EFFECT OF PHOSPHATE LIMITED GROWTH ON DRUG RESISTANCE OF *PSEUDOMONAS AERUGINOSA*

A thesis submitted by

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TO MY WIFE

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The nutritional requirements of *P. aeruginosa* NCTC 6750 were defined using a chemically defined medium (C.D.M.) buffered with 3-N-morpholinopropane sulphonic acid (MOPS). Utilisation of MOPS as a source of sulphur by strain 6750 necessitated medium reformulation and the use of acid ammonium phosphate buffer when sulphate requirements were investigated.

The C.D.M. was used to study the response to Polymyxin B (PB) and EDTA of batch and chemostat cultured cells grown in media containing graded amounts of phosphate.

The response of batch grown cells to PB was dependent upon the medium phosphate content and the drug concentration used. Cells treated with 10 i.u. ml^{-1} PB showed increasing sensitivity as the medium phosphate concentration increased to a certain level above which the cells were apparently more resistant to the drug. Evidence suggested this resistance was in part due to cell envelope components released into the medium interacting with PB. Phosphate limitation of growth determined resistance.

Phosphate or carbon limited chemostat grown cells showed increasing sensitivity to PB and EDTA as the dilution rate was increased.

Phosphate limited chemostat cells, at D = 0.17 hr^{-1}, were more sensitive to PB as the growth medium phosphate was increased, except when viable count was studied, when increasing resistance was seen at higher phosphate levels.

Batch and chemostat (D = 0.17 hr^{-1}) grown cells showed increasing sensitivity to 785μg ml^{-1} EDTA as the growth medium phosphate content increased over the range tested.

Inner and outer membrane preparations from batch and chemostat cells grown in various levels of phosphate were chemically analysed. Quantitative changes in outer membrane lipopolysaccharide, divalent cations and lipids and inner membrane lipids were detected. Phosphate depletion resulted in replacement of phospholipids by fatty acids and neutral lipids. Qualitative changes in outer membrane proteins were also noted. Some of these changes were possibly responsible for the altered sensitivity to PB and EDTA.

Key words: *Pseudomonas aeruginosa*, chemically defined media, phosphate limitation, drug resistance.

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ABBREVIATIONS

^{o}C	degrees centigrade
C.D.M.	chemically defined medium
c.f.u	colony forming units
C-lim	carbon (glucose) limited medium
cm	centimetre
D	dilution rate
D.P.G.	diphosphatidylglycerol
E_{470}	optical density at 470 nanometers
EDTA	Ethylenediaminetetra-acetic acid
F.A.N.	fatty acid and neutral lipid
g	gramme
mg	milligramme
µg	microgramme
Xg	relative centifrugal force
hr(s)	hours(s)
i.m.	inner membrane
i.u.	international units
XK	X,000 daltons
K.D.O.	2-keto-3 deoxyoctonic acid
L	litre
ml	millilitre
Ln	logarithm to the base e
log	logarithm to the base 10
L.P.S.	lipopolysaccharide
M	molar concentration
mM	millimolar concentration
µM	micromolar concentration
Mg^{2+} lim	magnesium limited medium

min	minute
MOPS	3-N-morpholinopropane sulphonic acid
n.m.	namometre
o.m.	outer membrane
P	phosphorus
P.B.	Polymyxin B
P.E.	phosphatidylethanolamine
P.G.	phosphatidylglycerol
P.I.	phosphatidylinositol
P.L.	phospholipid
PO_4^{3-}	phosphate
P.S.	phosphatidyl serine
R.E.L.	readily extractable lipid
S.D.S.	sodium dodecylsulphate
S.D.S. P.A.G.E.	sodium dodecylsulphate polyacrylamide gel electrophoresis
TLC	thin layer chromatography
Tris	tris (hydroxymethyl) aminomethane buffer
v/v	volume for volume
w/v	weight for volume

ORIGIN AND SCOPE OF THE WORK

Growth of *P. aeruginosa* in Mg^{2+} depleted chemically defined medium resulted in increasing resistance to Polymyxin B and Ethylene diamine tetra acetic acid (EDTA) (Brown and Melling, 1969a,b). Kenward (1975) extended this work and investigated the cell wall chemical changes associated with resistance of Mg^{2+} depleted *P. aeruginosa*.

The effect of phosphate depletion in batch culture upon the sensitivity of *P. aeruginosa* to Polymyxin B and EDTA was studied by Boggis (1971). He showed that batch grown cells became more resistant to these antimicrobials as the phosphate content of the growth medium was reduced. Phosphate depletion has been shown to produce profound changes in the cell wall chemical composition of both Gram-positives (Ellwood and Tempest, 1972) and Gram-negatives (Minnikin and Abdolrahimzadeh, 1974) and such changes were correlated with the increased resistance of *P. fluorescens* to Polymyxin B (Dorrer and Teuber, 1977).

It was decided in this study to define a simple salts medium which could be used to quantify the nutritional requirements of *P. aeruginosa* and use this medium to study the effect on sensitivity to Polymyxin B and EDTA of changes in the medium phosphate content of both batch and chemostat cultures.

During this study methods became available which permitted the isolation and purification of inner and outer membranes of *P. aeruginosa* (Hancock and Nikaido, 1978 and Hancock and Carey, 1979). Slight modifications of these techniques were used to isolate and purify these membranes from batch and chemostat grown cells and to study the chemical changes associated with growth in phosphate depleted media. It was expected to correlate chemical changes with alteration in the sensitivity of the cells to Polymyxin B and EDTA.

1. INTRODUCTION

1.1. The Gram-negative Envelope

1.1.1. Introduction

The Gram-negative cell envelope has been thoroughly investigated recently and many reviews comprehensively cover the subject. The envelope has been reviewed by Costerton et al (1974), Costerton (1977), Nikaido and Nakae (1979). Specialised reviews on envelope chemistry include those by Martin (1966), Braun et al (1974) and Rogers et al (1980). Recent structural reviews include those by Costerton et al (1974), Nikaido and Nakae (1979) and Wright and Tipper (1979). Biogenesis of envelope proteins have been reported by Di Rienzo et al (1978) and Osborn and Wu (1980) and genetics by Stocker and Mäkelä (1978) and Hall and Silhavy (1981).

The envelope structure shown in Fig. 1 was described by Costerton and Cheng (1975) and serves as a good working model. The envelope consists of the inner membrane (I.M.), the peptidoglycan layer, the periplasmic space, the outer membrane (O.M.) and in addition in some organisms especially *P. aeruginosa* an outer slime layer.

Fig. 2 shows in more detail the architecture of the Gram-negative cell wall especially that of the outer membrane.

1.1.2. Inner (cytoplasmic) Membrane

The inner membrane (I.M.) is an osmotic barrier which contains the protoplasmic contents and has the appearance of the typical double 70^{o}A track membrane. Plasmolysis shows 200-400 adhesion areas where the I.M. is attached to the outer membrane (Bayer, 1968).

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

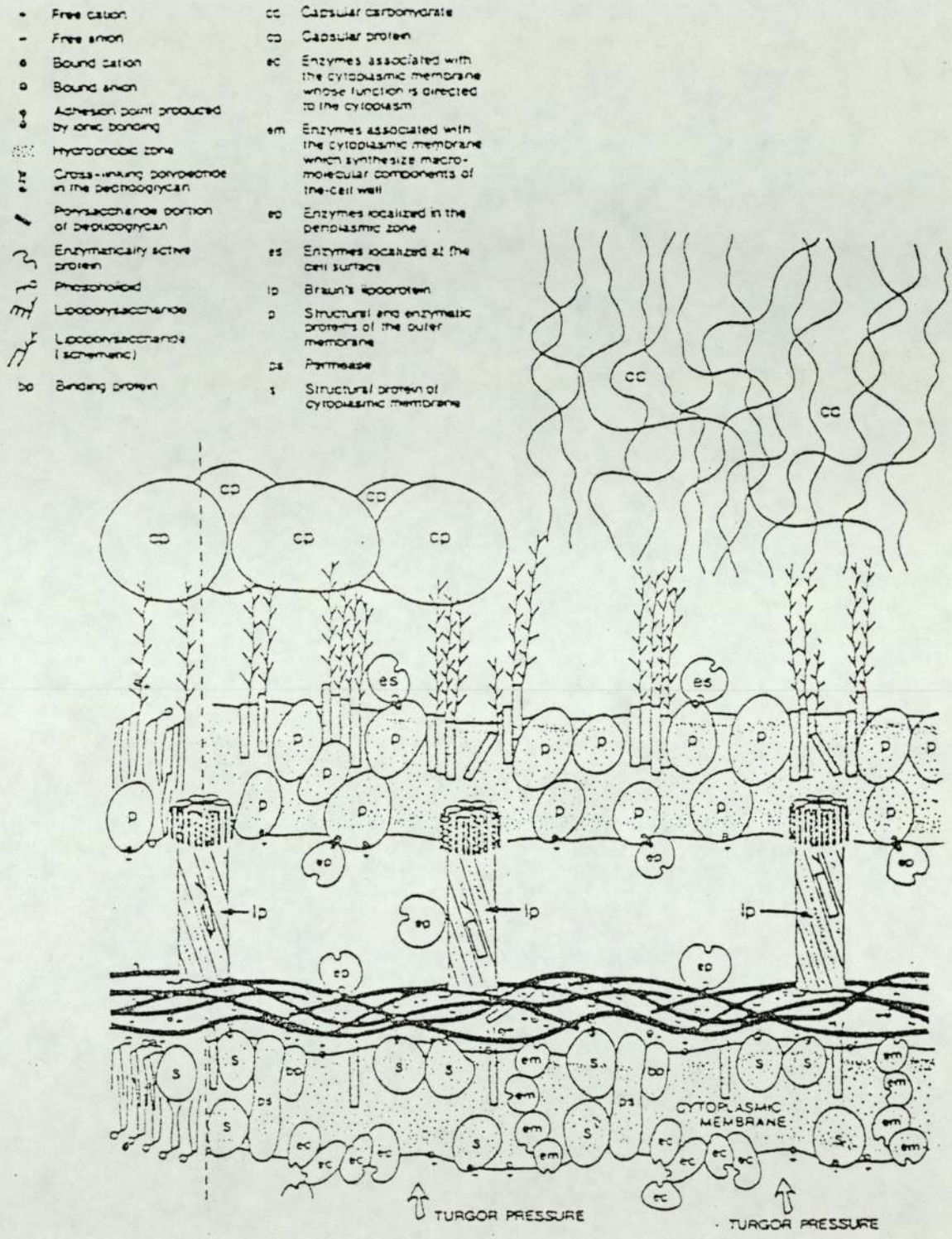
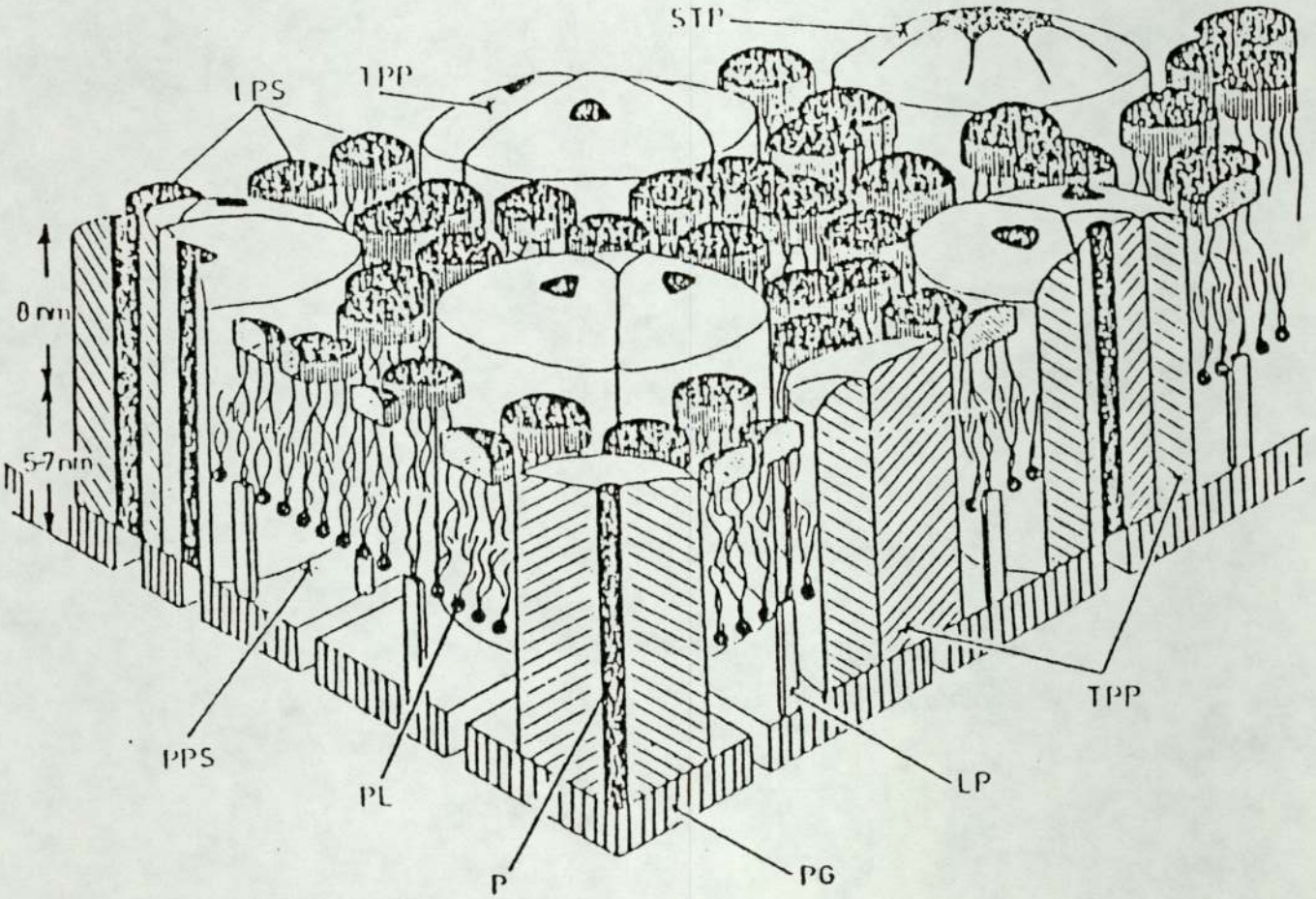


Fig. 2 Tentative structure of the Gram-negative cell wall

(Brown, Gilbert and Klemperer, 1979, adapted from Nikaido and Nakae, 1979)



LPS, Lipopolysaccharide; TPP, trimer of porin protein; STP, specific transport protein; PPS, periplasmic space; PL, phospholipid; P, hydrophilic pore; PG, peptidoglycan; LP, Braun's lipoprotein. The oligosaccharide chains extending from the LPS into the external environment are omitted for visual clarity.

Freeze fracture (a method free from shrinkage artefacts) shows the main I.M. fracture plane is through the hydrophobic interior of the membrane (Van Gool and Nanninga, 1971). 5-10nm diameter particles are seen on the inner fracture face and represent the inner surfaces of proteins (Salton and Owen, 1976) embedded in the half membrane or those pulled through the half membrane as the two halves separate. Depressions are seen on the exterior leaflet and probably correspond to the inner leaflet particles, although there may be less particles than pits.

1.1.2.1. Composition of the Inner Membrane

Separation of the inner and outer membranes of S. typhimurium by Osborn et al (1972a,b) showed the I.M. composition to be two thirds protein and one third phospholipid (P.L.) with no lipopolysaccharide (L.P.S.). The protein content of the I.M. is lower than the O.M. (Mizuno and Kageyama, 1978). The P.L. content of the I.M. differs quantitatively but not qualitatively from the O.M., the I.M. showing, under the conditions used, higher proportions of phosphatidyl glycerol (P.G.) and diphosphatidyl glycerol (D.P.G.) and lower concentration of phosphatidyl ethanolamine (P.E.) (Lugtenberg and Peters, 1976).

The I.M. shows considerable enzymatic activity compared to the O.M. ATPase and glycerol-3-phosphatase, succinate and D lactate dehydrogenases are located in the inner leaf of the E. coli I.M. (Salton, 1967). Cytochromes, and cytochrome oxidase are also found in this membrane (Salton, 1978).

The unit membrane structure of the I.M. has been described by Robertson (1964) who envisaged a 3 layer structure the middle layer being a lipid bilayer with hydrocarbon chains in end to end contact and with hydrophilic ends pointing outwards and in contact with protein molecules

on the outside. It is now realised that the outer surface is not entirely covered by protein and that globular proteins sometimes penetrate into and through the lipid bilayer, thus making the membrane asymmetric with respect to lipids and protein. The fluid mosaic model proposed by Singer and Nicolson (1972) accounts for most of the properties of the I.M. (Fig. 3).

The degree of association of proteins with the lipid matrix differs and two groups, the peripheral and integral proteins are now recognised. Peripheral proteins easily dissociate from the membrane by mild treatment (e.g. change of pH, ionic strength) and are relatively soluble in aqueous media and do not require lipids for activity. The integral proteins require harsher treatment e.g. detergent, or organic solvents to remove them and usually remain attached to lipid following extraction and if completely freed of lipid they are insoluble and frequently enzymatically inactive.

The membrane is fluid and components may move freely under certain restrictions. Phospholipids associated with integral proteins are less mobile otherwise lipids in each half of the bilayer move freely and rapidly by lateral or rapid displacement. Although transmembrane movement occurs P.L. asymmetry is maintained (Rothman and Lenard, 1977).

1.1.2.2. Inner Membrane function

The I.M. is actively involved in the synthesis and transport of many of the molecules found outside the membrane such as phospholipids (P.L.), proteins (Pr.) and lipopolysaccharide (L.P.S.).

The enzymes involved in the synthesis of the 'O' polysaccharide and core region of L.P.S. are found in the I.M. (Osborn et al, 1972b) and these components are synthesised in the membrane and translocated

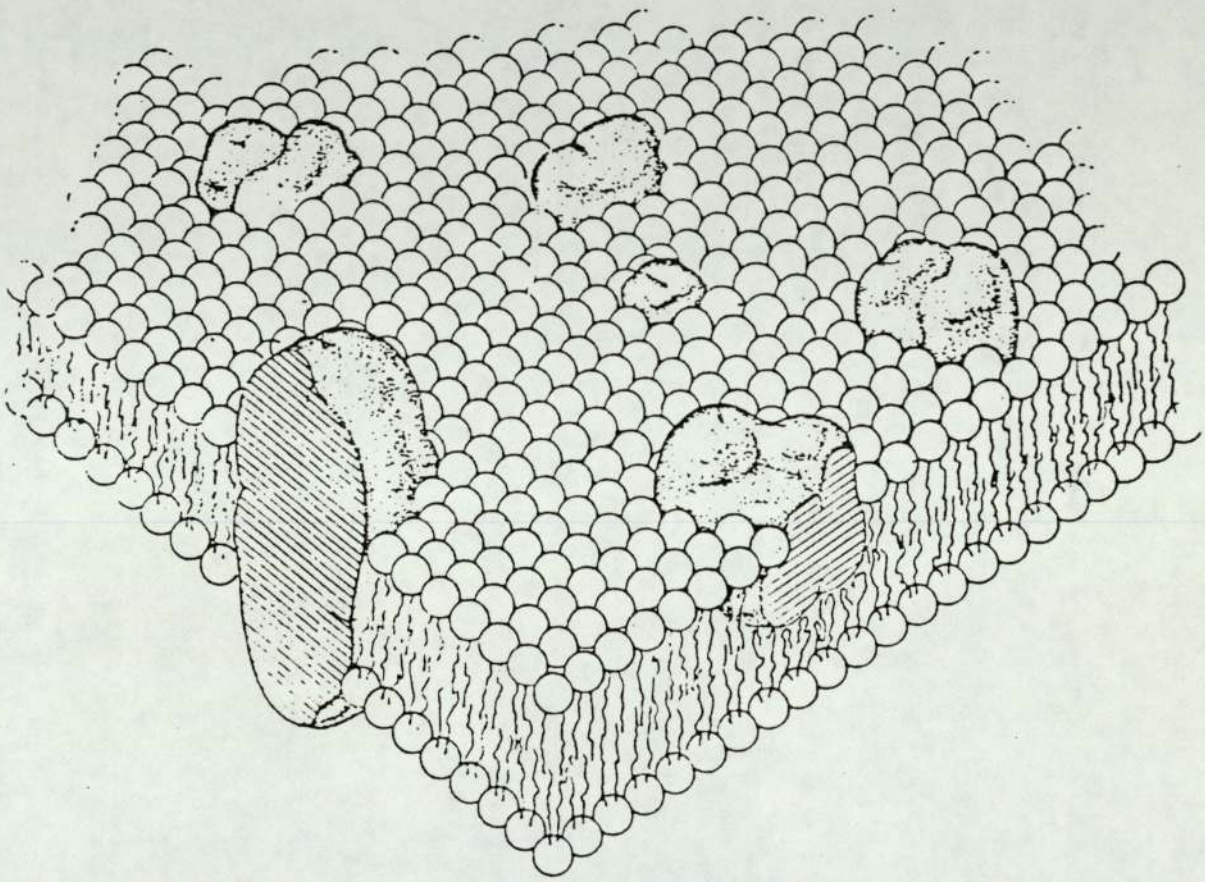


Fig. 3. The lipid-globular protein mosaic model with a lipid matrix (the fluid mosaic model); schematic three-dimensional and cross-sectional views. The solid bodies with stippled surfaces represent the globular integral proteins, which at long range are randomly distributed in the plane of the membrane. At short range, some may form specific aggregates, as shown.

through it to the O.M. Outer membrane and periplasmic protein precursors are synthesised on polysomes attached to the I.M. and translocated (Nikaido and Nakae, 1979). The enzymes of P.L. synthesis are integral proteins of the I.M. and cyclopropane fatty acid synthetase is also found in this membrane as an extrinsic protein (Taylor and Cronan, 1979)

Peptidoglycan synthesis occurs in the cytoplasm and intermediates are converted to lipid soluble derivatives in the I.M. (Braun, 1975). The I.M.'s function in the electron transport chain, oxidative phosphorylation and the transport of metabolites such as sugars, and amino acids have been reviewed by Rogers et al (1980).

There is little evidence that the I.M. is an important barrier to antibiotics (Brown, 1975), many antibiotics using natural permease systems to cross.

1.1.3. Periplasmic Space

This is the electron transparent zone between the inner and outer membrane. Stock et al (1977) suggested this space occupied 20-40% of the total cell volume in normal unplasmolysed *E. coli* and *Salmonella typhimurium*, however Nikaido (1979) found the space was less than 5% of the cell volume except in starved or stationary phase cells where an increase to 13% occurred. The periplasm is bridged by small zones of contact between the inner and outer membranes (Bayer, 1968). Osmotic shock or spheroplast formation results in the release of periplasmic macromolecules which are usually low molecular weight proteins. The periplasmic enzymes have been reviewed by Heppel (1971) and consist of 3 main groups whose functions are (1) to chemically prepare substrates for diffusion throught the I.M., (2) inactivation of antibiotics,

(3) to act as carriers between membrane bound enzymes and substrates e.g. binding proteins. These enzymes include, phosphatases, phosphodiesterases, β lactamases, nucleases, deoxyribonucleoside catabolizing enzymes, cytochrome c_2 and binding proteins for such substrates as sulphate, phosphate, galactose, glutamine, histidine and lysine. The presence of many negatively charged macromolecules in the periplasmic space results in a Donnan equilibrium of approximately -30mv across the outer membrane which facilitates the exit of diffusable anions (Stock et al, 1977).

1.1.4. Peptidoglycan Layer

The structure, biosynthesis and growth of the peptidoglycan layer has been extensively reviewed (Wright and Tipper, 1979; Mirelman, 1979 and Rogers et al, 1980). Electron micrographs of thin sections of Gram-negative organisms reveal a 20-30Å thick peptidoglycan layer sandwiched between the outer and inner membrane and separated from the O.M. by a 3.0nm gap. The peptidoglycan is attached to the O.M. by peptidoglycan associated proteins (Braun, 1975; Lugtenberg et al 1977), and forms a single or bimolecular layer surrounding the cell.

Peptidoglycan is easily isolated from Gram-negative bacteria by heating with 4% sodium dodecyl sulphate (SDS) followed by digestion of attached lipoprotein by trypsin (Braun, 1975). The peptidoglycans of different bacterial species have been classified into different chemotypes by Schleifer and Kandler (1972) and Ghuysen (1968). All Gram-negatives and many Gram-positives are of Type I consisting of chitin-like glycan chains interlinked into a 2D or 3D mesh by crosslinking repeated peptide subunits (Fig. 4). The covalent link between the glycan and peptide involves carboxyl groups of N-acetyl muramic acid. The

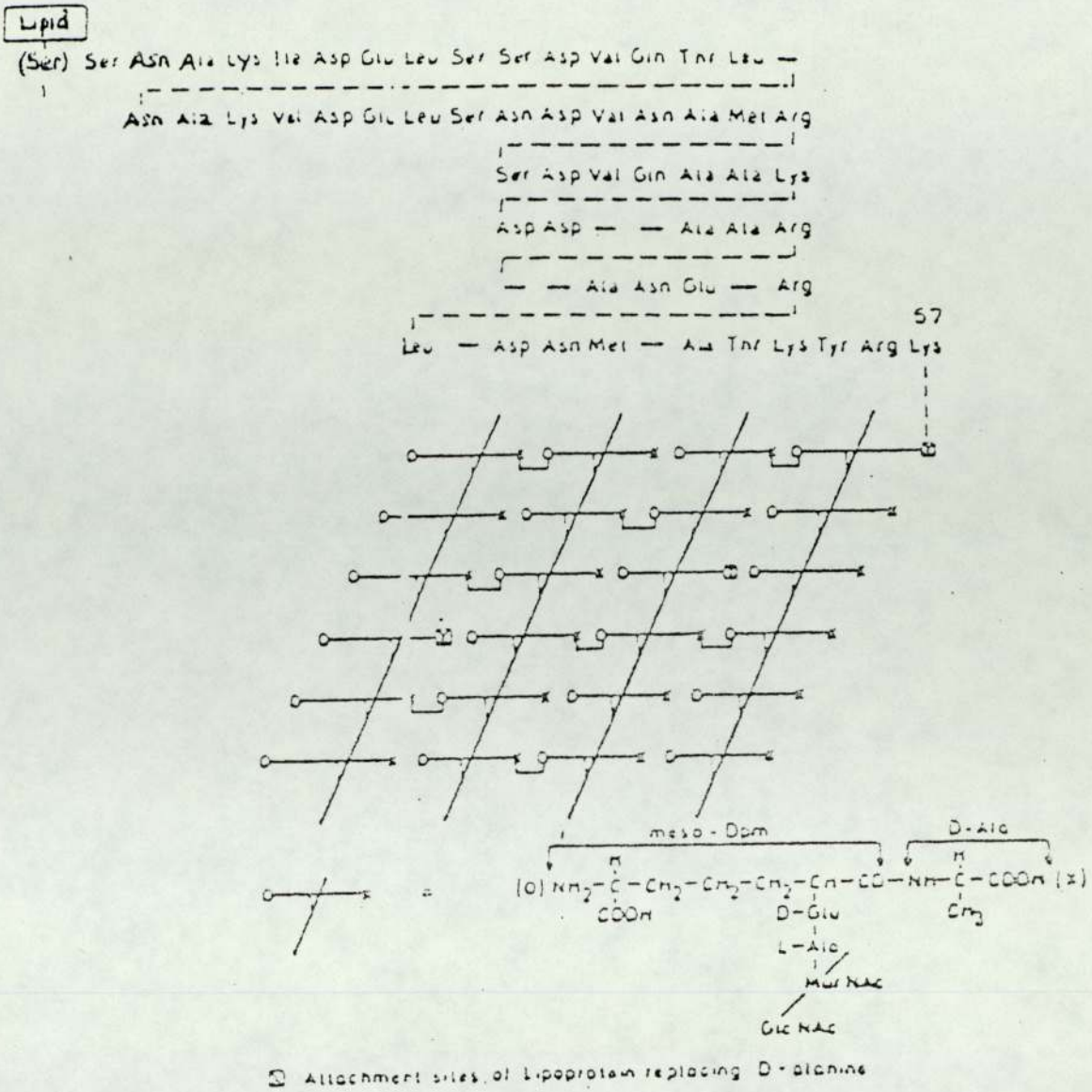


Fig 4. The lipoprotein-murein complex of E. coli (Braun, 1973).

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.

glycans consist of linear strands of alternating residues of N-acetyl glucosamine and N-acetyl muramic acid attached by β(1→4) linkage (Ghuysen, 1968; Tipper and Strominger, 1965). More than 40 species of Gram-negative and Gram-positive organisms surveyed by Wheat and Ghuysen (1971) failed to show hexosamines other than D glucosamine and D glucomuramic acid. Estimation of glycan chain length is complicated by the autolytic action of peptidoglycan degrading enzymes which results in underestimation of the chain length. E. coli chain lengths of 30-60 disaccharide units (Schindler et al, 1976) probably indicate an average chain length of 50-100nm. Substitution of the D-lactyl group of N-acetyl muramic acid by a short peptide unit is seen in most peptidoglycans. The peptides are usually tetrapeptides composed of L-alanine-D-isoglutamic acid-meso-diaminopimelic acid-D-alanine, although tripeptides lacking the terminal D-alanine do occur. The tetrapeptide sequence is unique in being an alternate L-D-L-D sequence. Peptide subunits covalently link glycan chains by "bridges". The amount of cross linking varies with species being higher (90%) in S. aureus than E. coli (25%).

Peptidoglycan has been shown to be associated with O.M. proteins such as Braun's lipoprotein (Braun and Rehn, 1969), and porin proteins in several Gram-negative organisms. These will be considered in the section on O.M. composition.

1.1.4.1. Function of the peptidoglycan

Peptidoglycan has been considered one of the major components concerned with strength and rigidity of the Gram-negative O.M. Conversion of a marine pseudomonad to a mureinoplast resulted in maintenance of its rod shape despite the peptidoglycan layer not being intact (Forsberg et al, 1970) and murein sacculi retained the shape of the cell from which they were derived (Forsberg et al, 1972). It is unlikely that peptido-

glycan is solely responsible for structural rigidity since the extreme halophile Halobacterium salinarium is devoid of muramic acid but grows as a rod (Steensland and Larsen, 1969). Other molecules such as protein and lipoproteins are also important in maintaining cell rigidity (Henning et al, 1973 and Schnaitman, 1971).

The damaging effects on growth of E. coli K12, Pr. mirabilis and P. aeruginosa during growth with ampicillin and lysozyme indicate that murein is also important in barrier function (Burman et al, 1972). Peptidoglycan barrier function was also shown in studies upon the effect of tetracycline and streptomycin resistance in P. aeruginosa.

1.1.5. The Outer Membrane

Much of the detailed structure and composition of the Gram-negative cell envelope has recently been made possible by the discovery of methods of separating in a relatively pure form the individual envelope components e.g. O.M., peptidoglycan and I.M. Some of these methods have been summarised by Rogers et al (1980).

The separation of the outer and inner membranes has been made possibly because the O.M. has a higher density than the I.M. (Miura and Mizushima, 1968) possibly due to its L.P.S. content. Miura and Mizushima (1968) were the first workers to separate the inner and outer membranes of E. coli by lysis of spheroplasts, produced by E.D.T.A. and lysozyme treatment, in the presence of $MgCl_2$ and subsequent dialysis against E.D.T.A. pH 7.0. The resulting membranes were separated by sucrose density gradient centrifugation for 4 hours at 140,000g.

Alternative methods of cell breakage including the French press or E.D.T.A. lysozyme lysis (Osborn et al, 1972), to give membrane fragments

which may then be purified by sucrose density centrifugation, have been developed. Solubilisation of the I.M. by Sarkosyl (Filip et al, 1973) to leave the outer membrane has also been described.

Using these methods relatively pure membranes may be obtained (e.g. 2% I.M. contamination of the O.M. and 10% O.M. contamination of the I.M.). Purity of the fractions may be assessed by estimation of enzymatic activity of the fractions. The I.M. has a large variety of enzymes including succinic and lactic dehydrogenase, ATPase, NADH dehydrogenase, whereas in the outer membrane only phospholipase activity has been found (Albright et al, 1973).

The study of isolated O.M. has shown the main components to be L.P.S., P.L. and proteins.

1.1.5.1. Lipopolysaccharide

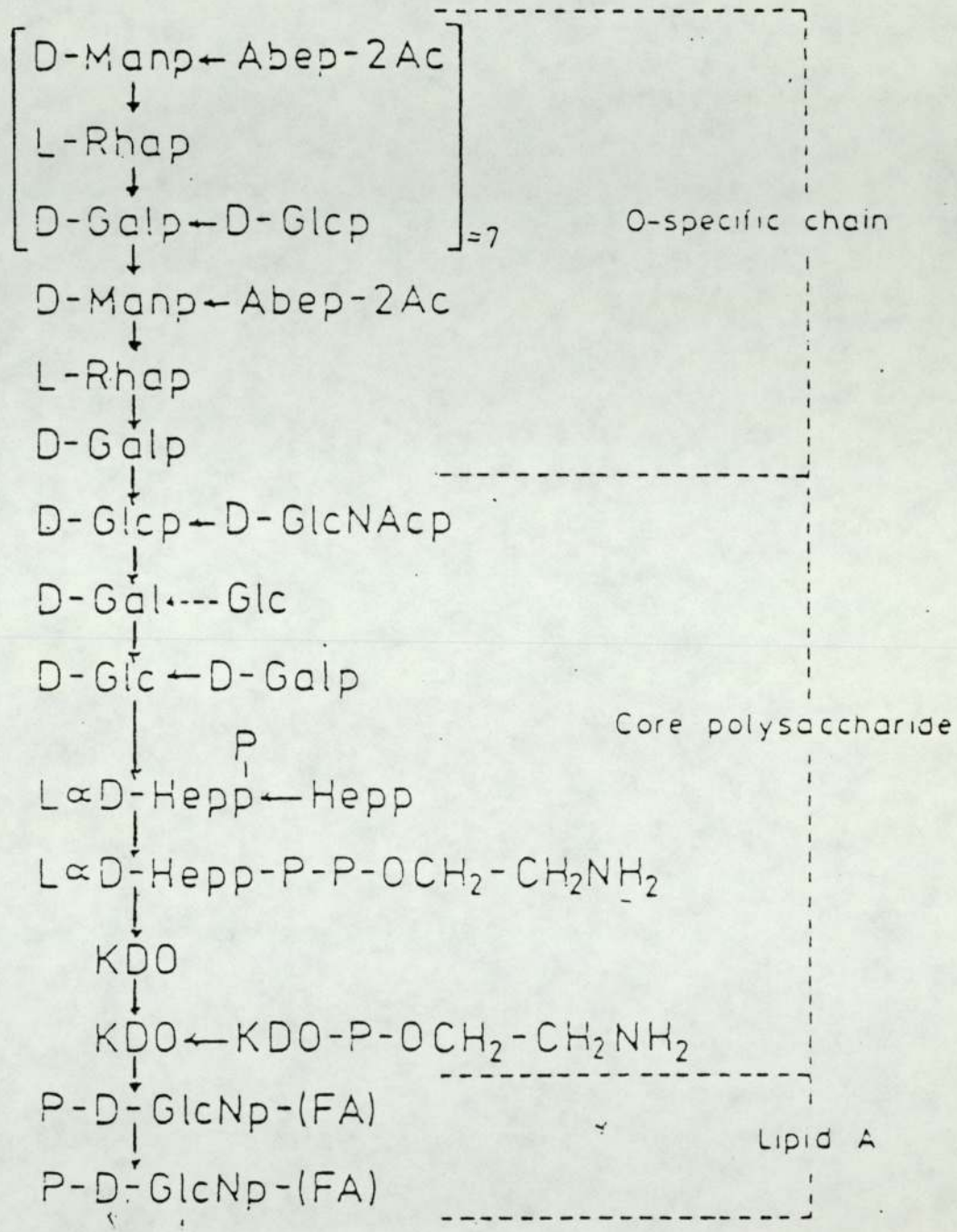
L.P.S. is a major component of the Gram-negative O.M. and is found only in the O.M. (Nikaido and Nakae, 1979). Its structure, biosynthesis and function have recently been reviewed (Wilkinson, 1977; Osborn, 1979 and Nikaido and Nakae, 1979).

Much work has been done on L.P.S. from E. coli and Salmonella typhimurium and care must be taken in equating the L.P.S. of other organisms to these two genera, although evidence would suggest that the general architecture is the same.

The classical model for bacterial L.P.S. is that of the wild type Salmonella typhimurium (Fig. 5). The 'S' form L.P.S. has 3 covalently linked segments viz. side chain, core and lipid A. The side chain is responsible for the 'O' antigenic specificity and is absent in rough strains. The core is divisible into an outer region attached to the side

Fig. 5 Structure of S. typhimurium lipopolysaccharide

(LÜderitz 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

chain and an inner region attached to Lipid A. The side chains from different smooth strains show considerable heterogenicity in number, composition and structure whilst the core and lipid A are essentially identical in all 'S'forms of Salmonella. The lipid A structure has been shown to be similar in a wide range of organisms including Pseudomonas spp (Drewry et al, 1973). Common hexoses such as D-glucose, D galactose, D mannose, 6 deoxyhexoses (L rhamnose and L fucose) and hexosamines (D glucosamine and D galactosamine) are widely distributed in bacterial L.P.S. L glycero-D-manno-heptose is the most common aldoheptose and KDO (3-deoxy-D-manno octulosonic acid) is usually present. L.P.S. extracted from P. cepacia contained no detectable K.D.O. (Manniello et al, 1979).

The lipid A basic structure is a backbone of β-1,6 linked disaccharides of glucosamine. The backbone is also ester linked with phosphate and ester and amide linked with fatty acids. The lipids contain D glucosamine, fatty acids and ethanolamine in varying proportions. The fatty acids vary with bacterial species, the major ones being 2OH 12:0 (2 hydroxydodecanoic acid) 3OH 12:0 (3 hydroxydodecanoic acid), 12:0 (Lauric acid), 16:0 (Palmitic acid) and 3OH 10:0 (3 hydroxydecanoic acid) (Hancock et al, 1970; Wilkinson and Galbraith, 1975). The evidence for cross linking of L.P.S. at the lipid A level by pyrophosphate bridges (Osborn, 1969 and Lüderitz et al, 1973) has not been substantiated by recent work (Mühlradt et al, 1977).

The lipid A is linked to the inner core by K.D.O. Glucose, rhamnose, galactosamine and alanine are usually present in the core of P. aeruginosa (Wilkinson and Galbraith, 1975). A partial structure for the core of P. aeruginosa NCTC 1999 has been elucidated (Drewry et al,

1975). The core heptose may contain glycero-D-manno heptose or D glycero-D-manno heptose. The core is particularly rich in phosphorus including ethanolamine ortho, pyro, and triphosphate as well as inorganic ortho and pyrophosphate.

The 'O' specific polysaccharide fraction has a variable composition depending on the strain. In P. aeruginosa rhamnose is the only major neutral sugar (Wilkinson and Galbraith, 1975). The side chains are particularly rich in amino compounds including glucosamine, and galactosamine (Wilkinson and Galbraith, 1975).

1.1.5.2. Lipids

The lipids of Gram-negatives have been well documented however, there is little information on the lipid composition of O.M.

Phospholipids are a major component of the O.M. of Gram-negatives, and these lipids represent >90% of the fatty acid containing structures in the enterobacteria (Cronan, 1979). The weight ratio of protein-L.P.S.-P.L. in the O.M. of wild type Salmonella typhimurium is 1:1:0.3 (Osborn et al, 1972) and the P.L:protein ratio is 1.5-2 times higher in the O.M. than in the I.M. (Mizuno and Kayeyama, 1978).

The major P.L.'s are P.E., P.G. and D.P.G. which have similar fatty acid compositions consisting of palmitic, myristic and palmitoleic acids. The O.M. of E. coli K12 is richer in saturated fatty acids, with palmitic 16:0 as the major acid, and P.E. than the I.M. The P.E. of the O.M. contained more saturated fatty acids than the P.E. of the I.M. (Lugtenberg and Peters, 1976). Osborn et al (1972) showed that the outer membrane of S. typhimurium had a larger amount (80% cf 60%) of P.E. than the I.M. The marine pseudomonad P. aeruginosa BAL-31 had an O.M. P.L. composition

of 78.9% P.E., 16.1% P.G. and an I.M. composition of 71.5% P.E. and 23.5% P.G. (Diedrich and Cota Robles, 1974).

Figures for membrane P.L. composition may be inaccurate due to intermembrane lipid transfer during membrane preparation (Tsukagoski and Fox, 1971) or during cocentrifugation (Devor et al, 1976).

The organisation of the P.L. in the O.M. is still unclear. The P.L. content of wild type S. typhimurium is insufficient to cover one side of the lipid bilayer structure, and this led to the proposed model of Smit et al (1975) that the outer leaflet of the O.M. is composed of protein and L.P.S. only. Mutation of the L.P.S. (Rd and Re mutants), or protein leads to a redistribution of the P.L. in the membrane leading to its appearance in the outer leaflet thus reducing membrane asymmetry. Osborn (1969) considered the O.M. to be a typical P.L. bilayer but that the bilayer structure was only present in small patches.

1.1.5.3. Proteins

In recent years the development of SDS polyacrylamide gel electrophoresis has lead to increased knowledge of the types and quantity of O.M. proteins especially those of Salmonella spp. and E. coli. Recent reviews of the Gram-negative O.M. proteins include those by Di Rienzo et al (1979), Osborn and Wu (1980) and Hall and Silhavy (1981), and the role of proteins and other O.M. components has been described by Nikaido and Nakae (1979) and Inouye (1979).

Gel electrophoresis of O.M. fractions reveals a few (4 or 5) prominent major protein bands (Schnaitman, 1970) and 10-20 minor proteins, although the term minor protein may be misleading since under certain conditions some of the minor proteins are produced in amounts equal to major proteins.

The nomenclature of the major proteins is confusing since the major research groups have until recently not standardised on a nomenclature system. A useful summary of these proteins is given by Osborn and Wu (1980) for S. typhimurium and E. coli (Table 1) and for P. aeruginosa in particular by Hancock and Carey (1979) (Table 2). Some of the nomenclature problems have arisen because of variation in the number of bands visualised by gel electrophoresis. These differences probably reflect differences in membrane preparation, the polyacrylamide used, and the temperature of SDS disaggregation employed, rather than major differences in O.M. proteins (Rogers et al, 1980 and Hancock and Carey, 1980). The outer membrane proteins may be considered in several groups.

The lipoproteins have been reviewed by Di Rienzo et al (1979) and Inouye (1979), and in E. coli may be the most abundant O.M. protein. This 7,000 molecular weight peptidoglycan associated protein forms 5-7% of the total cellular protein (Braun and Rehn, 1969). In enterobacteria it consists of 58 amino acid residues but lacks histidine, tryptophan, glycine, proline and phenylalanine. The protein is linked by the E-amino group of the C-terminal lysine to the carboxyl group of every 10 to 12th meso DAP acid residue of peptidoglycan. The N-terminal protein consists of glyceryl cysteine to which 3 fatty acids are attached. The amide linked fatty acid consists of 65% palmitate and the rest being monounsaturated fatty acids. The protein has a α helical structure (Braun, 1975) unlike the β structure of other outer membrane major proteins.

The lipoprotein also exists in a free form and dynamic equilibrium exists between the free and bound forms (Inouye et al, 1972; Hirasime

Table 1 Recommended genetic nomenclature for major outer membrane proteins of E. coli and S. typhimurium

(Osborn and Wu, 1980)

Gene	Map position (units)		Gene function	Protein nomenclature								
				Previous designations E. coli							S. typhimurium	Recommended uniform nomenclature
	E. coli	S. typhimurium		B	F	H	I	L	M	S		
ompA (tolG, con, tut)[a]	21	21	S[b]	B	TolG	II*	7	D	0-11	3a	33K	OmpA
ompB (cry)	74	74	R									
ompC (par, meoA)	47	46	S	A_2		Ib	4	c	0-8	1b	36K	OmpC
ompD	-[c]	28	S(?)								34K	OmpD
ompF (tolF, colB, coa, cry)	21	21	S	A_1	TolF	Ia	4	b	0-9	1a	35K	OmpF
nmpA	82.2	-[d]	?		E	Ic		e		NmpA		NmpA-B[d]
nmpB	8.6	-[d]	?							NmpB		
nmpC	12	-[d]	?							NmpC		NmpC[d]
LamB	90		S								44K	LamB
lpp (mlpA, lpm, lpo)	36.5		S	F		IV	11		0-18			Murein lipoprotein

[a] Previous gene designations are parenthesized

[b] S = structural, R = regulatory, ? = not known.

[c] No gene product corresponding to locus has been identified

[d] Tentative name, pending identification of structural gene locus.

Table 2 Nomenclature of *P. aeruginosa* outer membrane proteins

(From Hancock and Carey, 1979)

Name	Mol wt (10^3)							
	Mizuno and Kageyama	Hancock and Nikaido			Matsushita et al		Booth and Curtiss	Stinnett and Eagon
	8% Urea	11% A[a]	11% B[b]	14%	8% Urea	10%	11%	9%
D1	D (50)		49	46	4 (53)	4 (49)	I (56)	
D2				45.5				
E	E (45)	44	45	44	4 (48)		II (53)	
F (= porin)	F (33)	35	37	39	5 (34)	5 (31)	III (38)	A (43)
G	G (21)	21	22.5	25	6 (23)	6 (20)	IV (21)	
H1	H (21)	17	18.7	21	7 (19)	7 (18)	V (16)	B (16.5)
H2				20.5				
I (= lipoprotein)	I (8)			9-12	9 (9.7)	9 (8.7)		

a Using impure SDS

b Using ultrapure SDS

and Inouye, 1973). The free form exists exclusively in the O.M. In E. coli 7 x 10^5 protein copies exist, one third in the bound form and two thirds in the free form (Inouye et al, 1972).

Similar lipoproteins are present in Salmonella, Serratia (Braun and Wolff, 1970; Halegoua et al, 1974) and P. aeruginosa (Mizuno and Kayeyama, 1979). P. aeruginosa lipoprotein lacks proline, valine, isoleucine phenylalanine, tryptophan and cysteine (Mizuno and Kayeyama, 1978). It contains only 0.89 Mole % fatty acids and lacks ester linked fatty acids. Like that from E. coli the lipoprotein from P. aeruginosa does not produce transmembrane pores (Nakae, 1976b).

Some major O.M. proteins with apparent molecular weights in the range 30-40,000 show an altered molecular weight when solubilised in SDS at a different temperature (heat modifiable proteins). Protein 3a in E. coli (Schnaitman, 1973a,b), the 33K protein of S. typhimurium (Nikaido and Nakae, 1979) and proteins D1, D^2, F, G, and HI (Hancock and Carey, 1979) are 'heat modifiable'. The 40K protein 3b of E. coli K12 is 'heat modifiable' but is only produced when the culture is grown at temperatures above 37°C (Lugtenberg et al, 1977) and its function is unclear.

Protein 3a has an increased β structure when solubilised in SDS at low temperatures (Nakamura and Mizushima, 1976), and up to 25% of the lysine residues are converted to allysine (Diedrich and Schnaitman, 1978). The structural genes for both these proteins map at the same position (20-21 min.) on their respective chromosomes (Foulds, 1974 and Nikaido and Nakae, 1979). Its function is not clear but omp A mutants are deficient in F pilus mediated conjugation (Skurray et al, 1974) and because of its peptidoglycan association it may play a

significant role in cell morphology and O.M. integrity (Sontag et al, 1978).

1.1.5.3.1. Peptidoglycan associated proteins

The porins are 32-37K dalton outer membrane proteins which are linked covalently to peptidoglycan such that these proteins constitute part of the insoluble complex when cell envelopes are solubilised in 2% SDS below 70°C. These matrix proteins may be released from peptidoglycan by heating above 70°C or by extraction at 37°C in SDS containing 0.5M NaCl. (Nikaido and Nakae, 1979). These proteins have the unusual property of forming aqueous filled transmembrane diffusion pores when added to P.L.-L.P.S. mixtures (Nakae, 1976a,b; Hancock et al, 1979; Hancock and Nikaido, 1978), and are termed porins or matrix proteins. The number of porin molecules per cell is usually about 10^5 (Rosenbusch, 1974). The number of porin species per cell may vary. E. coli B produces a single porin (Rosenbusch, 1974) whereas in other E. coli up to 10 major proteins may be formed under differing circumstances. Omp F, Omp C, Lam B and protein 2 are porins and MRB, Nmp A-B and NmpC are thought to be porins (Hall and Silhavy, 1981). S. typhimurium LT2 has 3 porins 34K, 35K and 36K (Tokanaga et al, 1979). The 35K is homologous with the OmpF of E. coli and the 36K with OmpC of E. coli. 39K and 36K proteins of Pr. mirabilis have also been reported as porins (Nixdorff et al, 1977). P. aeruginosa protein F of molecular weight 35-39K, has been purified and characterised as a porin protein (Hancock et al, 1979). A glucose inducible protein D1 also has porin function (Hancock and Carey, 1980).

The relative amounts of E. coli Omp F and Omp C protein produced is influence by the medium used to grow the organisms, in particular the fermentable carbon source used (Bassford et al, 1977).

Infra red spectroscopy of porin proteins reveals considerable β structure in contrast to the common α structure of intrinsic membrane proteins (Rosenbusch, 1974). Electron microscopy of negatively stained protein-peptidoglycan complex reveals porin protein molecules arranged hexagonally in a lattice with 7.7nm repeating units, each unit composed of 3 porin molecules (Yu et al, 1979). The hexagonal lattice covers in excess of 60% of the outer peptidoglycan surface (Steven et al, 1977). Isoelectric focusing of purified porins (Ishii and Nakae, 1980) suggests that the porin trimers are formed from homologous subunits only (Palva and Randall, 1979) rather than both homologous and heterologous subunits (Ichihara and Mizushima, 1979). Evidence for penetration of the thickness of the O.M. is shown by their phage receptor function (Datta et al, 1977).

The functions and properties of the major porins of E. coli and Salmonella spp have been extensively reviewed by Osborn and Wu (1980) and Hall and Silhavy (1981) and only brief mention of their function will be made here. Omp C, Omp D and Omp F porins are effective in conferring permeability to sugars, disaccharides, amino acids, inorganic ions and β lactam antibiotics (Osborn and Wu, 1980). Nmp A-B and Omp F are involved in nucleoside uptake (Hall and Silhavy, 1981). Protein 2 restores amino acid and sugar uptake in porin deficient Omp B mutants (Pugsley and Schnaitmann, 1978) and Lam B mediates uptake of maltodextrins and can act as a non specific pore for glucose and lactose (von Meyenberg and Nikaido, 1977).

Some disagreement amongst workers has occurred about the specificity of transmembrane diffusion channels and this has been reviewed by Nikaido et al (1980). The work of Nakae (1976a,b) using E. coli and S. typhimurium

showed that porin protein when reconstituted into P.L.-L.P.S. bilayers produced channels which allowed the diffusion of sugar, sugarphosphates, nucleotides, amino acids and polyethylene glycols provided that their molecular weights was less than 600. These and other results led to the conclusion that porin channels were largely nonspecific and acted as molecular sieves. However, mutational loss of porin 1a did not decrease the rate of transport of various amino acids and sugars (Lutkenhaus, 1977) but reduced the rate of transport and hydrolysis of nucleotides and Van Alphen et al (1978) suggested that this demonstrated a specificity of this pore for nucleotides. Subsequent study of the flux rate of compounds into lysosomes containing channel forming proteins (Nikaido et al, 1980) has further supported the nonspecific nature of porins although configurational specificity has not been completely ruled out. Much of the flux rate data can be explained on the physicochemical properties of the solute such as size, hydrophobicity and charge.

The λ receptor protein which has been assumed to produce specific channels whose specificity for transport of oligosaccharides of the maltose series is based on configurational properties does show non-specific porin like properties towards small solutes (Nikaido et al, 1980). These authors postulate that penetration of small solutes does not require specific solute binding sites as required for oligosaccharides of the maltose series.

Cell permeability experiments have shown that E. coli and S. typhimurium exclude oligosaccharides (Decad and Nikaido, 1976) and peptides (Payne and Gilvarg, 1968) of approximately 600 daltons. Lysozyme treatment of cells failed to alter this exclusion limit suggesting that the O.M. was involved rather than the peptidoglycan

sieve (Nikaido and Nakae, 1975). Reconstituted vesicles containing protein were permeable to sucrose whereas vesicles lacking protein were impermeable implicating proteins in the exclusion mechanism.

Similar experiments with P. aeruginosa suggest a larger exclusion limit of 6000 ± 3000 daltons (Nikaido and Nakae, 1975; Hancock and Nikaido, 1978) although incorporation of porin protein F into lipid bilayers suggested that a low proportion of the pores were open in vivo (Benz and Hancock, 1981). Other O.M. proteins have been found associated with peptidoglycan in E. coli (Rosenbusch, 1974), Enterobacteriaceae (Lugtenberg et al, 1977) and P. aeruginosa (Mizuno and Kayeyama, 1979). Mizuno and Kayeyama (1979) found O.M. protein F and H associated with peptidoglycan however, Hancock et al (1981a) found that protein F was peptidoglycan associated but that protein H was actually two proteins H1 and H2 and only protein H2 was non covalently attached to peptidoglycan.

Other non-porin proteins have been demonstrated in Gram-negatives In E. coli these include the Omp A, protein 3b and lipoprotein which have been considered previously. In P. aeruginosa several other proteins have been discovered whose ability to produce transmembrane pores has not been detected to date. Protein D1 (M wt 46K) is an inducible protein produced under conditions which result in the induction of the high affinity glucose uptake system (Hancock and Carey, 1980). Protein D2 (M wt 45.5K), E (M wt 44K) and protein G (M wt 25K) are of unknown function. Protein H1 has recently been found in polymyxin and E.D.T.A. resistant strains are to be produced when P. aeruginosa is grown under magnesium limitation. Under these conditions it may become the major protein (Nicas and Hancock, 1980). Proteins D1, D2, E, G and H1 are released as the protein-lipopolysaccharide complex when P. aeruginosa is treated with E.D.T.A./Tris (Hedstrom et al, 1981).

Although the functions of some of the minor proteins are unknown some are important in cell growth, nutrient uptake, phage and colicin reception.

In E. coli vit B_{12} uptake involves a 60K protein which also binds E type colicin and phage BF23 (Di Masi et al, 1973 and Bradbear et al, 1976). Iron uptake systems in E. coli include ton A (78K) protein for Fe^{3+} ferrichrome uptake, cit (80.5K) for Fe^{3+} citrate uptake and feu B (81K) for Fe^{3+} enterochelin uptake (Konisky, 1979, Hancock et al, 1976). Protein tsx (27K) is involved in nucleoside uptake and is a receptor protein for phage T6 and colicin K (Hantke, 1976).

1.1.6. Outer Surface Layers

The outer surface of many Gram-positives and Gram-negatives exhibit arrays of subunits either on the O.M. (Glauert and Thornley, 1969) or on layers external to the O.M. (Watson and Remsen, 1970).

P. aeruginosa may possess a slime layer covering the cell. It is not referred to as a capsule since it does not form a distinct surface layer and may be dispersed in liquid media (Liu, 1979). The chemical composition of slime layers has been reviewed by Brown (1975) and Liu (1979). The composition appears variable and may reflect different extraction procedures and conditions of growth. Hyaluronic acid, mannose, glucose, RNA, DNA and other minor components including protein and glucosamine have been detected in slime (Warren and Grey, 1955; Bonde et al, 1957; Eagon, 1962 and Brown et al, 1966).

Slime of mucoid clinical isolates of P. aeruginosa associated with cystic fibrosis produced polysaccharides similar to marine algae, containing heteropolymers of mannuronic acid and guluronic acid in differing proportions depending on the site of isolation (Evans and Linker, 1973 and Linker and Jones, 1964).

The role of slime in infection is unclear although it is more toxic than L.P.S. to mice (Sensakovic and Bartell, 1974), and is antiphagocytic to rabbit polymorphonucleocytes (Schwarzmann and Boring, 1971). Slime glycolipoprotein has a protective effect in passive and active immunisation (Bartell et al, 1970) but a deleterious effect on host defences (Lynn et al, 1977).

Slime had a greater blocking effect on polymyxin than E.D.T.A. for P. aeruginosa grown in gluconate as a carbon source (Brown and Scott Foster, 1971). Glucose grown cells failed to produce slime.

1.1.7. Outer membrane asymmetry

1.1.7.1. Lipopolysaccharide

Studies by Mühlradt and Golecki (1975) using ferritin-labelled anti-lipopolysaccharide antibody to locate L.P.S. has shown that L.P.S. is present only the outer half of the outer membrane bilayer.

1.1.7.2. Phospholipid

Analysis of the O.M. composition of deep rough mutants of S. typhimurium indicated that protein loss resulted in a 70% compensatory increase in P.L. (Smit et al, 1975). These strains had P.L. distributed between the two havles of the outer membrane whereas in smooth and rough strains the P.L. is insufficient to cover on side of the membrane. Smit et al (1975) proposed that normally P.L. is present in the inner half of the leaflet only and this has been supported by Kamio and Nikaido (1976) who showed P.L. degradation by phospholipase C only occurred in intact deep rough mutants of S. typhimurium.

1.1.7.3. Protein

CN Br-activated dextran treatment of intact S. typhimurium resulted in 10 out of 13 O.M. proteins becoming dextran linked (Kamio and Nikaido, 1977), indicating exposure on the outer surface of the outer membrane.

Since matrix protein remains attached to peptidoglycan after treatment at 60°C with SDS it is likely that some of this protein penetrates through the bilayer to interact with peptidoglycan. Although there is evidence of protein asymmetry in the O.M. the exact arrangement of proteins is still uncertain.

Figure 6 shows the model proposed by Smit et al (1975) demonstrating outer membrane asymmetry.

1.1.8. Outer membrane functions

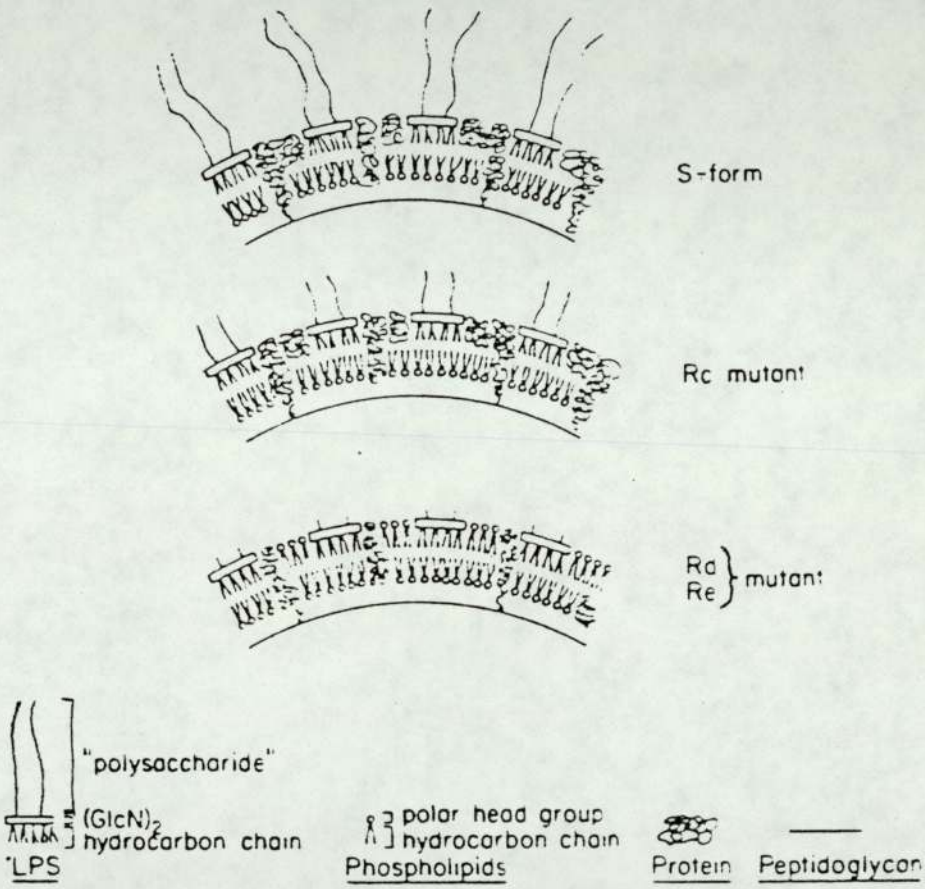
The O.M. of noncapsulate and non slime producing organisms is exposed to the surrounding environment and is therefore important in the cell's interaction with the environment. The O.M.'s function in cell-cell interaction during conjugation has been reviewed by Manning and Achtman (1979) and its role in chemotaxis by Hazelbauer,(1979). The outer membrane is hydrophilic with a contact angle of 21° for S. typhimurium and this is further reduced to 16.5° by increasing the 'O' chain of L.P.S. (Cunningham et al, 1975). Hydrophobicity is important in penetration of molecules into the cell and in resisting phagocytosis (Cunningham et al, 1975). The role of O.M. in exclusion of antibiotics and other molecules has been reviewed by Brown (1975) and Nikaido and Nakae (1979). There are at least two general non specific pathways for diffusion of small molecules across the outer membrane, one for hydrophilic compounds and one for hydrophobic compounds.

1.1.8.1. Hydrophilic compounds

The L.P.S. and lipid bilayer structure of the O.M. constitute a barrier to hydrophilic molecules, however transport mechanisms exist. Some small molecules such as iron are actively transported by specific transport proteins (Wayne et al, 1976). Of a less specific nature is the transport

Fig. 6 Schematic representation of outer membrane structure of S. typhimurium

(Smit et al, 1975)



of small hydrophilic molecules via the porin pores (Nikaido and Nakae, 1979). The specificity and rate of transport being dependent upon molecular size (Nikaido and Nakae, 1979), charge (Stock et al, 1977) and for molecules approaching the pore size limit, their hydrophobicity (Nikaido and Nakae, 1979).

1.1.8.2. Hydrophobic compounds

Transmembrane diffusion of small hydrophobic molecules occurs readily across most membranes (Danielli, 1952). However, penetration of hydrophobic molecules through the O.M. of wild type Gram-negatives such as E. coli and S. typhimurium is hindered partly by the hydrophilic polysaccharides and partly by the close packing of the L.P.S. caused by the lack of P.L. in the outer leaflet of the O.M. (Nikaido and Nakae, 1979). Deep rough mutants have an increased P.L. content in the outer leaflet of the O.M. thus making penetration of hydrophobic molecules into the O.M. easier than in wild type strains. Once access to the outer leaflet of the O.M. has been attained hydrophobic compounds may traverse the membranes by oil/water partitioning in the various aqueous and lipophilic layers and by passive diffusion (Stein, 1967).

1.2. The action of Polymyxin and E.D.T.A. on Bacteria

Much of the work in this study involves the effects of nutrient depletion on the action of Polymyxin (P.B.) and E.D.T.A. The next two sections will review recent findings concerning the action of these drugs.

1.2.1. Polymyxin

1.2.1.1. Introduction

The polymyxins, circulins and octapeptins are closely related agents which have been extensively studied in an attempt not only to understand their mode of action but also to study the structure and function of biological membranes.

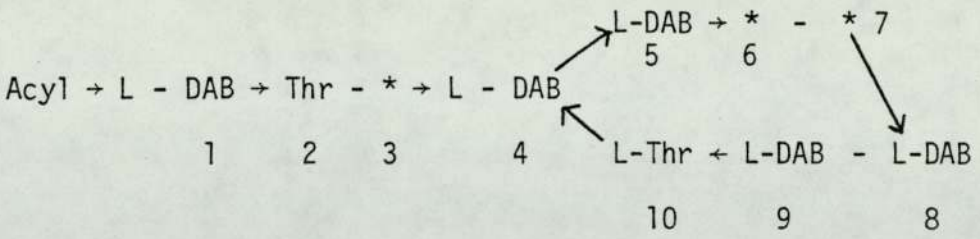
Newton (1956) reviewed the early work on the mode of action of polymyxins and an excellent review by Storm et al (1977), has recently updated the literature on polypeptide antibiotics.

The polymyxins are broad spectrum antibiotics produced by Bacillus polymyxa and have greater activity against Gram-negatives than Gram-positives. Natural resistance occurs in Proteus mirabilis, Serratia marcescens and Providencia spp.

The polymyxins are decapeptide antibiotics of molecular weight approximately 1200 which exhibit membrane activity. The structures of the polymyxins are shown in Fig. 7. The peptides are cyclised through the α amino and carboxyl groups of the 2,4 diamino butyric (Dab) residue at position 4. The molecule is rich in Dab with 4 residues in the peptide ring. Two variable regions exist in the ring at positions 6 and 7. Position 6 is either D-leucine or D phenylalanine and position 7 is L threonine or L leucine. A third variable region is found in the peptide

Figure 7 Structure of Polymyxins

(from Storm et al, 1977)



Polymyxin	Acyl Group	3	6	7
A	MOA	D-DAB	D-Leu	L-Thr
B1	MOA	L-DAB	D-Phe	L-Leu
B2	IOA	L-DAB	D-Phe	L-Leu
D1	MOA	L-Ser	D-Leu	L-Thr
E1	MOA	L-DAB	D-Leu	L-Leu
E2	IOA	L-DAB	D-Leu	L-Leu

DAB	2,4 diaminobutyric acid
MOA	6 methyloctanoic acid
IOA	6 methylheptanoic acid
Leu	Leucine
Thr	Threonine
Phe	Phenylalanine
Ser	Serine

side chain attached to the ring and except in Polymyxin D1 this region contains L or D Dab. The fourth variable region in the fatty acid residue attached via an amide bond to the peptide chain. This residue is either 6 methyl heptanoic acid or 6 methyl octanoic acid. These variable regions affect the biological properties and structural configuration of the molecules (Storm et al, 1977).

Modification of the polymyxin molecule has been used to understand its activity. Alteration of the ring structure reduces or even eliminates its activity (Nakajima, 1967). Polymyxin E derivatives are generally less effective membrane perturbants than Polymyxin B indicating that the major structural requirements for membrane disruption is the cyclic peptide moiety (Mohan et al, 1962). Intermediate fatty acid chain lengths C:8-14 produce the most biologically active molecules and the lower activity of molecules with longer chain lengths may be due to the exclusion by the outer membrane of hydrophobic molecules (Nikaido and Nakae, 1979).

Polymyxins exert a wide range of effects on cell morphology and biochemistry. Washed suspensions of sensitive organisms when treated with Polymyxin E rapidly released 260nm absorbing material, whereas resistant organisms showed no such leakage (Few and Schulman, 1953 and Newton, 1953a). P. aeruginosa, however, showed continued release of equimolecular amounts of pentoses and phosphates (Newton, 1956). These results suggested that death was not a primary event but rather a secondary polymyxin induced event. Early events following treatment of S. typhimurium preloaded with ^{14}C methyl-α-D-glucopyranoside (αMg) included loss of cell viability and inhibition of uracil incorporation with efflux of αMg and inhibition of RNA and DNA synthesis followed later by effects on respiration, LPS, peptidoglycan and protein synthesis (Teuber, 1974).

Release of periplasmic enzymes following treatment with Polymyxin occurred from E. coli, P. fluorescens and S. typhimurium but not from resistant organisms such as Pr. mirabilis and Serr. marcescens (Cerny and Teuber, 1972). Leakage of cellular constituents from P.B. treated E. coli is dependent upon the strain used, growth conditions and phase of cell growth when treated with the drug (Cerny and Teuber, 1971). Stationary phase cells released less than 3.5% of the total cellular proteins whereas log phase cells lost up to 68% protein. Cells grown in rich media released no cytoplasmic membrane enzymes or proteins within 30 minutes and the periplasmic enzymes were released at a slower rate and to a lesser degree than from glycerol grown cells. These results show that polymyxin action results in rapid permeability changes of the cytoplasmic membrane to charged or polar molecules followed later by secondary effects disrupting enzyme syntheses and transport resulting in irreversible death of the cells.

Electron microscopic studies on the gross bacterial changes due to P.B. treatment suggested that concentration of drug may be important. At concentrations <10μg/ml protuberances appear at the cell surface of E. coli B (Wahn et al, 1968), but little intracellular change is demonstrable. At concentrations >10μg/ml Wahn et al (1968) observed protuberances rapidly followed by cell autolysis which was first apparent as a brightening of the nuclear area and then cytoplasmic destruction. Using 100μg of colistin (Kaye and Chapman, 1963) observed 3 stages of change, a disaxialisation and margination of nuclear material with invasion of the nuclear area with cytoplasmic material but no loss of cellular contents (0-30 min.). This was followed by a loss of nuclear material and increasing electron density of cytoplasmic constitutents and agglomeration, but no loss of cytoplasmic constituents (30-120 min.) and finally a loss of cytoplasmic constituents (120-150 min.).

The covalent linkage of P.B. to agarose beads (Laporte et al, 1977) has identified the important role of the O.M. in polymyxin action. Immobilised polymyxin was unable to penetrate the O.M. of E. coli SC 9251, although its growth was inhibited by 1mg/ml (equivalent to <1.5μg/ml polymyxin). Respiration of E. coli and its spheroplasts and B. subtilis protoplasts was inhibited by polymyxin agarose, but respiration of whole cells of B. subtilis was unaffected. These results suggest that polymyxins do not have to enter the bacterial cell to affect growth nor do they move back and forth across the cytoplasmic membrane. Thus confirming that effects on nucleic acid and protein biosynthesis are secondary to membrane damage The differential effect of immobilised polymyxin on B. subtilis and its protoplasts indicates the possible role of the O.M. of Gram-negatives in polymyxin activity.

The evidence suggesting cytoplasmic membrane disorganisation is supported by findings on the effect of polymyxins on membrane cellular activities such as respiration and oxidative phosphorylation. An immediate decline in oxygen uptake following polymyxin treatment of E. coli and S. typhimurium occurs (Wahn et al, 1971; Teuber, 1974). The extent of inhibition is proportional to the concentration of drug used. Although stimulation of oxygen consumption did not occur at any concentration of P.B. using S. typhimurium, E. coli, B. subtilis or E. coli spheroplasts (Storm et al, 1977; Teuber, 1974), endogenous respiration was stimulated in P. aeruginosa to an extent dependent on the level of P.B. (Newton, 1956) and Polymyxin E (Newton, 1953b), to which the cells were exposed. At concentrations of drug above bactericidal levels endogenous respiration was inhibited. Inhibition of respiration following P.B. treatment results in depression of intracellular ATP levels (Storm et al, 1977).

1.2.1.2. Effect of ions on Polymyxin activity

Ions have been shown to alter the activity of polymyxins in both the test situation and under conditions of nutrient limitation. In certain conditions such as M.I.C. determinations these two situations interact (Brown, 1975).

Leakage of 260nm absorbing materials from washed cells of P. aeruginosa was prevented in the presence of magnesium and this protective effect was reversible by addition of 2 molecules of citrate per atom of magnesium (Newton, 1953c). 0.05MCa^{2+} reduced the fluorescence of washed P. aeruginosa when treated with DANSP (1 dimethylaminonaphthalene 5 sulphonyl Polymyxin), indicating a reduced uptake of the fluorescent labelled drug (Newton, 1955). The fluorescence of cells treated with polymyxin and a fluorescent probe N-tolyl-α-naphthylamine-8-sulphonic acid was controllable by the addition of bivalent ions to the test suspension. The order of effectiveness was $Mg^{2+}>Sr^{2+}>Ca^{2+}>Ba^{2+}$. Trivalent ions ($La^{3+}$ or Ce^{3+}) were effective at lower concentrations and uranyl ions were protective against P.B. (Newton, 1954). This ionic activity agrees with the reversal of charge on "phosphate colloids" (Bungenburg de Jong, 1949). The effect of the calcium content of human serum on the activity of colistin against E. coli and P. aeruginosa revealed that serum was only bactericidal for P. aeruginosa and only then if the calcium concentration was decreased by dilution or chelation with Na_2Mg E.D.T.A. (Davies et al, 1971a,b). It is likely that the protective effect of divalent cations is due to competition between polymyxin and the cations for charged groups (e.g. phosphates) near the cell surface.

The role of divalent cations of the cell wall in protecting cells from the action of polymyxins has also been investigated. Using whole cells of E. coli and P. aeruginosa and glucose containing liposomes (Hsu Chen

and Feingold, 1972) showed that P.B. cidal activity was inhibited by Ca^{2+} and Mg^{2+} of 2×10^{-3}M or less however, cation concentrations up to 2×10^{-2}M did not protect liposomes suggesting that the protective effect of ions is an indirect one and that by "enforcement" of the cell wall structure access of the antibiotic to its target site in the cytoplasmic membrane may be prevented. This ionic strengthening of the bacterial cell wall has been reported by Leive (1974). The binding of P.B. to lipid monolayers was inhibited at high Na^{+} and Ca^{2+} concentrations in the aqueous phase.

The role of univalent ions in protecting cells from the action of polymyxins is less clear. Newton (1954) failed to show competition between Na^{+}, Li^{+} and NH_4^{+} and polymyxin. Feingold et al (1974) found that 40nM P.B. was absorbed per μmole PO_4^{3-} in E. coli liposomes when 0.05M NaCl was present but no drug absorption occurred when 1M NaCl was used, but Teuber and Bader (1976a) failed to show reduction of polymyxin activity in 1M NaCl against Acholeplasma laidlawii sensitized to P.B. by fusion with phosphatidyl glycerol or cardiolipin.

The ionic composition of the test medium upon subsequent sensitivity of cells to P.B. is of importance. Whole cells and spheroplasts of P. aeruginosa and B. megaterium have been studied in this respect by Klemperer et al (1979). Whole cells of P. aeruginosa in ammonium and potassium acetate suspension medium showed most lysis, whilst sodium acetate gave some protection and sodium chloride suspensions showed little P.B. lysis. However, when viability rather than lysis was considered NaCl suspended cells were most susceptible. Lysis of spheroplasts of P. aeruginosa showed susceptibilities potassium acetate > sodium acetate > ammonium nitrate > ammonium chloride, ammonium hydrogen phosphate and calcium acetate. Neither monovalent cations nor ionic strength affected

bactericidal activity or O.M. disruptive effect (β lactamase leakage) of polymyxin sensitive S. typhimurium (Vaara, 1981a). Also polymyxin binding was unaffected by ionic strength up to lethal polymyxin concentrations. Polymyxin binding by isolated LPS was only slightly affected by ionic strength. The polymyxin resistant pmrA mutant of S. typhimurium's response to P.B. was very dependent upon ionic strength (Vaara, 1981a). High ionic strength media inhibited absorption of P.B., O.M. disruptive effects and binding to L.P.S. Polymyxin antagonism by Mg^{2+} correlates with an electron microscopically observed reduction in the number of cell wall projections caused by the action of the drug on sensitive cells (Koike et al, 1969).

The role of ions in polymyxin M.I.C. determinations is complicated by the fact that growth media may contain $>10^{-3}$M of divalent ions. Water washing of agar reduced the M.I.C. to polymyxin (Hanus et al, 1967), and this correlated well with Mg^{2+} and Ca^{2+} removal. Ion agar gave larger zones of inhibition than conventional agar to polymyxin (Bechtle and Scherr, 1958). An increase in the M.I.C. of most of 13 species of pseudomonads was shown by addition of Ca^{2+} or Mg^{2+} to Mueller Hinton agar. Supplementation of M.H. broth with Ca^{2+} or Mg^{2+} had little effect on the M.I.C. of colistin for E. coli but increase the M.I.C. for most nonfermenting strains studied.

The effect of nutrient depletion of anions and cations upon the susceptibility of P. aeruginosa has been reviewed (Brown, 1975). Brown and Melling (1969a, b) showed that batch grown P. aeruginosa grown in a simple salts medium without added divalent cations except Mg^{2+} were resistant to P.B. at low Mg^{2+} concentrations. Increased sensitivity occurred when the Mg^{2+} content of the medium was increased. These results confirmed by Boggis (1971) and Boggis et al (1979) who extended the

initial work to study the effect of other cations on restoration of sensitivity of P. aeruginosa to P.B. The order of effectiveness was $Ca^{2+} > Mg^{2+} > Ba^{2+} > Mn^{2+} > Sr^{2+}$ and effects due to Al^{3+} Zn^{2+} and Fe^{2+} were negligeable. The change in lysis rate per quantity of cation in the medium was less for P.B. than E.D.T.A. suggesting a relatively non-specific association between the P.B. target site in the wall and the ions. The cell wall content of Mg^{2+} and Ca^{2+} in P. aeruginosa was related to the availability of these cations in the growth medium. P.B. resistant cells had a lower Mg^{2+} and Ca^{2+} content. P.B. resistant P. aeruginosa were shown to have one tenth of the Mg^{2+} cell wall content of polymyxin sensitive strains (Brown and Watkins, 1970). Polymyxin sensitive P. aeruginosa were shown to have 10 fold higher cell wall Ca^{2+} and Mg^{2+} concentrations than polymyxin resistant Pr. vulgaris (Brown and Wood, 1972). Profound cell wall protein changes have been shown to occur in polymyxin resistant mutants of P. aeruginosa or in cells made resistant by growth in Mg^{2+} deficient media (Nicas and Hancock, 1980). These cells were polymyxin resistant and showed enhanced levels of outer membrane protein H1. In all strains the cell envelope Mg^{2+} content varied inversely with the amount of protein H1 (Nicas and Hancock, 1980).

Media contents other than ions may markedly affect the susceptibility of micro-organisms to P.B. Growth of P. aeruginosa on branched chain amino acids, their acyl derivatives, D-glucose L-glutamate or M.H. medium affected their resistance to P.B. and colistin to a variable degree (Conrad et al, 1979). Bacteria grown on branched chain acyl derivatives became more susceptible to P.B. and colistin. Further support for this resistance being due to altered lipid composition was seen when P. aeruginosa was grown on a range of amino acids as sole

carbon source. The M.I.C. to P.B. ranged from 2 U/ml for isobutyrate grown cells to 975 U/ml for L glutamate grown cells (Gilleland and Conrad, 1980). E. coli grown on cystine were more resistant to P.B. than those grown on nutrient agar (Hugo and Ellis, 1978).

1.2.1.3. Polymyxin and the Outer Membrane

There is much evidence for the importance of the O.M. of Gram-negative bacteria being involved in the activity of the Polymyxins. Gram-positives which lack the complex outer layers seen in Gram-negatives are generally less susceptible to these antibiotics. The importance of the O.M. as an exclusion barrier to antibiotics has been reviewed by Leive (1974) and Brown (1975). Proteus strains are resistant to very high concentrations of polymyxin. Such tolerance might be due to O.M. or I.M. resistance. The importance of the former was shown by Teuber (1969) who converted resistant Pr. mirabilis into its L-form and spheroplasts by the use of Penicillin G. L forms were sensitive to 50µg P.B. when grown in the presence of penicillin. Transfer to penicillin free media resulted in the appearance of bacillary forms accompanied by growth on media containing 50µg P.B. Addition of 5 and 50µg/ml P.B. to log phase spheroplasts grown in broth had little effect on the bacillary forms but a complete and immediate cessation of growth of the spheroplasts occurred. The spheroplasts were 400 X as susceptible to P.B. as the bacillary form. Low temperature resistance of Pr. mirabilis and its L-forms and spheroplasts was shown to be due to the O.M. and not the I.M. which remained sensitive to P.B. at all temperatures (Teuber and Bader, 1977). Liposomes prepared from wild type P.B. resistant Pr. mirabilis, a sensitive wild type mutant, and the wild type made sensitive by growth in sulphadiazine showed extensive and similar disruption by P.B. Analysis of the lipid composition of each strain used to produce the liposomes showed the lipids to be almost identical suggesting that the cell

envelope of the wild type protects the sensitive cytoplasmic membrane from disruption by P.B.

