969).

pH Meter, Corning-EEL Model 5: Evans Electroselenium Limited, Halstead, Essex, England.

Phase-contrast microscope, 'Wild' model M20, binocular: Micro Instruments (Oxford) Ltd., Oxford, OX1 2PH.

'Chromoscan 200' and 'Scan 201' thin layer chromatogram scanning and integrating equipment: Joyce, Loebel & Co. Ltd., Gateshead, NE11 OUJ.

Ballotini beads: Ballotini Glass Manufacturing Co., Pontefract Road, Barnsley, Yorkshire.

Mickle disintegrator: The Mickle Laboratory Engineering Co., Gomshall, Surrey.

Camera model M030001: Vickers Ltd., Breakfield Coulsdon, Surrey, with Ilford FP4 film.

Solvent resistant membranes: Millipore Corporation, Bedford, Massachusetts, U.S.A.

Large membrane filter and pre-filter: Sartorious, Göttingen, West Germany.

Chemostat: constructed by the university glass blower according to the design described by Gilbert & Stuart (1977).

2.1.5 Glassware

All glassware was Pyrex brand. For cleaning, it was immersed in 5% (v/v) Decon 90 (Decon Laboratories Ltd., Brighton BN 4 1EQ) overnight. It was then rinsed with distilled water, 1% (v/v) HCl, six times in distilled water, and twice in deionised distilled water. It was then dried at 60°, covered with aluminium foil and sterilised by heating at 160° for 3 hr.

2.2 Bacteriological Methods

2.2.1 Measurement of growth

Bacterial growth was determined by measuring optical density (OD).

When a monochromatic beam of light enters a suspension of bacteria, some of it will be scattered and diverted from the light path. The scattering is caused by reflection and refraction at the cell surface-medium interface. This can be quantified by measuring the undeviated light emerging from the suspension. At relatively low cell concentrations, the incident light (I_o) and the emerging light (I) are related by the Beer-Lambert law

$$\text{Log}_{10} \frac{I_o}{I} = elc$$

where e is the extinction coefficient which is specific for each particular organism, l the distance the light travels through the culture, and c the concentration of bacteria in the suspension.

Spectrophotometers which give readings in terms of OD($\log_{10} \frac{I_o}{I}$) are considered to be the most suitable instruments for turbidity measurements (Monod, 1949).

2.2.1.1 Choice of wavelength. The light scattered by a bacterial suspension is directly proportional to the ratio of cell size to the wavelength of the incident light (Koch, 1961). This implies that the shorter the wavelength used, the more sensitive the instrument will be to OD changes. Another consideration is that at the selected wavelength, light absorption by the medium ingredients, cell pigments and metabolic products should be at a minimum, preferably zero.

For non-pigmented organisms, 420 nm is the lowest practical wavelength to avoid interference by proteins and nucleic acids

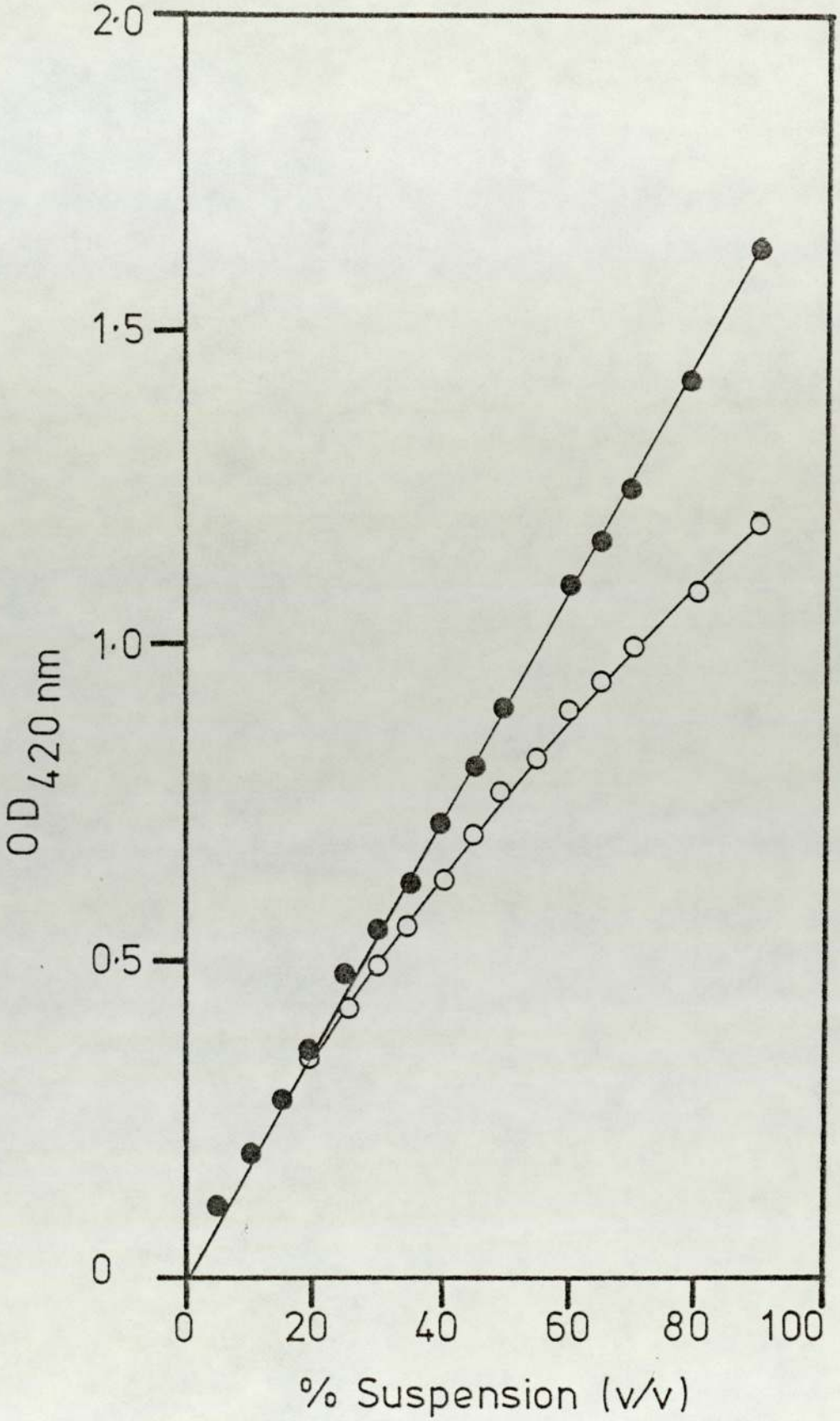
which absorb at <380 nm (Hodges, 1973, Bacillus megaterium; Handley, Quesnel & Sturgiss, 1974, Escherichia coli). Therefore 420 nm was the wavelength used in this study for growth measurements.

2.2.1.2 Relation between optical density and cell concentration. To determine the point at which the ratio OD/cell concentration deviated from the Beer-Lambert law, the OD at 420 nm of a series of dilutions of a stationary phase culture of 5887 grown in CDM (Table 1) and resuspended in CDM without glucose were measured. A plot of OD against % of original cell suspension showed that the relationship was obeyed up to an OD of 0.35 (Fig. 9). When the same suspensions were diluted with water to give OD readings below 0.35 linearity was restored up to an OD of 1.8.

The relatively low readings observed in dense cultures were probably due to secondary light scattering (Koch, 1961). Another possibility is that the shielding of the more distant cells by the bacteria nearest to the light source may obstruct their full contribution to the light scattering.

2.2.2 Batch cultures for the study of growth and resistance

The appropriate CDM was inoculated from CDM slopes (study of growth) or NA slopes (study of resistance) using 25 ml of medium in 100 ml conical flasks. These were incubated at 37° and shaken over 5 cm at 120 throws min^{-1}. Unless otherwise stated, the inoculum was grown in CDM, centrifuged at 30° at 10,000 g for 2 min and washed three times with a prewarmed medium free of the nutrient under investigation to prevent "carry over" of nutrients. Samples were taken at timed intervals with a Pasteur pipette and the OD measured. The sample was returned to the flask after each reading, unless it had been diluted. Table 1 shows the basic CDM used in the earlier stage of this study.


2·0
1·5
1·0
0·5
0
OD 420 nm
0
20
40
60
80
100
% Suspension (v/v)

2.2.3 Comparison of oxygenation of batch and chemostat cultures of P. mirabilis 5887

The OD of 5887 grown in 50 ml chemostats as batch cultures were compared with that of 25 ml batch cultures in 100 ml conical flasks and used as an indicator of efficiency of aeration. Growth conditions and measurements were as described in sections 2.2.2 & 3.3, except that media were directly inoculated with appropriate volumes of the inoculum culture. Inoculum and growth CDM were sufficient to support the growth theoretically to an OD of 10 (Table 6, p.91).

Fig.10 shows that the batch culture grew exponentially up to OD 4.0, and to a maximum OD of 7.5, while chemostat batch cultures grew exponentially up to OD 2.0, and to a maximum OD of 3.0. It seems that aeration of batch cultures was more efficient than that of chemostat cultures.

2.2.4 Media for nutrient-depleted cultures

To prepare nutrient-depleted cultures, CDM were designed to ensure that cells would cease growing exponentially at the required OD due to reduction in the concentration of the chosen nutrient. Glucose-depleted (C-dep) cells become stationary immediately. Magnesium-depleted (Mg-dep) or phosphate-depleted (P-dep) cells continue to grow slowly and were harvested after one more doubling. All other ingredients were in excess and were sufficient to allow 2 to 3 more doublings.

Table 2 shows the medium for small batch cultures for resistance studies, allowing exponential growth of C-dep cells to an OD of 0.6 and of Mg-dep and P-dep to an OD of 0.2 - 0.3. Table 3 shows the medium for 9 litre batch cultures for chemical

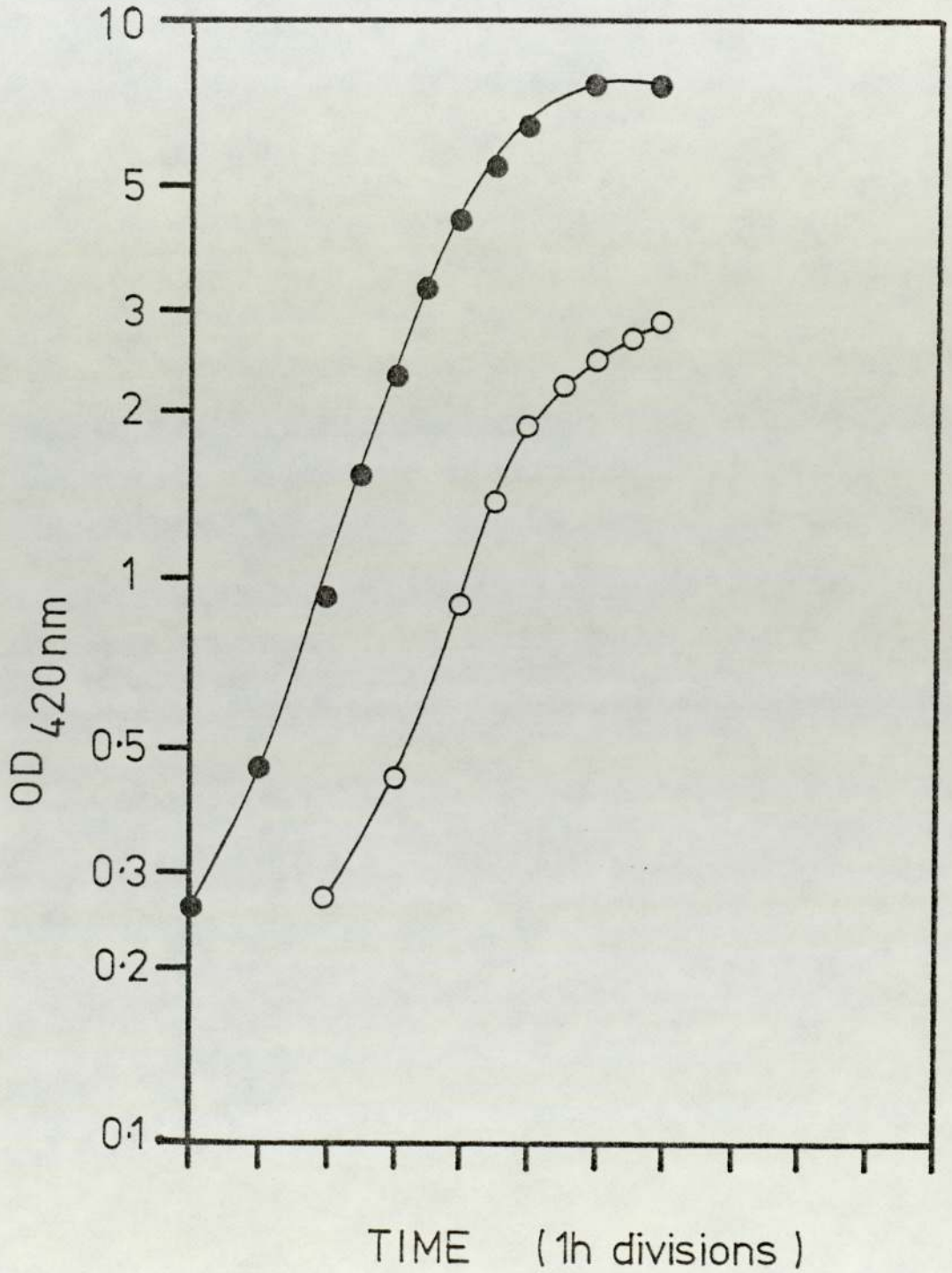

10
5
3
2
1
0·5
0·3
0·2
0·1
OD 420nm
TIME (1h divisions)

Table 2

Composition of chemically defined medium and resuspending media used in small batch cultures for resistance studies of P. mirabilis 5887 and RBH

Components of CDM (M)	Growth CDM			Resuspending media		Inoculum CDM
	C-dep	Mg-dep	P-dep	Glucose & Magnesium free	Glucose & Magnesium & Phosphate free	
Glucose	*2×10^{-3}	1.5×10^{-2}	1.5×10^{-2}	0	0	5×10^{-3}
Nicotinic acid	6×6^{-5}	6×6^{-5}	6×10^{-5}	6×10^{-5}	6×10^{-5}	6×10^{-5}
NaCl	5×10^{-5}	5×10^{-5}	5×10^{-5}	5×10^{-5}	5×10^{-5}	5×10^{-5}
KCl	10^{-1}	10^{-1}	10^{-1}	5×10^{-5}	5×10^{-5}	10^{-1}
$(NH_4)_2SO_4$	5×10^{-4}	5×10^{-4}	5×10^{-4}	5×10^{-5}	5×10^{-5}	3×10^{-5}
$MgSO_4.7H_2O$	1×10^{-4}	*2×10^{-6}	1×10^{-4}	0	0	3×10^{-5}
$(NH_4)_2HPO_4$] $NH_4H_2PO_4$]	10^{-1}	10^{-1}	*4×10^{-3}	0.5×10^{-1}	0	0.5×10^{-1}
MOPS	0	0	2.5×10^{-2}	0	2.5×10^{-2}	0

pH 7.0 $\pm$ 0.1

*The concentration of nutrient resulting in depletion.

analysis, which permitted exponential growth of C-dep cells to an OD of 1.0 and of Mg-dep cells to an OD of 0.6. The very large excess of KCl over theoretical requirements was used to reduce the number of Sc variants in these cultures (see section 3.5).

2.2.5 Growth of 9 litre batch cultures for chemical analysis

The compositions of the inoculum, Mg-dep and C-dep CDM used for the chemical analysis of 5887 and RBH are shown in Table 3. CDM (3 l) was filtered through an 0.2 µm membrane filter (size 142 mm, covered with a prefilter, size 132 mm) fitted on a stainless steel 142 mm disc filter holder (Gelman Hawksley, Northampton) into each of 3 x 5 l previously-autoclaved round-bottomed flasks, with magnetic followers inside. These flasks were then placed in a water-bath at 37° over magnetic stirrers.

To reduce the number of small colony (Sc) variants present in the cultures, the inoculum was prepared by adding a loopful of culture frozen in liquid N_2 (section 2.1.1) to about 80 ml of NB containing 5 ml of 1 M-glucose, and covering with a layer of paraffin to reduce diffusion of O_2, as Sc variants are non-fermentative in the O/F test (section 3.5.1). After overnight incubation at 37°, 2 ml of this culture was transferred to the inoculum CDM, which was then incubated overnight, washed and resuspended as described in section 2.2.2. 3 ml of this suspension (OD 5.0) was inoculated into each of the 3 l CDM. The speed of the magnetic follower was adjusted so that the vortex extended down to the follower. This provided a large surface area for gaseous exchange between the atmosphere and the culture, due to the numerous air bubbles resulting from the vortex touching the follower.

Table 3

Chemically defined medium (CDM) used for 9 L cultures of P. mirabilis 5887 and RBH

Components of CDM (M)	C-dep CDM	Mg-dep CDM	Inoculum CDM
Glucose	*3.5×10^{-3}	1.5×10^{-2}	4×10^{-2}
Nicotinic acid	6×10^{-5}	6×10^{-5}	6×10^{-5}
NaCl	5×10^{-5}	5×10^{-5}	5×10^{-5}
KCl	10^{-1}	10^{-1}	4×10^{-1}
$(NH_4)_2SO_4$	5×10^{-5}	5×10^{-5}	2×10^{-4}
$MgSO_4.7H_2O$	1×10^{-4}	*1×10^{-5}	2×10^{-4}
$(NH_4)_2HPO_4$] $NH_4H_2PO_4$]	10^{-1}	10^{-1}	10^{-1}

pH 7.0 ± 0.1

*Concentration of the nutrient resulting in depletion.

The growth of cultures was followed as described in section 2.2.2, until one hour after glucose-depletion in C-dep CDM or five hours after the onset of magnesium-depletion in Mg-dep CDM. The maximum OD reached by both depleted cultues was ca 1.0. At that stage, samples were taken for testing sensitivity to polymyxin (section 2.2.6) and for checking the absence of Sc variants. While waiting for these results the cultures were stored at 4° (36 hr).

2.2.6 Measurement of resistance to antibacterial agents

Depleted cultures were centrifuged at 10,000 g for 2 min and resuspended in a prewarmed glucose- and magnesium-free medium

for C and Mg-dep cultures, or glucose- magnesium- and phosphate-free medium for P-dep cultures, at an OD of 0.2 (Table 2). Cells suspensions were then equilibrated for 30 min at 37°. After that, 0.1 ml samples of antibacterial agents were added to 0.9 or 9.9 ml samples of the cell suspensions. The same volumes of water were added to control samples of cells. The mixtures were shaken at 37° and sampled at known times. To stop the activity of the antibacterial agents rapidly, each sample taken was immediately diluted $^{1}/10$. Because of the high concentration exponent of phenol, phenol-treated cells were diluted in NB. Polymyxin was quenched by dilution in lecithin-glycerol broth and cetrimide and chlorhexidine in lecithin-tween broth (Kohn et al., 1963a, b).

2.2.7 Viable counts

Serial dilutions (10^{-1} or 10^{-2}) were made in NB to give 30 - 300 colonies per plate, when 0.1 or 0.25 ml samples were spread by a sterile spreader on the surface. Plates were incubated at 37° for 36 - 48 h. To prevent swarming, plates were prepared from Oxoid NA (ONAA) or Gibco NA (GNAA) plus Lab M agar 2.3% (final agar concentration in these excess agar plates was 3.8%). They were dried for 1½ h at 55°.

At each dilution level three or five replicate plates were used. The viable count was calculated from the mean number of colonies per plate. The homogeneity of counts of each set of replicates was tested by χ^2 test as described by Meynell & Meynell (1970).

2.2.8 Preparation of cells and cell walls for chemical analysis

The cells from the 9 l cultures were harvested by centrifugation at 7,500 g for 20 min at 4° and washed once with

0.2% KCl (Strange & Shon, 1964) and twice with cold sterile distilled water. The washed pellet was resuspended with distilled water to 50 ml.

To determine the dry weight of the whole cells, accurately measured volumes of the cell suspension were added to a tared centrifuge tube and centrifuged at 7,500 g for 20 min. The pellets were dried over P_2O_5 *in vacuo* to constant weight. The remaining whole cell suspension was stored frozen until required for cell wall preparation.

To prepare cell walls, 5 - 10 mg of ribonuclease and deoxyribonuclease were added to thawed whole cell suspension which was gently shaken to ensure dispersal of cells in the water. 8 ml samples were placed in each of 4 tubes designed to be used with the Mickle tissue disintegrator, and 1.5 ml of water and 8 ml of 0.1 - 0.2 mm Ballotini beads were added. The tubes were stoppered with rubber bungs.

Preliminary experiments, sampling at regular intervals and monitoring the appearance of cells by phase-contrast, had indicated the most efficient programme for cell breakage. The suspensions were violently agitated in the Mickle tissue disintegrator for 120 min at 4°. During agitation, the temperature in the tubes rose from 4° to 20°. The glass beads were filtered off, and the residual whole cells removed by centrifugation at 3,500 g for 20 min. The pellets were bulked, resuspended and then subjected to a further 90 min agitation. The broken cell suspensions were bulked and centrifuged at 13,000 g for 45 min. The resulting pellet consisted of three layers. These layers were gently washed off separately and examined under the microscope. The bottom layer contained residual whole cells left from the first

centrifugation. The upper layers contained small and large cell wall fragments, and large membrane particles. The two top layers were bulked and washed three times with water. The residual whole cells were discarded.

Crude cell walls were purified by incubating with trypsin as described by Gray & Wilkinson (1965). The cell walls were then washed once with 0.2% KCl (Strange & Shon, 1964) and twice with water. The final clean pellet was resuspended in 25 ml water. To measure the dry weight, 12.5 ml of this suspension was placed into each of two tared centrifuge tubes, and centrifuged at 13,000 g for 45 min. The pellets were then dried to constant weight over P_2O_5 *in vacuo*.

2.3 Chemical Assay Procedures

2.3.1 Extraction of lipids from whole cells and cell walls

Accurately weighed (about 50 mg) samples of dried whole cells or cell walls were suspended in 50 ml of chloroform : methanol solvent (2 : 1, v/v) containing 0.01% (w/v), β-hydroxytoluene as anti-oxidant (Brown & Watkins, 1970). These samples were extracted for 45 min in a shaking water bath at 37°. The cells or cell walls were removed by centrifugation, and re-extracted for another 45 min with fresh solvent (50 ml). The two extracts were pooled and the volume reduced under vacuum to about 50 ml using a rotary evaporator operating at 40°. The lipid extract was washed twice with water in a separating funnel. The lower layer of washed lipid extract was then separated and evaporated to about 5 ml. This 5 ml was filtered through 0.2 μm solvent-resistant membrane to remove residual cells or cell walls. The filter was washed with 3 x 5 ml of solvent, and the extract and washings bulked and evaporated to dryness at 40°, as described before, using the rotary evaporator. The readily extractable lipids (REL) in the evaporating flask and extracted cells or cell walls in the centrifuge tubes were dried over P_2O_5 *in vacuo* to a constant weight. The weighed REL was taken up in about 1 ml of solvent, and 20 ml of diethyl ether at -20° added to precipitate β-polyhydroxybutyrate [ether insoluble fraction (EIF)] (Brown & Watkins, 1970). The β-polyhydroxybutyrate was centrifuged in a tared centrifuge tube, and the ethereal extract evaporated to dryness at 40°. The ether-soluble lipids (phospholipids, free fatty acids and neutral lipids) were dried over P_2O_5 *in vacuo* to a constant weight. The weighed ether-soluble lipids were taken up in 1 ml of solvent, and 20 ml

of acetone at -20° was added to precipitate the phospholipids (PL). The PL were removed by centrifugation in a tared centrifuge tube, and the acetone evaporated to dryness at 40°. The acetone-soluble extract, containing the free fatty acids and neutral lipids (FAN), was dried over P_2O_5 in vacuo to a constant weight.

2.3.2 Assay of phospholipids

The PL fractions were taken up in 0.5 ml of chloroform and stored at -20° until required. Ascending thin-layer chromatography was used to fractionate them into their component PL. Glass plates, 20 x 20 cm, were spread with a 0.25 mm layer of Silica Gel PF_{254} (E. Merck, Darmstadt, West Germany). The slurry was prepared by mixing 40 g of silica gel with 100 ml of 0.2% (w/v) aqueous sodium acetate; the plates were air-dried overnight (Minnikin & Abdolrahimzadeh, 1971) and then activated by heating at 70° for 90 min. They were ruled into nine 20 x 2 cm channels. Two channels were loaded with standards in duplicate and six channels with samples (Fig.11). The plates were developed in a lined chromatography tank using solvent B [chloroform - methanol - acetic acid - water (80 : 18 : 5 : 5)] described by Minnikin & Abdolrahimzadeh (1971). After development, the plates were air-dried, sprayed with 15%(w/v) ammonium sulphate, and the spots visualized by charring at 160° for 3 h.

The proportions of individual PL present were determined by densitometry. Integration readings were calculated for each peak, and expressed as a percentage of the integration reading obtained for all the peaks in a sample (Fig. 12).

Fig. 11 Phospholipid Assay: developed chromatogram of sample and standard phospholipids.

Sp, sample; St, standard phospholipids.

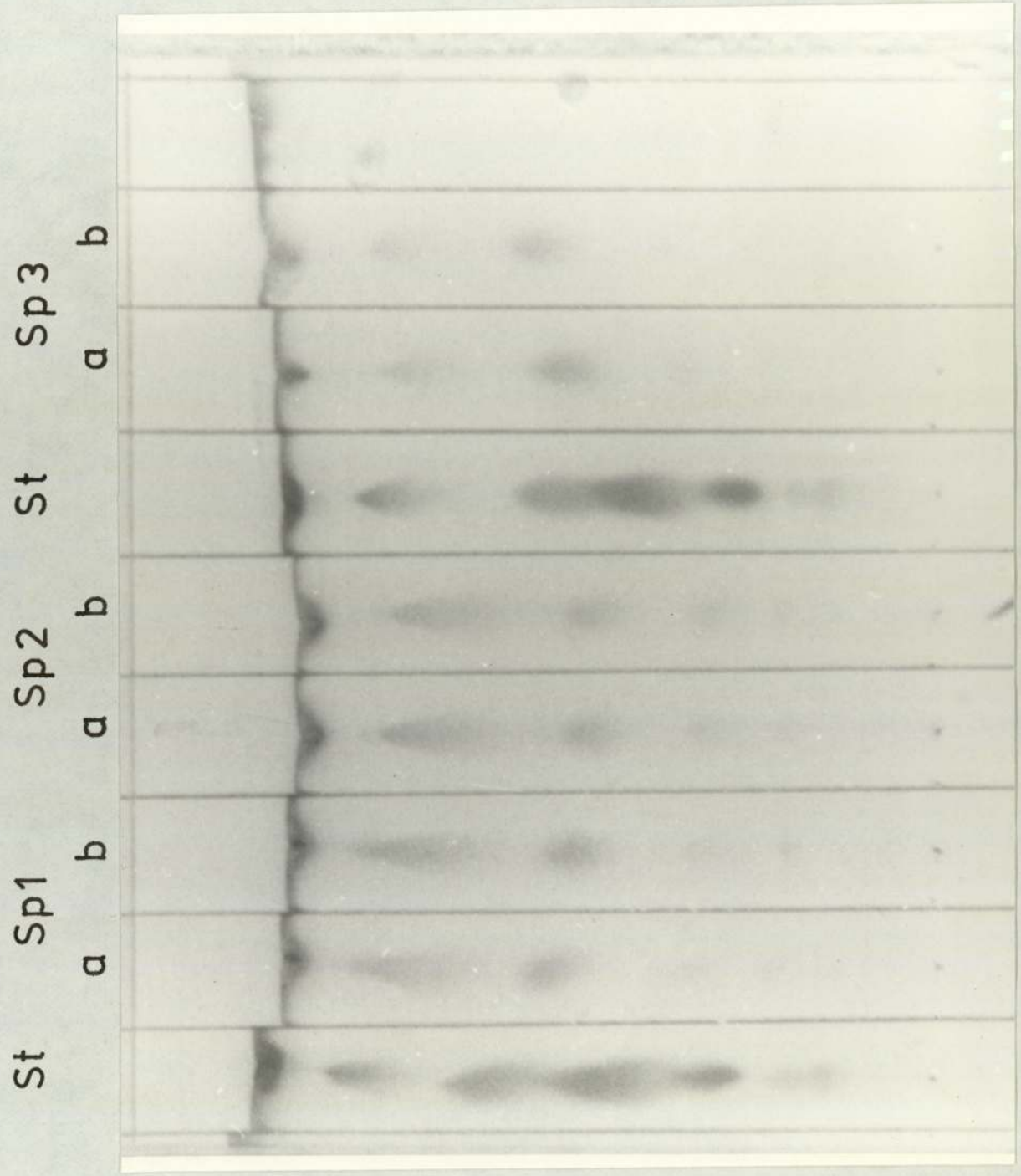
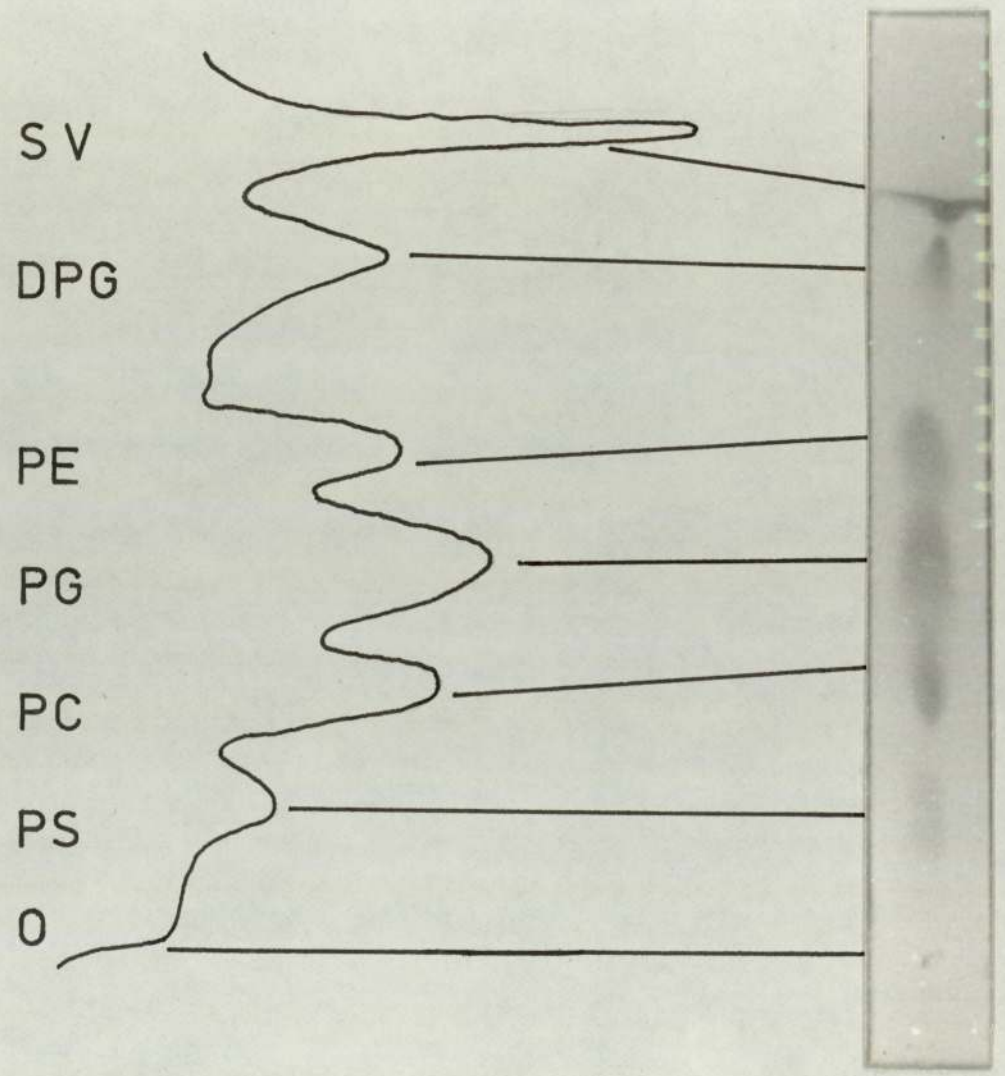

St
Sp1
a
b
Sp2
a
b
St
Sp3
a
b

Fig. 12. Comparison of densitometer chart recording with the corresponding thin layer chromatogram of phospholipid standards. SV, solvent front; DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PC, phosphatidylcholine; PS, Phosphatidylserine; O, origin.


S V
DPG
PE
PG
PC
PS
O

2.3.3 Assay of 2-keto-3-deoxyoctonic acid

The whole cell content of 2-keto-3-deoxyoctonic acid (KDO) was assayed using the method described by Weissbach & Hurwitz (1959).

A series of standard solutions of KDO were prepared in 0.05 M H_2SO_4 with concentrations ranging from 0 to 60 µg ml^{-1}, treated appropriately and the absorbance measured at 550 nm. A linear calibration line was obtained (Fig.13).

Accurately weighed whole cell samples (50 mg) were hydrolysed with 5 ml 0.05 M sulphuric acid in powder ampoules. The ampoules were sealed and placed in a boiling water bath for 30 min. After cooling, the hydrolysates were centrifuged and supernatants filtered through 0.2 µm millipore membrane filters. 0.25 ml samples were taken from the filtrates and assayed as described by Weissbach & Hurwitz (1959).

Since all 2-keto-3-deoxy sugar acids would react positively with thiobarbituric acid (Ellwood, 1970), all whole cell samples were assayed with and without an internal standard (0.25 ml 30 µg ml^{-1}).

The reproducibility of the assay was assessed by measuring the absorbance at 550 nm of five replicate samples from an acid hydrolysate of a sample of whole cells (Table 4).

2.3.4 Assay of calcium, magnesium and manganese

Standard solutions were prepared in 1% (v/v) HCl containing 1% (w/v) lanthanum chloride. The La^{3+} ions were added to prevent suppression of Ca^{2+} ion absorbance by phosphate ions (Willis, 1961). Since the same cell preparation was used to assay all 3 cations, the standard solutions for all cations were made up in 1% (w/v) La^{3+}.

Fig. 13. 2-keto-3-deoxyoctonic acid (KDO) calibration curve.