If the O.M. of Gram-negative bacteria is disrupted by the action of polymyxins then periplasmic enzymes which are sited between the outer and inner membrane should be released. 5'nucleotidase, 3'nucleotidase, ribonuclease 1, acid phosphatase and alkaline phosphatase were rapidly released from E. coli β following P.B. treatment (Cerny and Teuber, 1971).

The disruption of the O.M. by polymyxin has been shown in a number of permeabilisation studies. Pr. mirabilis growing in the presence of P.B. gave lower counts when plated on MacConkey agar than L-B agar (Sud and Feingold, 1970). No effect on viability was seen for P.B. and deoxycholate (D.O.C.) separately but when added together a 3 log fall in viable count was noted. Pre-treatment with D.O.C. prior to drug treatment produced no kill but D.O.C. treatment following P.B. resulted in rapid loss in viability. Polymyxin was also shown to act synergistically with normal serum (Sud and Feingold, 1975) on Pr. mirabilis and additively with Polysorbate 80 (Brown et al, 1979a) on P. aeruginosa. Polymyxin susceptible S. typhimurium strains lost their O.M. permeability barrier to lysozyme and periplasmic proteins upon treatment with P.B. However, their pmrA mutants (polymyxin resistant) retained the permeability barrier to these molecules (Vaara, et al, 1981). Sensitization of the pmrA strain to D.O.C. required a P.B. concentration 10X higher than that required for the parent strain. The lethal effect of E. coli O111:B4 Westphal endotoxin is neutralisable by P.B. (Rifkind and Palmer, 1966 and Rifkind, 1967). The endotoxin from sensitive strains was neutralised 5X more effectively than that from resistant strains.

Inhibition of phage T3, T4 and T7 absorption to E. coli B was shown following treatment with a lethal dose of P.B. (Koike and Kyoko, 1971). Since the receptors for these phages are L.P.S. which is located only in the O.M. this and the previously mentioned endotoxin neutralisation effects suggest interaction between P.B. and the O.M. especially the L.P.S.

The involvement of the O.M. in P.B. activity has also been shown microscopically. Koike et al (1969) showed that following treatment of E. coli B and P. aeruginosa P29 with P.B. and Polymyxin E blebs appeared extending from the outer surface. Although these blebs were seen in untreated cells their number was greater in treated cells, and the number of blebs was related to drug concentration. Similar projections were seen following treatment of P. aeruginosa and the number of projections was reduced following Polymyxin antagonism by the addition of Mg^{2+} ions (Gilleland, 1977). Proteus spp. rendered sensitive to Polymyxin by treatment with sulphonamide developed blebs from the L.P.S. layer (Handley et al, 1974).

Freeze etching of Polymyxin treated S. typhimurium showed blebs and projections arising from the smooth upper fracture face of the O.M. (Schindler and Teuber, 1975). The inner fracture face has also been shown to be involved in bleb formation (Lounatmaa and Nanninga, 1976).

A comparison of freeze etched resistant and sensitive P. aeruginosa revealed that the outer leaflet of the O.M. lacked particles when grown in the presence of P.B. However, the sensitive strain grown in the absence of P.B. showed an outer leaflet studded with particles. Resistant isolates grown in the absence of P.B. resembled the sensitive strain. Polymyxin B sensitive R and S strains of S. typhimurium developed O.M. projections but those of the rough (L.P.S. deficient)

mutant were smaller and flatter (Lounatmaa et al, 1976). R and S strains showed vesicular bulges from the O.M. Asymmetry of the O.M. is important in the formation of the O.M. projections since purified L.P.S. free from P.L. showed no projections or blebs when treated with P.B. although extensive fragmentation of L.P.S. sheets was seen indicating interaction with P.B.

It seems a characteristic of P. aeruginosa that the concave outer membrane layer possesses less particles than those of other Gram-negatives. E.D.T.A. extraction of P.aeruginosa (Roberts et al, 1970) showed the liberated complex to be composed of protein, L.P.S. and lipid and visualisation of the complex revealed spherical units (7nm) and rodlets (20 x 7nm) of identical size to the O.M. particles (Rogers et al, 1969). The evidence presented in the previous paragraphs shows that the O.M. is important in the action of polymyxins and also in the resistance of strains to these drugs. Much work has been carried out to elucidate the components of the inner and outer membranes which affect the action of P.B.

There is no evidence that membrane proteins act as receptors for polymyxins.

Lopes and Inniss (1969) examined electron microscopically the interaction of P.B. and L.P.S. extracted from E. coli 018. P.B. treatment resulted in complete disaggregation of material into short sections. Stable noncovalent complex formation between L.P.S. from S. typhimurium and P.B. has been demonstrated (Bader and Teuber, 1973). The primary interaction was considered to be between polymyxin and the negatively charged K.D.O. - lipid A portion of the L.P.S., 3 moles of polymyxin being bound per mole of L.P.S. These findings were supported by

Morrison & Jacobs (1976) using 'heptoseless' mutants of S. minnesota and S. typhimurium mutants (Schindler and Osborn, 1979). A stable complex was formed between 1 mole of Polymyxin B and 1 mole of lipid A. The interaction was reversible and probably involved ionic and hydrophobic interaction. Polymyxin resistant pmr A mutants of S. typhimurium and their extracted L.P.S. bound less P.B. than their parent strains and their L.P.S. (Vaara et al, 1979). The pmrA mutants had more ethanolamine containing compounds (P.E. and diPethanolamine) than the parent L.P.S. and contained 4-6 times more 4-ARAN (4 amino-4-deoxy-L-arabinose) as a substituent of the ester linked lipid A phosphate than the parent strain (Vaara and Vaara, 1981, and Vaara et al, 1981). Polyacrylamide gel electrophoresis showed complex formation between P.B. and L.P.S. of resistant Serr. marcescens 08 but dissociation and degradation in L.P.S. of sensitive Bizio strains (Brown and Tsang, 1978).

The importance of lipids in the action of P.B. is shown by several workers using differing techniques. Polymyxin E complexed strongly with monolayers of lipids extracted from P. denitrificans. Moderate complex formation also occurred with D.P.G. but lecithin failed to react with the drug (Few, 1955). Few (1955) suggested that the positively charged choline group of lecithin effectively shields the ionized phosphate group preventing electrostatic bonding between phosphate and amino groups of the antibiotics. Teuber (1973) showed that phosphatidic acid and P.G. form stable complexes with P.B. as judged by migration on paper chromatographs. Strong binding ofP.B. also occurred to phospholipid micelles of P.E., P.G. and D.P.G. (Rosenthal et al, 1976). The binding of mono N(^{14}C) acetyl P.B. to lipid monolayers formed from lipid A of Pr. mirabilis or pure individual lipids showed attachment of P.B. to the acidic negatively charged lipids (P.G., P.S., D.P.G.) was high (Teuber and Miller, 1977). Binding to P.E. was less strong and binding

to P.C. and the positively charged lipid stearylamine was poor. Absorption of drug to a mixture of P.E., P.G. and D.P.G. in proportions similar to E. coli total lipids gave similar results. Fusion of lipids from S. typhimurium to Acholeplasma laidlawii B resulted in a change of the organism from P.B. resistant to sensitive (Teuber and Bader, 1976a) The P.B. sensitivity depended upon the composition of the lipid vesicle. Only P.G. and D.P.G. or mixtures of these with P.C. or P.E. showed any effect on increasing susceptibility. Replacement of P.E., D.P.G. and P.G. by ornithine amide lipid by P. fluorescens under conditions of P-limitation and the resulting increasing resistance to P.B. shows the importance of these lipids especially P.G. and D.P.G. in the susceptibility of bacteria to P.B.

The efflux of trapped glucose from liposomes have been used to study Polymyxin-phospholipid interactions. Hsu Chen and Feingold (1973) and Feingold et al (1974) showed that liposomes prepared from P.E. were sensitive to P.B. but those prepared from N substituted analogues and P.C. were resistant. The degree of protection to P.E. containing liposomes was dependent upon the molar % of the N methyl analogue in the liposome and the antibiotic concentration. However, these liposomes contained 50% cholesterol and sometimes diacetyl phosphate and Imai et al (1975) have since shown that incorporation of cholesterol into liposomes from E. coli P.L. caused a decrease in P.B. sensitivity. When E. coli P.L. or purified phospholipid mixtures were used to prepare liposomes, those prepared from E. coli total lipids, purified E. coli P.E. and D.P.G. and a mixture of P.E. and P.G. were sensitive to P.B. Liposomes prepared from Streptococcus sanguis lipids, egg lecithin and sheep erythrocytes were less susceptible. Pure P.E. could not be studied since it did not form sealed liposomes. P.C. liposomes were sensitive if they contained

a negatively charged molecule such as D.P.G., and no appreciable sensitivity was shown by liposomes containing P.G. or D.P.G.

The use of such artificial systems as liposomes and lipid monolayers has been criticised by various workers since they do not represent the true situation in whole bacterial cells and it has been suggested that cell envelope analyses of bacteria resistant to P.B. might help to substantiate the role of various membrane components in P.B. entry to the cell. However, caution must be applied to this approach since changes in the composition of a particular membrane component may be misleading since the envelope is a dynamic structure and alterations in one component may result in compensatory changes in another component.

There is little evidence that loss of O.M. proteins result in P.B. resistance in fact Mg^{2+} depleted P. aeruginosa was shown to have an increased amount of an outer membrane protein H1 associated with the increased resistance to the drug (Nicas and Hancock, 1980). The loss of outer membrane proteins reported by Gilleland and Lyle (1979) for P.B. resistant P. aeruginosa probably cannot account for this resistance since further investigation of the strain by Conrad and Gilleland (1981) has shown that these resistant organisms also show changes in R.E.L. and P.L. content. Gilleland and Conrad (1980) could not correlate protein K.D.O., carbohydrate or O.M. protein composition with the resistance of P. aeruginosa strains grown on different carbon sources described by Conrad et al (1979). Lipid changes have been reported in P. aeruginosa by some workers and these results support liposome and lipid monolayer results which suggests that the P.L. composition of the envelope is important in the action of P.B. Although Brown and Wood (1972) showed P.B. sensitivity correlated with the P.L. content in the wall fractions of Pr. vulgaris and Klebsiella aerogenes, Kenward et al (1979) could not correlate changes in the P.L. and R.E.L. with the response of carbon

depleted and carbon/Mg^{2+} depleted P. aeruginosa to P.B. They found sensitivity was more dependent upon the cation content of the O.M. Lipid alterations were not reported in Proteus (Sud and Feingold, 1970) or in E. coli (Dame and Shapiro, 1979).

1.2.1.4. Polymyxin and the Inner Membrane

Direct evidence for the activity of P.B. on the I.M. is scanty although from the early studies of Newton (1956) its action on this membrane was suggested. The problem has been the technical one of production of uncontaminated I.M.'s. Indirect evidence of the role of I.M. was obtained by Newton (1955) using DANSP which bound to protoplasts of M. lysodeikticus and B. megatarium. Inner and outer membranes of known P.L., L.P.S. and protein composition were used by Teuber and Bader (1976b) to study their action with a mono-N acetyl ^{14}C derivative of P.B. The two membranes bound equivalent amounts of P.B. per mole of lipid phosphate when corrected for P.L. and L.P.S. content.

1.2.1.5. Mechanism of Polymyxin Action

The mechanism of action of the Polymyxins has still not been fully elucidated. Although polymyxin has been shown to affect the permeability of the cytoplasmic membrane it is likely that the interaction of the molecule with the O.M. is sufficient to cause disruption of the O.M. resulting in osmotic instability of the cytoplasmic membrane. The importance of divalent cations in their activity suggests competition between the polymyxin molecule and Mg^{2+} or Ca^{2+} for binding sites on the charged PO_4^{3-} groups of the membrane P.L. and L.P.S. Electrostatic interaction between peptides and lipid phosphates are also essential for activity, however, hydrophobic interaction between the fatty acid side chain and lipids of the membranes are also important.

Reviewing the literature on the effect of the environment upon the resistance of P. aeruginosa to antibacterial agents and also the role of the envelope in such resistance led Melling and Brown (1975) and Brown (1975) to suggest "a common P. aeruginosa-specific initial site(s) for action" for P.B., E.D.T.A. and gentamicin. This hypothesis was based upon the linked change in sensitivity to E.D.T.A. and P.B. upon Mg^{2+} and PO_4^{3-} depletion and the effect of cations especially Mg^{2+} on the sensitivity of P. aeruginosa to gentamicin. The lack of sensitivity of other Gram-negatives to these factors suggests that such a site is reduced or lacking in these organisms. Aminoglycoside uptake in these other Gram-negatives is probably via the hydrophilic (porin mediated) pathway (Hancock, 1981a).

This "common site" suggestion has recently been supported by other workers. Induction of O.M. protein H1 either by growth of P. aeruginosa under conditions of Mg^{2+} depletion or the use of mutants which overproduced H1 decreased the sensitivity of P. aeruginosa to P.B., E.D.T.A., gentamicin and streptomycin (Nicas and Hancock, 1980 and Hancock et al, 1981b). The normally observed permeabilisation to lysozyme and nitrocefin of the O.M. of P. aeruginosa by gentamicin and streptomycin was totally inhibited by 1mM Mg^{2+}.(Hancock et al, 1981b)and phosphate (Hancock, 1981b). The binding site proposed for aminoglycosides is a Mg^{2+} binding site on the L.P.S. and a proposed model for the initial uptake of aminoglycosides and probably P.B. and E.D.T.A. has been proposed by Hancock (1981b). This model is shown in Figure 8.

Models have recently been proposed for polymyxin/membrane interaction. Hartmann et al (1978) propose that the paraffin tail of the polymyxin molecule reacts with the hydrophobic elements of the membrane leaving the charged peptide ring lying flat on the membrane outer surface. This

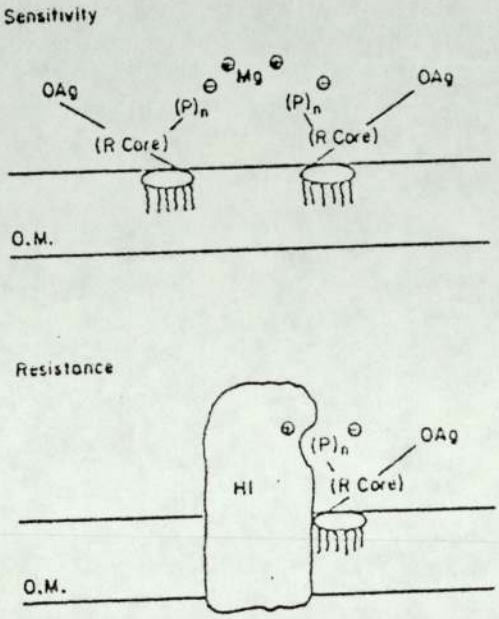


Fig 8. Model illustrating the proposed mechanism of resistance to aminoglycosides, polymyxin B and EDTA in *Ps. aeruginosa* strains with high levels of protein H1 (O.M., outer membrane; R, Core, the heptose; KDO, rough core region of the LPS; O Ag, the somatic antigen of *Ps. aeruginosa* LPS; $(P)_n$, the polyphosphate portion of the *Ps. aeruginosa* LPS; this phosphate is negatively charged at neutral pH and is attached to the KDO-Heptose region of the LPS and may be as many as 12–15 moles of phosphate/mole LPS; H1-major outer membrane protein H1 which appears in large amounts in the outer membranes of specific mutants and in strains grown in Mg^{2+} deficient media). It is proposed that the crosslinking of the negatively charged polyphosphate regions of the LPS by Mg^{2+} is important for outer membrane stability in sensitive cells. EDTA by removing Mg^{2+}, and the highly cationic antibiotics aminoglycosides and polymyxin B by displacing Mg^{2+}, lead to disruption of the outer membrane permeability barrier (see Hancock *et al.*, 1981; Nicas & Hancock, 1980). It is proposed that in cells with high levels of protein H1, this protein replaces Mg^{2+} in the outer membrane (an inverse correlation between the level of protein H1 in the outer membrane and the level of cell envelope Mg^{2+} has been demonstrated). Protein H1 thus protects the polyphosphate site on the LPS from attack by aminoglycosides, polymyxin B and EDTA and makes the cell relatively resistant to these agents.

insertion of the tail results in expansion and asymmetric distortion and tilting of the lipid chains and changes in fluidity and permeability of the membrane occur. In contrast to this El Mashak and Tocanne (1980) suggest penetration of the whole Polymyxin molecule into the membrane resulting in lateral expansion and disorganisation of the membrane.

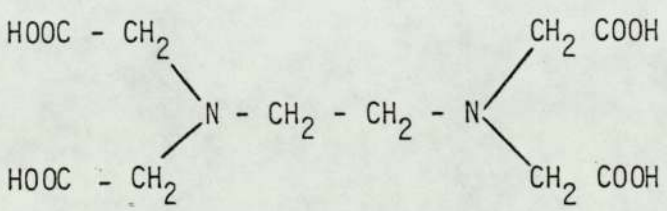
An alternative suggestion is that the polymyxin molecule might bind to the O.M., L.P.S. and or P.L. and penetrate the outer membrane through protein porins. Resistance to the drug in this case would be due to loss of outer membrane porin proteins. There is little evidence for this later method at the moment.

The mechanism of penetration of the Polymyxin molecule into the cytoplasmic membrane remains uncertain.

1.2.2. E.D.T.A.

E.D.T.A. is a tetrabasic molecule which acts on the O.M. of Gram-negative bacteria (Wilkinson, 1975). Its structure is shown in Fig. 9. Its activity is due to its metal ion chelating properties.

Figure 9 Structure of E.D.T.A.



The general properties of E.D.T.A. have been reviewed by Russell (1971) and its action on Gram-negatives by Leive (1974). The sensitivity of P. aeruginosa to this agent has been considered by Wilkinson (1975). In this section the action of E.D.T.A. on Pseudomonas spp. will specifically be considered and only where appropriate will reference to its action on other Gram-negatives by considered.

Surprisingly in view of their resistance to other antimicrobials pseudomonads especially P. aeruginosa are remarkably susceptible to the lytic bactericidal activity of E.D.T.A. Other Gram-negatives are less susceptible to E.D.T.A. unless treated in combination with tris buffer (tris (Hydroxymethyl)-amino methane). E.D.T.A.-tris induced lysozyme sensitivity resulted in rapid lysis of E. coli and Azotobacter spp. but not of Klebsiella aerogenes (Repaske, 1958). Bacillus spp. and Sarcina lutea but not S. aureus and S. albus were sensitive to lysozyme-tris-E.D.T.A. treatment (Repaske, 1958).

1.2.2.1. E.D.T.A. induced lysis and leakage

E.D.T.A. is rapidly and irreversibly bactericidal to P. aeruginosa although such treatment rarely results in sterilisation of the culture and a number of cells (dependent upon the density of the test population) will survive (Gray and Wilkinson, 1965a). These survivors do not appear to be genetically resistant to E.D.T.A. Turbidity changes do not always correlate with changes in viability. Loss of viability without turbidity may occur (Neu et al, 1967) as well as changes in turbidity without viability changes (Matula and Macleod, 1969). Brief exposure to E.D.T.A. results in changes in the O.M. rather than the I.M. since in Gram-negatives, other than pseudomonads, lysozyme and cold shock both of which involve cell components between the I.M. and O.M. require pre-

treatment with E.D.T.A. Pre-treatment with E.D.T.A. is also required for the action of such cytoplasmic active drugs as valinomycin on E. coli (Bhattacharyya et al, 1971). Treatment of P. aeruginosa with E.D.T.A., however, produces osmotically fragile forms (osmoplasts) which rapidly lyse unless stabilised in hypertonic solutions of sucrose or 0.5M NaCl (Gray and Wilkinson, 1965a). Osmoplasts can be restored to osmotically stable forms by addition of multivalent cations especially Ca^{2+}, Mg^{2+} and Zn^{2+} in the proportion found in the cell wall, although the cells remain leaky and have low viability (Asbell and Eagon, 1966a,b). Lysis by E.D.T.A. of P. aeruginosa results in leakage of 260nm absorbing material which is rich in phosphorus and pentoses. Leakage is generally less rapid than loss of viability (Gray and Wilkinson, 1965a). Release of periplasmic enzymes from P. aeruginosa by treatment with E.D.T.A. has not been demonstrated. although it might be inferred from the results of spheroplasting or cold shocking other Gram-negatives with lysozyme-tris-E.D.T.A. or tris E.D.T.A. respectively (Malamy and Horecker, 1961; Neu, 1969).

The increasing permeability of the O.M. resulting in increased sensitivity to a wide range of agents has been demonstrated. These substances include quaternary ammonium compounds (MacGregor and Elliker, 1958), lysozyme (Repaske, 1958),enzyme substrates (Wilkinson, 1975) and drugs (Neu and Winshell, 1970). The effect of E.D.T.A. on the sensitivity of E. coli to ampicillin, chloramphenicol, tetracycline and sulphamethoxazole-trimethoprim appears to be additive (Neu and Winshell, 1970) whilst these effects on P. aeruginosa are synergistic (Weiser et al, 1969).

1.2.2.2. Factors affecting E.D.T.A. activity

The enhanced activity of E.D.T.A. in combination with other compounds such as lysozyme (Repaske, 1956), tris (Repaske, 1958) and antibiotics (Leive, 1974) has been mentioned previously.

Increasing alkalinity generally results in an increase in the activity of E.D.T.A. Increasing the pH of phosphate buffer from 6 to 8 resulted in an increased lysis rate for several pseudomonad strains (Shively and Hartsell, 1964). The greater lysis of P. aeruginosa by E.D.T.A. in phosphate buffer compared with distilled water is probably due to the fact that an unbuffered solution of disodium E.D.T.A. is acidic and under acidic conditions the chelating properties of E.D.T.A. are poor. Chelation pH optima for Ca^{2+} and Mg^{2+} are 7.5 and 10.0 respectively.

The response of P. aeruginosa to E.D.T.A. is dependent upon the age of the test culture. The percentage lysis of P. aeruginosa exposed to E.D.T.A. fell from 74% for early log phase cultures to 36% for 48 hour old cultures (Repaske, 1958).

Much of the work involving E.D.T.A. lysis of cells has been performed in complex media making interpretation of results difficult. Using a defined glucose salts medium with sufficient Mg^{2+} to allow growth to be finally depleted by carbon Brown and Melling (1969a,b) showed that stationary phase batch grown P. aeruginosa were resistant to E.D.T.A. treatment. As the Mg^{2+} concentration in the medium was increased the sensitivity to E.D.T.A. increased. Calcium was able to replace magnesium in part. These results have subsequently been confirmed by Boggis (1971) who extended this original work to show that the order of effectiveness of cations in inducing sensitivity to E.D.T.A. was Mg > Mn > Ca > Ba > Sr and Zn had no effect. Fe^{2+} had a protective effect. The authors

suggest two sites for E.D.T.A. action, one near the surface and one less accessible probably the cytoplasmic membrane. This is supported by Cheng et al (1973) who showed that spheroplasts of P. aeruginosa were insensitive to actinomycin D unless treated with E.D.T.A. Work with E.G.T.A. (Ethyleneglycol bis (2 aminoethyl) tetra acetic acid) which specifically chelates Ca^{2+} ions suggested that Ca^{2+} was associated with the deeper part of the envelope (Boggis, 1971). P. aeruginosa grown in Mg^{2+} depleted continuous culture at low dilution rates was more resistant to E.D.T.A. than carbon depleted cells (Dean et al, 1976). Mg^{2+} depleted P. aeruginosa were more resistant to E.D.T.A. than cells grown in Mg^{2+} rich media. These resistant cells showed replacement of cell wall Mg^{2+} by protein H1, (Nicas and Hancock, 1980).

Cells stored for several hours before E.D.T.A. treatment were less sensitive to E.D.T.A. lysozyme than freshly harvested and washed cells (Repaske, 1958). The addition of 0.15M NaCl to E.D.T.A./tris increased the survival of buffer washed cells from 13 to 23% and decreased the lysis by E.D.T.A./lysozyme (Goldschmidt and Wyss, 1967). Changes in size and surface properties of cells and isolated walls caused by washing in media of different ionic strength markedly affects their response to E.D.T.A. (Matula and Macleod, 1969).

The true effect of temperature upon lysis and leakage of cellular material by E.D.T.A. treatment is difficult to determine since cells cold shocked show lysis and leakage anyway. Vancomycin adsorption to Flavobacterium spp. was greater at $37^{o}C$ than $5^{o}C$ for cells treated with E.D.T.A./tris (Haslam et al, 1969). However, permeability changes in E. coli induced by E.D.T.A. were complete within 15 seconds at either $4^{o}C$ or $37^{o}C$.

1.2.2.3. The effect of E.D.T.A. on cell membranes

The sensitisation of cells to the action of other compounds and the release of periplasmic enzymes following E.D.T.A. treatment suggests its activity is on the O.M. of bacteria. Its strong metal chelating properties indicate that permeabilisation of the membrane may result from extraction of cations.

Magnesium and calcium are the predominant divalent cations in the cell walls of Gram-negatives. The proportions of each cation vary with species but in P. aeruginosa Mg^{2+} is more plentiful than Ca^{2+} (Eagon, 1969). The extaction of Ca^{2+} from Rhizobium trifolli and Zn^{2+} from S. enteritidis by E.D.T.A. has been reported (Humphrey and Vincent, 1962). From a comparison of lysis of P. aeruginosa by various chelating agents Wilkinson (1970) concluded that Mg^{2+} was more important than Ca^{2+}, although these findings have been disputed by Leive (1974). The supplementation of medium low in Mg^{2+} with extra Mg^{2+} resulted in increasing sensitivity of P. aeruginosa to E.D.T.A. (Brown and Melling, 1969b), but Ca^{2+} only partly restored sensitivity and other ions were less effective. Tris solubilised only trace amounts of Ca^{2+} and Mg^{2+}, however, combination with E.D.T.A. resulted in the synergistic extraction of 82.7% and 42.0% of the envelope Mg^{2+} and Ca^{2+} respectively (Cox and Eagon, 1968). Stationary phase Mg^{2+} limited E. coli retained Mg^{2+} following exposure to E.D.T.A., however exponential phase cells lost similar amounts of Mg^{2+} as cells grown in Mg^{2+} plentiful medium (Lusk et al, 1968). The introduction of the RP1 plasmid into P. aeruginosa resulted in E.D.T.A. resistance (Kenward et al, 1978). Changes in cell wall composition were also noted. The authors suggest that the lack of lysis of $RP1^{+}$ compared with $RP1^{-}$ cells grown under identical conditions indicates that Mg^{2+}

and Ca^{2+} are located deeper in the cell wall of $RP1^{+}$ cells and are involved to a greater extent in cross linking of peptidoglycan than in $RP1^{-}$ cells. This deep seated position would make the cations less accessible to E.D.T.A.

Although this work has shown that E.D.T.A. binds cations and that Ca^{2+} and Mg^{2+} are the major cations involved this does not prove conclusively that such binding is the cause of the induced permeability changes.

1.2.2.4. Organic complex release by E.D.T.A.

Release of cell envelope components as complexes by E.D.T.A. coupled with ion extraction may be the answer to the question of cell permeabilisation. Between 33 and 50% of E. coli L.P.S. and 2% protein was lost following treatment with E.D.T.A. (Leive et al, 1968). The composition of these complexes vary with their origin i.e. whole cells or envelopes and the method used to isolate the envelope fractions. Isolation of P. aeruginosa cell walls resulted in loss of L.P.S. (Roberts et al, 1967) and the extent of extraction was affected by trypsin used in wall purification (Gray and Wilkinson, 1965b). Cell wall extracted complex from P. aeruginosa did not contain P.L.'s (Rogers et al, 1969) however, that obtained from whole cells did, having a composition protein 60%, L.P.S. 30% and loosely bound lipid 10%. P.L. was detected in the lipid fraction (Roberts et al, 1967).

SDS PAGE of N N'dimethyl formamide (DMF) extracts of P. aeruginosa cell envelopes revealed 16 proteins with 3 major proteins M wt 43,000 (A), 16,500 (B) and 72,000 (C). Electrophoresis of these E.D.T.A. extracted protein/L.P.S. complexes reveal that protein A and B were major components. Proteins A and B were thought to be glycoproteins and protein A resembled the endotoxin reported by Homma and Suzuki (1966).

Further purification and analysis of the P. aeruginosa L.P.S. complex by Hedstrom et al (1981) showed the complex to be similar to that of the O.M. and contained O.M. proteins D1, D2, E, G and H1. Proteins F (porin) H2 and I (lipoprotein) were not detected.

The extraction of L.P.S. from cells other than P. aeruginosa under controlled conditions suggests that L.P.S. release may not solely be responsible for E.D.T.A. induced permeabilisation. $PmrA^+$ strains of S. typhimurium showed greater E.D.T.A. induced lysozyme and deoxycholate sensitivity than $pmrA^-$ strains although E.D.T.A. released identical amounts of L.P.S. (9-11%) (Vaara, 1981b). Ultracentrifugation of extracts from E. coli revealed two fractions, one containing L.P.S.-protein-P.L. and the other containing almost pure L.P.S. (Leive et al, 1968). The release of the pure L.P.S. fraction was less in E.D.T.A. resistant mutants suggesting that this fraction is associated with membrane permeability changes. Differential release of L.P.S. was suggested by Gmeiner and Schlecht (1979) and Gmeiner et al (1980). who showed that rough mutants of S. typhimurium lost less L.P.S. (the amount depending on the L.P.S. chain length) than wild type cells despite the fact that these mutants had a 4 fold higher L.P.S. content. Strains grown on poor medium with mainly Mg^{2+} as the divalent cation released less L.P.S. The authors suggested that non-releasable L.P.S. was related to the lipid bilayer areas of the membrane, whereas releasable fractions were associated with divalent cations in protein rich areas of the membrane.

1.2.2.5. Electron microscopic changes associated with E.D.T.A. action

The complexes liberated by E.D.T.A. treated P. aeruginosa visualised in negatively stained preparations are seen as 7nm diameter spherical

units or rodlets (20 x 7nm) composed of spherical units (Stinnett et al, 1973). Such dimensions match the size of particles seen in freeze etched cells. Freeze etching of osmoplasts revealed that approximately half of these particles had been extracted from the concave outer membrane layer (Gilleland et al, 1973) suggesting that these particles correspond to the site of complex extraction by E.D.T.A. Freeze etching of 'restored' cells formed from osmoplasts in the presence of Mg^{2+} and E.D.T.A. extracted complex showed the outer membrane layer crowded with disorganised particles. Self repair of osmoplasts by growth in the absence of extracted complex gave cells whose O.M. showed a normal regular particle distribution on freeze etching. P. aeruginosa grown in Mg^{2+} plentiful medium were E.D.T.A. sensitive and showed a typical particle organisation. However, cells grown in Mg^{2+} deficient medium (E.D.T.A. resistant) showed a crowded particle arrangement which persisted after E.D.T.A. treatment (Gilleland et al, 1974).

1.2.2.6. Cell wall chemistry of E.D.T.A. sensitive and resistant Pseudomonas spp.

In an attempt to discover the reason for resistance of certain pseudomonads to E.D.T.A. cell wall analysis of resistant and sensitive strains has been performed. In general resistant strains show lower phosphorus levels and higher carbohydrate levels than sensitive strains (Gray and Wilkinson, 1965). Analysis of cell envelopes from P. aeruginosa grown in Mg^{2+} sufficient (E.D.T.A. sensitive) and Mg^{2+} deficient (E.D.T.A. resistant) media showed no difference in total protein content (Gilleland et al, 1974) but the Mg-deficient cells contained 1.5% phosphorus compared with 1.83% phosphorus for those grown in Mg^{2+} plentiful media. The Mg^{2+} deficient cells had a higher carbohydrate (15.2%) and K.D.O. (1.5%) content than envelopes from Mg^{2+} sufficient

cells (12.7% and 1.3% respectively). Despite showing no differences in total protein qualitative differences were noted by SDS PAGE. Envelopes from Mg^{2+} plentiful cells showed protein A (43,000), B (16,500). C (72,000) and E (100,000) whereas Mg^{2+} depleted cells had almost no protein E but protein D (50,000) was detected.

1.2.2.7. Mode of action of E.D.T.A.

Evidence suggests that a primary event is chelation of essential metal cations bound in the cell envelope. In P. aeruginosa at least it would appear that Mg^{2+} is an essential metal cation. The extraction of cations is rapidly followed by the partial solubilisation of the cell envelopes of sensitive bacteria. The solubilisation involves the O.M. components chiefly but not the peptidoglycan and although the cytoplasmic membrane may be slightly affected its function remains largely unaffected.

The reasons for the increased susceptibility of P. aeruginosa has been considered by Wilkinson (1975). The L.P.S. core polysaccharide of P. aeruginosa is highly substituted with polyphosphate residues which have strong metal binding capacity. Binding of L.P.S. to protein and phospholipids by cation bridges is important in membrane stability and cation removal by E.D.T.A. will result in breakage of the cross linked bridges releasing protein/L.P.S./lipid complexes and also creating highly anionic regions in the membrane. Repulsive forces may then serve to open up the membrane further to the disruptive action of E.D.T.A.

1.3. The role of Phosphorus in the biological activity of bacteria

The work described in this study involves the effects of variation of PO_4^{3-} on the sensitivity of P. aeruginosa to drugs. A review of the responses of cells to various levels of phosphorus will therefore be given.

1.3.1. Phosphorus requirements of bacteria

Bacteria have a requirement for phosphorus for incorporation in a wide range of compounds including nucleic acid, phospholipids, cell wall polymers etc. Occasionally it may be stored as polymetaphosphate. Only a small amount of the total phosphate required by a cell apppears in the form of diffusible organic phosphates such as ATP. Under laboratory conditions the cells phorphorus requirements may be met by inorganic PO_4^{3-} or alternatively by organic PO_4^{3-} or P.L.

1.3.2. Phosphate uptake

Phosphate (PO_4^{3-}) uptake has been studied in prokaryotes (Rosenberg et al, 1969; Rosenberg et al, 1979; Mitchell, 1954; Harold and Baarda, 1966; Friedberg, 1977; Lacoste et al, 1981), and eukaryotes (Burns and Beever, 1977). Biphasic uptake systems have been described for P. aeruginosa, E. coli, S. faecalis and Neurospora crassa and single uptake systems for Micrococcus pyogenes and Micrococcus lysodeikticus. PO_4^{3-} transport studies suggest a mechanism of repression-derepression with growth in an excess of Pi resulting in a slowing down of the rate of PO_4^{3-} uptake.

Lineweaver-Burk plots for PO_4^{3-} uptake by P. aeruginosa reveal high and low affinity systems with K_m 1.1μM, V max. 12.5nmol/mg dry wt per min. and K_m 10μM, V max. 22.2nmol/mg dry wt. per min. (Lacoste et al, 1981).

These results are comparable with those of E. coli of K_m 0.7 M and 9.2 M (Medveczky and Rosenberg, 1971). The accumulation of PO_4^{3-} in P. aeruginosa was increased by Mg^{2+} and reduced by other divalent cations such as Mn^{2+}, Ni^{2+} and Co^{2+} (Lacoste et al, 1981) and similar results have been reported for E. coli and B. cereus (Medveczky and Rosenberg, 1971 and Rosenberg et al, 1969). The high and low affinity systems of E. coli and P. aeruginosa can be distinguished by substrate specificity, osmotic shock sensitivity and energy requirements. The high affinity PO_4^{3-} transport system of P. aeruginosa has a much wider specificity than the low affinity system. Chemically related analogues such as phosphite, pyrophosphate, arsenate, and methylphosphonate inhibit the high affinity system, but arsenate (which inhibits oxidative phosphorylation and depletes the ATP pool) is the only one of these inhibitory to the low affinity system. Phosphonates affect PO_4^{3-} uptake to varying degrees depending on their chemical composition. Amino methyl phosphonate is less inhibiting than methyl phosphonate. These compounds are not hydrolysed by alkaline phosphatases and the extent of repression of PO_4^{3-} uptake is dependent upon the external PO_4^{3-} concentration derived from C-P bond cleavage (Lacoste et al, 1981). Arsenate inhibits both the Pst (phosphate specific transport) and Pit (Pi transport) systems of E. coli (Rosenberg et al, 1977) but in P. aeruginosa it competes with PO_4^{3-} in the high affinity system and is an uncompetitive inhibitor of PO_4^{3-} uptake in the low affinity system. (Lacoste et al, 1981). The low affinity system of P. aeruginosa is similar to the Pit system of E. coli in being cold shock resistant and sensitive to uncouplers of oxidative phosphorylation such as 2,4 dinitrophenol and carbonyl cyanide m-chlorophenyl hydrazone.

1.3.3. Phosphate depletion

In adapting to low concentrations of PO_4^{3-} in their surroundings micro-organisms produce major changes in their content of non nucleic acid phosphorus containing cellular components. The phosphorus content of cells is approximately 1.5% of the dry weight but this figure increases as the growth rate increases and varies inversely with the temperature. This variation has been shown to reflect the RNA content of the cell (Tempest, 1969). Bacteria exhibit a stoichiometry between Mg^{2+}, K^+, PO_4^{2-} and RNA which is characteristic of different groups of bacteria (Tempest, 1969). Gram-positive species have a Mg:K:RNA nucleotide:PO_4 ratio of 1:13:5:13 except under PO_4^{3-} limiting conditions when the ratio is 1:4:5:8. This later ratio is similar to that found in Gram-negative bacteria of 1:4:5:8. In this case the ratio is independent of growth rate, temperature, and growth limiting substrate (Pirt, 1975).

Growth of E. coli (Mallette et al, 1964) and P. aeruginosa (Hou et al, 1966) under conditions of P-depletion failed to demonstrate the 'maintenance energy' requirement shown for glucose. Both organisms proliferated under conditions of P-starvation indicating the utilisation of endogenous PO_4^{3-} for growth. Chemical analyses performed on P-starved P. aeruginosa (Hou et al, 1966) showed increased protein and DNA and decreased RNA during starvation suggesting that the PO_4^{3-} released as a result of RNA degradation was utilised for DNA synthesis. Addition of PO_4^{3-} to starved cells resulted in an increase in optical density, viable count, dry weight protein and RNA and DNA content. The percentage RNA increase was particularly high suggesting preferential resynthesis of ribosomal material degraded during P-starvation. Sucrose density gradient centrifugation of cell free extracts of starved, non starved and 'refed' cells showed a decrease in 70S ribosomes in the 24 hour starved cells which were restored in the 'refed' cells.

1.3.4. Cell wall changes

The most marked effect of P-limitation is seen in cell wall composition changes in micro-organisms especially Gram-positives. Whilst some species such as M. lysodeikticus and S. aureus varied little with growth rate the bacterial wall content of B. subtilis var niger decreased as P- and Mg-limited cells were grown at increasing growth rates in the range 0.05-0.3hr^{-1}, (Ellwood and Tempest, 1972). Examination of the cell walls of Gram-positives has revealed that under normal growth conditions their walls are composed of teichoic acid, a unique polymer not found in the walls of Gram-negatives. Teichoic acid is a polyol phosphate polymer which in conjunction with peptidoglycan forms an integral part of the wall structure. The teichoic acids may exist as wall or membrane acids. The wall acids are PO_4^{3-} polymers of ribitol or glycerol but the membrane teichoic acids are glycerol phosphate polymers only. Growth of B. subtilis under P-limiting conditions at a dilution rate of 0.3hr^{-1} and pH of 7.0 resulted in replacement of the wall teichoic acid, but not the intracellular teichoic acid by teichuronic acid, a polymer containing galactosamine and glucuronic acid but lacking PO_4^{3-} (Ellwood and Tempest, 1972). A teichuronic acid type polymer was found in M. lysodeikticus and S. aureus grown under similar P-limited conditions. Grant (1979) showed that P-starved B. subtilis 168 might utilise teichoic acid as a P-source since slower growth following P-limitation was accompanied by a loss of teichoic acid from the cell walls, which was not accounted for by increased PO_4^{3-} compounds secreted into the medium. Teichoic acid PO_4^{3-} added to the medium rapidly disappeared and cell growth was stimulated.

The sodium chloride content of the medium has a significant effect on the cell wall composition of P-limited B.subtilis. Increased sodium chloride resulted in walls exhibiting increased affinity for Mg^{2+} and an

altered wall composition. The teichuronic acid content was decreased and replaced by teichoic acid. Cells grown in 6% NaCl under P-limited conditions lacked teichuronic acid having teichoic acid as the sole anionic polymer (Ellwood and Tempest, 1972). In contrast to the work of Ellwood and Tempest (1972), Wright and Heckels (1975) showed that the walls of P-limited Bacillus W23 grown at a dilution rate of $0.1hr^{-1}$ had teichuronic acid but at a growth rate of $0.3hr^{-1}$ the wall phosphorus content was increased and the fall in galactosamine and glucuronic acid suggested the presence of teichoic acid. It is possible that at the higher dilution rate that the extracellular PO_4^{3-} concentration would be close to a nonlimiting value hence teichoic acid synthesis may commence.
The pH of the P-limiting medium also affects the cell wall composition. A progressive reduction in pH from 8.0-5.0 for B. subtilis resulted in decreased teichuronic acid and an increased teichoic acid content (Ellwood and Tempest, 1972). P-limited chemostat grown B. subtilis var niger NCIB 8058 showed a higher content of lysyl phosphatidyl glycerol at pH 5.1 than at pH 7.0 and B. subtilis var niger NCIB 3610 had no phosphatidyl ethanolamine or lysyl phosphatidyl glycerol at pH 7.0 but both of these were present in substantial proportions at pH 5 in P-limited cells. Evidence with other micro-organisms suggests that lysyl P.G. is produced in response to an increasing proton concentration in the external environment. Transition from log to stationary phase in batch grown (probably P-limited) B. subtilis (Marburg) resulted in a fall of P.E. counterbalanced by an increase in diglucosyl diglyceride (D.G.) and a phosphorus free peptidolipid (X) (Minnikin et al, 1972). This strain in P-limited chemostat culture however, was practically devoid of P.E. and had reduced proportions of acidic phospholipids P.G. and D.P.G. with increased D.G. and Lipid X. These results support the theory proposed by Minnikin et al (1971) of interchangeability of P.E. and D.G.

Replacement of polar lipids by P-free lipids has been shown in P-limited cultures of Candida utilis (Johnson et al, 1973) and in Streptomyces species (Zyuzina et al, 1979; and Kazymbekova and Konova, 1979). In the later case the P-free ornithine amide lipid represented 56% of the total polar lipids and the P.E. content fell from 47.9% in non P-limiting conditions to 15.9% under P-limitation.

Alterations in the cell walls of Gram-positive organisms have been shown to affect phage binding which is intimately involved with receptors on the surface of the cell envelope. Transition from P-limitation to K-limitation in a chemostat showed that phage SP50 adsorption to B. subtilis W23 was increased in relation to the replacement of wall teichuronic acid with teichoic acid (Archibald and Coapes, 1976). Transition from K -limited growth to P-limited growth resulted in phage binding at a constant level until a low level of teichoic acid was reached suggesting that newly incorporated material does not immediately appear at the cell surface in a phage adsorbable form. In contrast to this Streptococcus lactis MC8 bound phage with high efficiency when grown P-limited conditions (Archibald et al, 1978).

Changes in the cell envelope of Gram-negatives have also been reported following P-depletion in batch and continuous culture. Ames (1968) found the P.L. content of S. typhimurium to be reduced from 72.9 to 47.5μM/g dry weight under conditions of P-depletion, although the proportions of each P.L. was not greatly altered. The P.L. content of P-limited P. diminuta was greatly reduced and only a trace of P.G. was found. Phosphatidyl glucosyl diglyceride (P.G.G.) was absent and acidic and neutral glycolipids formed the bulk of membrane polar lipids of the envelope (Minnikin et al, 1974). This organism however, differs in many respects from typical pseudomonads in having a low PO_4^{3-} content,

not synthesising P.E. or D.P.G. although it is likely that P.G.G. being similar in function substitutes for P.E. and possibly D.P.G. Similar decreases were reported for P. fluorescens (Minnikin and Abdolrahimzadeh, 1974). Mg-limited chemostat cultures contained P.E., P.G. and D.P.G. with enhanced levels of P.G. and D.P.G. but no ornithine lipid was detected. In cells from P-limited ($D = 0.1hr^{-1}$) culture the ornithine lipid was the major lipid but small proportions of the other 3 lipids were present. At $D = 0.2hr^{-1}$ no P.L. were detected only ornithine amide lipid. Changing back and forth from P- to Mg-limitation resulted in typical lipid alterations. At both dilution rates a P-free lipid was detected which co-chromatographed with glucuronosyl diacylglycerol.

A similar replacement of P.L. was found in batch grown P-limited P. fluorescens by Dorrerand Teuber (1977). The amount of ornithine amide lipid increased with increasing culture age. Analysis of P-limited and non-limited control inner and outer membrane fractions revealed a fall in P.E. of 50% in both inner and outer membrane fractions of P-limited cells. Falls of 50% and 25% in P.G. and D.P.G. levels for O.M. and I.M. fractions were recorded. Ornithine amide lipid was not detected in the control cells but was higher on a molar basis than P.G. and D.P.G. in the P-limited membrane fractions. This increase in ornithine amide lipid was associated with resistance to P.B.

Dean et al (1976) studied chemostat grown P. aeruginosa under P-, C- or Mg-limiting conditions. P-limited cells had lower wall P.E., phosphorus and K.D.O. levels than C- or Mg-limited cells.

Phosphate depletion produced major changes in the P.L. and fatty acid and neutral lipid composition of whole cells of E coli with or without the resistance plasmid RP1 (Gilbert and Brown, 1978).

The P-limited cells had one third the P.L. content of the C- and Mg-limited cells, and a slightly reduced K.D.O. content. The decrease in P.L. occurred mostly in P.E. and D.P.G. but small changes were seen in levels of P.G. and P.C. The P.L. loss was compensated for by an increase in the fatty acid neutral lipid composition.

Phosphate concentration in the growth medium affected polysaccharide production by Aerobacter aerogenes (Duguid and Wilkinson, 1953). Media containing 6mg PO_4^{3-}/50ml or above gave maximal growth and minimal polysaccharide production. 0.2mg PO_4^{3-}/50ml gave less cell growth and nearly maximal polysaccharide production. Further decreasing the PO_4^{3-} content failed to increase the polysaccharide production. This PO_4^{3-} reduction resulted in an increase in capsular size. P-depleted cells could be distinguished from N- and SO_4-depleted cells by the amount of intracellular volutin production on subculture to fresh media containing PO_4^{3-}.

E. coli mutants lacking ompF and ompC are unstable and readily revert to stable strains which regain one or both of these porin proteins or contain a new O.M. porin e. Protein e has not been recorded in wild type E. coli. Growth of mutants under N-, C- or P-limitation followed by SDS PAGE of O.M. proteins showed that protein e was inducible under conditions of P-limitation (Overbeeke and Lugtenberg, 1980). Protein e is not, however, specific for PO_4^{3-} being able to function as a pore for several ions, amino acids, sugar nucleosides, monophosphates, and bis(paranitrophenyl) phosphate (Van Alpen et al, 1978). However, it is likely that protein e is synthesised in an attempt to scavenge PO_4^{3-} containing nutrients under P-limiting conditions and Overbeeke and Lugtenberg (1980) suggest that the e-pore is more effective than ompF or ompC pores for PO_4^{3-} and/or phosphorus containing nutrients.

1.3.5. Phosphate and resistance

The literature contains few reports on the effect of P-depletion on the antimicrobial susceptibility of bacteria, although in view of the dramatic cell envelope changes described previously alteration in the permeability properties of bacteria might be expected.

Boggis (1971) showed that in batch culture P. aeruginosa was more resistant to both P.B. and E.D.T.A. as the PO_4^{3-} content of the medium was decreased. Similarly Dean et al (1976) showed that P-limited chemostat grown P. aeruginosa were more sensitive to 50 U/ml P.B. than either C- or Mg-limited cells at all growth rates studied (0.05-0.5hr^{-1}). The P-limited cells showed a decreased sensitivity over a dilution rate of 0.05-0.1hr^{-1} and a subsequent increase in sensitivity at dilution rates above 0.1hr^{-1}. P. fluorescens was susceptible (M.I.C. 10µg/ml) to P.B. when grown in normal media, although a few resistant cells were seen in stationary phase cultures (Dorrer and Teuber, 1977). During growth in P-depleted medium resistant organisms appeared. These had M.I.C.'s to P.B. of >128µg/ml and the accompanying cell wall changes have been described. These cells were shown to bind less P.B.

The effect of various nutrient depletions on resistance of E. coli to 3 chlorophenol, 4 chlorophenol and 2 phenoxyethanol were studied by Gilbert and Brown (1978a). These compounds uncouple oxidative phosphorylation from respiration by increasing the cytoplasmic membrane permeability to protons. P- and Mg- depleted cells were much less sensitive to these agents than C-depleted cells and the presence of the RP1 plasmid had little effect on the P- or Mg-depleted cells.

1.3.6. Phosphate limitation and extracellular enzyme synthesis

Phosphate limitation leads to the derepression of synthesis and excretion of PO_4^{3-} mobilising enzymes by a range of organisms. The principle enzymes involved are the alkaline phosphatases and ribonucleases. Alkaline phosphatase is produced in maximal amounts at the end of the growth phase (Torriani, 1960 and Cheng et al, 1970). The specific activity of alkaline phosphatase in cell free extracts of P. aeruginosa increased several hundred fold during a 24 hour period of Pi starvation (Hou et al, 1966). The enzyme is strongly inhibited by the presence of PO_4^{3-} in both Gram-positive and Gram-negative species (Glew and Heath, 1971 and Torriani, 1960). Growth of E. coli in PO_4^{3-} concentration of >10 mole resulted in failure to detect the presence of the enzyme (Torriani 1960). Unlike other enzymes alkaline phosphatase was not produced by B. licheniformis grown under Mg-, S-, N- and C-limitation in a chemostat at dilution rates 0.05-0.8hr^{-1} (Wouters and Buysman, 1977). It was only produced under P-limiting conditions and its rate of synthesis was dependent upon growth rate up to a dilution rate of 0.3hr^{-1}. The alkaline phosphatase was partly cell bound.

Batch culture of P-limited B. licheniformis showed secretion of 30-40% of the detectable alkaline phosphatase into the medium. In chemostat grown B. licheniformis the amount secreted was related to pH At pH 6.8 30-40% was secreted and this amount was independent of growth rate. At pH 9 the amount secreted rose to 60% when the growth rate was less than 0.3hr^{-1} (Wouters and Buysman, 1980). Protoplast formation released less than 10% of the cell bound activity most remaining membrane bound (Wouters and Buysman, 1980). Alkaline phosphatase activity is associated with the I.M. and is related to divalent cation (Cheng et al, 1970; Glew and Heath, 1971). This PO_4^{3-} scavenging enzyme is active

on a range of PO_4^{3-} esters including p-nitrophenyl phosphate, β glycerophosphate, adenosine-5'-phosphate, glucose-6-phosphate, glucose-1-phosphate, ATP and sodium pyrophosphate and its action results in the liberation of inorganic phosphorus.

Ribonuclease like alkaline phosphatase is produced by Bacillus lichenformis only under P-limiting conditions but unlike alkaline phosphatase it is formed exclusively extracellularly (Wouters and Buysman, 1980). Other enzymes such as amylase and protease are decreased under conditions of P-limitation (May et al, 1968). Addition of PO_4^{3-} to the suspension medium containing PO_4^{3-} depleted cells resulted in inhibition of RNase activity over a 30 minutes period suggesting that direct repression by phosphate may not necessarily be involved but that an intermediate metabolite might be involved.

Penicillinase production by B. licheniformis was related to growth rate but unlike S-, N- and C-limited cells its production by P-limited cells fell off near the maximum specific growth site (Wouters and Buysman, 1977). Since this organism produces penicillinase constitutively its rate of production greatly increases with growth rate and the reason for the levelling off under P-limiting conditions is unknown. At growth rates $< 0.4hr^{-1}$ P-limited cells produce more penicillinase than Mg-, S-, N- or C-limited cells.

Since such enzymes as alkaline phosphatase and ribonuclease are produced only under P-limiting conditions it is probable that orthophosphate acts as a regulator, however P-limited cells must contain a certain level of orthophosphate otherwise oxidative phosphorylation would be impeded and it is possible that another cellular component such as ADP is the effector. This is supported by the finding that P-limited E. coli have a low energy charge (Chapman and Atkinson, 1977).

P. aeruginosa produces a variety of extracellular products which may play a role in infection. These include at least two haemolysins, lecithinases and proteases. During toxigenic studies it has been shown that the amount of PO_4^{3-} present in vitro affects the production of these toxins.

Extracellular production of these toxins when grown on simple salts medium was critically controlled by the PO_4^{3-} content of the medium (Liu, 1964). The PO_4^{3-} content of the medium needed to be 0.003% for lecithinase production, 0.005-0.01% for haemolysin production and 0.01-0.2% for protease production. This control of lecithinase by PO_4^{3-} is not, however, common to other pseudomonads since P. fluorescens and P. reptilivora readily produce lecithinases in media rich in PO_4^{3-} such as Trypticase Soy agar (Esselmann and Liu, 1961).

P. aeruginosa produces two extracellular haemolysins, a heat stable haemolytic glycolipid 2-O-α-L-rhamno-pyranosyl-α-L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate, and a heat stable phospholipase (Phospholipase C). Heat stable haemolysin production was detected well into the stationary phase of P. aeruginosa grown in glycerol peptone medium (Johnson and Boese-Marrazzo, 1980). Assay of the haemolysin production showed that 22 units/ml were produced in media lacking PO_4^{3-} and this fell to 11 units/ml in media containing 0.05M PO_4^{3-} although the optical density increased. The synthesis and secretion of the heat labile phospholipase C was repressed during early log phase when P. aeruginosa was grown in tryptose minimal medium. Depletion of PO_4^{3-} later in the growth cycle resulted in phospholipase C production (Stinson and Hayden, 1979). Addition of inorganic PO_4^{3-} to the medium before phospholipase C production delayed the appearance of the enzyme

for 6 hours but addition during active enzyme secretion stopped production of the enzyme within 60 mins. Phospholipase C is controlled by end product repression since PO_4^{3-} addition inhibits production of the enzyme and addition of glucose does not. It has been suggested by Stinson and Hayden (1979) that phospholipase C may be a component of the PO_4^{3-} scavenging mechanism of P. aeruginosa. In conditions where inorganic PO_4^{3-} is depleted the phospholipase genes are derepressed and synthesis of the enzyme occurs and phosphatides are degraded.

1.3.7. Adenylate Pool

The adenylate nucleotide pool is important in micro-organisms since adenine nucleotides are involved in every metabolic sequence and in most metabolic energy transductions (both chemical and mechanical) and are intermediates in the synthesis of nucleic acids. The adenylate energy charge (ATP + $\frac{1}{2}$ ADP/ATP + ADP + AMP) is a measure of the amount of metabolic energy stored in the adenine nucleotide pool. Enzyme response patterns have shown that in vivo the energy charge must be stabilised in the range 0.8 - 0.95 during growth and normal metabolism, and these values have been verified in nearly all organisms examined.

Phosphate depletion has been shown to affect the intracellular ATP level and the energy charge of the adenylate pool, generally reducing both parameters. E. coli NCTC 5928 grown under P-limited conditions in a chemostat showed a low level of intracellular ATP 0.5m mole/mg dry wt. compared to the normal E. coli figures of 2.9 - 10.8 m mole/mg dry weight (Damoglou and Dawes, 1967). Similar results were shown by E. coli when progressively depleted of PO_4^{3-} in low PO_4^{3-} medium. ATP, GTP and CTP levels fell over a period of 3 hours to less than 30% of their original levels and other this same period UTP levels fell to less than

15% of the original level (Nazar et al, 1972).

The effect of P-limitation on cellular energy levels and sporulation was investigated by Hutchison and Hanson (1974). Resuspension of exponentially growing B. subtilis 168 in P-limited medium showed a reduction in the growth rate and an immediate drop in ATP level and adenylate energy charge. 5-6 hours after resuspension in the P-limited medium refractile forespores were seen. This was some 3 hours in advance of their observation in the control culture. In contrast when depleted in tryptophan and PO_4^{3-} although the ATP concentration fell it remained 3-6 times higher than the levels seen in PO_4^{3-} limited cells and the energy charge did not change for several hours under these conditions. Refractile forespores were not seen indicating that PO_4^{3-} limitation is not solely responsible for derepression of sporulation genes. The smaller change in ATP levels and charge seen with B. subtilis might indicate prevention of protein synthesis with the resulting decrease in energy requirement. This would indicate that B. subtilis macromolecular synthesis is less strongly inhibited than in E. coli when the energy charge is lowered.

1.3.8. Phosphate and Secondary Metabolism

The amount of PO_4^{3-} remaining in the environment (Gentry et al, 1971) and accumulated by the cell (Gupta et al, 1977) may influence the survival of organisms and their ability to complete the process of secondary metabolism. In batch culture secondary metabolism immediately follows vegetative growth and results in the production of synthetases which transform small molecules into secondary metabolites. The duration of secondary metabolism is similar to that of vegetative growth (Weinberg, 1970). Generally secondary metabolism has narrower limits for pH, redox potential, temperature, trace metals and inorganic PO_4^{3-} than cell

division. The production of secondary metabolites by fluorescent pseudomonads has been reviewed by Leisinger and Margraff (1979). These metabolites include lipids, phenazines, pyroles, indoles, amino acids, peptides and pterines. Weinberg (1970) concluded that successful completion of secondary metabolism was necessary for long term survival based on the observation that the PO_4^{3-} concentration that inhibited pyocyanin formation caused glycerol grown cells to die early in the stationary phase but this does not agree with the findings of Leisinger and Margraff (1979) that wild type P. phenazium grown on P-limited media lost viability more rapidly than the pigmentless mutants after cessation of growth. Low levels of PO_4^{3-} at the end of growth trigger phenazine production which is inhibited at higher PO_4^{3-} concentrations (Leisinger and Margraff, 1979). High PO_4^{3-} levels inhibit and low PO_4^{3-} levels stimulate cyanogenesis (Meganathan and Castric, 1977), aflatoxin production (Gupta et al, 1977) and streptomycin, bacitracin, chlortetracycline, actinomycin, novobiocin, oxytetracycline, neomycin, nystatin and polymyxin production (Haavik, 1974). However, bacitracin production by B. licheniformis which fell as the PO_4^{3-} concentration increased from 0.2g/L to 1.0g/L rose as PO_4^{3-} was increased to 10-20g/L indicating no feedback inhibition mechanism and also indicating that the removal of trace metals essential for secondary metabolism by PO_4^{3-} as suggested by Weinberg (1970) was not important in bacitracin formation.

The effect of PO_4^{3-} and other parameters of the cell environment on the longevity of P. aeruginosa has been invesigated (Gentry et al, 1971). P. aeruginosa grown in glycerol medium containing PO_4^{3-} in the range 0.5-700mM lost viability between the 3rd and 10th day of incubation However, cells grown in concentrations of 0.1-0.25mM PO_4^{3-} still contained 10^9 viable cells per ml 4 to 5 weeks later and 0.6×10^8 at 3 months.

The importance of PO_4^{3-} concentration and whether or not PO_4^{3-} is growth limiting on culture pH was shown since in PO_4^{3-} lethal cultures a marked decrease in pH occurred following cessation of logarithmic growth and death occurred only in cultures containing lethal PO_4^{3-} concentration and low pH. When cells from lethal PO_4^{3-} cultures were exchanged with cells from non-lethal PO_4^{3-} cultures at various times they died only if continuously exposed to the lethal PO_4^{3-} concentration during the dying period.

Chromate and arsenate which possess similar chemical structures to PO_4^{3-} are unable to alter the PO_4^{3-} lethal effect but vanadate completely protects cells from the lethal action of PO_4^{3-} even when added 22 hours after inoculation of cells into PO_4^{3-} lethal media (Gentry et al, 1971).

1.3.9. Phosphate and Plasmid RP1

The presence of RP1 plasmid affects the nutritional and cell envelope composition of PO_4^{3-} depleted cultures. Dean et al (1976) showed that in the chemostat P-limitation favours growth of the plasmid free organism probably because of the additional PO_4^{3-} requirement for replication of the plasmid DNA.

The nutritional requirements of E. coli with or without the plasmid have been shown to differ (Klemperer et al, 1979a). Cells with the plasmid required higher levels of P, Mg and K to reach the same absorbence as R^- cells. R^+ cells had a shorter doubling time than R^- cells although the change in growth rate with PO_4^{3-} concentration was the same for both cultures at low PO_4^{3-} concentrations. Studies on uptake kinetics revealed that R^- cells have a dual PO_4^{3-} uptake system of high and low affinity but in the R^+ cells only the high affinity mechanism was detected.

2. MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Test Organism

The test organism used was Pseudomonas aeruginosa N.C.T.C. 6750 obtained from the National Collection of Type Cultures, London, as a freeze dried culture. The organism was reconstituted in nutrient broth at 37^{o}C for 24 hours. Strain purity was checked by plating on nutrient agar. Two colonial types were identifiable. The rough colony type having a typical pseudomonad form was chosen for further work and maintained on nutrient agar slopes at room temperature. Strain identity was checked at regular intervals. P. aeruginosa 6750 was 'O' serotype polyagglutinable, bacteriophage type 44/F8/109/352/1214.

2.1.2. Glassware

Glassware was treated using a standard procedure:

(i) 24 hour immersion in cold 5% Decon (Decon Labs. Ltd.) followed by rinsing in distilled water.

(ii) 24 hour immersion in 1% v/v hydrochloric acid.

(iii) 6 washes in glass distilled water.

(iv) 3 washes in distilled deionised water.

For iron limitation experiments the glassware was washed with 0.1% EDTA (B.D.H. Ltd.) following the six distilled water washes and finally washed in 3 changes of distilled deionised water.

2.1.3. Chemicals

The chemicals used in the preparation of all media except that for iron and glucose limitations were Analytical Reagent Grade (B.D.H. Ltd.).

For iron limitation, Aristar reagents (B.D.H. Ltd.) and Ultrar grade (Hopkins and Williams, Chadwell Heath) were used, when available. Aristar glucose was used throughout.

Glass distilled deionised water was used for media preparation.

Polymyxin B sulphate (Aerosporin) was obtained in vials containing 500,000 international units, a gift from Burroughs Welcome and Company, London. Stock solution at 50,000 I.U. ml^{-1} was prepared in distilled water and stored at $4^{o}C$ for up to one week.

E.D.T.A. (ethylene diaminetetra-acetic acid, disodium salt) was obtained from Hopkin and Williams Ltd.

Trypsin, Ribonuclease, 2 keto-3-deoxyoctonic acid (KDO) and phospholipids (Diphosphatidyl glycerol, Phosphatidyl ethanolamine and Phosphatidyl glycerol) were obtained from Sigma Chemical Company, London.

2.1.4. Buffer Solutions

For most experiments using defined media, MOPS (3-N-morpholinopropane sulphonic acid), a zwitterionic buffer (Hopkins and Williams Ltd.) was used at a concentration of 0.06M. The pH of the buffer was adjusted to pH 7.8 using 10M NaOH. For sulphate limitation work ammonium acid phosphate buffer (0.0075M) was used. This buffer was a 40:1 molar ratio of $(NH_4)_2HPO_4$ and $NH_4H_2PO_4$.

2.1.5. Media

Nutrient broth (Oxoid C.M.1.) and nutrient agar (Oxoid C.M.3.) were used.

The composition of the chemically defined medium (C.D.M.) used in this study is in Table 3. 25ml of medium was prepared in 100ml Ehrlenmeyer flasks which were incubated overnight at 37°C to check sterility before use.

Table 3

Composition of Chemically Defined Medium*

Constituent	Final Concentration
$(NH_4)_2SO_4$	4.0×10^{-2}M
Na_2HPO_4	3.2×10^{-3}M
Glucose	4.0×10^{-2}M
KCl	6.2×10^{-4}M
NaCl	5.0×10^{-4}M
$FeSo_4.7H_2O$	6.2×10^{-6}M
$MgSO_4.7H_2O$	4.0×10^{-4}M
MOPS	6.0×10^{-2}M
Deionised water	-

*This medium was used for all limitation studies except sulphate.

The pH before inoculation 7.8 was adjusted with 10M NaOH.

The composition of the chemically defined medium for sulphate limited growth is shown in Table 4.

Table 4

Composition of Chemically Defined Medium used for Sulphate Limitation Studies

Constituent	Final Concentration
Na_2HPO_4	$3.2 \times 10^{-3}M$
NH_4Cl	$4.0 \times 10^{-2}M$
NaCl	$5.0 \times 10^{-4}M$
$FeCl_2.4H_2O$	$6.2 \times 10^{-6}M$
KCl	$6.2 \times 10^{-4}M$
$MgCl_2.6H_2O$	$4.0 \times 10^{-4}M$
Glucose	$4.0 \times 10^{-2}M$
Na_2SO_4	$5.2 \times 10^{-5}M$
Acid Ammonium Phosphate	$7.0 \times 10^{-3}M$

The individual media constituents were prepared as 100 times and 25 times stock solutions and stored at room temperature. The ammonium sulphate, potassium chloride, sodium chloride and magnesium sulphate solutions were sterilised by autoclaving at 121°C for 15 minutes. All other solutions were sterilised by membrane filtration through 0.2μ membranes.

In order to prevent ferrous iron precipitation, 1ml conc. hydrochloric acid was added to every litre of $FeSO_4.7H_2O$ stock solution prior to sterilisation.

The neutralisation media used for viable count estimations were nutrient broth (Oxoid C.M.1.) for E.D.T.A. and lecithin/glycerol broth (Kohn et al, 1963) for Polymyxin.

The lecithin/glycerol broth was prepared as below:

Medium I		Medium II	
Nutrient broth	0.8g	Lecithin	0.5g
Water	100ml	Glycerol	4.0g
	pH 6.8	Medium I	100ml
			pH 6.8

The egg yolk lecithin (B.D.H. Ltd.) was dissolved in the minimum amount of absolute alcohol and a small volume of medium I added. The alcohol was evaporated by heating over a boiling waterbath. The glycerol was added and the remaining medium I added. The complete medium was dispensed in 9ml amounts and autoclaved at 121^{o}C for 15 minutes.

2.1.6. Apparatus

Spectrophotometers

Optical density measurements were made using a Unicam S.P. 1800 U.V. Spectrophotometer (Pye-Unicam, Cambridge).

Spectrophotometer cuvettes

1cm path length semi-micro optically matched glass cells were used for optical density measurements made at 470 nm. These were obtained from Chandos Intercontinental, Stockport.

Water Deioniser

Deionised water was produced following distillation of tap water in an Autostill 8+ (Jencons, Hemel Hempstead) using an Elgastat B126 deioniser (The Elga Group, Buckinghamshire).

Shaking Waterbath

A Mickle reciprocating thermostatically controlled waterbath obtained from Cam Lab Ltd., Nuffield Road, Cambridge and a Grant SS 30 shaking waterbath from Grant Instruments, Cambridge were used in this study.

pH Meter

pH measurements were carried out on a pH meter 22 (Radiometer, Copenhagen) with a type E5021 micro electrode. Sample volumes were 50 µl. Radiometer buffer type S1510 pH 7.383 ± 0.005 at 37°C was used as a reference standard.

Filtration Systems

Sterifil Aseptic Filtration System (Millipore U.K. Ltd., London) were used in conjunction with 47mm diameter 0.2 µ membrane filters supplied by Millipore or V. A. Howe, London. 25mm diameter, 0.2 µ membranes (Millipore and V. A. Howe) were used in Swinnex filters (V. A. Howe, London).

Eppendorf pipettes and tips

Eppendorf pipettes delivering volumes of 1000, 500, 250, 100, 90, 75, 50, 25, 10 and 5 µl, and disposable tips were obtained from Anderman and Company, East Molesey, Surrey.

Centrifuges

The centrifuges used in this work were the MSE super minor bench centrifuge for general work, the MSE High Speed 18 (Measuring and Scientific Equipment Ltd., Crawley) and I.E.C. B20 (International Equipment Co., USA) for harvesting cells for cell wall analyses. The MSE Superspeed 50 was used for the density gradient centrifugation of inner and outer membranes.

2.1.7. Membrane Filter Preparation

Brown, Farwell and Rosenbluth (1969) prewashed their membrane filters to remove light absorbing chemicals. Before assembly, membranes were washed three times in deionised water.

2.2 BASIC EXPERIMENTAL METHODS

2.2.1. Reproducibility of Growth and Lysis Parameters

2.2.1.1. Total Counts

Total counts were performed on stationary phase P. aeruginosa grown in C.D.M. The culture was killed by addition of 5% v/v formaldehyde solution. Counts were done in "Improved Neubauer" chambers with a chamber depth of 0.1mm. The culture was diluted to give a count of approximately 10 cells per small square. Before counting the cells were allowed to settle for 30 minutes, whilst the chamber was stored in a petri dish containing moist blotting paper to prevent drying. The chamber was viewed using a phase contrast microscope. Five replicate counts were made in five counting chambers. The results are shown in Table 5 and subjected to an analysis of variance (Table 6).

Table 5

Total Counts per Slide for Five Replicate Counts

Slide / Replicates	1	2	3	4	5
1	572	560	566	568	578
2	578	572	568	572	560
3	566	567	576	560	574
4	580	566	562	568	566
5	572	574	560	576	568
Totals T	2868	2840	2832	2844	2847

n = number of counts per slide = 5

m = number of slides = 5

m.n. = total number of observations = 25

1. $\Sigma x^2 = 8101721.00$

2. $\frac{\Sigma T^2}{n} = 8100998.60$

3. $\frac{(\Sigma x)^2}{n.m} = 8100854.44$

Table 6

Analysis of Variance of Five Replicate Counts

Source of Variance	Sum of Squares	Degrees of Freedom	Mean Squares	Variance Ratio (F)
Between Slides	(2) - (3) 144.16	m - 1 4	36.04	0.998
Within Slides	(1) - (2) 722.4	n.m - m 20	36.12	

Tabulated values of 'F' for 4/20 degrees of freedom are 2.87 and 4.43 at the 5% and 1% levels respectively. Therefore the variation between counts is not significantly greater than the variation within counts.

2.2.1.2. Reproducibility of Viable Count Estimations for P. aeruginosa

0.1ml of five replicate tenfold dilutions of a log phase culture of P. aeruginosa were surface spread onto five nutrient agar plates. The plates were counted following incubation at 37°C for 48 hours. The results are shown in Table 7 , and were submitted to an analysis of variance (Table 8).

Table 7

Colony Counts per Plate for Five Replicate Counts

Plate \ Count	1	2	3	4	5
1	115	106	102	100	114
2	110	108	117	93	109
3	113	105	116	101	105
4	105	117	114	92	114
5	114	106	97	112	106
Totals T	557	542	546	498	548

n = number of observations per count = 5

m = number of counts = 5

n.m. = total number of observations = 25

1. $\Sigma x^2 = 290911.00$

2. $\frac{\Sigma T^2}{n} = 290087.40$

3. $\frac{(\Sigma x)^2}{n.m} = 289659.24$

Table 8

Analysis of Variance of Five Replicate Counts

Source of Variance	Sum of Squares	Degrees of Freedom	Mean Squares	Variance Ratio (F)
Between Counts	(2) - (3) 428.16	m - 1 4	107.04	2.59
Within Counts	(1) - (2) 823.6	nm - m 20	41.18	

The tabulated values of 'F' for 4/20 degrees at 5% and 1% levels are 2.87 and 4.43 respectively. Therefore the variation between counts is not significantly greater than the variation within counts.

2.2.1.3. Reproducibility of Growth Rate Constants for Growth of *P. aeruginosa* in Chemically Defined Media on Different Days

The growth rate constants of five replicate cultures of *P. aeruginosa* grown in C.D.M. on three successive days was determined. Growth curves were constructed from optical density readings (E_{470}) taken at regular time intervals. The linear part of the growth curve (exponential growth phase) was considered in the calculation of the growth rate constants 'μ'.

The growth rate constant 'μ' is determined as follows:

$$\mu = \frac{\log N_2 - \log N_1}{t_2 - t_1} \qquad (1)$$

N_2 and N_1 and the E_{470} readings at times t_2 and t_1 respectively.

$$\mu = \frac{\log (N^2/N^1)}{t_2 - t_1} \qquad (2)$$

in the special case where $N_2 = 2N_1$ i.e. a doubling of the population, equation (2) becomes

$$\mu = \frac{\log 2}{t.d.} = \frac{0.301}{t.d.} \qquad (3)$$

where t.d. is the doubling time of the culture.

The calculated growth rate constant for the fifteen cultures are tabulated in Table 9 . The reproducibility was assessed by an analysis of variance (Table 10).

Table 9

Growth Rate Constant x 10^{-3} min^{-1} for Five *P. aeruginosa* Cultures on Three Successive Days

Flasks \ Days	1	2	3
1	4.667	4.178	5.145
2	4.894	4.561	4.778
3	5.017	4.561	4.934
4	5.017	4.778	5.017
5	4.778	4.778	4.895
Totals T	24.373	23.456	24.768

n = number of observations per day = 5

m = number of days 3

n.m = total number of observations = 15

1. $\Sigma x^2 = 351.759$

2. $\frac{\Sigma T^2}{n} = 351.534$

3. $\frac{(\Sigma x)^2}{n.m} = 351.354$

Table 10

Analysis of Variance of Growth Rate Constants on Three Successive Days

Source of Variance	Sum of Squares	Degrees of Freedom	Mean Squares	Variance Ratio 'F'
Between Days	(2) - (3) 0.182	m - 1 2	0.0910	4.89
Within Days	(1) - (2) 0.223	n.m - m 12	0.0186	

The tabulated values of 'F' for 2/12 degrees of freedom at 1% and 5% levels are 6.93 and 3.89 respectively. Therefore the variation within days is not significantly greater than the variation between days at the 1% level.

The mean was 4.84×10^{-3}/min which is equivalent to a doubling time of 62 min.

2.2.1.4. Reproducibility of Lysis by 40 i.u. ml^{-1} Polymyxin B for Replicate *P. aeruginosa* Cultures Grown in Phosphate Depleted C.D.M. on Three Consecutive Days

Five replicate cultures of *P. aeruginosa* grown in phosphate depleted C.D.M. were lysed by 40 i.u. ml^{-1} of Polymyxin B. The experiment was

repeated on three consecutive days.

The percentage lysis was determined following 60 minutes exposure to Polymyxin B.

$$\% \text{ lysis} = \frac{E_{470\ t=0} - E_{470\ t=60}}{E_{470\ t=0}} \times 100$$

where $E_{470\ t=0}$ is the optical density of the culture at time zero, $E_{470\ t=60}$ is the optical density of the culture after 60 minutes drug exposure. Correction was made for lysis of the control culture to which an equivalent volume of water was added.

The lysis results are shown in Table 11 and an analysis of variance is shown in Table 12.

Table 11

Percentage Lysis after 60 minutes Exposure to Polymyxin of Five Replicates of P. aeruginosa on Three Consecutive Days

Replicates \ Days	1	2	3
1	47.9	54.0	51.8
2	45.9	52.1	48.8
3	47.2	49.2	50.7
4	46.3	45.2	50.9
5	47.2	52.1	48.7
Totals T	234.5	252.6	250.9

n = number of observations per day = 5

m = number of days = 3

n.m = total number of observations = 15

1. $\Sigma x^2 = 36406.76$

2. $\frac{\Sigma T^2}{n} = 36349.56$

3. $\frac{(\Sigma x)^2}{n.m} = 36309.60$

Table 12

Analysis of Variance of Lysis Values

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	Variance Ratio 'F'
Between Days	(2) - (3) 39.96	m - 1 4	9.99	1.74
Within Days	(1) - (2) 57.2	n.m - m 10	5.72	

The tabulated values of 'F' for 4/10 degrees of freedom at the 5% and 1% levels are 3.48 and 5.99 respectively. Therefore the variation in lysis values between days are not significantly greater than the variation within days at these levels.

2.2.2. Spectrophotometric measurement

The turbidity of a bacterial suspension is the result of light scattered by the bacterial suspension. The proportion of incident monochromatic light failing to traverse a bacterial culture unchanged represents the sum of the light absorbed and of light scattered by the organism. Absorption is usually negligible unless pigments are produced. When measuring bacterial concentration either scattered light or undeviated light may be measured (Meynell and Meynell, 1971). In practice measurement of undeviated light includes some scattered light. For these studies undeviated light was used with the aid of a Unicam SP 1800 spectrophotometer.

2.2.2.1. Theory of measurement of undeviated light

At low bacterial concentrations the incident light intensity, I_o, and the transmitted light intensity, I, are related by Beer Lambert's Law:

$$I = I_o.10^{-\varepsilon lc}$$

ε = extinction coefficient, l = depth of suspension, c = bacterial concentration.

This equation becomes:

$$\log_{10} I_o/I = \varepsilon.\ l.\ c.$$

$\text{Log}_{10}\ I_o/I$ = optical density of the suspension.

The value of ε differs for different species (Brown, 1919) and for the same species treated in different ways (Spaun, 1962).

2.2.2.2. Selection of wavelength

Points to be considered when selecting a suitable wavelength for optical density measurements include:-

1. The medium constituents should exhibit little or no absorption.
2. Metabolic products should also exhibit little or no absorption.
3. The wavelength used should impart maximum sensitivity to changes in optical density.

A wavelength of <380nm should be avoided for growth curve experiments since proteins and nucleic acids absorb light in this range. Nutrient broth grown cultures are frequently measured at 650-660 nm since broth exhibits little light absorption at these wavelengths. Pyocyanin and fluorescein, pigments produced under certain conditions by P. aeruginosa, exhibit absorption maxima at 388 nm and 410 nm respectively. The absorption is minimal at 460 nm (Watkins, 1970). An absorption/wavelength scan on culture filtrate from cells grown in C.D.M. showed that at 470 nm absorption by pigments and metabolic products was negligible. It was decided to use this wavelength for spectrophotometric growth curve measurements.

2.2.2.3. Relationship between real and observed optical density for cultures of P. aeruginosa grown under various nutrient limitations

Stationary phase cultures of P. aeruginosa grown under various nutrient limited conditions were diluted in C.D.M. lacking glucose and the ion under test. Graphs of E_{470} against % of original suspension were plotted for each nutrient limitation. A typical graph is shown in Fig. 10 A linear relationship existed to an optical density which varied with the limitation. Above these E_{470} values the increase in optical density was less than predicted. Table 13 shows the optical density at which departure from linearity resulted for each limitation.

Fig. 10 Comparison for real and observed optical density for glucose limited P. aeruginosa

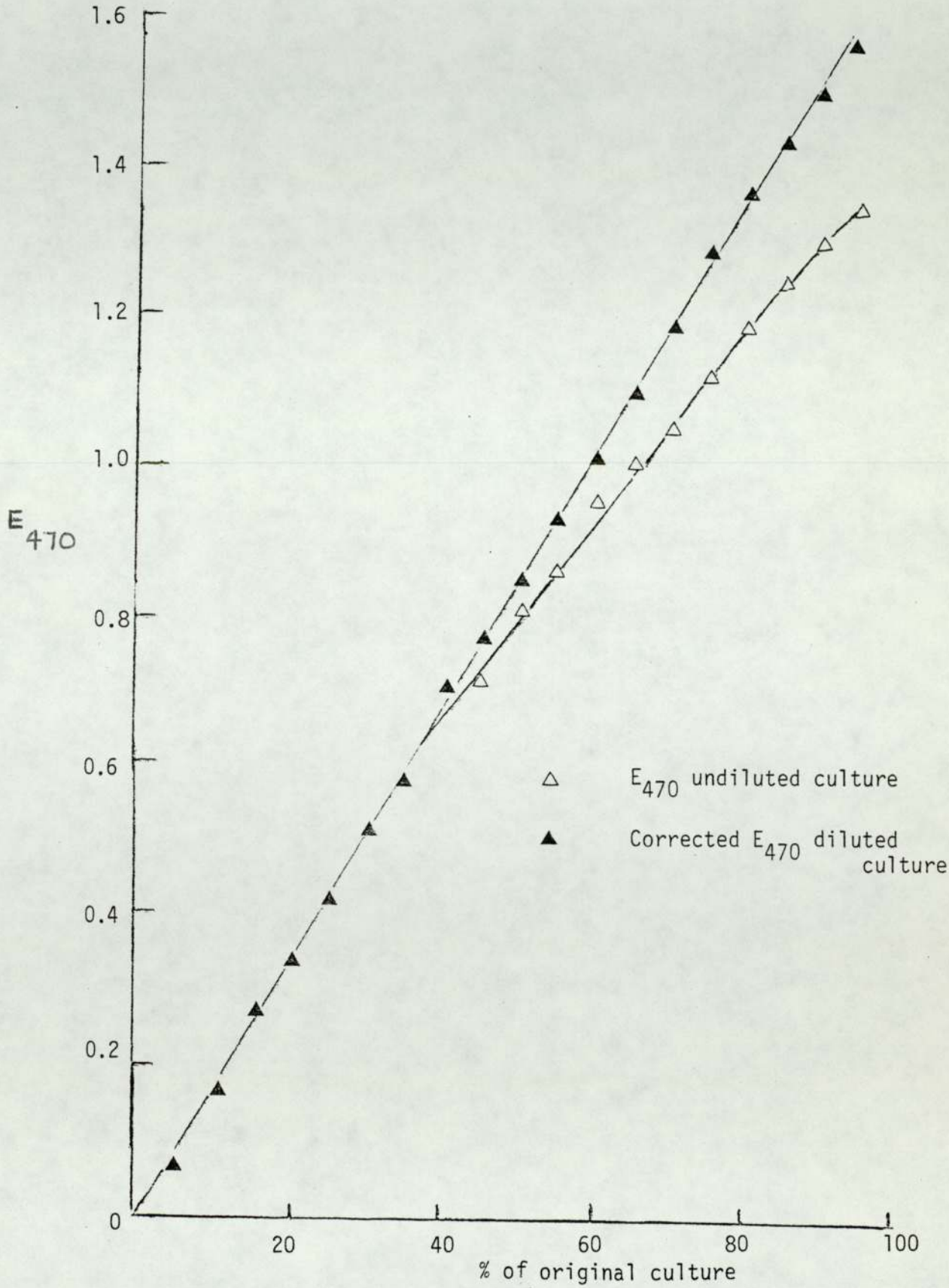


Table 13

Deviation from Beer Lambert's Law for P. aeruginosa grown under limited conditions

Limitation	Deviation from Linearity (E_{470})
NH_4^+	0.48
PO_4^{3-}	0.68
Glucose	0.65
SO_4^{2-}	0.58
Mg^{2+}	0.40
Fe^{2+}	0.60
K^+	0.44

If suspensions were diluted to give readings in the range 0.1 - 0.4 then linearity was restored, and Beer Lambert's Law obeyed over the range studied. In these studies it was decided that for optical densities (E_{470}) greater than 0.4 the culture would be diluted with the appropriate diluent before measurement.

2.2.2.4. Optical density measurement procedure

Initial work involved plotting growth curves i.e. the variation of E_{470} with time for cultures of P. aeruginosa depleted in various nutrients. Optical density measurements were made in optically matched 1cm path length semi-micro glass cuvettes. Readings were taken at various time intervals at the beginning of the growth curve and at half hourly intervals or less when departure from log-linear growth was approached. For optical densities up to an E_{470} of 0.4 the cultures were returned to the flask following optical density measurement. Above this optical density dilution of the culture was necessary in order that Beer Lambert's Law was obeyed and the contents of the cuvettes were then discarded.

Between readings cuvettes were rinsed with deionised distilled water and allowed to drain dry.

2.2.2.5. Relationship between Optical Density and Viable Count for *P. aeruginosa* grown in C.D.M.

25ml of C.D.M. was inoculated with *P. aeruginosa* and shaken at 120 throws/min. at 37°C. Optical density measurements were made and at the same time 1ml was removed for culture. Duplicate surface spread plates were made from appropriate tenfold dilutions using nutrient agar. Counts were made following incubation at 37°C for 48 hours. Growth curves obtained are shown in Fig. 11. Graphs with similar characteristics were obtained with growth rate constants for viable counts and E_{470} of 4.84×10^{-3} min^{-1}. The relationship between viable count and optical density was observed to be linear to an E_{470} of 2.0 and a viable count of 1.63×10^{9} organisms ml^{-1} (Fig. 12). The results obtained were used to calculate the correlation coefficient where:

$$r = \frac{c}{S^2x.S^2y}$$

c is the covariance $\varepsilon(x.y - \bar{x}\bar{y})/n-1$, '$S^2x$' the variance of the optical density readings $\varepsilon(x - \bar{x})^2/n - 1$, and '$S^2y$' the variance of the viable count determinations $\varepsilon(y - \bar{y})^2/n - 1$.

For the whole growth curve n = 16

correlation coefficient r = 0.9966

For log-linear growth phase n = 12

correlation coefficient r = 0.9982

The tabulated values of r for 30 and 22 degrees of freedom at 5% and 1% significance levels are 0.377, 0.495, and 0.475, and 0.625. Thus the correlation between optical density and viable counts was significant within these levels. Both growth curves exhibited similar characteristics and the growth rate constants were 4.84×10^{-3} min^{-1}.

Fig. 11 Comparison of colony count and E_{470} for *P. aeruginosa* grown in chemically defined medium

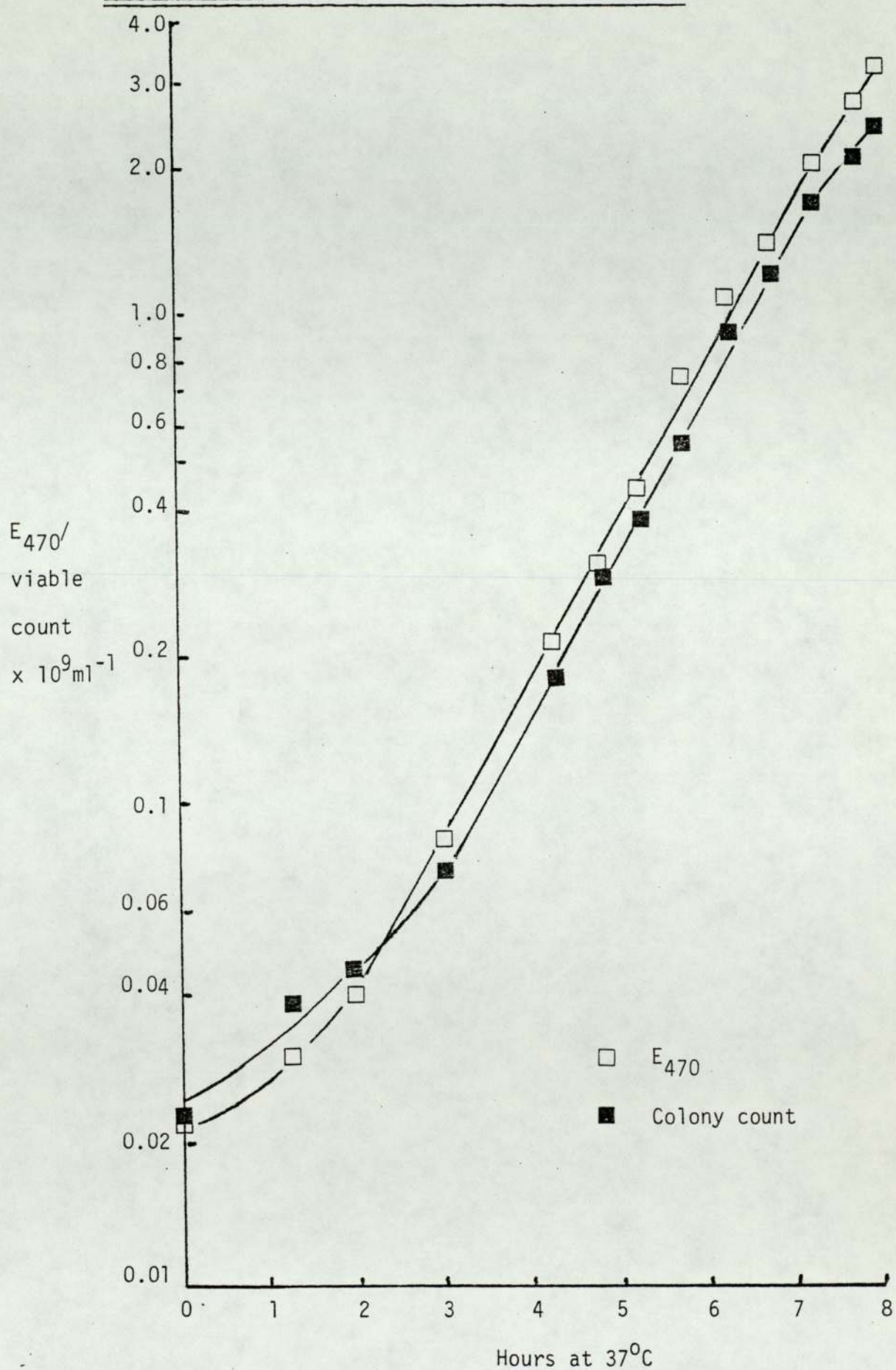
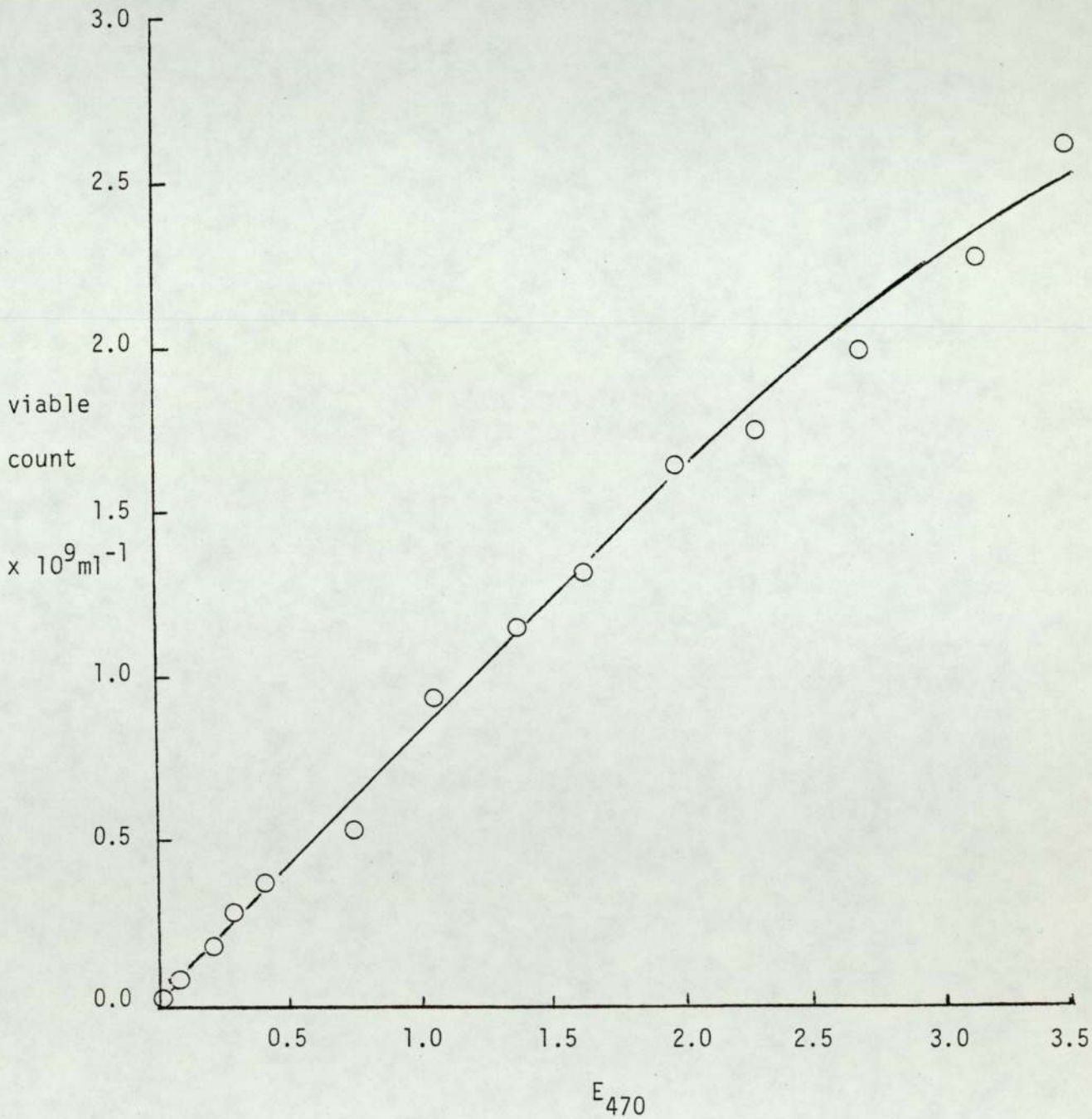


Fig. 12

Relationship between colony count and E_{470} for *P. aeruginosa* grown in chemically defined media



2.2.3. Production of cells for cell envelope chemical analyses

2.2.3.1. Preparation of 2L volumes of batch grown P. aeruginosa.

Approximately 10 litres of bacterial cells at an E_{470} of 0.8 was required to produce enough outer membranes for cell envelope chemistry. Cells were grown in 2 litre volumes of C.D.M. containing various concentrations of PO_4^{3-} in 5 x 5L conical flasks. The C.D.M. lacking glucose phosphate and iron was autoclaved at 121°C to sterilise it. Membrane filtered glucose, phosphate and iron solutions were added to the sterile basal medium. The complete medium was incubated at 37°C for 48 hours prior to use ot check for sterility. The formulation of C.D.M. used is shown in Table 14(a) and (b).

The medium was inoculated with a stationary phase culture of P. aeruginosa which was grown in 25ml of C.D.M. identical in formula to that of the large volume culture medium. The inoculated medium was incubated for approximately 18 hours at 37°C, aeration being provided by a magnetic follower. The speed of the follower was adjusted such that the vortex produced extended down to the follower. This ensured the production of air bubbles in the medium, and the maximum surface area for gaseous exchange. The cultures were removed for cell wall preparation 1 to 3 hours after cessation of growth.

Table 14 (a)

Media for 2L broth culture of P. aeruginosa

Constituent	Molarity (M)
$MgSO_4.7H_2O$	4.0×10^{-4}
NaCl	5.0×10^{-4}
KCl	6.2×10^{-4}
$(NH_4)_2SO_4$	4.0×10^{-2}
$FeSO_4.7H_2O$	6.2×10^{-6}
MOPS	6.0×10^{-2}
Water	-

Table 14 (b)

Constituent	Molarity				
	Medium				
	1	2	3	4	5
$(Na_2)HPO_4$	0.8×10^{-4}	1.33×10^{-4}	2.0×10^{-4}	2.67×10^{-4}	6.68×10^{-4}
Glucose	0.50×10^{-2}	0.46×10^{-2}	0.44×10^{-2}	0.40×10^{-2}	0.40×10^{-2}

2.2.3.2. Preparation of 10L volumes of chemostat grown P. aeruginosa

Preparation of 10 L volumes of bacterial cells was performed in a chemostat of working volume 705mls. The chemostat design was similar to that of the small volume chemostat described later. C.D.M. formulated as in Table 15 was used to prepare 14 litre volumes of media. 14L C.D.M. lacking glucose, phosphate and iron was prepared in a 20 litre aspirator and autoclaved at 121^{o}C for 2 hours. Sterile membrane filtered solutions of glucose phosphate and iron were added to the basal medium following autoclaving and the complete medium incubated for 48 hours prior to use to check for sterility. A dilution rate of 0.17 hr^{-1} was chosen for these experiments. At this low dilution rate 10 litres of cells were produced in approximately 72 hours. In order to prevent growth of the cells collected from the chemostat over this period the cells were collected in glass vessels whose walls were cooled with ethylene glycol circulated from a refrigerated waterbath. The temperature of the cells was 4 - 6^{o}C. Cells were harvested from the cooled vessels every 12 hours. The cells were centrifuged at 12,000 'g' for 15 minutes and resuspended in Tris buffer (30mM pH 8.0) and washed once in this medium.

Following centrifugation for 10 minutes at 16,000 'g' the cells were resuspended in 5ml of 20% sucrose - 30mM Tris-HCl (pH 8.0) and stored at -20^{o}C for subsequent use. When 10L of cells had been collected the 5ml portions were pooled for outer membrane preparation.

Table 15

Media for carbon and phosphate limited chemostat grown *P. aeruginosa*

Constituent	Carbon Limited growth	Phosphate Limited growth
Glucose	6.0×10^{-3}	4.0×10^{-2}
$(Na_2)HPO_4$	4.0×10^{-3}	1.5×10^{-4}
$(NH_4)_2SO_4$	4.0×10^{-2}	4.0×10^{-2}
NaCl	5.0×10^{-4}	5.0×10^{-4}
KCl	6.2×10^{-4}	6.2×10^{-4}
$FeSO_4.7H_2O$	6.2×10^{-6}	6.2×10^{-6}
$MgSO_4.7H_2O$	4.0×10^{-4}	4.0×10^{-4}
MOPS	6.0×10^{-2}	6.0×10^{-2}

2.2.3.3. Preparation of cell walls of *P. aeruginosa*

Whole cell suspensions were broken by passage three times through a cooled (4°C) French Pressure Press (Aminco, USA) at a pressure of 1,055 Kg/sq.cm. Following breakage the preparation was centrifuged at 3,000g for 20 minutes to remove unbroken whole cells. The supernatant was centrifuged at 17,000g for 15 minutes. The pellet was washed twice further with water. The preparation was then purified by enzyme digestion with trypsin and ribonuclease (0.5mg/ml) for 30 minutes at 44°C. Following digestion the cell walls were washed twice with 0.9% saline to remove adsorbed cations (Tempest and Strange, 1966) and twice with water. The purified cell walls were dried over phosphorus pentoxide *in vacuo* to constant weight.

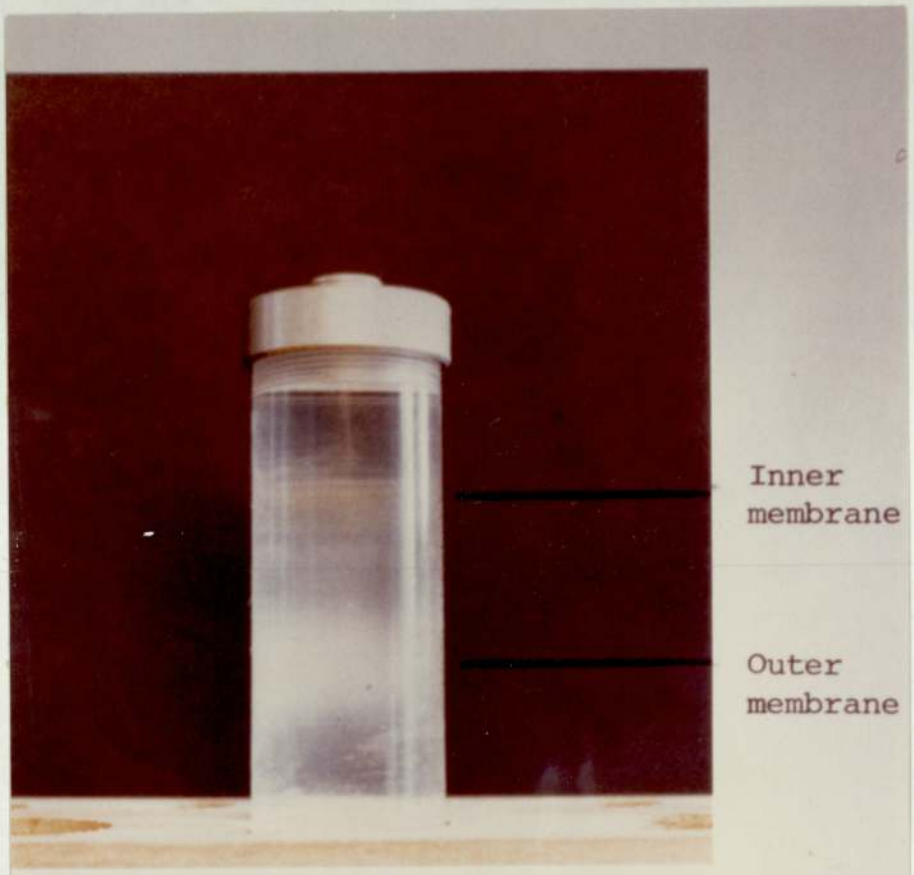
2.2.3.4. Outer membranes of P. aeruginosa

Inner and outer membranes of P. aeruginosa were prepared by a modification of the method of Hancock and Carey (1979).

The cells from 5 x 2L batch culture were harvested by centrifugation at 12,000g for 15 minutes, followed by washing in Tris-HCl buffer (30mM, pH 8.0). The cells were centrifuged at 16,000g for 10 minutes and the cell pellet resuspended in 25ml 20% sucrose - 30mM Tris-HCl (pH 8.0). The cells were cooled and ribonuclease 2mg and deoxyribonuclease 2mg were added. The cells were broken by 3 - 4 passages through a French Pressure Press. Following breakage, lysozyme 4mg was added and following shaking the enzyme digestion was allowed to proceed at $4^{o}C$ for 30 minutes. The envelope fragments were diluted in Tris-HCl and centrifuged at 3,000g for 10 minutes to remove unbroken cells. The supernatant was centrifuged at 38,000g for 60 minutes and the membrane pellet resuspended in 16ml 20% sucrose-Tris HCl. The suspension was homogenised in a tissue homogeniser (Jencons Ltd.). The membranes were then prepared for density gradient centrifugation as described by Cozens and Brown (1981). The membranes were layered into 20ml centrifuge tubes in the order: 2ml membranes in 20% sucrose-Tris HCl, 9ml of 60% sucrose-30mM Tris HCl pH 8.0, 9ml of 70% sucrose-30mM Tris HCl pH 8.0. The membranes were centrifuged overnight at 100,000g in a MSE 20^{o} angle rotor centrifuge. A typical membrane separation obtained is shown in Plate 1. The membrane fractions were removed from the density gradient from the top using a pipette. The separated fractions were washed in an equal volume of Tris-HCl and centrifuged at 38,000g for 1 hour. The membrane fractions were washed in Tris-HCl and centrifuged at 38,000g for 45 minutes. The pellets were finally homogenised in water to give a dry weight of approx.

PLATE 1

PLATE 1



Inner and outer membranes of P.aeruginosa separated by sucrose density gradient centrifugation

10mg ml^{-1}. Accurate dry weights were performed as described in section 3.2.1.5. The membrane fractions were stored until required in a deep freeze at -20°C.

2.2.3.5. Extraction of Lipids

The procedure used was that of Ames (1968). The lipids in the outer and inner membrane fractions were extracted in a monophasic system in methanol: chloroform and water (i.e. membrane fractions) (2:1:0.8v/v). The lipids were separated from the water soluble material by dilution of the extraction mixture with one volume of chloroform followed by one volume of water. The chloroform layer was filtered through phase separation paper (Whatman 1PS) into a tared tube. The remaining solution was re-extracted with methanol; chloroform and the extract filtered and pooled with the previous extract. The chloroform layers were evaporated to dryness on a rotary evaporator. The lipid was dried to constant weight in a vacuum dessicator over P_2O_5. The weight of readily extractable lipid (R.E.L.) was determined.

The dried lipid was dissolved in 1ml of choroform:methanol (2:1) and 20mls of diethyl ether added, and the solutions left overnight at -10°C. Centrifugation resulted in no deposit of ether-insoluble β poly hydroxybutyric acid in either the outer or inner membrane fractions. The ether was evaporated to dryness and the lipid residue dissolved in 1ml of chloroform:methanol (2:1) followed by addition of 20mls of acetone to precipitate phospholipids. Phospholipid precipitation was performed overnight at -10°C. Following centrifugation the phospholipid pellet was dried in a vacuum dessicator over P_2O_5 and the weight of phospholipid determined. The acetone fraction was evaporated to dryness on a rotary evaporator in tared glass tubes and the residue of fatty acid and neutral lipids dried to constant weight over P_2O_5 in a vacuum dessicator.

The phospholipids and fatty acid and neutral lipids were redissolved in 1ml chloroform:methanol (2:1) and stored at -10^{o}C.

2.2.4. Chemical assay procedures

2.2.4.1. Total protein

Outer and inner membrane preparations were assayed for total protein using the method of Lowry, Rosebrough, Farr and Randall (1951).

Bovine serum albumen standards in the range 0 to 250µg ml^{-1} were prepared. The mean of three separate sets of determinations of each standard was used to prepare the standard graph (Fig. 13).

Assay reproducibility was assessed using triplicate values from ten replicate samples of an unknown weight of outer membranes in a known volume of water. The results are shown in Table 16.

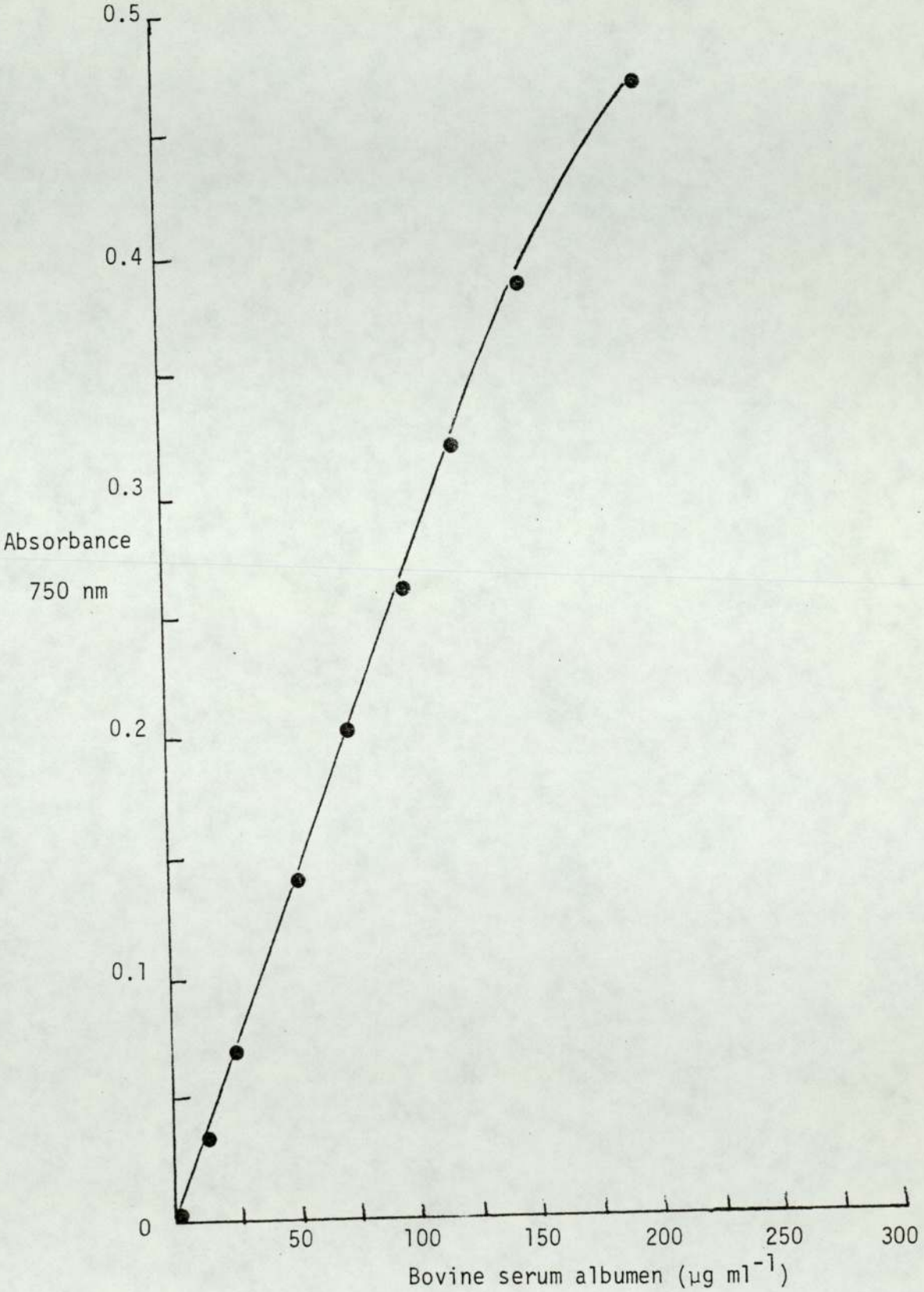
Table 16

Protein assay reproducibility

Replicates	Protein concentration µg ml^{-1}
0.099	38.0
0.099	38.0
0.104	40.0
0.106	41.0
0.107	42.0
0.095	37.5
0.103	39.5
0.101	38.3
0.095	37.5
0.099	38.0
Mean	38.98
Coeff of variation	4.03%

Fig. 13 Protein Calibration Curve

(Lowry et al, 1951)



Outer membrane preparations were diluted to contain approximately 200µg per ml dry weight and assayed.

2.2.4.2. Assay of magnesium and calcium

Cell wall preparations were assayed by atomic absorption spectroscopy for magnesium and calcium, using a Pye-Unicam spectrophotometer (Pye-Unicam Instruments Ltd., Cambridge).

Standards containing calcium and magnesium were prepared in 1% v/v hydrochloric acid containing 1% w/v Lanthanum ions. Lanthanum ions were added to overcome suppression of absorbance of Ca^{2+} ions due to phosphate ions (Willis, 1961). 0.5ml of cell wall fractions containing 10mg dry weight ml^{-1} were digested in 2ml of concentrated nitric acid containing Lanthanum ions (30µg ml^{-1}). The samples were boiled to dryness and when cool the ash was dissolved in 0.4ml of concentrated hydrochloric acid. The solution was transferred to a 10ml volumetric flask and the digestion tube washed with a solution of 1% La^{3+} in 1% hydrochloric acid. The volume was finally adjusted to 10ml. If necessary samples were diluted in analar water. Fig. 14 shows the standard calibration curve.

The reproducibility of the assay was determined by measuring the calcium and magnesium content of 5 replicates of an unknown weight of envelopes in a known volume of water. The results are shown in Table 17.

Fig. 14

Atomic absorption spectrophotometric assay of magnesium and calcium

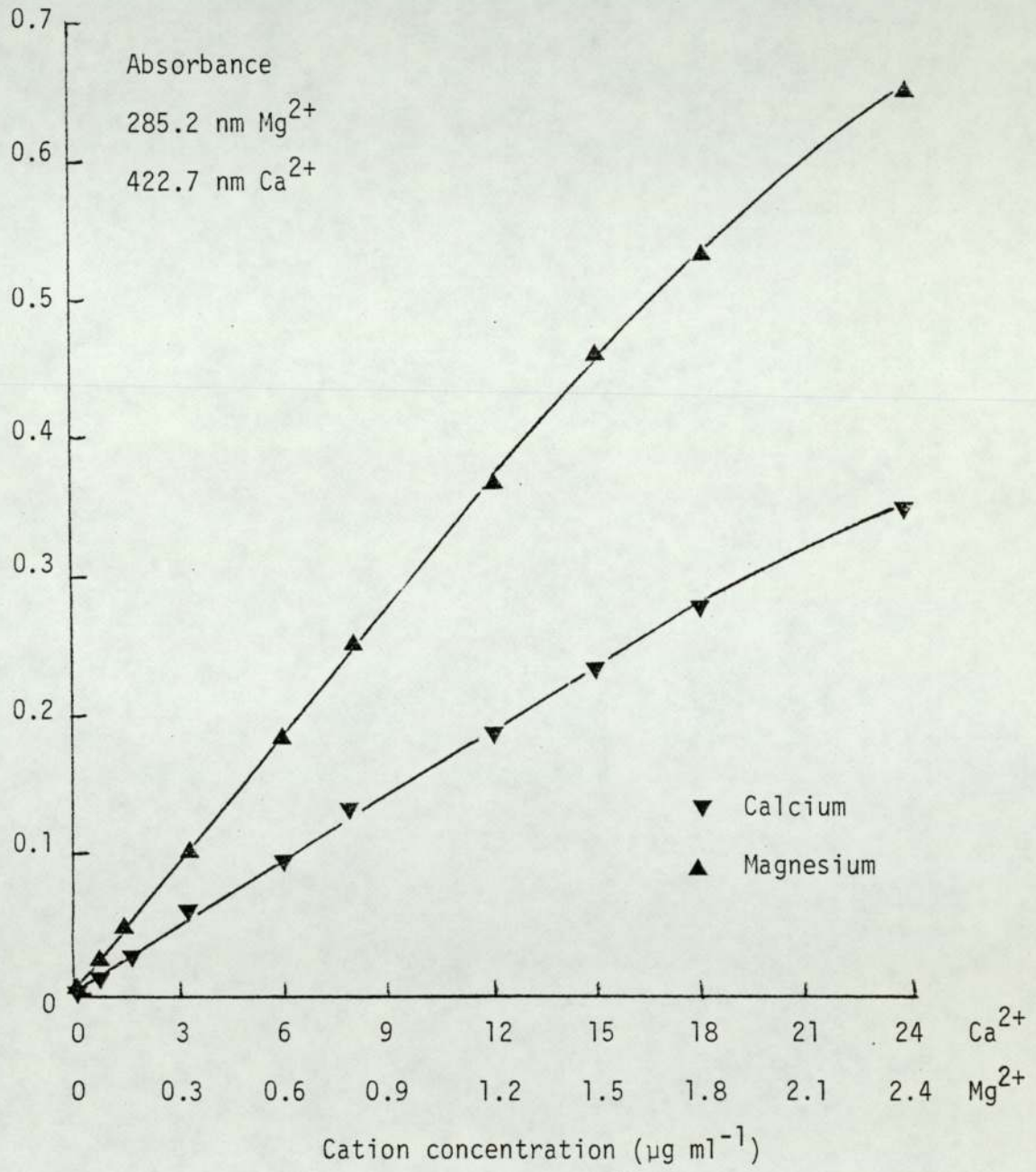


Table 17

Reproducibility of calcium and magnesium assays

Replicates	Magnesium $\mu g\ ml^{-1}$	Calcium $\mu g\ ml^{-1}$
1	0.65	7.2
2	0.62	7.0
3	0.65	7.4
4	0.63	7.0
5	0.63	7.3
Mean	0.64	7.18
Coefficient of variation	2.10%	2.49%

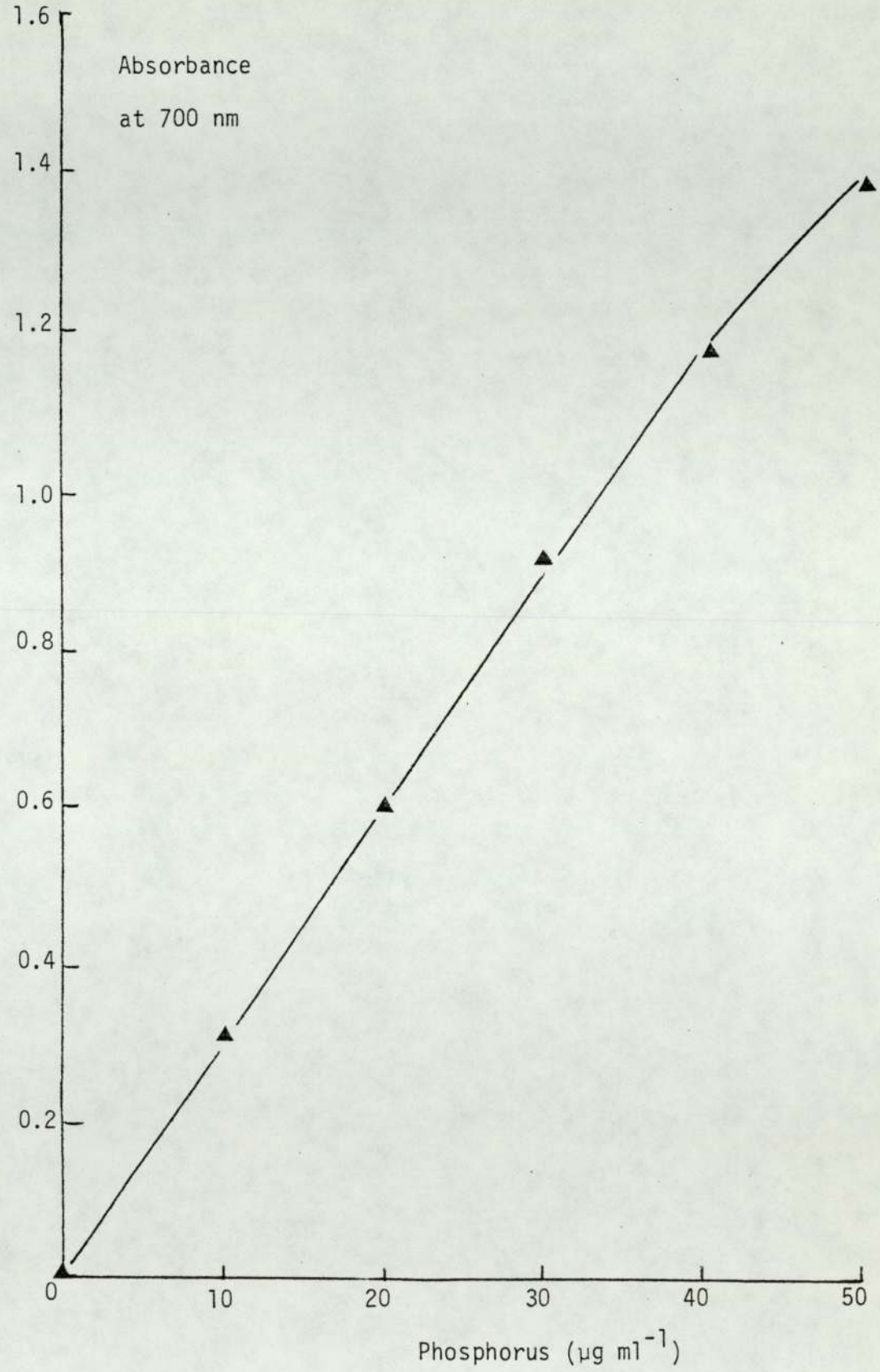
2.2.4.3. Total phosphorus

Outer membranes were assayed for total phosphorus content by the method of Baginski, Foa and Zak (1967). Standards and unknowns were assayed in triplicate and the mean values taken.

0.1ml samples of the membrane fractions were digested to dryness in concentrated nitric acid containing $50mgL^{-1}$ anhydrous calcium carbonate. The residue was redissolved in ascorbic acid/trichloroacetic acid and ammonium molybdate added. The blue colour was allowed to develop for 45 minutes and the reaction terminated by addition of sodium arsenite-sodium citrate. The absorbance was read at 700 mμ, and the standard curve shown in Fig. 15.

Fig. 15 Phosphorus Calibration Curve

(Baginski et al, 1967)



The reproducibility was assessed by determining the P content of 5 replicate samples of an outer membrane preparation suspended in a known volume of water. The results are shown in Table 18.

Table 18

Reproducibility of phosphorus assay

Replicates	Phosphorus $\mu g\ ml^{-1}$
1	20
2	17
3	18
4	21
5	17
Mean	18.6
Coefficient of variation	9.7%

2.2.4.4. Phospholipid phosphorus

Phospholipid phosphorus was determined by the method of Baginski, Foa and Zak (1967). The standard curve shown in Fig. 15 was used and the phospholipid phosphorus determined at the same time as the total phosphorus.

The phospholipids were extracted from 0.1ml of membrane fractions by shaking with absolute alcohol/ether (3:1) followed by centrifugation

at 6000g for 2 minutes. The supernatant was evaporated to dryness and the residue digested in nitric acid and then treated as described in the total phosphorus assay.

C.D.M. Phosphate content assay

To determine the phosphate content of media used in this study a method used in the Clinical Chemistry laboratory at Selly Oak Hospital, Birmingham, U.K. was used (Dr. P. Scott, personal communication).

Reagents

Tween 20 (1%)

Absolute alcohol

Ammonium molybdate solution (2.5%)

Malachite green solution in 40% HCl (0.06%).

The working phosphate reagent was prepared in a 100ml volumetric flask; 10ml ammonium molybdate; 10ml malachite green; 1ml Tween 20 and 0.5ml absolute alcohol; water to 100ml. This reagent is allowed to stand for 10 minutes before use and was prepared fresh daily.

Three ml of the working phosphate reagent was added to 10µl of sample. The mixture was incubated for 10 minutes at 37^{o}C. The resulting colour of the test and working standards (0 - 2.5 mmol/l) were read at 640nm.

The standard curve (not shown) was linear up to 2.5mmol/l and test results were read off directly.

2.2.4.5. K.D.O. (2-keto-3-deoxyoctonic acid)

The method of Ellwood (1970) which was based on the method of Weissbach and Hurwitz (1959) was used.

Samples of cell envelopes (5-10mg) and outer membranes (2mg) were hydrolysed with 0.1 NH_2SO_4 (1ml) at 100^o for 30 minutes. The residues were sedimented by centrifugation at 38,000g for 20 minutes.

The method of Ellwood (1970) was modified in the following ways: samples were treated with periodic acid in H_2SO_4 for 20 minutes at 55^{o}C and following addition of thiobarbituric acid the solution was heated to 100^{o}C for 20 minutes.

A series of standards was prepared using the ammonium salt of 3 deoxyoctulosonic acid in the range 0 - 0.194μmole. The standard curve is shown in Fig. 16.

The extinction of the acidic butanol chromophore was measured in a spectrophotometer at 550nm.

2.2.4.6. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

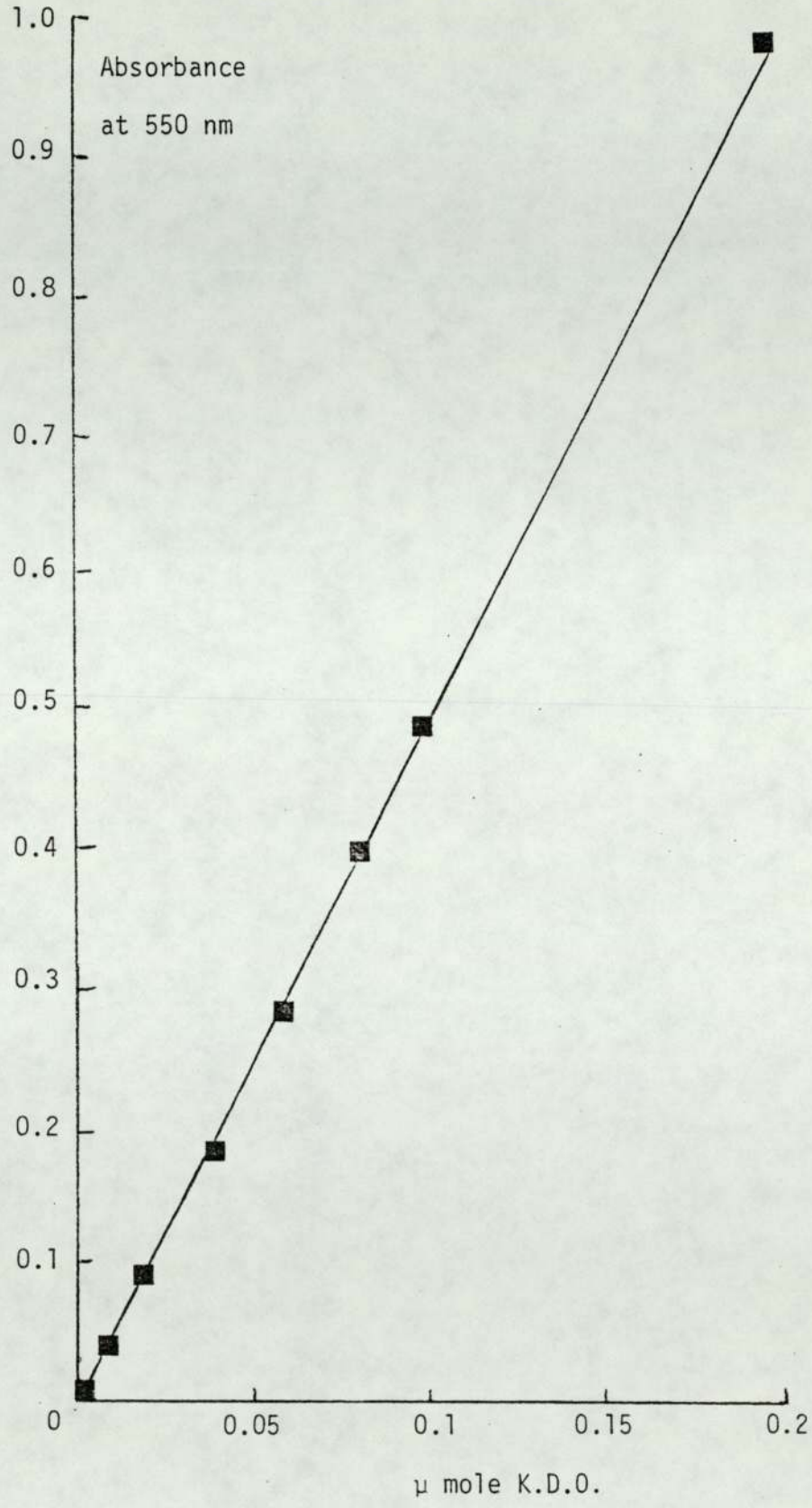
The method used for demonstration of the protein pattern of inner and outer membrane fractions was that of Lugtenberg et al (1975).

Two stock acrylamide solutions were used:- stock 1 (44% w/v acrylamide, 0.8% w/v N,N'-methylene-bisacrylamide (BIS)), stock 2 (30% w/v acrylamide, 0.8% w/v BIS).

Fig. 16

2 keto-3-deoxyoctonic acid (K.D.O.)

Calibration Curve (Ellwood, 1970)



	Running Gel	Stacking Gel
Stock 1	18.5ml	-
Stock 2	-	5ml
10% Amm. persulphate (APS)	0.2ml	0.1ml
10% Sodium dodecyl sulphate (SDS)	1.5ml	0.3ml
0.5M Tris (pH 6.8)	-	7.5ml
1.5M Tris	18.7ml	-
Distilled water	34.0ml	16.0ml
NNNN'Tetramethylethylene diamine (TEMED)	0.13ml	80µl

APS was made up fresh for each test. All other solutions were stable on storage.

Electrode Buffer		Sample Buffer	
Tris	6.0g	0.5M Tris	5.0ml
Glycine	28.8g	10% SDS	10.0ml
10% SDS	20.0ml	Mercaptoethanol	0.5ml
		Glycerol	5.0ml
		Distilled water	10.0ml

50µl of sample and molecular weight standards (Sigma Ltd.) (lysozyme m wt. 14,300, β lacto globulin 18,400 (36,800), bovine albumen (66,000), pepsin (34,700) and egg albumen (45,000)) was diluted with 50µl of sample buffer and 10µl bromophenol blue. The solution was boiled for 10 minutes. 20-80µl of solution was loaded onto the gel.

The electrophoresis was performed at room temperature at a constant (40mA) current. The electrophoresis was discontinued when the tracking dye had moved approximately 12cm.

Gels were stained with Brilliantblue R-250 overnight and destained with methanol/acetic acid.

2.2.4.7. Phospholipid assay

The phospholipids extracted from inner and outer membrane fractions were separated into their individual component lipids by ascending thin layer chromatography.

Glass plates 20 x 20cm were spread with 0.25mm layer of kiesel gel (Merck Ltd.). The slurry was prepared by mixing 60g kiesel gel with 120ml of water and the plates air dried overnight. Prior to use the plates were activated by heating at 70^{o} for 2 hours. The plates were loaded with the membrane fractions and standard phospholipids: phosphatidyl-ethanolamine, diphosphatidylglycerol and phosphatidylglycerol (Sigma Ltd.).

The plates were developed in a chromatography tank containing chloroform:methanol:water (65:25:4). The phospholipids were visualised by spraying with phosphate spray (Ames, 1968).

Densitometry measurements of the chromatograms were made using a Joyce Loebl Chromoscan 200. The proportion of each phospholipid as a percentage of the total phospholipid was determined by photocopying the densitometry peaks and weighing the cut-out peaks.

2.2.4.8. Outer membrane contamination

An estimate of the inner membrane contamination of the outer membrane was performed by assaying for the inner membrane succinic dehydrogenase by the method of Mizuno and Kageyama (1978). A mixture containing 60mM phosphate buffer (pH 7.2), 20µg dichlorophenolindophenol, 10µg phenazine methosulphate, 25mM succinate and the membrane fraction (20-100µg protein) in a volume of 1.0ml was incubated in a spectrophotometer cuvette. The absorbance at 600nm was measured for at least 10 minutes at room temperature ($25^{o}C$).

2.2.5. MOPS buffer

In the formulation of the chemically defined salts medium the choice of buffer was important. Inorganic buffers such as phosphate buffer have been widely used. These buffers suffer the disadvantage that media reformulation is often necessary if nutrient limitation involving an ion present in the buffer is to be studied. Boggis (1971) used ammonium acid phosphate buffer for limiation studies on K^+ and Na^+, and Na_2HPO_4/ KH_2PO_4 for Mg^{2+}, glucose, NH_4^+, SO_4^{2-}, Cl^-, and Fe^{2+} limitations. The presence of phosphate in his buffers necessitated a change of buffer to 3,6 endomethylene-1,2,3,6-tetrahydrophthallic acid (EMTA) for studies on phosphate limitation.

Since the aim of this study was to formulate a chemically defined medium for Pseudomonas aeruginosa such that any particular ion could be limited with a controlled excess of other ions it was decided to utilise a buffer which could be used for all ion limitation studies. MOPS - 3-(N-morpholino) propane sulphonic acid - a zwitterionic buffer was chosen because of its pKa of 7.2 at 20°C and useful buffering range of 6.5 - 7.9.

2.2.5.1. Buffering Range

The useful buffering range of MOPS was determined by potentiometric titration of the acid buffer with 0.1 N NaOH. The titration curve is shown in Fig. 17. The results confirmed that the pKa was 7.2 and a suitable buffering range was 6.5 - 7.8.

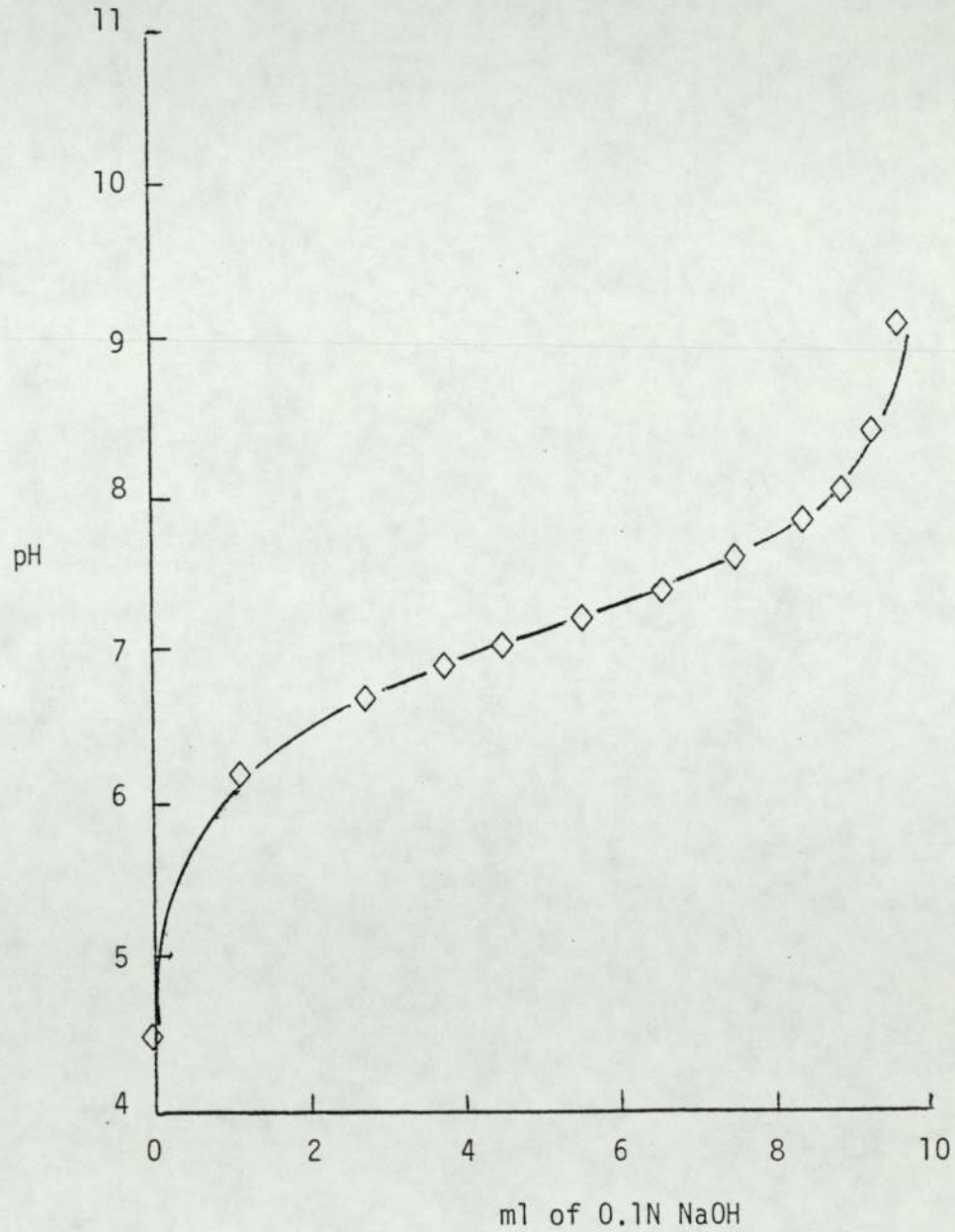
2.2.5.2. Toxicity of MOPS

The concentration of MOPS to be used in C.D.M. was required to provide adequate buffering capacity. However, the buffer concentration could not be high as toxic effects on the growing cells might occur.

Fig. 17

Potentiometric titration of acid

MOPS with 0.1N Sodium hydroxide



To investigate possible toxicity of MOPS three growth parameters were chosen for a range of MOPS buffer concentrations of 30mM, 60mM and 120mM. These parameters were the length of the lag phase of inoculated cultures, the effect on the doubling time of the cultures and the effect on the optical density at which log linear growth ceased. Toxicity of MOPS should exhibit itself by a change in one or more of these three parameters. For the toxicity studies C.D.M. low in phosphate (0.35×10^{-4}M) and C.D.M. high in phosphate (3.5×10^{-4}M) were chosen. Five flasks of media were prepared for each of three MOPS concentrations (30mM, 60mM and 120mM) in both of the media. An actively growing culture of P. aeruginosa grown in phosphate depleted C.D.M. was used to inoculate each flask. Cultures were shaken (120 throws/min) at 37°C and growth curves plotted. E_{470} values were taken every 10 minutes until the cultures were in log phase when readings were taken at 15 minute intervals. Growth curves were constructed for each MOPS concentration. From these graphs the lag time, doubling time and E_{470} onset could be determined. These values were submitted to an analysis of variance. Table 19 summarises the variance ratio (F) results.

Table 19

Variance Ratio (F) for Lag Period, Doubling Time and E_{470} (Onset) for P. aeruginosa grown in C.D.M. containing different amounts of PO_4^{3-}

	Variance Ratio		
	Lag period (mins)	Doubling time (mins)	E_{470} onset
PO_4^{3-} 0.35×10^{-4}	2.7	6.06	3.1
PO_4^{3-} 3.5×10^{-4}	3.0	3.03	2.22

The tabulated values of 'F' for 2/12 degrees of freedom at the 1% and 5% significance levels are 6.93 and 3.89 respectively. Therefore the variation between the values at the three parameters were no greater than the variation within the values at the 1% level. MOPS had no effect on the three parameters to a concentration of at least 120mM.

2.2.5.3. Buffering ability of 30mM, 60mM and 120mM MOPS

The previous experiment showed that a MOPS concentration of 120mM did not have a toxic effect on P. aeruginosa, judged by the three parameters tested. However, in order to achieve a suitable buffering capacity, the concentration of MOPS must not be too low. Its buffering ability deteriorates rapidly below pH 6.8. The aim of this experiment was to determine a suitable MOPS concentration which would maintain the culture pH above 7.0 when cells were grown to the typical optical densities likely to be used in subsequent experiments. Although MOPS does not appear toxic to P. aeruginosa, high buffer concentrations should be avoided.

Four batches of chemically defined media were produced containing 10×10^{-4}M glucose, 140×10^{-4}M glucose, 0.75×10^{-4}M phosphate and 7×10^{-4}M phosphate, at three MOPS concentrations of 30mM, 60mM and 120mM. The media was inoculated with a suspension of cells grown in C.D.M. low in phosphate or glucose, as appropriate.

Optical density measurements were made on samples removed at various time intervals and the culture pH at that time measured using a 413 pH meter (Radiometer Ltd.). The growth curves and pH measurements are shown in Figs. 18 and 19 , for glucose and phosphate systems respectively. A satisfactory pH was maintained throughout the growth cycle for cells grown in 10×10^{-4}M glucose, a pH drop of less than one pH unit being

Fig. 18 E_{470} and pH for *P. aeruginosa* grown in C.D.M. containing 140 x 10^{-4}M or 10 x 10^{-4}M glucose

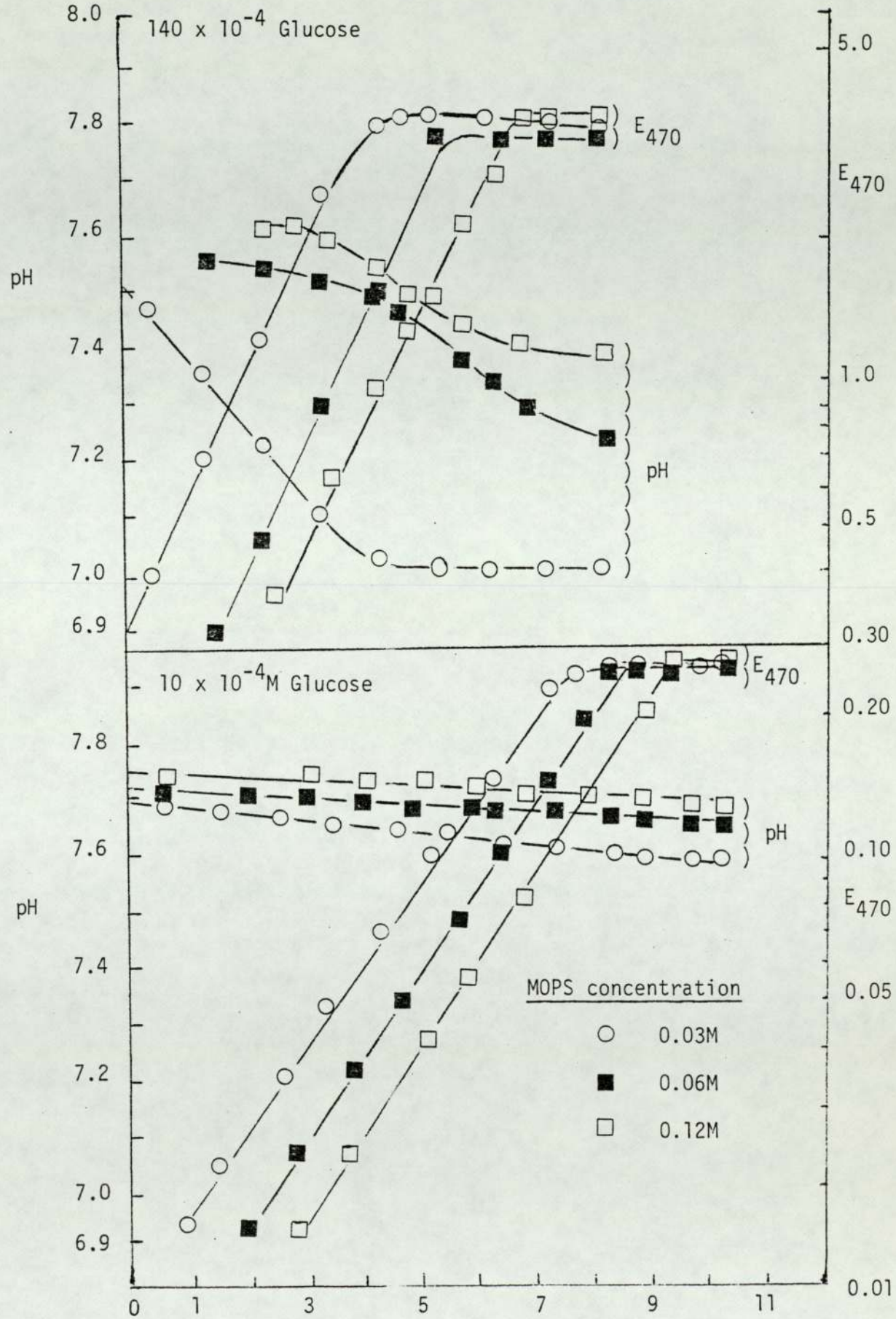
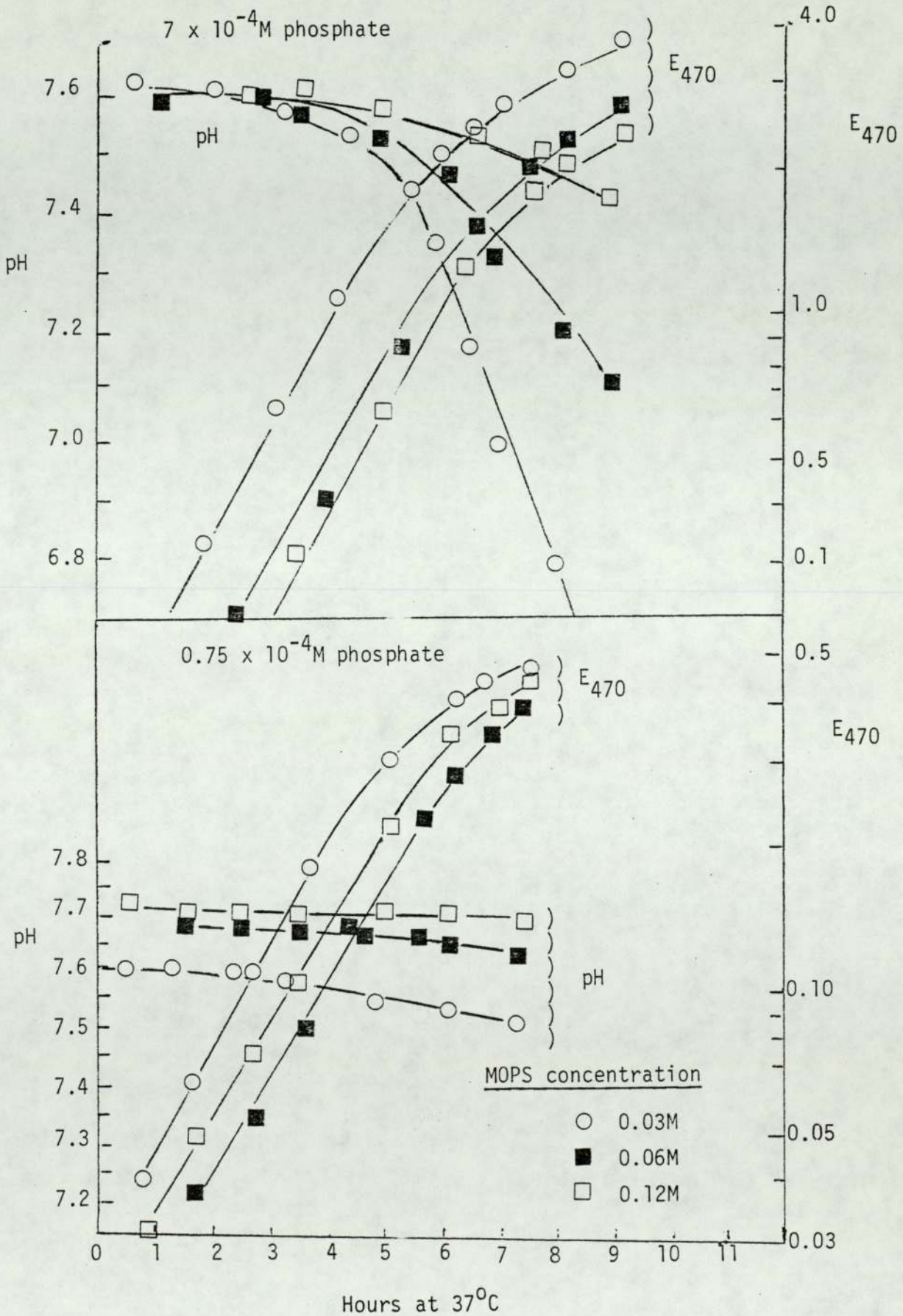


Fig. 19 E_{470} and pH for P. aeruginosa grown in C.D.M.containing 0.75 x 10^{-4}M or 7 x 10^{-4}M phosphate



observed for all MOPS concentrations tested (Fig.18). Growth in the presence of 140 x 10^{-4}M glucose (Fig.18) indicated that the pH of the media buffered with 30mM MOPS was 7.0 at the end of growth (E_{470} 3.6). This value was considered too borderline to be satisfactory although rarely would optical densities of 3 to 4 be used. the pH fell approximately 0.2 pH units and 0.4 pH units for cultures buffered with 60mM and 120mM MOPS respectively.

Cells grown in the low phosphate concentration were adequately buffered at all three levels, the pH falling less than 1 pH unit in all cases (Fig.19). Cells grown in high phosphate media showed a rapid fall in pH value as the optical density increased. The pH fell from pH 7.6 to pH 6.5 for cells buffered with 30mM MOPS. This was considered unsatisfactory buffering. Cells buffered with 60mM MOPS showed a pH fall from pH 7.6 to pH 7.2 as the optical density (E_{470}) approached 4. Cells buffered with 120mM MOPS showed a fall from pH 7.6 to pH 7.5.

These results suggested that a concentration of 60mM or above was satisfactory and this was adopted as standard.

2.2.5.4. Utilisation of MOPS as a Carbon or Nitrogen Source by P. aeruginosa

Since MOPS is an organic sulphonic acid, it is possible that P. aeruginosa could utilise it preferentially as a carbon or nitrogen source when starved of these two nutrients, thus reducing its buffering ability.

In order to test this, glucose depleted C.D.M. was prepared containing zero and 60mM MOPS as buffer. Ammonium depleted C.D.M. containing zero and 60mM MOPS was also prepared. The flasks were inoculated with P. aeruginosa grown in ammonium depleted and in glucose depleted C.D.M.

Fig. 20 Growth curves for P. aeruginosa grown in glucose free C.D.M. with zero or 0.06M MOPS

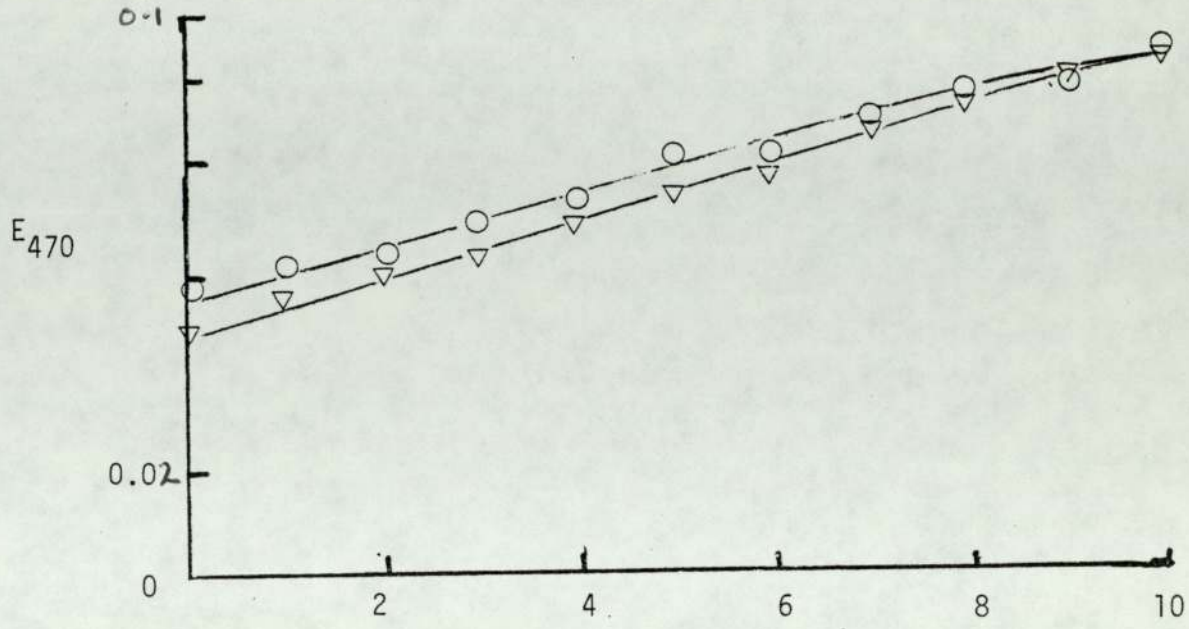
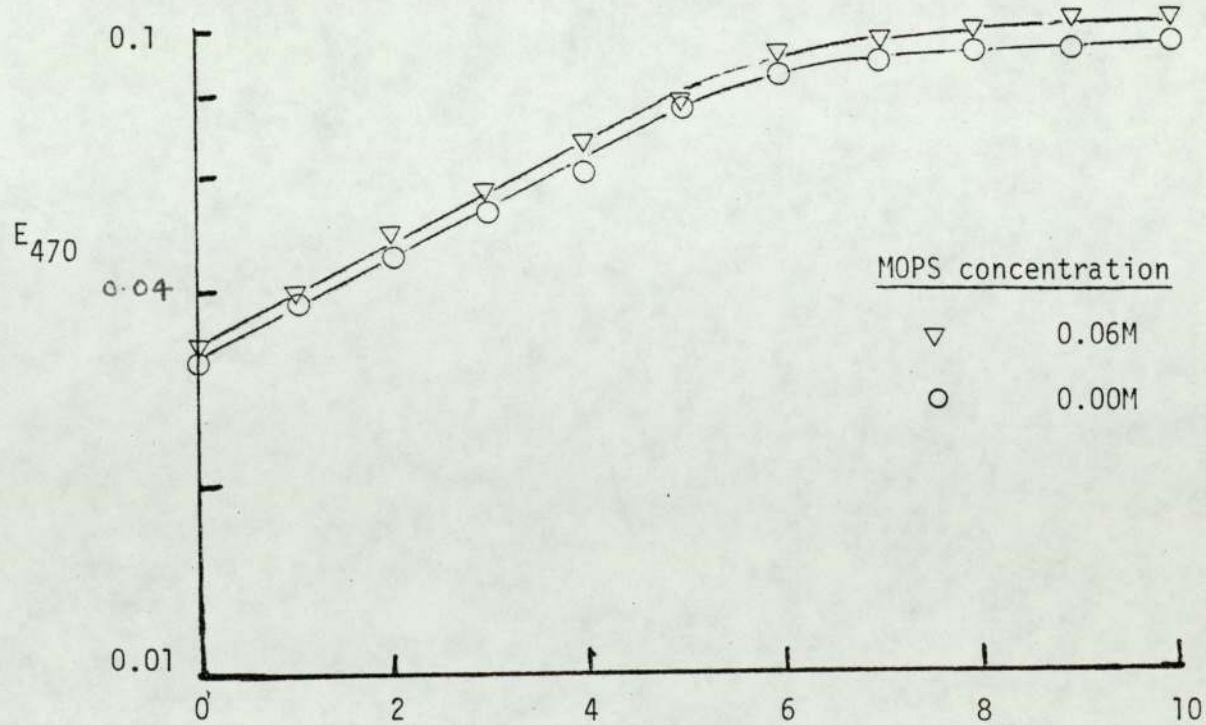


Fig. 21 Growth curves for P. aeruginosa grown in NH_4^+ free C.D.M. with zero or 0.06M MOPS



To avoid carry over of ammonia and glucose, the cells were centrifuged at 4,000g for 20 minutes, and washed three times in ammonia and MOPS-free C.D.M., and glucose and MOPS-free C.D.M. respectively. The optical densities of the cultures were plotted at hourly intervals. The results are shown in Figs. 20 and 21.

The cells grown in media containing zero MOPS grew as poorly as those containing 60mM MOPS, indicating the failure of the organism to utilise MOPS in the absence of a carbon or nitrogen source. The pH values of the unbuffered cultures were approximately 6.6 at the end of growth. These pH values were much below the pH of the buffered cultures, which were above 7.6. It was not considered that pH was the reason for the poor growth in the unbuffered cultures. The results would indicate that P. aeruginosa cannot utilise MOPS as a sole source of carbon or nitrogen.

2.2.5.5. Utilisation of MOPS as a source of sulphate

MOPS at 60mM was used successfully for nutrient limitation studies (described later) until sulphate limitation was investigated.

The results of sulphate limitation will be shown in a later section and only a brief summary will be made here. The results showed that E_{470} onset values obtained for a particular sulphate concentration were two to three times higher than those obtained by Boggis (1971). Extrapolation of the graphs of E_{470} versus added sulphate to zero added sulphate gave corresponding E_{470} values of 0.5 to 0.8, and indicated sulphate contamination levels in the medium of 0.75 - 1.3 x 10^{-5}M. E_{470} maximum values were also elevated above expected levels.

Such sulphate contamination was thought unlikely. Analysis of deionised water used to prepare media showed sulphate levels of

$<4 \times 10^{-6}$M. MOPS was also analysed at use dilution and found to contain $<0.1 \times 10^{-8}$ sulphate. The sulphate contamination arising from the chemical constituents of C.D.M. was found to be $<1.4 \times 10^{-8}$M.

The most likely cause of such high E_{470} values and contamination levels seemed to be the utilisation of MOPS as a source of sulphur.

This was confirmed by reformulation of the C.D.M., removal of MOPS and its replacement by 7.5mM acid ammonium phosphate buffer. When P. aeruginosa was grown in the reformulated medium containing no added sulphate, log linear growth ceased at an E_{470} of 0.086 compared with 0.53 when MOPS buffer was used.

When E_{470} versus added sulphate was plotted graphically much lower E_{470} onset and maximum values were obtained with ammonium acid phosphate buffer than with MOPS. Extrapolation of the graph to the X-axis gave levels for sulphate 'contamination' in the medium as 5.6μM sulphate for E_{470} maximum values. Compared with 50μM sulphate when MOPS was used. The results are summarised in Table 20.

The results indicated that MOPS could not be used as a buffer for investigation of sulphate limited growth using P. aeruginosa. For sulphate limited growth studies an alternative buffer such as ammonium acid phosphate must be used.

Table 20

E_{470} Onset, E_{470} Maximum Values and Sulphate Contamination Levels for P. aeruginosa Grown in C.D.M. Containing Zero Added Sulphate

	E_{470} onset	E_{470} max.
MOPS buffer 60mM	0.53	2.58
Acid ammonium phosphate 7.5mM	0.086	not done
Sulphate contamination (MOPS)	≡5μM	≡50μM
Sulphate contamination (ammonium acid sulphate)	≡5μM	≡ 5.6μM

2.2.6. Neutralisation of Polymyxin and E.D.T.A.

When performing viable count estimations to assess the antibacterial activity of any agent it is essential that the lethal process is arrested immediately the test sample is withdrawn. This necessitates the inclusion of a suitable inactivating agent in the recovery medium or diluent. With very few exceptions (notably phenolic disinfectants and alcohols) dilution alone is inadequate to stop antibacterial action. The neutralisating agent must not only be efficient in its inactivating ability but should also in itself not be inhibitory to the test micro-organism.

Lecithin glycerol has been recommended by Kohn et al (1963) for the neutralisation of polymyxin. Nutrient broth with added calcium was used by Brown and Melling (1969a) whereas Gray and Wilkinson (1965a) used nutrient broth without added calcium for the neutralisation of E.D.T.A.

This section describes tests which were performed to measure the efficiency of these neutralisers for viable count estimations.

2.2.6.1. Determination of neutraliser (inactivator) efficiency

P. aeruginosa 6750 was grown in phosphate depleted culture to an E_{470} of 0.2. The efficiency of the inactivators chosen: Lecithin/Tween 80 and Lecithin/glycerol for Polymyxin B and nutrient broth with and without the addition of 2.7μM calcium chloride dihydrate for E.D.T.A. was checked using a modification of the method described by Maurer (1974) which was based on B.S. 3286: 1960. This test ensured that the antimicrobial agent would be neutralised by the inactivator and that the inactivator was not itself inhibitory to the growth of the test organism.

The method of checking was summarised below:

Prepare:

A) 9ml inactivator broth and 1ml antimicrobial agent.

B) 9ml inactivator broth and 1ml sterile distilled water.

C) 10ml sterile distilled water.

D) a suitable dilution of the phosphate depleted P. aeruginosa was made to give an inoculum of approximately 10^4 organism ml^{-1}.

1) A, B and C were inoculated with 1ml of D.

2) As soon as possible after inoculation (<5 min) 1ml was withdrawn from each of A, B and C and ten fold dilutions prepared to give a plate count of approximately 100 colonies. The nutrient agar plates were incubated at 37°C for 48 hours, and the pattern of survivors compared.

3) A similar amount was withdrawn from each bottle at time 30 minutes and 60 minutes after neutralisation.

Interpretation

Similar numbers of colonies should grow from each of the three subcultures taken at the same time. Where A) gives a lower count than C), either the inactivator has failed to neutralise the agent which is therefore able to inhibit growth of the organisms or the inactivator itself is inhibiting growth. Where B) gives a growth lower than C) the inactivator itself is inhibiting growth.

The results of such neutraliser tests are shown in Table 21.

These results showed similar reductions in viable count from 698 to 630cfu ml^{-1} (9.7%) and 628 (10.0%) at 30 minutes for the water control and Lecithin/Tween inactivator respectively, indicating that the broth itself was not inhibitory. However the Lecithin/Tween failed to inactivate satisfactorily the Polymyxin at 10 I.U. ml^{-1} as shown by the fall in viable count at 30 minutes from 698 to 393cfu ml^{-1} (43.6% reduction). Lecithin/glycerol was a much better inactivator, again showing no inhibitory reaction itself. The viable count in the water control fell from 632 to 528 cfu ml^{-1} (16%) at 30 minutes and 516cfu ml^{-1} (18.3%) at 60 minutes compared with 632 to 600cfu ml^{-1} (5.1%) at 30 minutes and to 528cfu ml^{-1} (16.5%) at 60 minutes for the inactivator control. The cells treated with Polymyxin and inactivated suffered a fall in viable count of 632 - 534cfu ml^{-1} (15.5%) at 30 minutes and to 422 (33%) at 60 minutes. This suggests that if the neutraliser cannot fully neutralise the Polymyxin then damaged cells will die on prolonged storage in the presence of inactivator and unneutralised drug. Removal for plate counting at an early stage is preferred. The results suggest that the least fall in viable count will occur if cells are removed within the first 5 minutes of transfer to the neutraliser.