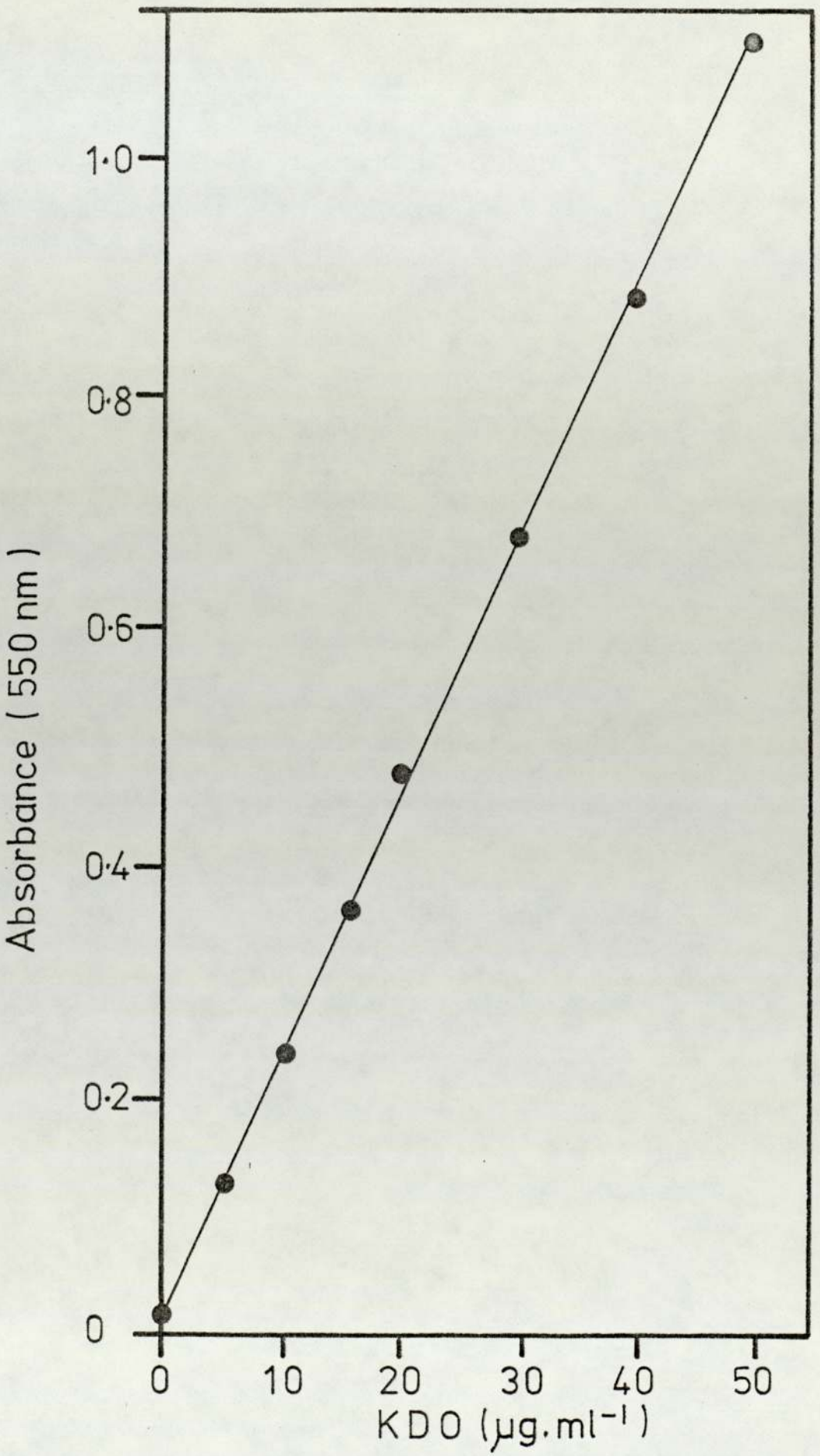

Absorbance (550 nm)
1·0
0·8
0·6
0·4
0·2
0
0
10
20
30
40
50
KDO (µg.ml⁻¹)

Table 4

Reproducibility of assay of 2-keto-3-deoxyoctonic acid

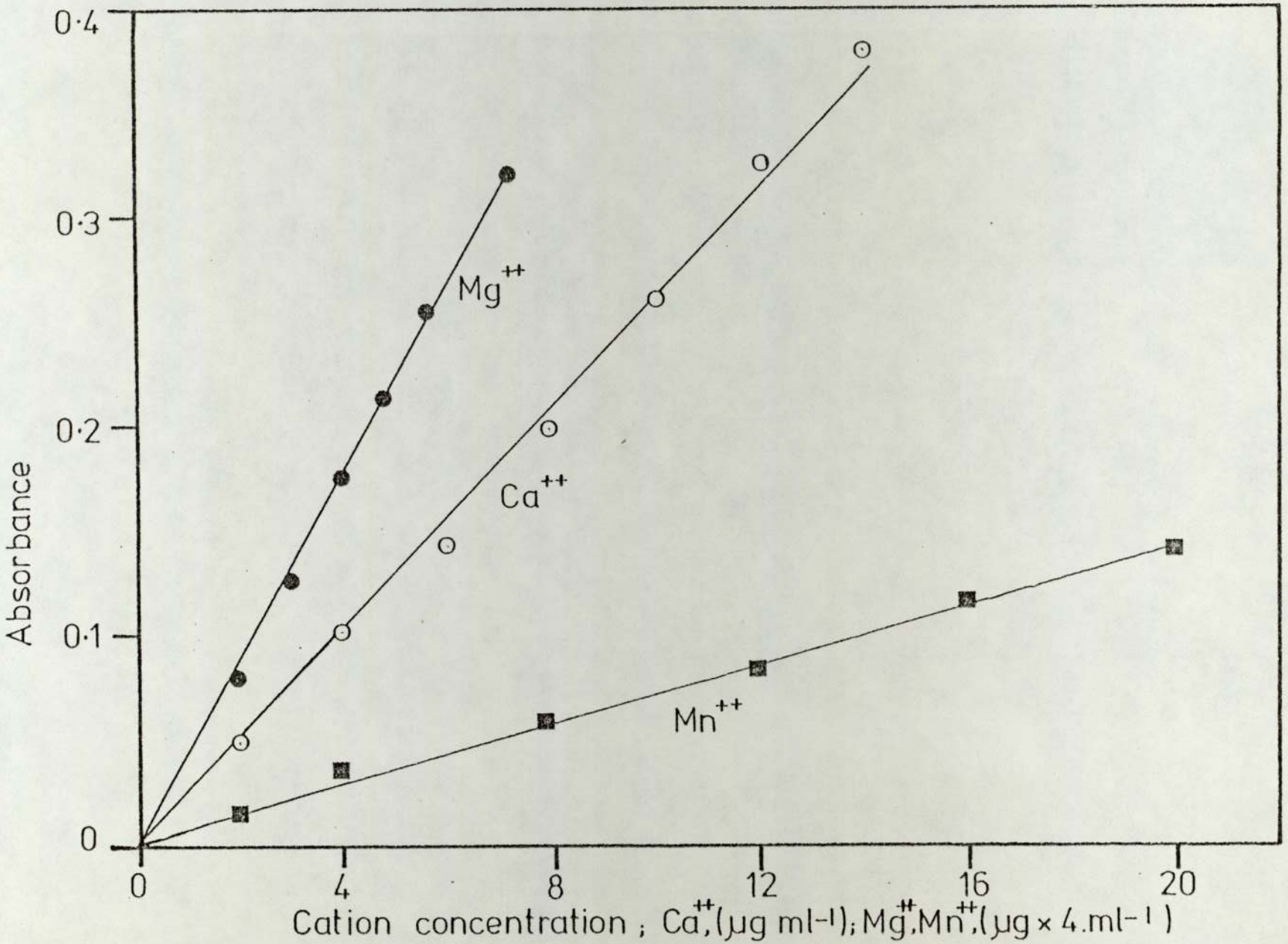
Replicates	Absorbance at 550 nm
1	0.320
2	0.316
3	0.330
4	0.348
5	0.313
Mean ± SD	0.325 ± 0.014
Coefficient of variation	4.3%

Standard solutions were assayed and calibration lines obtained (Fig.14). Each point is the mean value of 2 readings on the same sample.

The preparation of whole cells and cell walls for cation analysis was as follows: an accurately weighed sample (50 mg) of whole cells or cell walls was digested by boiling in 12 ml concentrated HNO_3, 3 ml aliquots added at a time. The samples were heated to dryness and on cooling, the ash was dissolved in 0.4 ml concentrated HCl and transferred to a 5 ml volumetric flask. The digestion tube was washed twice with 2 ml volumes of 1% HCl - 1% $LaCl_3$. The washings were added to the volumetric flask and the volume made up to 5 ml. This solution was then assayed directly or after suitable dilution.

The reproducibility of the assay was determined by measuring the cation content of five replicate samples of a cell wall

Fig. 14. Atomic absorption spectrophotometric assay of calcium, magnesium and manganese. Absorption wavelengths, Mg^{2+}, 285.2 nm; Mn^{2+}, 279.5 nm; Ca^{2+}, 422.7 nm.


0·4
0·3
0·2
0·1
0
Absorbance
Mg++
Ca++
Mn++
0
4
8
12
16
20
Cation concentration ; Ca++,(µg ml-1); Mg++,Mn++,(µg × 4.ml-1)

suspension. The results are shown in Table 5.

Table 5

Reproducibility of the assay for calcium, magnesium and manganese

Replicates	Calcium $\mu g\ ml^{-1}$	Magnesium $\mu g\ ml^{-1}$	Manganese $\mu g\ ml^{-1}$
1	5.5	1.1	0.13
2	5.5	1.12	0.12
3	5.6	1.11	0.13
4	5.8	1.12	0.11
5	5.4	1.13	0.12
Mean	5.56	1.11	0.122
Coefficient of Variation	2.7%	1.02%	6.8%

3. EXPERIMENTAL AND RESULTS

3.1 Nutrient Depletion Studies on P. mirabilis 5887 and RBH and Formulation of Chemically-Defined Media

It was the object of this study 1) to investigate and compare the nutritional requirements of two different strains of P. mirabilis, the polymyxin-sensitive RBH, and the polymyxin-resistant 5887; 2) to obtain data relating the extent of growth (OD at onset of depletion) to the concentrations in the medium of essential nutrients; 3) to formulate a defined medium which could be depleted of any desired component, but containing a controlled excess of all other components.

Experimental details of culture volumes, inocula, temperature, shaking rate and sampling procedures have been described in section 2.2.2.

3.1.1 Preliminary studies

Using the CDM described in Table 1 (p.66), which was based on the known requirements for Proteus (Wilson & Miles, 1966), the requirement for each ingredient was studied using RBH and a medium devised sufficient to support exponential growth theoretically to an OD of 10.0 (Table 6). The actual OD of stationary cells was found to be 6.5 - 8.0.

3.1.2 Effect of pH on the growth of P. mirabilis in CDM

A CDM sufficient to support growth of 5887 and RBH exponentially up to OD 2.5 was used (Table 7). Ammonium phosphate adjusted to different pHs was the buffering agent. Fig.15 shows the effect of pH on both doubling time and maximum OD for P. mirabilis RBH. There was no significant effect on doubling time over the pH range 5.5 to 7.3. However, above that there

Table 6

Chemically-defined medium with each ingredient sufficient to support growth of *P. mirabilis* RBH theoretically to OD 10

Ingredient	Molarity
glucose	4×10^{-2}
nicotinic acid	6×10^{-5}
NaCl	5×10^{-5}
KCl	3×10^{-2}
$(NH_4)_2SO_4$	2×10^{-4}
$MgSO_4$	2×10^{-4}
$(NH_4)_2HPO_4$ + $NH_4H_2PO_4$ pH 7.0	10^{-1}

Medium pH 7.0

was a gradual increase in doubling time up to 90 min at pH 7.8. Over the pH range 6.2 - 7.5 there was a small drop in the maximum OD from 3.2 to 2.5. When the initial pH was between 6.2 and 7.5, the pH only dropped 0.1 during growth.

In conclusion, it seems that a pH range of 6.2 - 7.5 would be suitable for the growth of *P. mirabilis* in CDM. pH 7.0 was used in the initial experiments, and is a suitable pH for testing a variety of antibacterial agents. It was therefore used throughout.

3.1.3 Nutrient requirements of *P. mirabilis* 5887 and RBH

Using concentrations of nutrients when required in excess which would support theoretical exponential growth to OD 10.0, and altering the salts to allow requirements to be studied in

Table 7

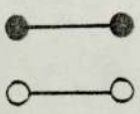
Chemically-defined medium used to investigate the effect of pH

Ingredient	Molarity
Glucose	9.5×10^{-3}
Nicotinic acid	6×10^{-5}
NaCl	5×10^{-5}
KCl	1×10^{-4}
$(NH_4)_2SO_4$	5×10^{-5}
$MgSO_4$	5×10^{-2}
$(NH_4)_2HPO_4$ + $NH_4H_2PO_4$	8×10^{-2}

detail (Table 8), the requirements of 5887 and RBH were compared. There was no significant difference in the relation between nutrient concentration and OD at the end of exponential growth for the 2 strains; only one set of graphs illustrating the results for each nutrient (Fig. 16-30) is therefore shown, compiled from the data for both strains together.

In all cases where a requirement for a nutrient could be demonstrated, a linear relationship was found between the OD at the end of exponential growth and the amount of added nutrient up to an OD of 4.0. The doubling time during exponential growth was the same, except at low concentrations of Mg^{2+} and phosphate and at the lowest concentration of glucose. The plot of OD: concentration of added nutrient did not always pass through the origin. In such cases, it was extrapolated back to the

Fig. 15. The effect of initial pH on doubling time (dt) and yield of *P. mirabilis* 5887.



, max OD

, dt

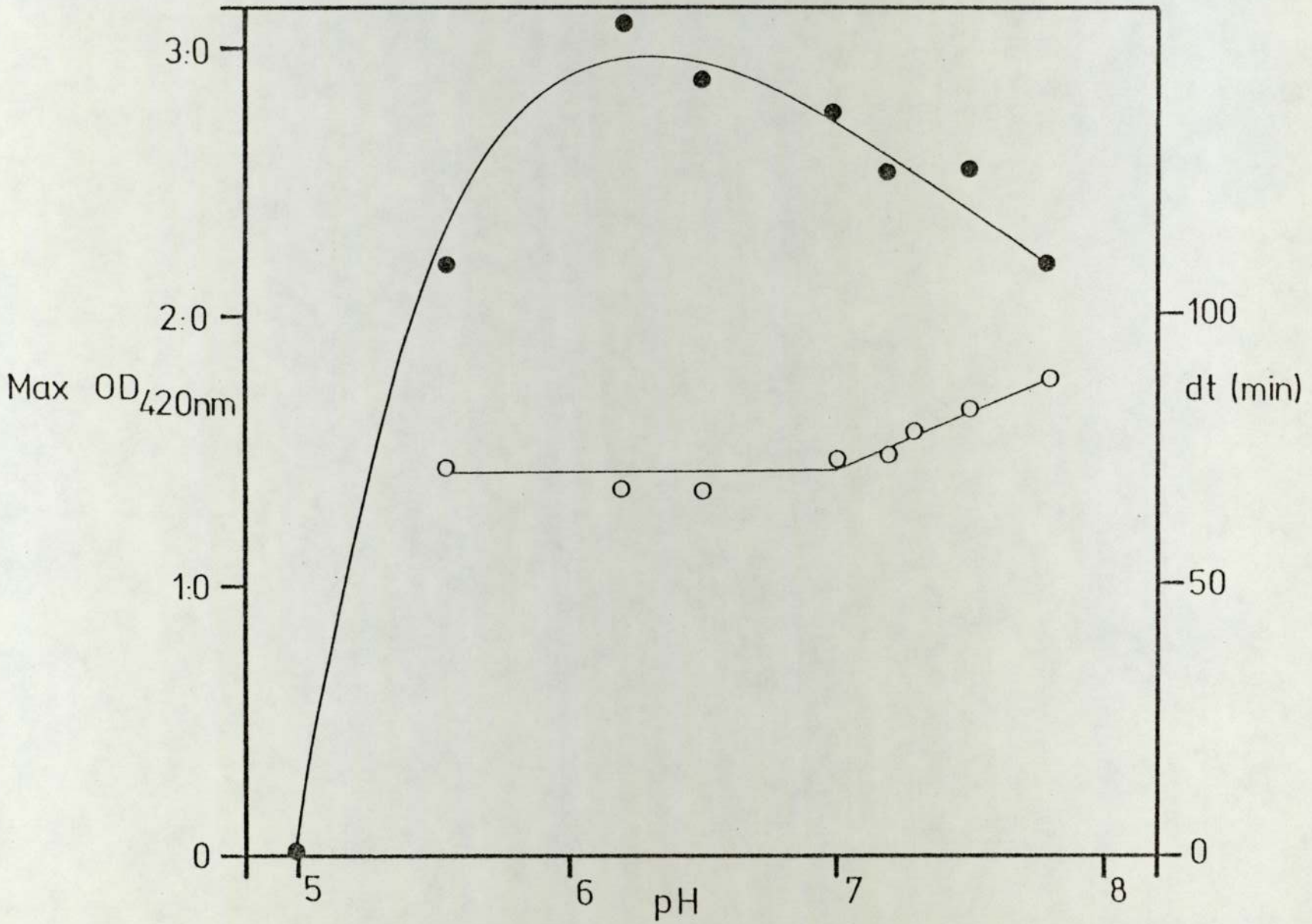

3·0
2·0
1·0
0
Max OD_{420nm}
100
50
0
dt (min)
5
6
7
8
pH

Table 8

Chemically-defined media used to quantify nutrient requirements of *P. mirabilis* 5887 and RBH

Compound used in medium	Medium component under investigation									
	Glucose	Nicotinic acid	Na^+	K^+	NH_4^+	Mg^{2+}	other cations(i)	Cl^-	SO_4^{2-}	$HPO_4^{2-}/H_2PO_4^-$
glucose	*	4×10^{-2}	4×10^{-2}	4×10^{-2}	4×10^{-2}	4×10^{-2}	4×10^{-2}	4×10^{-2}	4×10^{-2}	4×10^{-2}
nicotinic acid	6×10^{-5}	*	6×10^{-5}	6×10^{-5}	6×10^{-5}	6×10^{-5}	6×10^{-5}	6×10^{-5}	6×10^{-5}	6×10^{-5}
NaCl	5×10^{-5}	5×10^{-5}	0	5×10^{-5}	5×10^{-5}	5×10^{-5}	5×10^{-5}	0	5×10^{-5}	5×10^{-5}
KCl	3×10^{-2}	3×10^{-2}	3×10^{-2}	*	3×10^{-2}	3×10^{-2}	3×10^{-2}	0	3×10^{-2}	3×10^{-2}
$(NH_4)_2SO_4$	2×10^{-4}	2×10^{-4}	2×10^{-4}	2×10^{-4}	0	2×10^{-4}	2×10^{-4}	2×10^{-4}	0	2×10^{-4}
$MgSO_4$	2×10^{-4}	2×10^{-4}	2×10^{-4}	2×10^{-4}	2×10^{-4}	*	2×10^{-4}	2×10^{-4}	0	2×10^{-4}
$(NH_4)_2HPO_4$ / $NH_4H_2PO_4$ (ii)	10^{-1}	10^{-1}	10^{-1}	10^{-1}	0	10^{-1}	10^{-1}	10^{-1}	10^{-1}	*
NH_4Cl	0	0	0	0	*	0	0	0	0	0
Na_2HPO_4 / KH_2PO_4 (ii)	0	0	0	0	10^{-1}	0	0	0	0	0
other cations	0	0	0	0	0	0	*	0	0	0
K_2SO_4	0	0	0	0	0	0	0	3×10^{-2}	0	0
Na_2SO_4	0	0	0	0	0	0	0	0	*	0
$MgCl_2$	0	0	0	0	0	0	0	0	2×10^{-4}	0

*Component added at various concn., see Figs. 16-29.

(i) $CaCl_2$, $CoCl_2$, $MnSO_4$, $(NH_4)_2SO_4.FeSO_4$, $(NH_4)_6Mo_7O_{24}$ and $ZnSO_4$ all tested at 0.1 μg ml^{-1}

(ii) pH 7.0, concn refers to phosphate

x-axis. The intercept was a measure of the initial contamination of the medium from other constituents or glassware.

3.1.3.1 Glucose requirement is shown in Figs. 16 & 17. Growth ceases abruptly as glucose becomes depleted up to an initial glucose concentration of 8×10^{-3} M. Above that concentration, the total growth was greater but there was a progressive slowing of the growth above OD 4.0. The line in Fig. 17 passes through the origin. This indicates that there was insufficient material present as a contaminant and utilisable as a carbon source, to support visible growth.

3.1.3.2 Magnesium requirement is shown in Figs. 18 & 19. At the end of the exponential growth, growth did not cease abruptly as magnesium became limiting, but progressively slowed. The intercept on the x-axis represents an initial magnesium contamination of 2×10^{-6} M.

3.1.3.3 Sulphate requirement. Figs. 20 and 21 show the relationship between sulphate concentration and growth. The intercept on the x-axis in Fig. 21 corresponds to a sulphate contamination level of 5×10^{-6} M.

3.1.3.4 Nitrogen requirement. Growth curves and the relationship between OD at onset of limitation and NH_4Cl concentration in the medium are illustrated in Figs. 22 and 23. The shape of the growth curves is similar to that of glucose-depleted cultures; as nitrogen became limiting, the growth ceased abruptly. The contamination level of utilisable nitrogenous material was equivalent to about 1×10^{-3} M NH_4Cl.

3.1.3.5 Potassium requirement is shown in Figs. 24 and 25. The x-intercept indicates a potassium contamination of 2×10^{-5} M.

3.1.3.6 Phosphate requirement is illustrated in Figs. 26 and 27. The intercept on the x-axis indicates a phosphate contamination level of 10^{-3} M.

3.1.3.7 Sodium and chloride requirements. Fig. 28 shows that the addition of sodium and chloride to a medium had no effect on the extent of growth or its rate. It can be concluded that the requirements for sodium and chloride are satisfied by contaminating traces of these ions from other constituents of the medium.

3.1.3.8 Nicotinic acid requirement. Fig. 29 shows no difference between the growth curves of media containing no nicotinic acid or 6×10^{-5} or 6×10^{-4} M nicotinic acid.

3.1.3.9 Requirement for other trace cations. In all CDM tested, the relationship between concentration of limiting ingredient and growth departured from linearity at OD 4.0. This might have been caused by depletion of some essential trace elements. To test this possibility, cultures were grown in media supplemented with one of the following: $ZnSO_4.7H_2O$, $(NH_4)_6Mo_7O_{24}.4H_2O$, $CaCl_2.2H_2O$, $CoCl_2.6H_2O$, $MnSO_4.4H_2O$, $(NH_4)_2SO_4.FeSO_4.6H_2O$, or with a mixture of all of them, each at a concentration of 0.1 µg/ml. None of these cultures exhibited faster or more growth than the unsupplemented control (Fig. 30).

Fig. 16. The effect of glucose concentration on the growth of *P. mirabilis* 5887 and RBH.

Glucose conc^{n}. (M) ● , 1×10^{-1}; ○ , 4×10^{-2}; ■ , 2×10^{-2}; □ , 1×10^{-2}; ▲ , 8×10^{-3}; △ , 5×10^{-3}; ◆ , 3×10^{-3}; ◇ , 1×10^{-3}; ◑ , 5×10^{-4}; ◐ , 3×10^{-4}; ◪ , 2×10^{-4}.

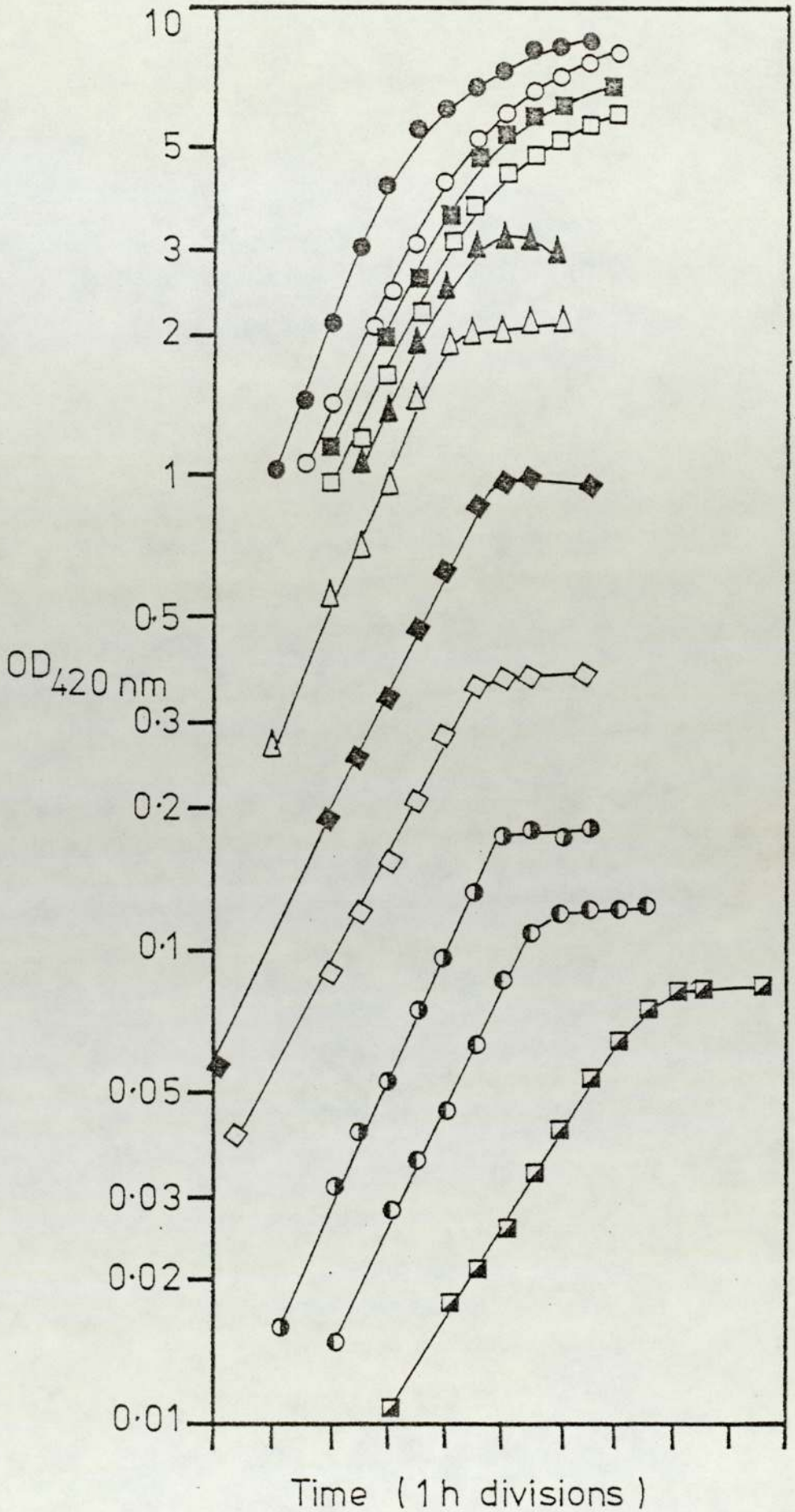

10
5
3
2
1
0·5
0·3
0·2
0·1
0·05
0·03
0·02
0·01
OD420 nm
Time (1 h divisions)

Fig. 17. The effect of glucose concentration on the maximum OD reached during exponential growth.

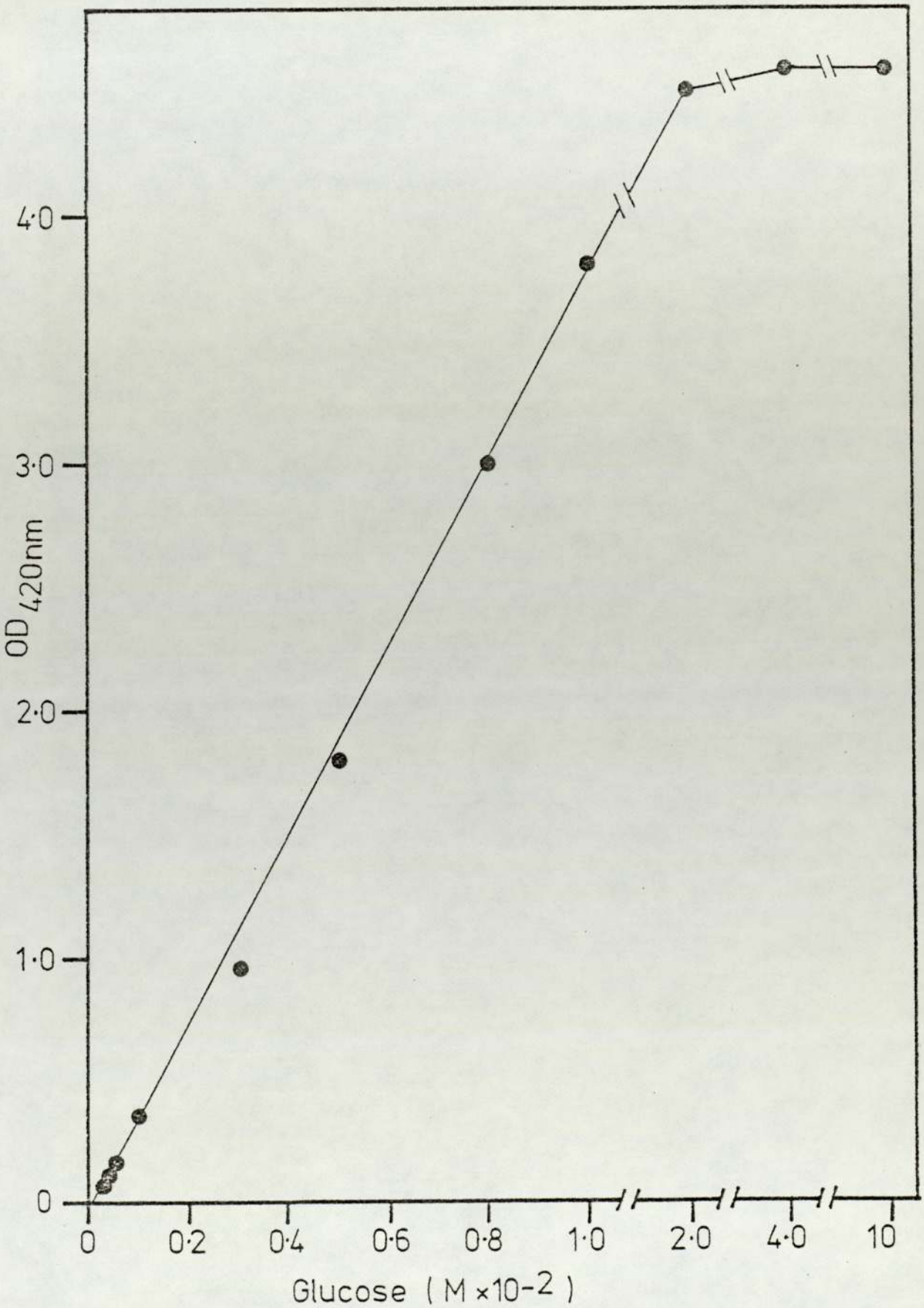
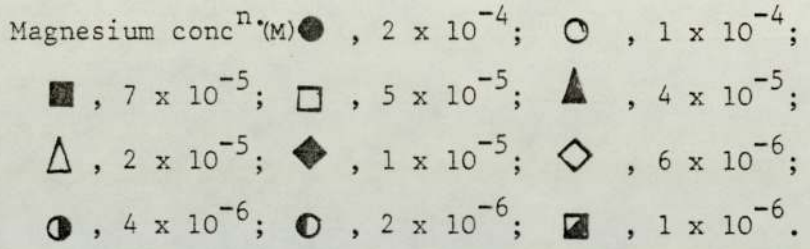

4·0
3·0
2·0
1·0
0
OD 420nm
0
0·2
0·4
0·6
0·8
1·0
2·0
4·0
10
Glucose (M ×10-2)

Fig. 18. The effect of magnesium concentration on the growth of *P. mirabilis* 5887 and RBH.



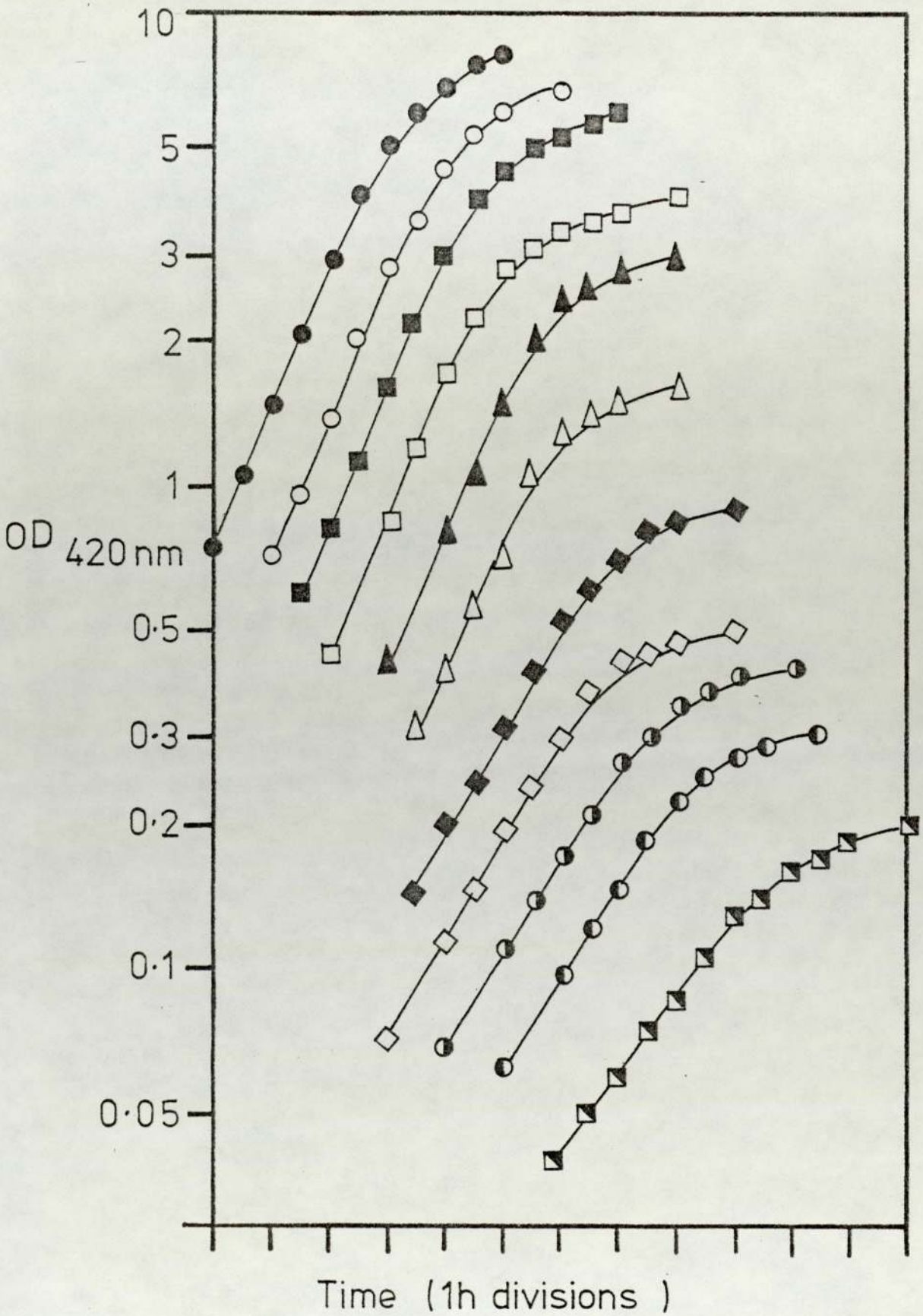

10
5
3
2
1
OD 420 nm
0·5
0·3
0·2
0·1
0·05
Time (1h divisions)

Fig. 19. The effect of magnesium concentration on the maximum OD reached during exponential growth.

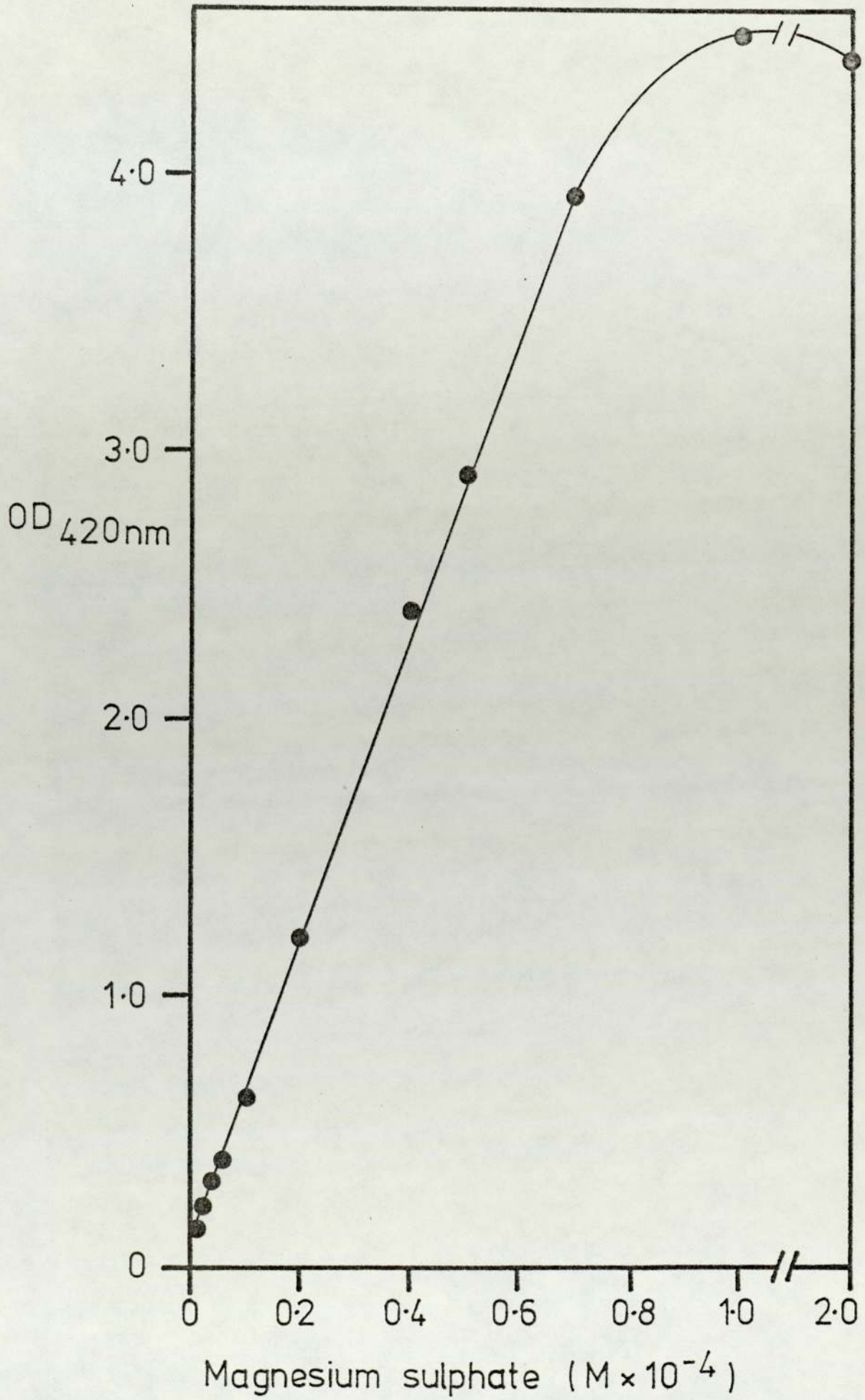

4·0
3·0
OD 420nm
2·0
1·0
0
0
0·2
0·4
0·6
0·8
1·0
2·0
Magnesium sulphate (M × 10⁻⁴)

Fig. 20. The effect of sulphate concentration on the growth of *P. mirabilis* 5887 and RBH.

Sulphate conc^{n}. (M); ● , 6×10^{-4}; ○ , 4×10^{-4}; ■ , 2×10^{-4}; □ , 1×10^{-4}; ▲ , 5×10^{-5}; △ , 3×10^{-5}; ◆ , 2×10^{-5}; ◇ , 1×10^{-5}.