Table 21

The Neutralisation of Polymyxin B (10 $ium l^{-1}$) and EDTA (785µg) by Various Media

Neutraliser	Flask content	Initial inoculum cfu ml^{-1}	Time in neutraliser (min) 5	30	60
			cfu ml^{-1}		
Lecithin/Tween	Water + Water		704	630	Not done
	Water + Inactivator	698	690	628	Not done
	Inactivator + Poly.B(10iuml^{-1})		568	393	Not done
Lecithin/glycerol	Water + Water		606	528	516
	Water + Inactivator	632	642	600	528
	Inactivator + Poly.B(10iuml^{-1})		570	534	422
Nutrient Broth	Water + Water		640	660	640
	Water + Inactivator	620	620	660	740
	Inactivator + EDTA(785µgml^{-1})		630	650	700
Nutrient Broth + Ca^{2+}	Water + Water		640	660	640
	Water + Inactivator	620	600	640	680
	Inactivator + EDTA(785µgml^{-1})		640	620	660

On the basis of these results it was decided to reject Lecithin/Tween as a neutraliser in further studies on the action of Polymyxin B and to use Lecithin/glycerol.

The results for E.D.T.A. neutralisation suggest that the addition of Ca^{2+} to the nutrient broth does not enhance the neutralisation properties of the medium. In the presence of inactivator broth with or without added Ca^{2+} the viable count rose by 620 to 680cfu ml^{-1} (9.7%) and 620 to 740cfu ml^{-1} (19%) compared with 620 to 640cfu ml^{-1} (3%) for the water control at 60 minutes. Obviously cells were able to multiply in the broth in the one hour neutralisation period. The E.D.T.A. treated cells were adequately neutralised at five minutes and prolonged holding in neutraliser allowed an increase in viable count from 620 to 700cfu ml^{-1} (12.9%) at 60 minutes for nutrient broth and from 620 to 660cfu ml^{-1} (6.5%) for nutrient broth and Ca^{2+} treated cells.

In view of these results it was decided to use nutrient broth as the neutraliser since the addition of Ca^{2+} was of no value and made the preparation of the broth more tedious. To avoid errors in assessment of survivors following E.D.T.A. treatment it was decided to neutralise for five minutes only.

2.2.6.2. Neutralisation of Polymyxin B and E.D.T.A. following lysis experiments

The previously described test showed that Lecithin/glycerol was a satisfactory neutraliser for Polymyxin and nutrient broth was satisfactory for neutralisation of E.D.T.A. under the artificial test conditions of the B.S. 3286: 1960 test. Their ability to neutralise these two agents satisfactorily during typical lysis work was tested.

Neutralisation of the agents might be easy immediately following exposure to the drugs but neutralisation following 60 minutes exposure to Polymyxin B and 180 minutes exposure to E.D.T.A. might be more difficult due to interaction of the drug with the cells.

To test neutralisation under typical test conditions the following experiments were performed. P. aeruginosa 6750 was grown in several flasks of phosphate depleted C.D.M. to an E_{470} of 0.2. To 24ml of culture in one flask (A) was added 1ml of sterile distilled water. To another flask (B) was added 1ml of Polymyxin B (250 iu ml^{-1}). The flasks were shaken for 5 seconds and 1ml aliquots removed from each flask. 1ml from flask A was added to 9ml of inactivator and 1ml added to 9ml of water. This procedure was repeated with samples from flask B. Samples were removed from the bottles after 2 minutes, 30 minutes and 60 minutes. The culture flasks were shaken at 120 throws/minute at 37°C for one hour as performed for a typical Polymyxin lysis experiment. 1ml samples were then taken from each flask and the neutralisation test repeated. Colony counts were performed after incubation for 48 hours at 37°C on nutrient agar.

This procedure was repeated for control and cultures treated with E.D.T.A. (785µg ml^{-1}) except that the flasks were shaken for 180 minutes.

The results of the neutraliser tests on Polymyxin treated cells are shown in Fig. 22 and those for E.D.T.A. treated cells in Fig. 23.

The results show that samples removed immediately following exposure to Polymyxin B were rapidly neutralised by the inactivator broth. After 5 minutes in inactivator broth the viable count had fallen from 640 -620cfu ml^{-1} (3.7%). The count fell to 500cfu ml^{-1} (21%) after one

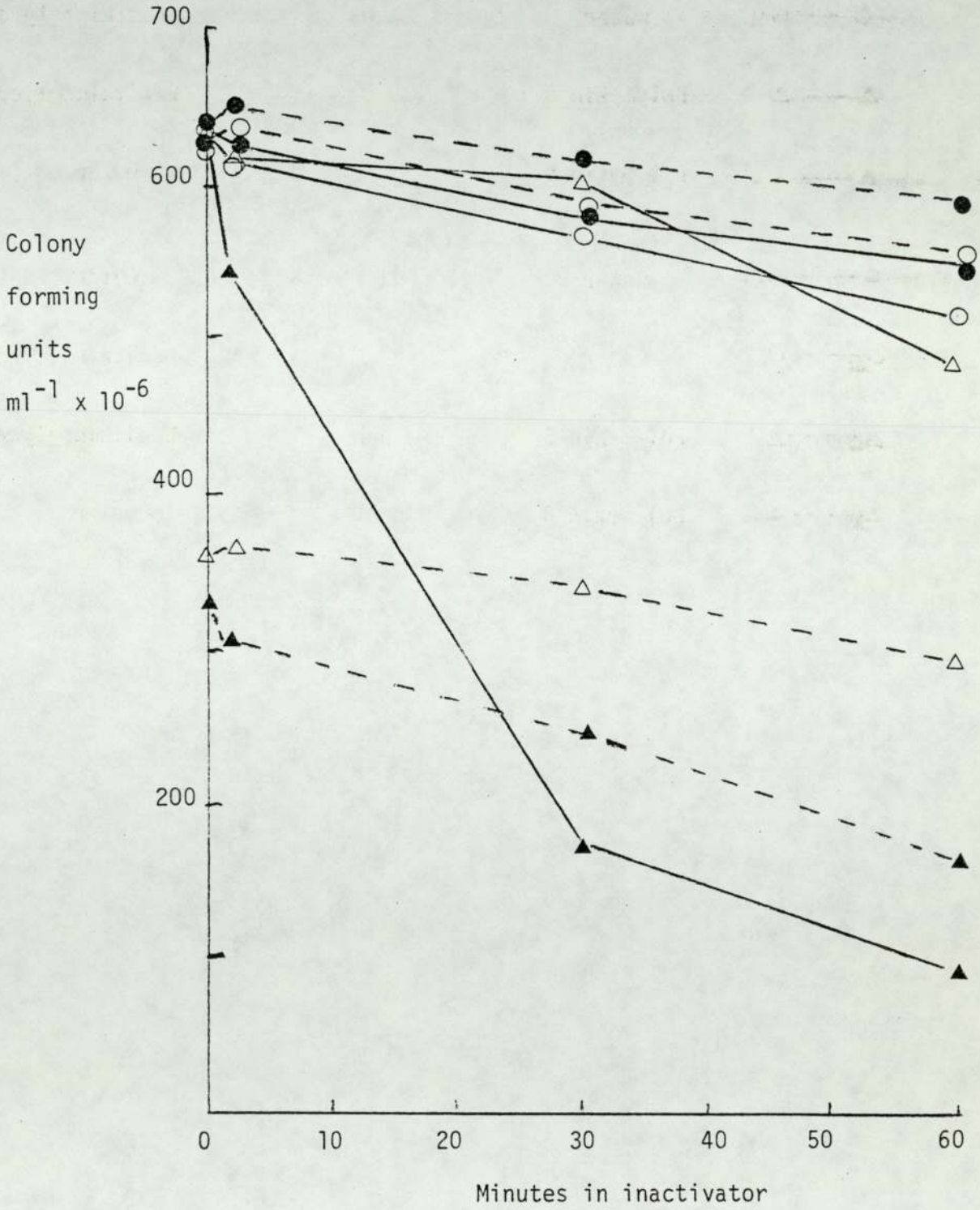
Key

Symbol	Additive to culture	Time in culture (min.)	Inactivator
● —— ●	water	0	water
○ —— ○	water	0	Lecithin/glycerol
△ —— △	Polymyxin B	0	Lecithin/glycerol
▲ —— ▲	Polymyxin B	0	water
● - - - - ●	water	60	water
○ - - - - ○	water	60	Lecithin/glycerol
△ - - - - △	Polymyxin B	60	Lecithin/glycerol
▲ - - - - ▲	Polymyxin B	60	water

Fig. 22

Neutralisation of Polymyxin B (10 I.U. ml^{-1})

by Lecithin/glycerol media



hour in neutraliser. The Polymyxin treated sample which was not neutralised showed a rapid fall in viable count. A fall in viable count from 640 to 90cfu ml^{-1} (85.9%) after one hour was observed.

Following shaking of the flasks for one hour a lower initial count was demonstrated. The difference between inactivated and non-inactivated culture was less marked presumably because most of the Polymyxin had been utilised in killing the cells. Inactivated cultures showed a fall in viable count from 366 to 324cfu ml^{-1} (6.0%) compared with a fall from 310 to 174cfu ml^{-1} (43.8%) for the non inactivated culture after 60 minutes in neutraliser or water. The fall seen in inactivated cultures was greater for those exposed to Polymyxin for 60 minutes than for those exposed for only a few seconds before neutralisation. This may have been because the Polymyxin was more easily neutralised before it had interacted with the cell. After one hour's exposure to the drug some of the drug will have interacted with the cell and may be less readily inactivated or inactivation may occur but the cells be so damaged that viability is lost.

Exposure to E.D.T.A. followed by immediate neutralisation in nutrient broth resulted in an increase in viable count after 60 minutes from 628 to 700cfu ml^{-1} (11.1%). However the control showed no increase. Following 180 minutes exposure to E.D.T.A. the viable count had fallen. The control showed an increase from 600 to 635cfu ml^{-1} (5.8%) during the 60 minutes 'neutralisation' period compared with the increase in the viable count of the neutralised cells from 520 to 580cfu ml^{-1} (11.5%). The water/nutrient broth cultures showed an increase of 16.5% from 635 cfu ml^{-1} to 740cfu ml^{-1} at 60 minutes, and a 7% increase from 715cfu ml^{-1} to 765cfu ml^{-1} in 60 minutes following 180 minutes incubation.

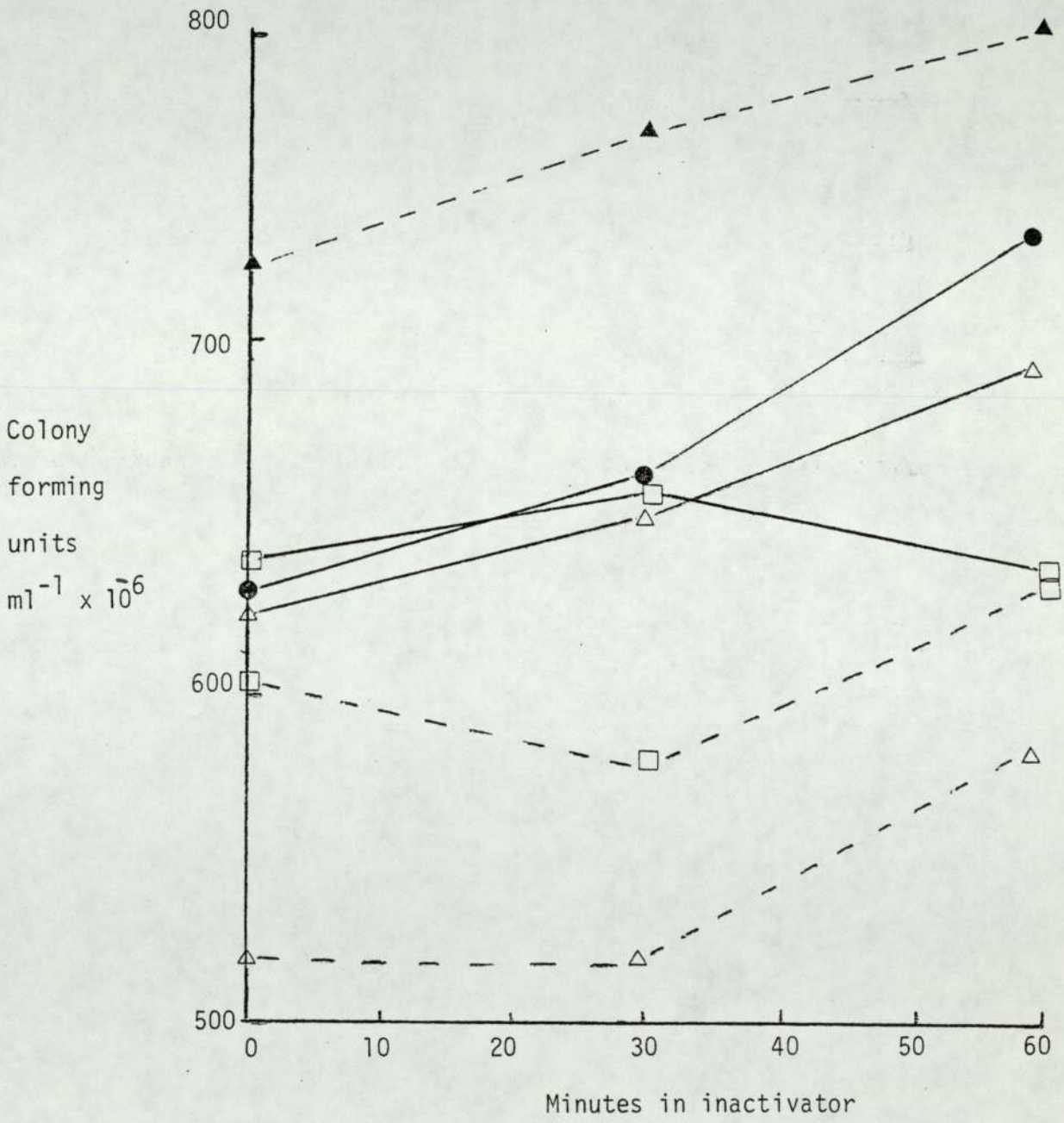
Key

Symbol	Additive to culture	Time in culture (min.)	Inactivator
□——□	water	0	water
●——●	water	0	nutrient broth
△——△	E.D.T.A.	0	nutrient broth
□---□	water	180	water
▲----▲	water	180	nutrient broth
△---△	E.D.T.A.	180	nutrient broth

Fig. 23

Neutralisation of E.D.T.A. (785µg ml^{-1})

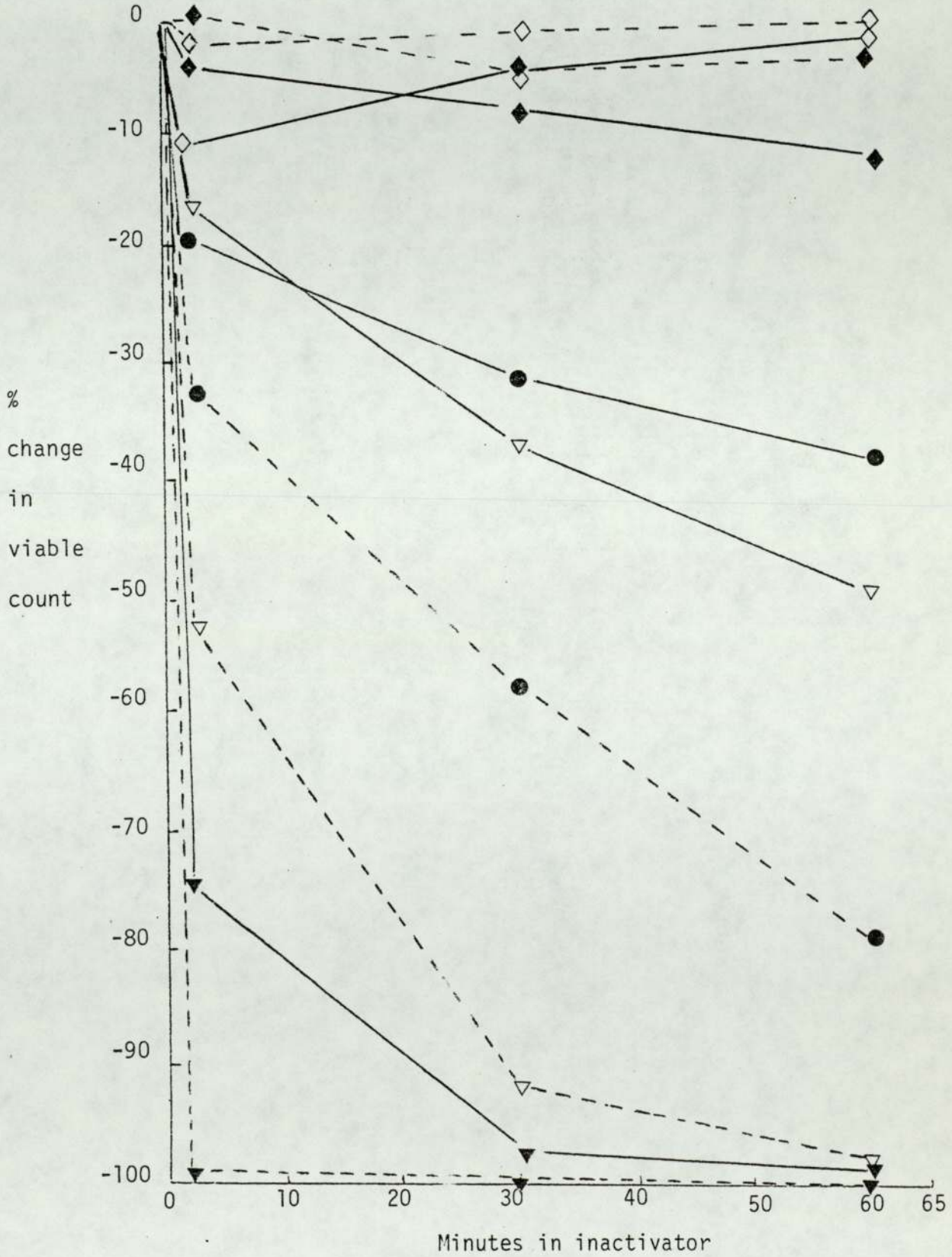
by nutrient broth



Key

Symbol	Polymyxin B	Neutraliser
◇——◇	0.0 i.u./ml	lecithin 1
◆——◆	1.0 i.u./ml	lecithin 1
●——●	10.0 i.u./ml	lecithin 1
▽——▽	100.0 i.u./ml	lecithin 1
▼——▼	500.0 i.u./ml	lecithin 1
◇- - -◇	0.0 i.u./ml	lecithin 2
◆- - -◆	1.0 i.u./ml	lecithin 2
●- - -●	10.0 i.u./ml	lecithin 2
▽- - -▽	100.0 i.u./ml	lecithin 2
▼- - -▼	500.0 i.u./ml	lecithin 2

Fig. 24 Neutralisation of Polymyxin B by two batches of lecithin/glycerol media



Both of these results suggest that the inactivator will work satisfactorily under experimental conditions.

2.2.6.3. Batch variation in neutraliser media

Poor reproducibility of viable count results following Polymyxin treatment of P. aeruginosa occurred at a time when a new batch of neutraliser medium was in use. To investigate the variation in neutralisation ability between different batches of neutraliser, broth made from two different batches of Lecithin was tested. Apart from different batches of Lecithin, other media constituents and preparation methods were identical.

9ml amounts of the two batches of inactivator broth were prepared and submitted to an inactivator test as previously described except that a range of Polymyxin concentrations was used from 0 to 500 $i.\mu ml^{-1}$. The neutraliser and Polymyxin were mixed for 15 minutes before addition of a culture of phosphate depleted P. aeruginosa. Samples were removed for viable count estimations at 0, 30 and 60 minutes.

Fig. 24 shows that a difference may occur with different batches of Lecithin. The differences become more pronounced at higher concentrations of Polymyxin. In order to minimise such problems a new 100g of Lecithin was purchased and utilised for all viable count experiments. A neutraliser test was carried out before each new batch of neutraliser broth was used.

2.2.6.4. Ability of Lecithin/glycerol neutraliser to inactivate a range of Polymyxin B concentrations

This was tested by the standard neutraliser test for a range of Polymyxin concentrations of 0 - 500 iu ml^{-1} using an inoculum of phosphate limited P. aeruginosa. The results are shown in Fig. 25 . For Polymyxin

Polymyxin concentration

◇ 0 i.u. ml^{-1}

♦ 1.0 i.u. ml^{-1}

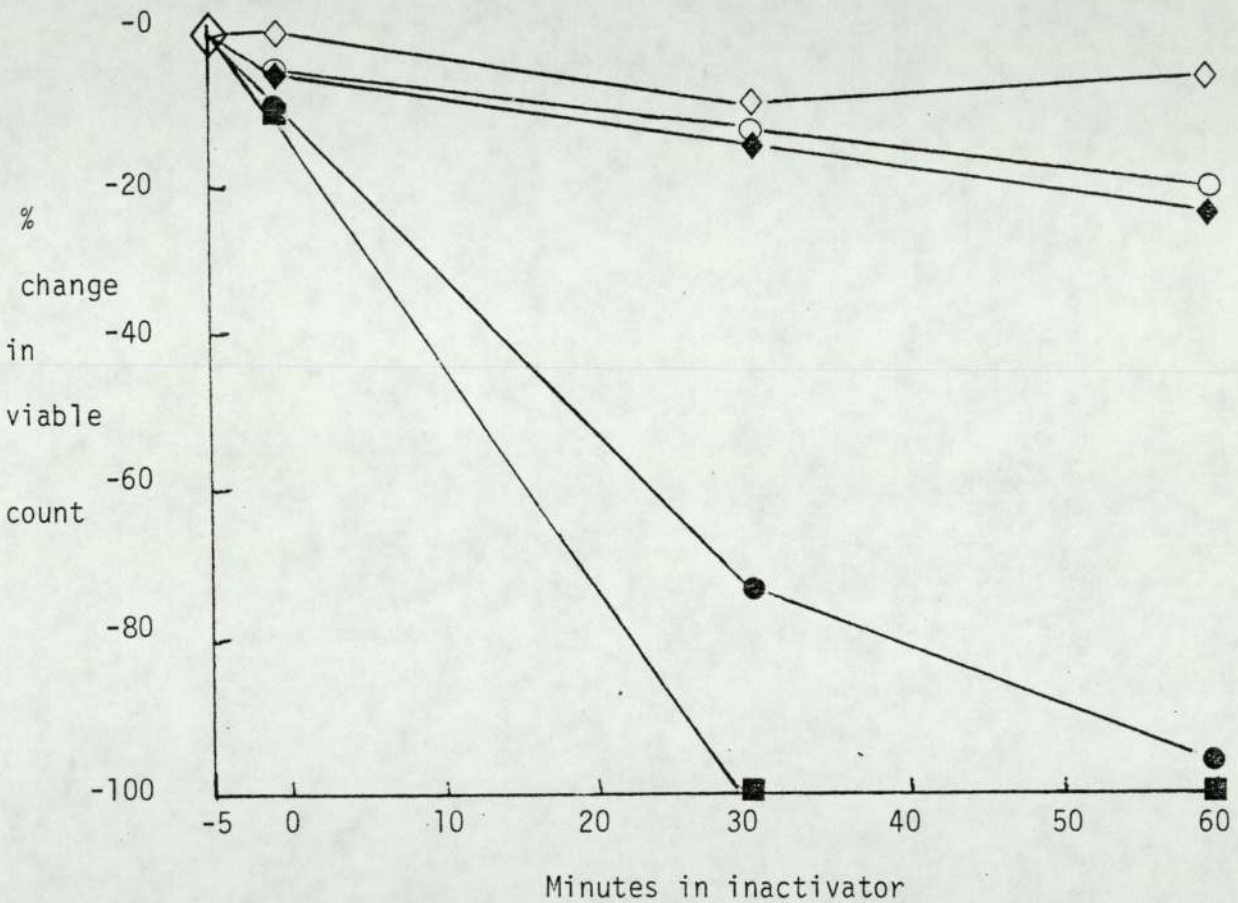
○ 10.0 i.u. ml^{-1}

● 100.0 i.u. ml^{-1}

■ 500.0 i.u. ml^{-1}

Fig. 25

Neutralisation of Polymyxin B



concentrations up to approximately 10 iu ml^{-1}, neutralisation appeared satisfactory. Poor neutralisation was obtained at Polymyxin concentrations of 100 iu ml^{-1} and 500 iu ml^{-1} as judged by fall in viable counts in excess of 90%.

3. EXPERIMENTAL AND RESULTS

3.1 NUTRIENT LIMITATION STUDIES

Boggis (1971) showed that a linear relationship existed between E_{470} onset and the limiting ion constituent for Mg^{2+}, glucose, NH_4^+, PO_4^{3-}, SO_4^{2-}, Fe^{2+} and K^+. P. aeruginosa 6750 appeared to have no requirement for Na^+ or Cl^-. These ions were found as contaminants in the media and on the glassware used. From his work chemically defined media limited in one medium constituent only and formulated to achieve a required cell concentation could be prepared. Such data also permitted the use of a controlled excess of non-limiting nutrients.

In formulating a chemically defined medium for this study several changes had to be made to the media devised by Boggis (1971). The reason for this was the replacement of acid ammonium phosphate buffer by MOPS buffer. Ammonium sulphate was used as the sole source of ammonium ion and disodium hydrogen phosphate as the source of phosphate. Unlike Boggis's medium it was decided to add ferrous sulphate to the medium for all optical densities studied. A ten times excess of all other media constituents was used for each limitation studied.

3.1.1. Inoculation Procedure

A standard inoculation procedure was adopted for all batch culture limitation studies. The culture of P. aeruginosa to be used was plated from the storage slope onto nutrient agar and cultured for 18-24 hours at 37°C. A single colony was then inoculated into a 25ml volume of C.D.M. low in molarity for the ion under test. A volume of this dilution was taken and added to each flask such that an E_{470} of approximately 0.02 would be achieved by 9.00am the next morning. Growth curves were plotted by measuring the E_{470} at various time intervals. Optical densities were

performed on undiluted culture in sterile glass semi-micro optically matched cuvettes using C.D.M. as a blank. Following reading the sample was returned to the flask. Above an E_{470} of 0.4 the sample was diluted with C.D.M. to give a value of E_{470} between 0.1 and 0.2. Following reading, this culture was discarded. The cuvettes were rinsed with distilled deionised water and drained between readings.

From the resulting growth curves two parameters could be determined

(1) E_{470} onset. This is the optical density where departure from log-linear growth occurred.

(2) E_{470} maximum. This is the optical density measured 18 hours following the onset of depletion.

E_{470} maximum and E_{470} onset were identical for glucose limited cells.

The period of 18 hours following onset of limitation was chosen as it was a convenient period (usually overnight). Different workers have chosen different times to record E_{470} maxima Kenward (1975) chose 3 hours after the growth rate became non exponential as E_{470} max. whereas Boggis (1971) took E_{470} max. values 36-38 hours after the start of incubation. Since most of his growth curves were plotted over about 20-24 hours this meant that his E_{470} max. values were taken approximately 16-20 hours after the onset of limitation.

To test whether the culture was depleted in the nutrient under test the appropriate nutrient was added to the culture 2-4 hours after the onset of limitation. An increase in growth rate to approximately that of the original rate coupled with the expected rise in E_{470} as judged by the amount of the nutrient added was taken to indicate that the culture was depleted in that nutrient.

3.1.2. Growth in chemically defined medium

The formula of chemically defined medium has been given previously (materials section). Fig. 26 shows the result of growth of P. aeruginosa 6750 in C.D.M. Log linear growth was maintained to an E_{470} value of 4.2 after which growth slowed presumably due to oxygen depletion. The doubling time was 62 minutes.

3.1.3. Growth limitation with ammonium ion

Figure 27 shows the growth curves for a range of concentrations of 2×10^{-3}M to 18×10^{-3}M added NH_4^+. As NH_4^+ became limiting the growth rate decreased abruptly followed by a slow increase in E_{470} with time. The exponential growth rate was dependent upon the initial NH_4^+ concentration below 6×10^{-3}M NH_4^+. A linear relationship existed between initial NH_4^+ concentration in the medium and E_{470} onset to an NH_4^+ concentration of 8.4×10^{-3}M (Fig. 28). The relationship between E_{470} max and added NH_4^+ concentration is shown in Fig. 29. Linearity was maintained to an E_{470} max of 3.65 corresponding to an NH_4^+ concentration of 13×10^{-3}M NH_4^+. Extrapolation of E_{470}max. curve to the x-axis showed NH_4^+ contamination from other media constituents of about 1×10^{-3}M NH_4^+.

3.1.4. Growth limitation with potassium

The growth curves for K^+ depleted cultures are shown in Fig. 30 for added K^+ concentrations of 1.25×10^{-5} - 12.5×10^{-5}M. Following exponential growth the growth rate decreased gradually. Fig. 31 shows the relationship between E_{470} onset and added K^+ in the range 1.25×10^{-5} - 17.5×10^{-5}M. Linearity was demonstrated to an E_{470} of 1.55 corresponding to a K^+ concentration of 9.5×10^{-5}M.

Fig. 26

Growth of P. aeruginosa in C.D.M.

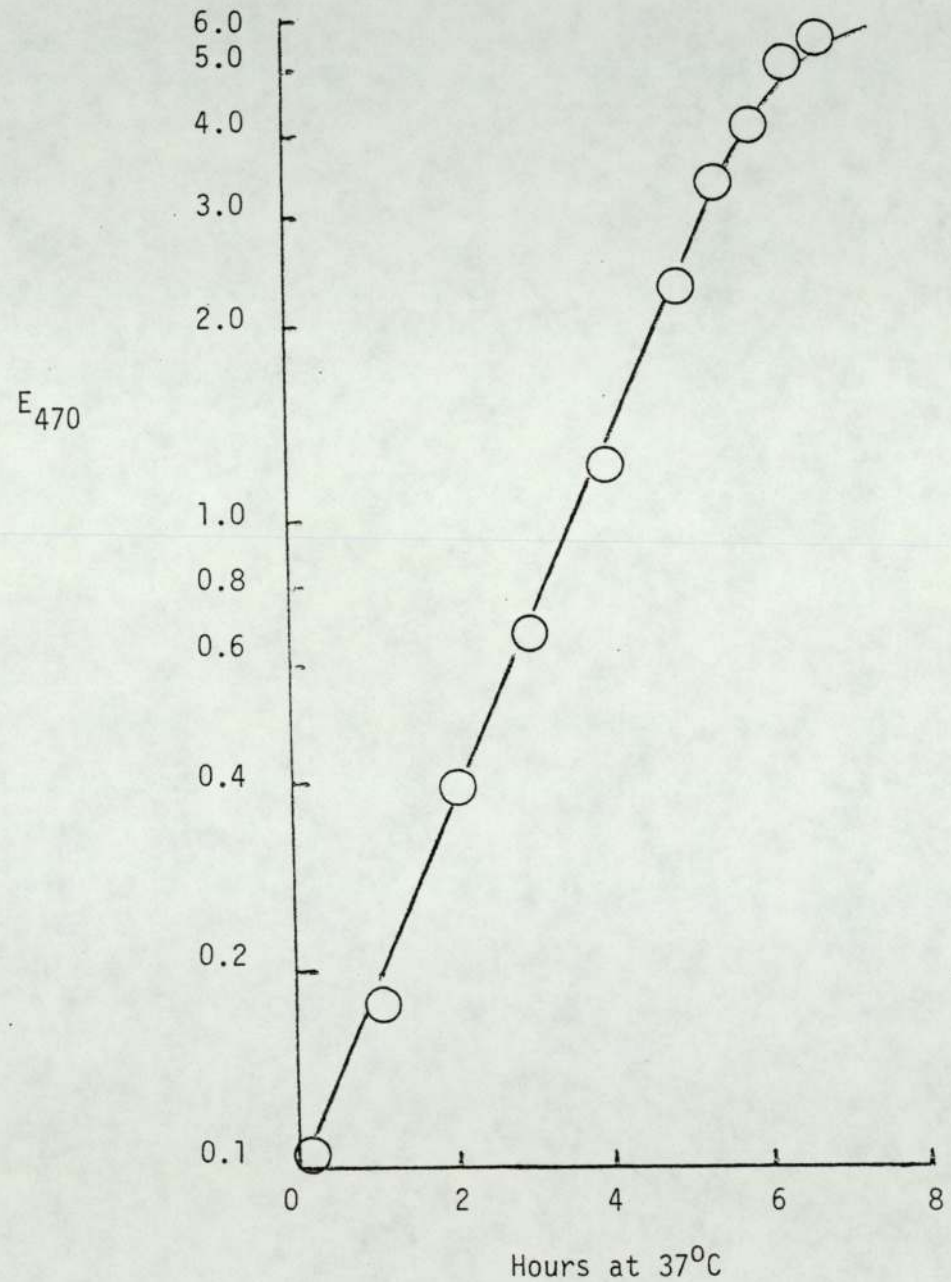


Fig. 27 Growth of P. aeruginosa in C.D.M. containing graded concentrations of NH_4^+

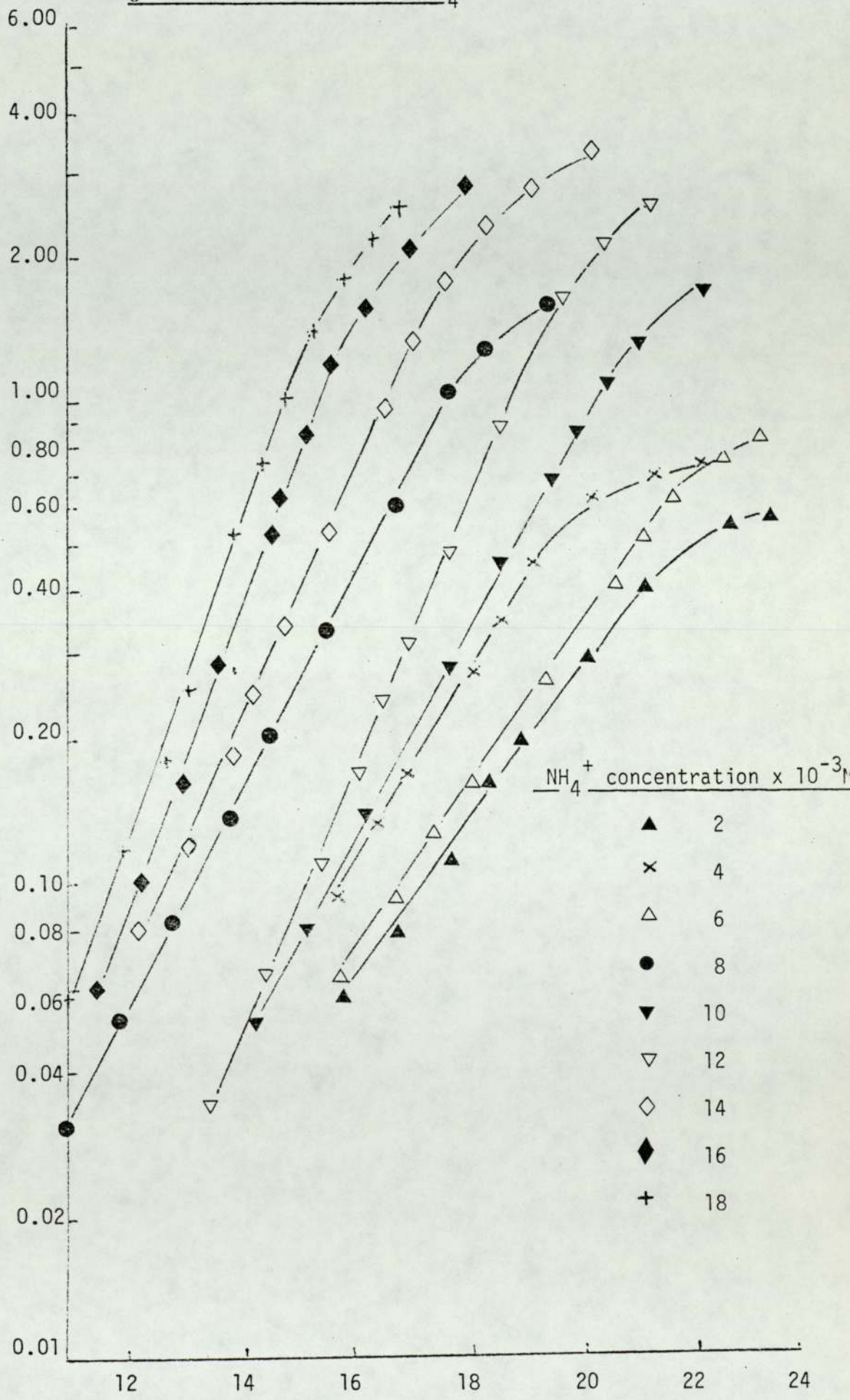


Fig. 28

Relationship between onset of non-linear growth of *P. aeruginosa* and initial NH_4^+ concentration

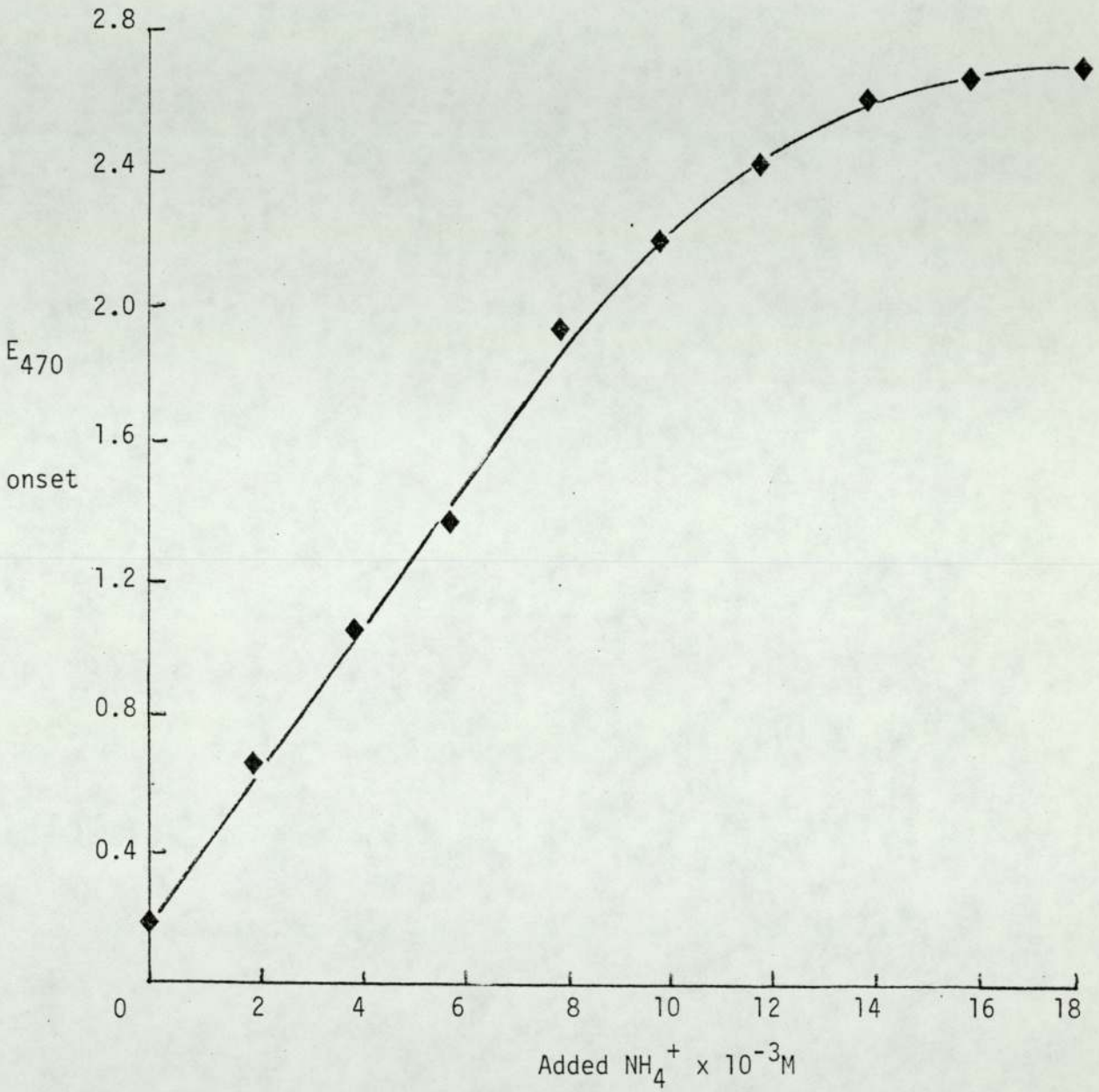


Fig. 29

Relation between maximum growth of P. aeruginosa and initial NH_4^+ concentration

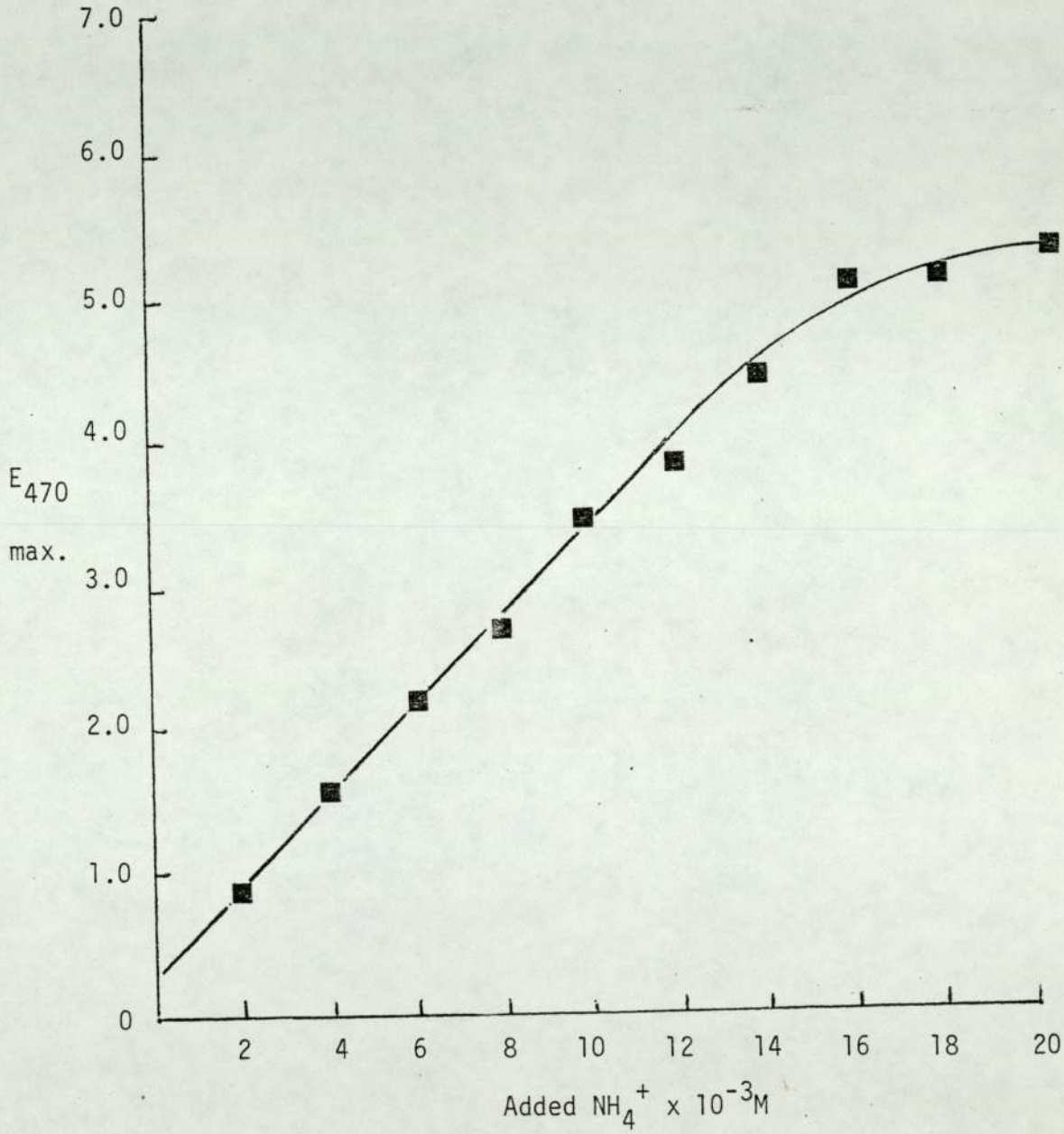


Fig. 30 Growth of P. aeruginosa in C.D.M. containing graded concentrations of K^+

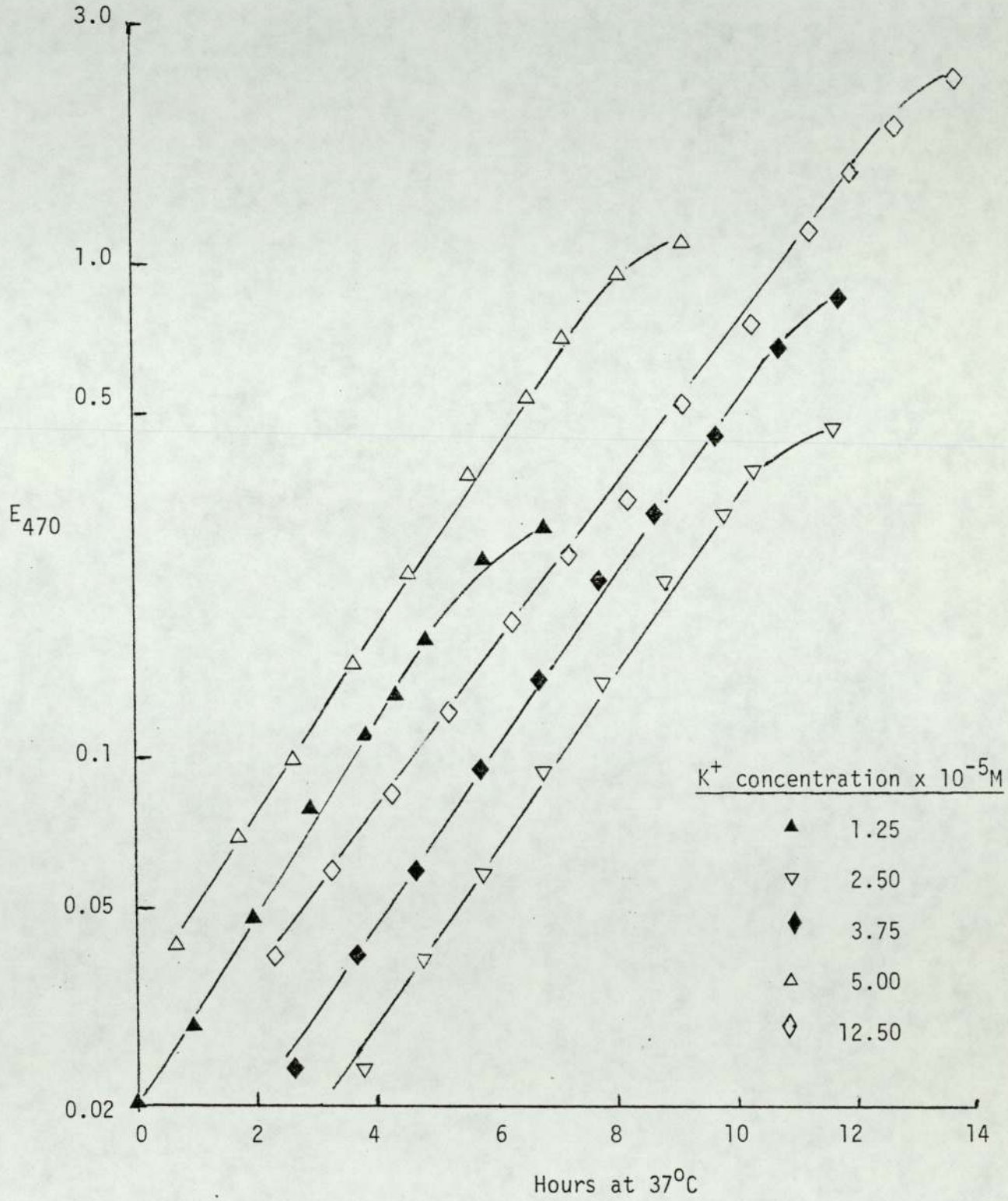
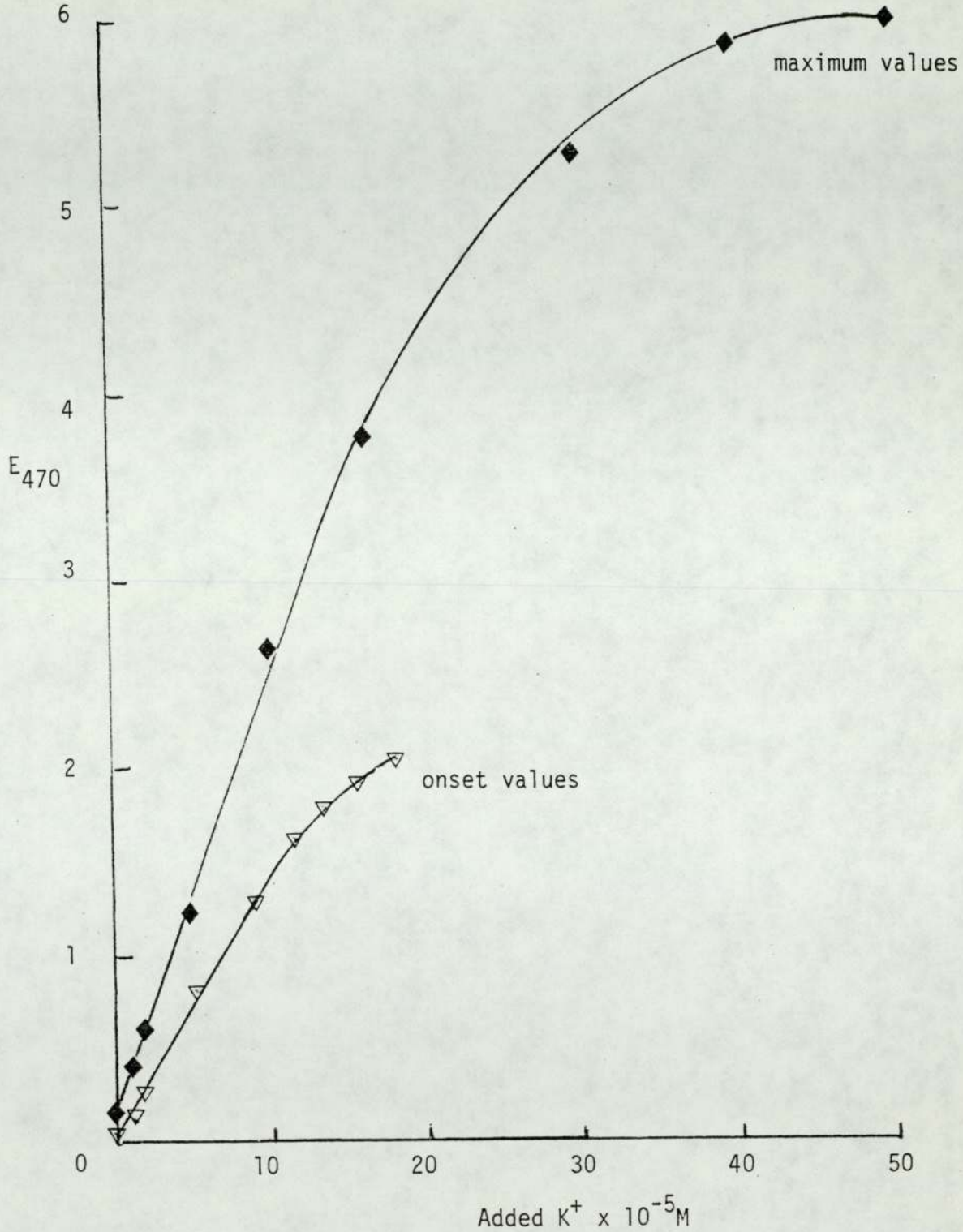


Fig. 31 Relationship between onset of non-linear growth and maximum growth of *P. aeruginosa* and initial K^+ concentration



The relationship between E_{470} and added K^+ is also shown in Fig. 31. Linear growth was maintained to an E_{470} max. of 2.45 corresponding to a K^+ concentration of 10.7×10^{-5}M. Extrapolation of the onset and maxima curves gave K^+ contamination levels of 0.2×10^{-5} and 0.6×10^{-5}M respectively.

3.1.5. Growth limitation with magnesium

The relationship between E_{470} and added Mg^{2+} for Mg^{2+} concentrations of 1.0×10^{-5} - 5.0×10^{-5}M is shown in Fig. 32 . Following the cessation of exponential growth the growth rate showed a gradual decrease but did not cease altogether. The relationship between E_{470} onset and added Mg^{2+} is shown in Fig. 33 , for Mg^{2+} concentrations of 0.2×10^{-5} to 14.5×10^{-5}M. Linearity was maintained to an E_{470} value of 1.7 corresponding to an initial Mg^{2+} concentration of 7×10^{-5}M. Fig. 33 also shows the relationship between E_{470} and added Mg^{2+} concentration. Linearity was maintained to an E_{470} of 3.5 corresponding to a Mg^{2+} concentration of 9.2×10^{-5}M. Extrapolation of the onset and maxima curves gave Mg^{2+} contamination levels of 0.1×10^{-5}M.

3.1.6. Growth limitation with iron

Initial experiments with iron limited cultures suggested that P. aeruginosa 6750 had no requirement for iron. E_{470} values for added Fe^{2+} were far higher than predicted from Boggis (1971). C.D.M. without added Fe^{2+} supported growth to an E_{470} of approximately 0.5. Calculation of the possible Fe^{2+} contamination from the other medium constituents revealed that as prepared C.D.M. should contain less than 4×10^{-9}M Fe^{2+}. Analysis of MOPS revealed that at use dilution it contained 0.64×10^{-9}M Fe^{2+}. When the glassware washing procedure was

Fig. 32 Growth of P. aeruginosa in C.D.M. containing graded concentrations of Mg^{2+}

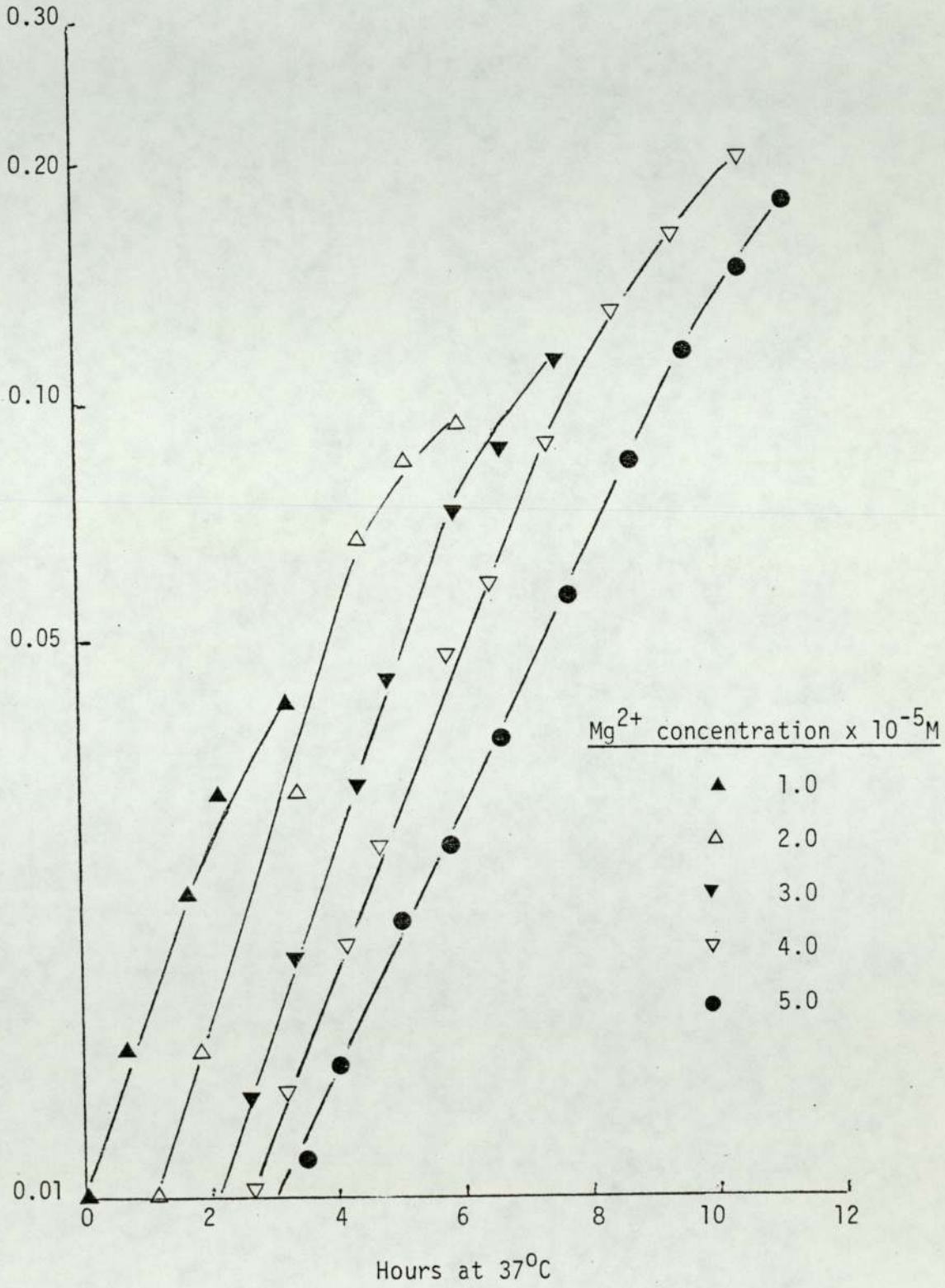


Fig. 33 Relationship between onset of non-linear growth and maximum growth of *P. aeruginosa* and initial Mg^{2+} concentration

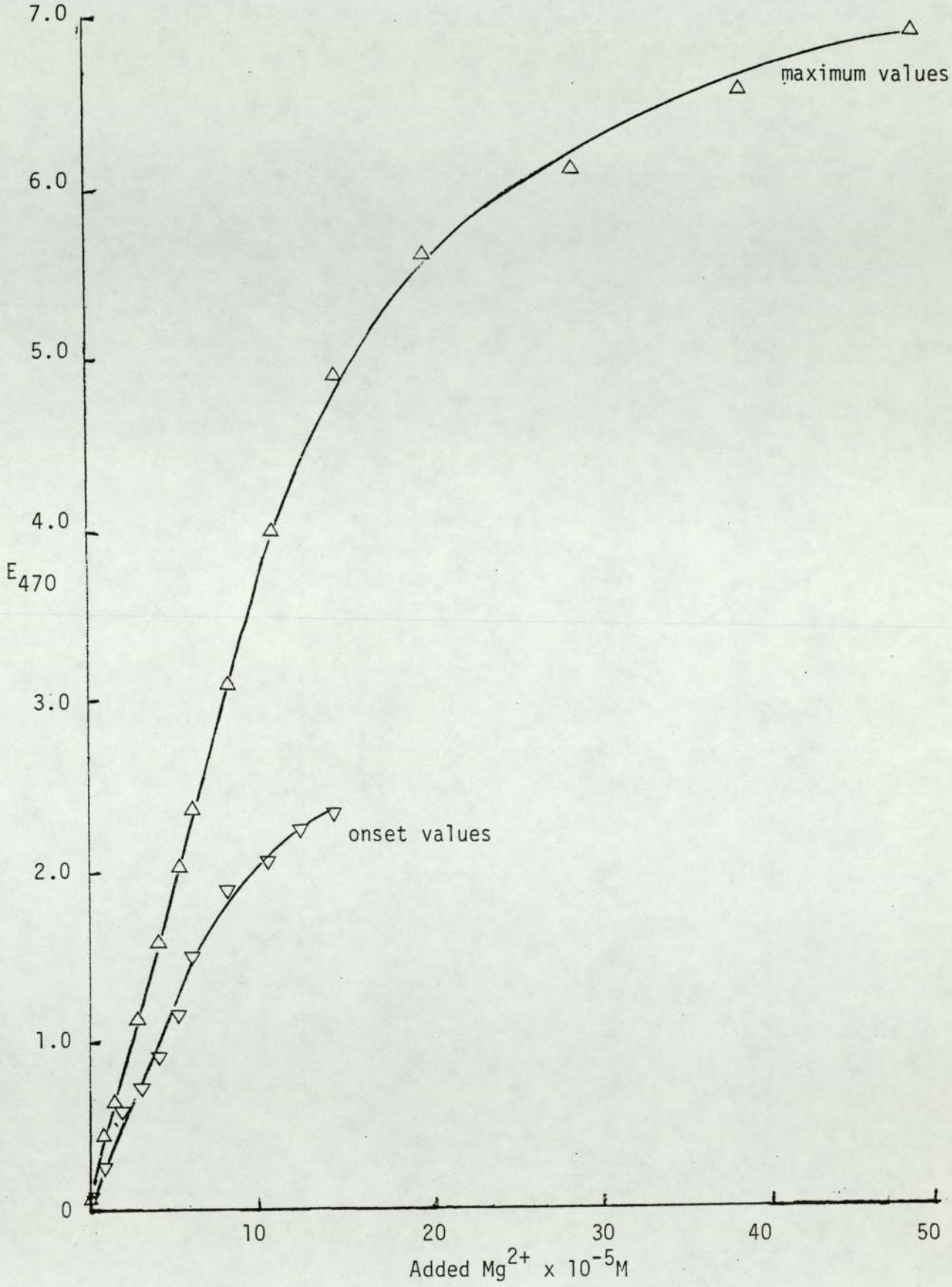


Fig. 34 Growth of P. aeruginosa in C.D.M. containing graded concentrations of Fe^{2+}

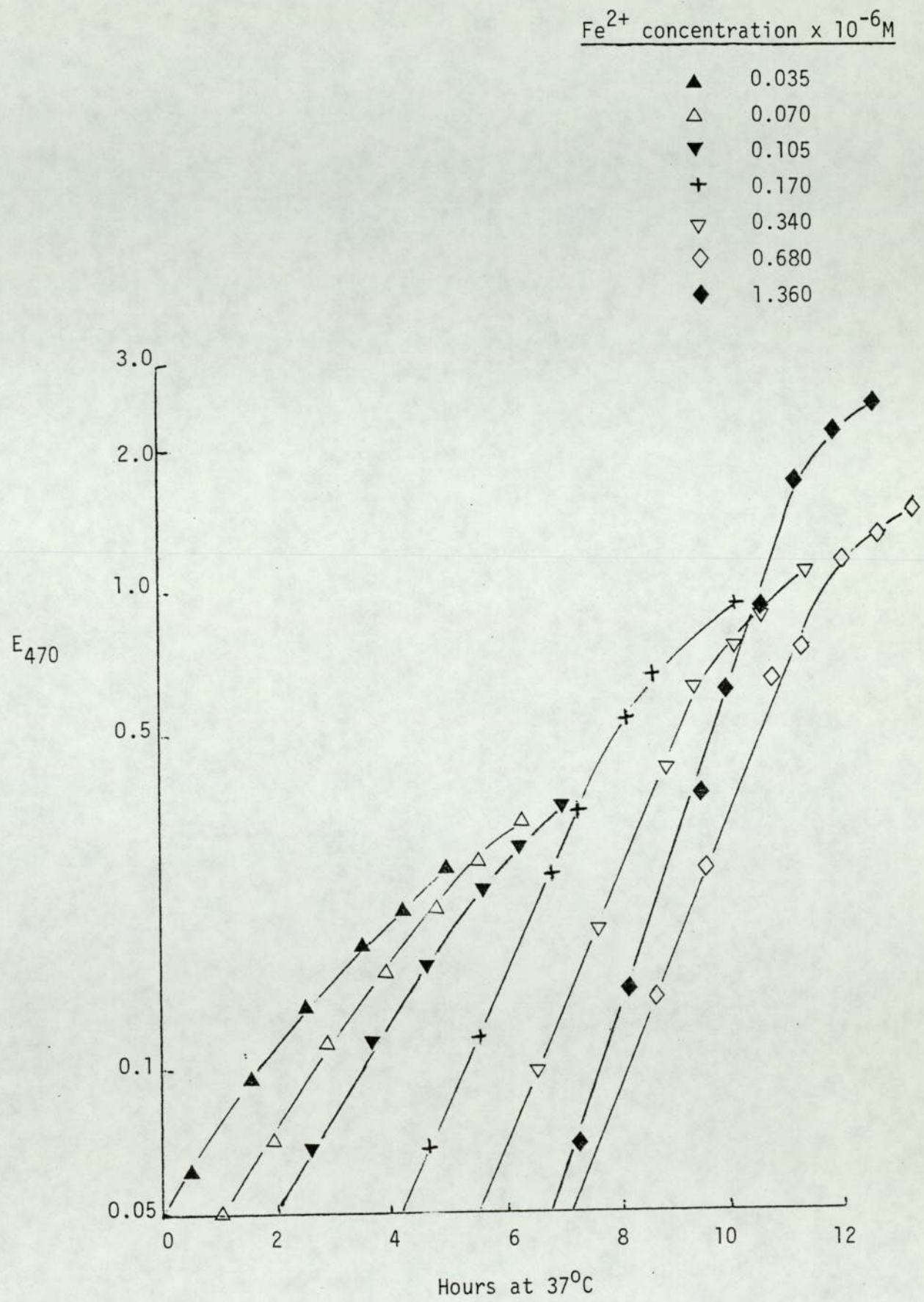
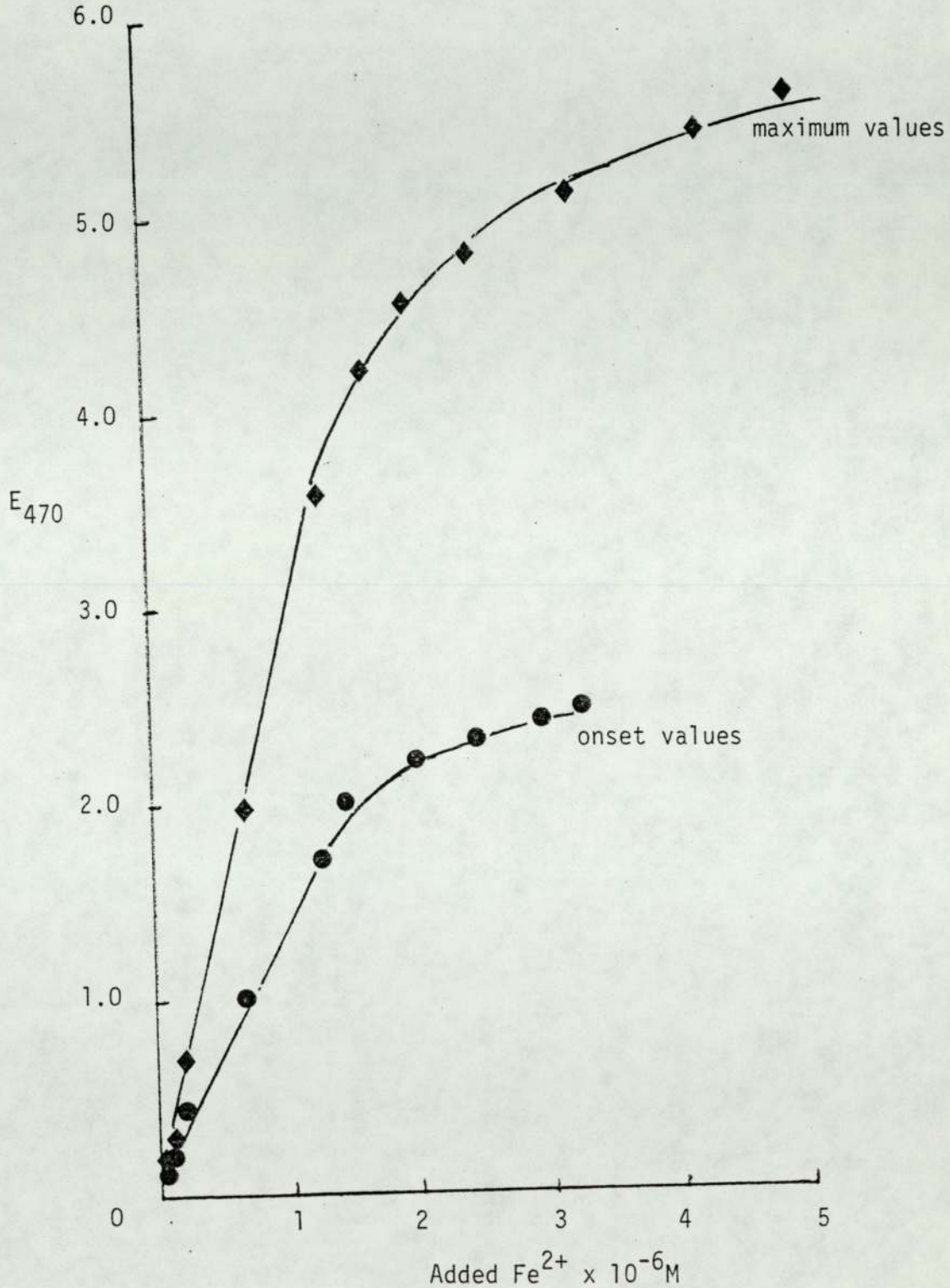


Fig. 35 Relationship between onset of non-linear growth and maximum growth of *P. aeruginosa* and initial Fe^{2+} concentration



altered to give an E.D.T.A. wash followed by further washes with deionised distilled water then growth in C.D.M. without added Fe^{2+} reached an E_{470} onset of 0.2 only. This value was reduced to 0.1 by the subsequent use of Aristar or Ultrar grade chemicals wherever available to make C.D.M.

Fig. 34 shows the growth curves for initial Fe^{2+} concentrations of 0.035×10^{-6}M to 1.36×10^{-6}M. Following the cessation of exponential growth the growth rate exhibited a gradual decrease. Fig. 35 shows the relationship between E_{470} onset and add Fe^{2+} for a range of Fe^{2+} concentrations from 0.035×10^{-6} - 3.3×10^{-6}M. Linearity occurred to an E_{470} onset of 1.7 corresponding to an initial Fe^{2+} concentration of 1.1×10^{-6}. Fig. 35 also shows the relationship between E_{470} max and added Fe^{2+} over the same range of Fe^{2+} concentrations. Linearity was maintained to an E_{470} of 3.4 corresponding to an initial Fe^{2+} concentration of 1.16×10^{-6}M. Fe^{2+} contamination was estimated at 0.1×10^{-6}M by extrapolation.

3.1.7. Growth limitation with phosphate

Fig. 36 shows the relationship between E_{470} and added phosphate in the range of PO_4^{3-} concentrations 0.35×10^{-4} to 3.4×10^{-4}M. The exponential growth rate was dependent upon initial PO_4^{3-} concentrations below 3.4×10^{-4}M. Following exponential growth the growth rate gradually decreased but did not cease altogether. The relationship between ED_{470} and added PO_4^{3-} for a range of PO_4^{3-} values from 0.35×10^{-4} - 12×10^{-4}M is shown in Fig. 37 . The relationship between E_{470} onset and added PO_4^{3-} was linear to a PO_4^{3-} concentration of 5.6×10^{-4}M corresponding to an E_{470} onset of 1.7. Fig. 37 also shows the relationship between E_{470} and added PO_4^{3-}. Linearity was maintained to an E_{470} max of 3.80 corresponding to a PO_4^{3-} concentration of 4.4×10^{-4}M. Extrapolation of the onset and maximum curves shows PO_4^{3-} contamination levels of 0.2×10^{-4}M.

Fig. 36 Growth of P. aeruginosa in C.D.M. containing graded concentrations of PO_4^{3-}

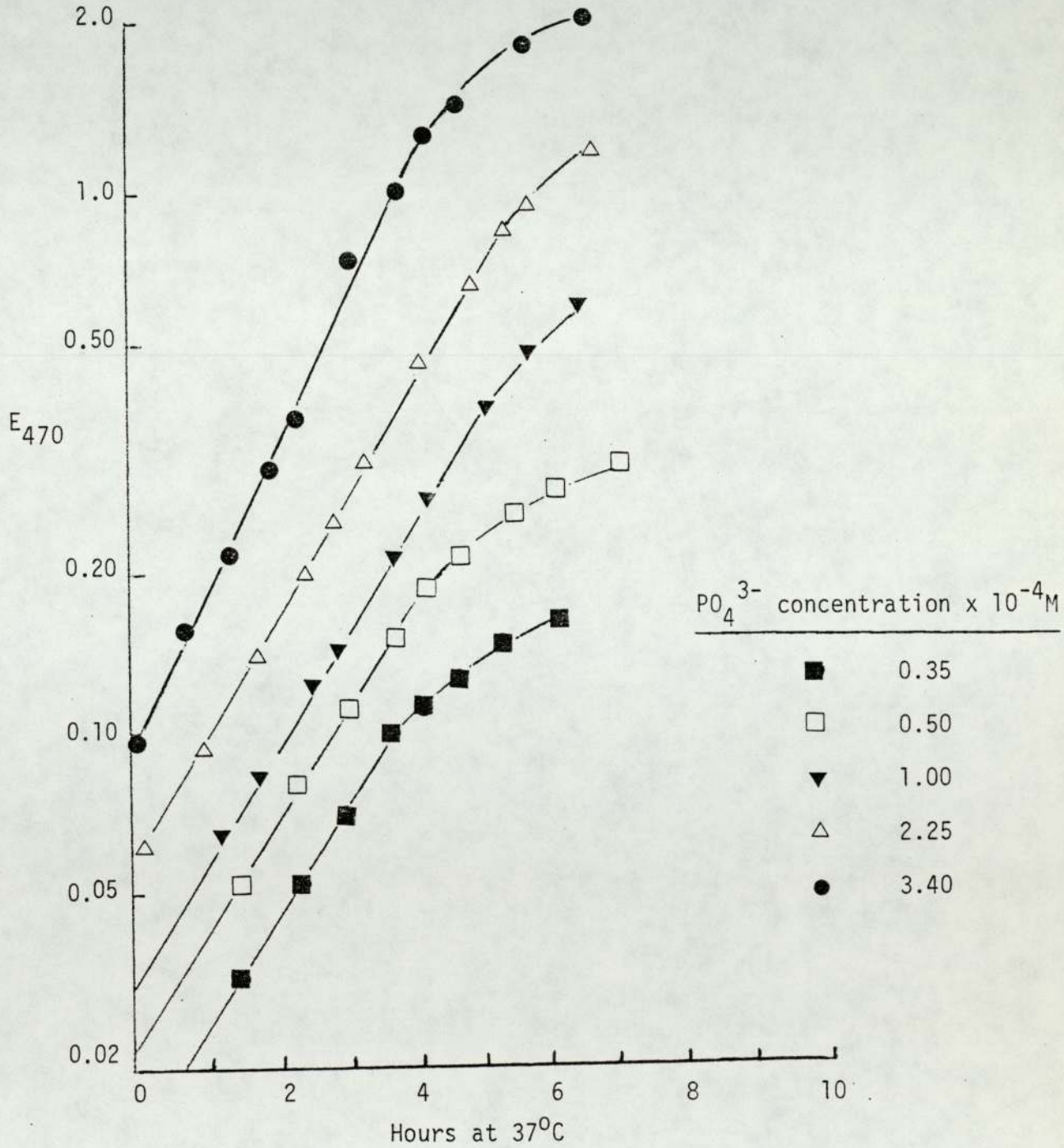
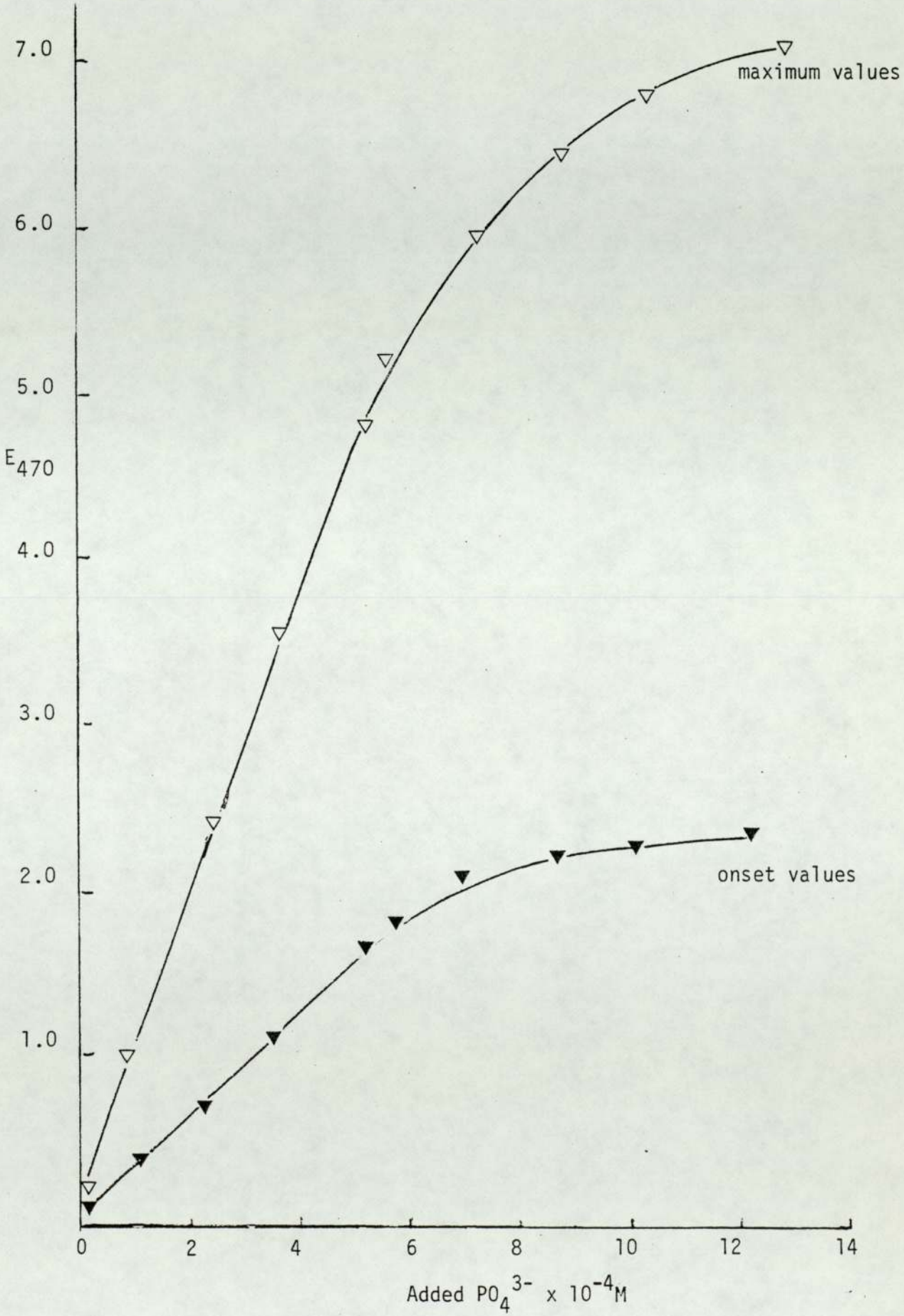


Fig. 37 Relationship between onset of non-linear growth and maximum growth of *P. aeruginosa* and initial PO_4^{3-} concentration



3.1.8. Growth limitation with glucose

Fig. 38 shows the characteristic growth curves for initial glucose concentrations of 4×10^{-4} to 80×10^{-4}M. Unlike the other nutrient limitations when glucose becomes depleted growth ceased abruptly followed by a gradual decline in E_{470}. Thus the E_{470} onset and maxima are coincident. Fig. 39 shows the relationship between E_{470} and added glucose for a range of glucose concentrations from 4×10^{-4} - 280×10^{-4}M. A linear relationship existed to an E_{470} of 4.1 corresponding to an initial glucose concentration of 165×10^{-4}M. Extrapolation of this graph gave glucose contamination of 0.0M.