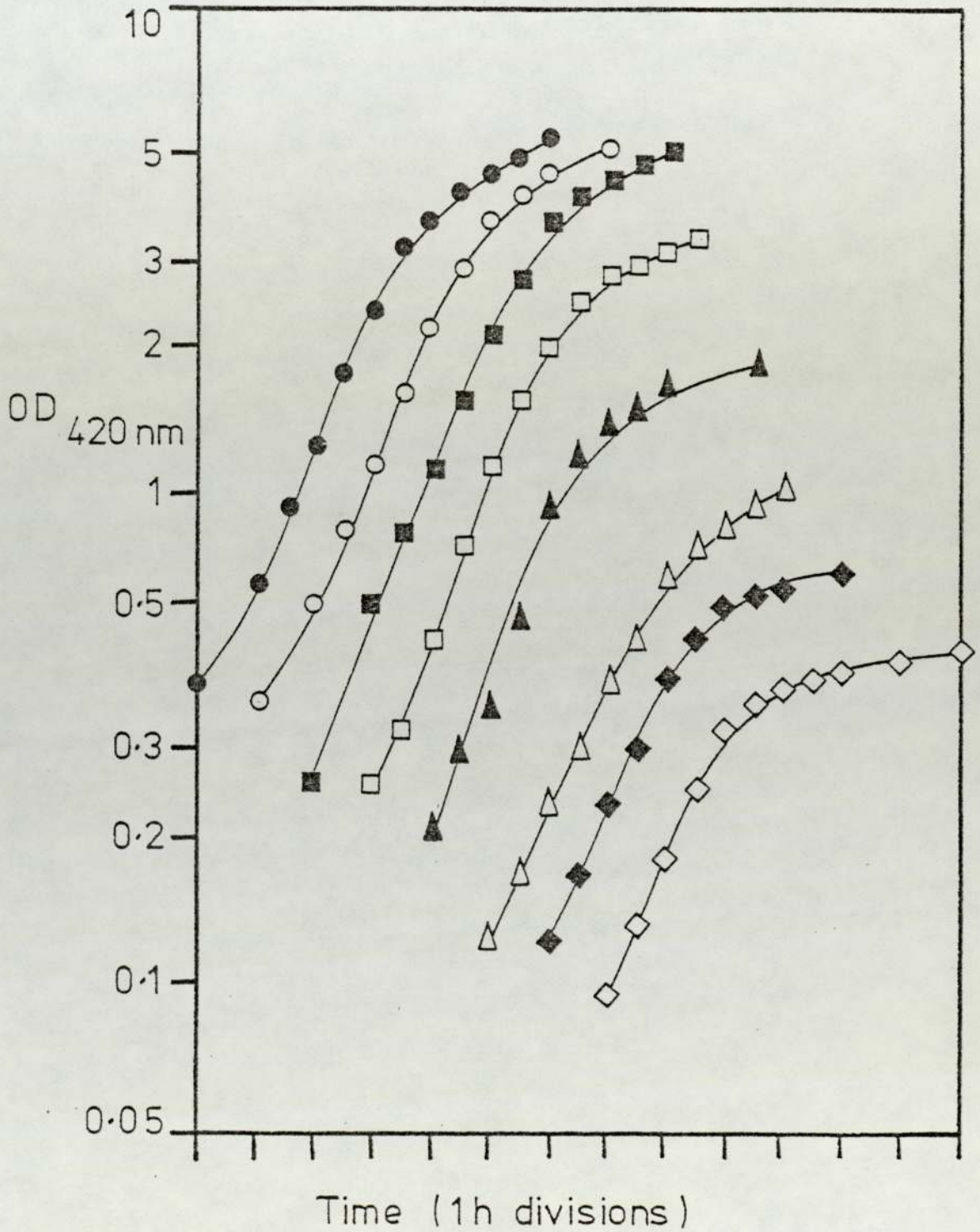

10
5
3
2
OD 420 nm
1
0·5
0·3
0·2
0·1
0·05
Time (1h divisions)

Fig. 21. The effect of sulphate concentration on the maximum OD reached during exponential growth.

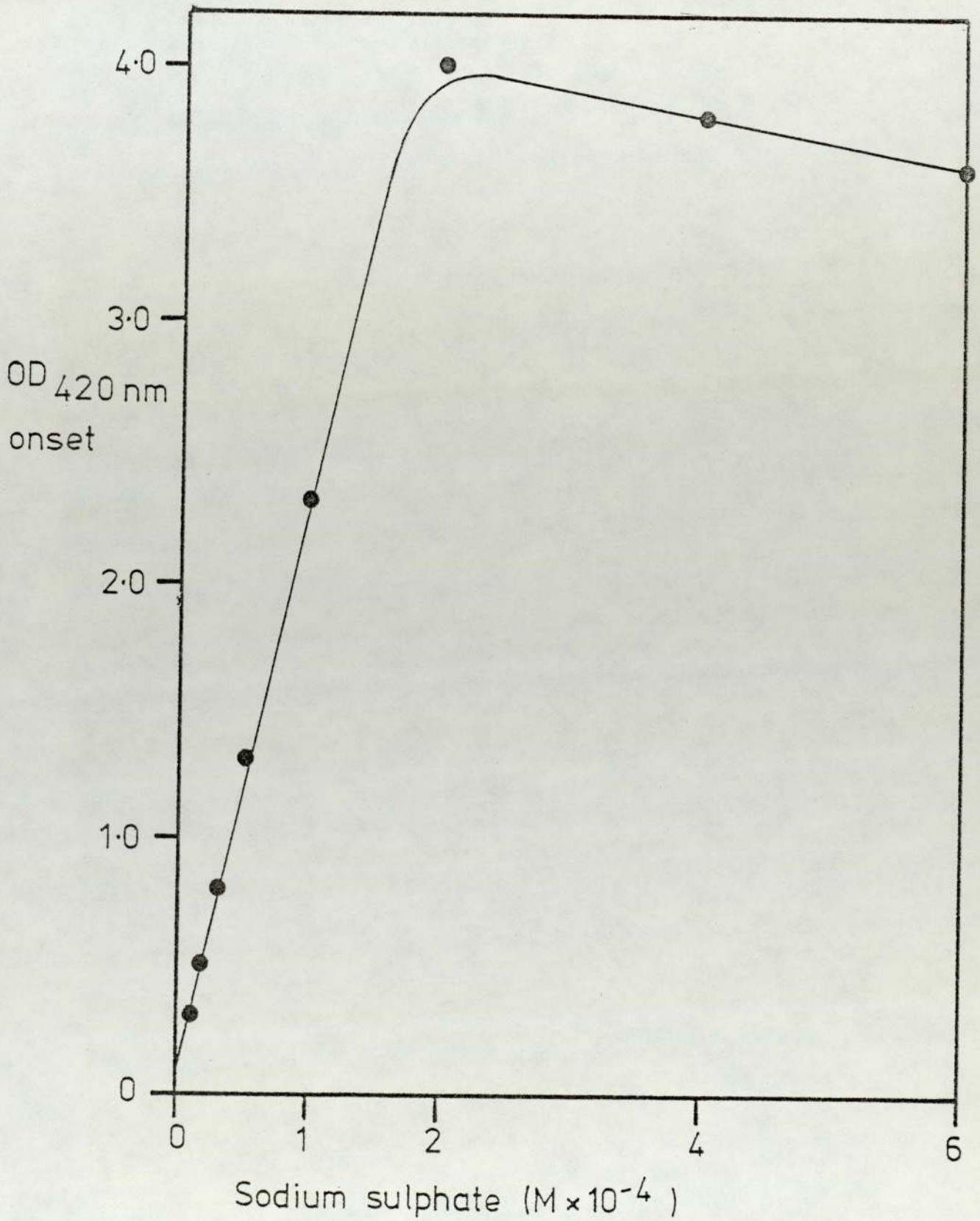

4·0
3·0
2·0
1·0
0
OD 420 nm onset
0
1
2
4
6
Sodium sulphate (M × 10⁻⁴)

Fig. 22. Effect of ammonium chloride concentration on the growth of *P. mirabilis* 5887 and RBH.

NH_4Cl concentration (M); ● , 6×10^{-2}; ○ , 4×10^{-2}; ■ , 2×10^{-2}; □ , 1×10^{-2}; ▲ , 8×10^{-3}; △ , 4×10^{-3}; ◆ , 2×10^{-3}; ◇ , 6×10^{-4}.

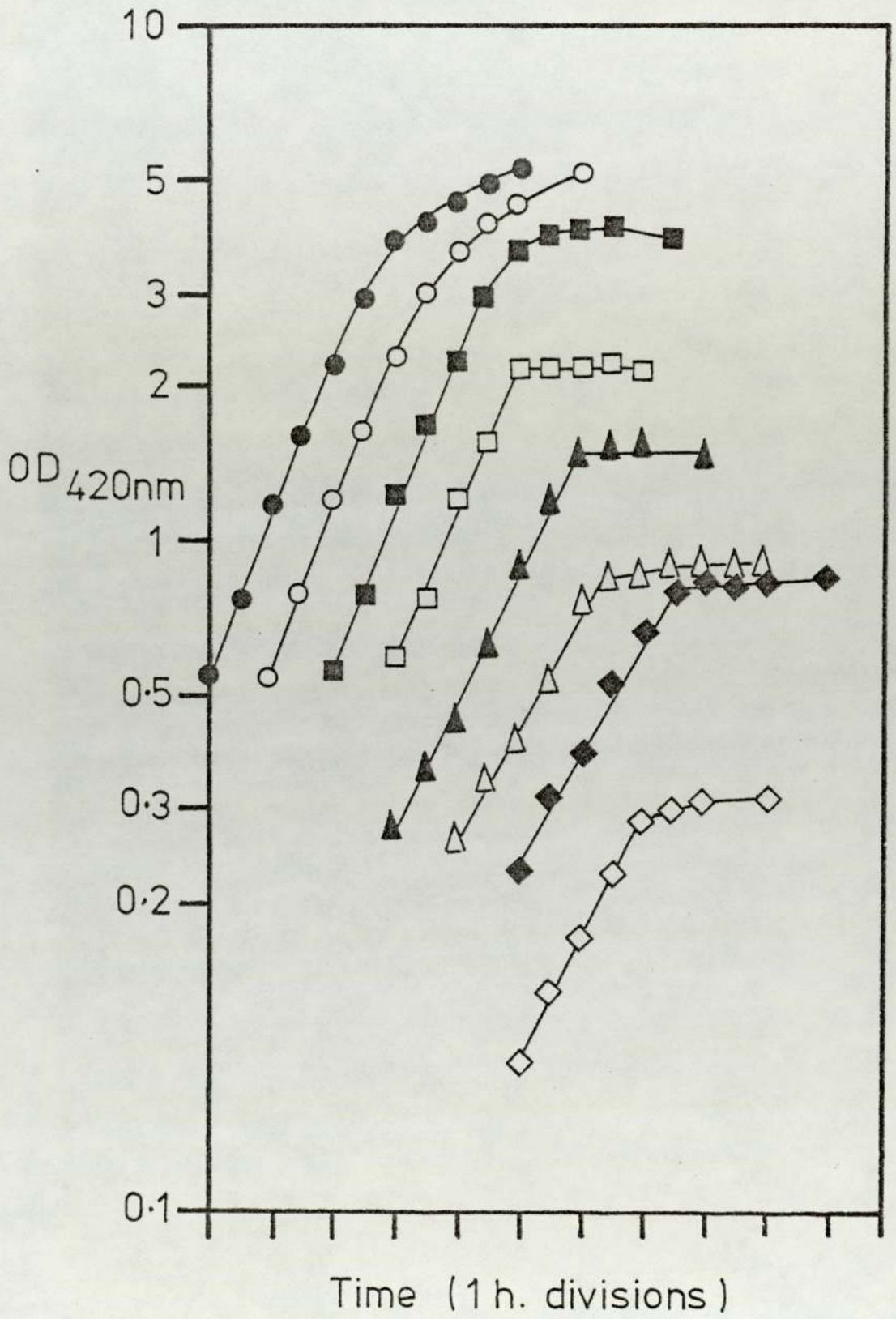

10
5
3
2
OD 420nm
1
0·5
0·3
0·2
0·1
Time (1 h. divisions)

Fig. 23. The effect of ammonium concentration on the maximum OD reached during exponential growth.

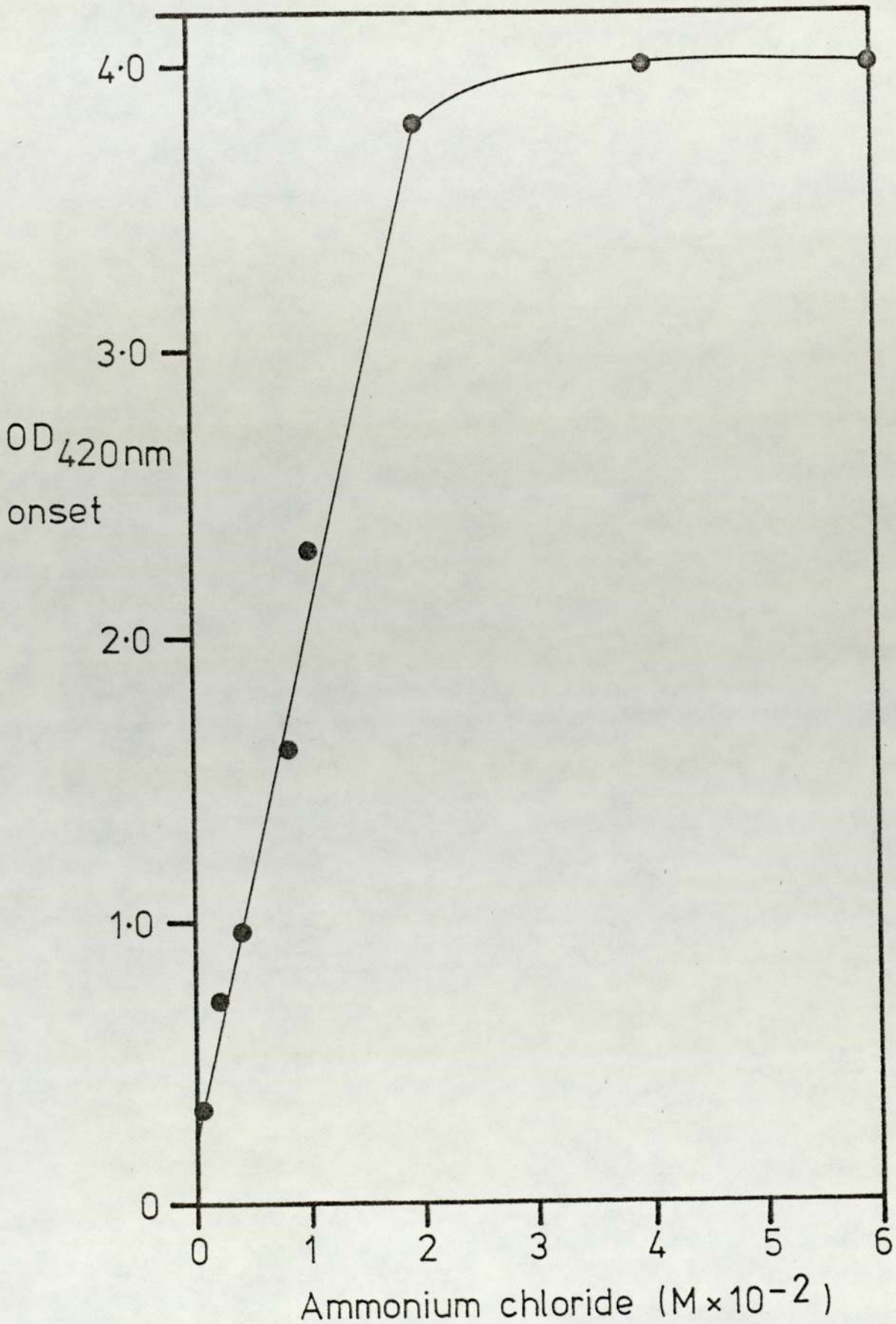

4·0
3·0
2·0
1·0
0
OD 420nm onset
0
1
2
3
4
5
6
Ammonium chloride (M × 10⁻²)

Fig. 24. The effect of potassium concentration on the growth of *P. mirabilis* 5887 and RBH.

Potassium ion conc^{n}. (M); ● , 3×10^{-2}; ○ , 5×10^{-4}; ■ , 3×10^{-4}; □ , 2×10^{-4}; ▲ , 1.5×10^{-4}; △ , 1×10^{-4}; ◆ , 8×10^{-5}; ◇ , 5×10^{-5}; ◑ , 3×10^{-5}; ◐ , 1×10^{-5}.

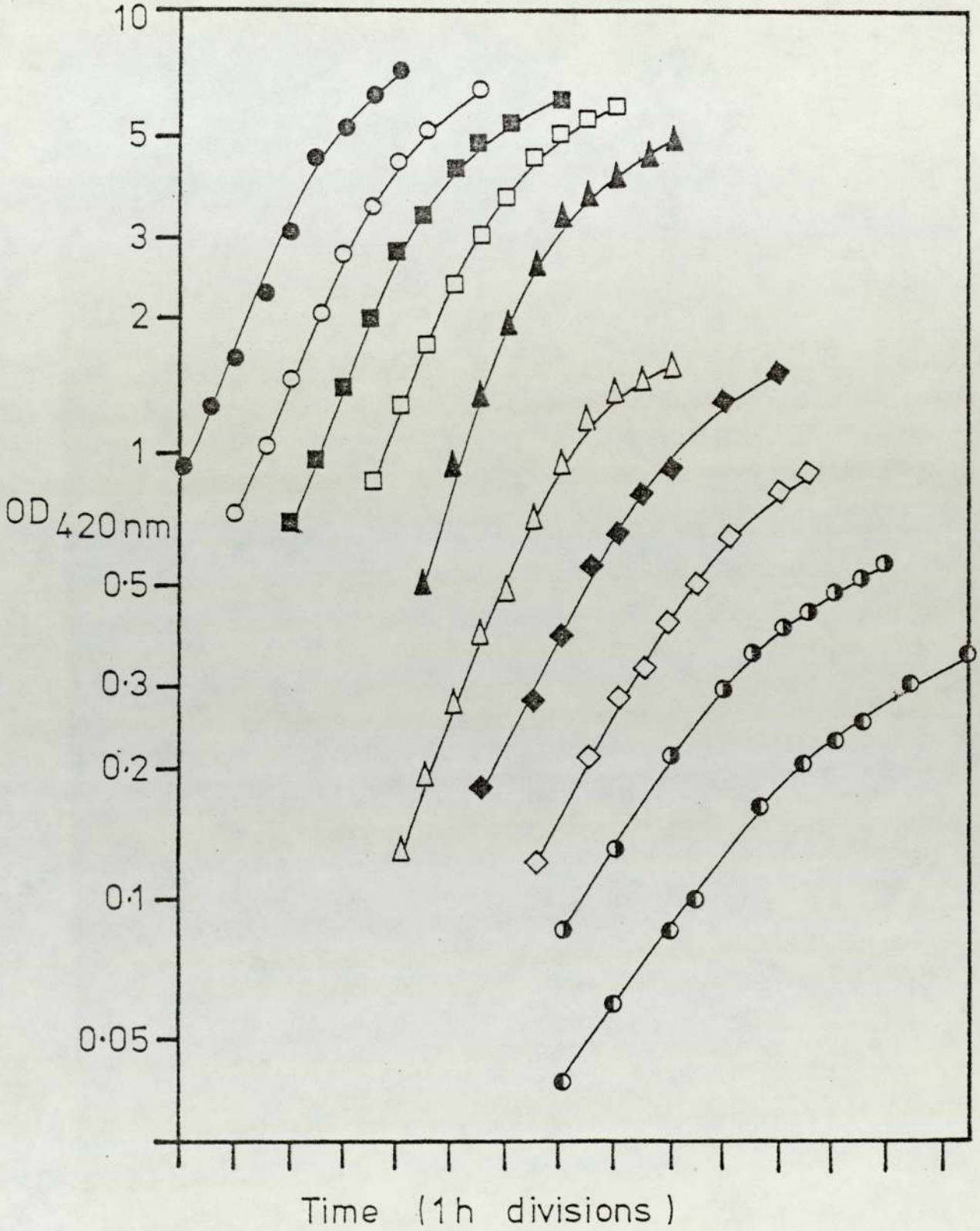

10
5
3
2
1
OD 420 nm
0·5
0·3
0·2
0·1
0·05
Time (1 h divisions)

Fig. 25. The effect of potassium concentration on the maximum OD reached during exponential growth.

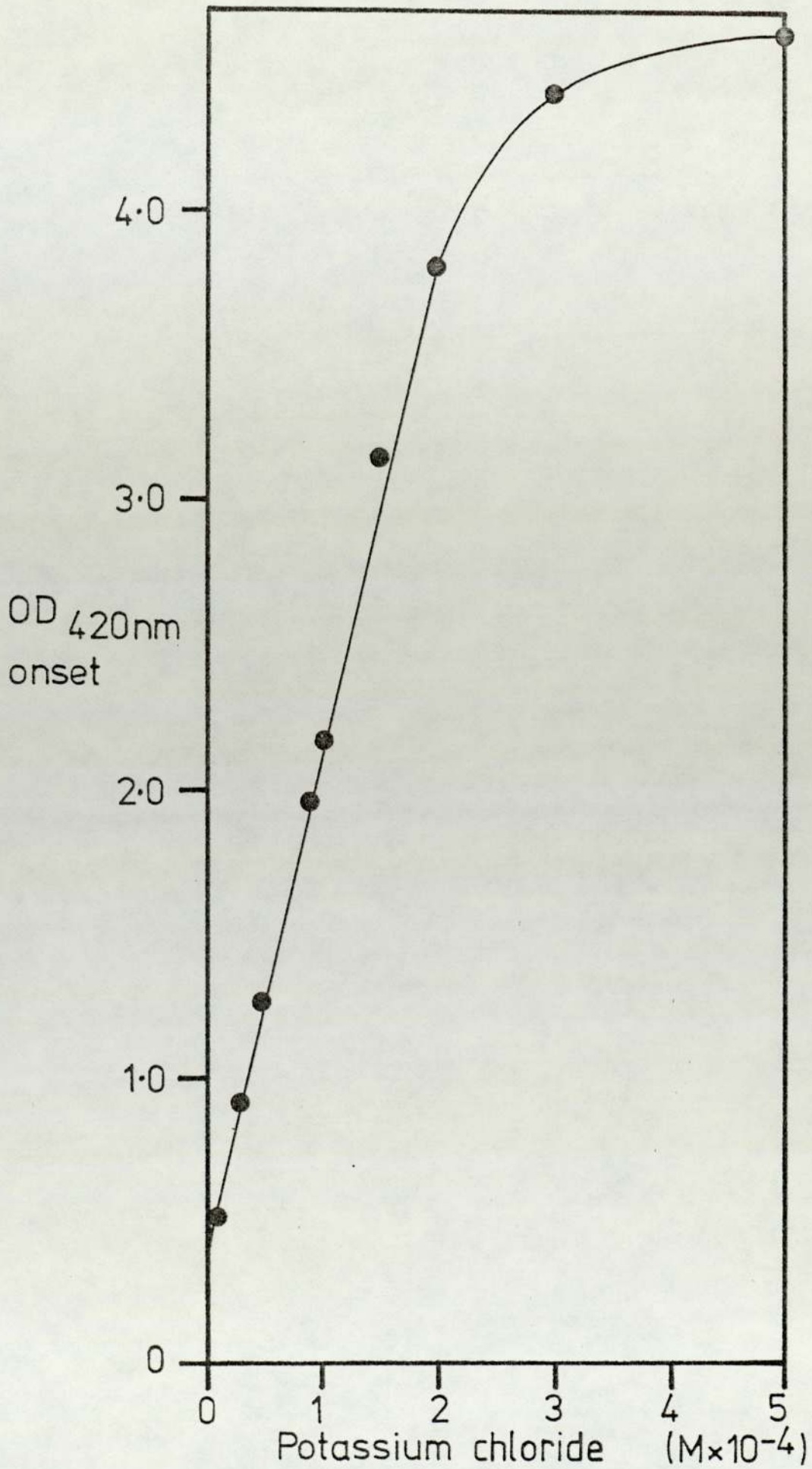

4·0
3·0
2·0
1·0
0
OD 420nm onset
0
1
2
3
4
5
Potassium chloride (M×10⁻⁴)

Fig. 26. The effect of phosphate concentration on the growth of *P. mirabilis* 5887 and RBH.

Phosphate conc$^{n.}$ (M); ● , 2×10^{-1}; ○ , 1×10^{-1}; ■ , 8×10^{-2}; □ , 6×10^{-2}; ▲ , 4×10^{-2}; △ , 2×10^{-2}; ◆ , 8×10^{-3}; ◇ , 4×10^{-3}; ◕ , 2×10^{-3}; ◐ , 1×10^{-3}.

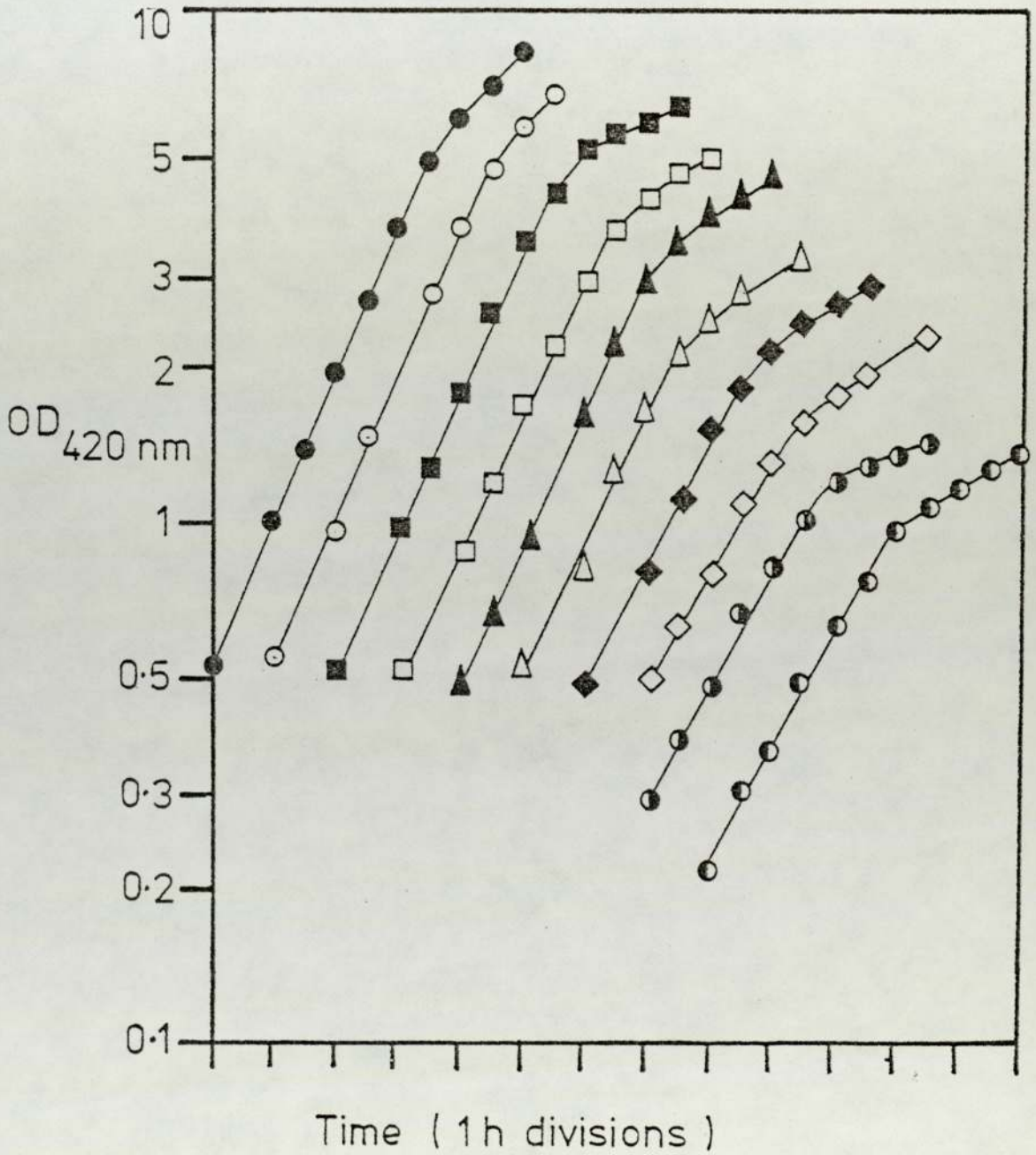

10
5
3
2
1
0·5
0·3
0·2
0·1
OD 420 nm
Time (1 h divisions)

Fig. 27. The effect of phosphate concentration on the maximum OD reached during exponential growth.

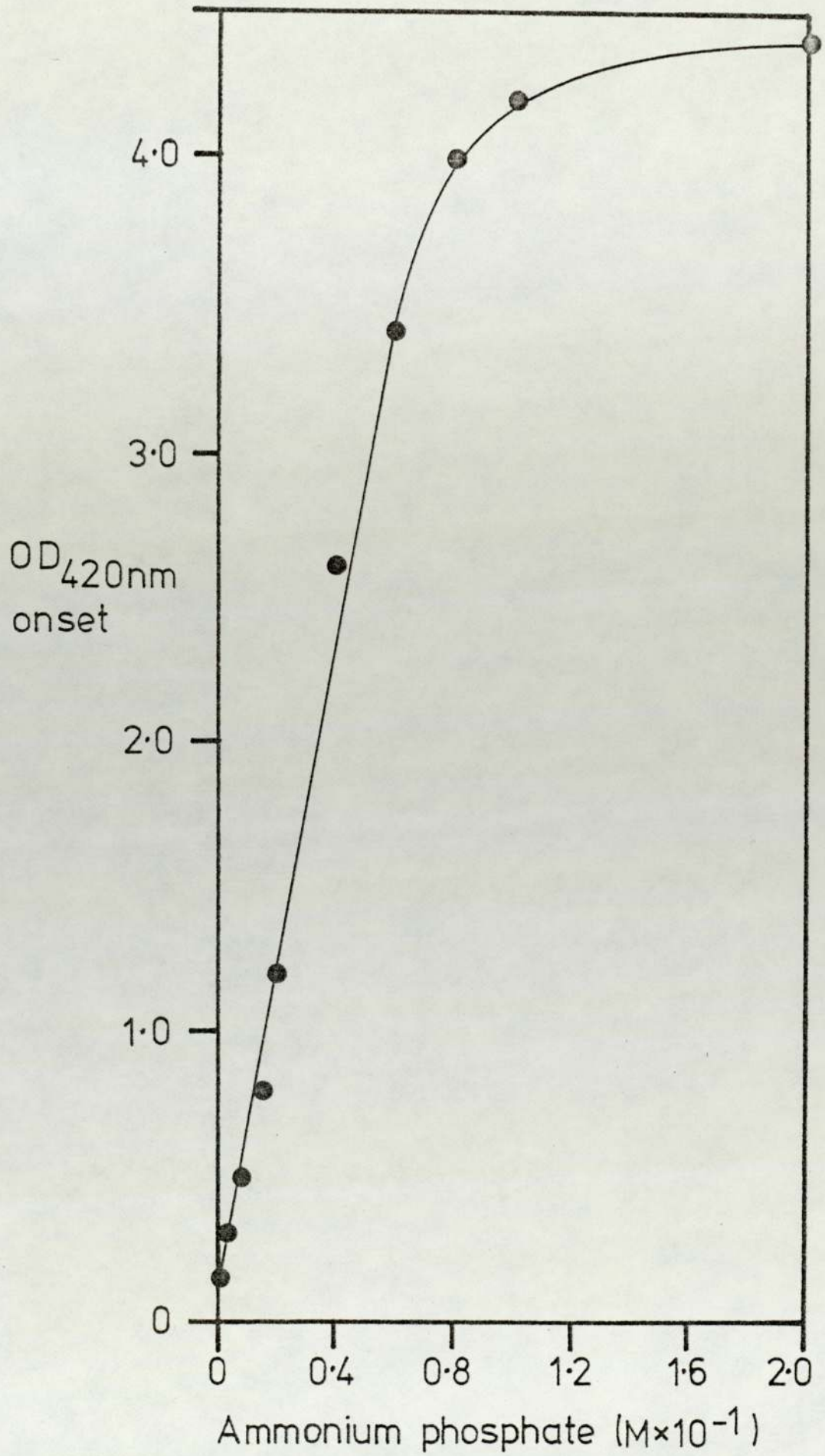
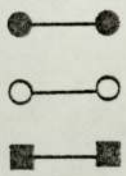

4·0
3·0
2·0
1·0
0
OD420nm onset
0
0·4
0·8
1·2
1·6
2·0
Ammonium phosphate (M×10⁻¹)

Fig. 28. The effect of added sodium and chloride on the growth of *P. mirabilis* 5887 and RBH.



, no added NaCl

, no added Na^+, Cl^- (3×10^{-2}M)

, Na^+ (5×10^{-5}M), Cl^- (3×10^{-2}M)

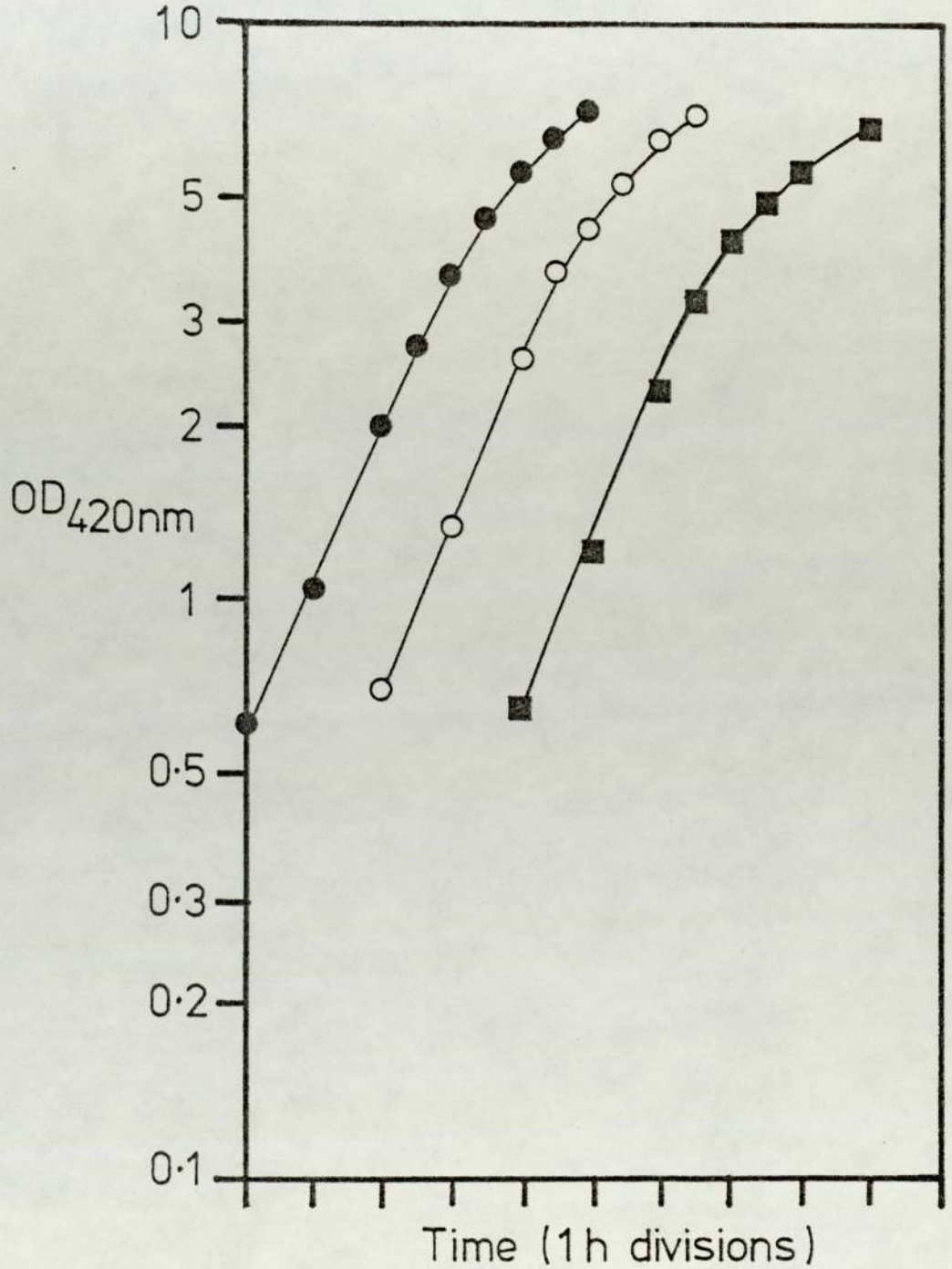
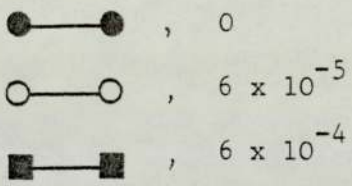

10
5
3
2
1
0·5
0·3
0·2
0·1
OD420nm
Time (1h divisions)

Fig. 29. The effect of nicotinic acid on the growth of *P. mirabilis*.

Nicotinic acid concentration (M)



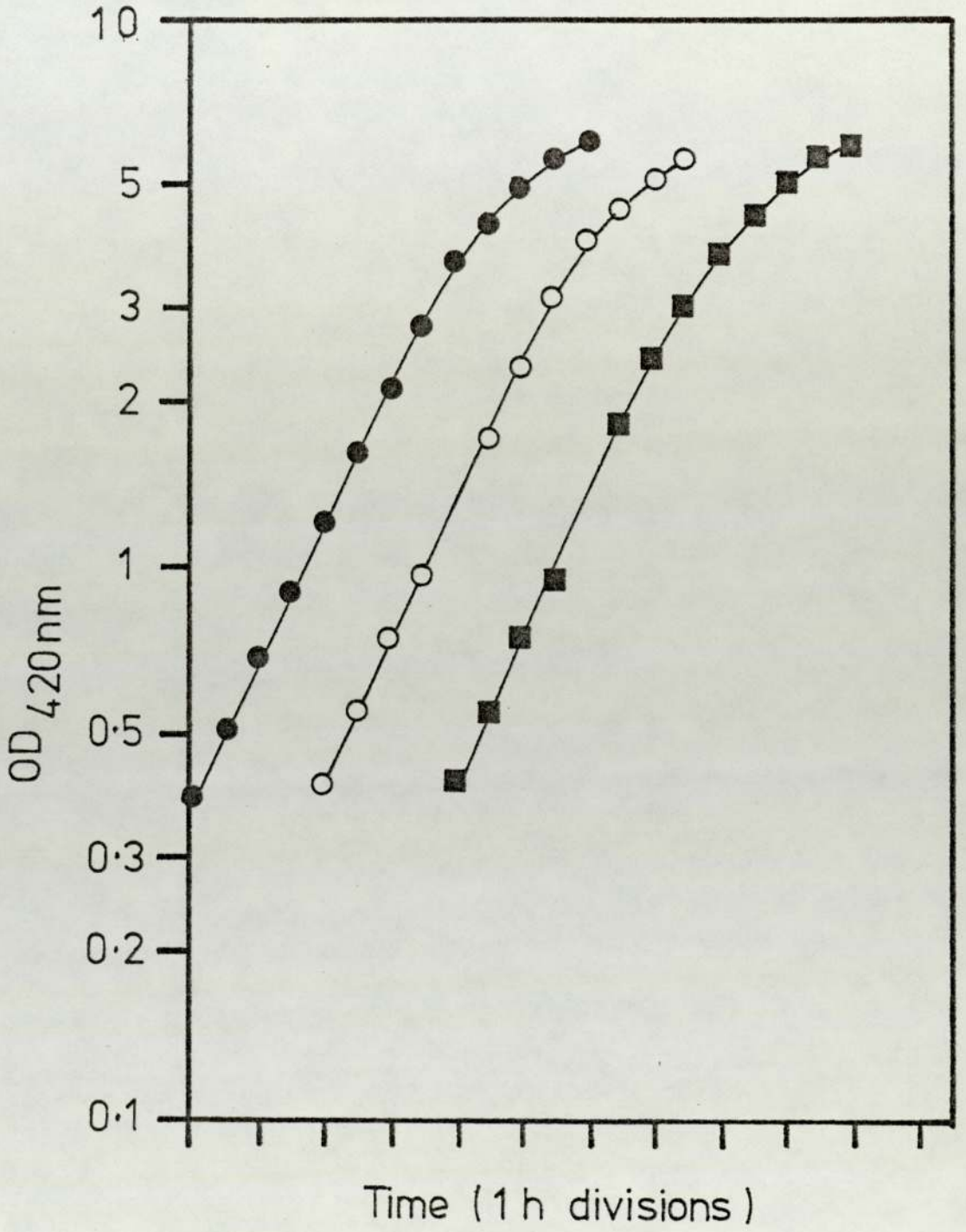
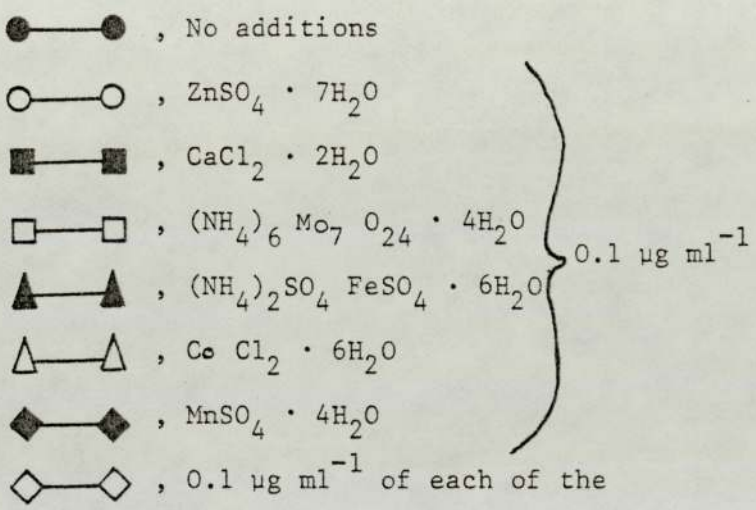
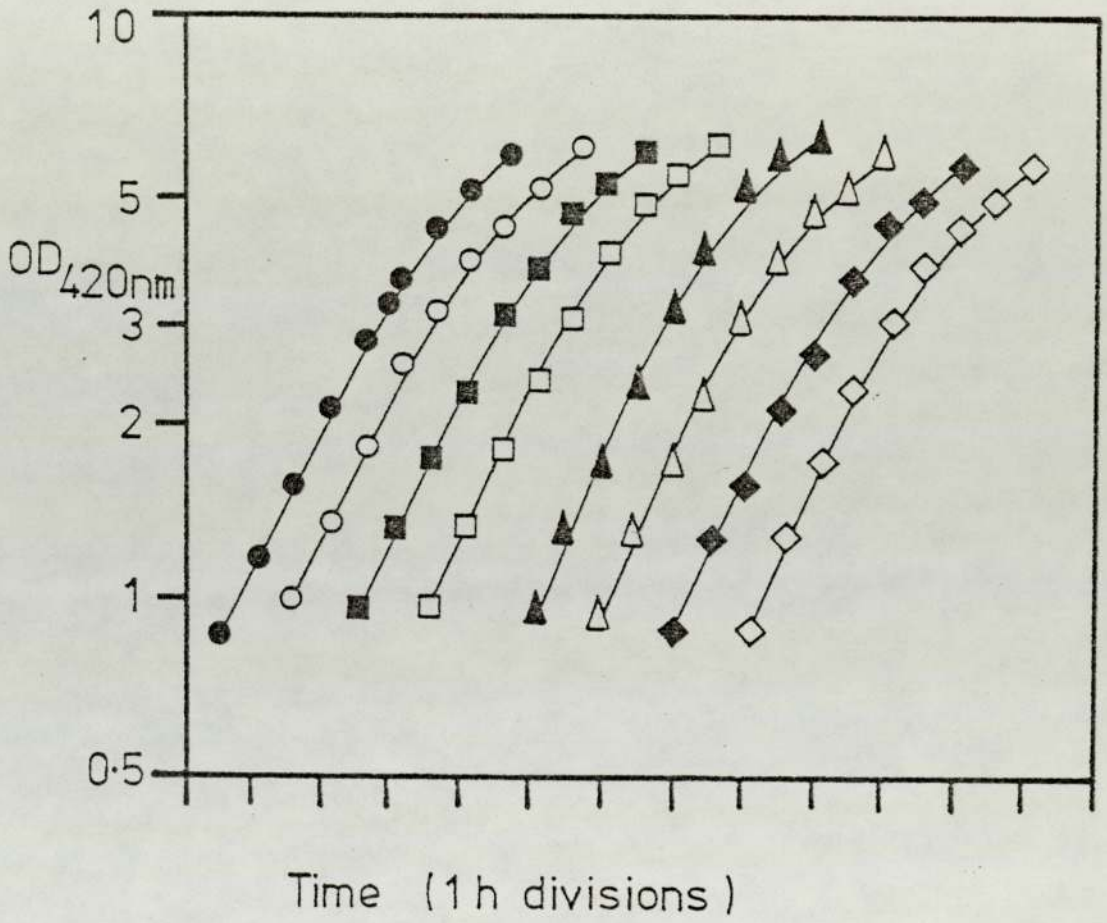

10
5
3
2
1
0·5
0·3
0·2
0·1
OD 420nm
Time (1 h divisions)

Fig. 30. The effect of added trace cations on the growth of *P. mirabilis* 5887 and RBH.




10
5
OD_{420nm}
3
2
1
0·5
Time (1 h divisions)

3.2 Resistance to Antibacterial Agents of Nutrient-Depleted *P. mirabilis* 5887 and RBH grown in Batch Culture

The cultures used were depleted of glucose or magnesium or phosphate, and resuspended to OD 0.2 in the same medium lacking the depleting nutrient and glucose (section 2.2.6). As high concentrations of ions may inhibit the action of membrane-active agents (Klemperer *et al.*, 1979), the phosphate and potassium concentrations in the resuspending media were reduced.

0.1 ml of each of the antibacterial agents tested was added to 9.9 ml of cell suspension to give a final concentration of: 3 μg ml^{-1} cetrimide, 2.5 mg ml^{-1} phenol, 5 μg ml^{-1} chlorhexidine and 250 u ml^{-1} polymyxin. 0.1 ml water was added to 9.9 ml of cell suspension and used as a control.

3.2.1 Effect of resuspending medium

Cultures depleted of Mg^{2+} were resuspended in phosphate-buffered CDM for testing. This was not suitable for P-dep cells, which were tested in MOPS-buffered CDM. The presence of phosphate appeared to cause some increase in resistance to cetrimide (Table 9).

3.2.2 Resistance studies on *P. mirabilis* 5887

3.2.2.1 Effect of cetrimide on the resistance of C-dep, Mg-dep and P-dep cultures of *P. mirabilis* 5887 is shown in Fig. 31. It can be seen that Mg-dep or P-dep cells are much more resistant than C-dep. Typical curves are shown, the resistance of C-dep cells varied in different experiments. Part of this variation was due to different resuspending media. However, some variation within the same system of testing existed although the relative

Table 9

Effect of resuspending medium on survival of carbon depleted P. mirabilis 5887

Resuspending medium / Drug	% survival in buffered CDM		
	phosphate buffer	MOPS buffer	MOPS + phosphate buffer
cetrimide 3 μ ml^{-1} 90 min	36.4 ± 4	11.5 ± 9	63.9 ± 20
phenol 3.5 mg ml^{-1} 90 min	15.3 ± 9	30.5 ± 20	34.2 ± 45
chlorhexidine 5 μ ml^{-1} 120 min	29.8 ± 19	23.2 ± 18	24.1 ± 25

Media and methods in sections 2.2.4 and 2.2.6.

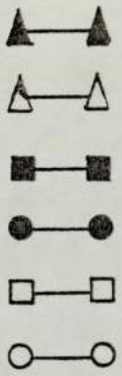
Means and SD of two experiments.

sensitivity of cells remained the same. The variation was not due to clumping of the cells by cetrimide. This might suggest that the variation was due to other physical properties of the cetrimide itself.

3.2.2.2 Effect of phenol is illustrated in Fig. 32. P-dep or Mg-dep were slightly more resistant to the antibacterial activity of phenol than the C-dep cells.

3.2.2.3 Effect of chlorhexidine is shown in Fig. 33. The response of Mg-dep and C-dep cells towards chlorhexidine did not differ significantly. P-dep cells were much more resistant. Although there was day-to-day variation in cell sensitivity, the relative sensitivities of different cells in the same experiment remained the same.

Fig. 31. Effect of cetrimide on *P. mirabilis* 5887 grown in batch culture.



, Mg-dep

, Mg-dep control

, P-dep

, P-dep control

, C-dep

, C-dep control.

Fig. 31-33
Resuspending medium

A Phosphate-medium
B MOPS-medium

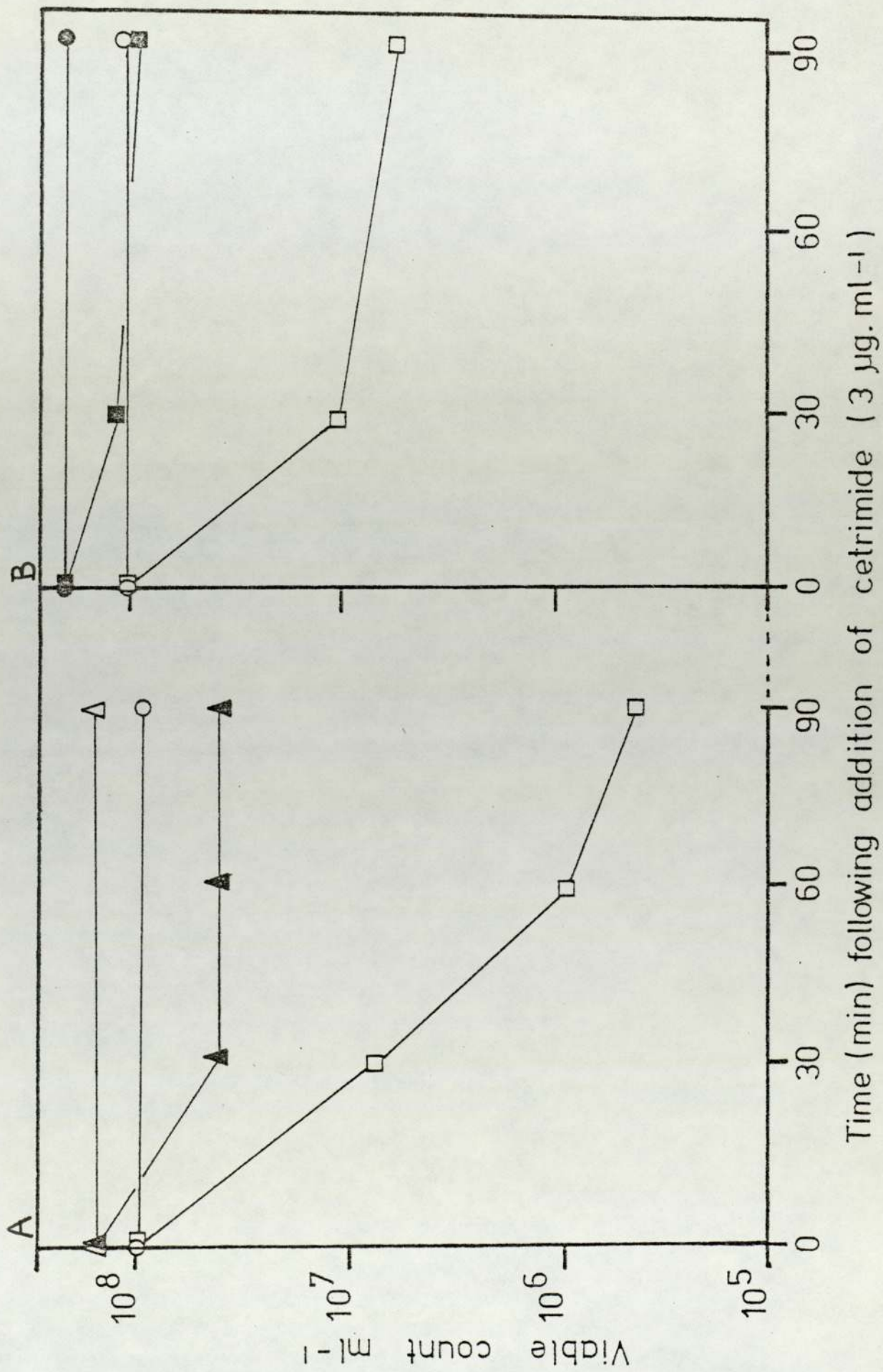
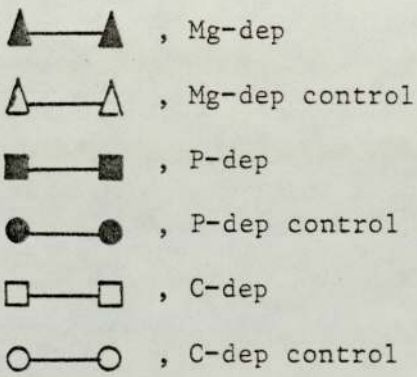

A
B
Viable count ml-1
10^8
10^7
10^6
10^5
0
30
60
90
0
30
60
90
Time (min) following addition of cetrimide (3 µg.ml-1)

Fig. 32. Effect of phenol on *P. mirabilis* 5887 grown in batch culture.



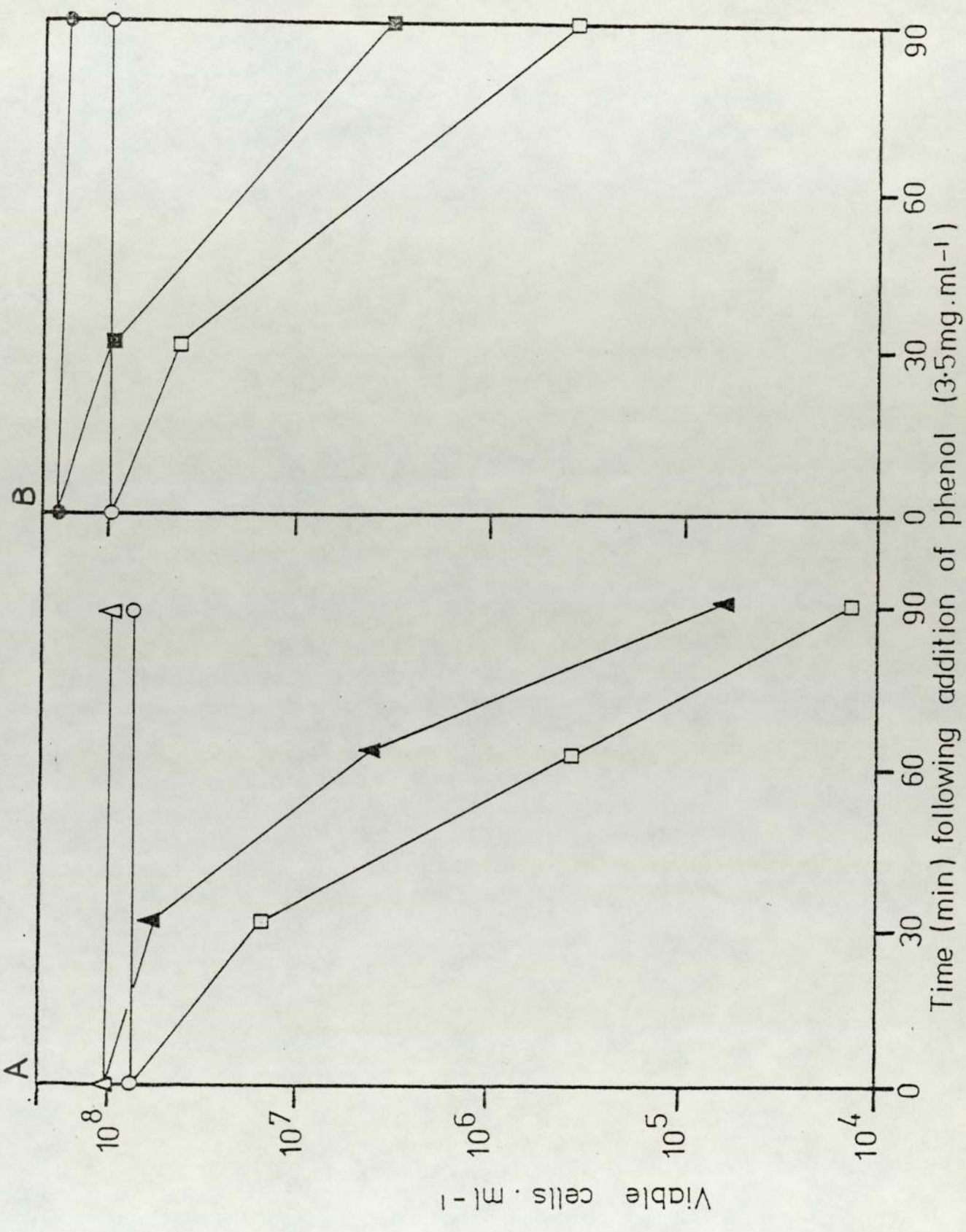
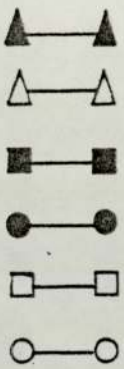

A
B
Viable cells . ml⁻¹
10⁸
10⁷
10⁶
10⁵
10⁴
0
30
60
90
0
30
60
90
Time (min) following addition of phenol (3·5mg.ml⁻¹)

Fig. 33. Effect of Chlorhexidine on *P. mirabilis* 5887 grown in batch culture.



, Mg-dep

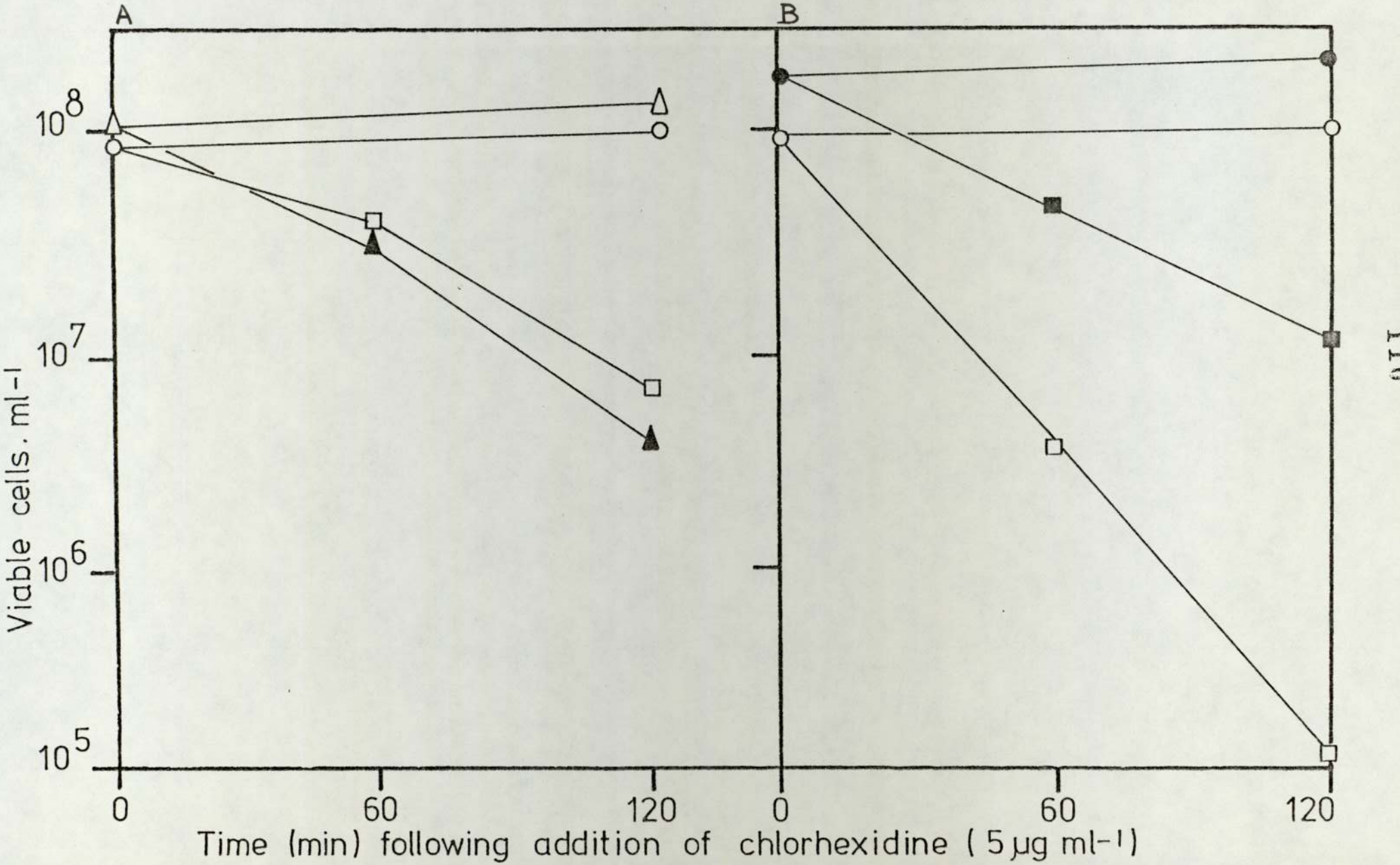
, Mg-dep control

, P-dep

, P-dep control

, C-dep

, C-dep control


A
B
10^8
10^7
10^6
10^5
Viable cells. ml^{-1}
0
60
120
0
60
120
Time (min) following addition of chlorhexidine (5 µg ml^{-1})

3.2.2.4 Effect of polymyxin. Table 10 shows that C-dep cells of 5887 are very resistant to high concentrations of polymyxin.

Table 10

Effect of polymyxin on C-dep cultures of P. mirabilis 5887

time (h)	% survival			
	polymyxin (u ml^{-1})			control
	710	10^3	2×10^3	
½	94	-	-	-
1	98	-	-	-
1½	100	64	59	140
24	-	18	30	29

3.2.3 Resistance studies on P. mirabilis RBH

3.2.3.1 Effect of cetrimide on RBH was different from its effect on 5887. Mg-dep RBH was more sensitive to cetrimide than C-dep. P-dep RBH was more resistant than C-dep, the same as 5887 (Fig. 34). As with 5887, variation was observed in the sensitivity of C-dep RBH to cetrimide. Like 5887, RBH sensitivity of C-dep RBH resuspended in MOPS-buffered medium was always more sensitive to cetrimide than when resuspended in phosphate-buffer. The results for RBH were always reproducible, the day-to-day variation found with 5887 was not found with RBH.

3.2.3.2 Effect of phenol on C-dep, Mg-dep and P-dep P. mirabilis RBH is shown in Fig. 35. The phenol survival graphs of RBH have almost the same pattern as those of 5887. Mg-dep and P-dep cells were more resistant to phenol than C-dep ones.

3.2.3.3 Effect of chlorhexidine on C-dep, Mg-dep and P-dep cells of P. mirabilis RBH is shown in Fig. 36. There was no difference in the susceptibilities of Mg-dep and C-dep cells. P-dep cells were more sensitive to chlorhexidine than C-dep cells, unlike P-dep cells of 5887.

3.2.3.4 Effect of polymyxin is shown in Fig. 37. It can be seen that Mg-dep and P-dep cells were much more resistant than the C-dep cells.

3.2.4 Summary of results

The actions of antibacterial agents on P. mirabilis 5887 and RBH are summarised in Tables 11-14. Because different resuspending media were used for Mg-dep and P-dep cells, these figures cannot be compared. The P-dep cells were resuspended in MOPS buffer and may be compared with cells grown in a chemostat (in CDM containing 0.1 M-KCl) (section 3.4.4). However, because of day-to-day variation in results, actual figures are only comparable for experiments done on the same day, i.e. within Tables, for each organism. Relative sensitivities of different kinds of cells always remained the same.

Fig. 34. Effect of cetrimide on *P. mirabilis* RBH grown in batch culture.

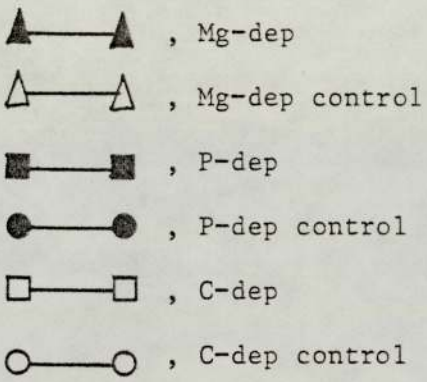


Fig. 34-37
Resuspending medium
A Phosphate-medium
B MOPS-medium

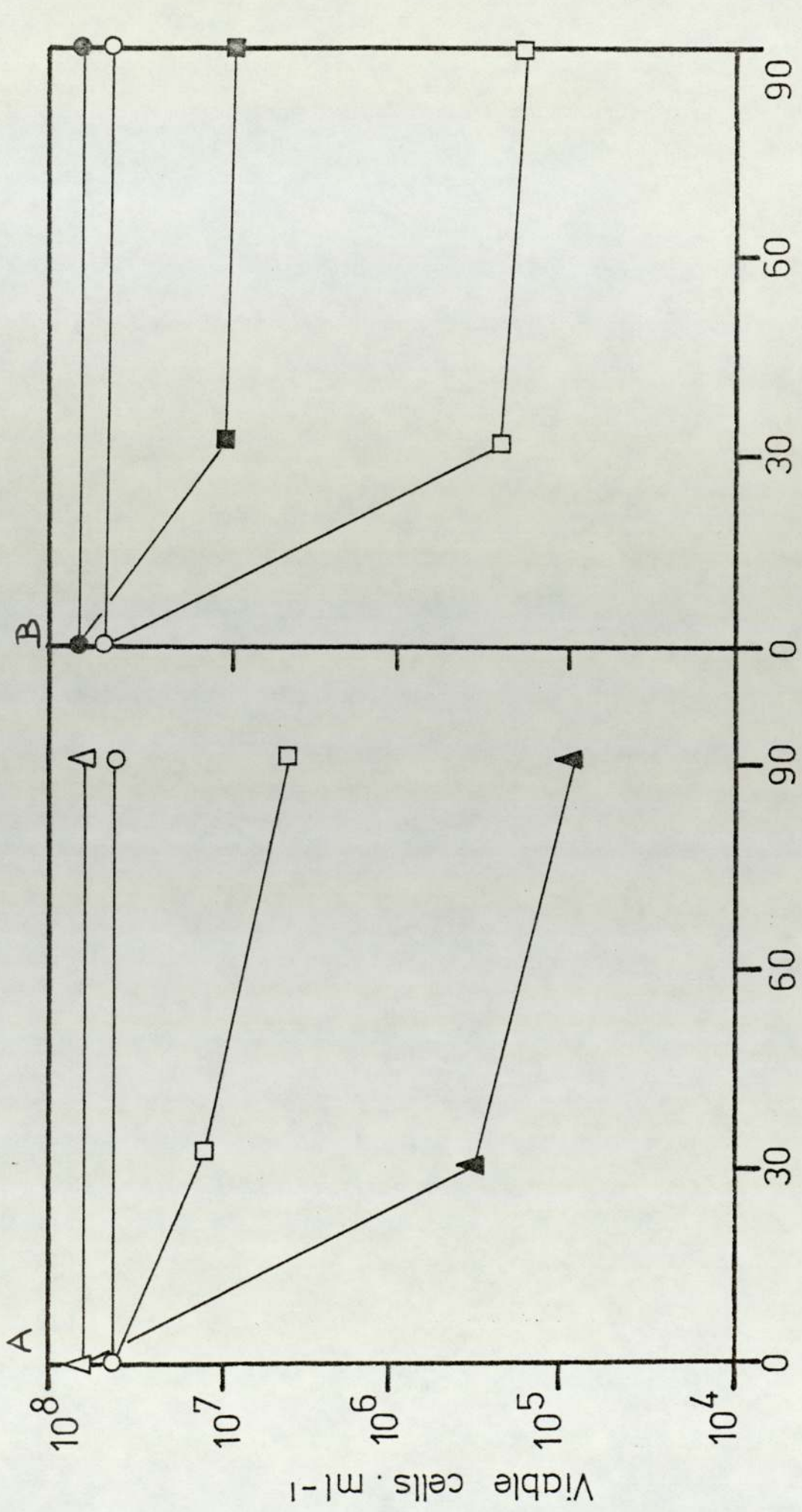
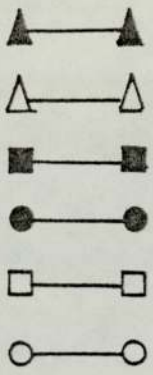

A
B
10^8
10^7
10^6
10^5
10^4
Viable cells . ml-1
0
30
60
90
0
30
60
90
Time (min) following addition of cetrimide (3µg.ml-1)

Fig. 35. Effect of phenol on *P. mirabilis* RBH grown in batch culture.



, Mg-dep

, Mg-dep control

, P-dep

, P-dep control

, C-dep

, C-dep control

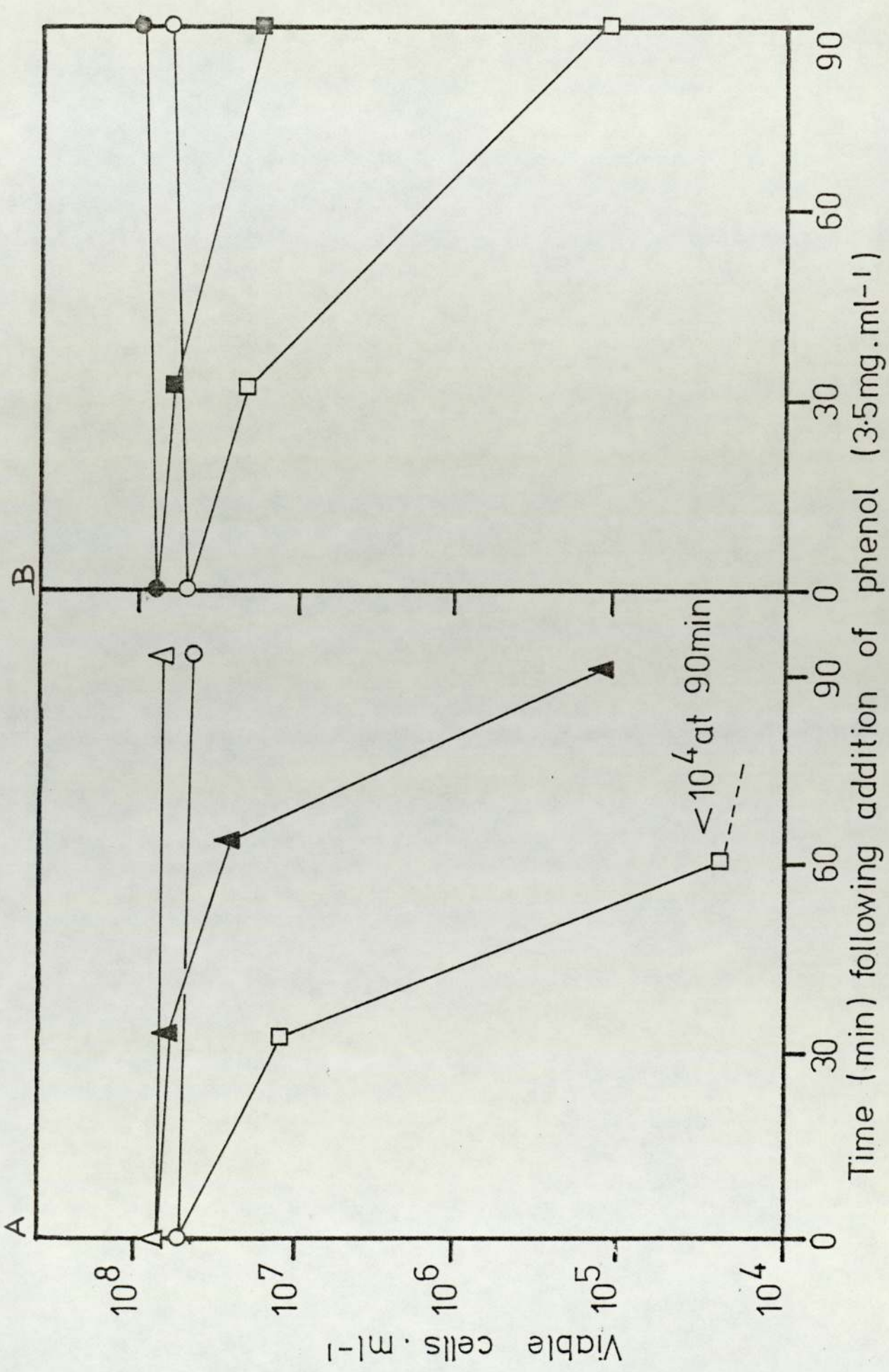
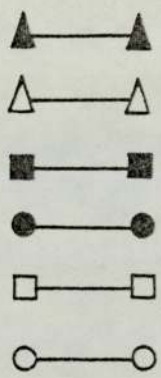

A
B
Viable cells . ml-1
10^8
10^7
10^6
10^5
10^4
$<10^4$ at 90min
0
30
60
90
0
30
60
90
Time (min) following addition of phenol (3·5mg.ml-1)

Fig. 36. Effect of chlorhexidine on *P. mirabilis* RBH grown in batch culture.



, Mg-dep

, Mg-dep control

, P-dep

, P-dep control

, C-dep

, C-dep control.