3.1.9. Growth limitation with sulphate

The problems caused by the utilisation of MOPS buffer by P. aeruginosa 6750 have been mentioned earlier (section 2.2.5). During sulphate limitation experiments this problem was recognised by (1) the culture attaining a far higher optical density than that predicted. An initial SO_4^{2-} concentration of 2.5×10^{-5}M was required by Boggis (1971) to attain an E_{470} onset of 0.5 whereas in these experiments only 0.1×10^{-5}M SO_4^{2-} were required. (2) In C.D.M. containing no added sulphate E_{470} onset values of 0.5 and E_{470} max values of 2.58 were obtained indicating SO_4^{2-} contamination of 1.3×10^{-5}M. These results could be explained by the breakdown of MOPS by P. aeruginosa. Sulphate limited growth was obtained by substitution of ammonium acid phosphate buffer for MOPS. Fig. 40 shows the growth curves for cultures grown in initial SO_4^{2-} concentrations of 0.25×10^{-5}M - 11.35×10^{-5}M. Fig. 41 shows the relationship between E_{470} onset and max values for sulphate in the range 0.25 to 18×10^{-5}M SO_4^{2-} for cells grown in C.D.M. containing MOPS and ammonium acid phosphate buffer.

Fig. 38 Growth of P. aeruginosa in C.D.M. containing graded concentrations of glucose

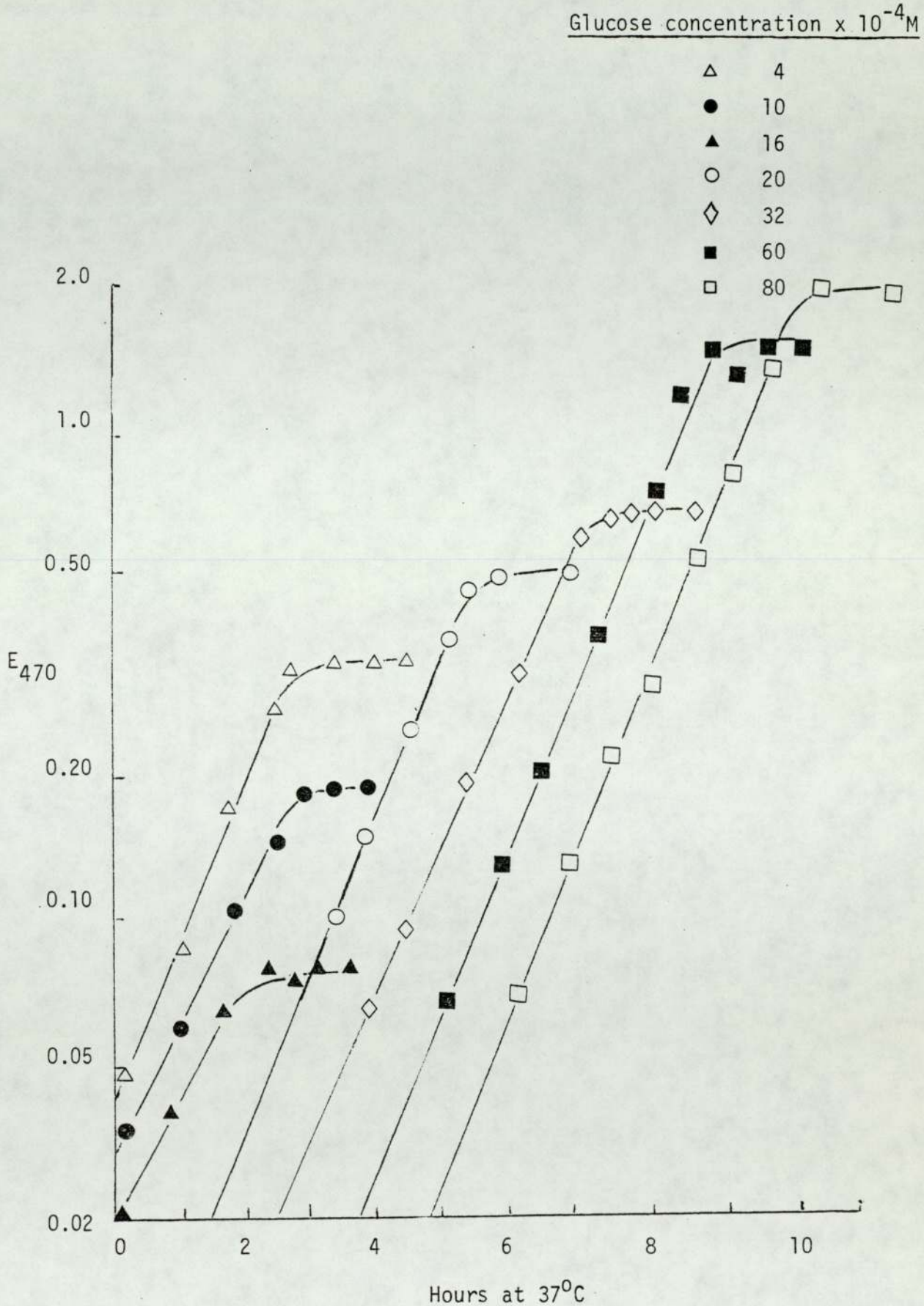


Fig. 39 Relationship between maximum growth of *P. aeruginosa* and initial glucose concentration

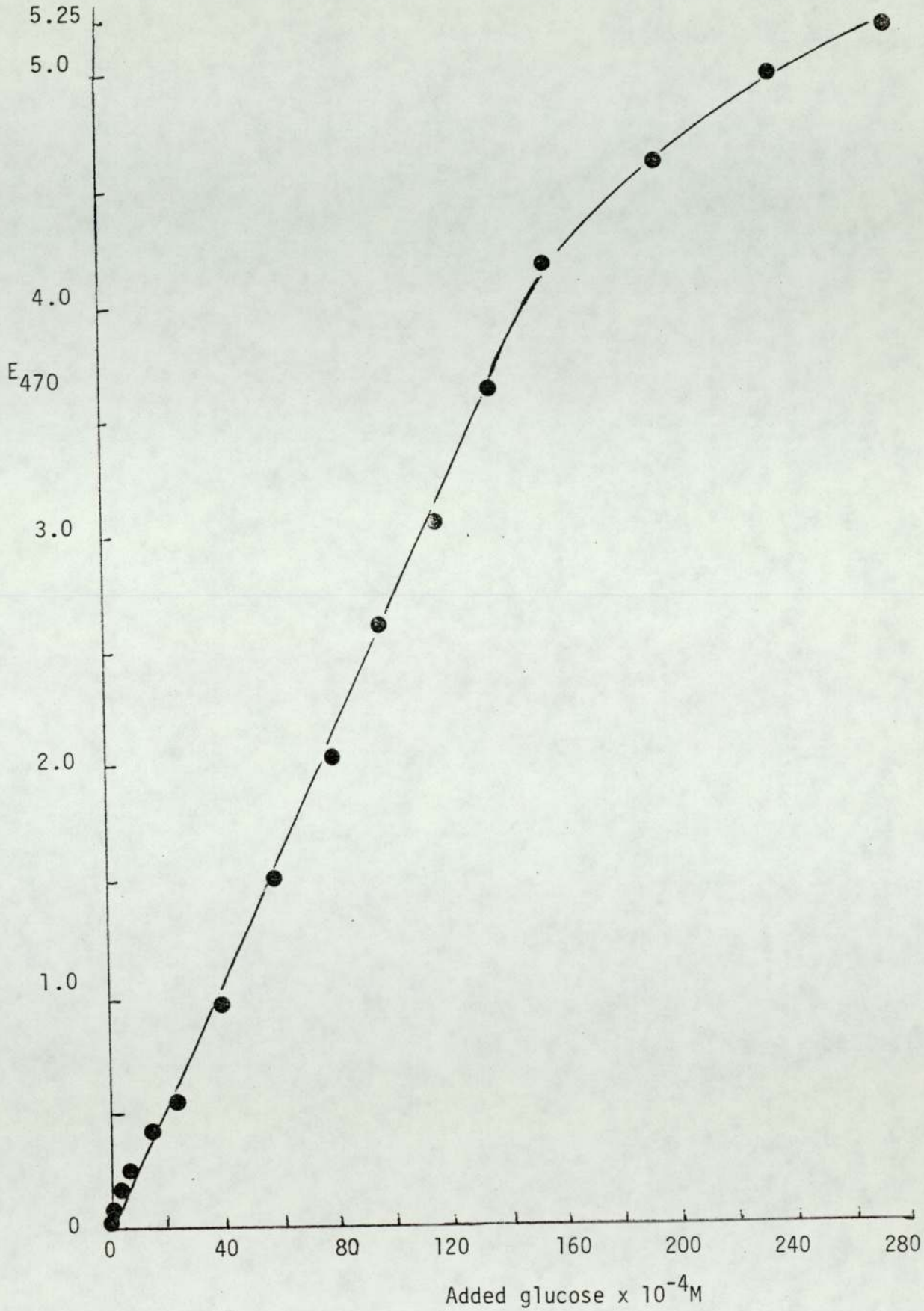


Fig. 40 Growth of P. aeruginosa in C.D.M. containing graded concentrations of SO_4^{2-}

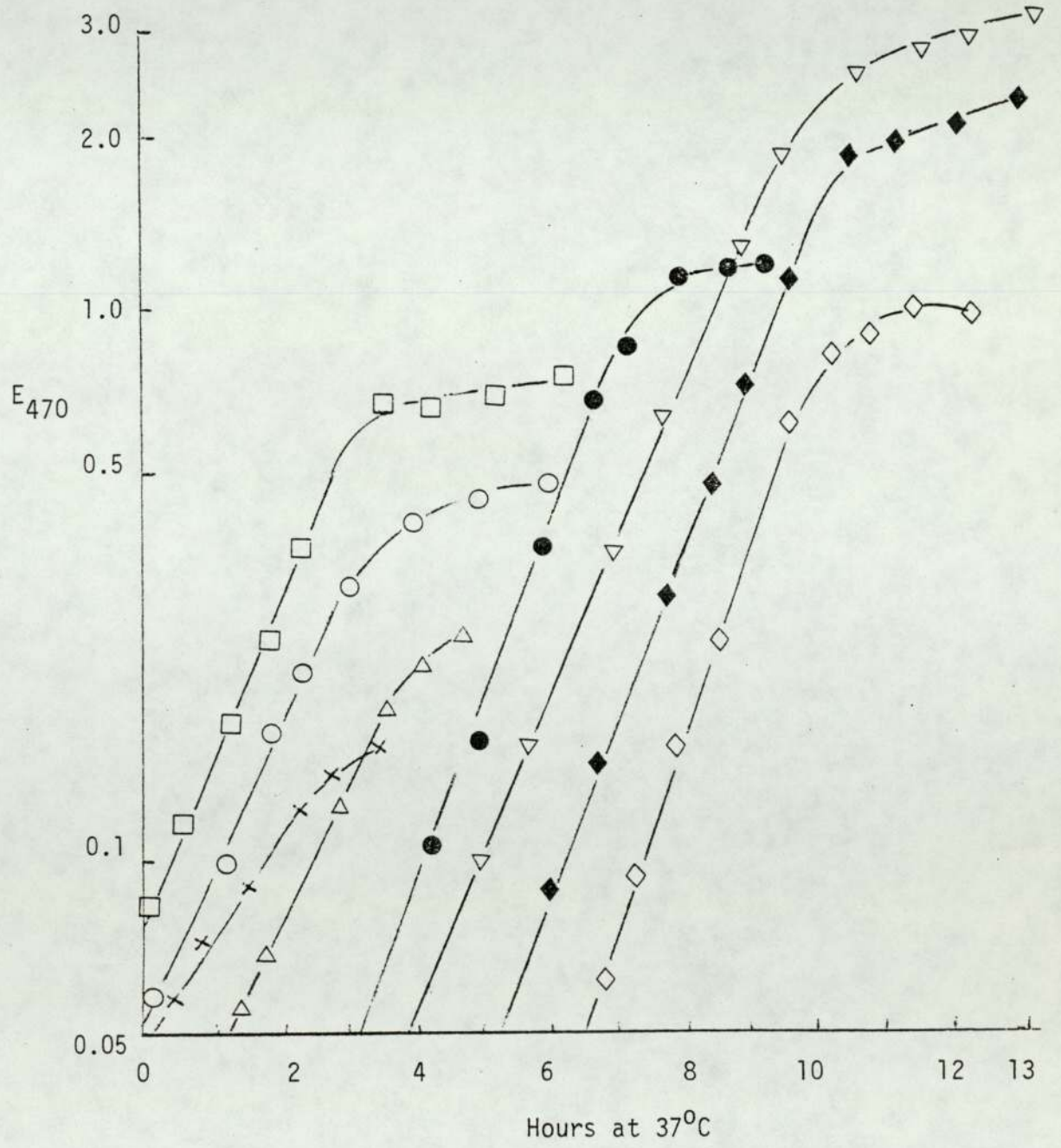
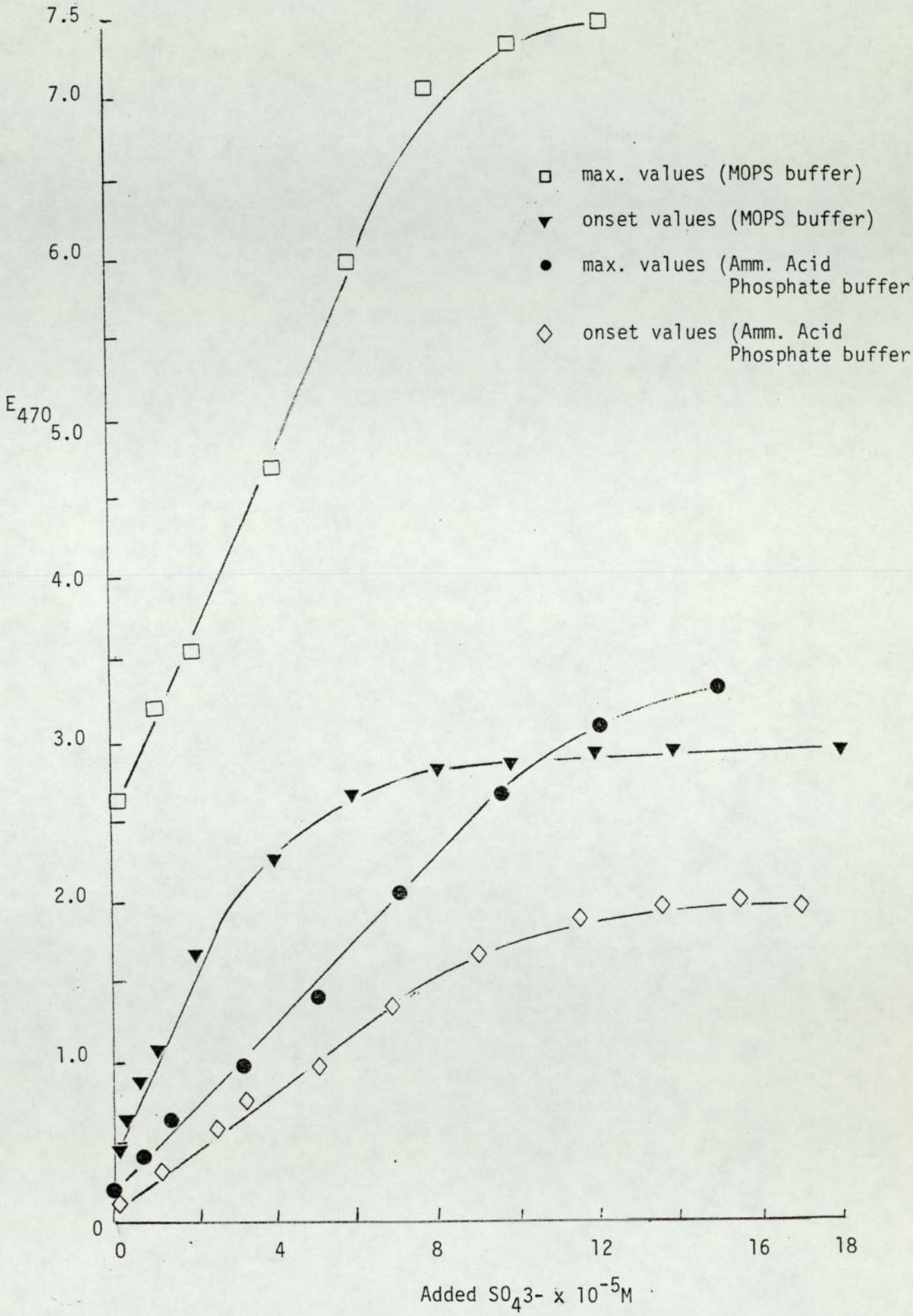


Fig. 41 Relationship between onset of non-linear growth and maximum growth of *P. aeruginosa* and initial SO_4^{2-} concentration



Using MOPS buffer linearity was maintained to an E_{470} of 1.8 (onset value) and 6.5 (max value) whereas when the medium was reformulated to contain ammonium acid phosphate buffer the linearity was achieved to onset and maximum values of 1.3 and 2.50 respectively. These values corresponded to SO_4^{2-} concentrations of 7.5×10^{-5}M and 9.5×10^{-5}M. Apparent sulphate contamination was 0.8×10^{-5}M when ammonium acid phosphate buffer was used and 0.5 to 5.0×10^{-5} when MOPS buffer was used.

3.1.10. Summary of limitation studies

The work done on nutrient limitation showed that a linear relationship exists between E_{470} onset and the limiting nutrients Mg^{2+}, glucose, NH_4^+, SO_4^{2-}, PO_4^{3-}, Fe^{2+} and K^+. Thus a chemically defined medium may be prepared which will allow the E_{470} of a culture to be varied to the required level for any particular nutrient. The limitation curves also allow the medium to be formulated containing a controlled excess of nutrients.

Table 22 summarises the media formulated from these experiments to give a single nutrient limitation. The results of the nutrient limitations are shown in Table 23.

Linearity in this experiment was obtained to different E_{470} values depending on the nutrient studied. However the values obtained for E_{470} were in the range 1.3 - 1.9. The value of E_{470} onset of 1.3 for SO_4^{2-} limitation is slightly lower than the rest and may reflect the reformulation of the medium necessary to study sulphate limitation. For sulphate limitation MOPS was replaced by ammonium acid phosphate buffer and all nutrients containing SO_4^{2-} were replaced by their chloride

Table 22

Notes

All concentrations expressed in Moles.

All nutrients other than that under test are in 10X theoretical excess.

Media adjusted to pH 7.8 with NaOH.

* 1ml l^{-1} conc HCl added to prevent precipitation of stock.

** Sulphate molarity to attain E_{470} onset 1.0 using MOPS.

*** Sulphate molarity to attain an E_{470} onset 1.0 using acid ammonium phosphate.

Table 22

Composition of C.D.M. used to obtain an E_{470} onset of 1.0

CONSTITUENT	LIMITATION					
	Glucose	NH_4^+	PO_4^{2-}	K^+	Mg^{2+}	Fe^{2+}	SO_4^{2-}
$(NH_4)_2SO_4$	4×10^{-2}	4×10^{-3}	←	4×10^{-2}	→	→	-
$Na_2H\ PO_4$	←	3.2×10^{-3} →	3.2×10^{-4} ←	3.2×10^{-3}	→	→	→
Glucose	4×10^{-3}	←	4×10^{-2}	→	→	→	→
KCl	←	←	6.2×10^{-4} →	6.2×10^{-5} ←	←	6.2×10^{-4}	→
NaCl	←	←	5×10^{-4}	→	→	→	→
$FeSO_4.7H_2O$*	←	←	6.2×10^{-6}	→	→	0.62×10^{-6}	-
$MgSO_4.7H_2O$	←	←	4×10^{-4}	→	4×10^{-5} ←	4×10^{-4}	→ -
NH_4Cl	-	-	-	-	-	-	4×10^{-2}
$FeCl_2.4H_2O$	-	-	-	-	-	-	6.2×10^{-5}
$MgCl_2.6H_2O$	-	-	-	-	-	-	4×10^{-4}
Na_2SO_4	-	-	-	-	-	-	0.8×10^{-5}** 5.2×10^{-5}***
MOPS	←	←	0.06	→	→	→	→
Acid Ammonium Phosphate	-	-	-	-	-	-	7×10^{-3}

Table 23

E_{470} onset, E_{470} max. values for linear growth and ion contamination for individual nutrients in C.D.M.

Limiting ion	Linearity maintained to		Ion contamination (M)
	E_{470} onset	E_{470} max.	
PO_4^{3-}	1.7	3.80	0.2×10^{-4}
Mg^{2+}	1.7	3.50	0.1×10^{-5}
Fe^{2+}	1.7	3.40	0.1×10^{-6}
NH_4^+	1.9	3.65	1.0×10^{-3}
Glucose	-	4.00	0.00
K^+	1.55	2.45	$0.2\text{-}0.6 \times 10^{-5}$
SO_4^{2-}	1.3 *	2.50*	0.8×10^{-5}*
	1.8**	6.50**	$0.5\text{-}5.0 \times 10^{-5}$**

* Using acid ammonium phosphate buffer

** Using MOPS buffer

salts. Values of E_{470} max were generally in the range 3.4 to 4.00. Values outside this range occurred for K^+ and SO_4^{2-} limitations. For all experiments a 10X excess of the nonlimiting nutrients was used. The maximum E_{470} attained for all limitations was 6.0 to 7.0.

3.2. The effect of phosphate upon lysis and viability of batch grown *P. aeruginosa* treated with Polymyxin B

3.2.1. Practical Procedures

3.2.1.1. Adjustment of Initial Optical Density

The preceding work (Section 3.1) on formulation of a chemically defined medium for *P. aeruginosa* 6750 enabled a medium to be prepared to study the effect of phosphate depletion on the sensitivity to EDTA and Polymyxin B (PB). Since it is likely that the lysis by these agents will be affected by the cell concentration, experiments were performed to formulate a medium which would allow cells grown under different degrees of PO_4^{3-} depletion to cease growth at a predetermined E_{470}. For these experiments cessation of growth was controlled at E_{470} 0.2. Work by Brown and Melling (1969a) studying the effect of magnesium limitation on the lytic action of EDTA had shown that when cells grown under different levels of magnesium depletion finally ceased growth due to glucose depletion the more severely magnesium depleted cells had a higher glucose requirement to attain a given E_{470}. Such cells also showed a variable lag phase which was inversely proportional to the magnesium concentration in the medium. It seemed likely that cells which were growing slower (due to nutrient depletion) had greater glucose requirements for cell respiration.

Experiments were performed to determine the glucose requirements of cells grown under different levels of phosphate depletion such that for lysis experiments the growth medium formulation could be adjusted such that cell growth ceased at an E_{470} of approximately 0.2.

Cells were grown in C.D.M. containing 8 x 10^{-4}M glucose with added phosphate in the range 0.1 to 0.24 x 10^{-4}M and the E_{470} max. recorded. Fig. 42 shows the relationship between E_{470} max. and the initial amount of PO_4^{3-} in the medium. The E_{470} max. achieved was dependent upon the PO_4^{3-} in the medium and suitable adjustment of the PO_4^{3-} would allow an E_{470} of 0.2 to be attained.

Cells were similarly grown in C.D.M. containing 0.1 x 10^{-4}M PO_4^{3-} but with varying amounts of glucose in the range 8 - 16 x 10^{-4}M. Fig. 43 shows the relationship between the final E_{470} obtained and the amount of glucose in the medium. Thus a final E_{470} of 0.2 could be obtained by adjustment of the glucose content of the medium.

3.2.1.2. Media for lysis studies

The medium used in lysis studies was formulated to achieve an E_{470} of 0.2 prior to lysis. The medium contained a theoretical 5 x excess of non limiting nutrients:- 4 x 10^{-3}M $(NH_4)_2SO_4$; 4 x 10^{-5}M $MgSO_4.7H_2O$; 5 x 10^{-4}M NaCl; 6.2 x 10^{-5}M KCl; 0.62 x 10^{-6}M $FeSO_4.7H_2O$ and 0.06M MOPS in deionised distilled water. The initial pH was adjusted to pH7.4. The glucose content of the medium was adjusted to ensure an E_{470} of 0.2 for the variable amount of PO_4^{3-} in the medium.

3.2.1.3. Drug treatment

Two methods were used to investigate the lytic action of PB and E.D.T.A. upon PO_4^{3-} limited cells. The noncentrifugation method described previously by Brown and Melling (1969a) and Boggis (1971) using unwashed cells was performed and also a centrifugation method using washed cells. The inoculum history was identical for both methods. P. aeruginosa 6750 was grown in C.D.M. containing a low concentration of PO_4^{3-}. This culture was used to inoculate flasks containing C.D.M. with various

Fig. 42

The effect of PO_4^{3-} on E_{470} max. for PO_4^{3-} limited cells with (8×10^{-4}M) glucose

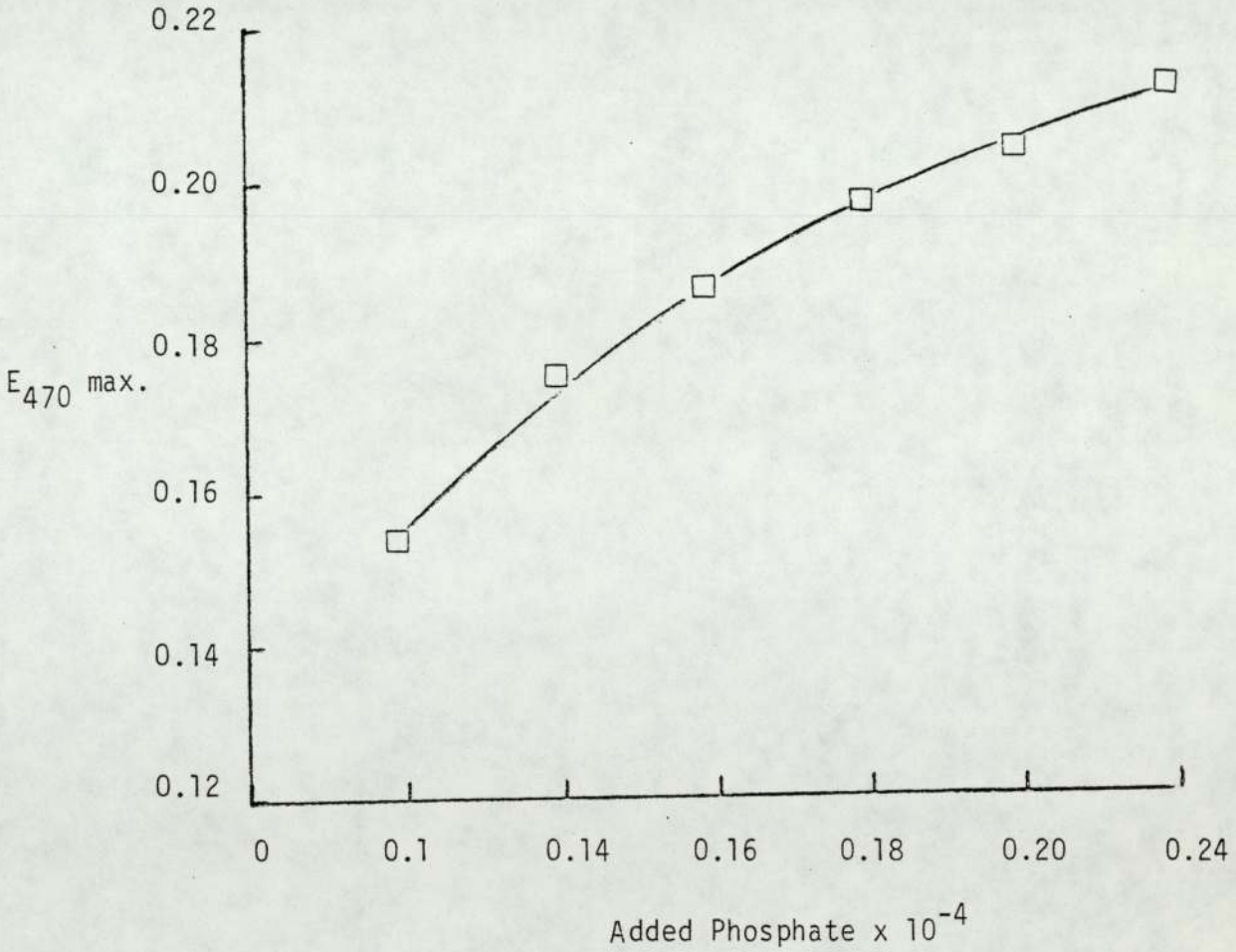
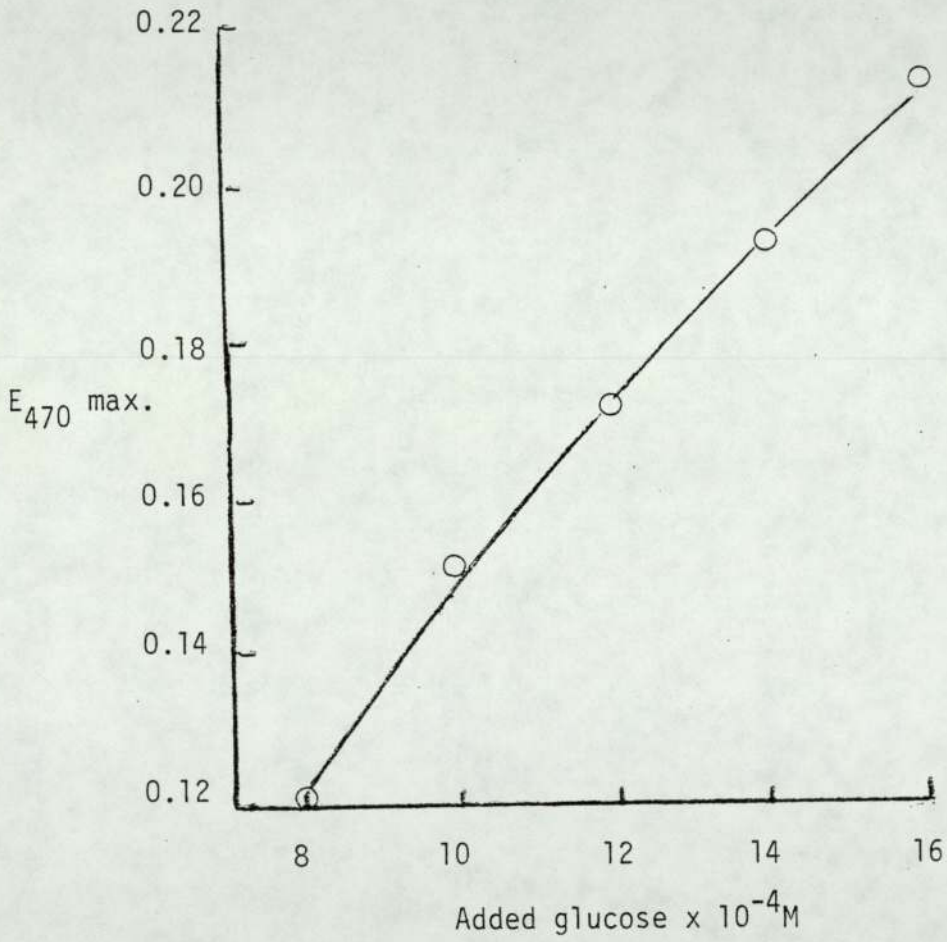


Fig. 43

The effect of glucose on E_{470} max. for glucose limited cells with (0.1×10^{-4}M) PO_4^{3-}



amounts of added PO_4^{3-}. The cells grew until glucose depletion intervened at an E_{470} of 0.2. As the cells neared glucose limitation the growth rate slowed dramatically. Growth was assumed complete when the growth rate was less than 2%/hour. The cultures were then treated by one or other of the two methods described below.

The noncentrifugation method was performed as below:
One hour following cessation of growth 24ml of culture (E_{470} 0.2) was rapidly transferred to a prewarmed 100ml Erhlenmeyer flask containing either 1ml of PB or E.D.T.A. The E_{470} values of the culture were read at time 0 minutes and at varying time intervals thereafter. Control flasks contained 1ml of distilled deionised water.

The centrifugation method used was as follows:
One hour following the cessation of growth the culture was centrifuged at 4,000 rpm for 20 minutes using prewarmed (37°C) centrifuge buckets. The cells were resuspended and washed 3 times in prewarmed C.D.M. lacking glucose. The formulation of the resuspension medium is described later. Following the third wash the cells were resuspended to an E_{470} of approximately 0.2 and allowed to equilibrate at 37°C in a shaking waterbath for at least one hour or until no further change in E_{470} occurred. To 24ml of culture was added 1ml of the appropriate agent (PB or E.D.T.A.) and the change in E_{470} recorded as described previously.

3.2.1.4. Total and colony counts

Samples were taken at the beginning and end of the lysis period for total and colony count estimations. The method of performing these estimations was as described previously (pages 82 and 83 respectively).

3.2.1.5. Dry weight estimations

These were performed as described by Meynell and Meynell (1970). One ml of the culture was treated with 10μl formalin and centrifuged at 4,000 rpm for 20 minutes in a weighed bijou bottle. The cells were washed once with 0.85% w/v saline + 1% formalin and once with distilled water. The deposited cells were dried for 24 hours at 105^{o}C and cooled in a dessicator over P_2O_5 before weighing.

3.2.1.6. Preparation of the lysis blank

The E_{470} of cells grown in C.D.M. and lysed to a value of 100% would not have the E_{470} of uninoculated C.D.M. To make the percentage lysis values following drug treatment more accurate an experiment was performed to determine the E_{470} of a culture lysed 100%. Cells at a range of optical densities up to 0.26 were disintegrated using a Dawes Soniprobe ultrasonic probe by the following method:-
The cells were cooled in a bath of ice/water and subjected to sonic disintegration for 4 x 1 minute intervals with 1 minute intervals between sonication to allow cooling. A sample of each set of sonicated cells was removed and Gram stained. The slide was examined for the presence of intact cells. None were found following this treatment. The optical density of the sonicated cells was E_{470} 0.040 to 0.046 although the final E_{470} following breakage showed no relationship to the initial E_{470}. Figures for % lysis were adjusted accordingly in lysis experiments.

Addendum

The formulation of the media used in this study resulted in cells grown in PO_4^{3-} levels up to 0.16 x 10^{-4}M being PO_4^{3-} limited for a variable time dependent upon the initial PO_4^{3-} concentration, growth eventually ceasing due to C-depletion. For PO_4^{3-} levels above 0.16 x 10^{-4}M cells were carbon depleted with a variable excess of PO_4^{3-}.

3.2.2. Factors affecting response to Polymyxin B

3.2.2.1. The effect of delayed exposure to Polymyxin B

Experiments performedusing cultures depleted to various degrees by PO_4^{3-} limitation to an E_{470} of 0.2 and subsequently lysed by 40 I.U. ml^{-1} of P.B. failed to show similar results to those obtained by Boggis (1971). He showed that at low levels of added phosphate P. aeruginosa was resistant to lysis by P.B. and that the cells became more sensitive as the amount of PO_4^{3-} in the growth medium was increased. In the present work lysis by 40 I.U. ml^{-1} of P.B. of cells grown in varying concentrations of phosphate gave results which suggested that the cells were equally sensitive to 40 I.U. ml^{-1} P.B. The cells, when treated with 40 I.U. ml^{-1} P.B. lysed approximately 60% in 60 minutes for PO_4^{3-} concentrations of 0.1×10^{-4}M to 10×10^{-4}M added PO_4^{-3}.

Delay between the cessation of bacterial growth and the addition of P.B. for lysis studies may have contributed to this difference. Boggis (1971) performed his lysis experiments one hour after growth ceased due to glucose depletion. In practice the identification of the cessation of growth was rather more difficult than expected. At higher levels of PO_4^{3-} in the C.D.M. growth ceased abruptly due to glucose depletion. However, at lower PO_4^{3-} levels this abrupt cessation of growth was less clearly seen. The cells growth rate slowed considerably such that it became difficult to measure changes in E_{470} even at hourly intervals. It was thus difficult to assess accurately one hour after growth ceased before commencing the lysis experiments.

To investigate the possible effect on lysis values of delay between cessation of growth and treatment with P.B. five replicate sets of five flasks were inoculated with P. aeruginosa. The C.D.M. used contained

2.5×10^{-4}M PO_4^{3-}. Growth in media containing this concentration of phosphate exhibited a clearly definable time at which growth ceased. One hour after cessation of growth cells in five flasks were treated with 40 I.U. ml^{-1} of P.B., and the percentage lysis after 60 minutes measured. The remaining flasks were reincubated at 37°C in the shaking water baths and sets of five flasks removed after 1 hour, 3 hours and 5 hours shaking. Treatment with P.B. and measurement of lysis was identical for all sets of flasks. The lysis results are shown in Table 24 and subjected to an analysis of variance (Table 25).

Table 24

% Lysis After 60 minutes for *P. aeruginosa* Following Delayed Exposure to 40 I.U. ml^{-1} Polymyxin B

Delay / Flasks	0 hr	1 hr	3 hr	5 hr
1	64.6	63.6	61.9	61.2
2	64.0	64.2	59.7	60.6
3	65.2	63.3	62.3	59.2
4	63.4	65.4	60.5	58.6
5	65.2	63.4	62.8	60.2
Totals T	322.4	319.9	307.2	299.8

n = number of observations per set = 5

m = number of hours delay = 4

n.m = total number of observations = 20

1. $\Sigma x^2 = 78122.53$

2. $\frac{\Sigma T^2}{n} = 78105.93$

3. $(\Sigma x)^2 = 78037.53$

Table 25

Analysis of Variance of Lysis Values

Source of Variance	Sum of Squares	Degrees of Freedom	Mean Squares	Variance Ratio 'F'
Between sets	(2) - (3) 68.40	m - 1 3	22.800	21.9
Within sets	(1) - (2) 16.60	nm - m 16	1.037	

The tabulated values of 'F' for 3/16 degrees of freedom at the 1% and 5% significance levels are 5.29 and 3.24 respectively. Therefore the variation between sets of flasks (i.e. delay in lysis) is greater than the variation within flasks at 1% and 5% levels.

Therefore in an attempt to standardise the time between cessation of growth and lysis was important. The results would suggest that delay reduces the sensitivity to P.B. although the decrease in lysis seen in this experiment was small. For cells grown in low levels of PO_4^{3-} some delay in determination of when growth ceased was inevitable. The cells would then have slightly lower lysis values than if the delay was minimised. It was decided that cells should be given one hour from when the E_{470} rise fell below 2.0% per hour before lysis.

3.2.2.2. The effect of repeated centrifugation on the E_{470}, colony count and total count during the 'holding period', and treatment with 10 I.U. ml^{-1} Polymyxin B

The effect of centrifugation on the three parameters, E_{470}, colony count, and total count during the one hour 'holding period' for cells grown in C.D.M. containing 0.13×10^{-4}, 0.22×10^{-4} and 1.00×10^{-4}M PO_4^{3-} was investigated using the following procedure.

Cultures were grown in several flasks to an E_{470} of 0.2. The cells were pooled and filtered through a 0.2μ membrane filter in a Millipore filtration unit. The cells were then washed in resuspension medium (C.D.M. minus glucose). The cells were again filtered and the filter reverse flushed using fresh resuspension medium. This final amount of medium was adjusted such that the resuspended cells had an E_{470} of 0.21 - 0.22. 29ml volumes of the washed cells were placed in separate centrifuge tubes and centrifuged at 4,000 rpm for 20 minutes. After each centrifugation one tube was removed and the pellet in each tube resuspended in the supernatant fluid. This was repeated until the cells in the final tube have undergone 5 centrifugations. From the resuspended cells in each tube 1ml of culture was taken for colony count, 0.1ml for total count and the E_{470} measured. The remaining culture was held for the one hour 'holding period' at 37°C with shaking. After one hour the colony count, total count and E_{470} were again measured. 24ml of the remaining culture was taken and treated with 1ml 10 I.U. ml^{-1} P.B. The % fall in E_{470} was recorded over one hour and the colony and total count determined at the end of this time. From the results the change in E_{470}, colony count and total count during the holding period and following P.B. treatment was determined for each set of centrifuged cells. The results are shown in Fig. 44 for the 'holding period' and

Lysis (E_{470})

▼ 0.13×10^{-4}M PO_4^{3-}

○ 0.22×10^{-4}M PO_4^{3-}

□ 1.00×10^{-4}M PO_4^{3-}

Total count

◇ 0.13×10^{-4}M PO_4^{3-}

◆ 0.22×10^{-4}M PO_4^{3-}

● 1.00×10^{-4}M PO_4^{3-}

Colony count

△ 0.13×10^{-4}M PO_4^{3-}

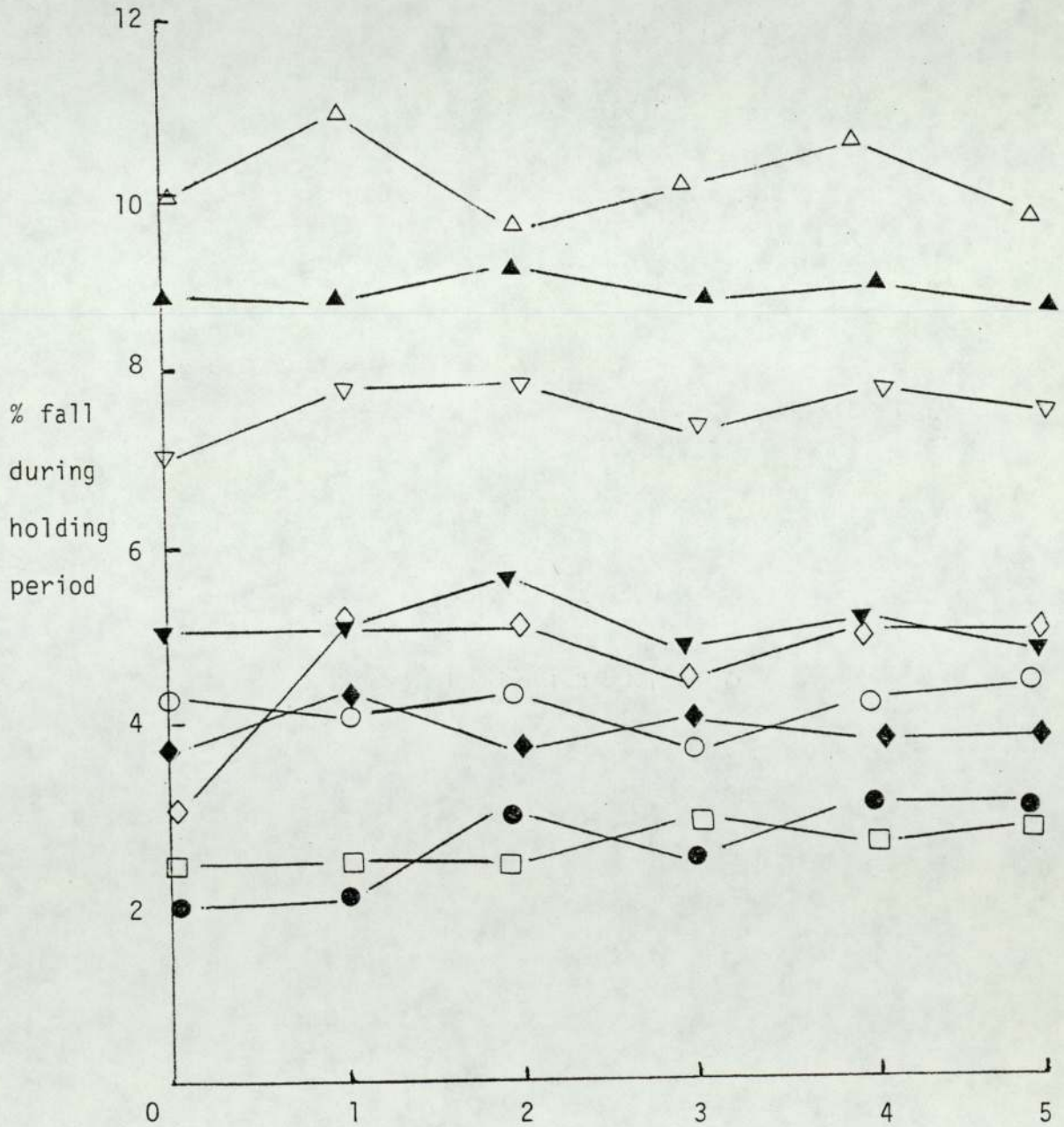
▲ 0.22×10^{-4}M PO_4^{3-}

▽ 1.00×10^{-4}M PO_4^{3-}

Fig. 44

The effect of centrifugation on % fall in E_{470}

total and colony count during the equilibration period



Lysis (E_{470})

♦ 0.13 x 10^{-4}M PO_4^{3-}

■ 0.22 x 10^{-4}M PO_4^{3-}

◇ 1.00 x 10^{-4}M PO_4^{3-}

Total count

△ 0.13 x 10^{-4}M PO_4^{3-}

▽ 0.22 x 10^{-4}M PO_4^{3-}

○ 1.00 x 10^{-4}M PO_4^{3-}

Colony count

▲ 0.13 x 10^{-4}M PO_4^{3-}

□ 0.22 x 10^{-4}M PO_4^{3-}

● 1.00 x 10^{-4}M PO_4^{3-}

Fig. 45

The effect of centrifugation on the E_{470}, total and colony count response to treatment with Polymyxin B (10 I.U. ml^{-1})

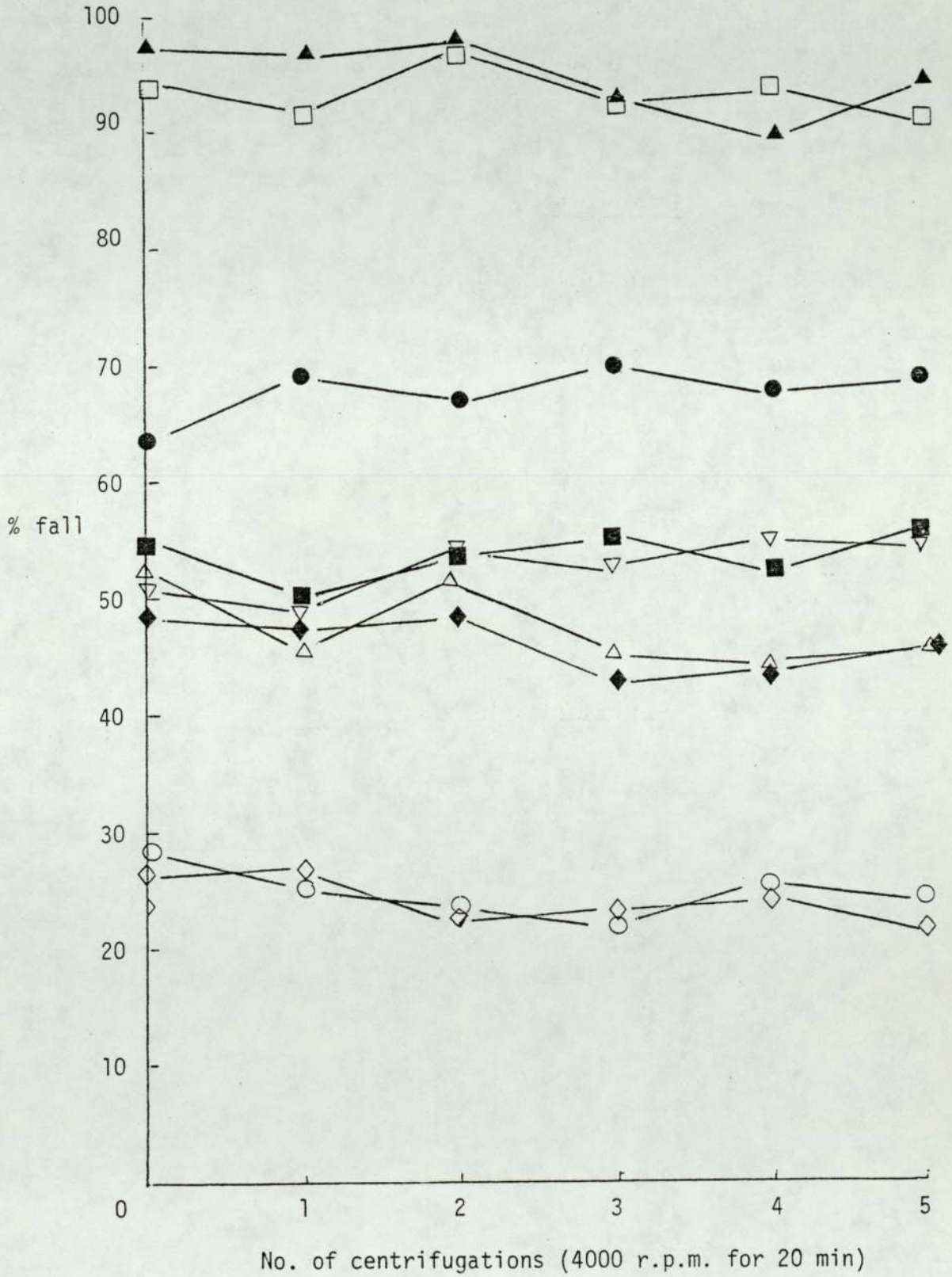


Fig. 45 for the P.B. treatment period. The effect of up to five centrifugations did not significantly affect the change in any of the 3 parameters tested during the 'holding period'. Up to 5 centrifugations had no effect on these parameters. The % change recorded for the 3 parameters is slightly higher than normal following treatment with 10 I.U. ml^{-1} P.B. This is probably because the cells were washed only once in resuspension medium, centrifuged a variable number of times and resuspended in the old supernatant which would have carried over medium constituents to alter the composition of the test menstruum. In routine experiments cells are resuspended in fresh medium.

3.2.2.3. The removal of phosphate during washing of cells

The centrifugation method of cell treatment prior to lysis was used since the repeated washing of the cells with fresh medium will remove contaminating growth media constituents and metabolites and enable lysis to be done in a defined medium. Although the previous experiment showed that up to 5 centrifugations had no deleterious effect on the cells this was a time consuming procedure. The minimum number of centrifugations necessary to reduce carry over from the initial growth to an acceptable level was investigated using cells grown in a PO_4^{3-} rich medium. The cells were then centrifuged and resuspended in C.D.M. minus PO_4^{3-} a further 3 times. The original medium and a sample of medium after each resuspension was assayed for phosphorus by the method of Scott described previously. Table 26 shows the results of the phosphorus assays. The results indicate that three washes gave a satisfactory removal of contaminating phosphate from the growth medium, and this procedure was adopted in future work.

Table 26

The Effect of Repeated Washing on Removal of Phosphate From the Original Growth Medium

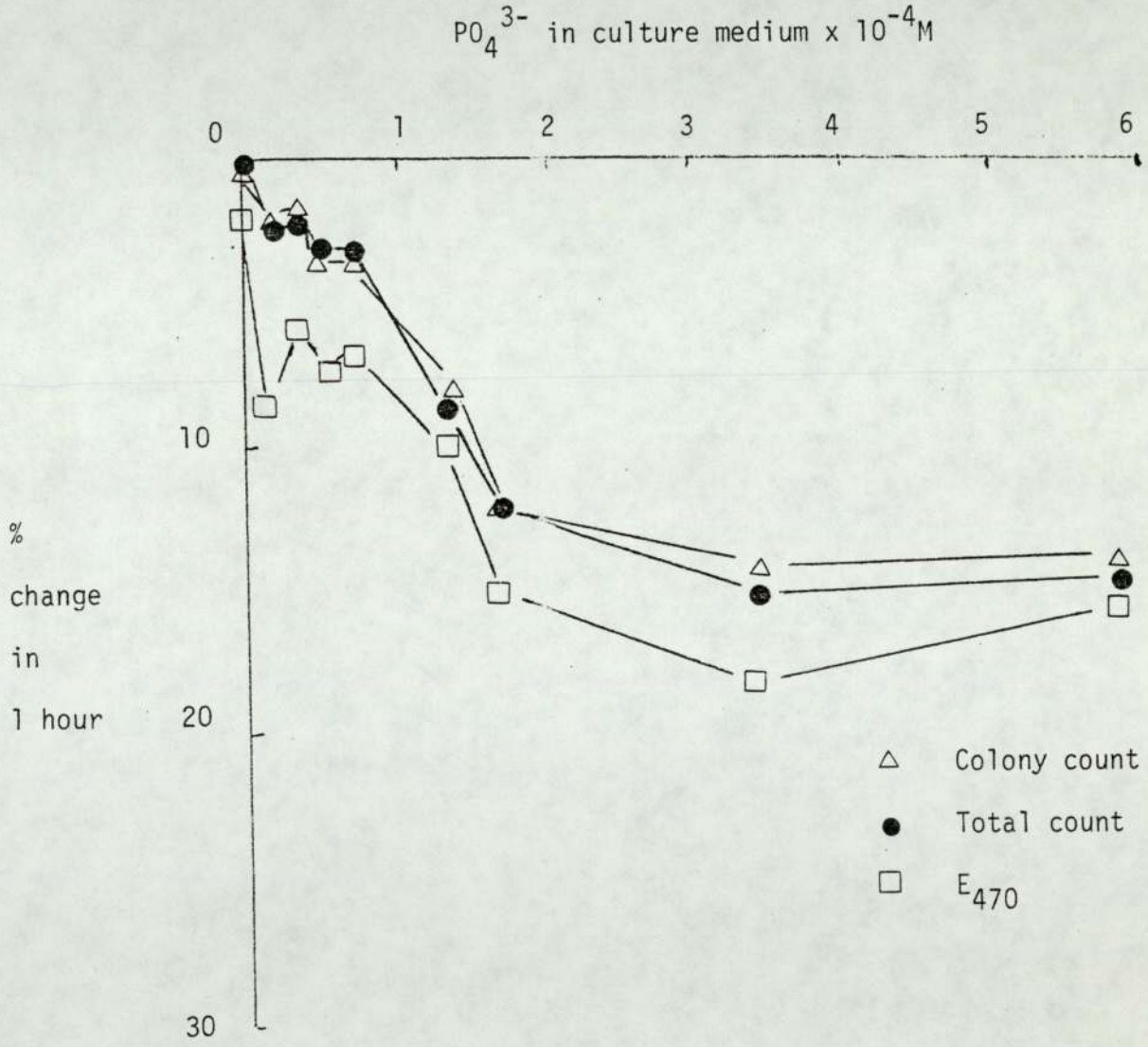
Medium	Conc. mM/litre
C.D.M. (high PO_4^{3-})	0.55
1st wash (C.D.M. - PO_4^{3-})	0.20
2nd wash (C.D.M. - PO_4^{3-})	0.08
3rd wash (C.D.M. - PO_4^{3-})	0.05

3.2.2.4. The effect of variation in phosphate concentration in the growth medium on E_{470}, total and colony count during the 'holding period'

Experiments on cells grown in C.D.M. containing graded amounts of PO_4^{3-} and subsequently processed by the centrifugation method previously described, showed that cells grown in PO_4^{3-} plentiful media and then transferred to C.D.M. minus glucose and PO_4^{3-} resuspension medium underwent greater lysis during the 'holding period' than did cells grown in low PO_4^{3-} C.D.M. This posed technical problems since cells grown in low PO_4^{3-} C.D.M. when finally resuspended to an E_{470} of 0.200 - 0.210 remained at an E_{470} 0.198 - 0.204 for the 1 hour 'holding period' and were at a satisfactory E_{470} for subsequent lysis. Cells grown in increasing levels of PO_4^{3-} however, suffered a variable fall in E_{470} during this 'holding period' of up to 18%. This resulted in E_{470} values well below the desired level of 0.2 at the start of the lysis period. Fig. 46 shows the results of the amount of PO_4^{3-} in C.D.M. culture medium on the subsequent fall in total count, colony count and E_{470} during the 1 hour holding period in C.D.M. minus glucose and PO_4^{3-}. Phosphate concentrations

Fig. 46

Effect of PO_4^{3-} in the growth medium on E_{470}, total and colony count during the equilibration period



Resuspension medium (C.D.M. lacking PO_4^{3-} and glucose)

in the growth media ranged from 0.1×10^{-4} to 6.0×10^{-4}M. The results show an increasing percentage fall in all three parameters studied for PO_4^{3-} in the range 0.1×10^{-4} - 1.75×10^{-4}M. Above 1.75×10^{-4}M PO_4^{3-} the % fall stayed approximately constant at 15%. Good agreement was maintained between fall in total count, colony count and optical density over the whole range of PO_4^{3-} concentrations studied.

3.2.2.5. The effect of variation in phosphate concentration in the resuspension medium on E_{470}, colony and total counts during the 1 hour 'holding period'

The previously described experiment showed that transfer of cells previously grown in PO_4^{3-} rich C.D.M. to C.D.M. minus glucose and PO_4^{3-} resulted in instability of the cells during the 'holding period' prior to treatment with drugs. In order to minimise these changes resuspension media containing graded concentrations of PO_4^{3-} were used.

Cells grown in PO_4^{3-} limited C.D.M. and PO_4^{3-} plentiful C.D.M. were centrifuged, washed and resuspended in the various resuspension media. E_{470}, colony and total counts were performed at the beginning and end of the 1 hour 'holding period'. Fig. 47 shows the effect of the PO_4^{3-} concentration in the range 0 to 7.0×10^{-4}M in the resuspension medium upon the percentage change in E_{470}, colony and total count. The cells grown in low PO_4^{3-} C.D.M. exhibited no significant change in either of the three parameters under test for any of the PO_4^{3-} concentrations tested. Cells grown in PO_4^{3-} rich C.D.M. however, showed significant changes in the parameters. There was a gradually progressive fall in all three parameters from approximately 14% to 7% as the PO_4^{3-} concentration in the resuspension medium was increased from 0.0 to 2.5×10^{-4}M. Above PO_4^{3-} concentrations of 2.5×10^{-4}M in the resuspension medium no significant change in the parameters studied

Lysis (E_{470})

▽ 0.1×10^{-4}M PO_4^{3-}

▼ 1.0×10^{-4}M PO_4^{3-}

Colony count

● 0.1×10^{-4}M PO_4^{3-}

○ 1.0×10^{-4}M PO_4^{3-}

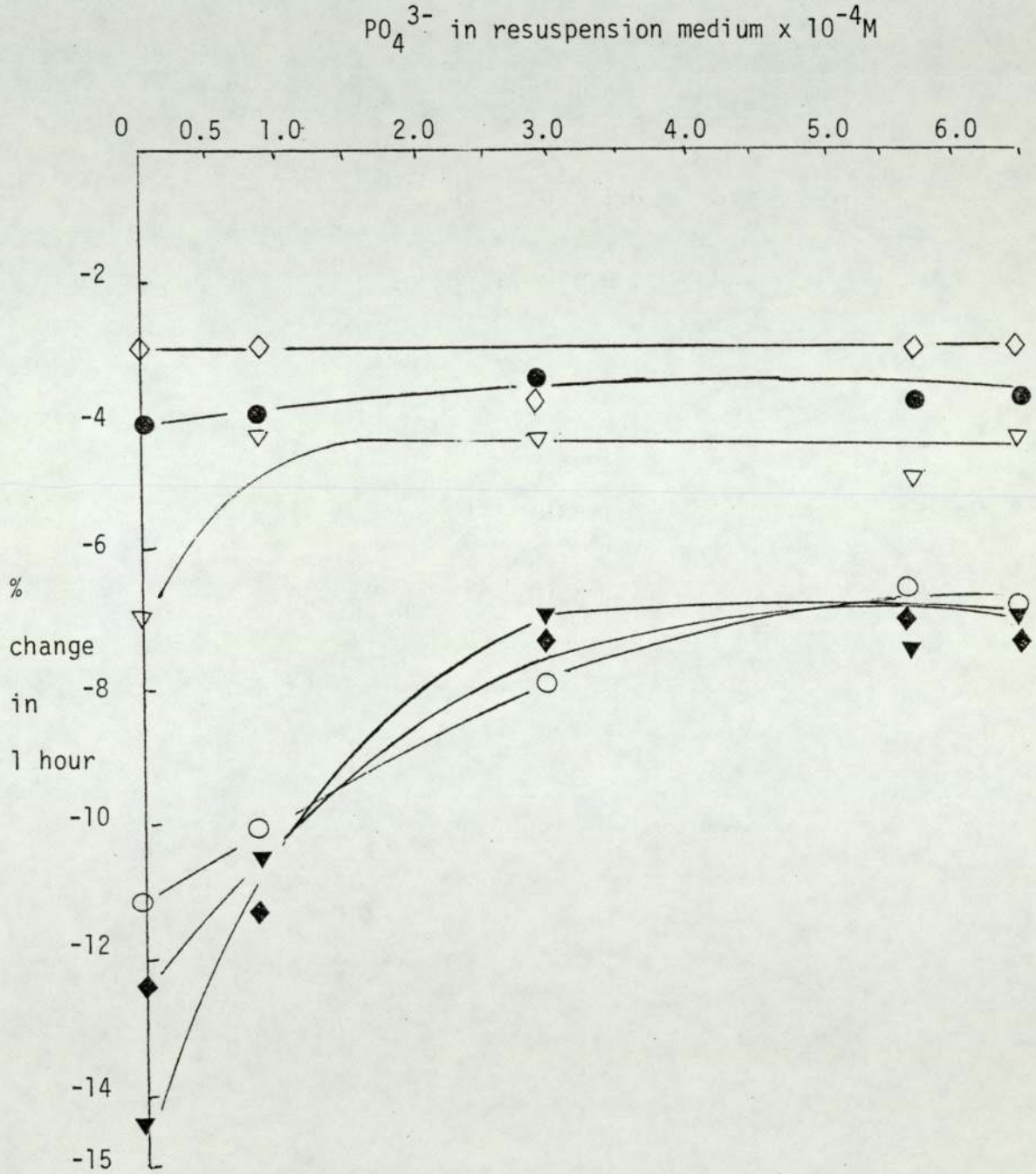
Total count

◇ 0.1×10^{-4}M PO_4^{3-}

◆ 1.0×10^{-4}M PO_4^{3-}

Fig. 47

The effect of PO_4^{3-} in the resuspension medium on E_{470} total and colony count during the equilibration period



was noted. There was good agreement between the % changes in the three parameters studied.

In order to minimise the effect of changes in these parameters especially E_{470} values, the resuspension medium in all cases was prepared containing 2.5×10^{-4}M PO_4^{3-}.

3.2.2.6. The effect of excess phosphate in the resuspension medium on lysis by 10 I.U. ml^{-1} Polymyxin B for cells grown in C.D.M. containing various phosphate concentrations

The possible effect of PO_4^{3-} in the resuspension medium on subsequent lysis by P.B. was studied since experimental technique was made easier if the resuspension medium contained 2.5×10^{-4}M PO_4^{3-}. High levels of PO_4^{3-} may have some inhibitory action on P.B. Also a criticism of the noncentrifugation method of measuring drug activity is that the lysis medium is of variable composition. Drug is added to each culture which contains a variable unused amount of the ion under test (PO_4^{3-}) and also a variable amount of metabolic products which might interfere with the action of P.B. These problems are removed by use of the centrifugation method since irrespective of the cells initial growth medium lysis is always performed in a standard medium.

P. aeruginosa was grown in C.D.M. containing 0.13×10^{-4}M PO_4^{3-}, 0.22×10^{-4}M PO_4^{3-} and 2.2×10^{-4}M PO_4^{3-}. The cells were harvested and washed in the normal manner. The washing and resuspension media were C.D.M. minus glucose but containing 0.0M, 0.52×10^{-6}, 0.52×10^{-5}, 0.52×10^{-4}, 0.52×10^{-3}, 0.52×10^{-2} and 0.52×10^{-1}M PO_4^{3-}. Final resuspension optical densities were adjusted such that following the 'holding period' all cultures had an E_{470} of approximately 0.2. The cells were then lysed with 10 I.U. ml^{-1} P.B. and the % fall in E_{470} after 60 minutes recorded in Table 27.

Table 27

The effect of phosphate in the resuspension medium on lysis of P. aeruginosa grown in C.D.M. containing various concentrations of phosphate

Polymyxin conc. I.U. ml^{-1}	Phosphate in resuspension medium (M)	Phosphate in growth medium x 10^{-4}M		
		0.13	0.22	2.2
10	0	31.7	36.8	16.7
	0.52×10^{-6}	33.0	36.0	16.8
	0.52×10^{-5}	32.6	35.6	16.4
	0.52×10^{-4}	36.2	38.2	17.2
	0.52×10^{-3}	36.6	38.8	17.0
	0.52×10^{-2}	37.0	39.4	17.1
	0.52×10^{-1}	36.4	39.0	17.6

Over the range of phosphate concentrations studied there was no signficant difference in the % lysis after 60 minutes for cells grown in the three media.

3.2.2.7. The effect of excess nutrients upon E_{470} following Polymyxin B treatment

The effect of excess nutrients in the lysis medium was tested using the noncentrifugation method testing cells grown in three concentrations of PO_4^{3-} (0.1, 0.25, 1.0 x 10^{-4}M) but with theoretical excesses of other nutrients at 5 and 25 times. Glucose was maintained at a concentration to achieve growth to E_{470} 0.2. The cells were lysed with 10 I.U. ml^{-1} P.B. Table 28 shows the results. These show that the % change in E_{470} and presumably other parameters not tested, such as total

count and colony count was dependent upon the excess of nutrients. These results are to be expected since high concentrations of ions have been shown to affect viability of untreated cells (Brown 1975). In routine experiments on the action of P.B. and E.D.T.A. a 5 X excess of nutrients was used.

Table 28

The Effect of Excess Nutrients on Lysis (E_{470}) by Polymyxin B

PO_4^{3-} conc. (x 10^{-4}M)	Nutrient Excess	
	5X	25X
	% lysis 60 min.	% lysis 60 min.
0.1	12	83
0.25	27	96
1.0	10	87

3.2.2.8. Effect of final culture pH on lysis by 10 I.U. ml^{-1} Polymyxin B

The chemically defined media for use in resistance studies was buffered with 0.06M MOPS. Results described previously had shown that for growth to an E_{470} 0.2 an expected fall of approximately 0.1 pH unit might occur. This small change was unlikely to have a major effect on lysis values. The effect of the final pH of the growth medium prior to treatment of the cells with 10 I.U. P.B. was investigated over a narrow pH range of 6.6 - 7.4, for cells grown in 0.1, 0.25, 1.0 x 10^{-4}M PO_4^{3-}. The pH was adjusted by alteration of the pH of the MOPS buffer used to prepare the C.D.M. A water control was also used. The results are shown tabulated in Table 29. The water controls lysed less than 1% and the

figures tabulated are adjusted for this. The results show no significant effect upon the lysis by P.B. over the pH ranged tested.

The pH of the growth medium may affect cell growth in several ways. The growth rate constant will alter as will the final cell mass. The bacterial response to growth in different pH's may be reflected in changes in the cell wall chemical structure or the overall cell wall negative charge thus altering the cells response to certain drugs. The effect of pH on the drug itself may also be an important factor.

Any such pH mediated alterations in the cell or activity of P.B. were not apparent as judged by changes in optical density readings following treatment with the drug.

Table 29

The effect of final pH of the growth medium upon lysis by 10 I.U. Polymyxin B

pH of C.D.M.	Phosphate conc. (x 10^{-4}M) in medium		
	0.1	0.25	1.0
	% lysis 60 min.	% lysis 60 min.	% lysis 60 min.
6.7	15.8	23.8	15.5
7.0	15.2	23.1	15.2
7.3	16.4	24.8	13.6
7.4	14.8	23.2	13.0

3.2.2.9. The effect of pH of the resuspension medium on lysis by 10 I.U. Polymyxin B

Cells grown in C.D.M. containing various PO_4^{3-} concentrations and processed by the centrifugation method prior to treatment with P.B. will have experienced a similar pH since the pH of the medium was controlled by alteration of the MOPS buffer. At the MOPS concentration used the pH fall was less than 0.1 pH unit. An experiment was performed to check the effect of the pH of the wash medium on the sensitivity of the cells to lysis by P.B. This pH effect was examined by growing cells in C.D.M. containing 0.1, 0.25 and 1.0 x 10^{-4}M PO_4^{3-} and washing and treating the cells with P.B. in resuspension media of varying pH. The effect on E_{470} after 60 minutes exposure to 10 I.U. ml^{-1} P.B. was determined. The pH values chosen were in the range 6.7 - 7.5. The results are shown in Table 30 . No significant difference was seen in % lysis over the pH range investigated.

Table 30

The effect of resuspension medium pH upon lysis by Polymyxin B

Resuspension medium pH	PO_4^{3-} x 10^{-4} in growth medium		
	0.1	0.25	1.0
	% lysis	% lysis	% lysis
6.75	28.4	33.5	22.7
7.10	25.5	30.0	20.5
7.30	26.5	33.0	20.0
7.50	25.0	29.8	19.0

Boggis (1971) showed that pH affected the activity of E.D.T.A. on cells grown in magnesium plentiful media, as judged by E_{470} values. There was little effect for pH values between 6.3 and 7.4. Above these pH values % lysis after 180 minutes increased sharply. This increase correlates well with increasing chelating activity of E.D.T.A. which is optimum for Mg^{2+} at pH 10.

In this work the initial pH of the PO_4^{3-} depleted and plentiful media was adjusted to pH 7.4 and this pH was also attained when preparing the resuspension medium.

3.2.3. Phosphate and Lysis of P. aeruginosa

3.2.3.1. Measurement of % lysis for resistance studies

Previous workers (Boggis, 1971, Kenward, 1975) had studied lysis by P.B. and E.D.T.A. for nutrient depleted cells. Fall in E_{470} following treatment with P.B. had been studied over a 60 minute period and for E.D.T.A. over a 180 minute period by both workers. Using cells grown in various PO_4^{3-} concentrations and treated with P.B. and E.D.T.A. showed that these lysis times were satisfactory. For comparative purposes these lysis times of 60 minutes and 180 minutes were used.

Cells grown in various degrees of PO_4^{3-} limitation were treated with P.B. or E.D.T.A. and E_{470} measurements taken at various time intervals. Results were calculated from the following formula:

$$\%\ \text{lysis}_{(x\ \text{min})} = \frac{E_{(o)} - E_{(t)}}{E_o} \times 100\%$$

where, E_o is the E_{470} of the culture at the beginning of drug treatment

E_t is the E_{470} of the culture at the end of drug treatment (60 minutes P.B., 180 minutes E.D.T.A.).

3.2.3.2. The effect of Polymyxin B concentration

P. aeruginosa was grown in C.D.M. containing two amounts of PO_4^{3-} (0.1×10^{-4}M and 3.0×10^{-4}M PO_4^{3-}), to an E_{470} of 0.2 and treated with P.B. at 10, 20, 40, 80, 120 and 160 I.U. ml^{-1}. Graphs of $\log_{10}$ E_{470} against contact time with the drug are shown in Fig. 48 for cells grown in 0.1×10^{-4}M PO_4^{3-} and Fig. 49 for cells grown in 3.0×10^{-4}M PO_4^{3-}. Values are corrected for lysis of a water inoculated control.

Fig. 48

The effect of Polymyxin B concentration upon lysis of *P. aeruginosa* grown in C.D.M. containing 0.1×10^{-4}M PO_4^{3-}

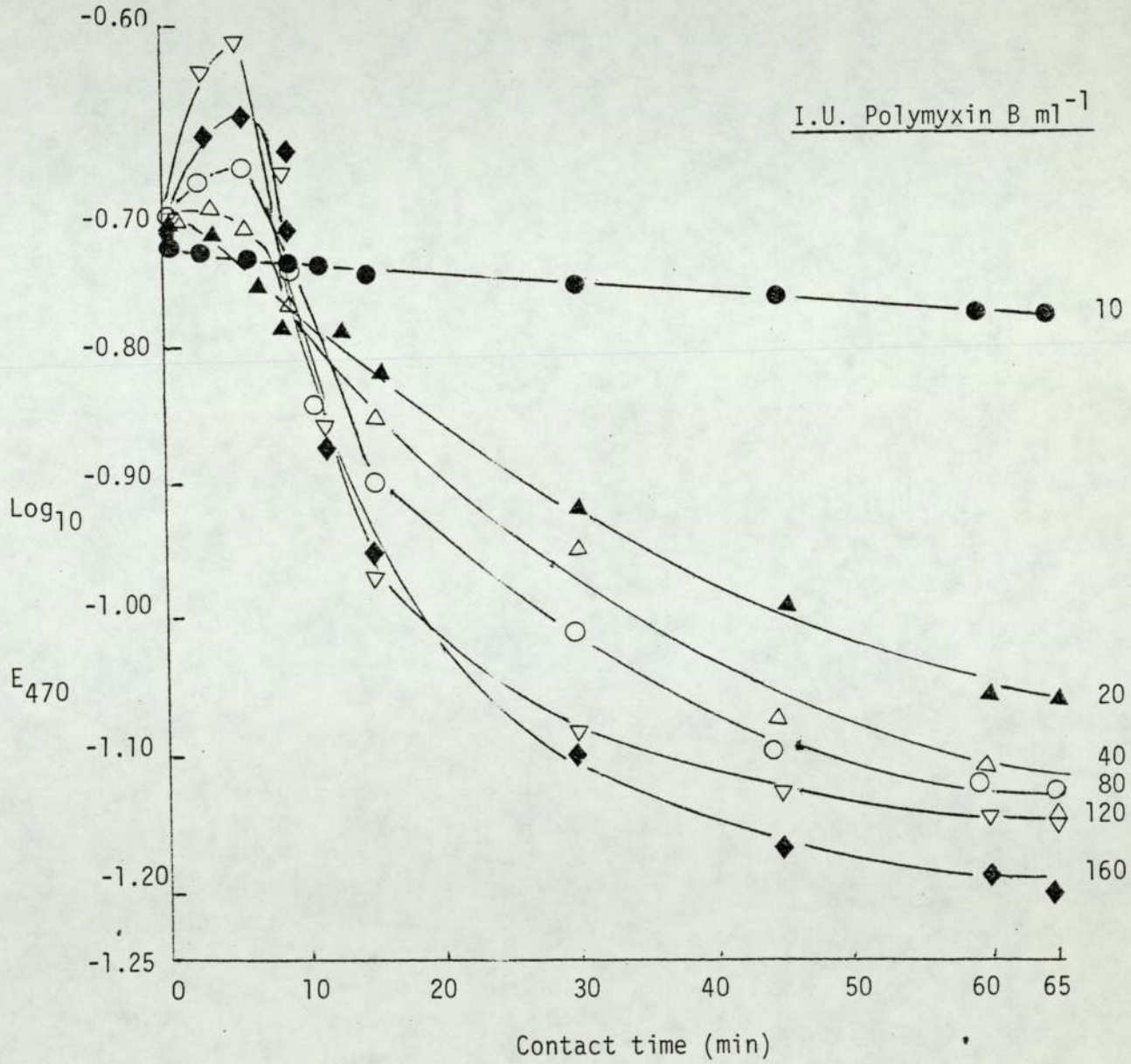
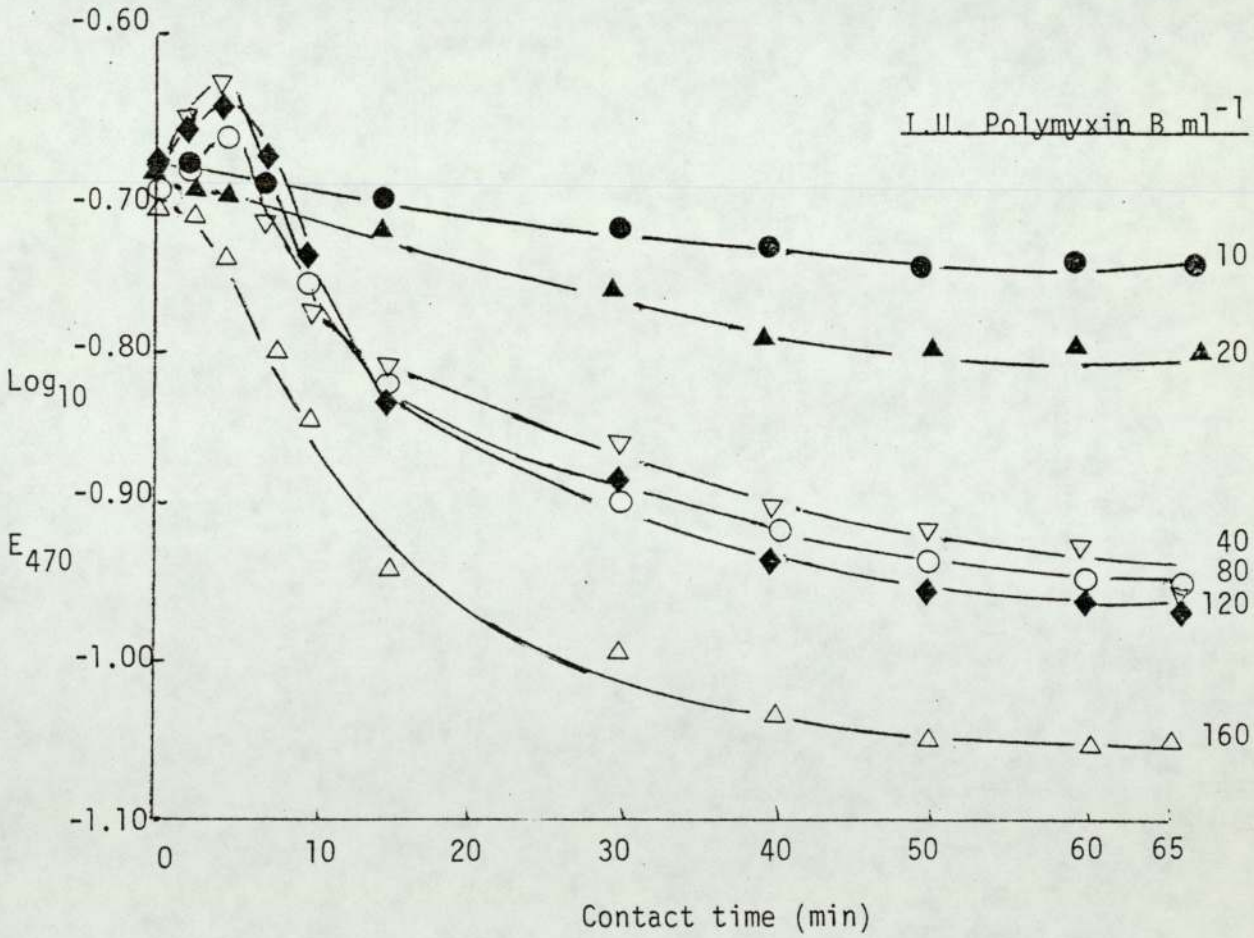


Fig. 49

The effect of Polymyxin B concentration upon lysis of *P. aeruginosa* grown in C.D.M. containing 3×10^{-4}M PO_4^{3-}



Cells grown in 0.1 x 10^{-4}M PO_4^{3-} (Fig. 48) showed increased sensitivity to P.B. up to 120 I.U. ml^{-1}. There was little difference between the sensitivity to P.B. at 120 and 160 I.U. ml^{-1}. Cells treated with 10 and 20 I.U. ml^{-1} of P.B. showed an exponential decrease with time. For cells treated with 40 I.U. ml^{-1} or more of P.B. the curves showed two portions. The first shows an initial increase in E_{470} for the first twelve minutes followed by an exponential decrease in E_{470}. The amount of the initial increase in E_{470} was dependent on P.B. concentration over the range of concentrations studied. Similar initial increases in E_{470} following drug treatment have been shown by Hugo and Longworth (1964) for chlorhexidine treated E. coli and S. aureus and by Brown and Melling (1969b) for P. aeruginosa treated with P.B. It is likely that the increase in E_{470} is caused by changes in light scattering properties of the cells when taking up the drug. The failure to observe an increase in E_{470} at the lower P.B. concentrations is probably due to small increases in E_{470} being counteracted by lysis.

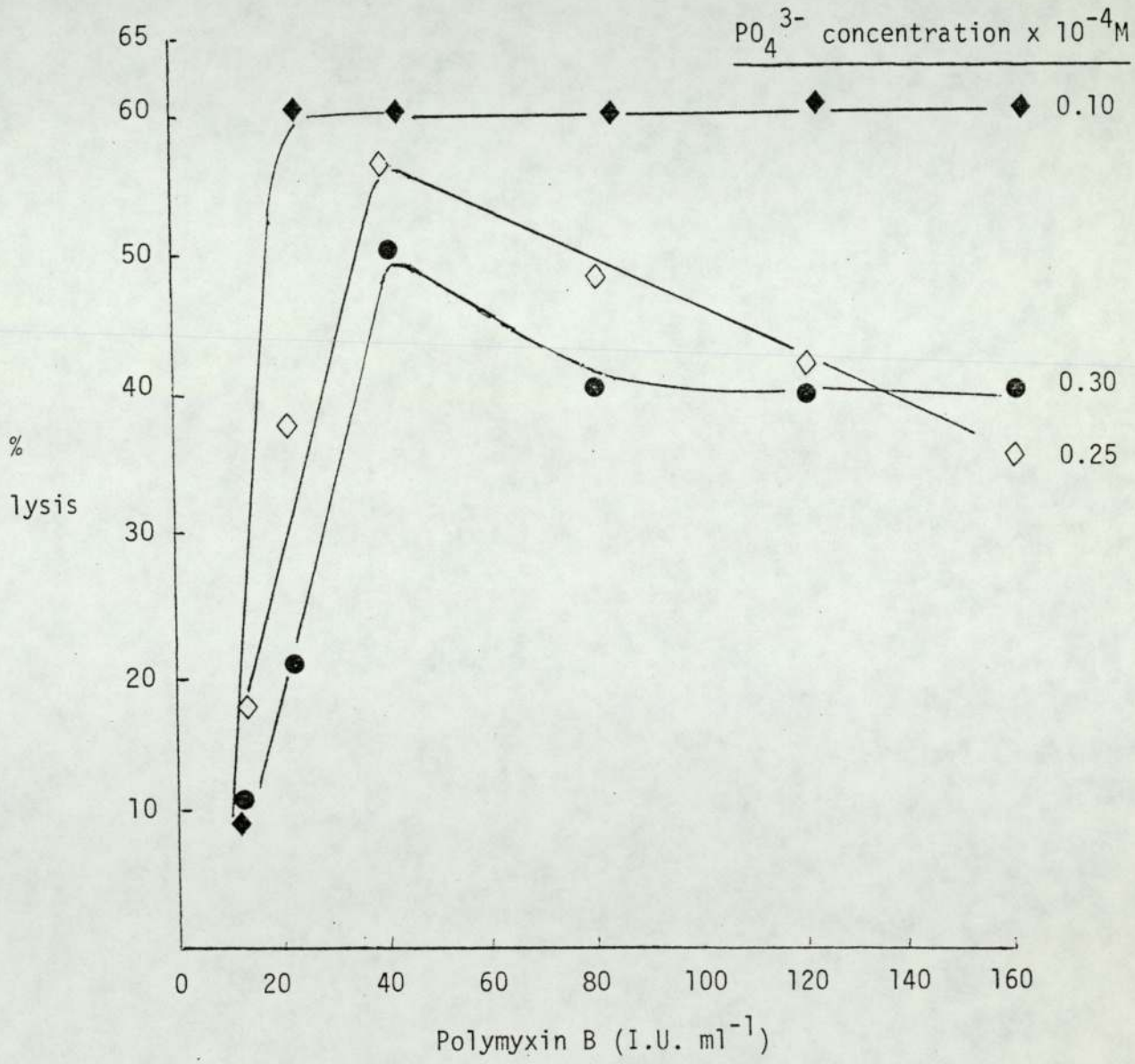
Fig. 49 shows that cells grown in 3 x 10^{-4}M PO_4^{3-} there was increasing sensitivity to P.B. over the range 10 - 40 I.U. ml^{-1}, but above 40 I.U. ml^{-1} a decrease in sensitivity was noted. The initial rise in E_{470} following P.B. treatment was less marked with cultures grown in this higher PO_4^{3-} concentration suggesting that less drug is taken up by cells grown in a plentiful supply of PO_4^{3-}. Similar results were obtained by Boggis (1971) for cultures grown in magnesium plentiful conditions. He showed that the decrease in E_{470} was greater for cells treated with 30 I.U. ml^{-1} than for those treated with 40 or 60 I.U. ml^{-1} of P.B. It seems likely that the effect of the initial increase in E_{470} value reduces the final % lysis result.

Fig. 50 shows the relationship between % lysis at 60 minutes and the P.B. concentration used to produce that lysis. The maximum lysis for PO_4^{3-} depleted cells (0.1×10^{-4}M PO_4^{3-}) occurred with 20 I.U. ml^{-1} of P.B. whereas that for cells grown in medium containing 0.25×10^{-4}M PO_4^{3-} and 0.3×10^{-4}M PO_4^{3-} (PO_4^{3-} plentiful medium) was 40 I.U. ml^{-1} P.B. Cells grown in PO_4^{3-} depleted C.D.M. showed little change in % lysis when concentrations of P.B. above 20 I.U. ml^{-1} were used. However, cells grown in higher concentrations of PO_4^{3-} showed a fall in % lysis as the concentration of P.B. was increased.

Fig. 51 shows the relationship between % lysis after 60 minutes exposure with increasing concentrations of P.B. and the amount of PO_4^{3-} originally present in the growth medium. Cells lysed with 10 I.U. ml^{-1} of P.B. showed increasing sensitivity to the drug as the PO_4^{3-} concentration was increased from 0.1×10^{-4} to 0.16×10^{-4}M. Above 0.16×10^{-4}M PO_4^{3-} the cells appeared to show an increasing resistance to 10 I.U. ml^{-1} P.B. Cells lysed with 20 I.U. ml^{-1} P.B. showed a maximum sensitivity to the drug when grown in PO_4^{3-} concentrations of 0.1×10^{-4}M. At PO_4^{3-} concentrations above this, increasing resistance to P.B. was exhibited. Similar results were obtained for cells lysed by 40, 80, 120 and 160 I.U. ml^{-1} P.B. Maximum sensitivity, shown by 60-65% lysis, occurred when cells were grown in 0.1×10^{-4}M PO_4^{3-}. Decreased sensitivity occurred for cells grown in higher concentrations of PO_4^{3-}. The decrease in sensitivity to P.B. with increased PO_4^{3-} growth levels was greatest for cells lysed with 20 I.U. ml^{-1} P.B. These cells exhibited a 40% decrease in lysis values. Cells lysed with 40 I.U. ml^{-1} P.B. showed the minimum decrease in sensitivity (approx 10%). The decrease in lysis for cells treated with 80, 120 and 160 I.U. ml^{-1} P.B. was 20, 25 and 27% respectively.

Fig. 50

The effect of Polymyxin B concentration on % lysis in 60 minutes for P. aeruginosa grown in C.D.M. containing various PO_4^{3-} concentrations



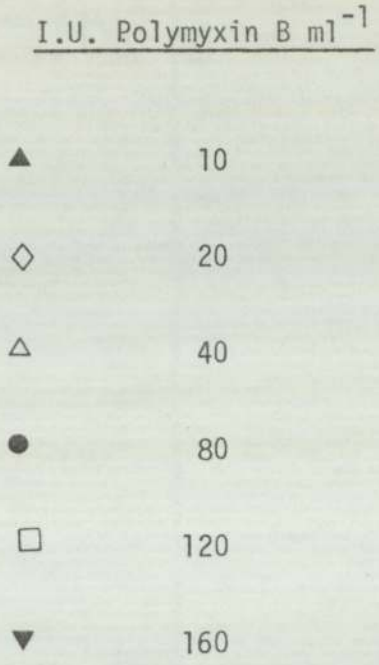
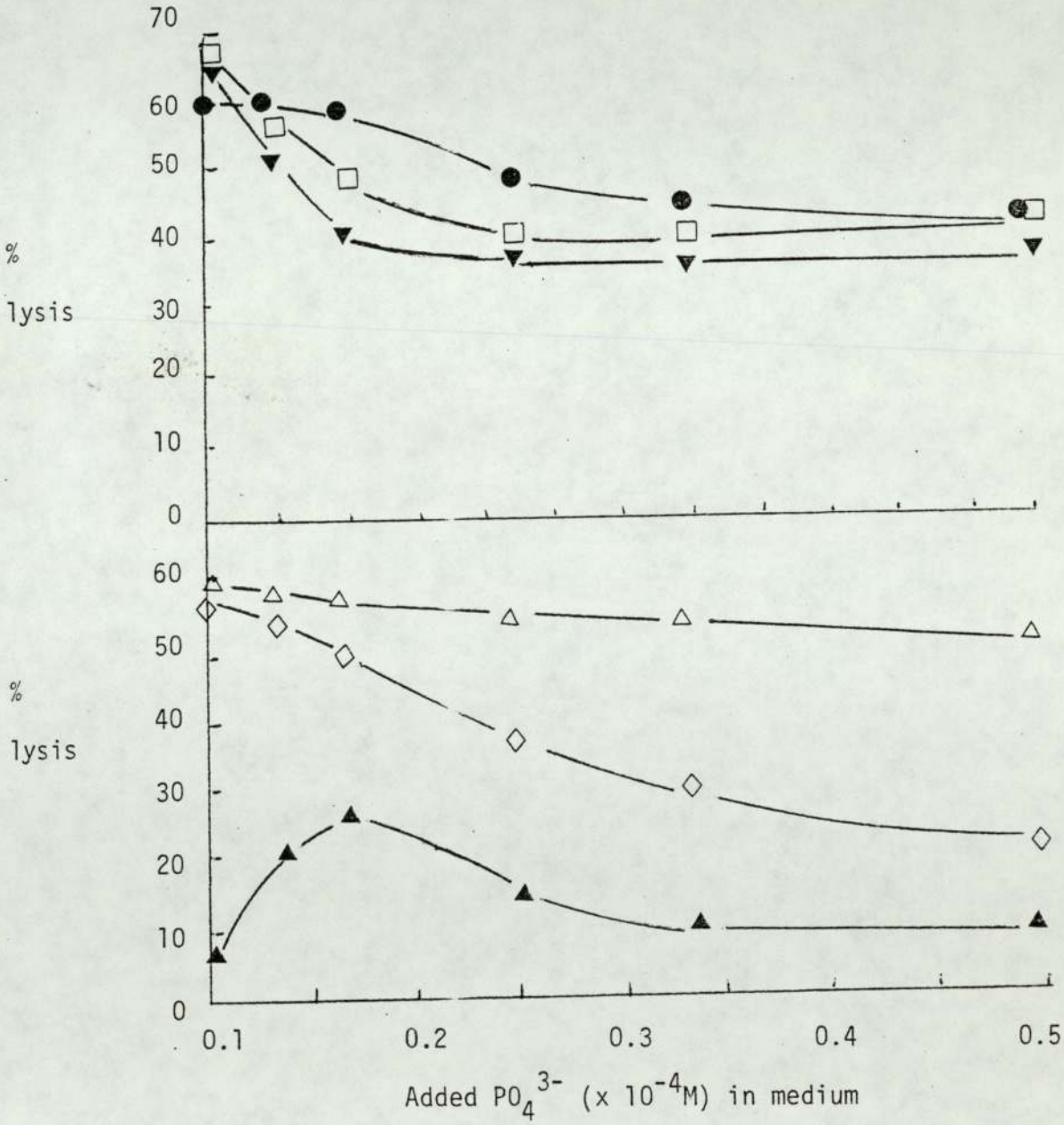

I.U. Polymyxin B ml^{-1}
10
20
40
80
120
160

Fig. 51

The effect of PO_4^{3-} concentration in the growth medium on % lysis of *P. aeruginosa* treated with various concentrations of Polymyxin B



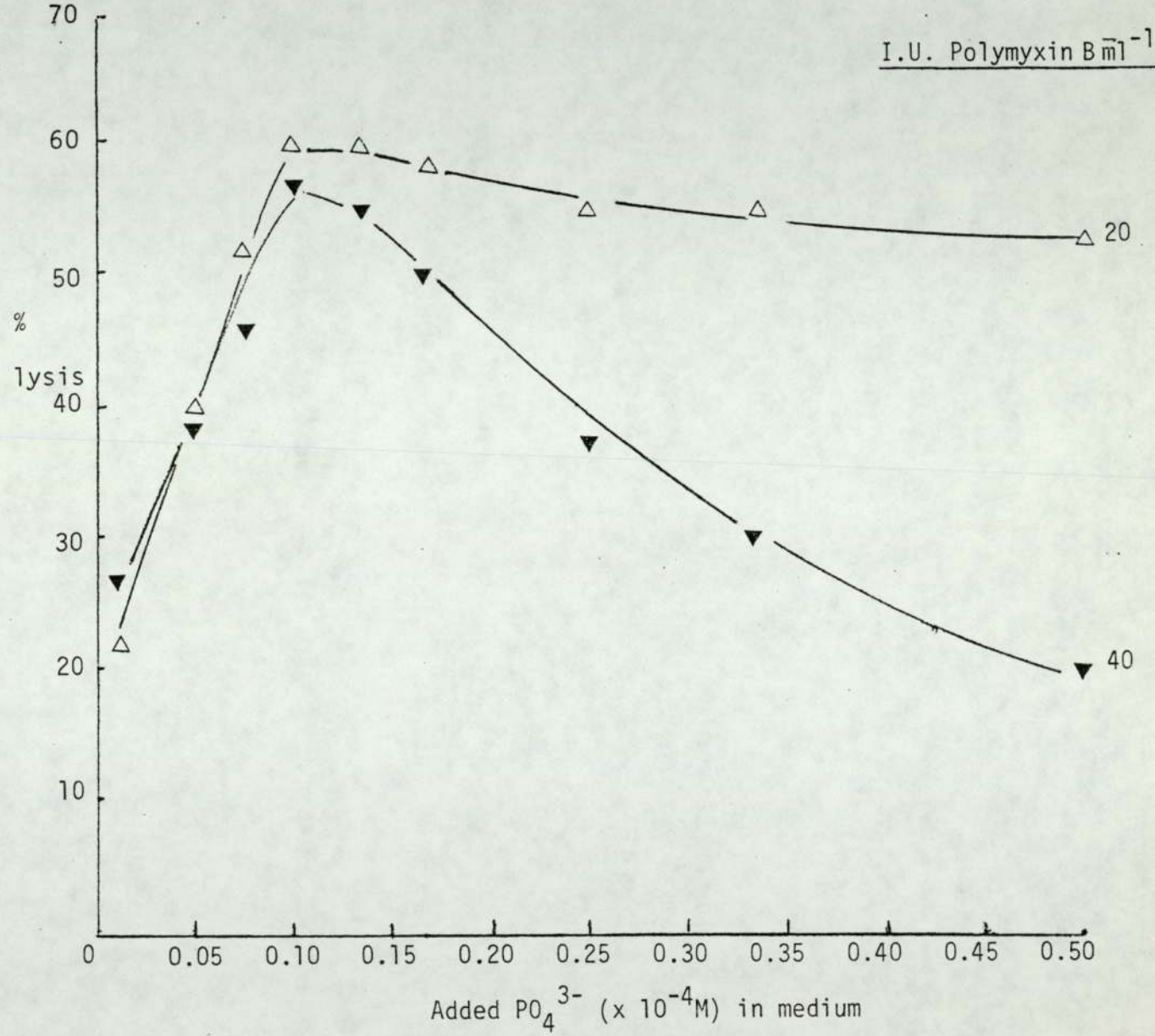
As previously mentioned cells grown at low PO_4^{3-} concentrations had an increased requirement for glucose. Below a PO_4^{3-} level of 0.1×10^{-4}M an increase in glucose concentration failed to permit growth to an E_{470} of 0.2. Thus using the noncentrifugation method PO_4^{3-} levels below 0.1×10^{-4}M could not be used if an initial E_{470} of 0.2 was required for lysis. However, the pooling, following centrifugation, of cultures grown in C.D.M. containing less than 0.1×10^{-4}M PO_4^{3-} and subsequent resuspension to an E_{470} of 0.2 enabled cells grown at lower PO_4^{3-} values to be studied.

Cells were grown in 0.01, 0.05 and 0.075×10^{-4}M PO_4^{3-} and following resuspension to E_{470} 0.2 they were lysed by 10, 20 and 40 I.U. ml^{-1} P.B. Lysis with 10 I.U. ml^{-1} P.B. was unsuccessful since in general centrifugation increased the % lysis compared with uncentrifuged cells and the lysis values obtained for cells grown in PO_4^{3-} below 0.1×10^{-4}M were higher than the values for centrifuged cells grown in 0.1×10^{-4}M PO_4^{3-}. The lysis values for treatment with 20 and 40 I.U. ml^{-1} P.B. were added to the previously obtained lysis values and the results shown in Fig. 52 . The results show that these cells were progressively more resistant to lysis by P.B. as the PO_4^{3-} concentration in the growth medium was lowered. The relationship was identical to that obtained for lysis by 10 I.U. ml^{-1} P.B. These results were comparable with those obtained for lysis using 40 I.U.ml^{-1}P.B. by Boggis (1971).

Since cells lysed with 10 I.U. ml^{-1} showed a pattern of increasing sensitivity to P.B., using thismethod, and subsequently a decrease in sensitivity as the PO_4^{3-} content in the culture medium was increased it was decided to study these cells in an attempt to explain this relationship.

Fig. 52

The effect of PO_4^{3-} in the growth medium on % lysis of *P. aeruginosa* treated with 20 and 40 I.U. ml^{-1} Polymyxin B



3.2.3.3. The effect of the phosphate concentration of the growth medium upon lysis by Polymyxin B.

Fig. 53 shows the effect on the lysis by 10 I.U. ml^{-1} P.B. of growth of P. aeruginosa in a range of PO_4^{3-} concentrations of 0.1 x 10^{-4}M to 0.48 x 10^{-4}M. Increasing sensitivity occurred with increasing PO_4^{3-} to a level of 0.16 x 10^{-4}M PO_4^{3-}. Above this level increased PO_4^{3-} concentration resulted in a decrease in sensitivity to P.B. Similar results were obtained irrespective of the method used to prepare the cells for lysis e.g. noncentrifugation or centrifugation method.

From the curves the rate constant 'K' for the lysis can be determined. Fig. 54 shows the relationship between the lysis rate constant 'K' (x 10^{-3} min^{-1}) and the amount of PO_4^{3-} in the initial growth medium. This shows a linear relationship between the rate of lysis and the PO_4^{3-} concentration in the medium to a PO_4^{3-} level of 0.166 x 10^{-4}M. After this the rate falls rapidly to a constant value of K = 1 x 10^{-3} min^{-1} at a PO_4^{3-} concentration of approximately 0.4 x 10^{-4}M, at which it remains to a PO_4^{3-} concentration of at least 1 x 10^{-4}M. The relationship between the lysis rate constant 'K' and % lysis is shown in Fig. 55 . There existed a linear relationship between % lysis and lysis rate constant to a % lysis value of 27.5.

3.2.3.4. The effect of phosphate on sensitivity to 10 I.U. ml^{-1} Polymyxin B (noncentrifugation method)

P. aeruginosa was grown in C.D.M. containing a range of added PO_4^{3-} from 0.1 x 10^{-4}M to 0.8 x 10^{-4}M. The final E_{470} was adjusted to 0.2 by alteration of the glucose content of the medium. Cultures were then treated with 10 I.U. ml^{-1} P.B. in the standard manner described previously.

Fig. 53

The effect of PO_4^{3-} concentration in the medium upon the lysis of *P. aeruginosa* by Polymyxin B (10 I.U. ml^{-1})

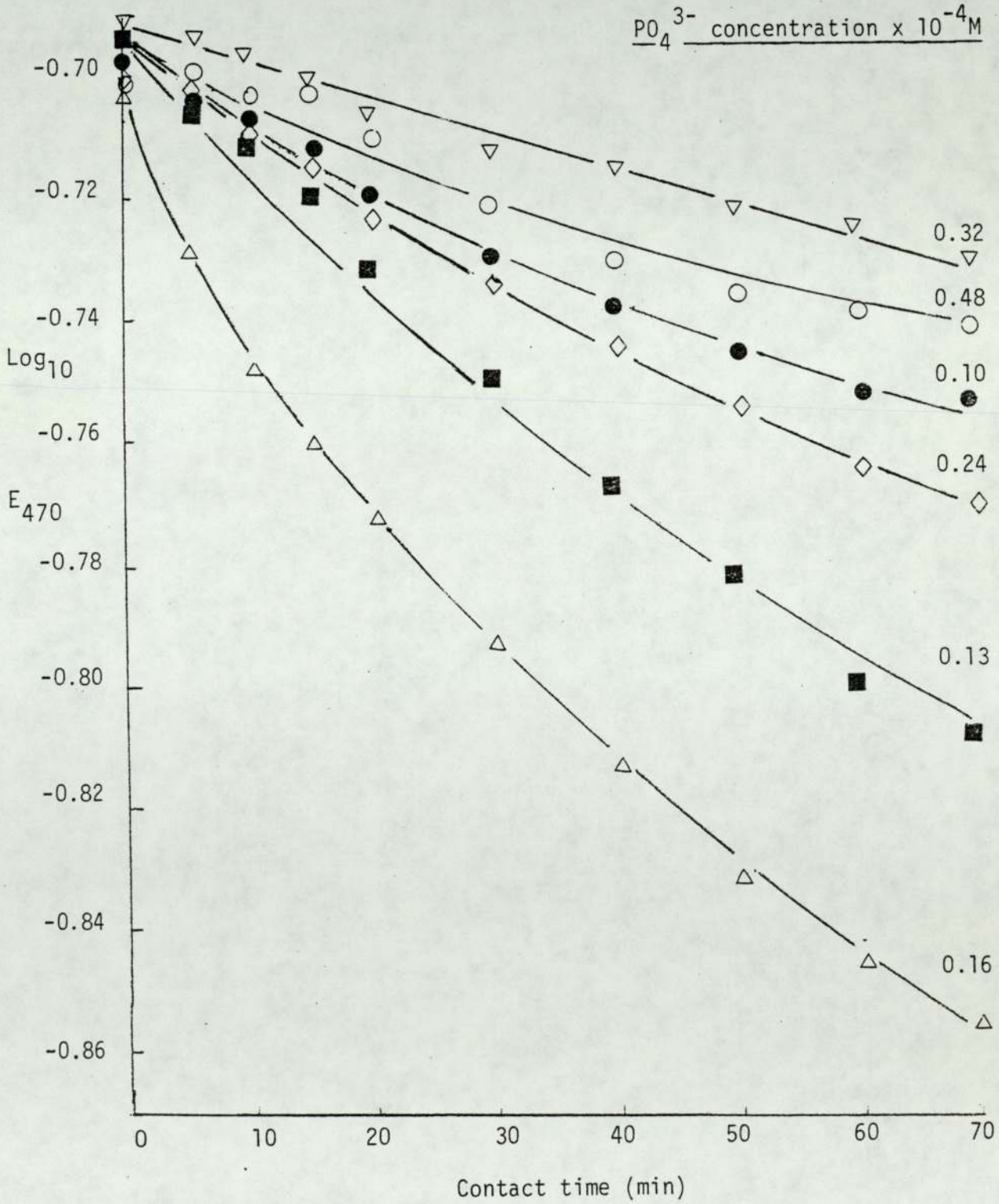


Fig. 54

The effect of PO_4^{3-} concentration in the medium upon the rate constant for lysis of *P. aeruginosa* by Polymyxin B (10 I.U. ml^{-1})

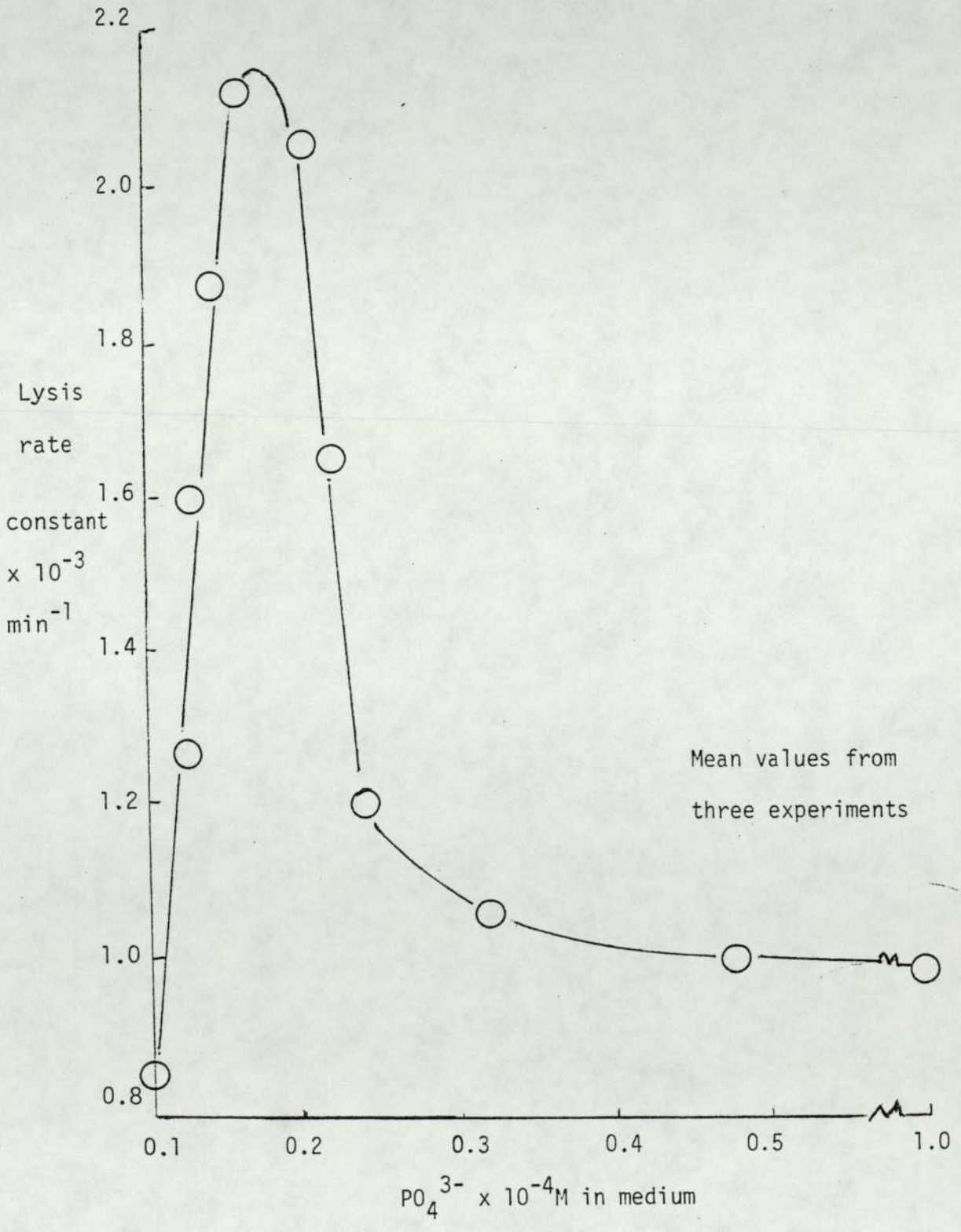
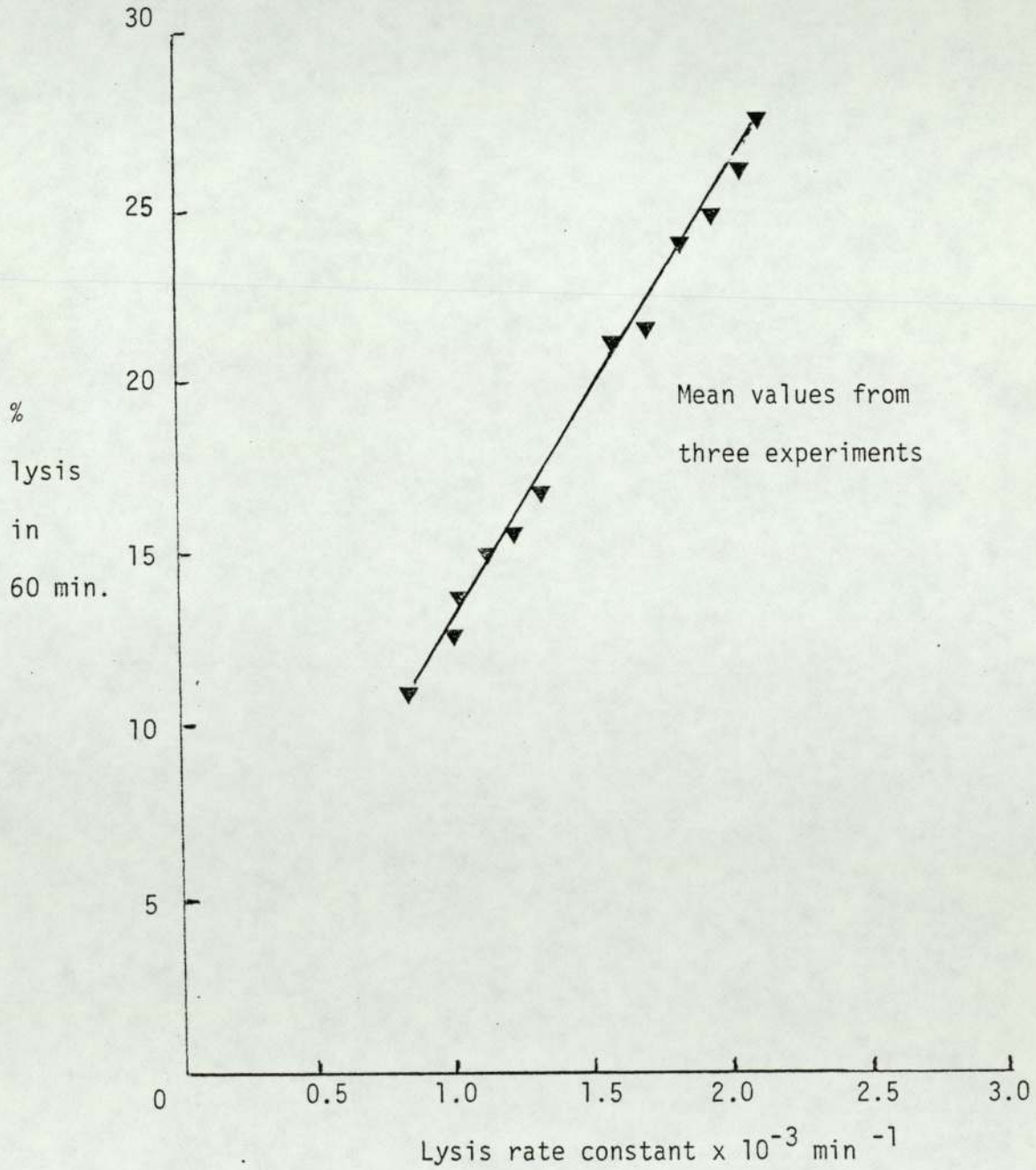


Fig. 55

Relationship between % lysis and rate constant for lysis of *P. aeruginosa* by Polymyxin B (10 I.U. ml^{-1})



At time 0 minutes 1ml of sample was removed for colony count estimation, 0.1ml for total count estimations and the E_{470} was measured. The E_{470} was determined over a 60 minute lysis period. After 60 minutes treatment a further 1ml of culture was taken for colony count and 0.1ml for total count. Colony counts were performed on duplicate plates following neutralisation in lecithin/glycerol neutraliser medium.

Fig. 56 shows the results of treatment with 10 I.U. ml^{-1} P.B. on the three parameters E_{470}, total and colony count for cells grown in increasing concentrations of PO_4^{3-}. For cultures grown in $0.1 \times 10^{-4}M$ PO_4^{3-} to $0.17 \times 10^{-4}M$ PO_4^{3-} an increase in sensitivity was noted from 10% to 25% for E_{470} and 12% to 24% for total count. A similar relationship existed for colony count but the change was 65% to 83% respectively. Above PO_4^{3-} concentrations of $0.17 \times 10^{-4}M$ a decrease in sensitivity was noted in all three parameters. The % change in E_{470} fell from 25% at $0.17 \times 10^{-4}M$ to 9% at $0.8 \times 10^{-4}M$. The corresponding fall in total count was 24% to 9%, and for colony count from 83% to 70%. The experiment shows good correlation between E_{470} and total count indicating that change in E_{470} was due to lysis and not to alteration in light scattering properties of the cells following treatment with P.B. The increased % change values noted for colony count indicates that many of the cells were still intact but had been so badly damaged that growth on nutrient agar was impossible.

3.2.3.5. The effect of phosphate on sensitivity to 10 I.U. ml^{-1} Polymyxin B (centrifugation method)

The previously described experiment was repeated but cells were prepared by washing and centrifugation as previously described. Fig. 57 shows the effect on the 3 parameters E_{470}, colony and total count of growth in increasing amounts of PO_4^{3-} followed by treatment with

Fig. 56

The effect of PO_4^{3-} concentration in the medium upon the % change in E_{470} total and colony count for P. aeruginosa treated with Polymyxin B (10 I.U. ml^{-1})

(Non-centrifugation method)

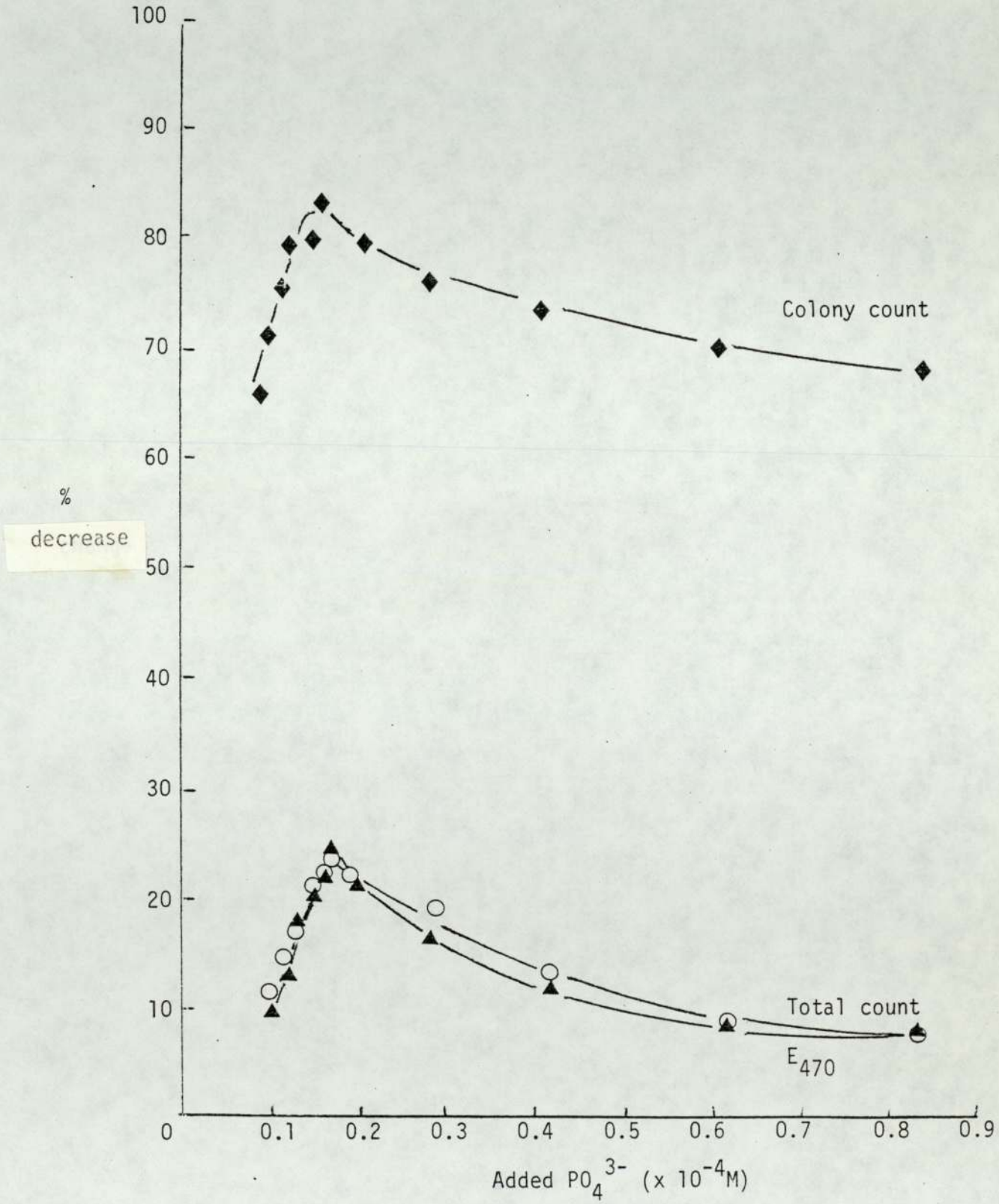
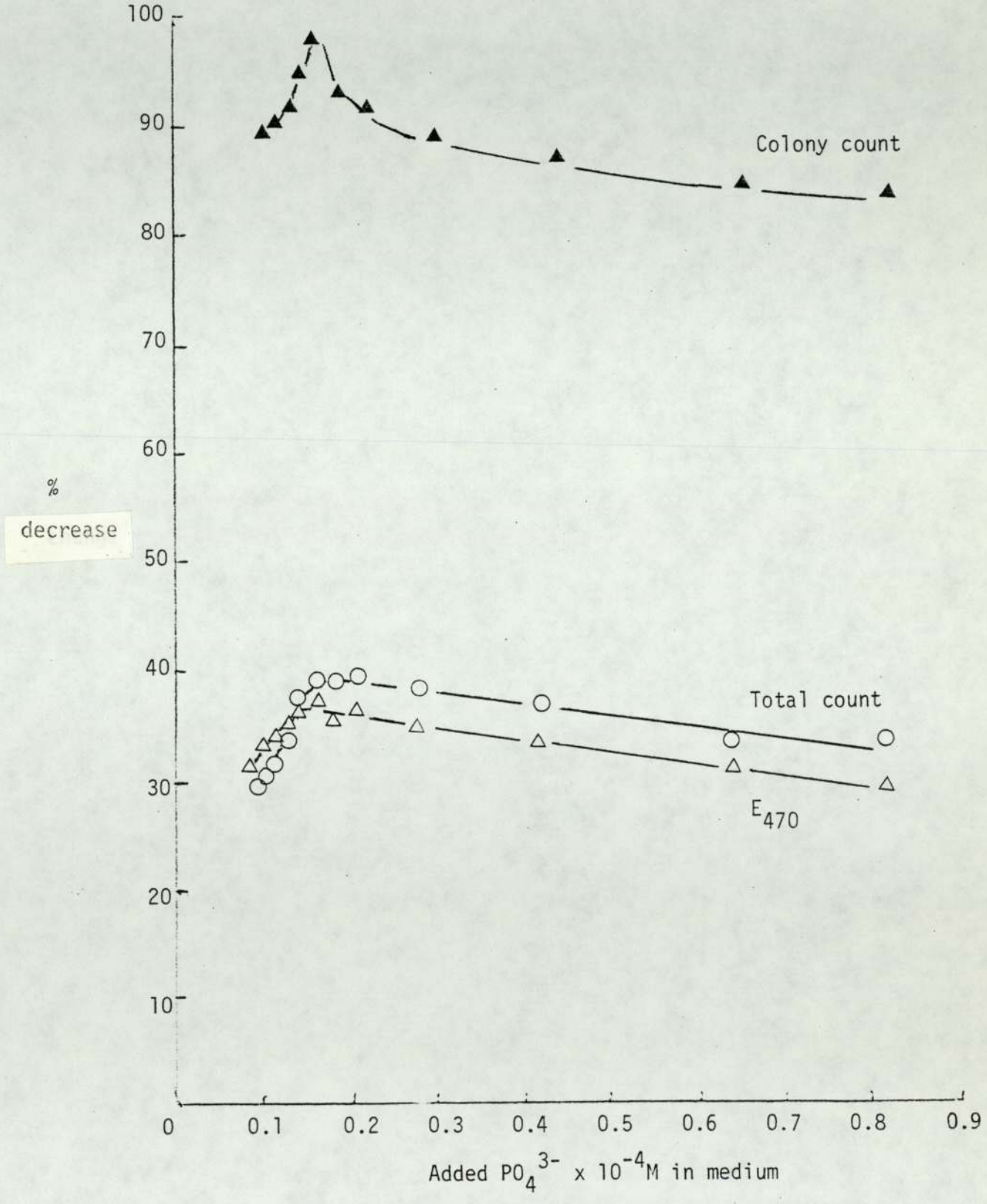


Fig. 57

The effect of PO_4^{3-} concentration in the medium upon the % change in E_{470}, total and colony count for *P. aeruginosa* treated with Polymyxin B (10 I.U. ml^{-1}) (Centrifugation method)



10 I.U. ml^{-1} P.B. Similar curves were obtained for the centrifugation method as for the noncentrifugation method. The % change in parameters was, however, altered. A similar increase in sensitivity to P.B. was recorded over the range of PO_4^{3-} from 0.1 x 10^{-4}M to 0.80 x 10^{-4}M. The % change in E_{470} rose from 30% to 38% whilst that of the total count rose from 29% to 40%, colony count changed from 87% to 98%. Above 0.16 x 10^{-4}M PO_4^{3-} a decrease in sensitivity was again observed. E_{470} fell from 38% to 32% as the PO_4^{3-} concentration rose to 0.8 x 10^{-4}M. The corresponding fall in total count and colony count was 40% to 34%, and 98% to 88% respectively.

The % changes seen were higher for cells having undergone the washing and centrifugation procedure. This may reflect the 'cleaner' medium in which polymyxin treatment was carried out by this method. Some inhibition of P.B. activity may occur due to metabolites found in the lysis medium when washing was not performed. Alternatively resuspension in fresh C.D.M. with an increased content of ions may have enhanced the activity of P.B. on the washed cells.

3.2.3.6. The effect of macromolecules released during growth on the lysis by 10 I.U. ml^{-1} polymyxin

The release of macromolecules e.g. proteins, lipopolysaccharides and protein-lipopolysaccharide-phospholipid complexes has been reported for both Gram-positive (Tsukagoshi et al, 1981) and Gram-negative organisms (Munford et al, 1980). Since P.B. interacts with both lipopolysaccharide (L.P.S.) and phospholipid (P.L.) in the bacterial envelope it is possible that in the test situation competition for P.B. may occur between cell envelope L.P.S. and P.L. and these materials shed into the growth medium prior to treatment with P.B.

To test this cells were grown in C.D.M. containing 0.1 and 0.5 x 10^{-4}M PO_4^{3-}. The cells were centrifuged at 6000 rpm for 20 minutes. The spent supernatant was saved. The cell pellet was divided into 8 equal portions. Cells in 4 of the 8 portions (A-D) were resuspended to E_{470} 0.2 in 12ml of spent C.D.M., and those from the remaining 4 portions (E-H) resuspended to E_{470} 0.2 in fresh C.D.M. containing PO_4^{3-} but lacking glucose. The formulation of this resuspension medium was described earlier. The 8 cultures were then shaken at 37°C for 1 hour to equilibrate. Concurrently eight further flasks (1-8) containing the following were shaken for 1 hour:

Flask 1 12ml resuspension medium

Flask 2 12ml resuspension medium + 1ml 250 I.U. ml^{-1} Polymyxin B

Flask 3 12ml spent culture medium

Flask 4 12ml spent culture medium + 1ml 250 I.U. ml^{-1} Polymyxin B

Flask 5 12ml resuspension medium)
Flask 6 12ml resuspension medium + 1ml water) Controls
Flask 7 12ml spent culture medium)
Flask 8 12ml spent culture medium + 1ml water)

After this hour the following additions were made to flasks 1-8:

Flask 1 + Flask E + 1ml 250 I.U. ml^{-1} P.B.

Flask 2 + Flask F

Flask 3 + Flask A + 1ml 250 I.U. ml^{-1} P.B.

Flask 4 + Flask B

Flask 5 + Flask G + 1ml water

Flask 6 + Flask H

Flask 7 + Flask C + 1ml water

Flask 8 + Flask D

All flasks with antibiotic contained a final concentration of 10 I.U. ml^{-1} P.B.

The flasks were shaken for a further 1 hour. E_{470} values were taken every 10 minutes and from the lysis curves obtained the % lysis values in 60 minutes were determined.

The results are shown in Table 31 . The lysis results shown are adjusted for lysis of the controls.

Table 31

Lysis of P. aeruginosa in 60 minutes by 10 I.U. ml^{-1} Polymyxin B

Flask	% lysis 60 min.	
	0.1 x 10^{-4}M PO_4^{3-}	0.5 x 10^{-4}M PO_4^{3-}
1	33.7	23.4
2	32.4	20.1
3	10.2	12.8
4	7.8	0.2

Results show that for cells grown in 0.5 x 10^{-4}M PO_4^{3-} the 1 hour exposure of P.B. to the spent culture medium (Flask 4) resulted in a dramatic loss of P.B. activity as shown by lysis of cells added subsequently. These cells lysed only 0.2%. Cells resuspended in spent medium (Flask 3) to which P.B. was added as in the normal drug testing proceudre showed by comparison a lysis of 12.8%. The difference in these values reflects inactivation of P.B. by the spent culture medium before addition of the cells. This large difference is not shown when clean resuspension medium was used, there being only 3.3% difference in the lysis between flasks 1 and 2. The results also reflect the difference in % lysis seen previously for cells treated by the centrifugation (23.4%) and the noncentrifugation (12.8%) methods. The apparent increase

in resistance for cells grown in higher PO_4^{3-} concentrations appears to be due in part to interaction between P.B. and constituents of the spent culture medium. It is not due to increased PO_4^{3-} in the medium as shown previously (p 184). Table 31 also shows that the effect of exposure of P.B. to spent medium had less effect on cells grown in a low concentration of PO_4^{3-} (0.1×10^{-4}M) than for cells grown at the higher PO_4^{3-} concentration (0.5×10^{-4}M).

3.2.3.7. The composition of the spent culture medium

The previous work suggests that the composition of the spent culture medium has an effect on the observed lysis when treated with P.B. It is likely that L.P.S. and/or P.L. or complexes of L.P.S. and P.L. may be involved. If this hypothesis is true then PO_4^{3-} depleted cells should release less P.L./L.P.S. into the culture medium than PO_4^{3-} plentiful cells. To test this, cells were grown to E_{470} 0.8 in batch culture media containing graded amounts of PO_4^{3-} (0.1, 0,17 and 0.5×10^{-4}M). Uninoculated C.D.M. containing zero PO_4^{3-} served as a control. 500ml of the spent culture was taken and the cells removed by centrifugation at 6000 rpm for 15 minutes followed by filtration of the supernatant through 0.45μ membrane filters. The culture supernatant was concentrated to approximately 20ml by rotary evaporation under vacuum. The 20ml portions were then dialysed against tap water for 48 hours and then freeze dried using an Edwards High Vacuum freeze dryer (Edwards Ltd., Crawley). 500mls of uninoculated C.D.M. was used as a control and treated as above. The freeze dried depositwas resuspended in 2ml distilled water. The samples were analysed for protein, K.D.O. and R.E.L. phosphorus and the results shown in Table 32.

Table 32

Chemical analysis of freeze dried culture supernatant of *P. aeruginosa* grown in various concentrations of phosphate

Medium PO_4^{3-} conc. ($\times 10^{-4}$)	% dry weight of lyophilised supernatant		
	Protein	K.D.O.*	R.E.L.P.**
0.00 (control)	nd	nd	nd
0.10	25.4	0.72	nd
0.17	15.4	0.95	nd
0.50	4.3	2.4	nd

* K.D.O. 2-keto-3-deoxyoctonic acid

** R.E.L.P. Readily extractable lipid phosphorus

nd not detected

3.3. The effect of phosphate upon lysis and viability of batch grown *P. aeruginosa* treated with E.D.T.A.

3.3.1. The effect of E.D.T.A. concentration upon lysis

P. aeruginosa was grown in C.D.M. containing a low level of PO_4^{3-} (0.1×10^{-4}M) and in C.D.M. containing excess PO_4^{3-} (1×10^{-4}M) to an E_{470} 0.2 and treated with graded amounts of E.D.T.A. from 0µg ml^{-1} to 1570µg ml^{-1}. Cells grown in low PO_4^{3-} medium showed no lysis at any level of added E.D.T.A. when corrected for lysis in control cultures. Fig. 58 shows the relationship between $\log_{10} E_{470}$ and time for cells grown in 1.0×10^{-4}M PO_4^{3-}. The cells showed increasing sensitivity to E.D.T.A. with increased E.D.T.A. concentrations.

Fig. 59 shows the relationship between % lysis in 180 minutes and E.D.T.A. concentration for the PO_4^{3-} depleted and plentiful cultures. Phosphate depleted cells showed complete resistance to E.D.T.A. up to the maximum concentration of E.D.T.A. tested (1570µg ml^{-1}). Cells grown in a PO_4^{3-} plentiful medium showed increased % lysis to a level of 33% at an E.D.T.A. concentration of 1570µg ml^{-1}. Linearity was maintained to an E.D.T.A. concentration of 785µg ml^{-1}. A concentration of 785µg ml^{-1} E.D.T.A. was chosen for subsequent work. This concentration of E.D.T.A. gave lysis differences of 0 - 30% for cells grown in low PO_4^{3-} and high PO_4^{3-} respectively.

3.3.2. The effect of phosphate content in the medium upon lysis of *P. aeruginosa* by 785µg ml^{-1} E.D.T.A.

Fig. 60 shows the relationship between lysis and PO_4^{3-} concentration for cells grown in C.D.M. containing 0.1×10^{-4}M to 1.7×10^{-4}M PO_4^{3-}. Increasing sensitivity to E.D.T.A. was exhibited as the initial PO_4^{3-} content of the growth medium was increased. The curves exhibited a

Fig. 58

The effect of E.D.T.A. concentration upon lysis of *P. aeruginosa* grown in C.D.M. containing 1.0×10^{-4}M PO_4^{3-}

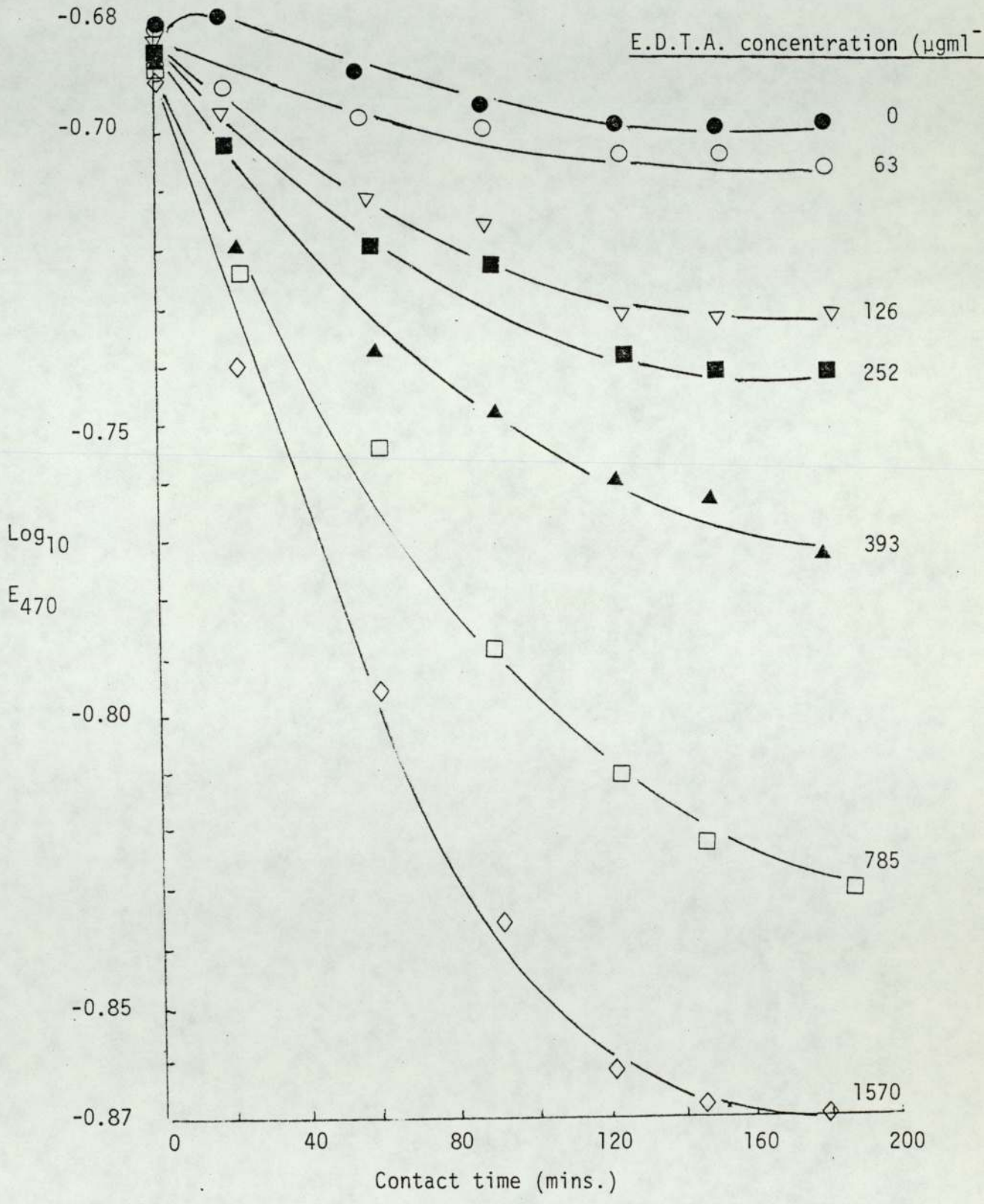


Fig. 59

The effect of E.D.T.A. concentration upon % lysis in 180 minutes for *P. aeruginosa* grown in C.D.M. containing 0.1 or 1.0 x 10^{-4}M PO_4^{3-}

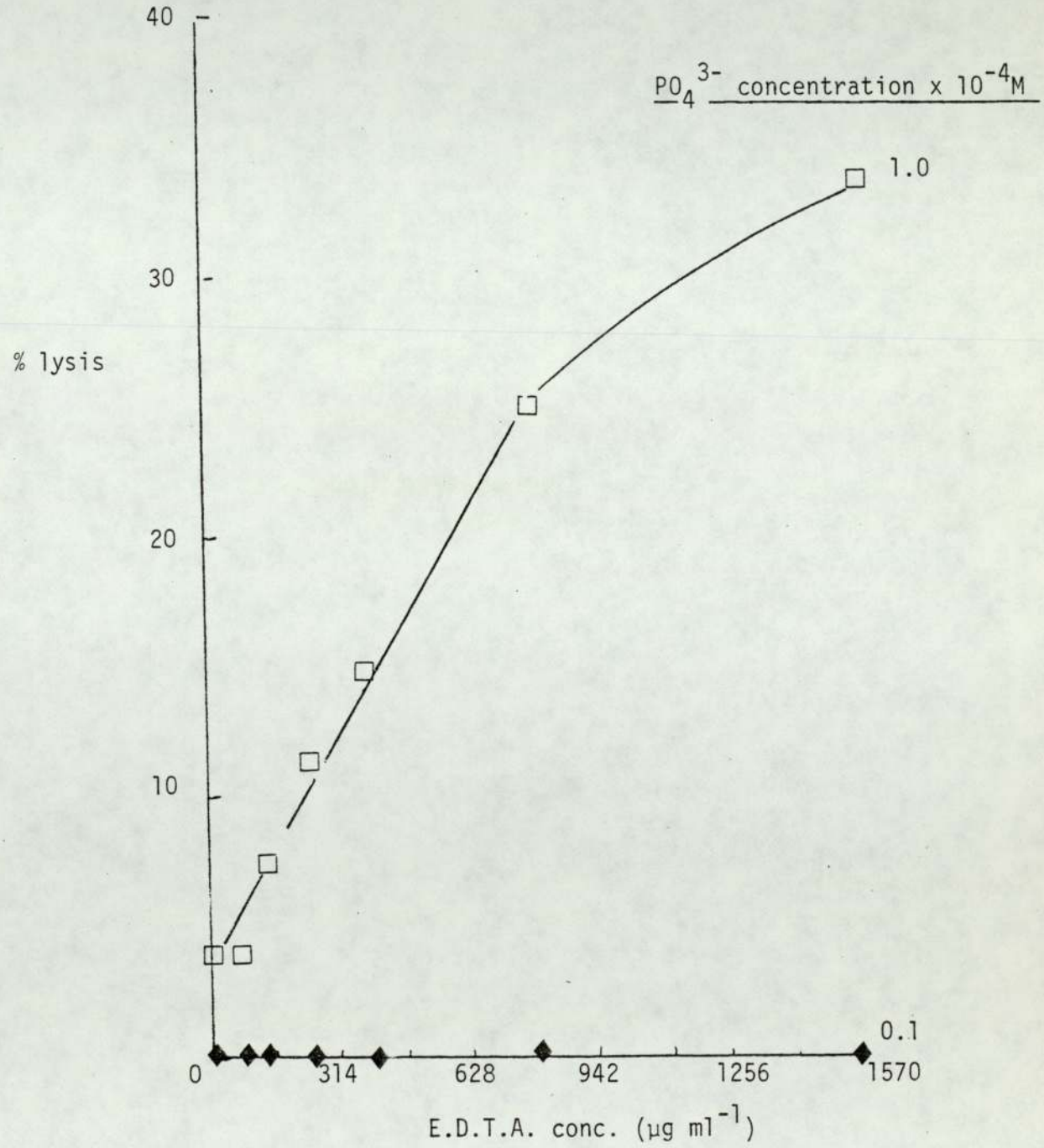
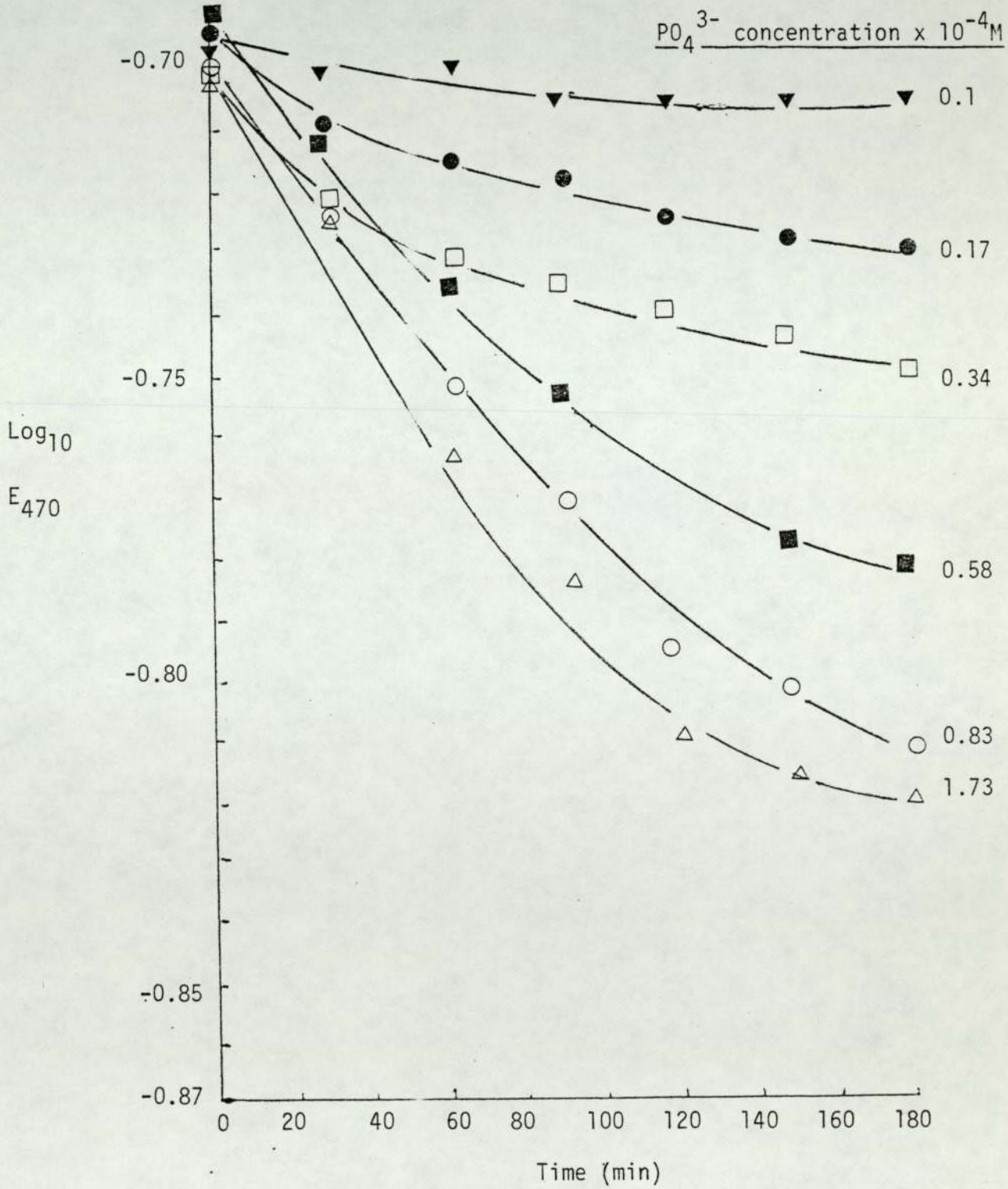


Fig. 60

The effect of PO_4^{3-} concentration in the medium upon lysis of *P. aeruginosa* by E.D.T.A. (785µg ml^{-1})



linear decline in E_{470} for approximately 60 minutes followed by an altered rate of decline up to 180 minutes. The decline in this later phase was approximately linear at lower PO_4^{3-} concentrations but became exponential at higher PO_4^{3-} concentrations. Fig. 61 shows the relationship between % lysis in 180 minutes and initial PO_4^{3-} concentration in the growth medium. The % lysis rose rapidly in a linear fashion from 0.1×10^{-4}M to 0.8×10^{-4}M PO_4^{3-}. Maximum lysis of 24.5% was shown at an initial PO_4^{3-} concentration of 0.83×10^{-4}M PO_4^{3-}. There was a slight decline in % lysis with increased PO_4^{3-} concentration. However, this decline was small compared with the decline seen for P.B. treated cells. A difference in sensitivity to P.B. and E.D.T.A. is seen since maximum sensitivity to P.B. was seen for cells grown in a PO_4^{3-} concentration of 0.16×10^{-4}M compared to 0.8×10^{-4}M PO_4^{3-} for E.D.T.A. treated cells.

Linear relationships existed for both primary lysis rate constant K (0 - 60 minutes) and the secondary rate constant K (60 - 180 minutes) as shown in Fig. 62 . The primary rate constant was linear to a value of 0.95×10^{-3} min^{-1} corresponding to a PO_4^{3-} concentration of 0.6×10^{-4}M PO_4^{3-}. The maximum rate constant was 1.15×10^{-3} min^{-1} at a PO_4^{3-} concentration of 1×10^{-4}M after which the rate constant fell progressively to approximately 0.7×10^{-3} min^{-1}. The secondary rate constant was linear to a value of 0.45×10^{-3} min^{-1} at a PO_4^{3-} concentration of 0.65×10^{-4}M and reached a maximum of 0.74×10^{-3} min^{-1} at a PO_4^{3-} concentration of 1.25×10^{-4}M PO_4^{3-}. Above this level of added PO_4^{3-} the rate fell slowly.

Linearity was shown between the lysis rate constants (both primary and secondary) and the % lysis of the culture by E.D.T.A. (Fig . 63).

Fig. 61

The effect of PO_4^{3-} concentration in the medium upon % lysis for *P. aeruginosa* treated with E.D.T.A. (785µg ml^{-1})

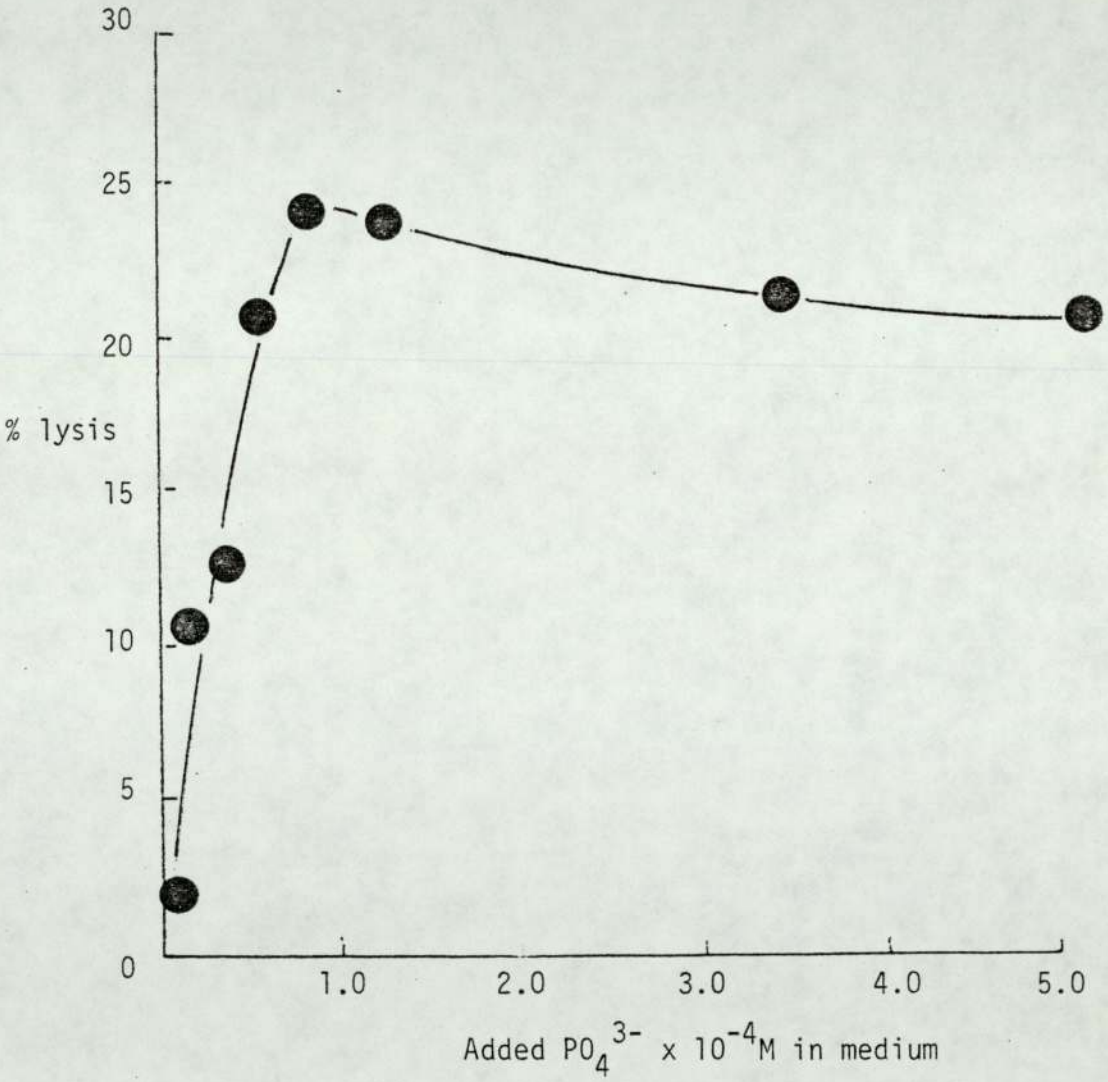


Fig. 62

The effect of PO_4^{3-} concentration in the medium upon the primary and secondary rate constants for lysis of *P. aeruginosa* by E.D.T.A. (785µg ml^{-1})

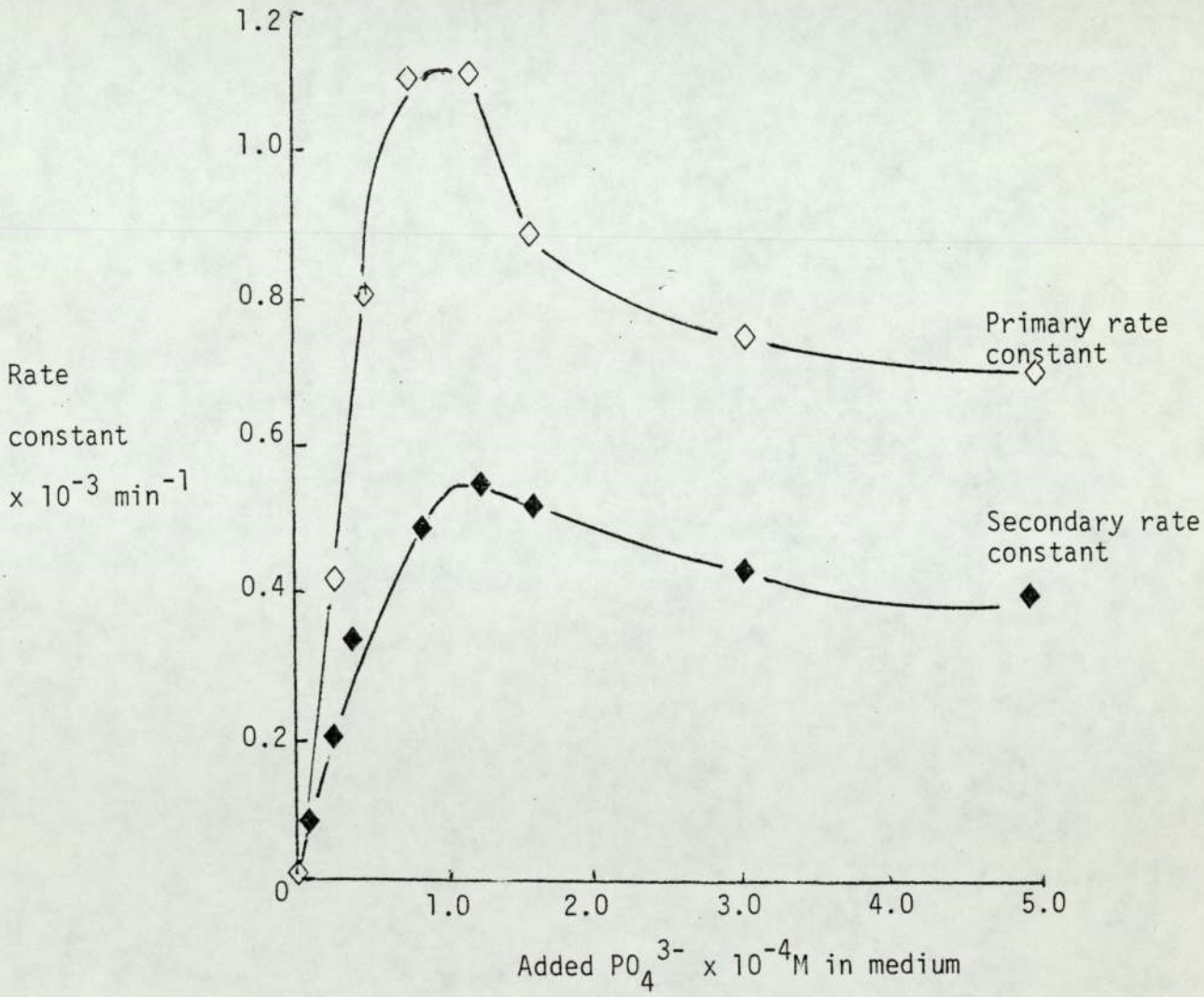
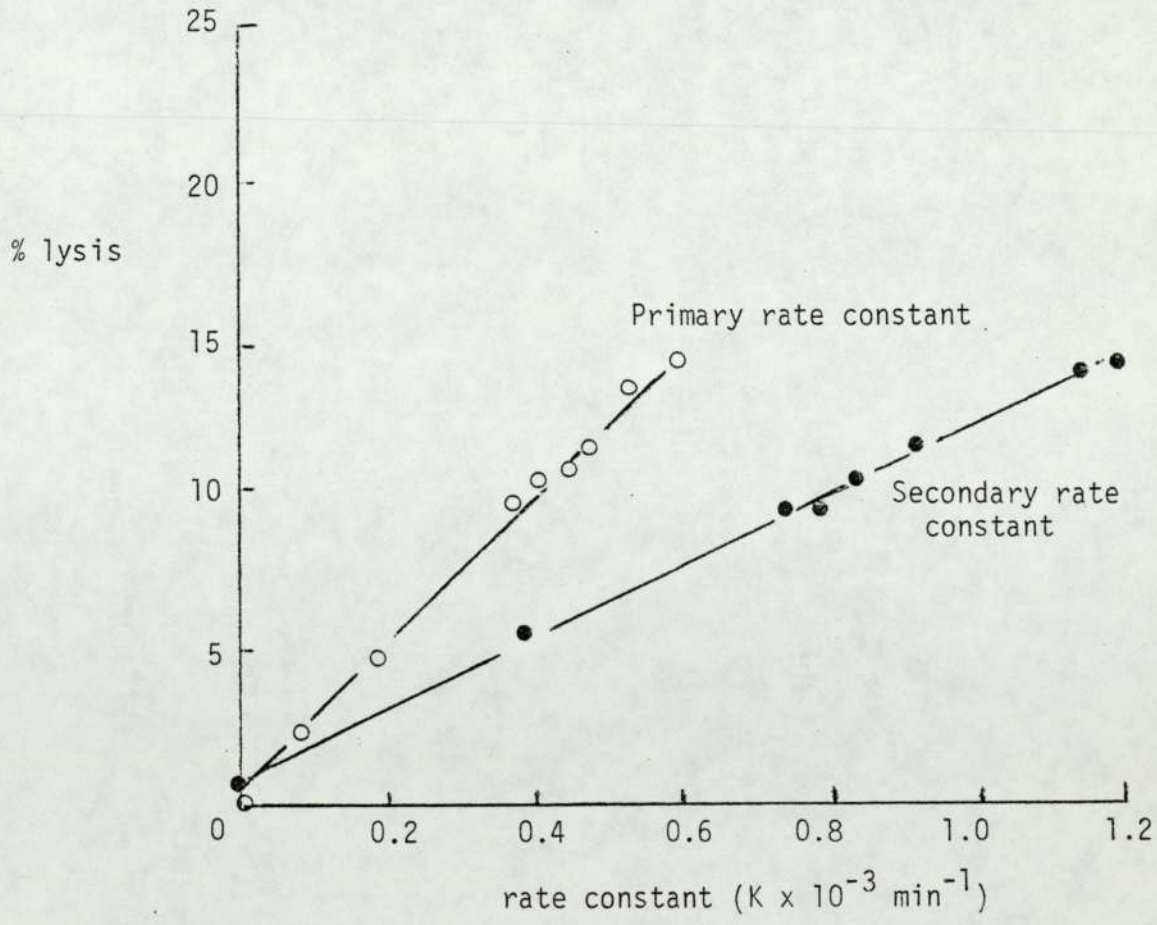


Fig. 63

Relationship between % lysis and primary and secondary rate constants for lysis of P. aeruginosa by E.D.T.A. ($785_{\mu}g\ ml^{-1}$)



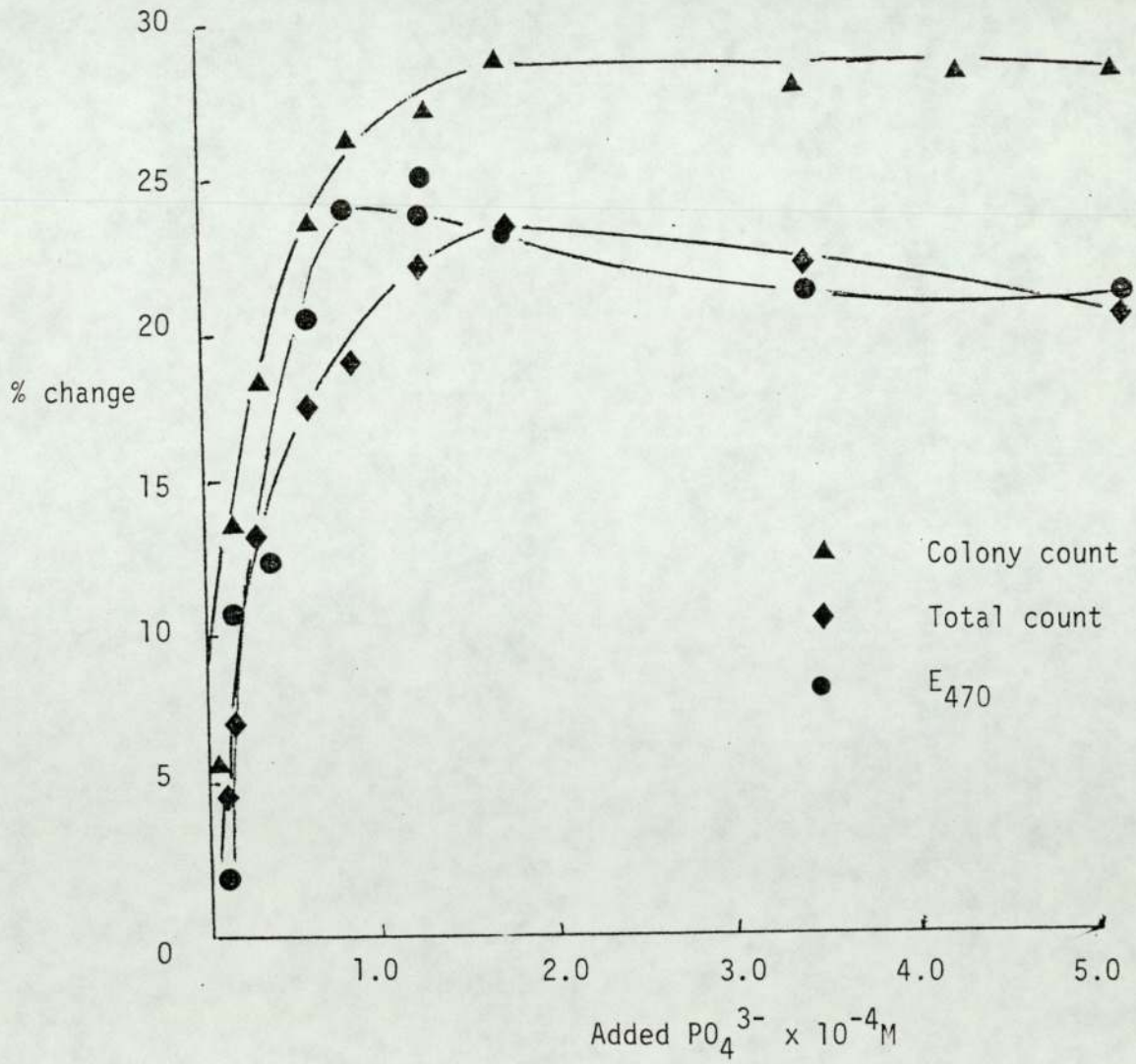
3.3.3. The effect of treatment with 785µg ml^{-1} E.D.T.A. on E_{470}, total and colony counts for growth in C.D.M. containing graded amounts of PO_4^{3-}.