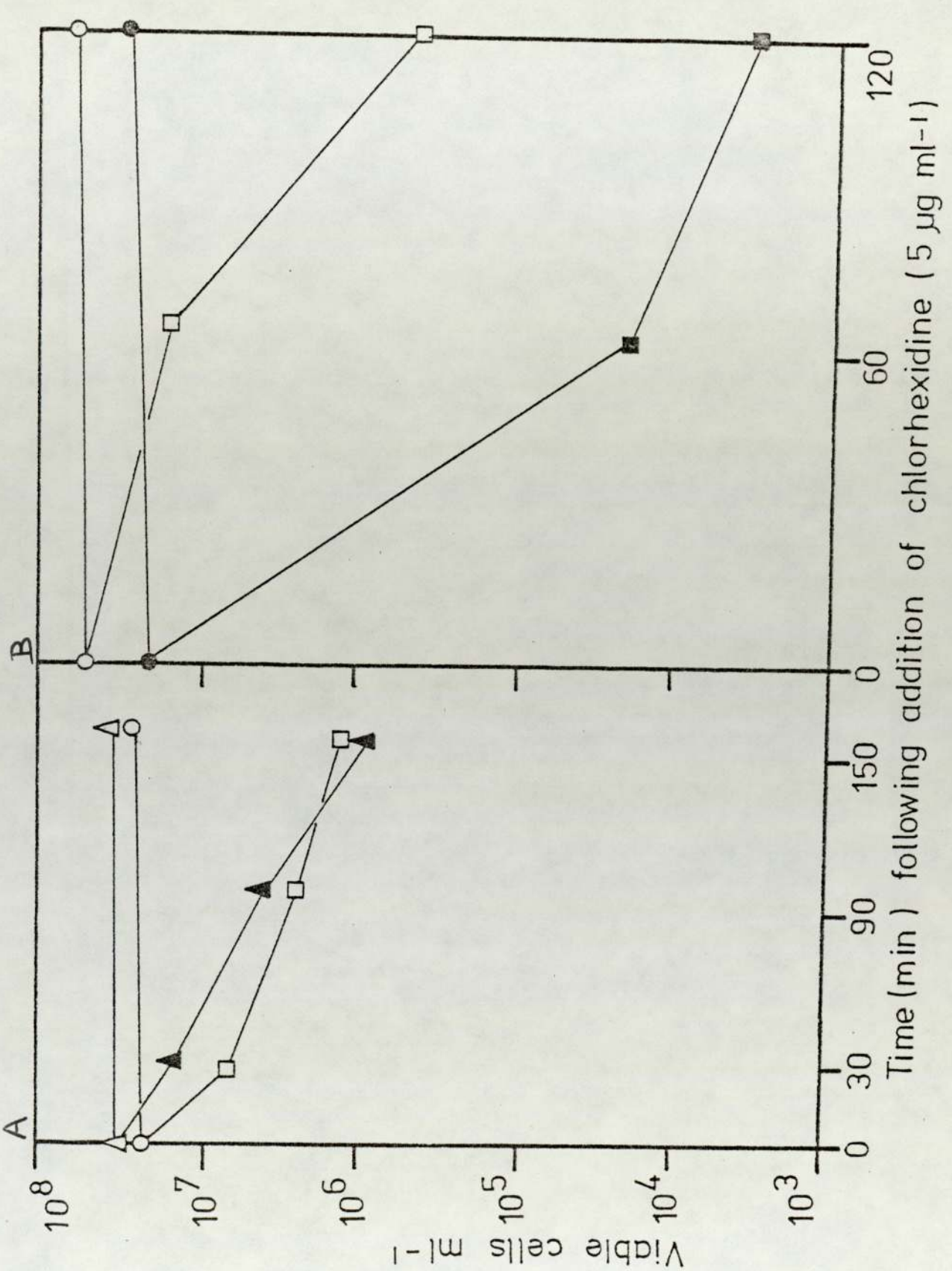
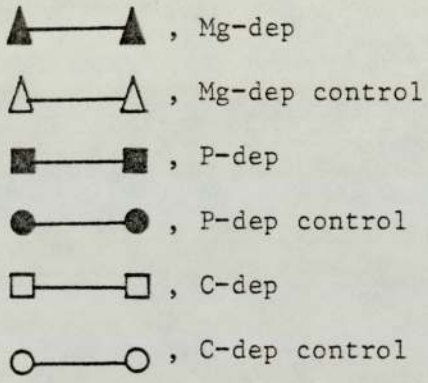

A
B
Viable cells ml-1
10^8
10^7
10^6
10^5
10^4
10^3
0
30
90
150
0
60
120
Time (min) following addition of chlorhexidine (5 µg ml-1)

Fig. 37. Effect of polymyxin on *P. mirabilis* RBH grown in batch cultures.



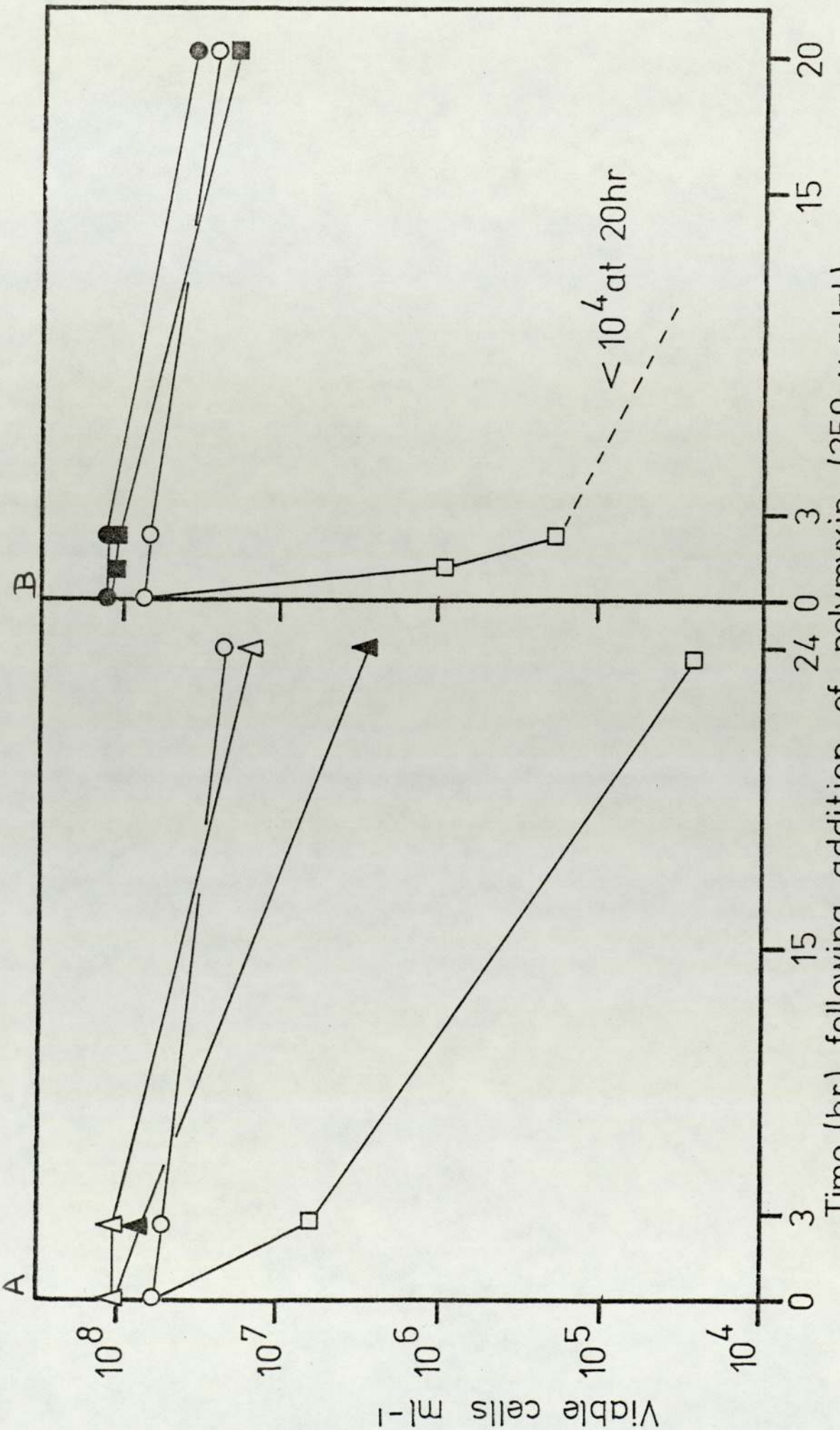

A
B
10^8
10^7
10^6
10^5
10^4
Viable cells ml^{-1}
0
3
15
24 0
3
15
20
Time (hr) following addition of polymyxin (250 u.ml^{-1})
$< 10^4$ at 20hr

Table 11

Resistance of P. mirabilis 5887 and RBH to cetrimide

a)

Cells	% Survival (90 min)	
	5887	RBH
C-dep	2.4 ± 2.5 (4)	17 ± 4 (3)
Mg-dep	24 ± 10 (4)	0.65 ± 0.6 (3)

b)

Cells	% Survival (90 min)	
	5887	RBH
C-dep	8.3 ± 7 (2)	1.3 ± 1 (3)
P-dep	47.0 ± 3 (2)	35 ± 18 (3)

Resuspending medium a) phosphate-buffered CDM

b) MOPS-buffered CDM

Cetrimide 3 μg ml^{-1}

Mean and SD given for 2 to 4 experiments (number in brackets) and includes data in Figs. 31 and 34.

Table 12

Resistance of P. mirabilis 5887 and RBH to phenol

a)

Cells	% Survival (90 min)	
	5887	RBH
C-dep	0.13 ± 0.26 (4)	0.02 ± 0.02 (2)
Mg-dep	0.78 ± 1.3 (4)	0.18 ± 0.02 (2)

b)

Cells	% Survival (90 min)	
	5887	RBH
C-dep	0.22 ± 0.3 (2)	3.0 ± 4 (2)
P-dep	2.7 ± 1.2 (2)	25 ± 10 (2)

Resuspending medium a) phosphate-buffered CDM

b) MOPS-buffered CDM

phenol 3.5 mg ml^{-1}

Mean and SD given for 2 to 4 experiments (number in brackets), and includes data in Figs. 32 and 35.

Table 13

Resistance of P. mirabilis 5887 and RBH to chlorhexidine

a)

Cells	% Survival	
	5887 (i)	RBH (ii)
C-dep	7.0 ± 0.2 (2)	13.0 ± 12 (4)
Mg-dep	5.3 ± 2.5 (2)	7.5 ± 4 (4)

(i) survival at 120 min

(ii) survival at 90 min

b)

Cells	% Survival (120 min)	
	5887	RBH
C-dep	0.065 ± 0.05 (2)	9.8 ± 9.8 (5)
P-dep	2.8 ± 3.6 (2)	2.2 ± 2.9 (5)

Resuspending medium a) phosphate-buffered CDM

b) MOPS-buffered CDM

Chlorhexidine 5 μg ml^{-1}

Mean and SD given for 2 to 5 experiments (number in brackets), and includes data in Figs. 33 and 36.

Table 14

Resistance of P. mirabilis 5887 and RBH to polymyxin

a)

Cells	% Survival (120 min)	
	5887*	RBH
C-dep	83 ± 16	0.02
Mg-dep	94 ± 24	76 ± 3

b)

Cells	% Survival (120 min)
	RBH
C-dep	0.2
P-dep	100

Resuspending medium a) phosphate-buffered CDM

b) MOPS-buffered CDM

polymyxin 250 u ml^{-1}

Mean and SD given for 2 experiments and includes data in Fig. 37.

*5887 C-dep resuspended in phosphate-buffered CDM:

% survival after 90 min + polymyxin 710 u ml^{-1} : 100

" 2000 u ml^{-1} : 59

(data from Table 10)

3.2.5 Minimum inhibitory concentrations of antibacterial agents for *P. mirabilis* 5887 and RBH

These were determined in NB using a serial dilution technique (Cruickshank, 1969) and are shown in Table 15.

Table 15

Minimum inhibitory concentrations of antibacterial agents for *P. mirabilis* and RBH

Antibacterial agent	Minimum Inhibitory Concentration	
	5887	RBH
Cetrimide ($\mu g\ ml^{-1}$)	12-15	8-9
Phenol ($mg\ ml^{-1}$)	1.5-2	1.5-2
Chlorhexidine ($\mu g\ ml^{-1}$)	9-10	8-9
Polymyxin ($u\ ml^{-1}$)	$>9.6 \times 10^4$	25-40

Mean of 2 experiments.

3.3 The Growth of P. mirabilis 5887 and RBH in a chemostat

The theory of bacterial growth in continuous culture has been discussed in section 1.3.

The growth constants (μ_m, K_s and Y) for glucose, magnesium and phosphate were calculated using data obtained in batch culture. They were the same for 5887 and RBH. In these conditions, the values of Y are constants. They are not necessarily so in continuous culture.

3.3.1 Calculation of the growth constants for glucose-limited cells

3.3.1.1 The yield constant, Y. Using CDM containing sufficient glucose to reach an OD of 1.0, the yield constant (Y) for glucose was determined by dividing the mean dry weight of three replicate cultures by the weight of glucose added. It was found to be 0.31.

3.3.1.2 The saturation constant, K_s, and the growth rate constant, μ_m were calculated from the growth rates of P. mirabilis 5887 grown in CDM containing different glucose concentrations (Fig. 16) (p. 97). According to Monod's equation (Monod, 1942; 1950):

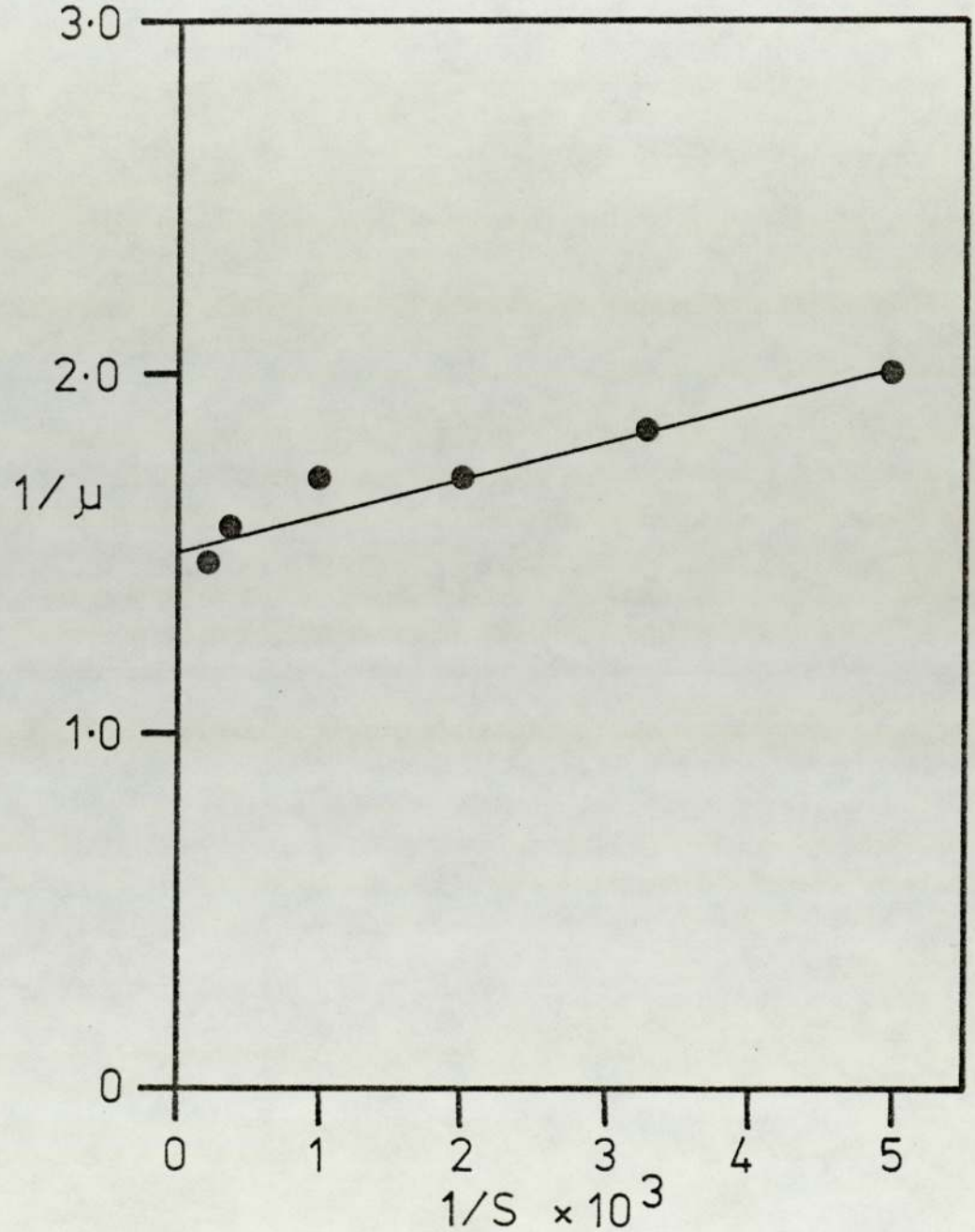
$$\mu = \mu_m\left(\frac{S}{K_s + S}\right)$$

and its rearrangement suggested by Lineweaver and Burke:

$$\frac{1}{\mu} = \frac{1}{\mu_m} + \frac{K_s}{\mu_m} \cdot \frac{1}{S}$$

Therefore, a plot of $\frac{1}{\mu}$ versus $\frac{1}{S}$ will give a graph with line of slope $\frac{K_s}{\mu_m}$ and intercept on the Y axis of $\frac{1}{\mu_m}$. Form Fig. 38, μ_m and K_s were calculated to be 0.66 and 7.2×10^{-5} respectively.

Fig. 38. Double reciprocal plot of *P. mirabilis* 5887 growth rate versus medium glucose concentration.


3·0
2·0
1·0
0
1/μ
0
1
2
3
4
5
1/S × 10³

3.3.2 Calculation of the growth constants for Mg-limited cells

3.3.2.1 The yield constant, Y. Three replicate CDM were used, each containing 10^{-5} M $MgSO_4$, (2.43×10^{-4} g l^{-1} Mg). Growth was followed until cells were apparently stationary four hours after onset of magnesium depletion. From the dry weight, the value of Y for Mg was determined to be 885.

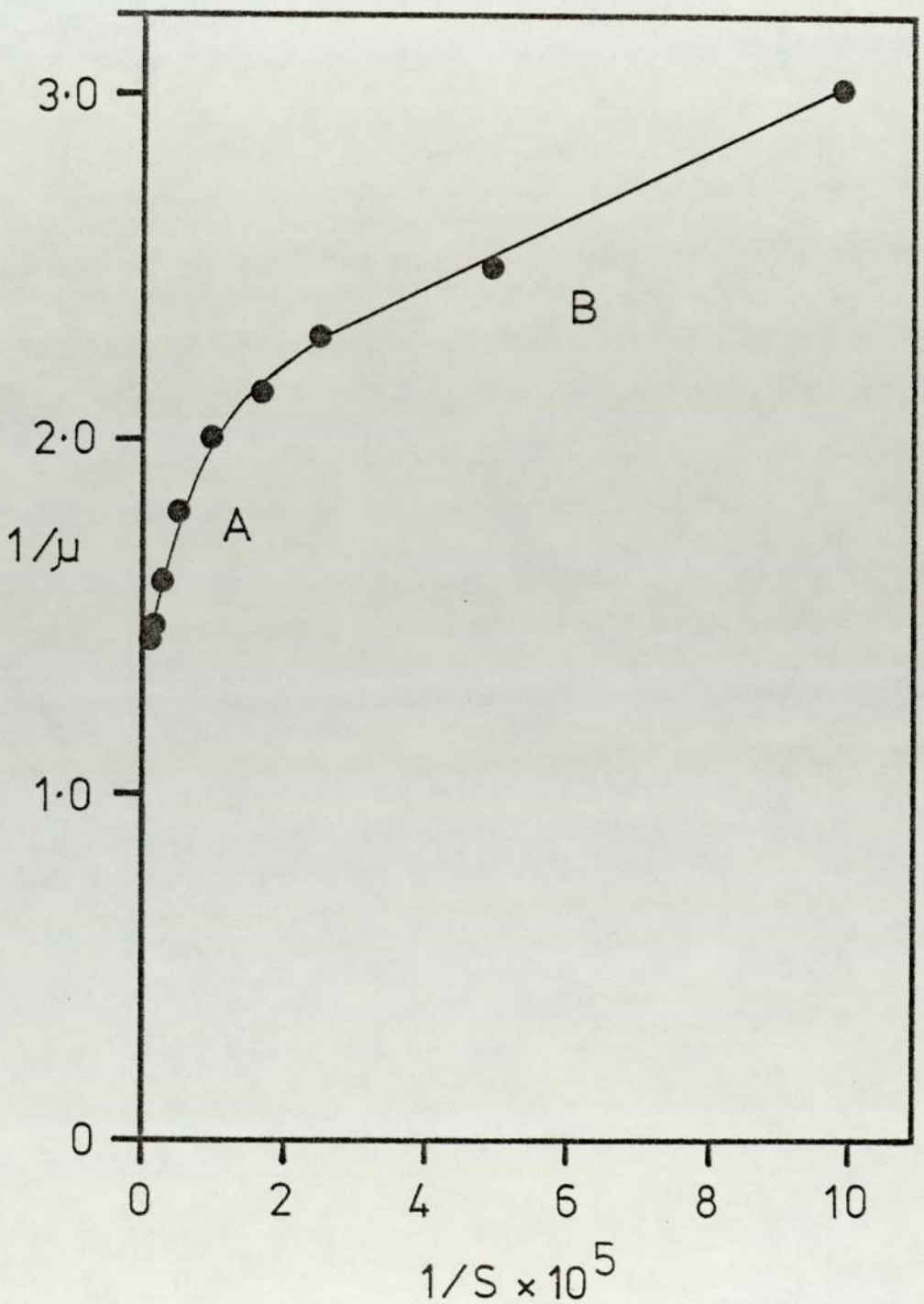
3.3.2.2 The saturation constant, K_s, and growth rate constant μ_m Growth rates of P. mirabilis 5887 at different initial magnesium concentrations of CDM were obtained from Fig. 18 (p. 99). The reciprocal values of S were plotted versus those of μ (Fig. 39). The graph appears to be biphasic indicating two different affinities for Mg^{2+}, similar to the results of Shehata & Marr (1971) for the growth of E. coli when phosphate-limited. K_s and μ_m were calculated for each part of the graph from the tangent to it and were as follows: A, $K_s = 4.3 \times 10^{-6}$ and $\mu_m = 0.7$; B, $K_s = 4.9 \times 10^{-7}$, $\mu_m = 0.49$. As the calculated values of S_r (section 3.3.4) were close to the point of inflection of the graph, the lower value for S_r was used in practice to ensure the cells were Mg-limited. The OD reached was 0.8 - 1.0.

3.3.3 Calculation of the growth constants for P-limited cells

3.3.3.1 The yield constant, Y was determined using three replicate CDM containing 8×10^{-3} M ammonium phosphate (0.223 g l^{-1} phosphorus). Growth was followed until cells were apparently stationary four hours after onset of phosphate depletion. The yield constant, Y for P was found to be 1.60.

3.3.3.2 The saturation constant, K_s, and growth rate constant, μ_m The growth rates of P. mirabilis 5887 at different phosphate concentrations in CDM were calculated from Fig. 26 (p. 107) and

Fig. 39. Double reciprocal plot of *P. mirabilis* 5887 growth rate versus medium magnesium concentration.


3·0
2·0
1·0
0
1/μ
A
B
0
2
4
6
8
10
1/S × 10^5

a graph of $\frac{1}{S}$ versus $\frac{1}{\mu}$ was constructed (Fig. 40). The K_s for P was calculated to be 8.5×10^{-4} and μ_m to be 0.65.

3.3.4 Media for chemostat cultures

Three types of media were prepared in 2 x 2 litre batches. These were glucose-limited (C-lim), magnesium-limited (Mg-lim), and phosphate-limited (P-lim) CDM. To produce similar concentrations of cells in each of the three media, the concentration in the reservoir (S_r) was calculated from the equation:

$$S_r = \frac{2\bar{x}Y - Y^2K_s \pm \sqrt{Y^4K_s^2 + 4\bar{x}Y^3K_s}}{2Y^2} \quad \text{at } D = D_m$$

using K_s, Y and the dry weight ($\bar{x}$) values for magnesium and phosphate (Table 16) (D = dilution rate, D_m = D at μ_m). These S_r values were determined to give OD 1.0; other ingredients were present in sufficient concentration to support the growth up to OD 4.0 (Table 17).

Initially, 0.1 M KCl was used in the three media. However, many Sc variants were isolated (section 3.5). 0.4 M KCl was then used in an attempt to reduce the number of Sc present.

Table 16

Growth constants and reservoir concentration for glucose, Mg^{2+} and phosphate

Media	limiting nutrient	Y	K_s (M)	$\bar{X}$ (mg ml^{-1})	S_r (M)
C-lim	Glucose	0.31	7.2×10^{-5}	0.17	3×10^{-3}
Mg-lim	Mg^{2+}	885	4.9×10^{-7}	0.25	1.2×10^{-5}
P-lim	Phosphorus	1.60	8.5×10^{-4}	0.19	0.12×10^{-1}

Fig. 40. Double reciprocal plot of *P. mirabilis* growth rate versus medium phosphate concentration.

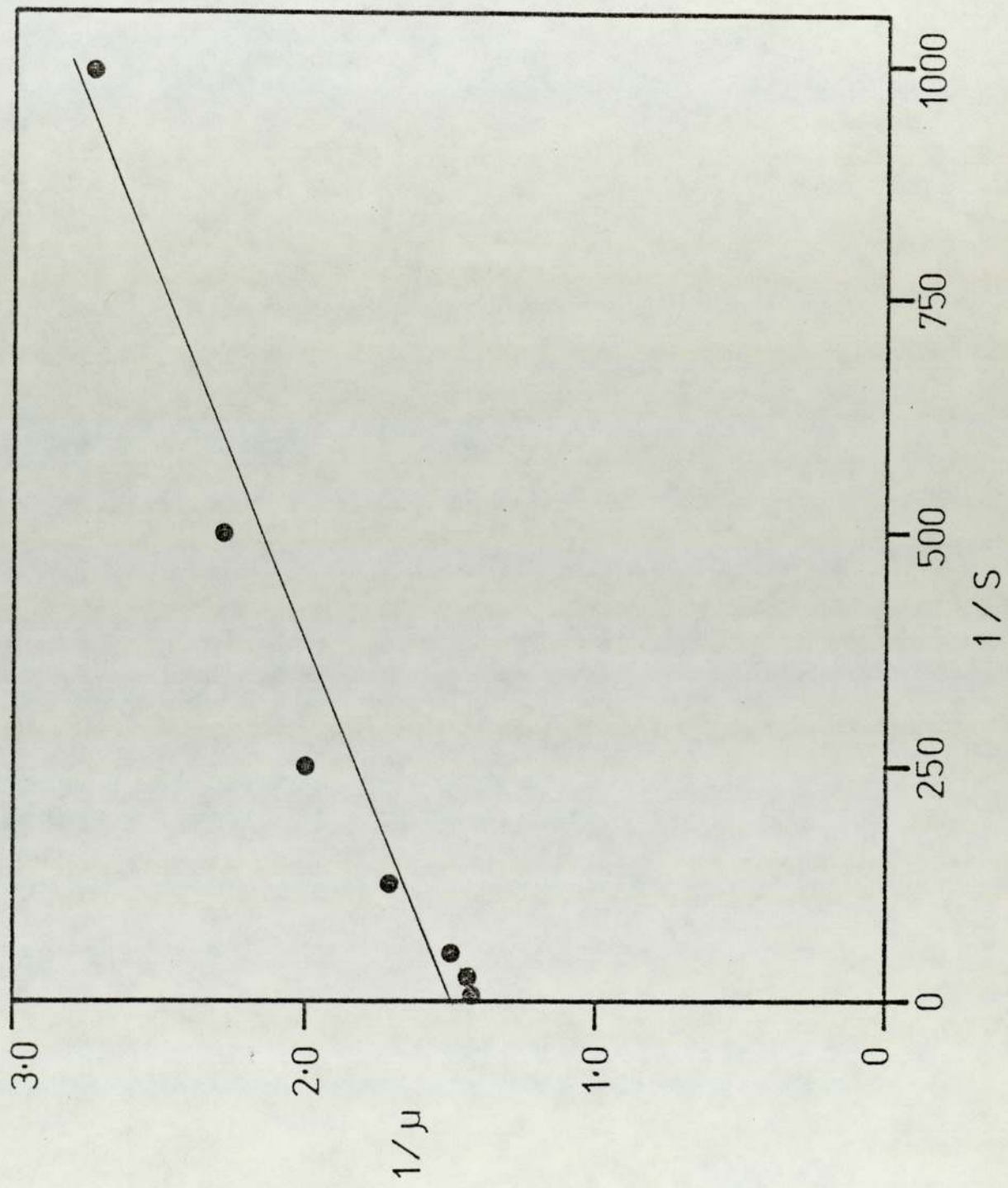

3·0
2·0
1·0
0
1/μ
0
250
500
750
1000
1/S

Table 17

Media used for chemostat cultures of P. mirabilis 5887 and RBH

Ingredient (M)	Medium		
	C-lim	Mg-lim	P-lim
Glucose	3×10^{-3}	1.4×10^{-2}	1.4×10^{-2}
Nicotinic acid	6×10^{-5}	6×10^{-5}	6×10^{-5}
KCl	10^{-1} or 4×10^{-1}	10^{-1} or 4×10^{-1}	10^{-1} or 4×10^{-1}
$(NH_4)_2SO_4$	5×10^{-5}	5×10^{-5}	5×10^{-5}
$MgSO_4$	1×10^{-4}	1.2×10^{-5}	1×10^{-4}
$(NH_4)_2HPO_4$] $NH_4H_2PO_4$]	10^{-1}	10^{-1}	0.12×10^{-1}
MOPS	0	0	2.5×10^{-2}

pH 7.0 ± 0.1

3.3.4.1 Preparation of media. 0.5 l of deionised distilled water was autoclaved for 45 min at 15 lb/in^2 in a 2 l bottle fitted with a cotton wool glass vent and a millipore filter apparatus. After autoclaving, a sterile 0.2 µm filter was aseptically placed on the filter holder and, by applying a vacuum at the vent, the medium components dissolved in 1½ l water, were filtered into the bottle. The filter holder was then aseptically replaced with a sterile plug.

3.3.5 Growth in a chemostat

Chemostats (50 ml) similar to that described by Gilbert & Stuart (1977) were used. Six chemostats were assembled and autoclaved. The culture vessels were immersed in water bath at 37°. Media were supplied to the vessels by a peristaltic pump and the air pump switched on. The air was saturated by passing

through sterile water before entering the vessels. The vessels were inoculated aseptically with several mls of P. mirabilis 5887 or RBH from a batch culture grown in CDM (2.2.2). The bacteria were allowed to grow as a batch culture overnight, and when a visible growth was observed in the vessel, the pump was set to a dilution rate of 0.05 h^{-1}. On changing the dilution rate, the chemostat was left to equilibrate for at least five complete changes of medium.

The pHs of cultures was found to remain at 7.0 ± 0.1 throughout the running of the continuous culture, and at all dilution rates.

3.4 Resistance of Chemostat-grown P. mirabilis 5887 and RBH to antibacterial agents

The method used to study the resistance of chemostat-grown P. mirabilis 5887 and RBH in different media and at different growth rates was essentially that described in section 2.2.6.

3.4.1 Resistance to polymyxin and cetrimide of P. mirabilis 5887

3.4.1.1 Effect of nutrient limitation and different potassium chloride concentrations in CDM on cultures growing at 0.05 h^{-1}.

Table 18 shows that the resistance of cells to polymyxin is greatest for C-dep > P-dep > Mg-dep cells. When grown in 0.4 M KCl, the C-lim cells were more sensitive to polymyxin than when grown in 0.1 M KCl. C-lim cells were more sensitive to cetrimide than Mg-lim and P-lim. When grown in 0.4 M KCl, the C-lim cells were more resistant to cetrimide than when grown in 0.1 M KCl CDM.

3.4.1.2 Effect of nutrient limitation at 0.5 h^{-1} is shown in Table 19. Nutrient limitation had no significant effect on the response of P. mirabilis 5887 at 0.5 h^{-1} to cetrimide. CDM containing 0.4 M KCl was used to grow the cells.

3.4.1.3 Effect of growth rate is shown in Table 20. There was no significant change in the sensitivity of C-lim, Mg-lim and P-lim cultures to cetrimide, but the sensitivity of C-lim culture to polymyxin was greatly increased with increase in growth rate.

Table 18

The effect of nutrient limitation on the sensitivity of *P. mirabilis* 5887 to polymyxin or cetrimide:

dilution rate 0.05 h^{-1}

Limiting Nutrient	Time (min)	% survival					
		0.1 M KCl*			0.4 M KCl*		
		No addition	polymyxin 250 u ml^{-1}	cetrimide 3 µg ml^{-1}	No addition	polymyxin 250 u ml^{-1}	cetrimide 3 µg ml^{-1}
C	20	-	33	22	-	-	-
	40	-	30	22	-	12	-
	80	-	32	12	-	4	-
	120	72	21	6	92 ± 33(4)	1.5 ± 0.5(3)	74 ± 2.5(2)
Mg	20	-	1.1	67	-	-	-
	40	-	0.5	79	-	-	103
	80	-	0.22	71	-	-	96
	120	59	0.12	67	105 ± 11(2)	-	93 ± 10(2)
P	20	-	15	107	-	-	-
	40	-	5	114	-	-	92
	80	-	3	95	-	-	99
	120	129	2	56	98	-	78 ± 16(2)

Method: see section 2.2.6. Mean ± SD given for 2 or more experiments, number of experiments in brackets.

*KCl in growth medium.

Table 19

The effect of nutrient limitation on the sensitivity of P. mirabilis 5887 to cetrimide: dilution rate 0.5 h^{-1}

Limiting nutrient	Time (min)	% survival	
		No addition	cetrimide 3 µg ml^{-1}
C	40	-	68 ± 16(2)
	80	-	40 ± 12(2)
	120	69 ± 19(4)	42 ± 17(2)
Mg	40	-	108
	80	-	98
	120	90 ± 4(2)	63 ± 46(2)
P	40	-	72
	80	-	87
	120	91 ± 25(2)	72 ± 2 (2)

Method: see section 2.2.6. Mean ± SD given for 2 or more experiments, number of experiments in brackets. Growth medium contained 0.4 M-KCl.

Table 20

The effect of dilution rate on the sensitivity of P. mirabilis 5887 to polymyxin or cetrimide

Limiting nutrient	% survival at 120 min					
	No addition		polymyxin 250 u ml^{-1}		cetrimide 3 µg ml^{-1}	
	0.05*	0.5	0.05	0.5	0.05	0.5
C	92 ± 33	69 ± 19	1.5 ± 0.5	0.11 ± 0.01	74 ± 25	42 ± 17
Mg	105 ± 11	90 ± 4	-	-	93 ± 10	63 ± 46
P	98	91 ± 25	-	-	78 ± 16	72 ± 2

Results are mainly from Tables 18 & 19, growth medium containing 0.4 M-KCl.

*dilution rate (h^{-1})

3.4.2 Resistance to polymyxin and cetrimide of P. mirabilis RBH

3.4.2.1 Effect of nutrient limitation and different potassium chloride concentration in CDM on cultures growing at 0.05 h^{-1} is shown in Table 21.

P-lim cells growing in CDM containing 0.1 M KCl were more sensitive to polymyxin than the C-lim and Mg-lim cells. When grown in 0.4 M KCl, C-lim and Mg-lim cells were more sensitive to polymyxin than when grown in 0.1 M KCl.

The resistance of Mg-lim and P-lim cultures to cetrimide was greater than C-lim. Different KCl concentrations during growth had no detectable effect.

3.4.2.2 Effect of nutrient limitation at 0.5 h^{-1} in CDM containing 0.4 M KCl is shown in Table 22. The Mg-lim culture of RBH was more resistant to polymyxin than the C-lim culture. There was no detectable effect of nutrient limitation on sensitivity to cetrimide.

3.4.2.3 Effect of growth rate is shown in Table 23 using cells grown in 0.4 M KCl. C-lim cultures become about 10 times more sensitive to polymyxin at 0.5 h^{-1} than at 0.05 h^{-1} and Mg-lim cultures become about 4 times more sensitive. There was no significant change in the response of C-lim, Mg-lim and P-lim cultures to cetrimide.

Table 21

The effect of nutrient limitation on the sensitivity of *P. mirabilis* RBH to polymyxin or cetrimide: dilution rate 0.05 h^{-1}

Limiting Nutrient	Time (min)	% survival					
		0.1 M KCl*			0.4 M KCl		
		No addition	polymyxin 250 u ml^{-1}	cetrimide 3 µg ml^{-1}	No addition	polymyxin 250 u ml^{-1}	cetrimide 3 µg ml^{-1}
C	20	-	38	24	-	-	-
	40	-	24	20	-	2.1 ± 0.2 (2)	-
	80	-	12	16	-	0.9 ± 0.01 (2)	-
	120	99	9	13	80 ± 8.5 (3)	0.4 ± 0.29 (3)	29 ± 18 (2)
Mg	20	-	32	197	-	-	-
	40	-	24	173	-	1.4	99
	80	-	20	134	-	0.88	83
	120	147	16	143	84 ± 10 (3)	0.6 ± 0.02 (2)	96 ± 5 (2)
P	20	-	2.3	67	-	-	-
	40	-	1.5	80	-	-	97
	80	-	0.89	46	-	-	84
	120	95	0.69	18	101 ± 4 (2)	-	77 ± 3 (2)

Method: see section 2.2.6. Mean ± SD given for 2 or more experiments, number of experiments in brackets.
*KCl in growth medium.

Table 22

The effect of nutrient limitation on the sensitivity of *P. mirabilis* RBH to polymyxin or cetrimide: dilution rate 0.5 h^{-1}

Limiting nutrient	Time (min)	% survival		
		No addition	polymyxin 250 u ml^{-1}	cetrimide 3 µg ml^{-1}
C	40	-	0.41	54
	80	-	0.041	41
	120	88 ± 22	0.028 ± 0.012	34 ± 20
Mg	40	-	3.5	85 ± 16
	80	-	0.62	86 ± 4
	120	94 ± 6	0.15 ± 0.05	80 ± 11
P	40	-	-	79
	80	-	-	69
	120	90 ± 1	-	70 ± 11

Method: see section 2.2.6. Mean ± SD given for 2 experiments.

Growth medium contained 0.4 M KCl.

Table 23

The effect of dilution rate on the sensitivity of *P. mirabilis* RBH to polymyxin or cetrimide

Limiting nutrient	% survival at 120 min					
	No addition		polymyxin 250 u ml^{-1}		cetrimide 3 µg ml^{-1}	
	0.05*	0.05	0.05	0.5	0.05	0.5
C	80 ± 9	88 ± 22	0.4 ± 0.3	0.03 ± 0.01	29 ± 18	37 ± 20
Mg	84 ± 10	94 ± 6	0.6 ± 0.02	0.15 ± 0.05	96 ± 5	80 ± 11
P	101 ± 4	90 ± 1	-	-	77 ± 3	70 ± 11

Results are from Tables 21, 22, growth medium containing 0.4 M KCl.

*dilution rate (h^{-1})

3.4.3 Summary of results

3.4.3.1 Resistance of chemostat-grown cells to cetrimide is summarised in Tables 24 and 25.

Table 24

Effect of nutrient limitation and dilution rate on resistance to cetrimide

Cells	% survival (120 min)			
	5887		RBH	
	0.05 h^{-1}	0.5 h^{-1}	0.05 h^{-1}	0.5 h^{-1}
C-lim	74	42	29	37
Mg-lim	93	63	96	80
P-lim	78	72	77	70

Data from Tables 20 and 23.

Cells grown in 0.4 M KCl and treated with cetrimide 3 µg ml^{-1}.

Table 25

Effect of KCl concentration in the growth medium on resistance to cetrimide

Cells	% survival (120 min)			
	5887		RBH	
	0.1 M-KCl	0.4 M-KCl	0.1 M-KCl	0.4 M-KCl
C-lim	6	74	13	29
Mg-lim	67	93	143	96
P-lim	56	78	18	77

Data from Tables 18 and 21.

Dilution rate 0.05 h^{-1}; cells treated with cetrimide 3 µg ml^{-1}.

3.4.3.2 Resistance of chemostat-grown cells to polymyxin is summarised in Tables 26 and 27.

Table 26

Effect of nutrient limitation and dilution rate on resistance to polymyxin

Cells	% survival (120 min)			
	5887		RBH	
	0.05 h^{-1}	0.5 h^{-1}	0.05 h^{-1}	0.5 h^{-1}
C-lim	1.5	0.11	0.4	0.03
Mg-lim	-	-	0.6	0.15

Data from Tables 20 and 23.

Cells grown in 0.4 M-KCl and treated with polymyxin 250 u ml^{-1}.

Table 27

Effect of KCl concentration in the growth medium on resistance to polymyxin

Cells	% survival (120 min)			
	5887		RBH	
	0.1 M-KCl	0.4 M-KCl	0.1 M-KCl	0.4 M-KCl
C-lim	21	2	9	0.4
Mg-lim	-	-	16	0.6

Data from Tables 18 and 21.

Dilution rate 0.05 h^{-1}; cells treated with polymyxin 250 u ml^{-1}.

3.5 Small Colony Variants of P. mirabilis

When P. mirabilis was grown in CDM and plated on ONAA, small colonies (Sc) were seen which were quite different in appearance from typical large colonies (Lc) (Fig. 41). These variants were not usually found from NB cultures. The occurrence of Sc variants was found to be related to the salt concentration in CDM.

Sc variants of large typical colonies (Lc) of P. mirabilis 5887, RBH, 7827, 8309, 60, 2100 and of P. morgani 10466 were isolated after growth in CDM containing low KCl and NaCl concentration (5×10^{-5} M of each). They were then maintained on NA slopes and their properties studied.

3.5.1 Characteristics of Sc variants

Sc variants were easily distinguished after 17 h at 37° on GNAA plates, or 36-48 h on ONAA plates. The colonies were 0.5 - 1 mm in diameter on GNAA or 0.1 - 0.25 mm on ONAA after 48 h incubation (Fig. 42), and were circular, entire, domed, smooth, opaque. They were not pigmneted but appeared slightly yellowish in colour on ONAA or greenish on GNAA. Lc swarmed on GNA or formed large colonies of 2 - 3 mm in diameter after 48 h at 37° on ONAA. These Lc were low irregular in outline, matt and translucent (Fig. 41).

In 24 h NB or CDM (KCl, 5×10^{-5} or 0.1 M; NaCl, 5×10^{-1} M) cultures examined by phase-contrast, both Lc and Sc appeared as short rods. However, Sc were often arranged in pairs, with some short chains (4 - 5 cells) and occasionally longer chains of up to 50 cells (Fig. 43). All Lc but no Sc, were found to be motile.

Fig. 41. P. mirabilis 5887 Lc and Sc on ONAA, incubated 48 h at 37°.

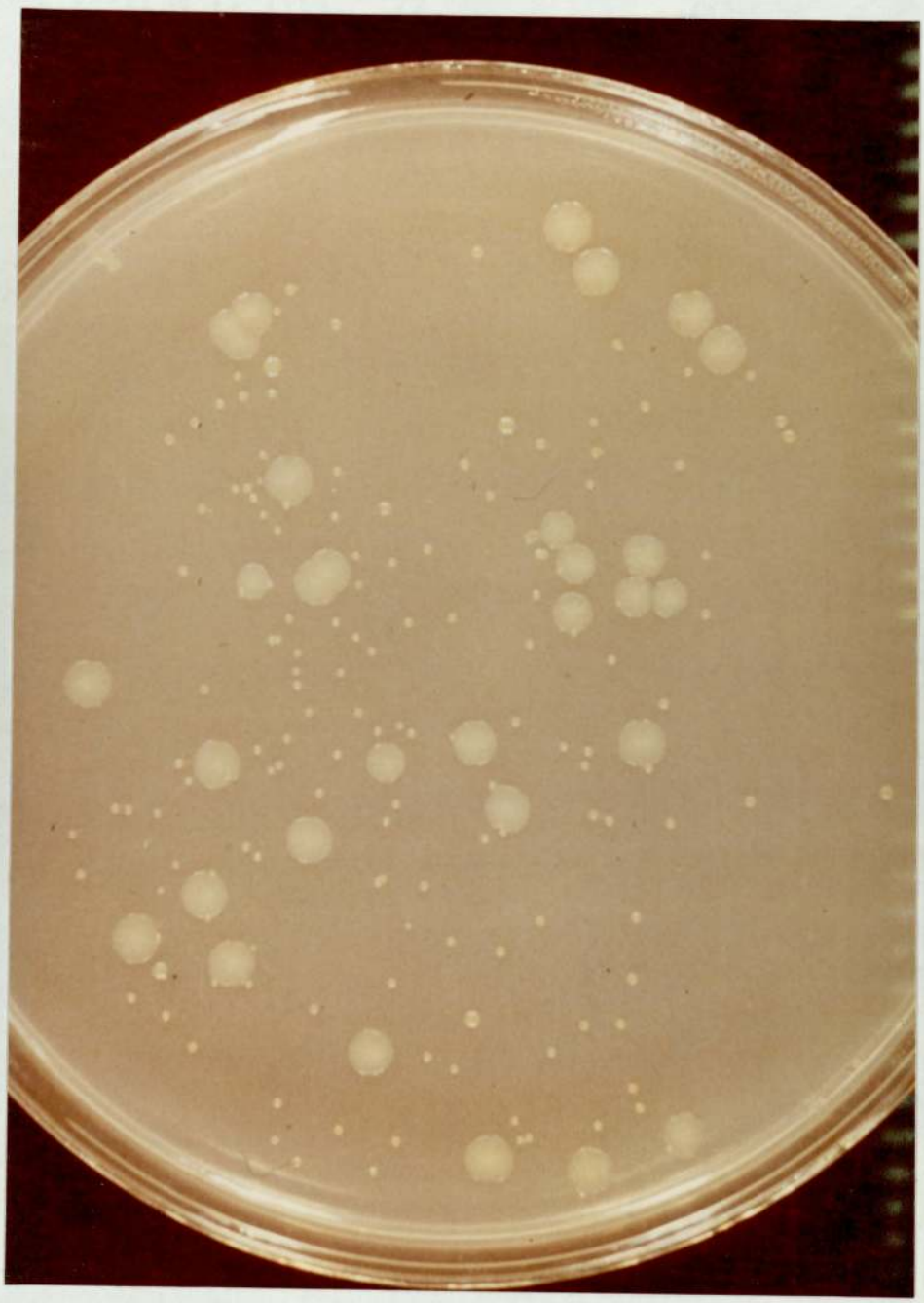
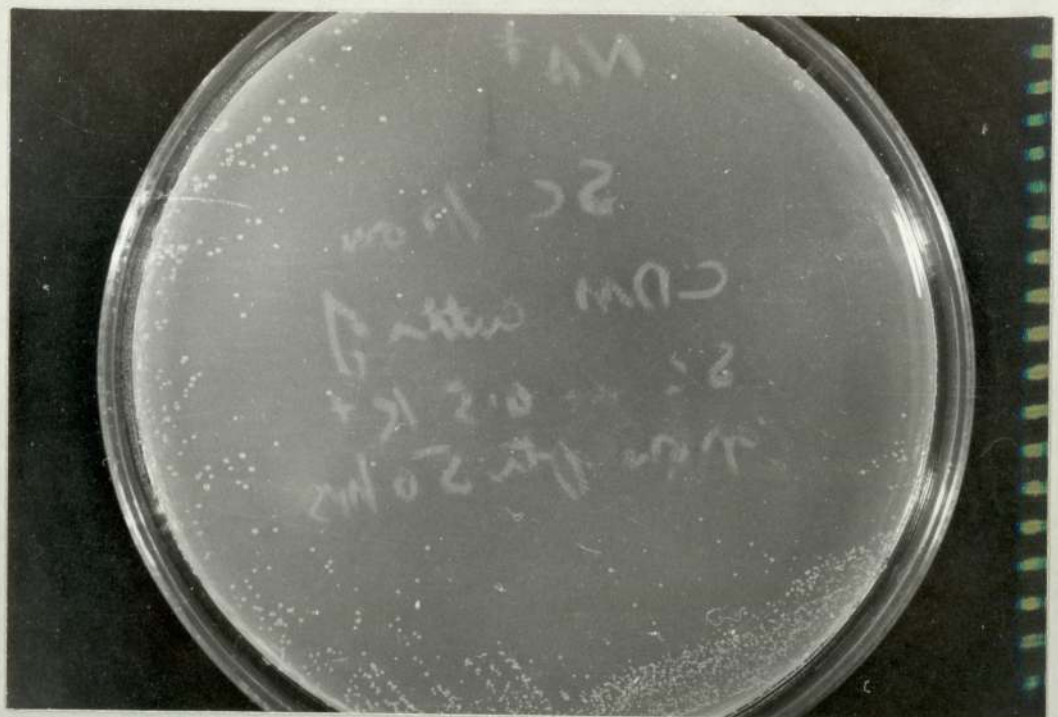
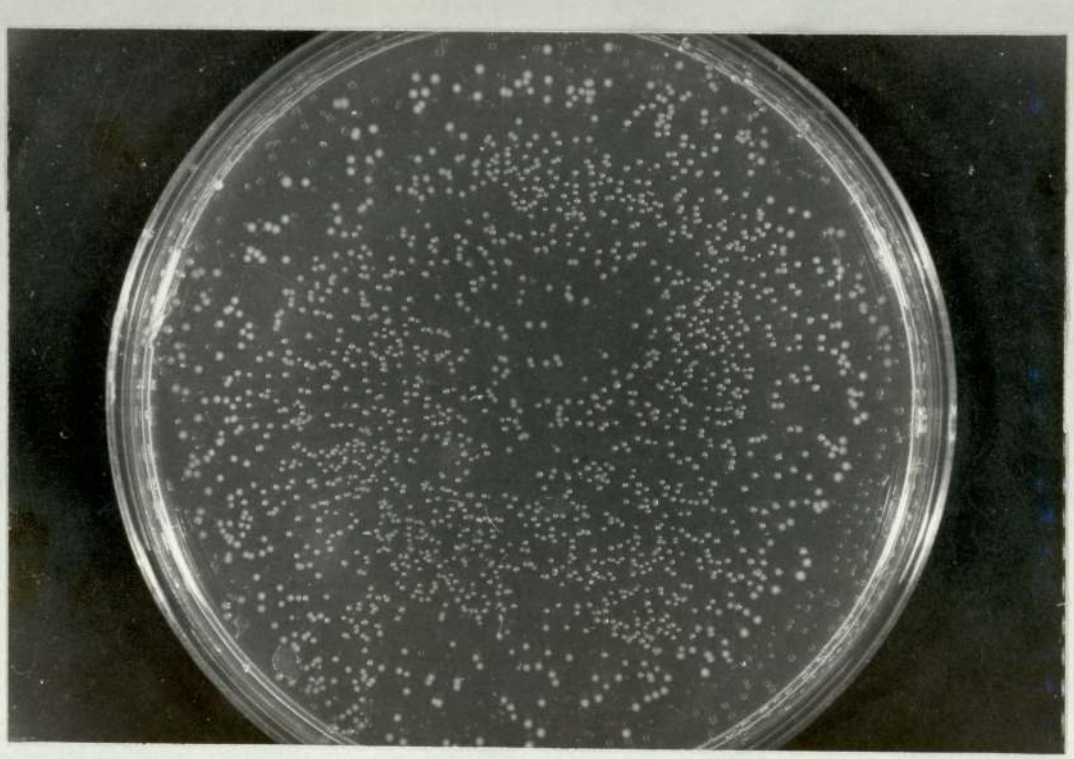


Fig. 42. *P. mirabilis* 5887 SC grown on (a) ONAA, (b) GNAA; 48h incubation at 37°.



a) ONAA

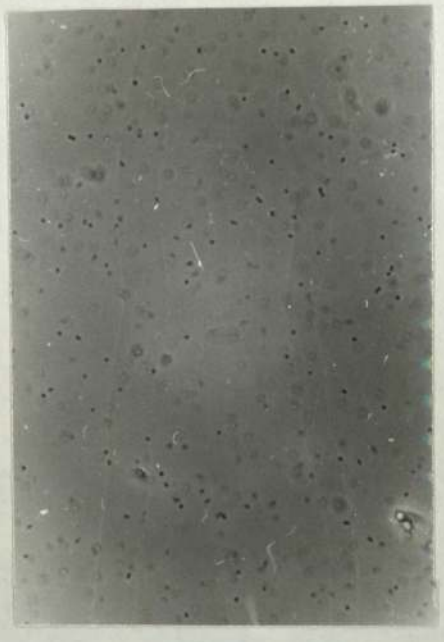


b) GNAA

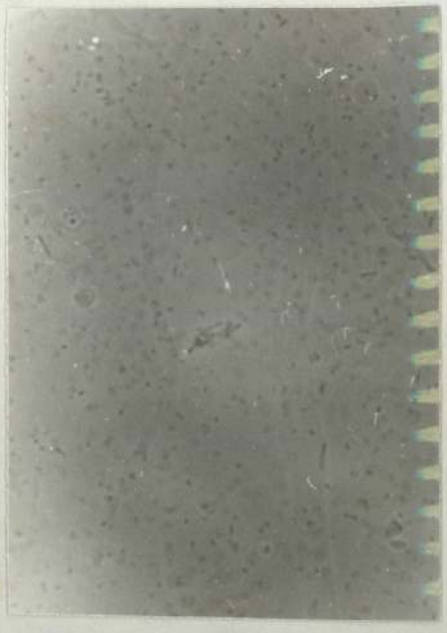
Fig. 43. *P. mirabilis* in NB, wet preparation seen by phase contrast microscopy. Magnification X600.

5887

LC



SC



RBH

LC

SC

Biochemical reactions are listed in Table 28. It can be seen that there are important metabolic differences, particularly a shift in the formation of haem compounds (Sc being catalase-negative and oxidase-positive, the opposite of Lc) and a failure of Sc to form gas from "sugars". Sc variants were urease-positive after 24 h incubation at 37^{o}, in comparison with a reaction in 2 h for Lc. Similar results were found for Sc variants of the other strains tested (Klemperer, personal communication); furthermore, in the O/F test, all Sc were only oxidative, whereas Lc can oxidise or ferment glucose.

Sc variants were always stable. No reversion to Lc was found, even after growth in 0.1 M or 0.2 M KCl-CDM, or serial subculturing on ONAA plates. Lc organisms are not stable, and give rise to Sc variants at random, apparently by spontaneous mutation. RBH cultures Lc, derived from single Lc colonies, which had been shown to be homogeneous by replica plating onto ONAA containing polymyxin 250 u ml^{-1} sufficient to kill Lc but not Sc cells, yielded variable numbers of Sc colonies on serial subculture.

3.5.2 The effect of KCl and NaCl on the occurrence of Sc variants

The effect of the salt concentration in CDM and NA plates on the occurrence of Sc variants is shown in Fig. 44a, b, c, d, e, f & g. Cultures of Lc in C-dep CDM (Table 2, p. 75) but with only 5×10^{-5} M KCl, yielded large number of Sc on subsequent plating, but when the concentration of either KCl or NaCl was increased to concentrations comparable to those in NB (at least 0.09 M NaCl from manufacturer's literature), the number of Sc found was low or undetectable. In all cases, 0.1 to 0.2 M-KCl or NaCl in the growth medium caused a great reduction in the number of Sc variants

Table 28

Biochemical characteristics of *P. mirabilis* Lc 5887, RBH and Sc variant of RBH

Test	Expected *P. mirabilis* results*	5887 Lc	RBH	
			Lc	Sc
Phenylalanine	+	+	+	+
Urease	+	+	+	+
Catalase	+	+	+	-
Oxidase	-	-	-	+
Indole	-	-	-	-
Sucrose	+	+	+	-
Glucose	AG	AG	AG	A
SIM(H_2S)	+	+	+	-
Gelatin	+	+	+	-
KCN	+	+	+	+
Citrate	d	+	-	-
Lactate	-	-	-	-
Mannitol	-	-	-	-

Tests carried out as described in Cowan (1974).

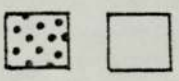
AG = acid + gas

*according to Cowan (1974)

+ = positive, - = negative, d = 79-21% of strains positive

Fig. 44. The effect of the concentration of KCl and NaCl in liquid and solid media on the occurrence of small colonies.

Cultures grown in NB  or CDM  (table 2) with varying concentrations of KCl or NaCl for 24h at 37° and plated on ONAA or GNAA.

 large colonies  small colonies

a) *P. mirabilis* 5887

b) *P. mirabilis* RBH

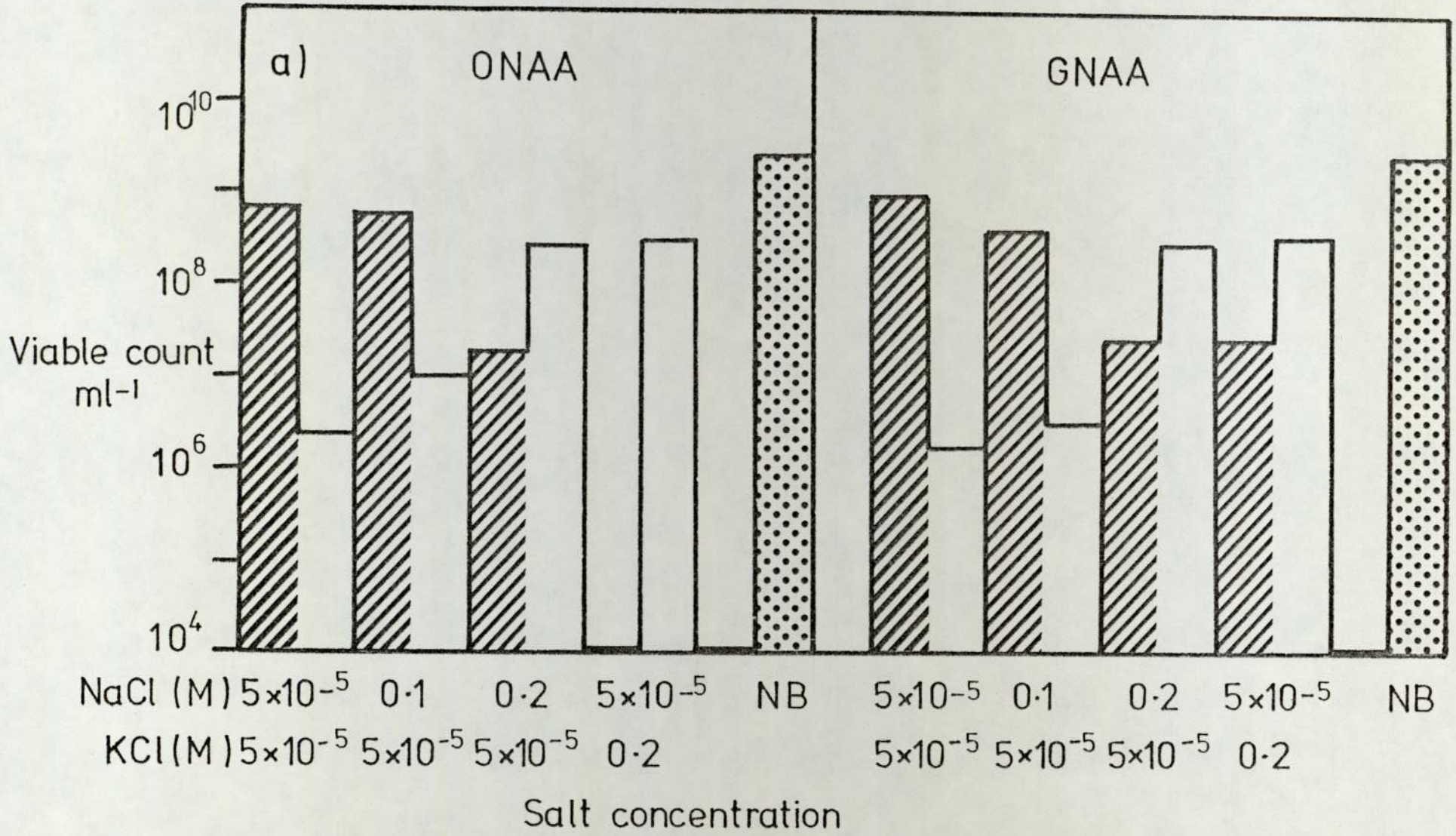
c) *P. mirabilis* 7827

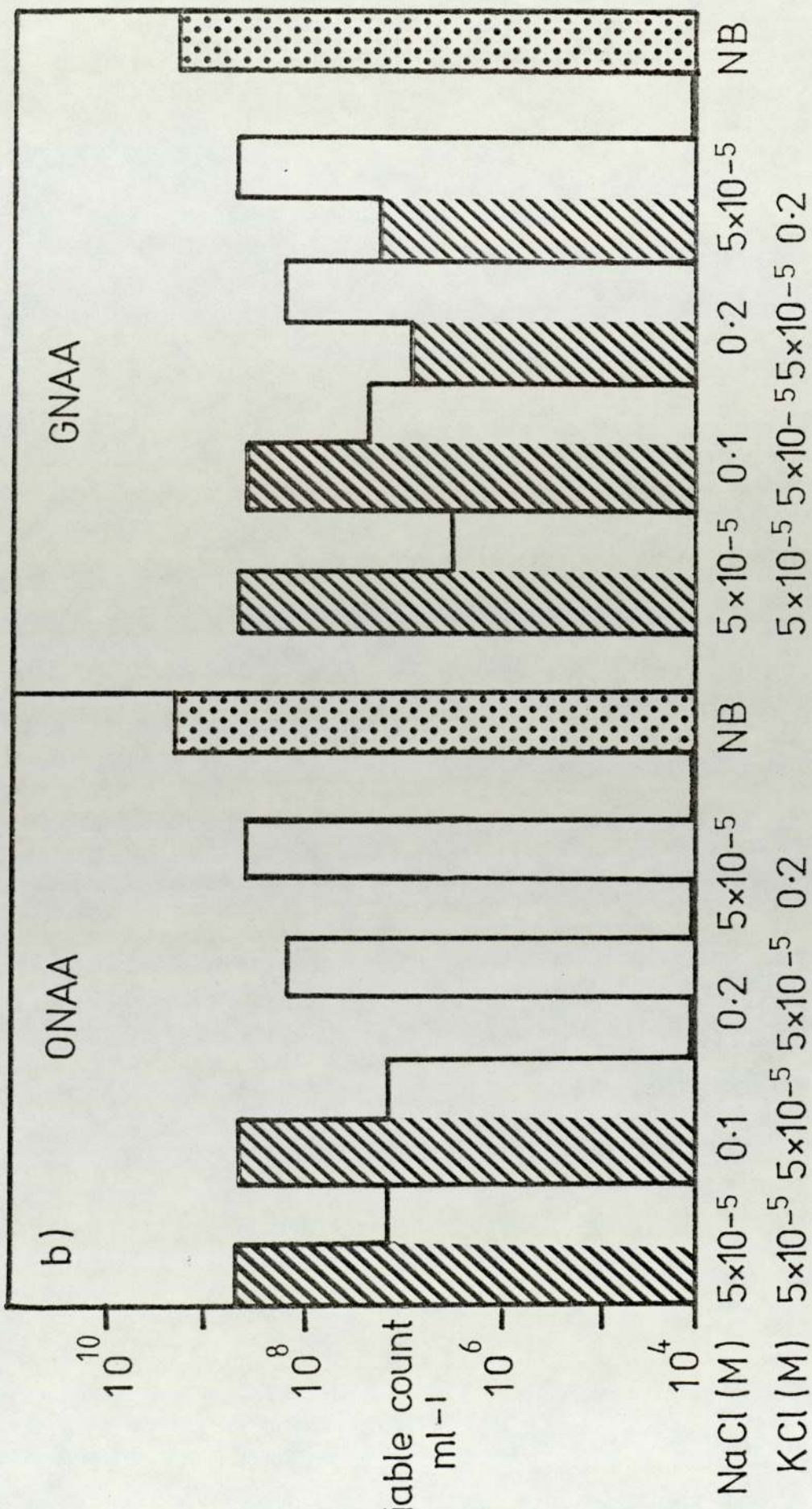
d) *P. mirabilis* 8309

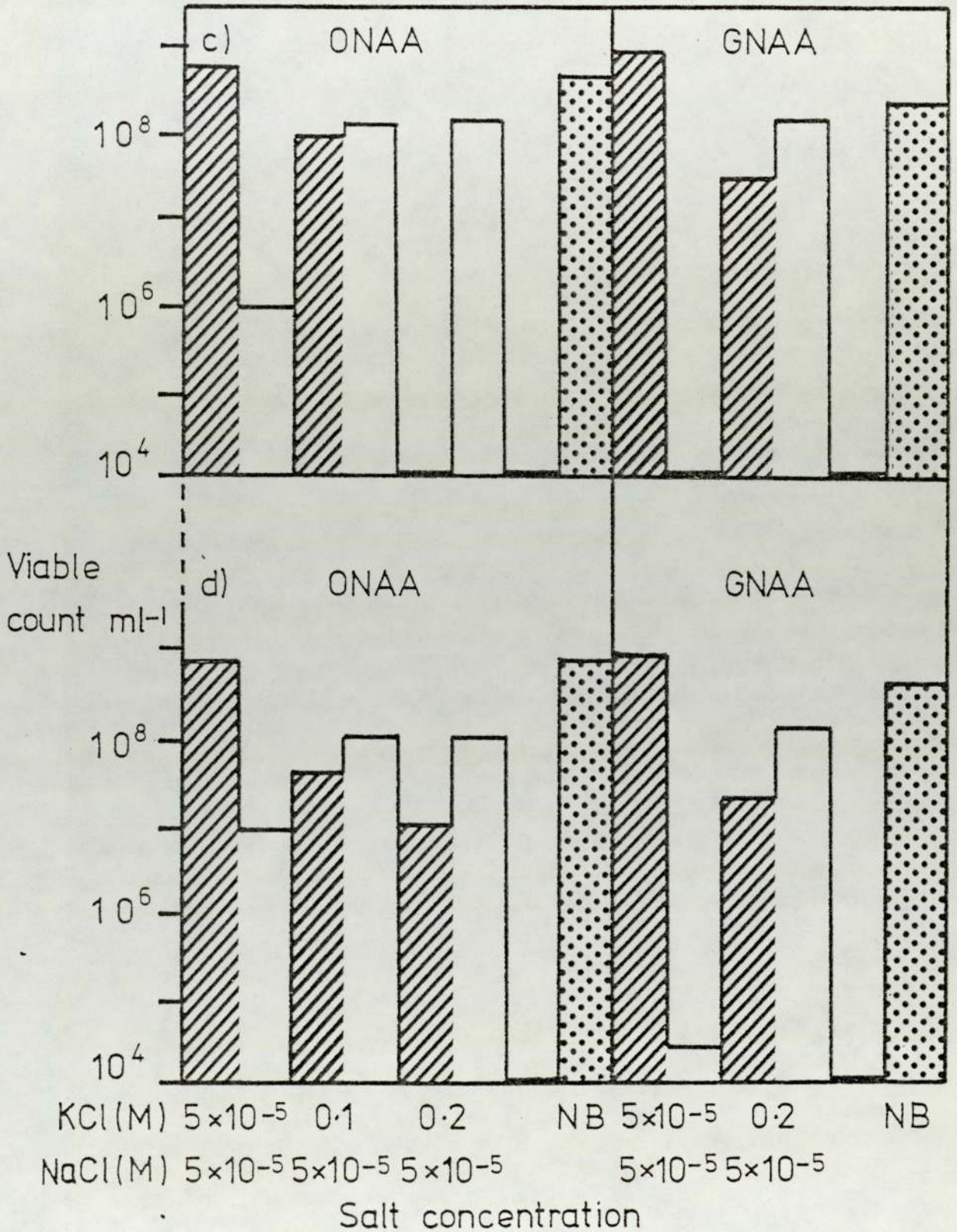
e) *P. mirabilis* 60

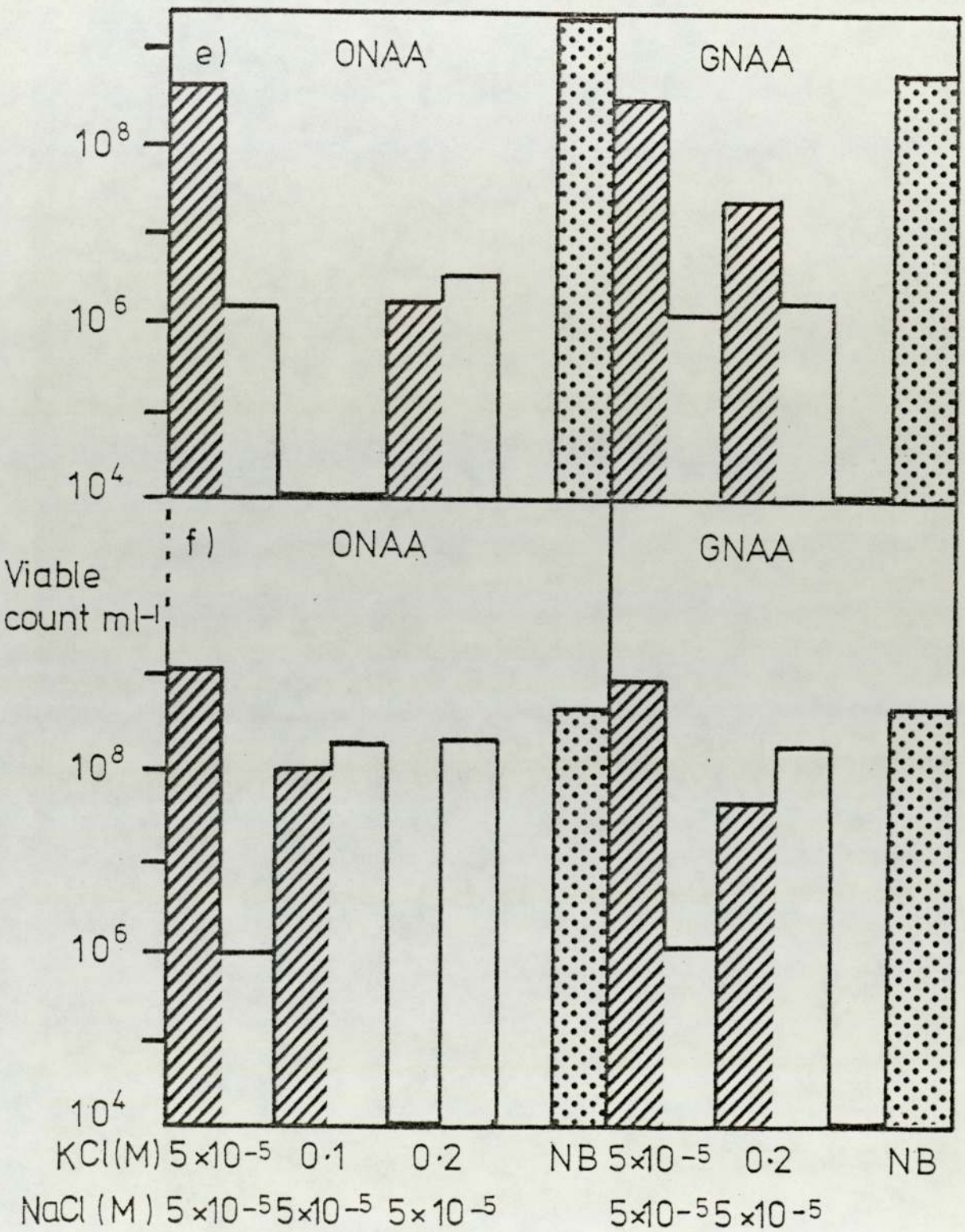
f) *P. mirabilis* 2100

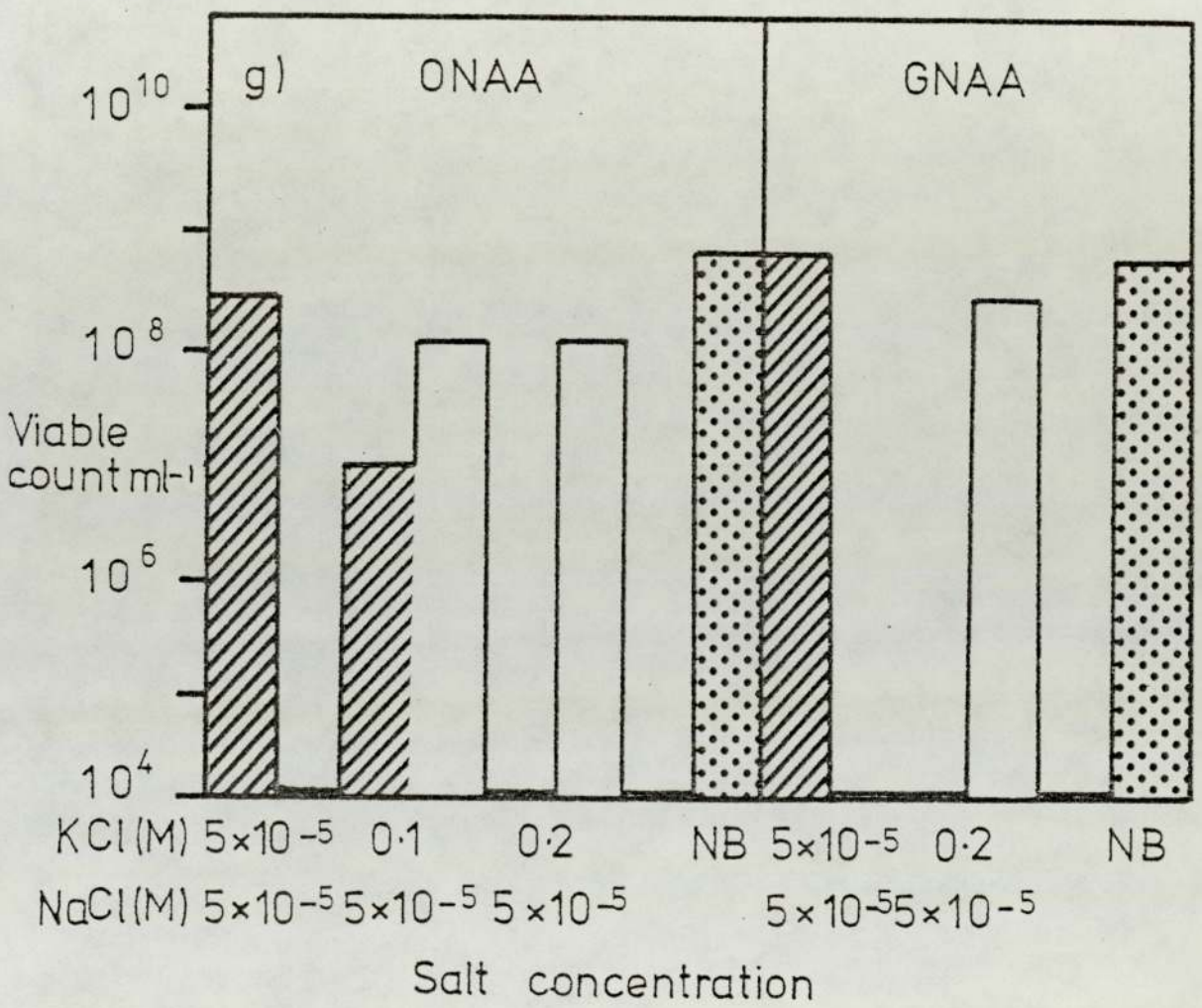
g) *P. morgani* 10466


a)
ONAA
GNAA
10^{10}
10^{8}
10^{6}
10^{4}
Viable count ml⁻¹
NaCl (M) 5×10⁻⁵ 0·1 0·2 5×10⁻⁵ NB 5×10⁻⁵ 0·1 0·2 5×10⁻⁵ NB
KCl (M) 5×10⁻⁵ 5×10⁻⁵ 5×10⁻⁵ 0·2 5×10⁻⁵ 5×10⁻⁵ 5×10⁻⁵ 0·2
Salt concentration


b)
ONAA
GNAA
Viable count ml⁻¹
10¹⁰
10⁸
10⁶
10⁴
NaCl (M)
KCl (M)
5×10⁻⁵
0·1
5×10⁻⁵
0·2
5×10⁻⁵
NB
Salt concentration


c)
ONAA
GNAA
10^8
10^6
10^4
Viable count ml-1
d)
ONAA
GNAA
10^8
10^6
10^4
KCl(M) 5×10-5 0·1 0·2 NB 5×10-5 0·2 NB
NaCl(M) 5×10-5 5×10-5 5×10-5 5×10-5 5×10-5
Salt concentration


e)
ONAA
GNAA
10^8
10^6
10^4
f)
ONAA
GNAA
Viable count ml-1
10^8
10^6
10^4
KCl (M) 5×10-5 0·1 0·2 NB 5×10-5 0·2 NB
NaCl (M) 5×10-5 5×10-5 5×10-5 5×10-5 5×10-5


g)
ONAA
GNAA
10^{10}
10^{8}
10^{6}
10^{4}
Viable count ml⁻¹
KCl (M) 5×10⁻⁵ 0·1 0·2 NB 5×10⁻⁵ 0·2 NB
NaCl (M) 5×10⁻⁵ 5×10⁻⁵ 5×10⁻⁵ 5×10⁻⁵ 5×10⁻⁵
Salt concentration

subsequently found, and they were never detected by direct plating from NB cultures. It can be seen that in most cases, Sc variants from CDM containing 0.2 M-KCl or NaCl appeared on GNAA, but not on ONAA plates, even after 50 h incubation. According to the manufacturer's literature, ONAA has NaCl (0.09 M) added, in addition to salts present in the protein digest. GNAA has no additional salt.