P. aeruginosa was grown in C.D.M. containing 0.1×10^{-4} - 5×10^{-4}M PO_4^{3-} to an E_{470} value of 0.2. The cells were then transferred to warmed flasks containing 785µg ml^{-1} E.D.T.A. 1ml aliquots were removed at time 0 minutes for estimation of colony count and 0.1ml taken for total count estimations. The E_{470} was recorded. 10 fold dilutions were made in nutrient broth for viable count estimations. The culture was exposed to E.D.T.A. for 180 minutes and similar samples were removed for viable count, E_{470} and total count estimations. From the results the % change in the 3 parameters for each initial PO_4^{3-} concentration can be determined. The results are shown in Fig. 64 . Good agreement was obtained between all 3 parameters with respect to change in 180 minutes and inital PO_4^{3-} concentration. The cells showed a large change in sensitivity for all 3 parameters for growth in PO_4^{3-} from 0.1 - 1.25×10^{-4}M PO_4^{3-}. Maximum lysis values of 22.5, 24.0 and 28.5% were obtained for total count, E_{470} and colony count respectively. Above a PO_4^{3-} concentration of 1.25 $\times 10^{-4}$M there was little % change in the 3 parameters.

The % change in colony count resembled much closer the values for E_{470} and total count for E.D.T.A. treated cells than for P.B. treated cells. Many more of the E.D.T.A. treated cells must have been less seriously damaged after treatment.

Fig. 64

The effect of PO_4^{3-} concentration in the medium upon the % change in E_{470} total and colony count for *P. aeruginosa* treated with E.D.T.A. (785µg ml^{-1})



3.4. The culture of *P. aeruginosa* in a chemostat

3.4.1. Theory of the chemostat

The sequence of changes undergone by a microbial population following inoculation into a nutrient medium have been well documented. A "lag" phase of variable length is followed by an "exponential" growth phase during which nutrients are consumed and metabolic end products are released into the medium. A fall in pH and/or oxygen solution rate may eventually be insufficient to support exponential growth and this coupled with the changing growth environment results in the culture entering the "stationary" phase. This growth cycle has been studied by Monod (1942, 1949).

During the exponential growth phase:

$$\frac{1}{x}\frac{dx}{dt} = \mu \left(= \frac{d \log_e x}{dt} = \frac{\log_e 2}{td}\right) \qquad (1)$$

where td is the culture doubling time, μ is the specific growth rate constant and x is the initial concentration of organisms. Generally μ and td are constant, but they are affected by such environmental characteristics as the concentration of various essential nutrients. The dependence of μ upon substrate concentration s was shown by Monod (1942, 1950) to be representable by the Michaelis-Menton type formula

$$\mu = \mu \text{ max} \left(\frac{s}{K_s + s}\right) \qquad (2)$$

where μ max is the maximum value of μ when s is nonlimiting and K_s is the saturation constant (equal to the growth limiting substrate concentration at 0.5μ max). In a batch culture where nutrients are initially in excess the growth rate is equal to μ max.

$$\frac{dx}{dt} = -Y \frac{ds}{dt} \qquad (3)$$

Y is termed the "yield factor". For the exponential growth phase in batch culture.

$$Y = \frac{\text{Weight of bacteria formed}}{\text{Weight of substrate consumed}} \qquad (4)$$

All continuous cultures begin as batch cultures. If fresh culture media is added at a sufficient rate to maintain the culture density at a submaximal level then unlike batch culture where growth ultimately ceases the culture should continue growth indefinitely.

The chemostat is a vessel of fixed volume V into which medium is pumped at a constant rate (f). The medium is formulated such that all substances required for growth except one are present in excess of growth requirement.

In such an arrangement the growth rate depends upon the medium flow rate and also on the dilution rate D.

$$D = f/_V \qquad (5)$$

In the continuous culture vessel organisms are growing and being simultaneously washed out. The relative rates of each process affects the net change in concentration of the organisms x.

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

If $\mu > D$, dx/dt is a positive value and the concentration of organisms will increase with time. dx/dt will be negative and cells washed out of the growth vessels if $\mu > D$. Steady state conditions will occur when μ equals D i.e. dx/dt is zero. Culture of organisms in the chemostat can be described by the equation:

$$D = \mu = \mu \max \left(\frac{s}{K_s + s} \right) = \frac{\log_e 2}{td} \qquad (7)$$

Provided that dilution rate is maintained constant the system is inherently self balancing. Within limits the growth rate of the culture can be adjusted by altering the dilution rate. However the specific growth rate (μ) cannot be made to exceed μmax, and above certain dilution rates (D_c), where D_c is nearly equal to μmax, progressive washout from the vessel will occur.

The general growth formulae derived by Monad (1942, 1950) have been shown in practice not to predict accurately the growth of cultures in the chemostat. No account is taken of diffusion effects, endogenous metabolism, the accumulate of storage products or variations in bacterial composition with growth rate all of which make the observed behaviour of cells in the chemostat different from the theoretical behaviour. (Pirt, 1975).

3.4.2. Preliminary experiments

The work described previously using batch cultures of P. aeruginosa grown under glucose limited and PO_4^{3-} limited conditions can be used to calculate such growth parameters as the yield constants, saturation constants and growth rate constants. From these parameters media for use in the chemostat may be formulated.

3.4.2.1. The Yield Constant, Y for glucose growth cells

Flasks containing media to give carbon limited growth to an E_{470} 0.2 were prepared. Following the cessation of growth in the media formalin was added to the culture to a concentration of 1%. A known volume of culture was then centrifuged at 4000 rpm for 20 minutes in a preweighed

tube. The cells were washed twice, first in 0.89% saline and finally in distilled water. The final cell pellet was dried at 105°C for 48 hours. Following drying the tube was cooled in a vacuum dessicator over P_2O_5. The tube was then weighed. The yield constant Y was calculated to be 0.54.

3.4.2.2. Saturation constant K_s and the growth rate constant μm for glucose grown cells

From the growth curves for P. aeruginosa obtained previously (Fig. 38) the saturation constant and growth rate constant were determined by construction of the graph $1/\mu$ against $1/s$ where μ is the growth rate constant and s is the substrate concentration (Fig. 65).

The Monod equation: $$\mu = \mu_m \left(\frac{s}{Ks + s}\right)$$

can be rearranged using the Lineweaver Burke formula:

$$\frac{1}{\mu} = \frac{1}{\mu_m} + \frac{K_s}{\mu_m} \times \frac{1}{s}$$

The slope of the line equals $\frac{K_s}{\mu_m}$ and the intercept on the Y axis equals $\frac{1}{\mu_m}$.

From Fig. 65 μ_m was calculated to be 0.725 and K_s to be 20μM for the high affinity uptake system. The μm and K_s for the low affinity uptake system was 0.820 and 1.1mM respectively.

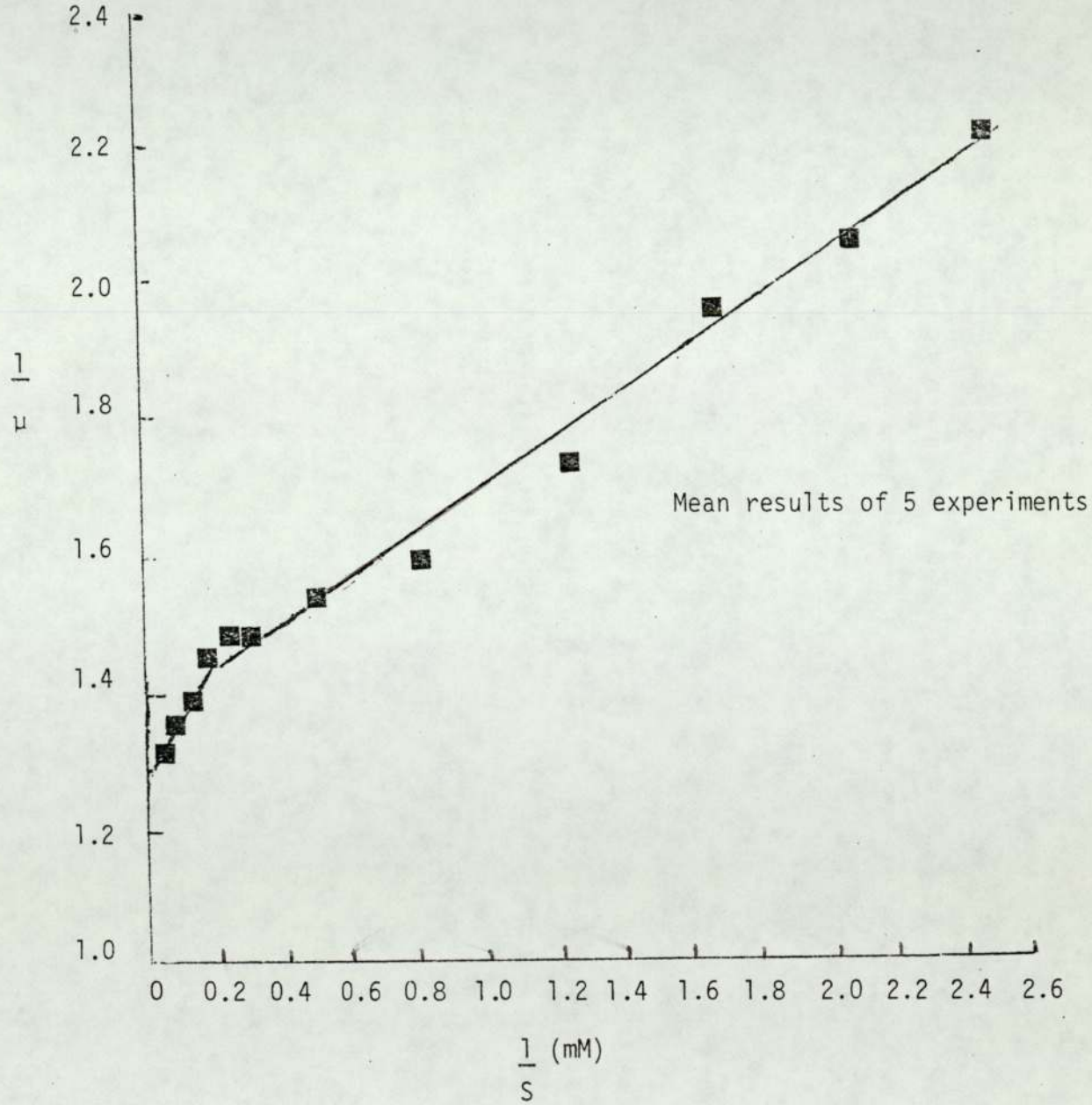
3.4.2.3. Determination of Sr

The concentration of growth limiting nutrient Sr may be determined from the formula:

$$Sr = \frac{2\tilde{x}Y - Y^2K_s \pm \sqrt{Y^4K_s^2 + 4\tilde{x}Y^3K_s}}{2Y^2}$$

Fig. 65

Double reciprocal plot of P. aeruginosa growth rate versus medium glucose concentration



when Y is the yield constant, K_s the saturation constant and $\tilde{x}$ is mg/ml of cells. Using this data media used for glucose depletion studies was formulated. All other medium constituents were present in a theoretical 10x excess (Table 33)

3.4.2.4. Determination of yield constant, growth rate constant and saturation constant for phosphate grown cells

The experiments described for glucose depleted growth were repeated using phosphate depleted media and the parameters yield constant Y, saturation constant K_s, and growth rate constant μ_m calculated. K_s and μ_m were calculated using data obtained from Fig. 66.

The yield constant was 44.6 and the values of μ_m and K_s for the low affinity uptake system were 0.75 and 36μM respectively and for the high affinity uptake system 0.47 and 1μM respectively.

This data was used to formulate phosphate depleted media for use in the chemostat. All other nutrients were added in a theoretical 10x excess (Table 33).

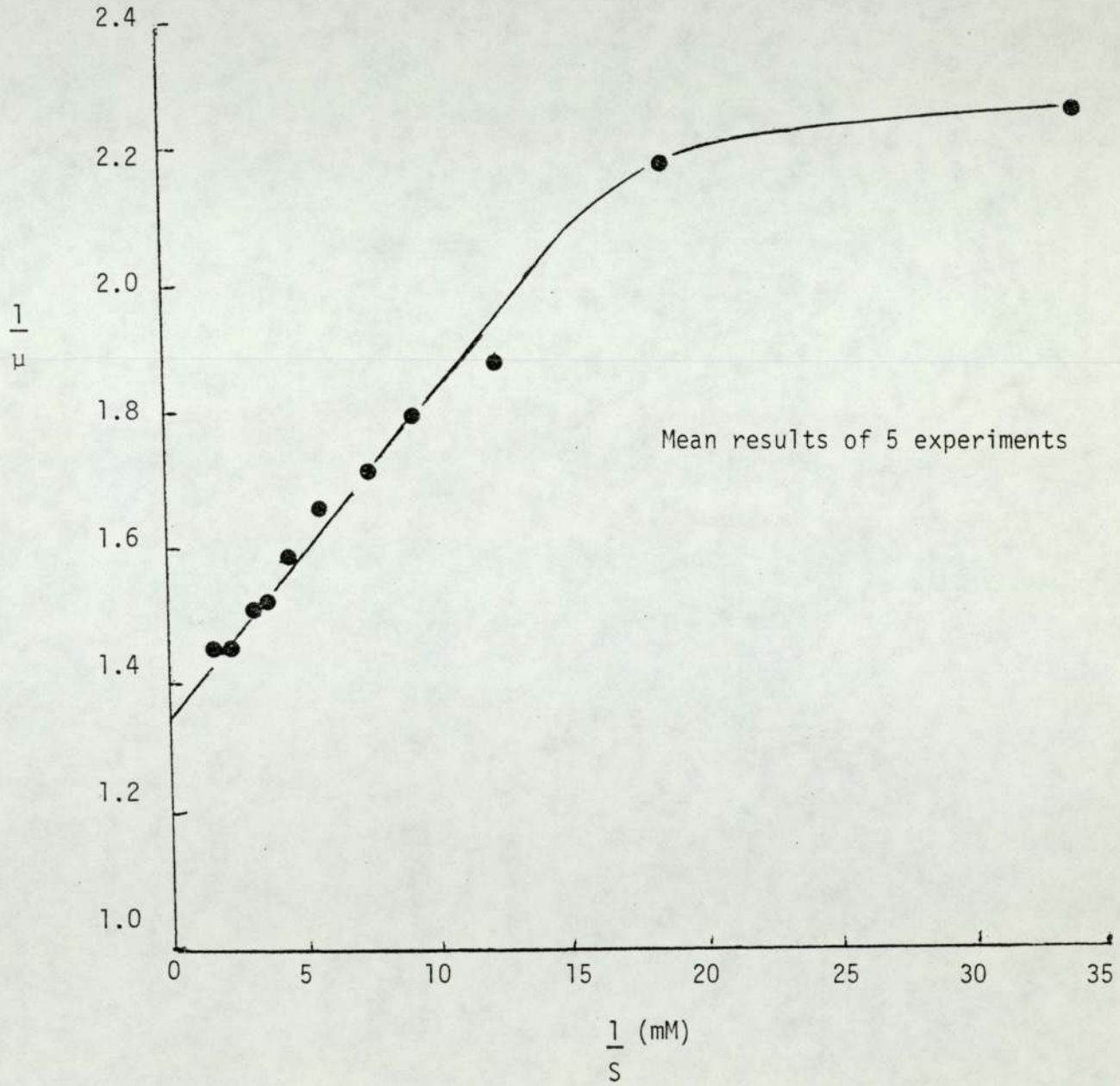
Table 33

Media formulation for studies using chemostat grown P. aeruginosa

Nutrient	Carbon limited growth	Phosphate limited growth
Na_2HPO_4	6.4×10^{-4}M	5.0×10^{-5}M
Glucose	8.5×10^{-4}M	8.0×10^{-3}M
$(NH_4)_2SO_4$	8.0×10^{-2}M	8.0×10^{-2}M
$MgSO_4.7H_2O$	8.0×10^{-4}M	8.0×10^{-4}M
$FeSO_4.7H_2O$	1.2×10^{-4}M	1.2×10^{-4}M
NaCl	1.0×10^{-3}M	1.0×10^{-3}M
KCl	1.2×10^{-3}M	1.2×10^{-3}M
MOPS	6.0×10^{-2}M	6.0×10^{-2}M

Fig. 66

Double reciprocal plot of *P. aeruginosa* growth rate versus medium PO_4^{3-} concentration



3.4.3. The chemostat design

Resistance studies were performed on cells grown in small 50ml working volume chemostats. The chemostat has been described by Gilbert and Stuart (1977).

The chemostats were suspended in a water bath at 37°C. Media was pumped from 2 litre flasks by a Watson Marlow pump using a delta drive (Watson Marlow Ltd., Cornwall). The pump speed could be adjusted to give any desired speed between 0 and 1000. The volume pumped was varied for any set pump speed by using pump tubing of various diameters (1mm to 6mm) or insertion of Y-pieces in the media lines and feeding from two or more reservoirs simultaneously. Sterile air was supplied to the chemostat by a HY-FLO pump (Gallenkamp Ltd., London) and adjusted to 150-200cc min^{-1} by a 'blow-off' valve. The air was humidified at 37.5°C by passage through deionised water via a glass sinter unit.

The chemostat was filled to working volume with the limitation medium and the pump stopped. P. aeruginosa was inoculated into the chemostat and grown as a batch culture overnight at 37°C. After overnight growth the pump was started and media fed to the chemostat at the desired rate. Before using the cells or if any change in media or growth rate had been made the chemostat was re-equilibrated for at least 5 complete media changes through the vessel. The dilution rate was checked by collection and measurement of the effluent flowing from the culture vessel for several 1 hour periods during each experiment. A rapid check of the flow rate was frequently made by timing the period for 50 drops of medium to be delivered into the vessel from the '50 dropper' pipette (Bilbate Ltd.).

3.4.3.1. Determination of the working volume of the chemostat

Six nominally 50ml chemostats were used for resistance studies. The working volume of each chemostat was determined as follows.

Air was pumped through the chemostats at constant rate (180cc min^{-1}). Water was pumped into the chemostats to give a dilution rate of 0.2 hr^{-1}. After 5 volume changes the water feed was switched off and the water left in each chemostat was poured into a previously weighed beaker and the weight of water determined. This procedure was repeated a further 5 times and the water in each chemostat weighed. The results are shown in Table 34 and were submitted to an analysis of variance (Table 35).

Table 34

Weight (g) of water in the chemostat on 5 successive weighings

Weight \ Chemostat	1	2	3	4	5	6
1	44.1	45.0	45.0	45.3	44.0	43.8
2	44.0	46.0	44.4	44.2	44.3	43.1
3	44.1	45.3	43.1	43.2	44.5	43.5
4	43.5	44.9	44.0	43.9	43.7	43.2
5	42.3	44.7	44.9	45.2	44.0	44.2
Totals T	218.0	225.9	221.4	221.8	220.5	217.8

n = number of weighings per chemostat = 5

m = number of chemostats = 6

n.m = total number of observations = 30

$$\Sigma x^2 = 58575.16 \qquad (1)$$

$$\frac{\Sigma T^2}{n} = 58565.02 \qquad (2)$$

$$\frac{(\Sigma x)^2}{n.m} = 58556.17 \qquad (3)$$

Table 35

Analysis of variance of working volume of 6 chemostats on 5 occasions

Source of variance	Sum of squares	Degrees of freedom	Mean squares	Variance ratio 'F'
Between chemostats	(2) - (3) 8.85	m - 1 5	1.770	4.184
Within chemostats	(1) - (2) 10.14	n.m - m 24	0.423	

The tabulated values of 'F' for 5/24 degrees of freedom at the 1% and 5% significance level are 3.9 and 2.62 respectively. Therefore the variation within chemostats is not significantly greater than the variation between chemostats at the 1 and 5% levels.

In future work the working volume of the chemostats was taken as 44.2mls.

3.4.4. The effect on growth parameters of change in dilution rate

3.4.4.1. The effect of dilution rate on glucose depleted P. aeruginosa

P. aeruginosa was grown in the chemostat in glucose depleted C.D.M. over a range of dilution rates (D, hr^{-1}) of 0.05 to 0.625 hr^{-1}. The dilution rates were chosen in random order and following alteration of

the dilution rate at least 5 volume changes were permitted before cells were removed for subsequent testing. At each dilution rate samples were removed for colony count estimations, total count and E_{470} determinations. 20ml volumes were removed for dry weight estimations. The methods of performing these estimations was as previously described.

The results of increasing growth rate from 0.05 to 0.625 hr^{-1} on total and colony count, E_{470} and dry weight values are shown in Fig. 67. Good agreement between the four parameters was shown. An increase in dilution rate from 0.05 to 0.35 had no effect on any of the parameters studied. At dilution rates in excess of approximately 0.4 hr^{-1} O.D. of the culture occurred at an increasing rate. In further experiments growth rates below 0.4 hr^{-1} were used.

3.4.4.2. The effect of dilution rate on phosphate depleted P. aeruginosa

The previous experiment was repeated using PO_4^{3-} depleted C.D.M. The dilution rate was adjusted in the range 0.025 hr^{-1} to 0.325 hr^{-1}. At each dilution rate 1ml samples were removed, as previously, for colony count, total count and E_{470} estimations and 20ml amounts removed for dry weight estimations.

Unlike the glucose depletion curves described previously the four parameters studied fell rapidly as the dilution rate was increased Fig. 68. At dilution rates above 0.325 hr^{-1} the equilibrium E_{470} was 0.04 which was too low to be of practical use in subsequent resistance studies. In view of this the dilution rate was not increased above 0.325 hr^{-1}. Addition of PO_4^{3-} to the chemostat following equilibration resulted in an increase in E_{470} of the culture at each dilution rate indicating that the culture was indeed PO_4^{3-} depleted.

Fig. 67

The effect of dilution rate on E_{470}, dry weight, total and colony count for P. aeruginosa grown in a carbon limited chemostat

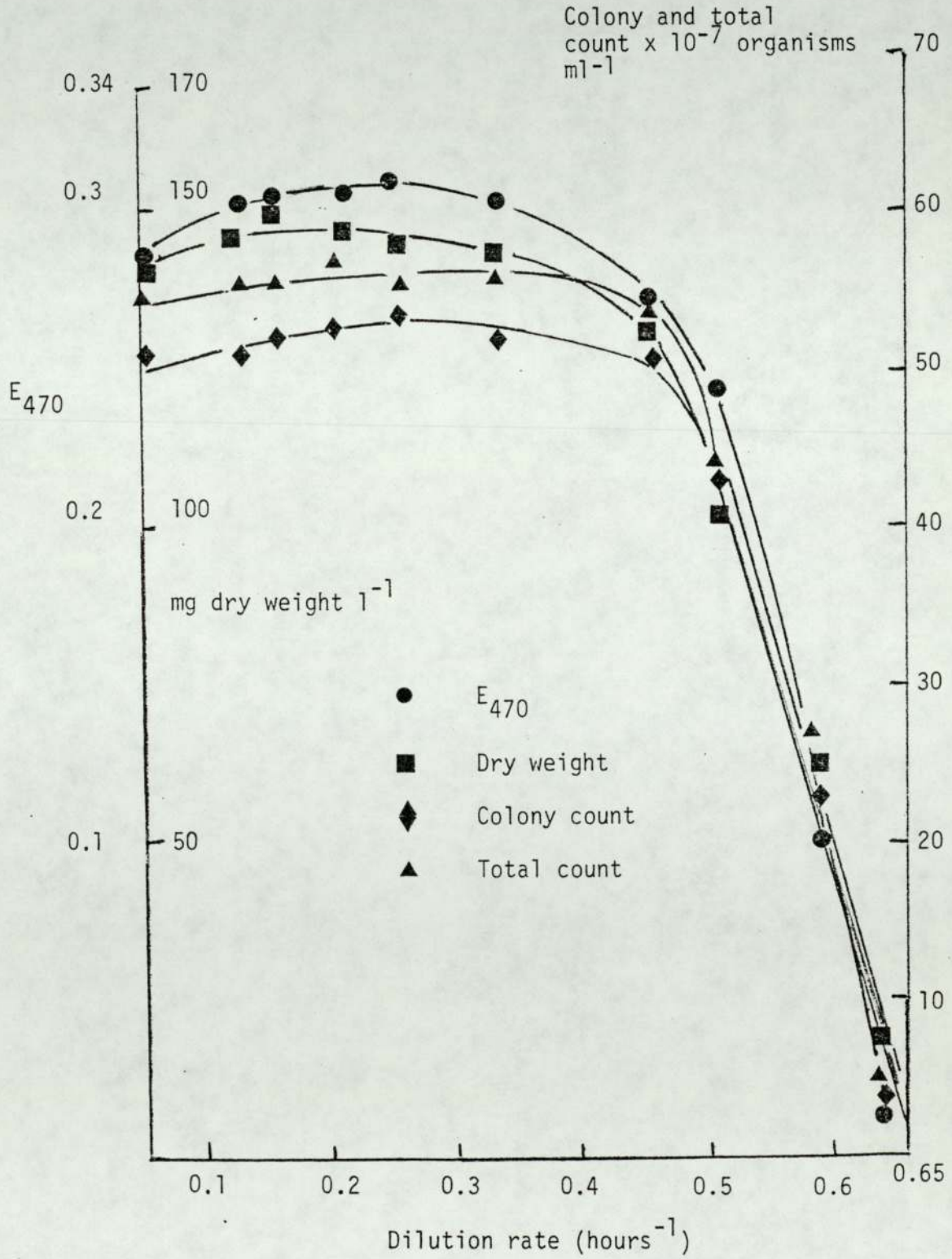
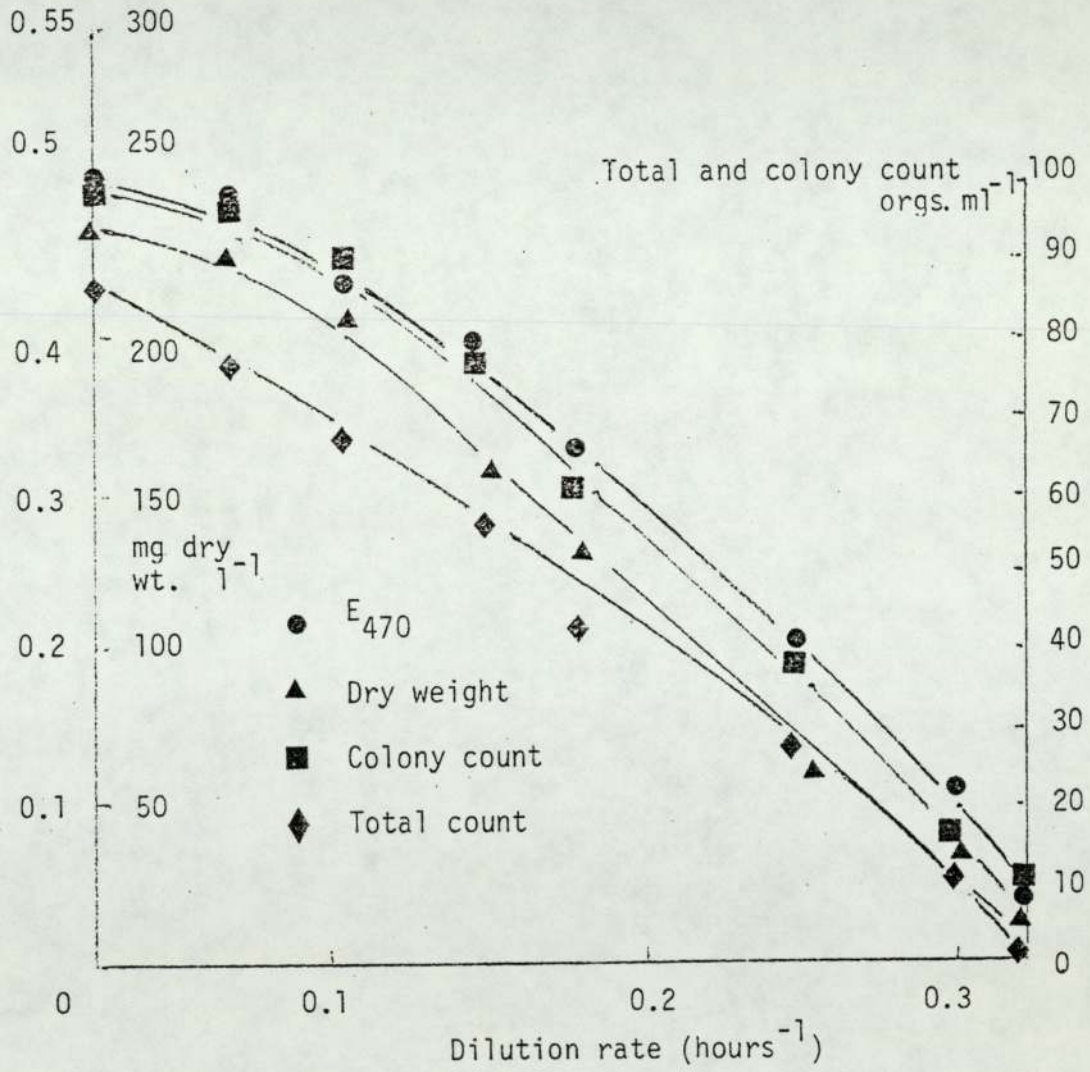


Fig. 68

The effect of dilution rate on E_{470}, dry weight, total and colony count for *P. aeruginosa* grown in a PO_4^{3-} limited chemostat



3.4.4.3. The effect of trace elements on the growth of phosphate depleted P. aeruginosa

The sharp fall in E_{470} and the other parameters studied for PO_4^{3-} depleted cells suggested that at faster growth rates (higher dilution rates) another nutrient may be limiting. Pirt (1975) suggested that trace elements may be of importance when cells are rapidly growing.

A mixture of trace elements was formulated as shown in Table 36. The concentration of the trace elements were as described by Evans et al (1970). The ions were added at the rate of 1ml litre^{-1} for calcium chloride, 0.1ml litre^{-1} sodium molybdate and 5.0 ml litre^{-1} of trace elements to the PO_4^{3-} depleted C.D.M.

Table 36
Trace elements added to PO_4^{3-} limited chemostat media

Ion	Compound	Concentration
Ca^{2+}	$CaCl_2$	0.294g 100ml^{-1}
Mo	MoO_3	0.014g 100ml^{-1}
Zn^{2+}	$ZnSO_4.7H_2O$	0.146)
Fe^{3+}	$FeCl_3.6H_2O$	0.900)
Mn^{2+}	$MnCl_2.4H_2O$	0.357)
Cu^{2+}	$CuSO_4.5H_2O$	0.030) g 100ml^{-1}
CO^{2+}	$CoCl_2.6H_2O$	0.080)
Bo_4^{3-}	H_3Bo_4	0.006)

The effect of dilution rate on the E_{470} for cells grown in C.D.M. with or without the addition of trace elements is shown in Table 37 for three dilution rates (0.25, 0.38 and 0.50 hr^{-1}).

The results show that the addition of trace elements failed to increase the E_{470} of the cells at the dilution rates tested, indicating that the culture was not depleted in any constituent of the trace element mixture.

Table 37

Effect of growth rate on E_{470} of P. aeruginosa grown in CDM containing trace elements

Dilution rate (hr^{-1})	E_{470}	
	C.D.M.	C.D.M. + trace element
0.25	0.200	0.202
0.38	0.080	0.075
0.50	0.012	0.013

This does not eliminate trace element deficiency since all of the trace elements mentioned by Pirt (1975) were not included. However all of the trace elements considered by Pirt (1975) as frequently essential for growth were included. Al, Si, V, Cr, Ni, As, Se, Sn, I, Be, F, Sc, Ti, Ga, Ge, Br, Zr and W are the elements not added but these are considered rarely to be essential for growth. The failure of the trace element mixture to improve the biomass makes such a deficiency very unlikely.

3.5. Resistance of chemostat grown *P. aeruginosa* to Polymyxin B

3.5.1. Resistance study procedure

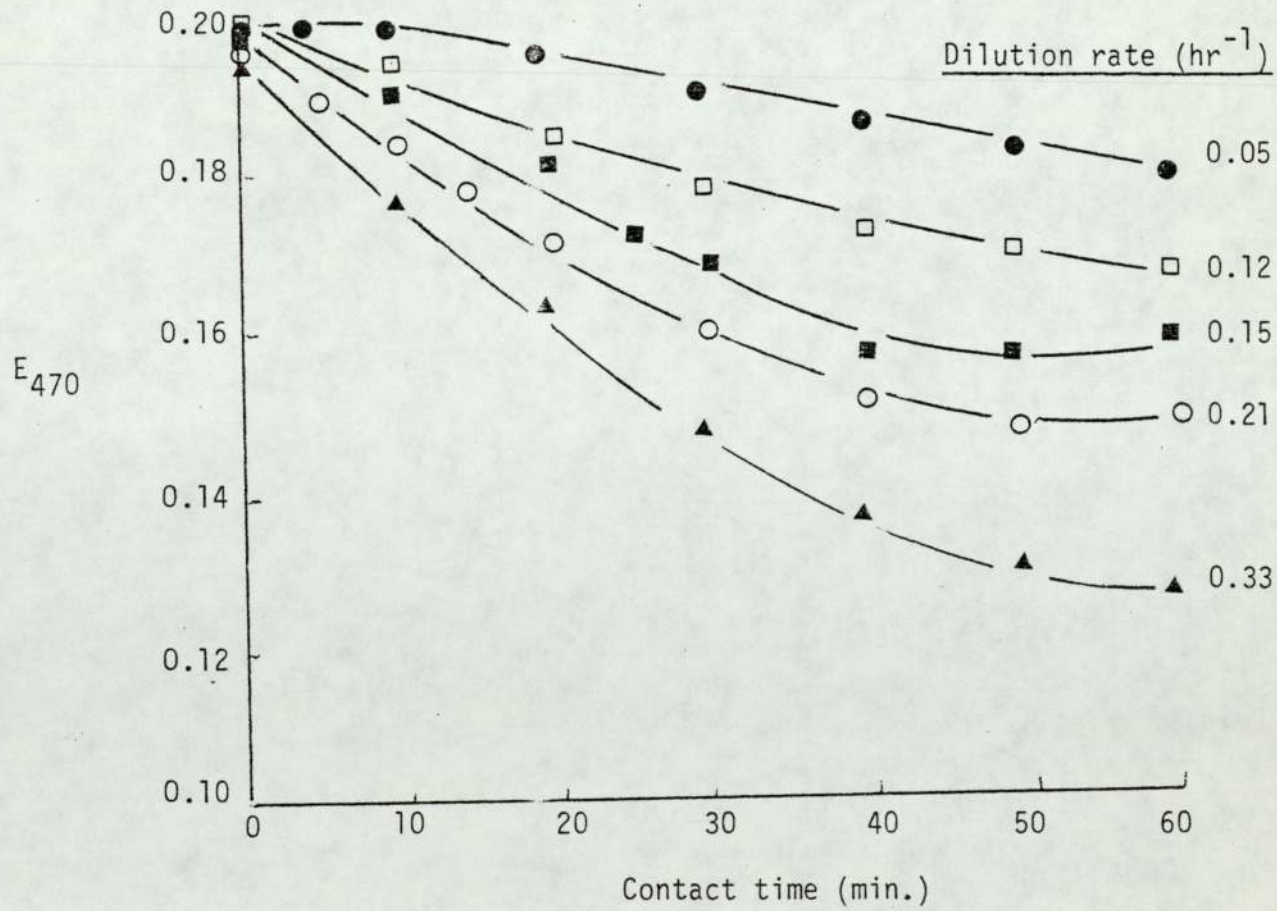
Cells grown at various dilution rates or under various limitations were removed from the chemostat for subsequent treatment. The cells were washed in the resuspension medium described previously for batch culture studies and treated by the centrifugation procedure described previously. Following resuspension to an E_{470} of 0.2 and 'holding' for 1 hour the cells were then treated in a manner similar to that described for batch studies with P.B. and E.D.T.A.

3.5.2. The effect of growth rate on the response of phosphate limited *P. aeruginosa* to Polymyxin B

Pseudomonas aeruginosa 6750 was grown in PO_4^{3-} limited C.D.M. formulated as shown in Table 33 . The dilution rate was adjusted over the range 0.05 hr^{-1} to 0.33 hr^{-1}. Following five volume changes cells were removed from the chemostat for further study. Following three washes in C.D.M. containing PO_4^{3-} (2.5×10^{-4}M) but lacking glucose the cells were resuspended in this medium to an E_{470} 0.2 and left to equilibrate with shaking for 1 hour. Before treatment with 10 I.U. ml^{-1} P.B. samples were removed for E_{470}, total and colony count estimations performed as previously described. The cells were then treated with P.B. and the E_{470} measured at various time intervals. Water was added to the control flasks. After 60 minutes exposure to the drug, samples were again removed and E_{470}, colony and total count estimations performed. Fig. 69 shows the result of increasing dilution rate on the E_{470} of PO_4^{3-} depleted cells. The rate and extent of lysis was dependent on the growth rate. Cells grown at higher dilution rates were more sensitive to P.B. At the lower dilution rate (0.05 hr^{-1}) a linear relationship existed

Fig. 69

The effect of dilution rate on the lysis by Polymyxin B (10 I.U. ml^{-1}) of chemostat grown PO_4^{3-} depleted *P. aeruginosa*



between E_{470} and dilution rate for the first 10 minutes following exposure to the drug. This relationship was not seen at dilution rates of 0.12 hr^{-1} or above. At all dilution rates a linear phase of lysis occurred between 10 minutes and 40 minutes. At dilution rates above 0.12 hr^{-1} a decrease in lysis was seen after 40 minutes.

Fig. 70 shows the relationship between the dilution rate and lysis rate constant (K) over the 10-40 minute lysis period. The lysis rate constant at each dilution rate was calculated from the fall in E_{470} over the 10-40 minute period. A linear relationship existed between K and the growth rate for growth rates from 0.05 hr^{-1} to 0.17 hr^{-1}. The relationship between dilution rate and % change in 60 minutes for the parameters E_{470}, total and colony count are shown in Fig. 71 . Linear relationships existed between % change in E_{470} and total count over the dilution rates studied. Good agreement was observed between the change in E_{470} and total count over the 60 minute exposure period. Larger % changes in colony count were observed. The change in colony count was linearly dependent upon dilution rate over the range of dilution rates 0.05 to 0.15 hr^{-1}. Above dilution rates of 0.15 the change was less marked. The % change in colony count approached a maximum of 98%.

The results show that as the dilution rate increased i.e. cells were dividing more rapidly, they became more sensitive to P.B. action.

3.5.3. The effect of growth rate on the response of glucose limited *P. aeruginosa* to Polymyxin B.

Pseudomonas aeruginosa 6750 was grown in glucose limited C.D.M. formulated as shown in Table 33 . The dilution rate was adjusted over the range 0.05 to 0.33 $hour^{-1}$. The cells were washed and resuspended

Fig. 70

The effect of dilution rate on the lysis rate constant for chemostat grown PO_4^{3-} depleted *P. aeruginosa* treated with Polymyxin B (10 I.U. ml^{-1})

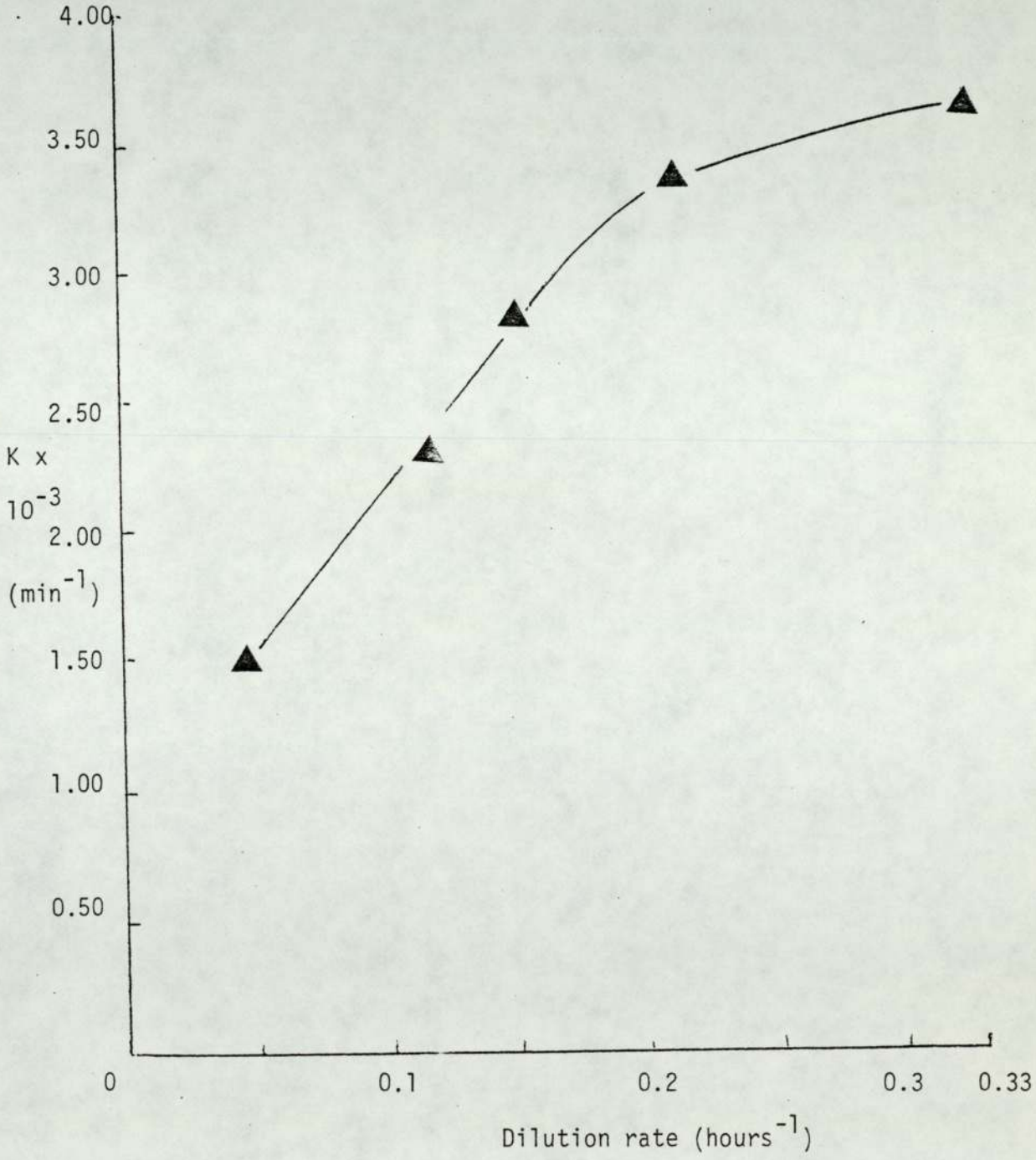
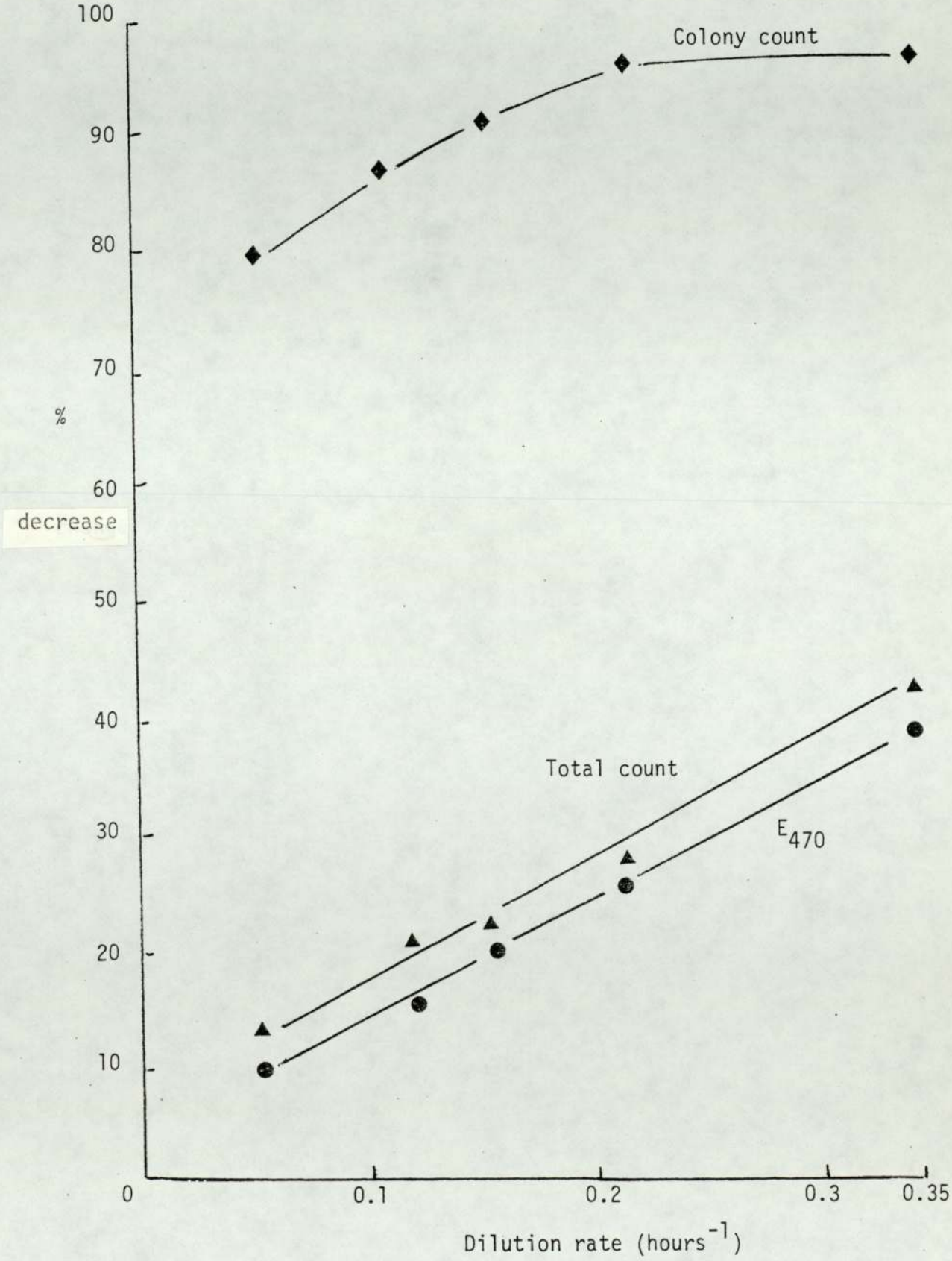


Fig. 71

The effect of dilution rate on E_{470}, total and colony count for chemostat grown PO_4^{3-} depleted *P. aeruginosa* treated with Polymyxin B (10 I.U. ml^{-1})



in C.D.M. lacking glucose but containing 2.5×10^{-4}M PO_4^{3-} to an E_{470} 0.2 as previously described. Samples were removed for E_{470}, total and colony counts before P.B. treatment. E_{470} values were determined at intervals over the 60 minute exposure period. After 60 minutes samples were removed for E_{470}, total and colony counts. Water was added to the control flasks.

Fig. 72 shows the change in E_{470} over the 60 minute exposure period as the dilution rate was increased. As the growth rate was increased so the rate of lysis was increased. A linear relationship over 60 minutes was shown at all dilution rates chosen. The fall in E_{470} over this 60 minute period was used to calculate the lysis rate constant at each dilution rate.

The effect on the lysis rate constant (K) of increasing dilution rate is shown in Fig. 73 . A linear relationship existed between the lysis rate constant and the dilution rate of a K value above 4.0×10^{-3} min^{-1} at a dilution rate of 0.3 hr^{-1}.

The change in colony count, total count and E_{470} with increasing dilution rate is shown in Fig. 74 . A linear relationship existed for E_{470}, colony and total count to a dilution rate of 0.25 hr^{-1} with corresponding changes during the 60 minutes drug exposure period of 37.0, 36.0, 61.0% respectively. Again good agreement was shown between the % change in total count and E_{470} over the 60 minute lysis period. As the growth rate of the cells increased they showed increasing sensitivity to 10 I.U. ml^{-1} P.B. as judged by changes in optical density, total and colony counts of the culture.

Fig. 72

The effect of dilution rate on the lysis by Polymyxin B (10 I.U. ml^{-1}) of chemostat grown glucose depleted *P. aeruginosa*

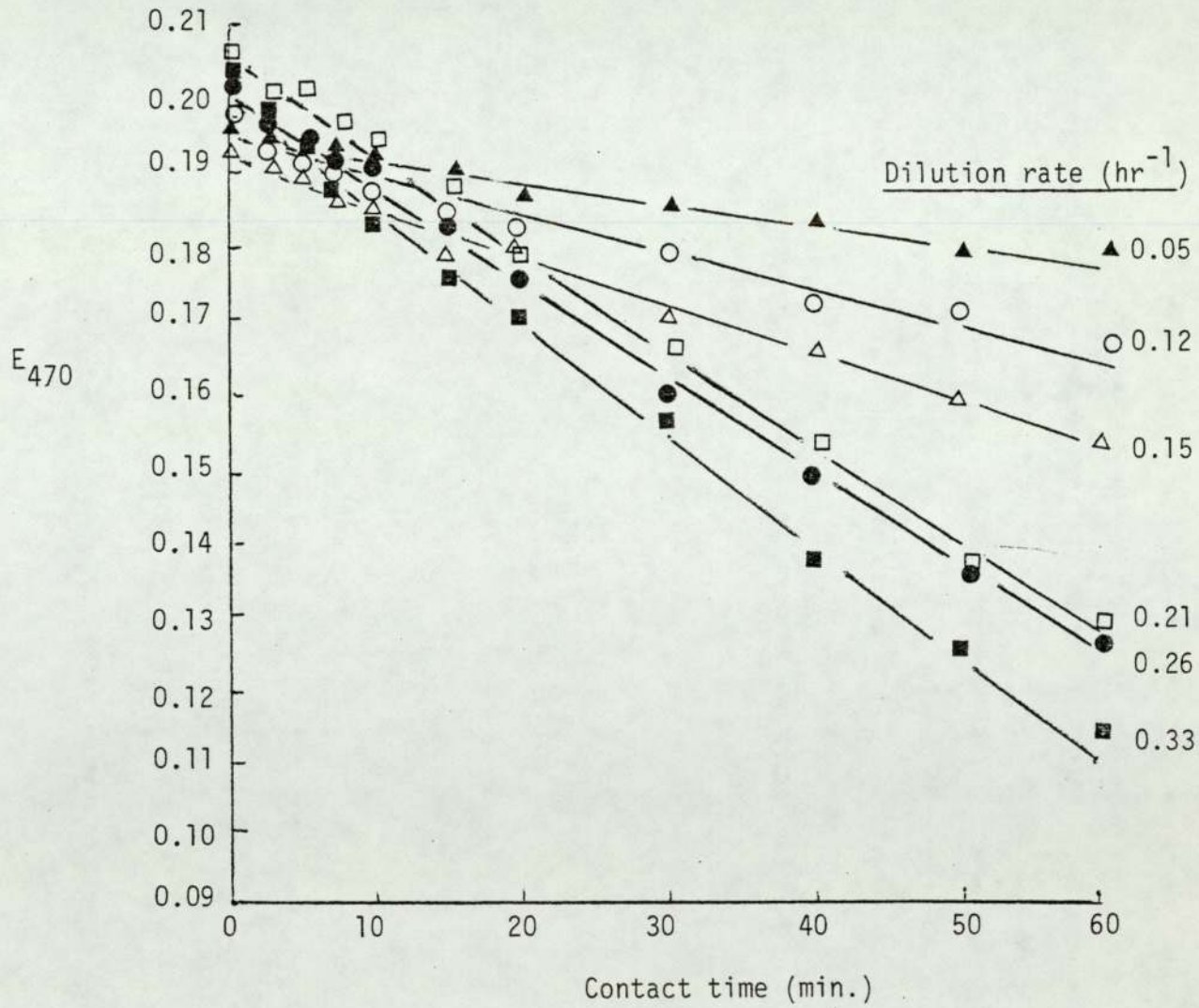


Fig. 73

The effect of dilution rate upon the lysis rate constant for chemostat grown glucose depleted *P. aeruginosa* treated with Polymyxin B (10 I.U. ml^{-1})

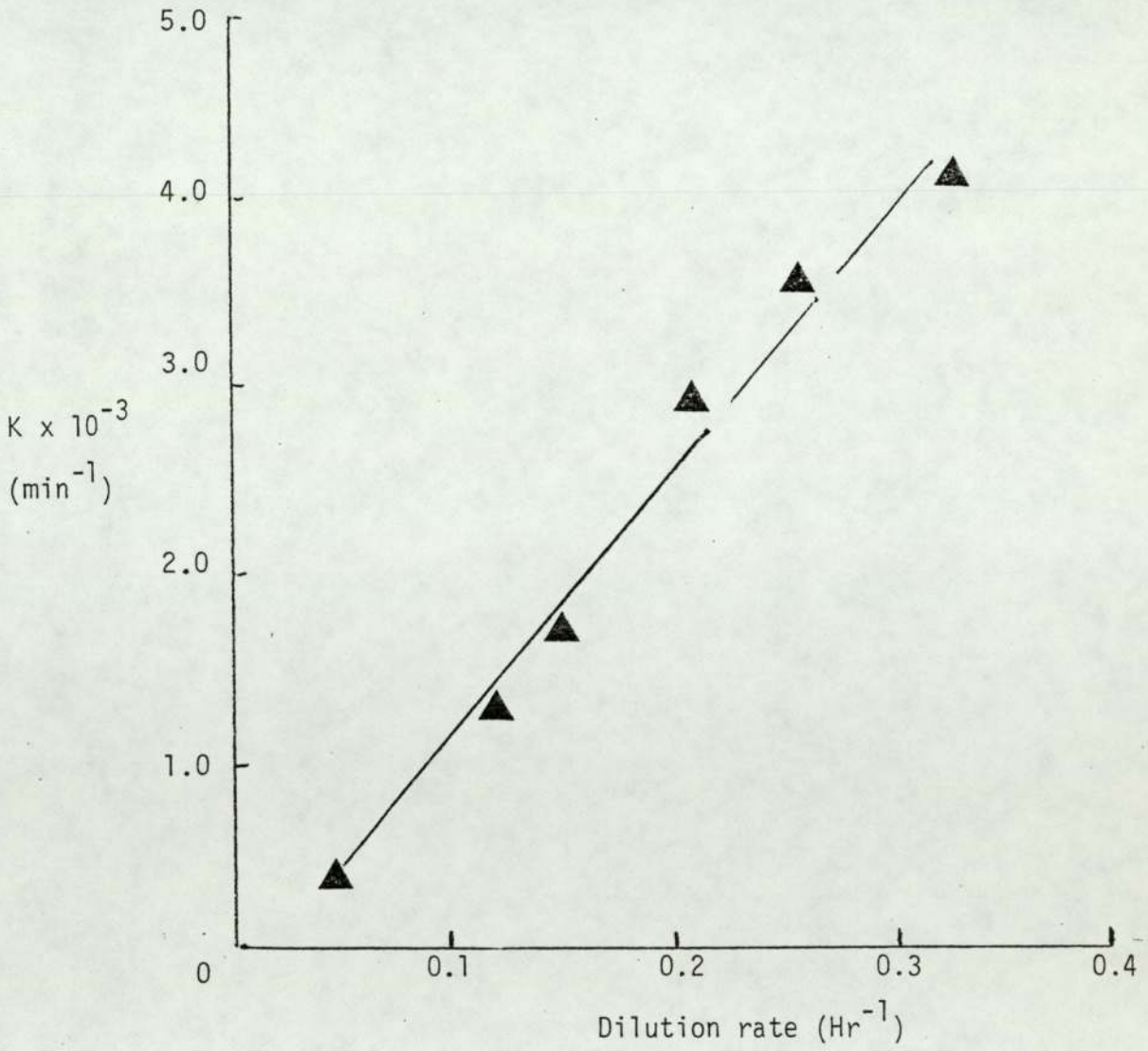
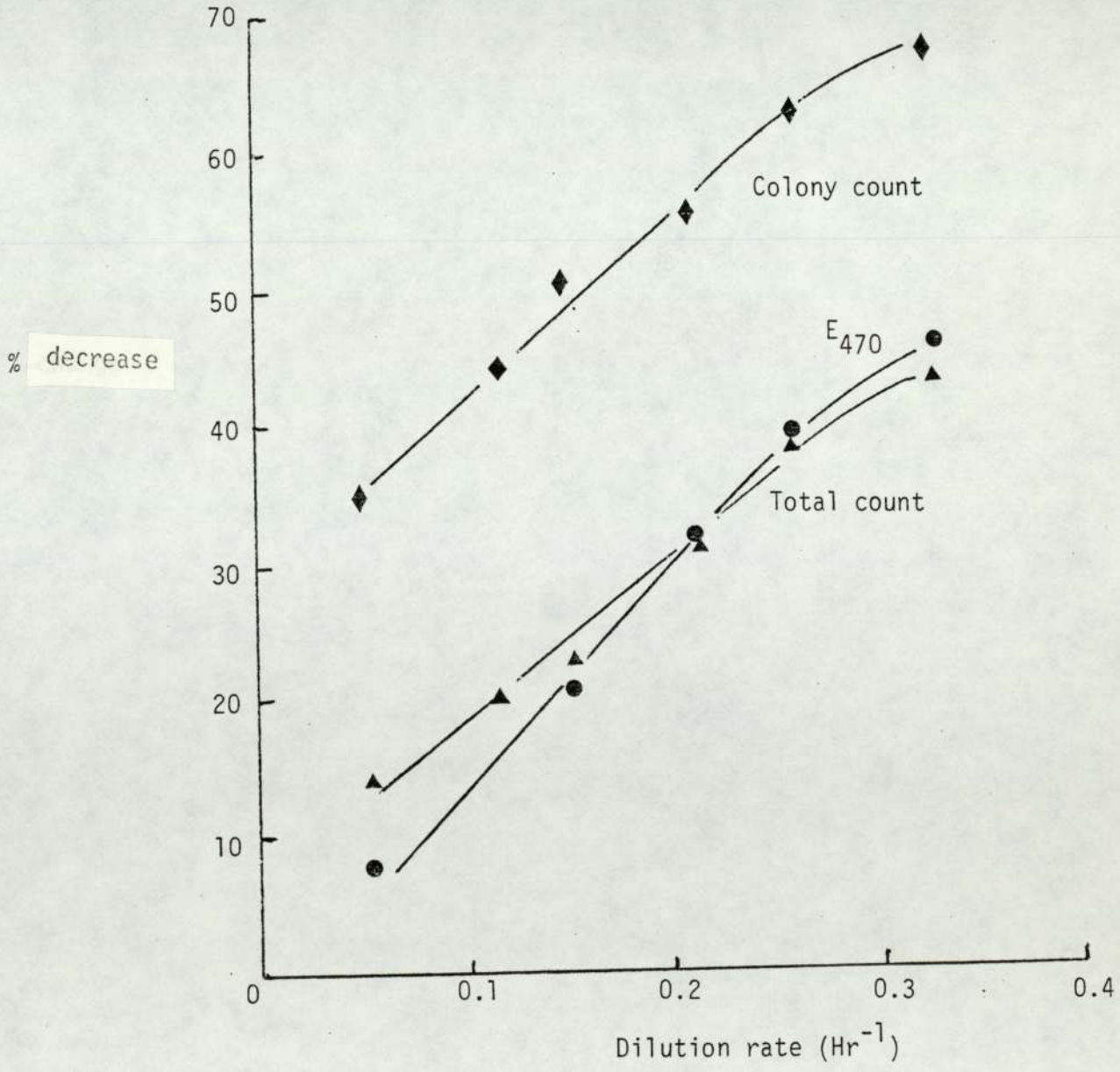


Fig. 74

The effect of dilution rate on E_{470}, total and colony count for chemostat grown glucose depleted *P. aeruginosa* treated with Polymyxin B (10 I.U. ml^{-1})



3.6. Resistance of Chemostat grown *P. aeruginosa* to E.D.T.A.

3.6.1. The effect of growth rate on the response of PO_4^{3-} limited *P. aeruginosa* to E.D.T.A.

The previous experiments reported for treatment of *P. aeruginosa* with P.B. were repeated treating the cells with 785µg ml^{-1} E.D.T.A. for 180 minutes at dilution rates from 0.07 to 0.60 hr^{-1}. In order that sufficient cells were available for resistance studies at the higher dilution rates the cells from identical chemostats were pooled. The E_{470} was determined initially and at various time intervals for the 180 minute exposure period. Total and colony counts were performed initially and after 180 minutes exposure to E.D.T.A.

The effect of increasing the dilution rate on the E_{470} during the 180 minute drug exposure period is shown in Fig. 75 . Increasing sensitivity to E.D.T.A. was shown with increasing dilution rate over the range tested. The lysis curves show three phases. Rapid lysis occurred between 0 to 30 minutes, with a slower lysis occurring between 30 and 90 minutes. Between 90 and 180 minutes little change was noted especially at the higher dilution rates. The relationship between the primary and secondary lysis rate constants and dilution rate is shown in Fig. 76 . The fall in E_{470} values in the first 30 minutes and between 30 and 90 minutes were used to calculate the primary and secondary lysis rate constants respectively. The primary rate constant showed a rapid increase over the range of dilution rates 0.07 to 0.17 hr^{-1} which was followed by a slower increase over the range 0.17 to 0.6 hr^{-1}. A linear relationship existed between secondary lysis rate constant and dilution rate over the range 0.07 to 0.6 hr^{-1}. The increase was less than that observed for the primary rate constant.

Dilution rate (hr^{-1})	
■	0.07
●	0.10
○	0.17
□	0.40
▽	0.50
▼	0.60

Fig. 75

The effect of dilution rate on the lysis by E.D.T.A. (785µg ml^{-1}) of chemostat grown PO_4^{3-} depleted P. aeruginosa

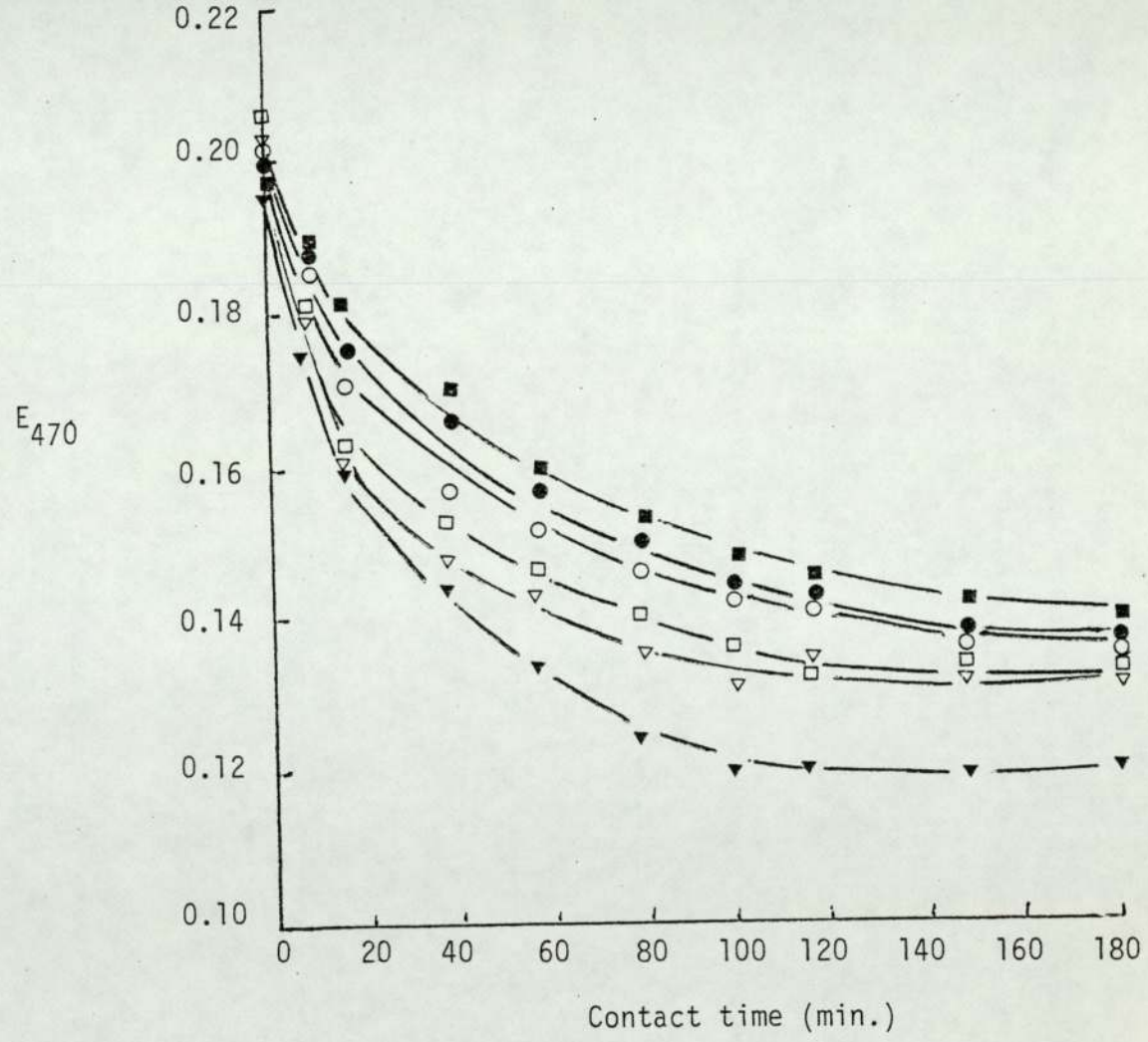


Fig. 76

The effect of dilution rate on primary and secondary lysis rate constants for chemostat grown PO_4^{3-} depleted P. aeruginosa treated with E.D.T.A. (785µg ml

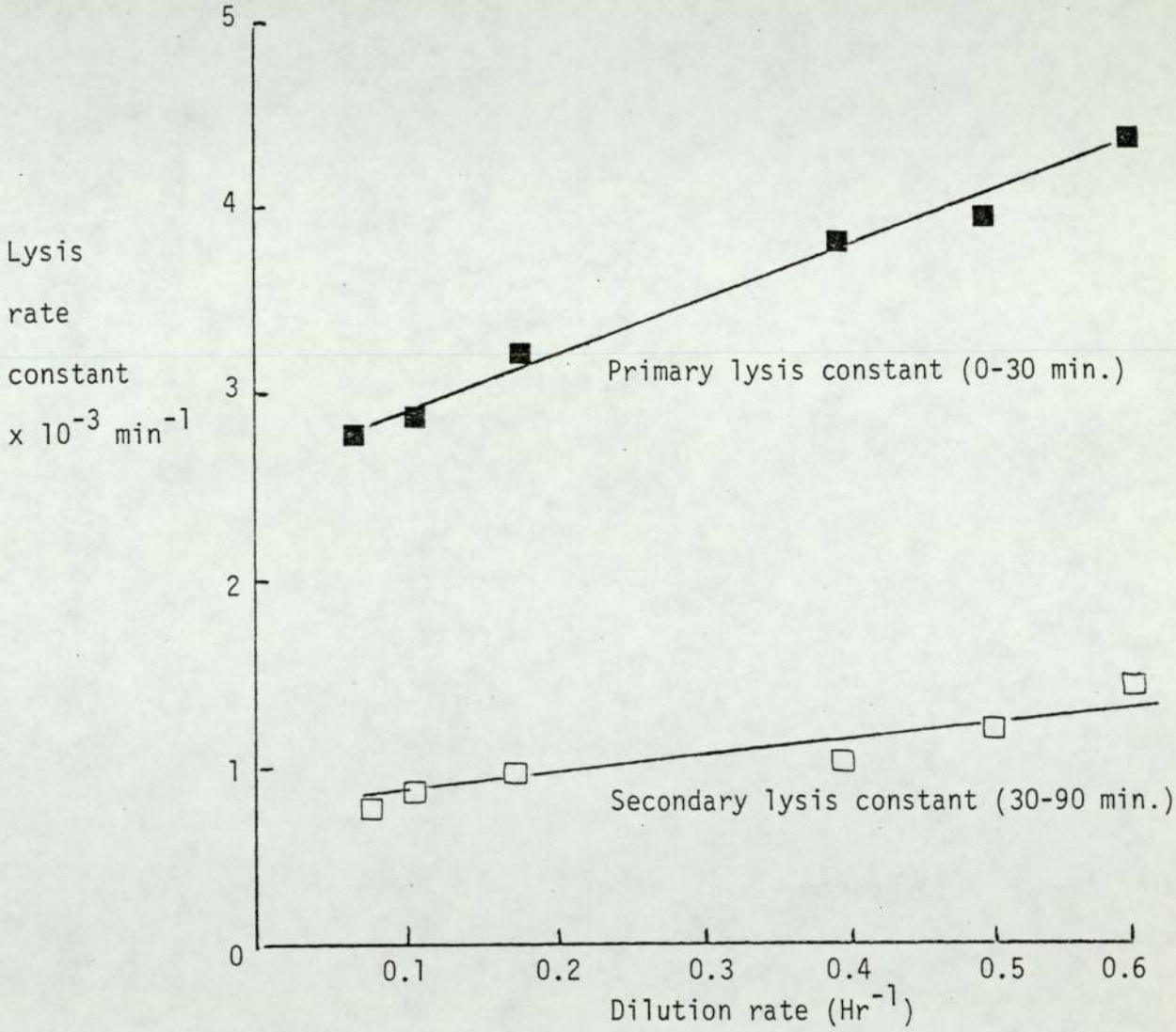


Fig. 77 shows the relationship between the dilution rate and % change in E_{470}, colony and total count over the 180 minute drug exposure period. Good agreement was shown between the change in these parameters as the dilution rate was increased from 0.07 to 0.175 hr^{-1}. At dilution rates above 0.2 hr^{-1} the % change in these three parameters was linear. The % change in colony count increased from 31 to 46.2% as the dilution rate increased from 0.2 to 0.6 hr^{-1}. The corresponding change in total count and E_{470} was from 28 to 42% and from 30 to 40% respectively.

3.6.2. The effect of growth rate on the response of glucose limited *P. aeruginosa* to E.D.T.A.

Glucose depleted cells grown at dilution rates 0.01 to 0.60 hr^{-1} were treated as described previously with 785 $\mu g\ ml^{-1}$ E.D.T.A. for 180 minutes. E_{470} total and colony count measurements were performed at intervals during the exposure period.

Fig. 78 shows the effect on the E_{470} of glucose limited cells of increasing the dilution rate in the range 0.05 hr^{-1} to 0.33 hr^{-1}. The lysis curves show two phases. Rapid lysis occurred from 0 to 30 minutes, but the rate was decreased from 30 to 180 minutes. The amount of lysis was dependent on the dilution rate, increased lysis occurring with increased dilution rate.

The relationship between the lysis rate constant (K) over the period 30 to 180 minutes and the dilution rate is shown in Fig. 79 . The lysis rate constant was calculated from the fall in E_{470} over the period 30 to 180 minutes. A linear relationship was evident between the lysis rate and dilution rate as the rate was increased to 0.24. As the dilution rate was increased the cells became more sensitive to the action of E.D.T.A. as shown in Fig. 80. Increasing sensitivity was shown in all three parameters colony count, E_{470} and total count.