Serial subcultures of 5887 and RBH showed that cultures with no detectable Sc may contain sufficient surviving cells for them to grow up again when salt conditions become favourable, and that salt concentrations sufficient to prevent the emergence of detectable numbers of Sc may not prevent the growth of those in the initial inoculum. Alternatively, more Sc variants may arise de novo by mutation. These results are shown in Fig. 45.

3.5.3 Effect of KCl concentration in CDM on the growth rate of Sc variants

To investigate the selective effect of salt concentration on Sc variants, the growth rate of Lc and Sc were compared at different KCl concentrations.

An inoculum CDM (Table 2) containing either 0.2 or 5×10^{-5} M-KCl was used for Lc and Sc variants respectively. For growth rate measurements, C-dep CDM (Table 2) containing different KCl concentrations were used. Different KCl concentrations had relatively little effect on the growth rate of Lc of 5887 and RBH, while Sc variants have prolonged doubling times at high KCl concentrations (Table 29).

Figure 45

The effect of KCl on the proportion of small colonies following serial subculture of P. mirabilis 5887 and RBH in CDM.

5887

Culture number	KCl (M)	% Sc
1	0.2	10.3
2	0.2	41.4
3	0.2	37.7
3	0.3	2.5
2	0.3	N.D
3	5×10^{-5}	99.7
3	0.1	1.1
3	0.2	N.D
3	0.3	N.D
2	0.4	N.D

RBH

Culture number	KCl (M)	% Sc
1	0.2	24.7
2	0.2	33.8
3	0.2	21.6
3	0.3	N.D
4	0.2	3.5
4	0.3	N.D
2	0.3	N.D
3	5×10^{-5}	99.3
3	0.2	0.3
3	0.3	N.D
		N.D
2	0.4	N.D

N.D = Sc not detectable

*inoculated from NA slope

Table 29

The effect of KCl on the doubling time of P. mirabilis 5887 and RBH, large (Lc) and small (Sc) variants

KCl (M)	Doubling Time (min) (a)			
	5887		RBH	
	Lc	Sc	Lc	Sc
5×10^{-5}	70.5 ± 2(2)	76 ± 7(2)	69 ± 4(2)	75 ± 5(5)
0.1	81 ± 8(4)	102 ± 1(2)	80 ± 7(4)	102 ± 9(6)
0.15	82 ± 6(3)	135	91 ± 7(3)	148
0.2	96 ± 15(3)	259 ± 6(2)	111 ± 6(3)	206
0.3	-	(b)	-	-
0.4	85 ± 3(2)	(c)	91	(d)

Method: see section 2.2.2; Lc cultures checked for absence of Sc.

(a) Mean ± S.D for 2 or more experiments; number of experiments in brackets.

(b) 1 doubling in 18 h.

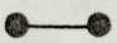
(c) 1 doubling in 21 h.

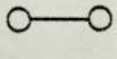
(d) no growth in 7 h.

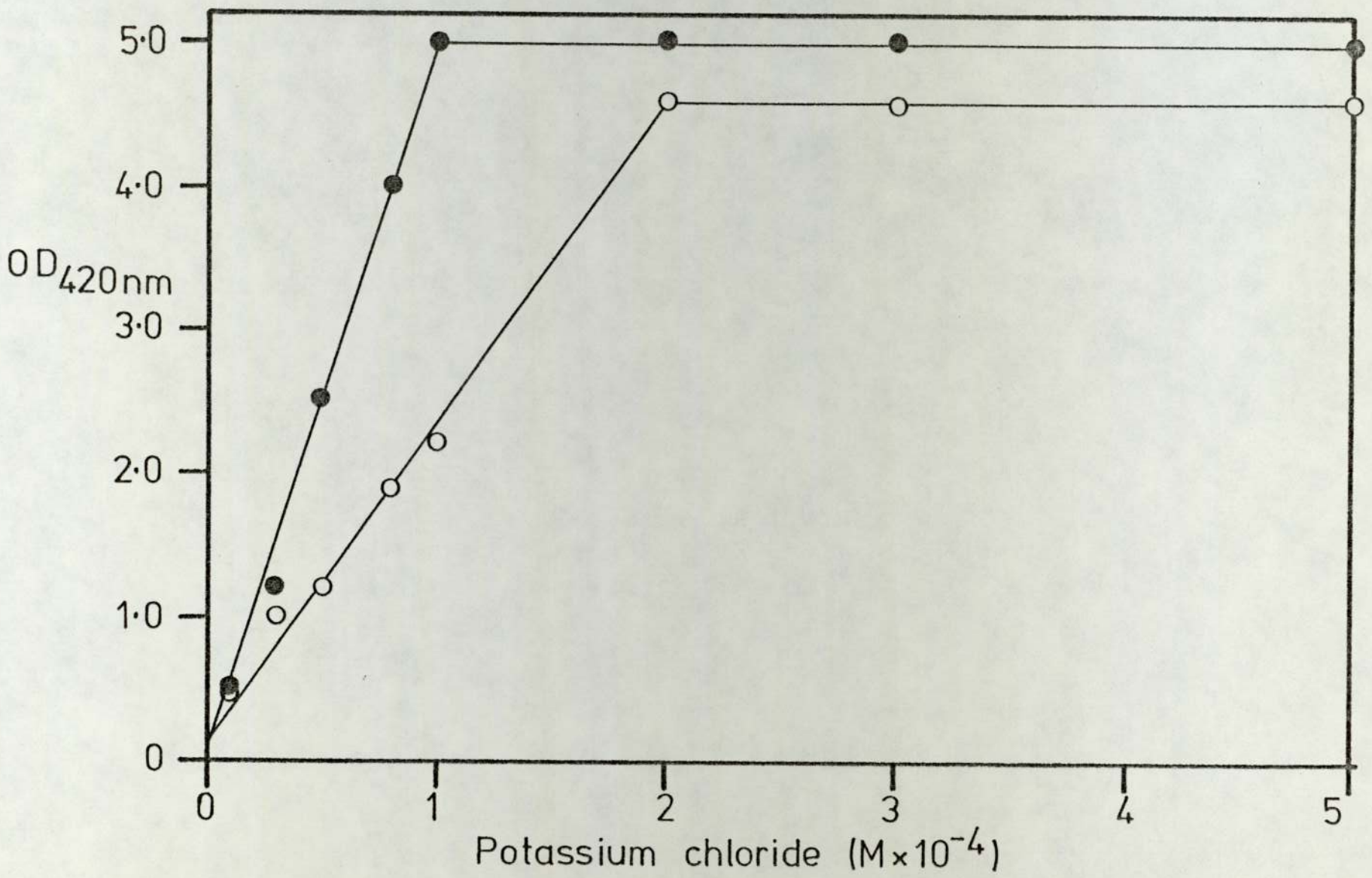
3.5.4 Relation between KCl concentration in CDM and the total growth of Sc variant

Fig. 46 shows the linear relationship between OD at the end of exponential growth of P. mirabilis RBH, Lc and Sc, and the KCl concentration in CDM. Growth conditions and media are as described in section 2.2.2 and Table 8 (p. 94). For the same amount of available KCl, the Sc variant reaches twice the OD of Lc, which suggests that the requirement of the Sc variants for potassium is much less than that of the Lc.

Fig. 46. Relation between KCl concentration in CDM and OD.


, RBH SC


, RBH LC


5·0
4·0
3·0
2·0
1·0
0
OD420nm
0
1
2
3
4
5
Potassium chloride (M×10⁻⁴)

3.5.5 Resistance of Sc variants to antibacterial agents

3.5.5.1 Resistance of Sc variants to polymyxin and disinfectants was compared with that of Lc, using the procedure described in section 2.2.6. C-dep CDM (Table 2) containing 5×10^{-5} and 0.2 M-KCl was used for Sc and Lc respectively.

Table 30 shows that Sc variants of 5887 and RBH were more sensitive to phenol than Lc, but much more resistant to polymyxin and chlorhexidine.

Table 30

Effect of polymyxin and other antibacterial agents on large (Lc) and small (Sc) colony variants of P. mirabilis 5887 and RBH

Antibacterial agent	Concn	Time of exposure (min)	% survival			
			5887		RBH	
			Lc	Sc	Lc	Sc
cetrimide	3 μg ml^{-1}	90	17 ± 14(3)	9 ± 6(3)	26 ± 13(2)	17 ± 8(2)
phenol	3.5 mg ml^{-1}	30	31 ± 22(2)	11	65 ± 7(2)	7 ± 1(2)
chlorhexidine	5 μg ml^{-1}	120	12 ± 11(2)	72 ± 11(2)	11 ± 4(2)	43 ± 4(2)
polymyxin	250 u ml^{-1} 2000 u ml^{-1}	180 90	- 60	- 100	15 ± 5(2)	104 ± 13(3)

Mean and SD for 2 to 3 experiments, number of experiments in brackets.

3.5.5.2 Sensitivity of Sc variants to antibiotics. Overnight cultures of Lc and Sc variants were spread on GNA plates and antibiotic multodiscs placed on the surface. Table 31 shows that all the Lc which were sensitive to the aminoglycosides or β-lactam

antibiotics formed Sc variants with increased resistance. The converse effect was found with tetracycline, the two strains with tetracycline-resistant Lc formed sensitive Sc variants. In addition, Lc and Sc were sensitive to sulphamethoxazole and sulphafurazole. All Sc variants of the seven strains were sensitive to tetracycline but resistant to all other antibiotics tested, except that of RBH which was sensitive to carbenicillin and ampicillin.

Table 31

Sensitivity of *P. mirabilis* and *P. morgani* Lc and Sc variants to antibiotics

Antibiotic (µg)	NCTC 5887		NCIB 10466		NCIB 2100		NCTC 60 NCTC 7827 NCTC 8309		RBH	
	Lc	Sc	Lc	Sc	Lc	Sc	Lc	Sc	Lc	Sc
Kanamycin 5 Gentamicin 10	R	R	S	R	S	R	S	R	S	R
Carbenicillin 100 Ampicillin 25	R	R	S	R	S	R	S	R	S	S
Ampicillin 2	R	R	R	R	R	R	S	R	S	R
Cephaloridine 25	R	R	R	R	S	R	S	R	S	R
Cloxacillin 5 Methicillin 10	R	R	R	R	S	R	R	R	R	R
Tetracycline 50	S	S	S	S	R	S	S	S	R	S

S zone > 2 mm from disc

R zone < 2 mm from disc

3.6 Chemical Analysis of Whole Cells and Cell Walls of C-depleted and Mg-depleted Cultures of P. mirabilis 5887 and RBH

The preparation of whole cells and cell walls and the chemical assay procedures used have been described in sections 2.2.5, 2.2.8 and 2.3.

Duplicate cultures were used; they were checked for Sc and found to contain less than 1% of Sc variants (see section 3.5).

The results of chemical analysis are presented in Tables 32 to 35 as percentage (mean and SD) dry weight. The following points of particular interest may be noted. The PL content of C-dep RBH cell walls is relatively low (Table 32a & b). Fractionation of the PL shows that this low PL content of C-dep RBH cell walls is due to a very low PE content (Table 33b). Fluctuations in the ratios PE/DPG and PG/DPG with nutrient depletions are shown in Table 33c. The appearance of PC in C-dep RBH whole cells is interesting (Table 33a & b). The presence of EIF in Mg-dep RBH whole cells and in some samples of C-dep RBH whole cells (Table 32a & b), and the low level of KDO in RBH (Table 34) are other features distinguishing RBH from 5887.

Cation analyses did not reveal any big differences between 5887 and RBH, though the drop in Mg^{2+} content of 5887 cell walls was very marked (Table 35).

Table 32

Lipids of whole cells and cell walls of P. mirabilis 5887 and RBH

a)

	Percentage dry weight of whole cells or cell walls							
	5887				RBH			
	whole cells		cell walls (iii)		Whole cells (ii)		Cell walls (iii)	
	C-dep (i)	Mg-dep (ii)	C-dep	Mg-dep	C-dep	Mg-dep	C-dep	Mg-dep
cell walls	11.5 ± 0.4	10.0 ± 0.3	-	-	10.6 ± 1	7.9 ± 0.5	-	-
REL	13.7 ± 1	16.4 ± 0.9	22.0 ± 9	21.4 ± 6	16.1 ± 3	19.3 ± 8	21.5 ± 4	21.2 ± 2
PL	4.3 ± 1	4.8 ± 2	4.8 ± 1	5.1 ± 2	3.8 ± 2	4.8 ± 4	2.6 ± 0.4	4.6 ± 0.3
FAN	9.5 ± 1.5	12.0 ± 0.4	17.8 ± 7	17.8 ± 3	8.9 ± 3	12.9 ± 5	18.4 ± 4	18.1 ± 4
EIF	ND	ND	ND	ND	ND*	4 ± 3 (iv)	ND	ND

ND = not detectable, i.e. < ca 2%

* but in a further 2 out of 4 samples 7% EIF was found

(i) 2 samples from each of 2 cultures

(ii) 2 samples from 1 culture and 1 sample from a second

(iii) 1 sample from each of 2 cultures

(iv) 2 samples from 1 culture

b)

	Percentage dry weight of total readily extractable lipid							
	5887				RBH			
	whole cells		cell walls		whole cells		cell walls	
	C-dep	Mg-dep	C-dep	Mg-dep	C-dep	Mg-dep	C-dep	Mg-dep
PL	32	29	23	23	24	25	12	22
FAN	69	73	80	85	59	67	86	86
EIF	ND	ND	ND	ND	ND*	21	ND	ND
total	101	102	103	108	83	113	98	108

Figures calculated from data in part a).

*See note about EIF in part a).

Table 33

Phospholipid fractions of whole cells and cell walls of cultures of P. mirabilis 5887 and RBH

(a)

	Percentage dry weight of total phospholipids							
	5887				RBH			
	whole cells		cell walls		whole cells		cell walls	
	C-dep	Mg-dep	C-dep	Mg-dep	C-dep	Mg-dep	C-dep	Mg-dep
DPG	28.2 ± 8	28.7 ± 4	50.9 ± 3	35.7 ± 1	29.5 ± 3	15.3 ± 2	50.4 ± 2	46.5 ± 1
PE	64.5 ± 8	57.5 ± 4	34.0 ± 3	39.2 ± 3	60.8 ± 3	66.1 ± 9	26.3 ± 3	41.3 ± 0.9
PG	10.7 ± 4	16.2 ± 3	9.3 ± 1	12.0 ± 0.5	5.9 ± 2	16.8 ± 9	15.8 ± 2	6.2 ± 1
PC	ND	ND	5.4 ± 1	7.0 ± 1.6	5.4 ± 3	ND	7.4 ± 2.6	5.6 ± 1
PS	ND	ND	ND	5.8 ± 0.7	ND	ND	ND	ND
total	103	102	98	100	102	98	100	100

ND = not detectable

Mean ± SD represents duplicate readings from each of two replicate samples

(b)

	Percentage dry weight of whole cells or cell walls							
	5887				RBH			
	whole cells		cell walls		whole cells		cell walls	
	C-dep	Mg-dep	C-dep	Mg-dep	C-dep	Mg-dep	C-dep	Mg-dep
DPG	1.2	1.4	2.4	1.8	1.1	0.7	1.3	2.1
PE	2.8	2.8	1.63	2.0	2.3	3.2	0.7	1.9
PG	0.46	0.78	0.45	0.61	0.22	0.81	0.41	0.29
PC	ND	ND	0.26	0.36	0.21	ND	0.19	0.26
PS	ND	ND	ND	0.30	ND	ND	ND	ND

Figures calculated from data in Tables 32a & 33a.

(c)

Ratio of PE or PG/DPG in whole cells or cell walls

	5887				RBH			
	whole cells		cell walls		whole cells		cell walls	
	C-dep	Mg-dep	C-dep	Mg-dep	C-dep	Mg-dep	C-dep	Mg-dep
PE/DPG	2.3	2.0	0.68	1.1	2.1	4.6	0.54	0.9
PG/DPG	0.38	0.56	0.19	0.34	0.2	1.2	0.32	0.14

Table 34

KDO content of whole cells of cultures of P. mirabilis 5887 and RBH

	Percentage dry weight of whole cells			
	5887		RBH	
	C-dep	Mg-dep	C-dep	Mg-dep
KDO	0.52 ± 0.09	0.6 ± 0.025	0.28 ± 0.04	0.31 ± 0.03

Mean ± SD Duplicate samples from each of two cultures.

Table 35

Cation contents of whole cells and cell walls of C-dep and Mg-dep cultures of P. mirabilis 5887 and RBH

	Percentage dry weight of whole cells or cell walls							
	5887				RBH			
	whole cells(i)		cell walls(ii)		whole cells(i)		cell walls(ii)	
	C-dep	Mg-dep	C-dep	Mg-dep	C-dep	Mg-dep	C-dep	Mg-dep
Mg^{2+}	0.15 ± .001	0.09 ± .02	0.29 ± .08	0.1 ± .004	0.12 ± .02	0.1 ± .02	0.19 ± .02	0.15 ± .03
Ca^{2+}	0.01 ± 0.0	0.01 ± .004	0.27 ± .05	0.35 ± .03	0.01 ± .001	0.01 ± .001	0.4 ± .04	0.3 ± .09
Mn^{2+}	0.001 ± .0001	0.001 ± 0.0	0.001 ± .0001	0.001 ± .0002	0.001 ± .0002	0.001 ± .0003	0.001 ± .0001	0.001 ± .0001

Means ± SD represent the following samples:

(i) 1 sample from each of 2 cultures

(ii) 1 sample from each of 2 cultures for Mg^{2+} and Mn^{2+};

3 samples from each of 2 cultures for Ca^{2+}

4. DISCUSSION

4.1 Nutrient Requirements of P. mirabilis 5887 and RBH

4.1.1 Introduction

Monod (1942) first demonstrated a linear relationship between the maximum population density of a culture and the concentration of added carbon source. Since then the growth requirements of many Gram-negative bacteria have been investigated in chemically-defined media. The nutrient depletion studies carried out on P. mirabilis 5887 and RBH showed that there was no difference in the nutritional requirements of both strains. Assuming that optical density can be used to indicate yield, the growth yield of the two strains was the same for all the nutrients investigated. The requirement for each nutrient is discussed below and compared with the requirements of other Gram-negative species.

4.1.2 Glucose

When all nutrients required for the growth of P. mirabilis 5887 and RBH in CDM were available in excess except glucose, growth ceased abruptly when the latter became depleted (Fig. 16) in cultures with low initial glucose concentrations. Cultures with high initial concentrations eventually showed a progressive decline in the growth rate. This indicates that above the concentration of 8×10^{-3} M, glucose was no longer the limiting nutrient (Fig. 16). The exponential growth rates of the cultures were independent of the initial glucose concentration except at the lowest concentration (2×10^{-4} M). A linear relationship existed between OD at the end of exponential growth and glucose concentration in the medium up to OD of 4.0, as shown in Fig. 17. This line extrapolates back through the origin which indicates that there

was no other carbon source present in the medium which can be used to produce an increase in the cell mass more than that produced by the added glucose.

The yield of P. mirabilis 5887 and RBH for glucose is similar to that of E. coli (Ismail, 1977), but slightly more than that of Ps.aeruginosa (Boggis, 1971).

4.1.3 Magnesium

The growth characteristics of magnesium-depleted cultures (Fig. 18) are markedly different from those of glucose-depleted ones (Fig. 16). The progressive decrease in the growth rate after the end of the exponential growth may be due to a corresponding decrease in the synthesis of RNA and protein. Tempest et al. (1965) observed that the RNA content of A. Aerogenes cells limited by magnesium decreased as the degree of limitation increased. A similar result was reported for Ps.putida (Sykes & Tempest, 1965). These results indicated that RNA synthesis, including ribosomal RNA was related to the magnesium content. McCarthy (1962) and Morgan et al. (1966) have shown that protein synthesis in magnesium-depleted E. coli is proportional to the ribosome content. Therefore it seems that at each successive division of magnesium-depleted cells, the cellular content of magnesium and ribosomes will progressively decrease causing a fall in the rate of protein synthesis resulting in a corresponding decrease in the growth rate.

Magnesium is also essential for the activity of many enzymes including those involved in the synthesis of cell wall components such as fatty acids (Knivett & Cullen, 1967), peptidoglycan (Garrett, 1969), lipopolysaccharide (Edstrom & Heath, 1967) and phospholipids (White et al., 1971). There is much evidence that

magnesium is a structural component of the Gram-negative outer membrane (Costerton et al., 1974), especially in Ps. aeruginosa (Brown & Melling, 1969a & b).

The growth yield of P. mirabilis for magnesium was similar to that of E. coli (Ismail, 1977) but much higher than that of Ps. aeruginosa (Boggis, 1971). It is possible that these differences can be attributed to the metabolic and structural differences between enterobacteria and pseudomonads.

Fig. 39 shows a biphasic curve for the relation between the reciprocal of the specific growth rate of exponential phase cells and the reciprocal of the initial magnesium concentration in the medium, unlike the linear relation found by Finch (1976) for Ps. aeruginosa. Shehata & Marr (1971) reported a similar relation between the growth rate of E. coli and phosphate concentration and suggested the presence of a dual transport system for phosphate of high and low affinity.

4.1.4 Nitrogen

The rapid cessation of the growth at the end of exponential growth after exhaustion of nitrogen indicates an essential requirement for nitrogen (Fig. 22). Nitrogen is involved in the synthesis of cellular components, in addition to protein, such as nucleic acids and some phospholipids.

A linear relationship was maintained between OD at end of exponential growth and nitrogen concentration in the medium up to OD of 4.0 (Fig. 23). The intercept of the line showed a contamination level of 1×10^{-3} M, equivalent to utilizable nitrogeneous materials from other medium ingredients. Over the range of NH_4Cl concentrations tested, it can be seen that the

growth rate was independent of NH_4 concentration added to the medium (Fig. 22).

4.1.5 Phosphate

The shape of the growth curves of phosphate-depleted cultures shown in Fig. 26 is similar to that of magnesium; as phosphate became limiting there was a progressive slowing of growth. The stoichiometry between the RNA, magnesium and phosphorus content of A. aerogenes (Dicks & Tempest, 1966) would explain this similarity. The gradual slowing of growth may be explained by the reduction in the rate of protein synthesis due to the effect of decreasing phosphorus content on the ribosomal RNA. The exponential growth rate of phosphate-depleted cultures was dependent upon the initial phosphate concentration in the medium (Fig. 40). A linear relationship exists between OD at end of exponential growth and phosphate concentration added to the medium up to OD of 4.0 (Fig. 27). The amount of phosphate present as contaminant from other medium ingredients was equal to 1×10^{-3} M.

The phosphate requirement of P. mirabilis was found to be very much higher than that of E. coli and Ps. aeruginosa; phosphate concentration of 10^{-3} M supported exponential growth of E. coli (Ismail, 1977) and P. mirabilis up to OD 4.0 and 0.15 respectively, while 10^{-4} M phosphate maintained the exponential growth of Ps. aeruginosa up to OD 0.35 (Boggis, 1971).

In addition to its many metabolic roles, phosphate is a constituent of the cell envelope of Gram-negative bacteria in phospholipids and lipopolysaccharide. However, the phospholipid content of whole cells of E. coli (Gilbert & Brown, 1978a) and Ps. aeruginosa (Kenward, 1975) were not significantly different from that of P. mirabilis 5887 and RBH.

4.1.6 Potassium

The growth curves of cultures during potassium-depletion (Fig. 24) are similar to those of cultures during magnesium and phosphate depletion. This may be correlated with the stoichiometry between RNA, magnesium, phosphorus and potassium in A. aerogenes (Dicks & Tempest, 1966), and explain the gradual decrease of growth on the same basis as for magnesium and phosphate depleted cultures. A linear relationship existed between OD at the end of the exponential growth and the added potassium concentration in the medium up to OD of 4.0 (Fig. 25). There was a contamination level of potassium presumably from other medium ingredients equal to 2×10^{-5} M as indicated from the x-intercept of the line.

The requirement of P. mirabilis 5887 and RBH for potassium is the same as that of E. coli (Ismail, 1977), but about half that of Ps. aeruginosa (Boggis, 1971). This may be due to metabolic differences between P. mirabilis and Ps. aeruginosa, since potassium, in addition of being one of the principle inorganic cations in the cell, also acts as cofactor for some enzymes (Stanier, 1977).

4.1.7 Sulphate

Sulphate as a source of sulphur is required for some amino acid syntheses, and as a constituent of some coenzymes such as coenzyme A and cocarboxylase (Stanier, 1977).

The shape of the growth curves of sulphate-depleted cultures is similar to that of magnesium, phosphate and potassium depletion curves. It can be seen in Fig. 20 that as sulphate became limiting there was a progressive decrease of growth. There was no significant difference in the sulphate requirements of

P. mirabilis, Ps. aeruginosa (Boggis, 1971) and E. coli (Ismail, 1977).

4.1.8 Nicotinic acid

Nicotinic acid is a growth factor required for the synthesis of the coenzymes nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) by organisms that cannot themselves synthesize the pyridine ring. These two coenzymes are essential in most dehydrogenase reactions. It was found to be an essential nutrient for Proteus (Fildes, 1938; Peclzar & Porter, 1940) though not all strains of a given species required it. Neither P. mirabilis 5887 nor RBH require added nicotinic acid.

4.1.9 Conclusion

P. mirabilis 5887 and RBH have the same nutritional requirements and can be grown in a simple CDM containing carbon source, an inorganic source of nitrogen (NH_4) and phosphorus (PO_4) and small quantities of magnesium, potassium and sulphate. Other ions such as sodium, chloride and other cations may be required in quantities minute enough to be satisfied by contaminants in other components of the medium. Nicotinic acid was not an essential constituent, but it was included in the subsequent CDM used in case its presence affected other cell properties. Their quantitative nutritional requirements were the same, and also the same as those of E. coli except that P. mirabilis had a significantly higher PO_4^{3-} requirement. P. mirabilis, like E. coli, had a lower requirement for glucose, Mg^{2+} and K^{+} than Ps. aeruginosa and no demonstrable Fe^{2+} requirement, possibly due to different metabolic pathways.

Extrapolating the line in Fig. 27 indicates that phosphate concentration of 0.2 M is required to support the exponential growth theoretically up to OD of 10. However to avoid a possible toxic effect, phosphate concentration of 0.1 M was used in CDM required to support the growth exponentially up to OD 10.0. Furthermore, 0.1 M phosphate was shown to be sufficient to support the growth to the same actual level as that obtained with 0.2 M (Fig. 26).

For all required nutrients, the relation between the OD at the end of exponential growth and nutrient concentration was linear up to OD 4.0, although a sufficient concentration of each nutrient was tested to theoretically maintain linearity up to OD 10.0. This may indicate that some additional limitation had been superimposed at ODs greater than 4.0. The possibility that depletion of some essential trace elements occurred above that OD was not supported by the results shown in Fig. 30, which suggested that the requirements for these trace elements could be satisfied by contaminants from other ingredients. In most of the growth studies, the pH was found to have dropped from 7.0 to 6.6 - 6.9 after maximum growth of OD 7.0 had been reached. This drop in pH on its own would be unlikely to affect the growth rate since between pH 6.2 and 7.3 the initial growth rate was found to be the same (Fig. 15). It seems probable that departure from linearity above OD 4.0 was mainly due to oxygen depletion and/or accumulation of toxic metabolites.

It should be noted that a comparison of the yield of different organisms using OD readings does not necessarily reflect similar cell dry weights. Different nutrient-depleted cells may have different sizes. Tempest et al. (1965) have found that the cell

size of chemostat-grown A. aerogenes cultures decreased with increasing magnesium-limitation. The decrease in the cell size would increase the surface area and hence the light scattering properties of the culture.

4.2 Resistance Studies on Nutrient-Depleted P. mirabilis 5887 and RBH grown in Batch Culture

4.2.1 Introduction

The activity of polymyxin and other antibacterial agents against different nutrient-depleted cultures of P. mirabilis 5887 and RBH was assessed by measuring the decrease in viability after contact with the drugs for a specified time. This method is a direct assessment of the bactericidal activity. Cells were not washed prior to the killing experiments to avoid any change in sensitivity due to the washing procedure (Brown, 1968). They were centrifuged and resuspended at a predetermined OD in prewarmed glucose and magnesium-free resuspending medium (for C- or Mg-dep cultures) or glucose, magnesium and phosphate-free resuspending medium (for C- or P-dep cultures), i.e. in a medium similar to the growth medium at the time of harvesting. However, the resuspending media contained much lower concentrations of potassium and phosphate than the growth medium, to avoid inhibition of the bactericidal activity by these ions, as potassium phosphate and to a lesser extent potassium chloride are moderately protective against the action of polymyxin B on Ps. aeruginosa (Klemperer et al., 1979). The cells were equilibrated for 30 min after resuspending and the OD was taken at the start and at the end of the 30 min to test the stability of the culture before the addition of the antibacterial agent.

The day-to-day variation in response of different cells to polymyxin and other agents used in this study may be in part due to the inactivating procedure. It is probable that different batches of egg-yolk leicithin varied in the efficiency with which

they inactivated polymyxin and other agents. However, Russell et al. (1979) found that simple dilution was sufficient to remove polymyxin, admittedly from polymyxin-resistant species. They also suggested the inclusion of inactivator in the serial dilutions and in the recovery medium.

In discussing the resistance to membrane-active agents, the role of the outer membrane must be considered. To reach the cytoplasmic membrane and exert their action, these agents must first penetrate the outer membrane. Therefore, the uptake and absorption of these agents at the outer membrane play a crucial role in the resistance mechanism. The outer membrane itself may be either non-adsorbing or adsorbing and acting as a barrier to penetration to the cytoplasmic membrane (Hamilton, 1971). The effect of absorption at the outer membrane will be a balance between the amount of drug absorbed and the amount passed on to the underlying layers, including the cytoplasmic membrane. Therefore, on the one hand, absorption of the drug at the outer membrane and then its passing to the cytoplasmic membrane may lead to sensitivity, on the other, too much absorption so that none is passed on may lead to resistance.

The MIC of cetrimide, phenol and chlorhexidine for P. mirabilis 5887 and RBH are the same (Table 15), but the susceptibilities of different nutrient-depleted cultures were different. Polymyxin and the other antibacterial agents used in this study are all membrane-active agents and are thought to exert their action at the same site. It seemed likely that resistance depended on similar factors. Ps. aeruginosa mutants selected for resistance to polymyxin are also more resistant to chlorhexidine (Watkins, 1970) and when selected for resistance to cetrimide become more

resistant to chlorhexidine and polymyxin (Klemperer, personal communication). Furthermore, sulphonamides are synergistic with both polymyxin (Greenfield & Feingold, 1972) and chlorhexidine (Quesnel et al., 1978), which may be due to sulphonamide-induced changes in the cell wall (Handley et al., 1974). However, the results shown here indicate that different nutrient depletions imposed on cultures result in different responses and may have affected differently the passage of these agents to their site of action at the cytoplasmic membrane.

4.2.2 Resistance of nutrient-depleted P. mirabilis 5887 and RBH to cetrimide

Fig. 31 and Table 11 show that Mg-dep and P-dep 5887 are significantly more resistant to cetrimide than C-dep. Similarly P-dep RBH is also more resistant than C-dep. However, Mg-dep RBH is more sensitive than C-dep RBH (Fig. 34 and Table 11). C-dep cells of 5887 and RBH varied in sensitivity in different resuspending media. Both were more resistant to cetrimide when suspended in phosphate buffer, than in MOPS (Table 9). However, day-to-day variation also occurred, but it did not affect the pattern of resistance of different nutrient-depleted cultures. It seems probable that some properties of cetrimide could contribute to such variation. It has been reported that cetrimide and other QACs cause clumping of cells in microbial suspension and can also cause the cells to adhere to the walls of the vessel containing the suspension (Sykes, 1965), but Smith et al. (1975) found little evidence to support either of these phenomena using E. coli treated with 0.1 mM cetrimide. In this study, using cetrimide concentrations very much lower than that, microscopical

examination of P. mirabilis 5887 and RBH cultures treated with cetrimide did not reveal any clumping. The possibility of cetrimide adhering to the glass containers was not investigated. It was also reported that for cetrimide is a mixture of QACs (Smith et al., 1975), the dissimilarity in action between cetrimide and CTAB was correlated with the presence of QACs of various chain lengths in cetrimide.

Bacterial resistance to QACs may be based on biochemical modifications of the cell envelope resulting in an alteration of the permeability barrier (Adair et al., 1971). EDTA reversed the resistance of Ps. aeruginosa to QACs (MacGregor & Elliker, 1958). It was postulated that the relative impermeability of Ps. aeruginosa to QACs was due to a high lipid content and that EDTA enabled the quaternary compound to penetrate this barrier and then reach its site of action at the cytoplasmic membrane. Brown & Richards (1965) have also shown that EDTA enhanced the activity of benzalkonium chloride (BC), chlorhexidine and polymyxin B on growing cultures of Ps. aeruginosa. Richards & Cavill (1976) using electron microscopy showed a pronounced stripping of the outer membrane of Ps. aeruginosa treated with BC (50 $\mu g\ ml^{-1}$). In the same study, it was shown that cells growing in the presence of BC (500 $\mu g\ ml^{-1}$) were more susceptible to the lytic action of 23 $\mu g\ ml^{-1}$ of EDTA than were cells that had been grown in the presence of BC and then grown overnight in its absence. These latter cells showed the same level of resistance to 23 $\mu g\ ml^{-1}$ EDTA as normal cells grown for the same length of time. They therefore suggested that when EDTA was added to cultures of Ps. aeruginosa growing in the presence of BC, EDTA was able to gain access to the cell structures, including cytoplasmic membrane,

on which it might have more potent effects. They also suggested that the resistance of Ps. aeruginosa to BC is at sites internal to the outer membrane, most likely at the cytoplasmic membrane. However, it is possible to interpret their results assuming that both BC and EDTA act on the outer membrane. Two mechanisms can therefore be suggested for the resistance of nutrient-depleted cultures to QACs: 1) the inability of the drug to penetrate the outer membrane barrier to its site of action, due to chemical and structural changes, 2) the unavailability or alteration of the target molecule at the cytoplasmic membrane. The resistance of P-dep 5887 and RBH to cetrimide may involve either or both of these mechanisms. The anionic lipids at the cytoplasmic membrane are involved in the interaction with the QACs including cetrimide (Riemersma, 1966) and Hugo (1967) found lipids were protective. Gilbert & Brown (1978a) found a decrease in the whole cell PL of E. coli following phosphate-depletion, associated with greater sensitivity to cetrimide. However, Anderes et al. (1971) found a reduction in the lipids of QAC-resistant cells of Ps. aeruginosa, which contradicts Hugo's hypothesis (1967).

Salt & Wiseman (1970) reported that the uptake of CTAB is an ion exchange process; it was found to compete with Mg^{2+} for sites at the cell surface. Mg-dep cells 5887 and RBH might be expected to have less Mg^{2+} bound to their surfaces (Tempest & Strange, 1966; Meers & Tempest, 1970) than would be expected to bind more CTAB than their respective C-dep cells; however, their resistance was very different. The presence of higher PG/DPG ratio (Table 33) in the cell wall of Mg-dep 5887 than after C-depletion may represent a permeability barrier owing to the lower proportion of anionic sites, hindering cetrimide from

reaching the CM and accounting for its higher resistance. A similar correlation was found for C-dep and Mg-dep RBH, the latter being more sensitive and having the lower PG/DPG. Mg-dep E. coli, like RBH, was more sensitive to cetrimide than C-dep cells (Ismail et al., 1977) and whole cell analyses showed a higher PG/DPG ratio in the more resistant cells (Gilbert & Brown, 1978a).

4.2.3 Resistance of nutrient-depleted P. mirabilis 5887 and RBH to phenol

The patterns of resistance of nutrient-depleted P. mirabilis 5887 and RBH to phenol were similar (Fig. 32, 35, Table 12). Sensitivities of different nutrient-depleted cells remained the same in spite of day-to-day variation. Different resuspending media seemed to have little effect (Table 9).

Cytoplasmic membrane damage is considered to be the primary lesion caused by phenol (Pullman & Reynolds, 1965). There is evidence to suggest that binding of phenol to lipids and/or protein (Judis, 1966) at the cytoplasmic membrane could account for its disruption. The uptake of phenol by the cells is related to its activity (Bean & Das, 1966). Protoplasts of M. lysodeikticus bind less phenol than the whole cells (Judis, 1966). This suggests that Gram-positive cell walls do bind a portion of phenol. Extraction of lipids from Ps. aeruginosa with petroleum ether did not alter its viability, but increased its sensitivity to phenol (Ivanov et al., 1964). Also, Srivastava & Thompson (1965, 1966), using synchronous cultures of E. coli, found that the cells were most sensitive to phenol when they were dividing and these cells had the least PL.

Mg-dep or P-dep may reduce the uptake of phenol, so that less is available at the cytoplasmic membrane. The cell wall of the more sensitive C-dep RBH contained less PL than Mg-dep (Table 32a, b). The cell wall of C-dep and Mg-dep 5887 contained the same amount of PL; this might account for the slight difference in their response to phenol. The PL content was markedly reduced by P-dep in E. coli (Gilbert & Brown, 1978a), and these cells were sensitive to phenol, unlike the P-dep 5887 and RBH.

Gilbert & Brown (1978b) found a correlation between the KDO content of chemostat-grown C-lim and Mg-lim Ps. aeruginosa and resistance to chlorinated phenols. No such correlation can be suggested for the resistance of Mg-dep 5887 and RBH. From Table 34 it can be seen that there was no difference in the KDO content of Mg-dep 5887 and RBH as compared with their respective C-dep cells. Furthermore, there was no clear difference in the sensitivity of 5887 and RBH to phenol which could be correlated with their different KDO content.

4.2.4 Resistance of nutrient-depleted P. mirabilis 5887 and RBH to chlorhexidine

The sensitivity of Mg-dep cells to chlorhexidine was almost the same as that of C-dep cells, for both 5887 and RBH (Fig. 33, 36, Table 13). However, P-dep 5887 was more resistant to chlorhexidine than the C-dep, while P-dep RBH was slightly more sensitive than C-dep RBH. Different resuspending media did not affect the response of C-dep 5887 to chlorhexidine significantly (Table 9), but there was day-to-day variation in the susceptibility of different cells of 5887 and RBH. However, this did not affect the pattern of resistance of different nutrient depleted cells.

The primary effect of chlorhexidine is the disruption of the cytoplasmic membrane by virtue of its lipophilic group (Hugo & Longworth, 1964). The reduction of the electrophoretic mobility of E. coli after addition of chlorhexidine indicates that, as a strong base, it reacts with charged groups on the cell surface (Hugo & Longworth, 1966). The activity of chlorhexidine was increased on increasing the pH, which was attributed to an increase in its uptake due to an increasing number of binding sites on the cell surface (Hugo & Longworth, 1964). There were also more cationic binding sites, as measured by H^+ binding, on C-dep E. coli than following Mg-depletion and these C-dep cultures were relatively sensitive to chlorhexidine (Ismail, 1977).

The differences in the PL fractions of cell walls and whole cells of Mg-dep 5887 and RBH compared with their respective C-dep cells (Table 33a, b & c) do not correlate with their similar sensitivities to chlorhexidine; however these cultures had very similar REL content (Table 32). Gilbert & Brown (1978a) found no difference in the REL of C-, Mg- and P-dep E. coli, but their resistances to chlorhexidine were different (Ismail , 1977). P-dep E. coli, like 5887, but unlike RBH, was more resistant to chlorhexidine than C-dep. The change in E. coli was correlated with a large fall in whole cell PL. However, the surface of P-dep cells was strongly anionic, which would be expected to be associated with increased sensitivity. These results show that P-depletion may affect the surface and the interior of the cell wall differently. Small differences at the two sites in 5887 and RBH following P-depletion might be sufficient to account for their big differences in resistance.

4.2.5 Resistance of nutrient-depleted P. mirabilis 5887 and RBH to polymyxin

From Table 10 and 14, it can be seen that C-dep 5887 is resistant to high concentrations of polymyxin, and 250 u ml^{-1} of polymyxin had almost no effect on either C-dep or Mg-dep cells. The effect of polymyxin on P. mirabilis RBH was completely different ; C-dep RBH was very sensitive to 250 u ml^{-1} polymyxin, Mg-dep and P-dep RBH were much more resistant (Fig. 37, Table 14). C-dep RBH resuspended in phosphate buffered-CDM was less sensitive to polymyxin than when resuspended in MOPS buffered-CDM, when compared at the same time (Fig. 37). This is similar to the effect of resuspending media on resistance to cetrimide (Table 9).

It has been proposed that the resistance of typical P. mirabilis strains to polymyxin is due to the impermeability of the outer membrane (Teuber, 1969; Sud & Feingold, 1970), preventing the polymyxin from penetrating the outer membrane and exerting its action at the cytoplasmic membrane. According to the asymmetric model of the outer membrane proposed by Nikaido & Nakae (1979), LPS and protein comprise the outer surface of this layer, while the inner surface is covered with PL and protein. It has been shown that "deep rough" mutants of S. typhimurium, whose LPS lacks most of the oligosaccharide chains, were much more sensitive than wild type to certain antibiotics and dyes, including polymyxin B (Roantree et al., 1969; Nikaido, 1976). Nikaido (1976) found that these compounds were usually hydrophobic or had molecular weights greater than 650. Antibiotics, whose activities were unaffected by the LPS were hydrophilic molecules of less than 650 daltons which apparently gained entrance to the periplasmic space through

water-filled protein-lined pores in the outer membrane. Thus the outer membrane would not be the barrier to such molecules that it would to hydrophobic molecules or hydrophilic ones of greater molecular weight. It was proposed that the difference in hydrophobic permeability between the smooth and rough mutants was due to a radical difference in the organization of the outer membrane, more specifically to the presence or absence of exposed PL at the surface (Nikaido, 1976). Polymyxin is amphiphilic and has a molecular weight of about 1200. It reacts with LPS (Rifkind, 1967; Teuber, 1973) as well as PL and its low partition coefficient may not reflect its true affinity toward the interior of the outer membrane (Nikaido, 1976).

It appears that LPS and PL may assume major roles in the initial binding of polymyxin to the outer membrane. The difference in the amount of LPS and their ability to complex with polymyxin B may reflect different degrees of antibiotic susceptibility of Ser. marcescens strains (Brown & Tsang, 1978). P. mirabilis 5887 contains about twice as much KDO as RBH (Table 34). This suggests that the resistance of 5887 to polymyxin may be due in part to more, and more closely-packed LPS in the outer membrane, allowing no PL to be exposed. It is also possible that the saccharide part of LPS may prevent access of polymyxin to the lipid A region of LPS or to the interior of the outer membrane by interacting with the hydrophilic part of polymyxin. The finding of Gmeiner (1975a & b) that P. mirabilis produces two types of LPS, with either long or short O-antigen side-chains both with more negatively-charged residues than other Gram-negatives lends credence to such a concept. However, if there is such a difference between the LPS of 5887

and RBH, it would not be detected by the KDO assay. The higher KDO content in 5887 also suggests that LPS acts as a barrier for polymyxin by interacting with the hydrophobic part of polymyxin, preventing its entry to the cytoplasmic membrane. This agrees with Tsang et al. (1976) who suggested that the ability of LPS to complex with polymyxins in resistant cells of Ser. marcescens may be the basis of their resistance to these antibiotics. However, polymyxin B has been found to inhibit the growth and respiration of E. coli by interacting only with the outer membrane (Laporte et al., 1977). Tsang et al. (1975) found that lower yields of LPS were obtained from untreated sensitive cells of Ser. marcescens and negligible yields were recovered from sensitive ones after treatment with polymyxin, in comparison with yields from treated and untreated resistant cells. They reported that a high protein/LPS ratio in the LPS components is indicative of the susceptibility of the organism to polymyxin. However, this increase seems mainly to be due to LPS breakdown and afforded no significant role for proteins associated with the LPS in resistance to polymyxin.

PL, as a component of the outer membrane, was also thought to be involved in the passage of polymyxin to the cytoplasmic membrane (Brown & Watkins, 1970), in addition to being a target molecule at the cytoplasmic membrane (Sud & Feingold, 1972). Low sensitivity to polymyxin was correlated with the low amounts of PL in the walls of Ps. aeruginosa, P. vulgaris and K. aerogenes (Brown & Wood, 1972). Higher PL content in the walls of P. mirabilis 5887 may exclude polymyxin from the cytoplasmic membrane. Mackenzie & Jordan (1970) reported that resistance to viomycin, a basic cyclic polypeptide, of a mutant of Rhizobium meliloti was accompanied by an accumulation of PL in the cell

envelope. However, Wade et al. (1975) observed no difference between the content of REL, total PL and individual PL in polymyxin B-sensitive and resistant whole cells of Ser. marcescens. The differences between these strains might exist in their cell wall PL. These authors also found that more unsaturated fatty acids were present in the PL of the sensitive strains. It was suggested than an increase of unsaturated fatty acids in sensitive strains might facilitate penetration of polymyxin B to its site of action by decreasing the hydrophobic interactions between membrane fatty acids and the 6-methyloctanoic acid of the polymyxin B. However, Sud & Feingold (1970) found no difference in the fatty acid composition of polymyxin B-sensitive and resistant P. mirabilis. In this study, fatty acids composition data was not obtained.

The literature concerning the specifity of polymyxin - PL interactions is contradictory. There is no evidence that the polymyxins interact exclusively with any particular PL, although they do show some preference for the acidic PLs (Storm et al., 1977). It can be seen (Table 33b) that C-dep 5887 cell wall has much more PL than RBH cell wall particularly DPG (an acidic PL) and PE (a zwitterion at physiological pH, but known to interact with polymyxin (Sud & Feingold, 1973). Furthermore, it is these PL which are increased in the cell wall of Mg-dep polymyxin-resistant RBH. C-dep 5887 cell wall has a slightly higher PE/DPG ratio than RBH cell wall. An increase in PE/DPG has been correlated with an increase in polymyxin resistance in Ps. aeruginosa following insertion of R-plasmid RP1 (Kenward et al., 1978) and also with resistance to gentian violet (Boman et al., 1974). The higher PL, particularly PE and PG in the whole cell of C-dep 5887 compared with C-dep RBH suggests that the target molecules

are available at the CM of C-dep 5887 and that its resistance is due to the impermeability of its cell wall to polymyxin. Whole cells of C-dep 5887 had a higher PG/DPG ratio than whole cells of C-dep RBH, but this may not be relevant to its greater resistance. Suling & O'Leary (1977) found that whole cells of polymyxin-resistant P. mirabilis PM5 had a lower PG/DPG ratio than the same organism, which had become more sensitive to polymyxin following the introduction of the R-plasmid R222. These results may in fact reflect the correlation between lipids of the outer membrane and antibiotic resistance.

The resistance of Mg-dep RBH to polymyxin is compatible with the results of Brown & Melling (1969), Boggis (1971) and Kenward (1975) who found that the sensitivity of Ps. aeruginosa decreases with a decrease in the Mg^{2+} concentration in the growth medium. Cations have been reported to antagonise the action of polymyxin. Newton (1954) concluded that the antagonism of polymyxin by Mg^{2+} and Ca^{2+} was probably due to competition for anionic sites on or within the cell wall. It is probable that in the absence of metal cations during growth, these may be replaced by some other compounds to stabilize the outer membrane. Wilkinson & Galbraith (1975) have suggested that polyamines and other similar organic cations may take the role of Mg^{2+} to some extent. The presence of these organic cations may prevent access of polymyxin to the initial binding sites or bind more tightly than polymyxin to the same sites. However, in this study, the cation content including Mg was almost the same for the different types of whole cells and cell wall except for the cell wall of Mg-dep 5887 which contained significantly less Mg^{2+} than the C-dep (Table 35). The KDO content of C-dep and Mg-dep cells was

the same for both strains, suggesting no role for LPS in the resistance of Mg-dep cultures. Minnikin & Abdolrahimzadeh (1974) and Minnikin et al. (1972) observed that B. subtilis and A. aerogenes increase the content of negatively-charged PL under conditions of Mg-depletion in batch cultures and Mg-limitation in continuous culture. This suggests that such cells will have a greater capacity for binding of the basically charged polymyxin. However, Kenward et al. (1979) found that Mg-dep cultures of Ps. aeruginosa possessed an increased amount of lysylphosphatidyl glycerol in the wall but showed little lysis with polymyxin. They also found that Mg-dep resistant cultures of Ps. aeruginosa possessed a reduced amount of PE, but these cells, when supplemented with Ca^{2+}, contained even less PE but were almost as polymyxin-sensitive as C-dep cells, with a high PE content. They concluded that the action of polymyxin upon Ps. aeruginosa could not be related to any one PL fraction in the wall. Although the REL content was the same, the PL content of the cell wall of Mg-dep RBH was more than that of C-dep RBH and comparable with that of both C- and Mg-dep 5887 CW (Table 32a & b). This is compatible with the results of Mackenzie & Jordan (1970) for Rhizobium meliloti. The PG/DPG ratio in the cell wall of Mg-dep RBH is about half that of C-dep RBH and the PE/DPG ratio is higher in cell walls of Mg-dep RBH than C-dep (Table 33c). This may indicate more binding capacity for polymyxin at the outer membrane of the Mg-dep cells. The presence of a higher amount of PE, even though DPG has fallen in Mg-dep cells of RBH than the C-dep cells (Table 33b) seems to indicate that the barrier of the outer membrane to polymyxin is the main cause of resistance. Brown & Melling (1969b) reported that initial increase in OD which occurs in cultures treated with

a high concentration of polymyxin did not occur with Mg-dep cultures, presumably because little polymyxin was taken up by the cells.

The resistance of P-dep RBH to polymyxin is consistant with the results reported by other workers. Boggis (1971) found that Ps. aeruginosa became resistant to polymyxin B under P-depleting conditions and its sensitivity was linearly related to the phosphate concentration in the medium. Dorrer & Teuber (1977) also found that P-dep Ps. fluorescens was resistant to polymyxin B. These authors found an increase in the cell envelope content of positively-charged ornithine amide lipid concomitantly with the increased resistance, but a decrease of PE, PG and DPG in both the cytoplasmic membrane and outer membranes. The PE content was reduced by approximately 30% in both membranes, the amount of PG and DPG was only 50% in the outer membrane and 70% in the cytoplasmic membrane. The binding of radioactive polymyxin B to intact cells and membranes isolated from these cells was diminished after P-depletion; in particular, adsorption of polymyxin to the outer membrane was greatly reduced. These results suggested that P-depletion reduced the negatively-charged binding sites for polymyxin. No chemical data for P-dep RBH was available. However, it seems reasonable to assume that as phosphorous is a major component of PL and LPS, changes in either would follow P-depletion and be likely to affect sensitivity to polymyxin.

4.3 Resistance Studies on Chemostat-grown P. mirabilis 5887 and RBH

4.3.1 Introduction

Chemostat cultures were resuspended after centrifugation in glucose-, Mg- and PO_4-free MOPS-buffered CDM before sensitivity testing. Therefore the resistance of those cells which had been grown in CDM containing 0.1 M KCl could be compared with batch culture cells resuspended in the same medium. Only the effect of cetrimide and polymyxin on chemostat-grown P. mirabilis 5887 and RBH was investigated, because these agents had yielded the more interesting results in batch cultures (sec. 2.1.1 & 2.2.1). Their effect was assessed by the same plate count method used for batch cultures. Percentage survival of the treated cultures was determined after a specific time. In addition, CDM containing 0.4 M KCl was used in many chemostat studies, to minimise the accumulation of Sc variants. 0.4 M KCl had a slight effect on the growth rate of Lc (sec. 3.5).

4.3.2 Resistance of chemostat-grown P. mirabilis 5887 and RBH to cetrimide

Chemostat-grown C-lim cells of 5887 grown in 0.1 M KCl had almost the same sensitivity to cetrimide as that of C-dep 5887 grown in batch culture (Tables 11, 18 & 25). The greater resistance of Mg- and P-lim chemostat cultures of 5887 (Tables 18 & 25) is compatible with the batch culture results (Table 11, Fig. 31).

Chemostat-grown C-lim cells of RBH were more resistant to cetrimide than the C-dep RBH batch culture (Table 11, 21 & 25). The slight increase in resistance of P-lim RBH compared

with C-lim cells is compatible with the batch culture results (Table 11, Fig. 34), but the higher resistance of Mg-lim cells of RBH is different. In batch culture, Mg-dep RBH was more sensitive to cetrimide than C-dep (Table 11). Although different resuspending media were used for Mg-dep and Mg-lim cells, these changes in response may also reflect different susceptibilities of growing and resting cells to cetrimide. This effect was not detected when other chemostat cultures of 5887 and RBH were compared with the respective batch cultures and C-dep 5887 was more sensitive to cetrimide when tested in MOPS instead of in PO_4-buffer (Table 9); the degree of resistance of Mg- and P-lim 5887 (Tables 18 & 25) and P-lim RBH (Tables 21 & 25) was not significantly different from those of batch cultures growing under the same conditions of nutrient depletion.

The higher resistance to cetrimide of C-lim 5887 grown in CDM containing 0.4 M KCl compared with cells grown in CDM containing 0.1 M KCl was not found with C-lim RBH (Tables 18, 21 & 25). This indicates that different KCl concentrations may cause different changes in C-lim 5887 and RBH. The higher resistance of C-lim 5887 grown in CDM containing 0.4 M KCl may be due to altered cell wall (see 4.3.3), which would affect the access of cetrimide to its site of action at the cytoplasmic membrane. Chemical analysis of cell walls prepared from cells of C-lim 5887 grown in different KCl concentrations would elucidate such alterations. This was not done in this study.

It can be seen (Tables 20, 23 & 24) that all chemostat-grown cultures of P. mirabilis 5887 and RBH, whether C- or Mg- or P-lim showed no change in their sensitivity to cetrimide with changing dilution rate. It is possible that the concentration of cetrimide used was too low to show such an effect.

4.3.3 Resistance of chemostat-grown P. mirabilis 5887 and RBH to polymyxin

Although different resuspending media were used, C-lim 5887 was more sensitive to polymyxin than C-dep 5887 batch culture (Table 14, 18 & 27). Mg-lim cells of 5887 appeared to be much more sensitive to polymyxin than the Mg-dep cells grown in batch culture. It is probable that this may be in part due to the MOPS-buffered resuspending medium used for the chemostat grown cells. C-dep RBH was also more sensitive to polymyxin when suspended in such media (Fig. 37). The difference in response to polymyxin between chemostat-grown and batch cultures 5887 may reflect different susceptibilities of the growing and resting cells. In this connection, it is interesting to note that Gmeiner et al. (1978) noted a great variation in the amount of lipoprotein linked to murein in differently grown cells of Proteus. In particular, it was absent from log phase broth-grown cells but appeared when these became stationary (Martin et al., 1972).

C-lim cells of RBH were more resistant to polymyxin than C-dep RBH batch culture cells (Tables 14 & 21). However, Mg-dep RBH, which was resuspended in a different medium, was more resistant to polymyxin than the Mg-lim chemostat grown cells of RBH. The P-lim cells of RBH were very much more sensitive to polymyxin than P-dep RBH cells grown in batch culture. Again, as with 5887, growing and resting cells of RBH show different susceptibility to polymyxin.

The higher sensitivity to polymyxin of Mg- and P-lim 5887 compared with the C-lim cells, and of Mg-lim 5887 compared with the P-lim cells may reflect changes in the outer membrane as a result of nutrient limitation which affected the uptake of polymyxin.

In contrast to the results in batch culture, P-lim cells of RBH were much more sensitive to polymyxin than C-lim cells. This is compatible with the results of Melling et al. (1974) who found that P-lim cells of Ps. aeruginosa were very sensitive to polymyxin compared with C-lim cells at a dilution rate of 0.1 h^{-1}.

Unlike its effect on sensitivity to cetrimide, a high KCl concentration (0.4 M) in the growth medium increased the sensitivity of all cultures tested to polymyxin. The presence of a high KCl concentration in the growth medium would increase the osmolarity and the internal ionic strength of the cells, owing to the accumulation of K^+ ions. The ionic strength within the cell affects the stability and behaviour of enzymes and other biological macromolecules (Stanier, 1977). Van Alphen & Lugtenberg (1977) showed that changing the osmolarity of the growth medium by the addition of high concentrations of NaCl, KCl or sucrose caused a drastic change in the ratio of the two peptidoglycan-associated major outer membrane proteins (b & c) of E. coli K12. Change in the amount of these two proteins, b and/or c can also influence the amount of a third protein (d) and the ratio KDO: cell envelope protein decreased slightly at high NaCl concentrations. These proteins have a high affinity for LPS in the outer membrane. These proteins have also been implicated in the formation of the aqueous pores in the outer membrane.

Phospholipid metabolism (Munro & Bell, 1973) and composition also vary with medium osmolarity. The fatty acid composition of stationary phase E. coli K12 (McGarrity & Armstrong, 1975) varied with the NaCl concentration, and in Staph. aureus the ratio DPG/PG and to lysylphosphatidyl glycerol increased with increasing NaCl. It is therefore likely that changes of KCl concentration in the

growth medium from 0.1 to 0.4 M may cause a variety of alterations in P. mirabilis. Because of the close association of outer membrane proteins with LPS and PL, it seems reasonable to assume that initial binding of polymyxin at the cell surface and subsequent penetration of the outer membrane through to the cytoplasmic membrane may be facilitated in the cells of 5887 and RBH growing in media containing 0.4 M KCl.

The sensitivity to polymyxin of C-lim 5887 and RBH growing at a dilution rate of 0.5 h^{-1} is higher than that of cells growing at 0.05 h^{-1}; this is compatible with the finding that the sensitivity of C-lim Ps. aeruginosa to 50 u ml^{-1} polymyxin was increased with increasing dilution rate (Melling et al., 1974). However, no change in the resistance of Mg-lim Ps. aeruginosa with dilution rate was reported, in contrast to the finding in this study. The sensitivity of Mg-lim cells of RBH to polymyxin was increased on increasing the dilution rate. In addition, these Mg-lim cells were more resistant to polymyxin than C-lim cells, particularly at a high dilution rate (Tables 23 & 26). This is consistent with the results of batch culture (Fig. 37, Table 14).

Gilbert & Brown (1978b) found that the fast-growing cultures of C- and Mg-lim Ps. aeruginosa were more sensitive to chlorinated phenols than the slower growing ones, and Mg-lim cells were generally more resistant than the C-lim ones. They also found that KDO content varied continuously with the growth rate of the cultures, slower-growing cells of Ps. aeruginosa containing more than the fast growing ones. C-lim cultures had a lower level of KDO than Mg-lim ones at all growth rates. The variation in KDO correlated significantly with the sensitivity of Ps. aeruginosa to chlorinated phenols. The less KDO contained by cells, the more

sensitive they were. The variation in sensitivity to these agents reflected exclusion from their site of action at the cytoplasmic membrane, by the outer membrane. Gilbert & Brown (1978b) suggested that an increased KDO content might result in a decreased drug uptake, and the LPS might form a barrier in the outer membrane against penetration of the phenols into the cytoplasmic membrane and cytoplasm. This mechanism of resistance to polymyxin was proposed for P. mirabilis (Teuber, 1969; Sud & Feingold, 1970), and is consistent with the results of Brown & Tsang (1978), who related LPS content of Ser. marcescens to resistance to polymyxin. It therefore seems reasonable to suggest such an explanation for the more resistant slow-growing C-lim and Mg-lim P. mirabilis 5887 and RBH. The presence of a higher KDO content in the polymyxin-resistant 5887 compared with the polymyxin-sensitive RBH grown in batch culture (Table 34) is evidence in favour of such explanation. Dean et al. (1976) also found that polymyxin-sensitive C-lim and P-lim Ps. aeruginosa cultures contained less KDO than the polymyxin-resistant Mg-lim ones. Furthermore, Tempest & Ellwood (1969) found that the KDO content of Mg-lim Enterobacter aerogenes decreased with increasing growth rate.

Gilbert & Brown (1978b) found that the PL content of C-lim and Mg-lim cells of Ps. aeruginosa decreased with increasing growth rate. This suggested that polymyxin interaction with PL may also be affected by changing the dilution rate. Although no chemical data from C-lim and Mg-lim cells of P. mirabilis 5887 and RBH growing at different growth rate are available, it seems possible that increased sensitivity of fast-growing P. mirabilis cultures to polymyxin might involve alteration of the outer membrane components such as PL and LPS. Because of the affinity of some

outer membrane proteins for LPS (Nakamura & Mizushima, 1975; Lugtenberg et al., 1976; Schmitges & Henning, 1976; Van Allen et al., 1977) these might also be affected.

4.4 Chemistry of C-depleted and Mg-depleted Batch Cultures of P. mirabilis 5887 and RBH

As described in section 2.2.5, the whole cells were stored at 4° for 36 h, harvested, washed with 0.2% KCl solution and cold water and then stored deep-frozen until used for cell wall preparations. This procedure, which was done to prevent physiological changes of the culture, may have caused a loss of cell wall and surface components. More losses were also likely to have occurred during cell wall preparation. The initial storage of the cells at 4°, while checking cultures for the absence of Sc variants, and polymyxin-sensitivity testing, would be expected to retard cell metabolism, but it may also have affected the cell envelope. Washing with KCl solution was to remove any loosely bound cations from the cell surface (Strange & Shon, 1964). The subsequent washings with cold water may have resulted in the loss of some envelope components concerned with permeability control of the cells. Leder (1972) found that the transport and permeability function of E. coli was affected significantly by the temperature and composition of the washing medium. It has also been reported that Pseudomonad spp lose periplasmic enzymes upon washing in various media (Cheng et al., 1970; Fitzgerald & Laslie, 1975). It seems possible that some outer membrane components were partially lost during the harvesting and washing procedures. Some of the cell walls may also have been lost during the removal of the glass beads by filtration. Considerable losses of components are likely to occur during the incubation of crude cell walls with trypsin, prior to the final washes in KCl and water, due to weakening of the cell surface by

the harvesting procedure (Kenward, 1975). The frequent washing involved in cell wall preparation may also have contributed to the low yield of the cell walls (Table 32).

The assays used for the lipid content of the cells and the cell walls were gravimetric. These were subject to the errors involved in the measurement of small weights (order of 1 - 10 mg). The estimation of the percentage composition of the individual PL in the total phospholipid fraction was densitometric. The method of Minnikin & Abdolrahimzadeh (1971) for the separation of the PL was closely followed. These authors found that the error in a quantitative densitometric assay was ± 5%. In this study, a similar densitometer was used, as also by Kenward (1975) for the separation of PL of Ps. aeruginosa. The washing of the lipid extract with water was to remove inorganic components which may be associated with the lipid. The variation observed between the batch results for REL and PL fractions may well be methodological, probably due to differences between extractions and weighings.

The REL contents of different cells and cell walls of 5887 and RBH were similar (Table 32a & b). Although EIF of β-polyhydroxy butyrate has no correlation with resistance to polymyxin, its presence in C-dep and Mg-dep cells of RBH (Table 32a & b) may be considered as distinguishing features from 5887.

With the exception of the cell walls of C-dep RBH, there was no difference in the PL content of other cells and cell walls. The lower PL content and the presence of low PE/DPG ratio (Table 33c) in the cell walls of C-dep RBH compared with Mg-dep RBH may be correlated with the sensitivity of RBH to polymyxin. Günther et al. (1975) showed that the PL content of E. coli whole cells was significantly altered by Mg-depletion. From Table 33a, b & c, it

can be seen that Mg-depletion caused considerable changes in the composition of PL fractions of Proteus. Higher PE/DPG and PG/DPG ratios were found in the cell walls of Mg-dep 5887 compared with cell walls of C-dep 5887. Whole cells of Mg-dep RBH contained more PG/DPG and PE/DPG compared with C-dep cells, but cell walls contained less PG/DPG and more PE/DPG compared with C-dep cell walls of RBH. A variation in the number of cationic binding sites at the cell surface could account for some changes in resistance. Several workers have correlated the cell lipid content and its various components with the resistance of various bacteria (Brown & Watkins, 1970; Brown & Wood, 1972; Suling & O'Leary, 1977). However, Kenward (1975) found that resistance of Ps. aeruginosa to EDTA and polymyxin observed upon Mg-depletion was not related to the PL content of the cell walls, but rather to the availability of cations which were required for cross-linking and stabilization of the wall. Chapman & Russell (1978) observed that pretreatment of Proteus species with EDTA did not alter their sensitivity to colistin, suggesting that outer membrane cations did not play a major role in resistance. Similarly, in this study, it seems unlikely that cations have a role in the resistance of 5887 and RBH. From Table 35, it can be seen that the cation content of the cells and cell walls of C-dep and Mg-dep 5887 and RBH are very similar. The Ca^{2+} content of the cell walls of C-dep and Mg-dep 5887 and RBH was almost the same. The only significant difference is in the cell walls of Mg-dep 5887, which contained less Mg^{2+} compared with C-dep cell walls. However, the resistance of Mg-dep 5887 to polymyxin is similar to that of C-dep 5887 (Table 14).

The polymyxins bind and disrupt the structure of PL and LPS aggregates (Storm et al., 1977). Interactions between polymyxin B and LPS may be either through hydrophobic interactions between 6-methyloctanoic acid and the lipid A moiety or through electrostatic interactions between the hydrophilic part of the polymyxin and the negatively-charged phosphate groups of the LPS core (Bader & Teuber, 1973). The binding and complex formation of polymyxin B with LPS was shown in the water-soluble LPS fractions isolated from the outer membrane of polymyxin-resistant strain of Ser. marcescens (Brown & Tsang, 1978). The assay for KDO gives an indication of the amount of core polysaccharide present in the LPS. The higher KDO content of C-dep 5887 than of C-dep RBH (Table 34) may correlate with the resistance of 5887 to polymyxin.

The O-antigen specific polysaccharide chain has also been shown to act as a barrier against the binding and penetration of polymyxin and other antibiotics (McQuillen, 1956; Sanderson et al., 1974). Teuber (1974) has correlated the length of the O-antigen chain of S. typhimurium species with sensitivity and binding to polymyxin. It is possible that the resistance of C-dep and Mg-dep 5887 and Mg-dep RBH to polymyxin might in part be due to an increase in the O-antigen side chain length, or some other change in the LPS. Such changes would not be reflected in the KDO assay. Mg-depletion did not cause any change in the KDO content of 5887 and RBH (Table 34).

4.5 Small Colony Variants of *Proteus*

Colonial variation is a well-known phenomenon, and there have been recent reports linking increases in resistance to benzalkonium chloride (Brown, 1977) and to gentamicin, colistin and polymyxin B (Annear, 1976) in small colony variants of *Ps. aeruginosa*. Dwarf-colony variants of *Staph. aureus* resistant to aminoglycoside antibiotics have also been described by Lacey (1968). Small colony variants of *Proteus* have been recognized for a long time (Felix, 1923). Belyavin (1951) suggested that these variants may have been the phase B variants described by him for *P. vulgaris*. The small colony (Sc) variants of *P. mirabilis* and *P. morgani* described in this study and of other *Proteus* species (Klemperer, personal communication) seem to resemble phase B organisms in both cellular and colonial morphology. However, the phase B organisms, in contrast to the Sc variants described here, were not stable and reverted to the typical phase A colonies. It was also noted that it was difficult to distinguish phase B colonies from the typical phase A ones on media containing a low concentration of NaCl. In this study, it was shown that low concentrations of both KCl and NaCl favoured the growth of Sc variants (Figs. 44 & 45). The reduction in number of these Sc variants by high concentrations of KCl or NaCl may be an effect of osmolarity rather than a specific effect; in agreement with this, the growth of Sc variants was also inhibited by a high concentration of sucrose (Klemperer, personal communication). Furthermore, the K^+ requirement of the Sc variants is about half that of Lc (Fig. 46) which may indicate a more efficient method of concentrating K^+ from the medium and thus the possibility that high KCl concentrations are also toxic to Sc variants.

The effect of NaCl is particularly important as its concentration is often adjusted in media for isolation of Proteus species, to prevent swarming. Naylor (1960) advocated media with no added NaCl. In such conditions, many Sc variants would grow and be indistinguishable from Lc. However, Kopper (1962) advocated 4% NaCl which would probably permit very few Sc variants to grow.

Serological analysis indicated that phase B cells were deficient in O-antigen side chain and had some of the characters of "rough" strains (Belyavin, 1951). There is now much evidence that alterations of the LPS of the outer membrane may affect sensitivity to drugs (Sanderson et al., 1974; Nikaido, 1976; Roantree et al., 1977). The different pattern of sensitivity of Sc variants to antibiotics and disinfectants compared with Lc (Tables 30 & 31) indicate major changes in the cell wall. Preliminary results with Sc variants of 5887 suggest a decrease in LPS (measured by a decrease in KDO of a broth-grown culture) and an increase in surface lipid (as indicated by an increase in the rate of migration following treatment with sodium dodecyl sulphate, using microelectrophoresis (Klemperer, personal communication). It is also of interest that the increase in resistance to aminoglycoside and β-lactam antibiotics, and in the case of RBH to polymyxin, is associated with an increase in sensitivity to tetracycline. A similar result was obtained with Ps. aeruginosa mutants selected for resistance to polymyxin by serial subculture (Brown, et al., 1972). The sensitivity of Sc variants to tetracycline may reflect an alteration in the cytoplasmic membrane where the transport process of tetracycline into the cell is located. Alterations in the cytoplasmic membrane are also indicated by their positive oxidase test.

In vivo, the small colony variants have been isolated from humans during *Salmonella* (Kauffman, 1941) and urinary tract infections (*Pseudomonas*: Voureka, 1951; *E. coli*: Borderon *et al.*, 1977); Borderon & Horodniceanu, 1978) as well as from Staphylococcal diseases (Wise & Spink, 1954; Goudie & Goudie, 1955; Quie, 1969; Borderon & Horodniceanu, 1976). It has been found that Sc variants of *Proteus* can grow on MacConkey agar media (Klemperer, personal communication) which indicates their ability to survive in the gut. In this case, their resistance to most antibiotics might cause clinical problems. However, the survival of these Sc variants in the urinary tract may be affected by the osmolality of urine (Anderson, 1976).

P. vulgaris is one of the test-organisms recommended in the Kelsey-Sykes test for disinfectants (Kelsey & Maurer, 1974). In this test the chemically-defined liquid medium described by Wright & Mundy (1960) is used. It contains 18 amino acids, glucose and other inorganic ingredients. According to the results reported in this study (Figs. 44 & 45, Table 29), the NaCl and KCl content of Wright & Mundy medium (NaCl *ca* 0.05 M, KCl *ca* 0.003 M) would not be sufficient to inhibit the growth of the Sc variants. Thus, the appearance of Sc variants in this medium may cause misinterpretation of the results. Tests in the presence of high concentrations of salt might be inconvenient. However, tests could be devised which would take advantage of the inability of Sc variants to ferment sugars.

It is probable that Sc variants of *Proteus* are metabolically more efficient than Lc, as they grow more readily in simple salts media in which growth factors required for Lc may be missing.

4.6 Conclusion

It has been shown that P. mirabilis 5887 and RBH are almost identical; the only distinguishing feature found was their different susceptibilities to polymyxin (Table 14). The nutritional requirements and the biochemical behaviour of both strains were similar (sec. 3.1, Table 28). However, chemical analysis revealed significant chemical differences between the two strains. The LPS content (measured as KDO) of C-dep cells of RBH was half that of C-dep 5887 (Table 34). The PL content of the cell wall of C-dep RBH was much less than that of C-dep 5887 cell wall (Table 32). The content of major individual PLs in whole cells of C-dep 5887 and RBH were almost the same, although C-dep RBH cells contained PC which was absent in C-dep 5887 cells (Table 33). The PE and DPG content were significantly higher in the cell wall of C-dep 5887 than C-dep RBH cell wall. All these differences in the LPS and PL between 5887 and RBH may have contributed to their different sensitivities to polymyxin (see sec. 4.2.5). Magnesium-depletion of both 5887 and RBH did not affect their LPS content (Table 34), but the content and composition of PL of Mg-dep 5887 and RBH cell walls were significantly different from their respective C-dep cell walls (Tables 32 & 33). These changes in the PL composition may have affected the initial binding and the subsequent penetration of polymyxin and other membrane-active agents through the outer membrane to their site of action at the cytoplasmic membrane, which resulted in different patterns of resistance for the Mg-dep cells compared to their respective C-dep cells.

P. mirabilis 5887 and RBH grown in batch or continuous culture showed marked changes in sensitivity to the antibacterial

agents used in this study. No consistent pattern of resistance was observed for the two strains; specific nutrient deficiency caused different sensitivity patterns. Table 36 summarizes the effect of nutrient depletion on the response of 5887 and RBH to the disinfectants used here (see Table 14 for polymyxin effect). In continuous culture, there was no significant difference in sensitivity to cetrimide between C-lim and Mg-lim cells at high and low growth rate. However, an increase in growth rate resulted in an increase in sensitivity to polymyxin; Mg-lim RBH were more resistant than C-lim, particularly at high growth rate.

Table 36

Disinfectant	5887		RBH	
	Mg-lim	P-lim	Mg-lim	P-lim
Cetrimide	+	+	-	+
Phenol	+	+	+	+
Chlorhexidine	=	+	=	-

Cells compared with C-dep.

+, Increased resistance; =, No change; -, Decreased resistance.

During the course of this study, Sc variants of P. mirabilis 5887 and RBH and of other Proteus species were detected during growth in CDM containing low concentration of salts (sec. 3.5). These variants had a different pattern of resistance to different antibiotics and other antibacterial agents from the typical Lc organisms (Tables 30 & 31).

In conclusion, the results presented here indicate not only the importance of the growth media, but also of the growth rate,

in affecting the cell envelope, which alters resistance in different ways for different drugs. These factors, therefore, have an important role in the standardisation of reproducible inocula for use in sensitivity testing and antibiotic assay.

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