Fig. 77

The effect of dilution rate on % change in E_{470}, total and colony count for chemostat grown PO_4^{3-} depleted *P. aeruginosa* treated with E.D.T.A. (785µg ml^{-1})

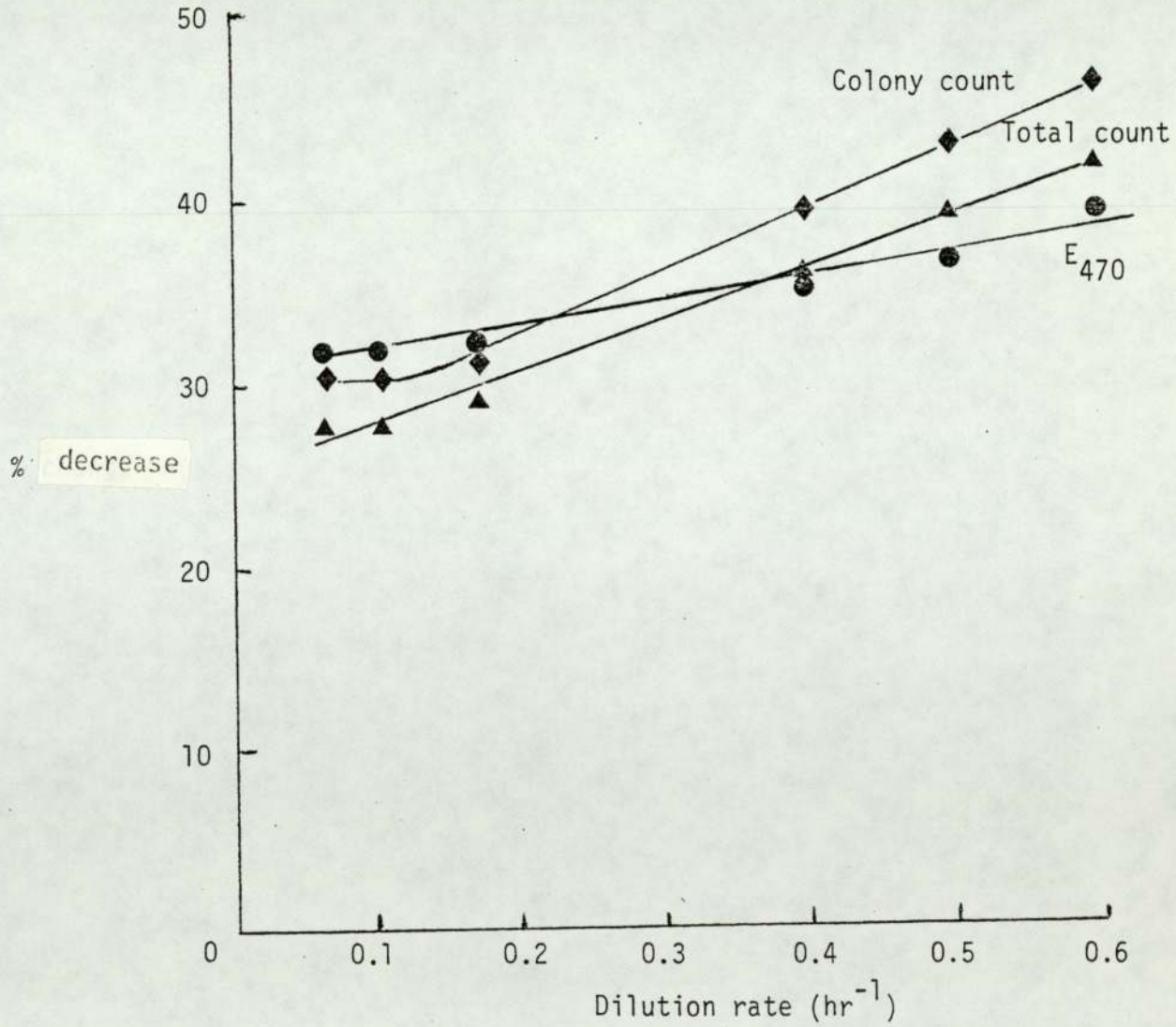


Fig. 78

The effect of dilution rate on the lysis by E.D.T.A. (785µg ml^{-1}) of chemostat grown glucose depleted *P. aeruginosa*

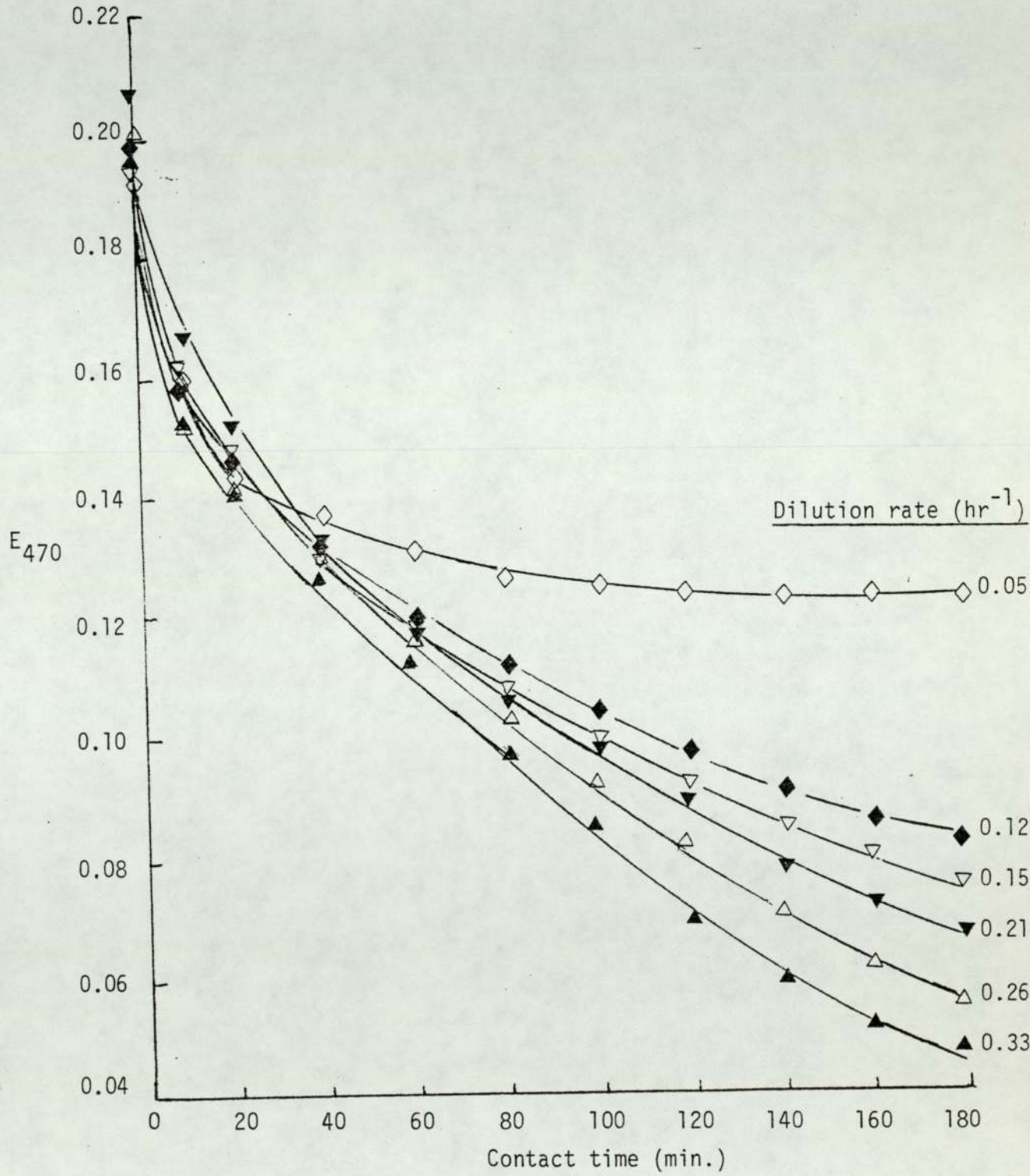


Fig. 79

The effect of dilution rate on lysis rate constant for chemostat grown glucose depleted *P. aeruginosa* treated with E.D.T.A. (785µg ml^{-1})

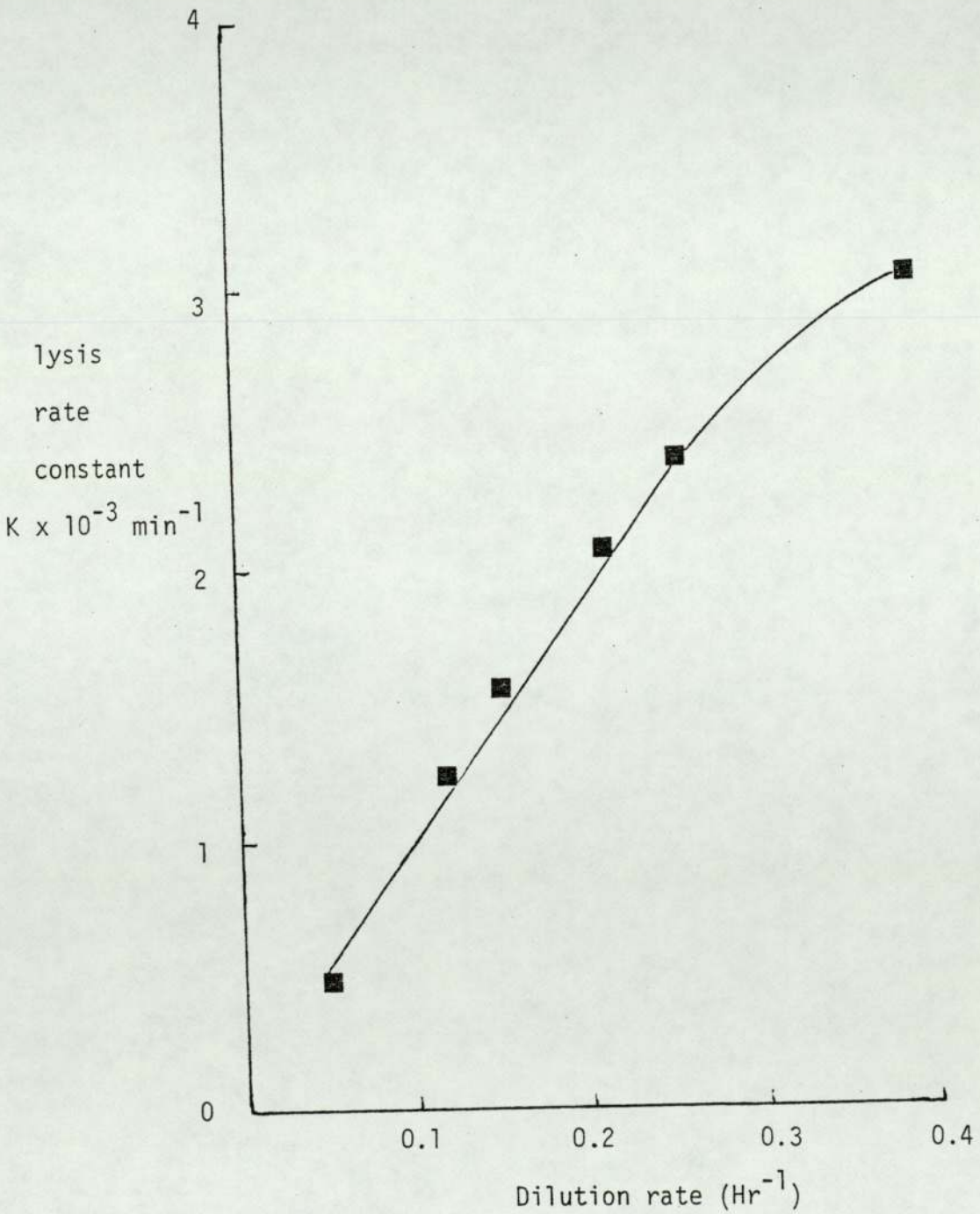
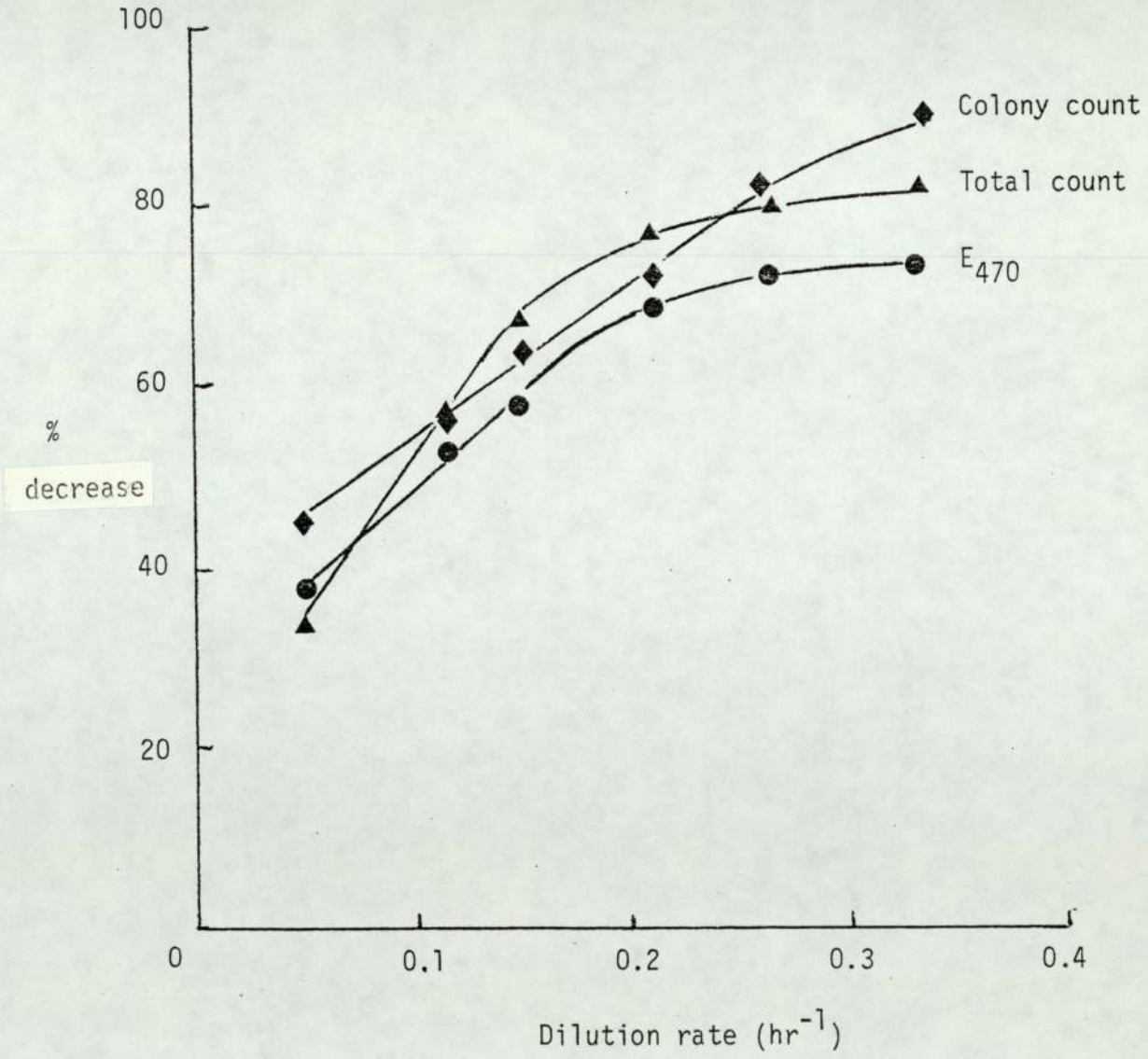


Fig. 80

The effect of dilution rate on E_{470}, total and colony count for chemostat grown glucose depleted P. aeruginosa treated with E.D.T.A. (785µg ml^{-1})



3.7. The effect of increasing phosphate on sensitivity to E.D.T.A. and Polymyxin B of chemostat grown P. aeruginosa

3.7.1. Practical procedure

As shown in an earlier section the resistance of batch grown P. aeruginosa to P.B. and E.D.T.A. is dependent upon the PO_4^{3-} concentration in the growth medium. Chemostat grown cells are subjected to much less variation in cultural conditions and thus their response to a particular drug may differ from that of batch grown cells even though the amount of limiting nutrient in the media used for both cultures was identical when growth commenced. Furthermore chemostat cells have stabilised for more generations at a particular growth rate.

In this section experiments were performed to determine the effect of increasing concentrations of PO_4^{3-} in the growth medium upon resistance to P.B. and E.D.T.A. The nature of growth in the chemostat does not permit the double limitation used previously in the batch cultures. However, C limited and PO_4^{3-} limited cells are obtainable and by manipulation of the PO_4^{3-} concentration should permit conditions closely resembling the batch conditions.

Pseudomonas aeruginosa was grown in PO_4^{3-} limited media and C limited media whose formulation has been previously described. The PO_4^{3-} concentration was adjusted between the amounts used under these two limitations. The flow rate was adjusted to a dilution rate of 0.17 hr^{-1} for all experiments.

Following 5 volume changes for each medium formulation a sample of the culture was removed and tested under batch culture conditions in a shaking flask to determine the limiting nutrient. The sample was divided into 3 and to one portion was added PO_4^{3-}. To a second portion was

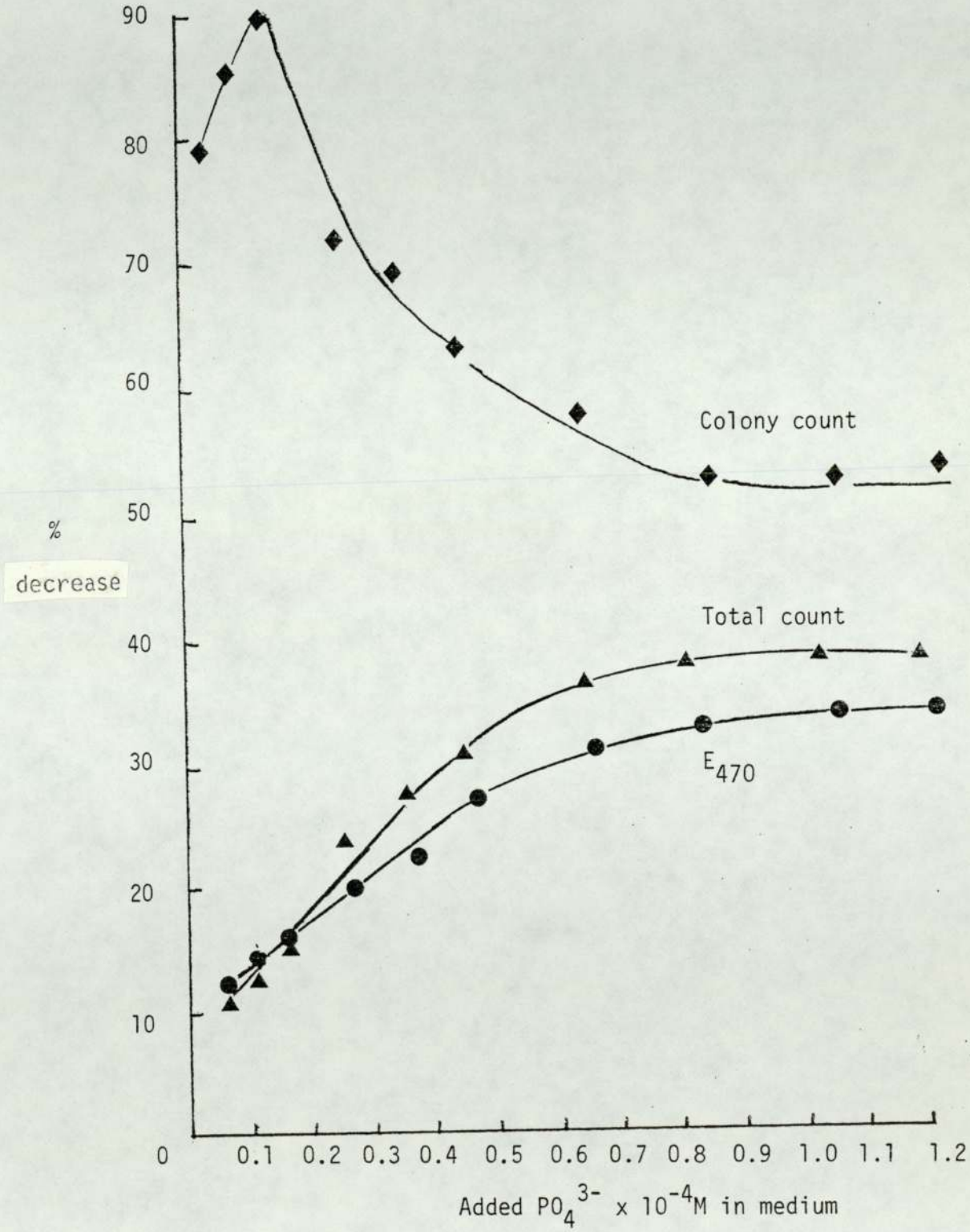
added glucose and to the third portion was added glucose and PO_4^{3-}. The change in E_{470} was measured every 30 minutes for 2-4 hours and any increase in E_{470} recorded. Following determination of the limiting nutrient samples were then removed and washed as described previously in C.D.M. containing PO_4^{3-} but lacking glucose. The cells were finally resuspended to an E_{470} 0.2 and equilibrated for one hour prior to treatment with P.B. (10 I.U. ml^{-1} and E.D.T.A. 75µg ml^{-1}). E_{470}, colony and total counts were repeated following 60 minutes treatment with P.B. and 180 minutes treatment with E.D.T.A.

3.7.2. The effect of treatment with 10 I.U. ml^{-1} P.B.

The result of treatment with P.B. are shown in Fig. 81 for a range of PO_4^{3-} concentrations from 0.07×10^{-4}M to 1.2×10^{-4}M. The results show a good correlation between E_{470} and total count. Linearity was maintained to a PO_4^{3-} concentration of 0.4×10^{-4}M PO_4^{3-} in the medium for total count and to 0.43×10^{-4}M for E_{470}. For both parameters a gradual decrease in % change occurred above 0.4 and 0.43×10^{-4}M. The maximum % change was 34% for E_{470} values at a PO_4^{3-} concentration of 1.0×10^{-4}M. The maximum for total count was 37% at a concentration of 0.9×10^{-4}M PO_4^{3-}. The change in colony count showed poor correlation with the change in total count and E_{470}. As seen with the batch culture results for P.B. treated cells the % change in colony count was considerably higher than the change in the other two parameters. A linear increase in sensitivity was seen for colony counts over a PO_4^{3-} concentration range of 0.07 to 0.2×10^{-4}M. The corresponding % change in colony count increased from 78% to 90%. At PO_4^{3-} concentrations above 0.2×10^{-4}M increased resistance was seen and the % change in colony count fell from 90% to a minimum of 53% corresponding to a PO_4^{3-} concentration of 0.9×10^{-4}M.

Fig. 81

The effect of PO_4^{3-} concentration in the medium upon the % change in E_{470} total and colony count for chemostat grown (D = 0.17 hr^{-1}) P. aeruginosa treated with Polymyxin B (10 I.U. ml^{-1})

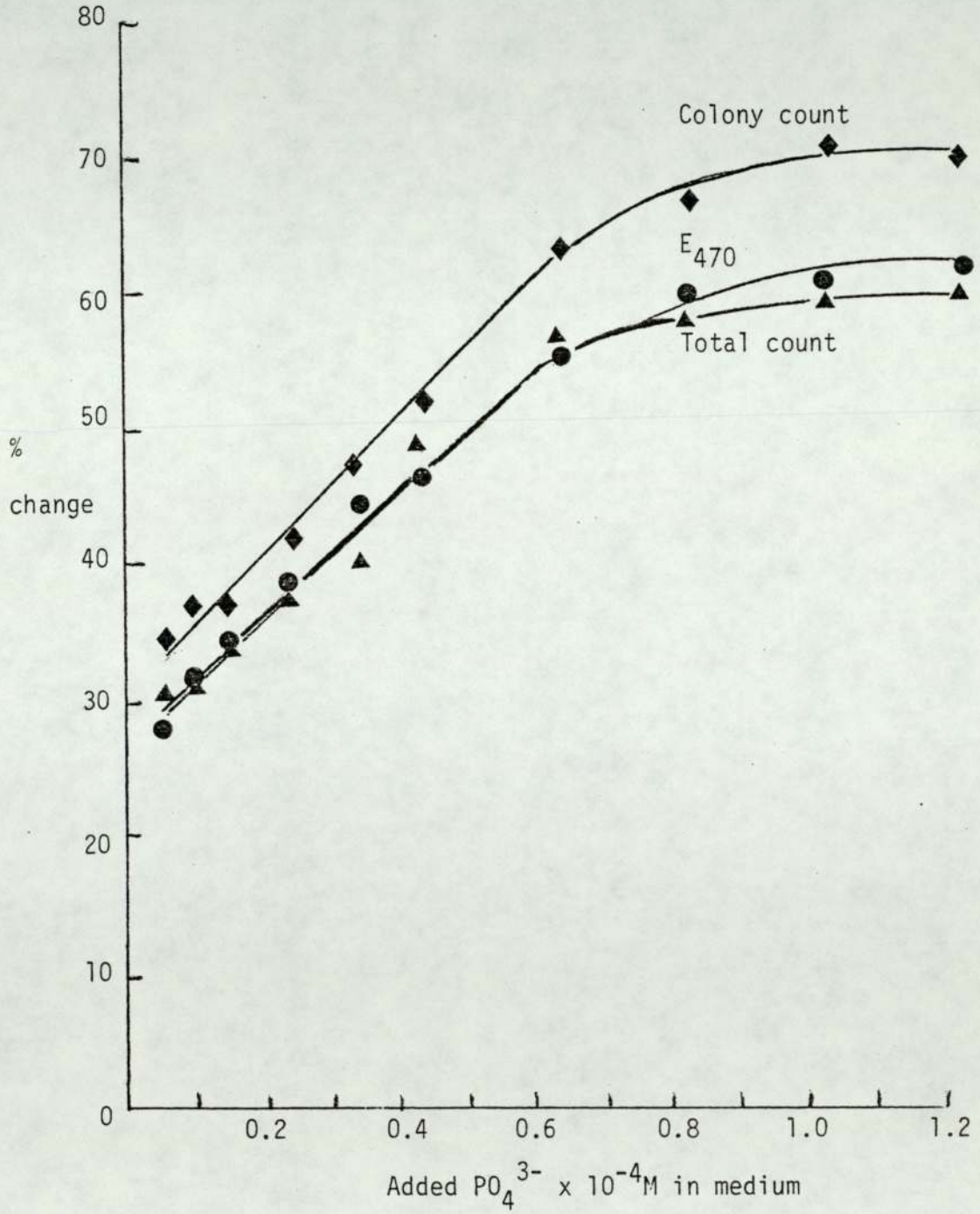


3.7.3. The effect of treatment with 785µg ml^{-1} E.D.T.A.

The changes in the three parameters under test for treatment with 785µg ml^{-1} E.D.T.A. are shown in Fig. 82 . Good agreement was obtained for the changes in all three. A linear increase in sensitivity occurred with increasing PO_4^{3-} in the culture medium to 0.65, 0.58 and 0.55 x 10^{-4}M PO_4^{3-} for colony count, total count and E_{470} respectively. The maximum % change for colony count was 69% at a PO_4^{3-} concentration of 1.0 x 10^{-4}M. The maximum values for total count and E_{470} were 57% at 1.0 x 10^{-4}M PO_4^{3-} and 59% at 1.05 x 10^{-4}M respectively. As with P.B. treated cultures the colony count showed the maximum change for any particular PO_4^{3-} concentration.

Fig. 82

The effect of PO_4^{3-} concentration in the medium upon the % change in E_{470} total and colony count for chemostat grown (D = 0.17 hr^{-1}) P.aeruginosa treated with E.D.T.A. (785µg ml^{-1})



3.8. Chemistry of cell walls and inner and outer membranes of P. aeruginosa grown in chemically defined media containing various amounts of phosphate

3.8.1. Introduction

Batch and chemostat cells were grown in media containing various amounts of PO_4^{3-} as shown in Table 38. For each PO_4^{3-} concentration duplicate sets of walls and membranes were prepared. The wall and inner and outer membrane fractions were prepared as described in sections The assays were performed in triplicate, except for lipid analyses, which were performed in duplicate. There was insufficient material to perform more than one lipid assay per sample of membrane. All assays were performed upon outer membranes except the cation assay where cell wall cation content only was performed. K.D.O. assays were performed on cell wall and outer membrane fractions. Owing to insufficient inner membrane material only protein and lipid assays were performed on these fractions There was insufficient material for an accurate determination of the quantitative composition of individual P.L., although T.L.C. was performed on inner and outer membrane fractions to identify individual lipids present.

The results are expressed as % dry weight of the sample assayed. For convenience the cultures were numbered 1-5 for batch grown cells and C^- and P^- for carbon and phosphate depleted chemostat cultures respectively.

Table 38

Phosphate composition of media used to grow cells for chemical analyses

Culture	Phosphate content x 10^{-4}M
1	0.80
2	1.33
3	2.00
4	2.67
5	6.68
C^-	40.00
P^-	1.50

This designation was used in the following Tables of results. The a and b subscripts denote replicate cultures.

[1] Assays performed on outer membrane fractions unless otherwise stated.

c.w. cell wall fraction assayed.

* Replicate fractions pooled for assay.

** L.P.S. in outer membrane fraction calculated using factor K.D.O. = 3.2% L.P.S. for strain 6750 (Wilkinson and Galbraith, 197

+ L.P.S. in wall calculated as for O.M.

Table 39

Chemistry[1] of batch cultures of P. aeruginosa grown in chemically defined media containing various amounts of phosphate

Assay \ Culture	1		2		3		4		5	
	a	b	a	b	a	b	a	b	a	b
Protein	52.2	56.7	54.7	55.2	54.2	54.8	52.3	54.1	53.2	51.8
P (Total)*	12.1		20.3		19.8		22.4		22.3	
R.E.L.P.*	2.4		8.1		8.9		10.0		12.6	
K.D.O.	0.58	0.65	0.71	0.73	0.69	0.72	0.73	0.74	0.72	0.74
L.P.S.**	18.1	20.3	22.2	22.8	21.6	22.5	22.8	23.1	22.5	23.1
K.D.O. (c.w.)	0.74	0.78	0.88	0.72	0.86	0.88	0.97	0.96	0.98	0.92
L.P.S.+	23.1	24.4	27.5	22.5	26.9	27.5	30.3	30.0	30.6	28.8
R.E.L.	38.2	36.0	37.2	36.5	32.8	34.2	33.1	32.4	32.2	33.4
P.L.	4.1	7.2	14.3	10.0	14.0	19.0	17.0	21.2	26.4	22.6
F.A.N.	32.1	28.0	20.6	25.2	16.7	14.0	13.9	10.2	8.0	7.6
Mg^{2+} (c.w.)	0.270	0.274	0.321	0.272	0.312	0.283	0.327	0.361	0.368	0.342
Ca^{2+} (c.w.)	0.243	0.253	0.257	0.247	0.252	0.264	0.264	0.258	0.267	0.263

Table 40

Chemistry of inner membranes from batch cultures of *P. aeruginosa* grown in chemically defined media containing various amounts of phosphate

Culture / Assay	1 a	1 b	2 a	2 b	3 a	3 b	4 a	4 b	5 a	5 b
Protein*	48.2		49.6		44.0		48.4		49.7	
R.E.L.*	56.4		56.8		60.2		54.6		58.0	
P.L.*	16.1		22.8		26.1		26.9		28.3	
F.A.N.*	38.1		30.8		30.7		31.0		30.2	

* Replicate fractions pooled for assay.

Table 41

Chemistry[1] of chemostat cultures of P. aeruginosa grown under phosphate or carbon limited conditions

Assay \ Culture	C^-		P^-	
Protein	55.4	53.7	56.2	55.1
P (Total)*	25.9		15.0	
R.E.L.P.*	16.5		6.7	
K.D.O.	0.58	0.57	0.48	0.44
L.P.S.**	18.1	17.8	15.0	13.8
K.D.O. (c.w.)	0.84	0.88	0.76	0.72
L.P.S.$^+$	26.3	27.5	23.8	22.5
R.E.L.	34.4	34.6	38.6	39.5
P.L.	28.0	24.7	11.2	14.6
F.A.N.	5.7	9.9	28.1	24.0
Mg^{2+} (c.w.)	0.402	0.397	0.342	0.368
Ca^{2+} (c.w.)	0.372	0.366	0.342	0.361

see Table 39 for details of symbols used

Table 42

Chemistry of inner membranes from chemostat cultures of *P. aeruginosa* grown under phosphate or carbon limited conditions

Assay \ Culture	C^-	P^-
Protein*	44.8	40.4
R.E.L.*	48.4	36.1
P.L.*	21.3	6.8
F.A.N.*	24.2	24.8

* Replicate fractions pooled for assay.

3.8.2. Changes in cell wall and outer membrane chemistry associated with growth in various levels of phosphate

Tables 39 and 41 show that proteins represented approximately 50% of the O.M. of both batch and chemostat grown cells. Growth in various levels of PO_4^{3-} produced little or no change in the quantitative protein composition of the O.M. fractions. C^-P^+ batch grown cells had an average O.M. protein content of 52.5% compared to a composition of 54.4% for P^-C^+ cells (Table 39). Carbon limited chemostat grown cells contained an average of 54.6% compared to 55.7% for P-limited cells (Table 41).

A reduction of PO_4^{3-} in the growth media resulted in a reduction to a variable extent in the total P and the amount of P containing O.M. components. The total P fell from 22.3% to 12.1% for C^-P^+ and P^-C^+ grown cells respectively (Table 39) whilst a decrease from 25.9% to 15.0% was evident for C- and P-limited chemostat grown cells respectively (Table 41).

The fall in total P resulted in a fall in K.D.O. and P.L. content. The O.M. K.D.O. fell 16.4% when C^-P^+ and C^+P^- cells were compared and a fall of 20% occurred in wall K.D.O. values (Table 39). A fall in O.M. K.D.O. of 20% and in cell wall K.D.O. of 14% resulted on changing for C-limited to P-limited chemostat cultures (Table 41).

The major portion of the fall in total P appeared to be due to a fall in P.L. content. A small fall in R.E.L. (11.6%) for batch grown cells was noted (Table 39) when C^-P^+ and C^+P^- cells were compared. Carbon limited chemostat grown cells, however, showed less R.E.L. (average 34.5%) than P-limited cells (average 39%) (Table 41). The changes in R.E.L. resulted from large changes in P.L. and F.A.N. composition as the PO_4^{3-}

content of the growth medium was reduced. P^-C^+ cells contained 5.6% P.L. compared with the 24.5% P.L. content of C^-P^+ cells. Accompanying this fall in P.L. content was an increase in the F.A.N. content of the O.M. from 7.8% for C^-P^+ cells to 30% for C^-P^+ cells (Table 39). Similar changes were seen when P-limited and C-limited chemostat cultures were compared. Outer membrane fractions from P-limited cells contained 12.9% P.L. compared with the 26.3% P.L. content of the O.M. from C-limited chemostat grown cells. A compensatory increase in F.A.N. accompanied this fall in P.L., P-limited cultures containing 26% F.A.N. compared to 7.8% for C-limited cells (Table 41).

Decreases in cell wall Mg^{2+} and Ca^{2+} content of 23.4% and 6.4% respectively were demonstrated as the PO_4^{3-} content of the medium was reduced (Table 39). A change from C-limited to P-limited growth resulted in falls of 11% for wall Mg^{2+} content and 4.6% for wall Ca^{2+}content (Table 41).

3.8.2.1. Qualitative changes in O.M. protein associated with growth in various levels of phosphate.

The O.M. protein profiles of the five batch grown cultures and C- and P-limited chemostat grown cells are shown in Plate 2. For comparative purposes an O.M. preparation from P. aeruginosa PAO1 grown in nutrient broth and molecular weight standards were included in the gels. The designation of the tracks is as shown below (Table 43).

Proteins of equivalent molecular weight to D1, D2, E, F and I of P. aeruginosa PAO1 were seen in strain 6750. Proteins D1 and D2 were resolved into 2 bands under all growth conditions although this less clearly seen on the photograph than on the gel. The porin protein D1 was present in high concentration under all growth conditions.

Plate 2

S.D.S. polyacrylamide gel electrophoresis of outer membrane proteins of P. aeruginosa

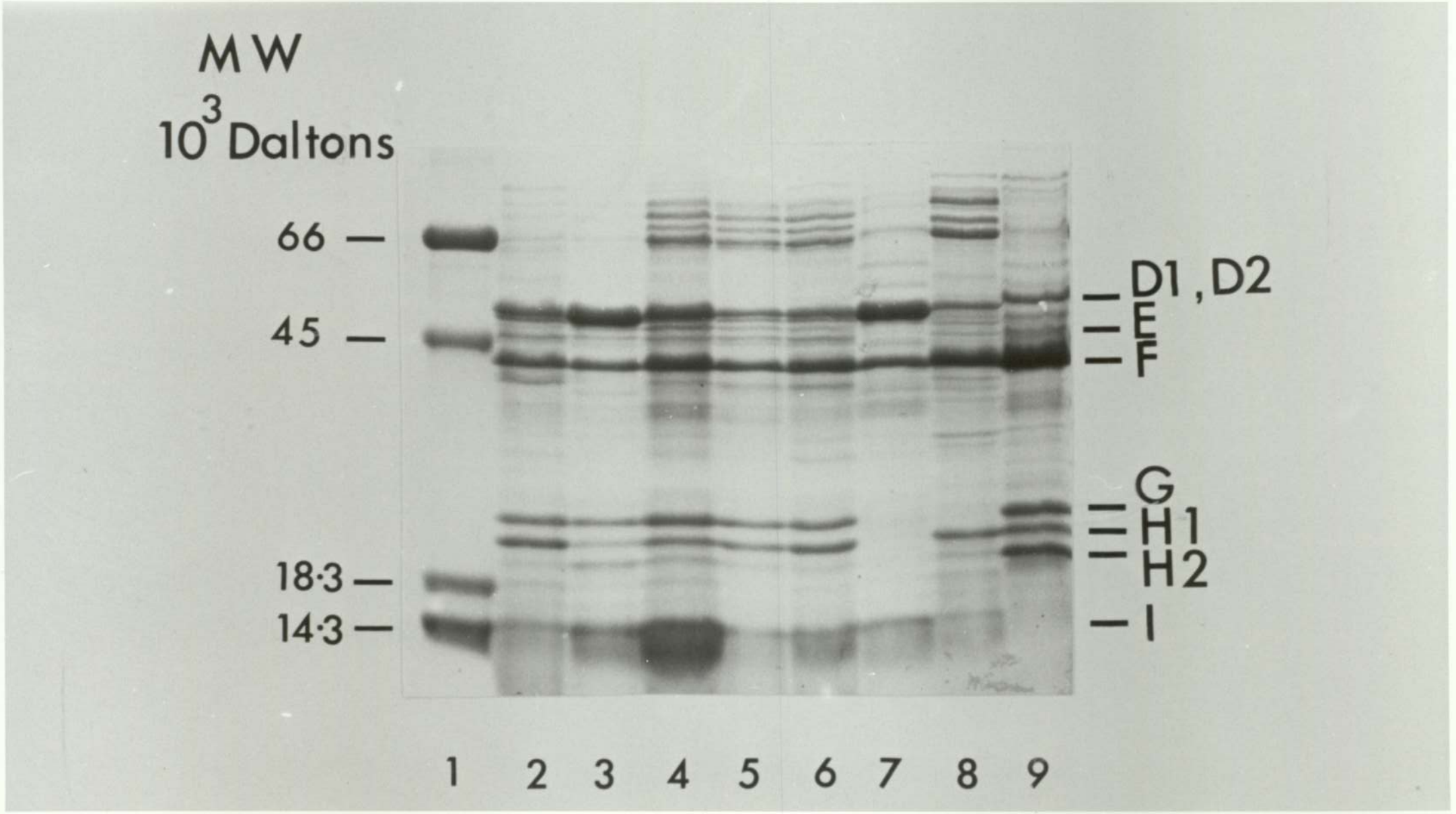

MW
10³ Daltons
66
45
18·3
14·3
D1, D2
E
F
G
H1
H2
I
1 2 3 4 5 6 7 8 9

Table 43

Designation of S.D.S. P.A.G.E. tracks

Track	Culture	C.D.M. PO_4^{3-} content x 10^{-4}M
1	M.wt. standard	-
2	1	0.80
3	2	1.33
4	3	2.00
5	4	2.61
6	5	6.68
7	P^-	1.50
8	C^-	40.00
9	PA01	nutrient broth

The major porin F was present under all growth conditions. Proteins G, H1 and H2 were barely detectable in chemostat grown cells under P-limited conditions (track 7). In chemostat grown cells under C-limited conditions G and H2 were also virtually undetectable whereas H1 was present in relatively large amounts (track 8). Proteins G, H1 and H2 were seen in all batch cultures. A protein cluster of high molecular weight (65-80K) was present in varying amounts under all growth conditions.

3.8.3. Changes in the inner membrane chemistry associated with growth in various levels of phosphate

The inner membrane fraction contained slightly less protein than the O.M. fractions. The I.M. of batch grown cells contained approximately 48% protein whilst that of chemostat grown cells contained approximately 42% protein (Tables 40 and 42). As demonstrated in the O.M. fractions

PO_4^{3-} changes in the growth medium produced no significant quantitative change in the I.M. protein composition of batch or chemostat grown cells.

No significant changes was seen in the R.E.L. composition of the I.M. of batch grown cells (Table 40), although the P-limited chemostat grown cells showed only 36.1% R.E.L. compared with 48.4% for C-limited cells. Batch grown cells showed a fall in the P.L. content of the I.M. as the PO_4^{3-} content of the medium was reduced. P^-C^+ cells contained 43% less P.L. than the C^-P^+ cells (table 40). However an approximately 68% difference was seen between C-limited and P-limited chemostat grown cells (Table 42). The F.A.N. content of chemostat grown cells revealed no significant change however. The F.A.N. content of P^-C^+ cells was 26.4% higher than that of C^-P^+ cells (Table 40). The interpretation of the differing response in batch and chemostat grown cells is difficult. It seems likely an error occurred in the estimation of the P.L. content of the I.M. of P-limited chemostat grown cells since addition of the figures for F.A.N. and P.L. do not equate to the R.E.L. content of the I.M. Some material may have been lost during the separation of the lipid fractions. There was insufficient material for a repeat assay to be performed.

4. DISCUSSION

4.1. Formulation of a chemically defined medium for P. aeruginosa

4.1.1. Introduction

"Pseudomonas aeruginosa is an organism well adapted to survive an environment selective because of antibiotics. Biologically active to an extreme degree it can live on simple substrates, survive for long periods under a wide range of environmental conditions apart from severe dehydration, and adapt to the presence of high concentrations of most antibiotics and antibacterial substances" (Gould, 1963). It is because P. aeruginosa possesses these characteristics and has been associated with infections due to contaminated disinfectants (Ayliffe et al, 1965), ophthalmic preparations (Theodore, 1951) and non-sterile preparations (P.H.L.S. Report, 1971) that it is now one of the recommended organisms for most tests upon the effectiveness of antimicrobial agents. Such tests include the modified Kelsey Sykes Test (Kelsey and Maurer, 1974), and the test for efficacy of preservatives (British Pharmacopoeia, 1980 and United States Pharmacopoeia, 1980). The two Pharmacopoeia also require tests to be performed which specifically detect this organism in pharmaceutical preparations.

Phenotypic variation amongst bacteria has been well recognised although it is only recently that the critical role of the growth medium has been appreciated (Brown and Melling, 1969a, b, and Ellwood and Tempest, 1972). Such variation has been shown to affect the response of organisms to antibiotics (Melling and Brown, 1975) and disinfectants (Ismail et al, 1977).

Since most bacteria are probably nutrient depleted in their natural ecosystems and when faced with a specific nutrient depletion the growing bacterium will produce an envelope characteristic of that depletion

(Brown, 1977) it seems reasonable to study the response of bacteria to inimical agents under such depleted conditions.

Poor reproducibility of test systems for disinfectants and antibiotics has resulted in research into methods of improving the situation and modification of such tests as the Kelsey-Sykes Test have resulted in the use of a chemically defined media (Kelsey and Maurer, 1974). However, in many of these tests the limitation if any of their inoculum is still not considered. Chemically rich media are still recommended for the Pharmacopoeia tests (British Pharmacopoeia, 1980 and United States Pharmacopoeia, 1980), and for antibiotic susceptibility testing overnight inocula from nutrient broth are still used. It is probable that during growth in a rich medium that the cell experiences a series of nutrient limitations resulting in diauxic growth and such limitations may modify their subsequent response to antimicrobial challenge.

If defined conditions for subsequent testing of antimicrobial activity or for studies including genetic and physiological work are to be produced a chemically defined medium as well as a defined test system is essential. The aim of this project was to define such a medium for P. aeruginosa which could be used by workers as a defined medium without limitation or as a medium in which one or more different nutrients may be limited with a fairly controlled excess of other constituents.

The nutritional requirements of many of the widely used research bacteria are not known. The nutritional requirements of S. typhimurium LT2 and E. coli B, B/r and K12 have recently been investigated by Neidhardt et al (1974) and for E. coli with or without the RP1 resistance plasmid by Klemperer et al (1979b). In the former paper, however, compete nutritional requirements were not determined.

4.1.2. Choice of buffer

The choice of buffer is central to the formulation of a synthetic medium. The possible requirement to produce a high cell density for subsequent analyses makes heavy demands on the buffering capacity of the medium and may frequently mean that certain ions, notably Fe^{2+} and Fe^{3+}, are near their solubility limits at neutral pH. Phosphate buffers have been widely used because of their favourable pK and because phosphate is a metal chelator providing a reservoir of metal ions and trace elements. Such a chemically defined medium was used by Boggis (1971) for use with P. aeruginosa. However, the use of phosphate buffer required media reformulation and the use of an alternative buffer when phosphate limitation studies are to be performed. Boggis (1971) reformulated his media to contain EMTA (3,6 endomethylene-1,2,3,6-tetrahydrophthallic acid). Tris (tris(hydroxymethyl) amino methane) has been used as an alternative. Evidence suggests that Tris is not a physiologically inert buffer and may affect the permeability of the cell wall (Voss, 1967). Tris was more effective than other buffers (e.g. phosphate buffer) in promoting the bactericidal activity of EDTA and HAPS (2 hydroxy 3 (dimethyl hexadecylammonio)-propane-1 sulphonate) for a range of Gram-negatives. Tris alone or in combination with EDTA causes release of UV absorbing material following short exposure (Neu et al, 1966).

At the beginning of this study MOPS (3-N-morpholinopropane sulphonic acid) was being used for studies with E. coli (Ismail, 1977) and Pr. mirabilis (Al-Dujaili, 1979). The use of this buffer has been reported by Neidhardt et al (1974). MOPS is one of a series of organic buffers devised by Good et al (1966) and possesses many of the requirements of a buffer quoted by these authors. Fig. 17 shows that MOPS has a useful buffering range of 6.5 - 7.8 and a pK 7.2 at $20^{o}C$.

Concentrations up to 80 x 10^{-3}M was not inhibitory to E. coli or S. typhimurium (Neidhardt et al, 1974). However, although a MOPS concentration of 75 x 10^{-3}M did not affect the yield of glucose limited E. coli the growth rate was reduced by 10% but below 50 x 10^{-3}M no detectable effect on either parameter was seen (Klemperer et al, 1979b). Table 19 shows that when tested at 30, 60 and 120 x 10^{-3}M in C.D.M. low in PO_4^{3-} (0.75 x 10^{-4}M) or high in PO_4^{3-} (7.0 x 10^{-4}M) no detectable effect on the three parameters lag period, doubling time or E_{470} onset was seen. MOPS at a concentration of 60mM permitted growth in glucose depleted C.D.M. to an E_{470} of 3.8 and in PO_4^{3-} depleted C.D.M. to an E_{470} of above 3.0 with a fall of only 0.3 and 0.4 pH units respectively (Figs.18 &19). Since under normal test conditions E_{470} values of <1.0 would be used 60 x 10^{-3}M MOPS showed adequate buffering capacity.

The use of 60 x 10^{-3}M MOPS and an initial pH of 7.6 would result in a final culture pH of 7.2 - 7.3. For lysis studies the initial pH was adjusted to 7.4 which resulted in a final pH of 7.3 - 7.4 at an E_{470} of 0.2. Polymyxin B lysis of P. aeruginosa was only slightly affected over the pH range 6.3 - 7.3 but lysis by EDTA increased rapidly as the pH rose above 7.3 (Boggis, 1971). Culture pH values of 7.2 were used by Neidhardt et al (1974) and Klemperer et al (1979b).

Since MOPS contains carbon, nitrogen and sulphur the possibility that P. aeruginosa could utilise the buffer as a C, N or S source under conditions where these elements were limiting was tested. E. coli W3110 could not utilise MOPS as a source of N or S (Klemperer et al, 1979b). S. typhimurium could not utilise MOPS as a C, N or S source but E. coli B whilst not using MOPS as C or N source showed diauxic growth under SO_4^{2-} limiting conditions (Neidhardt et al, 1974). MOPS was only utilised by E. coli B when the SO_4^{2-} source had been consumed thus making MOPS an

inappropriate buffer for SO_4^{2-} limitation studies. Figs. 20 and 21 show that P. aeruginosa could not utilise MOPS as a C or N source however, the high E_{470} values obtained under SO_4^{2-} depletion (Table 20) suggested that MOPS was utilisable as an SO_4^{2-} source, although no diauxic growth was detected. Replacement of the MOPS buffer by acid ammonium phosphate buffer resulted in lower E_{470} onset and maximum values also indicating that P. aeruginosa could utilise MOPS as a SO_4^{2-} source

The breakage of C-S bonds and the utilisation of the liberated S as a growth source is not common. No reference could be found to such a cleavage. The utilisation by bacteria of similar compounds such as phosphonates has been recorded although the cleavage of the C-P bond does not appear common (Tamari et al, 1977). These authors showed that E. coli TM1 could utilise 2-aminoethyl phosphonic acid but scarcely 1-aminoethyl phosphonic acid as a P-source. The culture of sewage organisms on 13 alkyl phosphonates yielded several strains of Pseudomonas capable of utilising these phosphonates as a P-source (Cook et al, 1978).

An additional advantage with MOPS is that it is a poor ion chelator and thus unlike phosphate buffer its use will not introduce extraneous metal ion contamination to the medium.

The nutritional requirements of P. aeruginosa will be detailed later but the reasons for the choice of the salts will be described here. At the beginning of this study only N, P and C limitations were to be investigated and so Mg^{2+}, Fe^{2+} and NH_4^+ was added to C.D.M. as their sulphate salts. A later decision to quantify all the requirements of P. aeruginosa meant that reformulation of the medium was necessary in order to study SO_4^{2-} depletion. For SO_4^{2-} depletion studies the chlorides of the other cations were used. This reformulation did not alter the

growth characteristics of the medium. The chloride salt was chosen since Boggis (1971) had shown that the chloride requirement of P. aeruginosa was satisfied by contamination from other salts. Similarly no requirement for Na^+ was found. Sodium hydrogen phosphate was used in preference to the K^+ salt since P. aeruginosa has a requirement for added K^+. The pH of MOPS was adjusted with NaOH rather than KOH for a similar reason. The use of Na^+ in excess is not without problems. Na^+ displaces loosely bound Mg^{2+} from the surface of A. aerogenes (Strange and Shon, 1964), whilst whole cell K^+ is related to the NaCl content of the medium (Tempest and Meers, 1968).

Glucose was used as a carbon source such that comparison with other workers (Boggis, 1971 and Keward, 1975) could be made. Fe^{2+} was the only trace element added since P. aeruginosa has an Fe^{2+} requirement (Boggis, 1971). Trace elements other than Fe^{2+} were not required for the growth of E. coli (Klemperer et al, 1979b). Many trace elements are added as contaminants with other media constituents.

This simple salts medium was easily prepared and could be stored for several months without deterioration. The salts were stored as concentrated stock solutions and diluted as required. At use concentration the MOPS buffer could be autoclaved in the medium but stock solutions could not be autoclaved and required filter sterilisation. Na_2HPO_4, glucose and $FeSO_4$ were also filtered and added aseptically to the autoclaved medium.

The doubling time of P. aeruginosa in C.D.M. was 62 minutes which compared well with the results of Boggis (1971) for P. aeruginosa. Growth in unlimited C.D.M. was linear to an E_{470} onset of 4.1 (Fig. 26).

4.1.3. Growth studies of *P. aeruginosa* in batch culture

4.1.3.1. Nitrogen (N)

The main role of N in cells is as a constituent of proteins which occur as enzymes or polymers. Purines, pyrimidines and some growth factors also contain nitrogen (Hawker and Linton, 1979). A variety of organic (e.g. amino acids) and inorganic (N_2 and NH_4^+ salts) N sources may be utilised. Cellular nitrogen constitutes up to 12% of the bacterial cell dry weight (Pirt, 1975).

Under conditions of N-limitation the biomass protein content is lower than that for C-limited cells (Light, 1972), which may reflect accumulation of energy reserves such as glycogen. A high C/N ratio favours the synthesis of exocellular polysaccharides by some organisms (Tempest and Wouters, 1981). The primary NH_4^+ uptake system involves glutamic acid and glutamate dehydrogenase. However, these enzymes have only low affinity for NH_4^+ and cannot function effectively at low NH_4^+ concentration. Such NH_4^+ limitation may result in derepression of an alternative high affinity NH_4^+ uptake system involving glutamine synthetase and glutamate synthase and glutamate dehydrogenase is over produced (Tempest and Wouters, 1981). Under N-limiting conditions *E. coli* and *K. aerogenes* derepress the synthesis of the high affinity system and also systems for the uptake of amino acids thus making them susceptible to drugs involved specifically in amino acid transport e.g. cycloserine (Tempest and Wouters, 1981).

As NH_4^+ became limiting the growth rate decreased followed by a slow increase in E_{470} with time (Fig. 27). The exponential growth rate was dependent upon NH_4^+ concentration up to 6×10^{-3}M NH_4^+. A linear

relationship existed between E_{470} onset and E_{470} max and the initial NH_4^+ content to E_{470} values of 1.9 and 3.65 respectively (Figs. 28 and 29). Linearity to at least E_{470} 0.6 was maintained in the studies of Boggis (1971) and to 0.83 in the studies of Ombaka (1980). To attain an E_{470} onset of 0.5 required the addition of 1.5×10^{-3}M NH_4^+ in this study and this corresponds well with the value of 1.5×10^{-3}M required to attain the same E_{470} by Boggis (1971).

Extrapolation of the E_{470} max curve to the x axis indicated a contamination level of 1×10^{-3}M NH_4^+ from other media constituents.

4.1.3.2. Potassium

Potassium is a major nutrient for micro-organisms and is present in large amounts (up to 2% of the dry weight of A. aerogenes (Tempest and Dicks, 1967)). The K^+ is largely bound up with the cellular RNA (Tempest, 1969). Thus factors which increase the growth rate and thus the RNA content of the biomass increase the cellular requirement for K^+. The K^+ content of bacteria also varies with the osmolarity of the medium (Tempest and Meers, 1968). K^+ is unique among the major nutrients in that it is contained within the cell in an unmodified and largely unbound state and may be exceedingly mobile (Pirt, 1975). The cytoplasmic K^+ concentration is much in excess of that in the growth medium and hence a large cellular energy expenditure must occur in order to concentrate K^+ against a large transmembrane K^+ gradient. K^+ limitation produces a heavy demand for respiratory energy (Pirt, 1975).

Following exponential growth the growth rate decreased gradually (Fig. 30). K^+ has a precise inter-relationship with the ribosome content of growing organisms. It is likely that K^+ limitation results in loss of protein synthesising ability resulting in the gradual reduction of the growth rate. K^+ depletion has been shown to lead to loss of proteins

synthesising ability in E. coli (Ennis and Lubin, 1961).

A linear relationship existed between E_{470} onset and E_{470} max and the amount of K^+ added to the growth medium. Linearity was maintained to an E_{470} onset of 1.55 and E_{470} max of 2.45 (Fig. 31). Fenton (1970) demonstrated a linear relationship between E_{470} onset and K^+ content of the medium to at least 0.6. In this study an E_{470} onset of 0.5 was attained by addition of 0.03×10^{-3}M K^+ to the medium which is in agreement with the figure of 0.026×10^{-3}M K^+ to achieve the same E_{470} shown by Fenton (1970). The slightly lower requirement for K^+ might result from the higher K^+ contamination level from other nutrients recorded by Fenton (1970) (0.004×10^{-3}M) compared with the K^+ contamination level of 0.002×10^{-3}M in this study.

4.1.3.3. Magnesium

Bacteria have an absolute requirement for Mg^{2+} and the basic Mg^{2+} requirements are similar for Gram-positives and Gram-negatives. Cellular Mg^{2+} content increases with the growth rate in a manner closely associated with changes in cellular RNA content (Tempest, 1969). Mg^{2+} is important in RNA synthesis (Cohn and Ennis, 1967) and membrane stability and permeability (Lederberg, 1956 and Asbell and Eagon, 1966a,b).

Mg^{2+} is also present in the cell walls in quantities that vary greatly with growth conditions (Tempest and Strange, 1966). Mg^{2+} is requires for activity by enzymes involved in the synthesis of such wall constituents as peptidoglycan, lipopolysaccharide and phospholipids (Hawker and Linton, 1979). Interaction of Mg^{2+} with the negatively charged components of LPS and PL is important for cell wall integrity (Costerton et al, 1974 and Wilkinson and Galbraith, 1975). Under

conditions of Mg^{2+} depletion the cell wall of P. aeruginosa is altered rendering the cells more resistant to PB and EDTA (Brown and Melling, 1969a,b) and gentamicin (Nicas and Hancock, 1980).

The growth rate of Mg^{2+} depleted cultures gradually decreased after exponential growth (Fig. 32). Since Mg^{2+} is involved with ribosomal content and protein synthesis it is likely that the decrease in growth rate was due to a fall in ribosomal content and a corresponding fall in protein synthesis. A linear relationship was shown between E_{470} onset and added Mg^{2+} to an E_{470} of 1.7 (Fig. 33). This value is higher than that obtained by Kenward (1975) (0.72), Finch (1976) (0.9), Ombaka (1980) (0.7) or Boggis (1971) (0.4). In the case of the first two workers the low E_{470} attained was probably due to limitation by some other nutrient. Iron was omitted from the medium by Boggis (1971) but the nature of the limiting nutrient in the medium used by Ombaka (1981) is unclear.

The high E_{470} max of 3.5 attained in this study (Fig. 33) is also higher than the E_{470} 2.35 achieved by Boggis (1971), E_{470} 1.3 Finch (1976), E_{470} 1.35 Kenward (1975). The amount of Mg^{2+} to attain an E_{470} of 0.4 in this study was 16×10^{-6}M which compares favourably with that reported by Kenward (1975) (12×10^{-6}M) and Finch (1976) (12.5×10^{-6}M). Extrapolation of the E_{470} onset curve to the x axis showed a magnesium contamination level of 1×10^{-6}M.

4.1.3.4. Iron

Iron is an essential element for micro-organisms. Although media lacking added iron have been used to grow bacteria these media will contain iron as an extraneous contaminant from glassware and other chemicals in the medium. A substantial decrease in the amount of iron may result from autoclaving media due to the formation of insoluble

salts. Such losses will rarely have a marked effect upon growth rate but may markedly affect cellular metabolism (Pirt, 1975).

Iron is required for the cofactors of enzymes involved in cellular redox functions, such as the haem pigments of cytochromes and catalase, ferredoxins and flavoproteins. Iron depletion results in disruption of oxidative phosphorylation in the respiratory chain (Light, 1972). Morphological changes in E. coli have been reported due to iron depletion (Rutledge and Winder, 1964).

The solubility of inorganic iron in aerobic conditions and at neutral pH values is approximately 10^{-15}M. This concentration of dissolved iron is too low for the requirements of many organisms. The result of iron depletion is the induction of synthesis and excretion in large amounts of iron chelating compounds called siderophores or siderochromes. P. fluorescens has been shown to produce an iron chelating fluorescent pigment Pyoverdine pf (Meyer and Hornsperger, 1978). P. aeruginosa responds to iron depletion by production of pyochelins (Cox, 1980) and enteric bacteria produce a large number of iron transporting systems (Braun et al, 1976). Until recently (Stiefel and Watt, 1979) no bacterial counterpart to the eukaryotic iron storage molecule ferritin had been discovered. A ferritin like compound (cytochrome b 557.5) produced from Azotobacter sp has been characterised by Stiefel and Watt (1979) as a bacterioferritin. Enteric bacteria derepress the synthesis of specific envelope proteins under iron limiting conditions and these proteins function in conjunction with enterochelin as a high affinity iron uptake system (Braun et al, 1976).

Iron is a limiting nutrient for bacteria invading human tissues. The role of iron in infection has been reviewed by Weinberg (1978).

In this study preliminary investigations failed to reveal any iron requirement for P. aeruginosa 6750. CDM lacking added iron supported growth to an E_{470} of approximately 0.5. Calculation from manufacturer's specifications of the theoretically possible iron contamination showed that the iron contamination added with other media constitutents was approximately 5×10^{-9}M Fe^{2+}. Thus contaminating Fe^{2+} levels from this sources was low. An iron limited culture was used in these experiments as the initial inoculum. Work has since shown that E. coli can accumulate large amounts of iron (McIntosh and Earhart, 1977). Such accumulated iron stores might result in some growth in media lacking added iron. A likely source of extraneous iron was the glassware. This was shown to be true since washing the glassware with EDTA plus the normal stringent wash procedure resulted in growth to an E_{470} of 0.2. Iron contamination was further reduced by the use of Aristar grade chemicals. These procedures resulted in the detection of an iron requirement for P. aeruginosa.

The growth rate following cessation of exponential growth declined gradually (Fig. 34). Linearity was maintained to an E_{470} onset of 1.7 and E_{470} max of 3.4 (Fig. 35). This onset value was higher than that attained by Ombaka (1980) of 1.02. To attain an E_{470} onset of 1.0 required 0.75×10^{-6}M added Fe^{2+} in this study and 0.9μM in Ombaka's (1980) study. Al Dujaili (1979) using Pr. mirabilis and Ismail (1977) using E. coli RP^- failed to show any requirement for added iron by these organisms.

4.1.3.5. Phosphorus

Phosphorus is normally supplied to cells as inorganic PO_4^{3-} although organic PO_4^{3-} may be used. It is incorporated into nucleic acids, PL, and cell wall polymers although it may be stored as polymetaphosphate.

The phosphorus content of bacteria is about 1.5% of the dry weight however, the content is dependent upon growth rate and temperature varying directly with the former and inversely with the latter (Pirt, 1975). This relationship is due to the stoichiometry which exists between Mg^{2+}. K^{+}, PO_4^{3-} and RNA (Tempest, 1969).

Phosphate limitation produces marked effects on cells. The effect of limitation is most marked in the cell walls of Gram-positives where teichoic acid is replaced by the non phosphorus containing teichuronic acid (Ellwood and Tempest, 1972). The replacement of PL by glycolipids has been demonstrated in P. diminuta (Minnikin et al, 1974) and P. fluorescens (Minnikin and Abdolrahimzadeh, 1974 and Dorrer and Teuber, 1977). Such changes in cell wall composition may result in altered sensitivity to antibiotics (Dorrer and Teuber, 1977), disinfectants (Gilbert and Brown, 1978a) and phages (Anderson et al, 1978). PO_4^{3-} depletion results in derepression of synthesis and excretion of PO_4^{3-} scavenging enzymes such as alkaline phosphatase and ribonuclease (Wouters and Buysman, 1977).

The depletion of PO_4^{3-} in the medium resulted in the gradual reduction in growth rate (Fig. 36). Decreased growth rate is probably due to a decreased rate of protein synthesis associated with a decreasing phosphorus content of the ribosomal RNA. The relationships between E_{470} onset and max and added PO_4^{3-} were linear to an E_{470} onset value of 1.7 and E_{470} max value of 3.8 (Fig. 37). Ombaka (1980) obtained a linear relationship to E_{470} onset of 1.0 and Boggis (1971) to at least 0.4. A similar requirement of 0.14×10^{-3}M added PO_4^{3-} was required to attain an E_{470} of 0.4 in this study and by Boggis (1971) and Ombaka (1980). Extrapolation of the E_{470} onset curve to the x axis showed extraneous PO_4^{3-} contamination of 0.02×10^{-3}M.

4.1.3.6. Carbon

Carbon is the basic structural component of all organic compounds and is therefore needed by microbes in large quantities. A variety of C sources may be used by heterotrophs including glucose, monocarboxylic acids and to a lesser extent amino acids, lipids and alcohols. In batch culture mixed C sources are utilised sequentially but in continuous culture their utilisation will depend upon the nature of the limiting constituent. Acetate is utilised by Pseudomonas spp (Ng and Dawes, 1973) before glucose under nitrogen limiting conditions but both sources are used simultaneously when the culture is C-limited (Pirt, 1975). Microbes utilise C-sources both for growth and other maintenance purposes such as cell material turnover, osmotic work, and cell mobility (Pirt, 1975). The considerable effect of ionic strength of the medium upon the maintenance energy requirements of the cell suggest a large proportion of this energy is required to perform work to maintain concentration gradients between the cell and its exterior environment.

Organisms including K. aerogenes (Tempest and Wouters, 1981) and P. aeruginosa (Dawes et al, 1976) possess dual systems for the uptake and metabolism of C substrates. A high affinity system is used in the presence of low concentrations of substrate and the low affinity system under conditions of C excess. Glucose is metabolised in P. aeruginosa by extracellular (periplasmic) and intracellular pathways. When glucose is in excess the low affinity system operates and the enzymes glucose dehydrogenase and gluconate dehydrogenase metabolise glucose via the extracellular pathway and gluconate and 2 oxogluconate accumulate in the medium since they are produced faster than they can be transported (Dawes et al, 1976). Under glucose restricted conditions the extracellular pathway enzymes are repressed and increase of high affinity enzymes occurs

and hexakinase and glucose 6-P-dehydrogenase enzymes are produced. This ensures that the restricted amount of glucose is taken up as rapidly as possible.

Growth of glucose depleted cells ceased abruptly as the nutrient became exhausted (Fig. 38). The doubling time of 60 minutes was in agreement with Boggis (1971), Finch (1976) and Kenward (1975). A linear relationship existed between E_{470} max and added glucose to an E_{470} of 4.1 (Fig. 39) at a glucose content of 165×10^{-4}M. Linearity to only 0.53 (Boggis, 1971), 1.4 (Finch, 1976) and 1.7 (Ombaka, 1980) was reported for glucose depleted cells. Examination of the growth curves of the latter two workers revealed that at higher glucose concentrations an abrupt cessation of growth was not apparent and the gradual cessation of growth suggests that the cells were not glucose depleted. Finch (1976) suggested his cultures were oxygen limited although his cultural conditions (25ml volumes in 100ml flasks shaken at 120 throws/minute at 37°C) were identical to the ones used in this study. Boggis (1971) did not add iron to his media and thus his cultures were possibly iron depleted at higher optical densities. 2.0×10^{-3} glucose was required to attain an E_{470} of 0.5 (Fig. 39) this agrees well with the amounts required by Boggis (1971) (1.8×10^{-3}M), Finch (1976) (2.0×10^{-3}M) and Kenward (1975) (1.9×10^{-3}M) to attain this E_{470}. Extrapolation of the max growth curve to the origin indicated that the medium contained insufficient amounts of any other suitable C source to support growth.

4.1.3.7. Sulphur

This is utilised as inorganic SO_4^{2-} but some organisms require organic S in the form of cysteine or methionine. Sulphur represents 0.3-0.4% of the dry weight of the cell. Sulphur is essential to all

living organisms and is present in the form of cysteine and methionine and as a number of coenzymes e.g. lipoic acid, coenzyme A, ferredoxin and thiamine (Pirt, 1975).

Sulphate limitation results in a lowering of the protein content of the cell envelope and in the sulphur content of soluble protein. Sulphate depletion of C. utilis resulted in loss of one site of oxidative phosphorylation in the respiratory chain (Light, 1972). A reduction in non haem iron proteins with which sulphide is bound in the electron transport chain also occurred. Similar results were obtained for sulphate depleted E. coli K12 (Poole and Haddock, 1975). Sulphate depletion also affects sensitivity to EDTA and PB (Klemperer, 1976).

The E_{470} onset and max values obtained in preliminary experiments with sulphate depleted media gave unexpectedly high results (Fig. 41). To achieve an E_{470} onset of 0.5 required only 1/25 of the sulphate required by P. aeruginosa in the studies of Boggis (1971). Extrapolation of the growth curve to the x axis indicated an SO_4^{2-} contamination level in the range 0.5 - 5.0 x 10^{-5}M and CDM without added SO_4^{2-} supported growth to an E_{470} onset of 0.5 and E_{470} max of 2.58 (Fig. 41). These results seemed not to be the result of sulphate contamination in the media which from manufacturers purity data and analysis ofthe deionised water used in this study was shown to be less than 1 x 10^{-6}M. This suggested MOPS buffer was being utilised as a source of sulphur by these cells. This was confirmed by reformulation of the medium, replacing MOPS by ammonium acid phosphate buffer (7 x 10^{-3}M).

Fig. 40 shows that as the cells became sulphate depleted the growth rate slowed progressively. Linearity between E_{470} onset and added SO_4^{2-}

was maintained to 1.3 and E_{470} max values were linear to an E_{470} of 2.55. The E_{470} onset values are in agreement with those of Ombaka (1980) who obtained linearity to an E_{470} of 1.35 at an added SO_4^{2-} concentration of 7.0×10^{-5}M (Fig. 41). To attain an E_{470} of 1.45 in this study 7.5×10^{-5}M SO_4^{2-} was required. However, Boggis (1971) achieved linearity to an E_{470} onset value of only 0.4 at an added SO_4^{2-} concentration of 2.0×10^{-5}M and linearity was maintained to an E_{470} max of 2.0 at an SO_4^{2-} concentration of 3.0×10^{-5}M. Thus Boggis (1971) required only 1/3 of the SO_4^{2-} concentration to attain a similar E_{470}. The reason for this lower requirement is unclear but extraneous SO_4^{2-} contamination in his media is unlikely. The attainment of only an E_{470} of 0.4 was probably due to the omission of iron from the medium. Using acid ammonium phosphate buffer instead of MOPS revealed an SO_4^{2-} contamination level of 8×10^{-6}M.

4.2. The effect of medium phosphate content on sensitivity of batch grown *P. aeruginosa* to Polymyxin B and EDTA

4.2.1. Introduction

Changes in optical density (E_{470}) total and colony count were used to measure the sensitivity of *P. aeruginosa* to PB and EDTA. Changes in E_{470} of treated cells might reflect changes in light scattering properties of the cells induced by drug action and not by true cell lysis. Microscopy of treated cells indicated that lysis had occurred. Nutrient limitation may also result in changes in size of the cells and thus suspensions with similar optical densities may not contain similar numbers of cells. Mg^{2+} limited *A. aerogenes* showed decreased cell size with increasing limitation (Tempest et al, 1965). Phase contrast microscopy of carbon and PO_4^{3-} plentiful and depleted cells used in this study failed to reveal differences in cell size or microscopic clumping which might affect E_{470} or colony count results. Colony counts of C-depleted PO_4 plentiful (C^-P^+) and PO_4^{3-} depleted carbon plentiful (P^-C^+) cells revealed that an E_{470} of 0.2 gave approximately 1.98×10^8 cfu ml^{-1} and 1.78×10^8 cfu ml^{-1} respectively. These figures were considered sufficiently close not to result in drug sensitivity changes due to inoculum size effects. Changes in E_{470} and total count gave good agreement in this study indicating optical density changes were due to lysis of the organism. Boggis (1971) also showed good correlation between E_{470} changes and total count changes.

The lysis curves following exposure to PB and EDTA were not log linear but for comparison with other workers (Boggis (1971) and Finch (1976)) the sensitivity of the cells was measured as the fall in selected parameter (E_{470} total or colony count) over a period of 60 minutes for PB treated cells and 180 minutes for EDTA treated cells.

4.2.2. The test situation

Two general approaches have been used to test the action of drugs on bacterial populations. These are a) procedures not involving harvesting of the strain (Brown and Garrett, 1964 and Brown and Melling, 1969a) and b) procedures using harvested cells (Finch, 1976 and Ombaka, 1980).

In the former the only stress which the organisms suffer is that due to interaction with the drug itself. This method may be criticised since for cells under different degrees of nutrient depletion interaction with the drug will occur in media whose composition and tonicity is variable especially with respect to nutrients in the media and metabolic products formed by growth in that medium. Such metabolic products, if they have any effect at all, will either antagonise or potentiate the action of the agent under test. Envelope macromolecules may also be shed at various stages of growth (Loeb and Kilner, 1979) and since these molecules may be the receptor molecules for the drug in intact bacteria some reduction in antimicrobial activity may result. Procedures involving harvesting may result in abnormal stresses on the cells which may affect both their survival during the harvesting and washing procedure and their subsequent response to drug treatment. Changes in composition of the drug testing menstruum compared with the growth medium may severely stress cells and the test medium composition may also affect the drug action.

The response of cells to changes in environment and various harvesting procedures has been comprehensively reviewed by Farwell and Brown (1971) and specific effects on drug sensitivity by Melling and Brown (1975) and Wilkinson (1975).

Centrifugation is normally considered relatively harmless but little data exists on this. Up to 5 centrifugations of cells grown in various PO_4^{3-} concentrations (Fig. 44) had no deleterious effect on the survival of cells during the "holding" period prior to treatment with PB or on subsequent treatment with PB (Fig. 45). Despite these findings it was decided that since 3 washings of the cell inoculum resulted in minimal carryover of PO_4^{3-} (Table 26) that this minimum number of centrifugations should be used.

In this study susceptibility to PB was tested with and without harvesting for batch cultures and with harvesting for chemostat cultures Susceptibility to EDTA was determined without harvesting for batch cultures and with harvesting for chemostat cultures.

The choice of resuspension medium has been shown to effect markedly such properties as optical density (by shrinkage or swelling), viability and leakage of cellular constituents (Farwell and Brown, 1971) and also the action of PB (Newton, 1953a, Klemperer et al, 1979a) and EDTA (Hague and Russell, 1974). Glycerol limited A. aerogenes died more rapidly when resuspended in a medium of higher or lower tonicity than the growh medium (Postgate and Hunter, 1962). Changes in optical density following resuspension have been demonstrated by Klemperer et al (1979a). Solutes which had restricted penetration into P. aeruginosa produced an initial increase in optical density followed by a decrease over the next hour. Increasing the NaCl content of the storage media resulted in less leakage of 260nm absorbing material although viability could not be correlated with loss of absorbing material. A mixture of monovalent and divalent cations which might be optimal for survival in resuspension medium may not be ideal to test lysis by drugs. Divalent ions are more antagonistic to PB than monovalent ions (Newton, 1954). Resuspension of P. aeruginosa

in buffered solutes is osmolar with 200mM NaCl showed that water, NH_4^+ or K^+ acetate resulted in increased lysis by PB, whilst Na^+ acetate gave moderate protection and NaCl gave little lysis (Klemperer et al, 1979a). However, when viability rather than lysis was considered the protective capacity of the solutes changed. Since chelation of divalent cations is an early event in the action of EDTA (Wilkinson, 1975) many workers have avoided the addition of divalent cations to their wash and test media. However since divalent cations (particularly Mg^{2+}) are needed for growth and it was intended to treat batch grown cells with EDTA without prior washing, divalent cations were used in the wash medium for chemostat grown cells for comparative purposes. Divalent cations in concentrations similar to that used in the growth medium was used in the resuspensions medium for measurement of PB activity.

The numbers of organisms in the test suspension has considerable effect on both survival during harvesting and activity of drugs. In general higher cell concentrations give better survival during washing procedures although cell concentrations $>10^7$ were necessary for this effect to be shown (Spangler and Winslow, 1943). The increased survival of high inocula may be due to the protective effect afforded to remaining cells of leaked cellular material from non viable cells (Farwell and Brown, 1971). Boggis (1971) showed that an initial E_{470} of 0.134 resulted in 60% lysis of cells treated with 40 units ml^{-1} PB whilst an E_{470} 0.228 resulted in 26% lysis. Large inoculum size dependent lysis changes were also seen for EDTA treated cells (Boggis, 1971). In order to eliminate such effects the E_{470} of harvested or non harvested cells was adjusted to $0.2 \equiv 2 \times 10^8$ viable cells ml^{-1} prior to treatment with EDTA or PB.

Although the pH of the growth medium has relatively little effect on cellular composition (Brown, 1975) low pH may result in altered membrane composition (Van Iterson and Op den Kamp, 1969). As with other stresses the effect of sudden changes of pH are more dramatic than gradual changes (Brown and Winsley, 1969). The survival of bacteria is generally better in buffered than unbuffered media (Postgate and Hunter, 1962). Changes in pH will also affect the action of PB and EDTA. Alteration in pH affected the binding of PB to phosphatidic acid membranes (Sixl and Galla, 1979) and the optimum pH for release of glucose from E. coli liposomes was pH 7-8 (Feingold et al, 1974). EDTA activity against P. aeruginosa increases as the pH increased from 7.1 - 9.2 (Wilkinson, 1967).

In this study changes of pH for both growth medium and washing and resuspension medium were minimised as much as possible. At cessation of growth (E_{470} 0.2) the medium pH was 7.3 - 7.4 and a washing medium of this pH was used. Table 29 shows that for cells grown to E_{470} 0.2 in a range of PO_4^{3-} concentrations the final culture pH over the range 6.6 - 7.4 had little effect upon the % lysis by 10 units ml^{-1} PB, and similar cells showed no significant change in PB lysis when the pH of the washing and resuspension medium was altered in the range 6.75 - 7.50 (Table 30).

To minimise the effect of temperature changes cultures were grown at 37°C and washing solutions, centrifugation and lysis was performed at 37°C.

Preliminary experiments showed that during the 1 hour equilibration period following washing and prior to drug treatment wide variations in the E_{470} values of cultures resulted. Cells grown in low PO_4^{3-} media and resuspended in CDM minus PO_4^{3-} and glucose showed less change than cells grown in higher PO_4^{3-} concentrations. Fig. 46 shows the effect of PO_4^{3-} concentration in the growth medium upon the stability of these cells

during the equilibration period and confirms the preliminary results. The good agreement between E_{470} total and colony count suggests that cells were dying during the equilibration period and not merely suffering changes in cell size or optical density properties due to media changes.

To stabilise these changes prior to lysis various concentrations of PO_4^{3-} were added to the resuspension medium and the effect on the 3 growth parameters tested. Fig. 47 shows that these effects can be minimised for cells grown in high PO_4^{3-} concentrations by addition of 2.5×10^{-4}M PO_4^{3-} to the resuspension medium. The effect of addition of PO_4^{3-} to the resuspension medium on cells grown in different levels of PO_4^{3-} and subsequently lysed to 10 units ml^{-1} is shown in Table 27. The addition of 2.5×10^{-4}M PO_4^{3-} to the resuspension medium had no detrimental effect on PB lysis.

The effect of gross nutrient excess on the subsequent lysis of cells by PB is shown in Table 28. The use of a 25X excess of nutrients greatly increased the % lysis compared to the effect of a 5X excess, presumably by affecting the ionic composition of the test medium.

In summary the composition of the washing and lysis medium used in this study was chosen to closely resemble the growth medium composition and should thus avoid stress on the cells due to media changes. It also enabled a comparison between unwashed and washed cells to be made although the resuspension medium did not have metabolic growth products or shed membrane fragments in it. Whilst the composition may be criticised for containing divalent cations its use did permit minimal stress conditions on the cells.

4.2.3. Sensitivity to Polymyxin B

A range of PB concentrations have been used by workers studying its effect on P. aeruginosa. 40 units ml^{-1} was used by Boggis (1971) and Finch (1976), 50 units ml^{-1} by Kenward (1975) and Dean et al (1976) and 32.5 units ml^{-1} by Ombaka (1980). It is only clear from the work of Boggis (1971) how the concentration of PB for use was chosen. He showed that 35 units ml^{-1} was maximal for lysis of Mg^{2+} plentiful cells and 50 units for Mg^{2+} depleted cells. He therefore chose 40 units ml^{-1} for subsequent studies. He did not determine the sensitivity of PO_4^{3-} depleted cells and for comparative purposes used 40 units ml^{-1} for treatment of these cells.

The lysis curves used to determine the optimal concentration of PB for C^+P^- and C^-P^+ cells are shown in Fig. 48 for cells grown in low PO_4^{3-} ($0.1 \times 10^{-4}M$) and Fig. 49 for cells grown in high PO_4^{3-} ($3 \times 10^{-4}M$). The lysis curves reveal several interesting features. Initial increases in E_{470} were seen for both sets of cells when PB up to 120 units ml^{-1} and 160 units ml^{-1} were used for PO_4^{3-} plentiful and depleted cells respectively. The increase in E_{470} was related to the concentration of drug to which the cells were exposed (Fig. 48). This phenomenon has been reported by Brown and Melling (1969b) and Boggis (1971) for Mg^{2+} depleted P. aeruginosa, and is probably related to altered light scattering properties of the cells upon uptake of the drug. Since sensitivity to PB was determined by changes in E_{470} at time 0 and time 60 minutes such initial increases in E_{470} might significantly affect the results if higher concentrations of PB are used. The initial increase in E_{470} was minimal for cells treated with 10 and 20 units ml^{-1} PB. The amount of PB used may have profound effects upon the % lysis observed for cells grown in CDM containing graded amounts of PO_4^{3-} (Fig. 50). Whilst the cells grown

in the 3 PO_4^{3-} concentrations 0.1, 0.25 and 0.3 x 10^{-4}M all exhibited an increased lysis over 60 minutes which was related to the PO_4^{3-} content of the growth medium at PB concentrations up to 20 i.u. ml^{-1}, above this PB concentration the amount of lysis achieved by a specific amount of PB was not so clearly dependent upon the medium PO_4^{3-} content. Above 120 i.u. ml^{-1} cells grown in 0.1 x 10^{-4}M PO_4^{3-} were still the most sensitive but cells grown in 0.25 x 10^{-4}M PO_4^{3-} were slightly more resistant than cells grown in 0.3 x 10^{-4}M PO_4^{3-} (Fig. 50). Thus the % lysis by PB is dependent upon the PO_4^{3-} concentrations in which the cells have been grown and also the concentrations of PB used. The relationship shown in Fig. 50 was not investigated using total or colony count as parameter but subsequent use of 10 units ml^{-1} showed a similar relationship existed between lysis (E_{470}) and total count. Although larger changes in colony count were evident for a given PO_4^{3-} concentration than changes in E_{470} the pattern observed between % change in any of the 3 parameters and PO_4^{3-} concentration was similar suggesting that the phenomenon described for E_{470} could well be shown by colony and total count estimations. The reason for this difference with different concentrations of PB is unclear.

The relationship between the PO_4^{3-} content of the growth medium and changes in E_{470} following exposure to PB at concentrations of 10 - 160 units ml^{-1} is clearly dependent upon the PB concentration used (Fig. 51). Cells grown in PO_4^{3-} concentrations up to 0.16 x 10^{-4}M exhibited increasing sensitivity to PB when 10 units ml^{-1} of PB was used. Above 0.16 x 10^{-4}M PO_4^{3-} the cells became less sensitive to 10 units ml^{-1} PB. Fig. 51 also shows that for cells grown in similar concentrations of PO_4^{3-} maximum sensitivity to concentrations of PB above 10 units ml^{-1} occurred at a PO_4^{3-} concentration of 0.1 x 10^{-4}M and above this concentration the cells became more resistant to PB.

The initial PO_4^{3-} concentration which can be used in the medium is controlled by the initial E_{470} selected for lysis studies. Thus below 0.1×10^{-4}M PO_4^{3-} in the growth medium the required E_{470} of 0.2 was not achieved. Fig. 51 suggests however that the relationship seen for treatment with 10 units ml^{-1} PB might be true for other PB concentrations if cells grown at lower PO_4^{3-} concentrations could be used. This was shown to be true for 20 and 40 units ml^{-1} PB when tested further Fig. 52. Higher PB concentrations were not tested. An initial E_{470} of 0.2 was obtained by growth in PO_4^{3-} to a minimum of 0.01×10^{-4}M PO_4 if cells were pooled following centrifugation and finally resuspended to an initial E_{470} of 0.2. Such cells when lysed with 20 or 40 units ml^{-1} PB showed the characteristics of cells treated with 10 i.u. ml^{-1} PB i.e. increasing sensitivity to PB as the PO_4^{3-} concentration was increased followed by a subsequent decrease in sensitivity. This relationship was similar to that obtained by Boggis (1971) for cells depleted by PO_4^{3-} and C.

Further studies upon PO_4^{3-} depletion were performed using 10 units ml^{-1} PB and typical lysis curves are shown in Fig. 53. The rate of lysis was dependent upon the PO_4^{3-} content of the growth medium up to 0.166×10^{-4}M. Above this level the lysis rate constant fell to a minimum of $1 \times 10^{-3} min^{-1}$ and above PO_4^{3-} concentrations of 0.4×10^{-4}M the lysis rate was independent of the PO_4^{3-} concentration of medium (Fig. 54).

Irrespective of the PO_4^{3-} medium content the lysis rate constant K was linearly dependent upon the % lysis in 60 minutes (Fig. 55).

The effect of PO_4^{3-} in the medium on colony and total count is shown in Fig. 56. The changes observed in E_{470} were accompanied by similar changes in total count. The % change values correlated well. A similar pattern was seen for colony count although the % change at any particular PO_4^{3-} concentration was higher than that observed for E_{470} or total count

indicating that cells not lysed were sufficiently damaged to prevent them being recoverable on the nutrient agar counting medium used.

When cells were harvested and washed prior to treatment with PB a similar relationship between PO_4^{3-} content and the three test parameters was noted (Fig. 57). The % change was larger for the harvested cells than for unwashed cells either indicating that the harvesting and washing procedure is slightly traumatic to the cells or that substances inhibitory to PB are present in the unwashed cell culture medium. Fig. 57 also shows that the decrease in PB sensitivity in all 3 parameters at PO_4^{3-} concentration above 0.16×10^{-4} is less in washed than unwashed cells supporting the hypothesis that unwashed cell culture medium contains macromolecules inhibitory to PB.

At high PO_4^{3-} concentrations a similar decrease in sensitivity to PB was noted by Boggis (1971) but he did not investigate this further. There is now much evidence to suggest that organisms especially Gram-negatives (Thorne et al, 1976; Munford et al, 1980 and Loeb and Kilner, 1979) release membrane fragments into the medium during the growth cycle. These fragments contain LPS, PL and proteins although the relative composition need not be representative of the O.M. as a whole (Loeb and Kilner, 1979). The ratio of polypeptides released by E. coli B was dependent upon the growth medium composition (Loeb and Kilner, 1979). Since PB uptake is dependent upon binding with O.M. components especially LPS (Bader and Teuber, 1973) it is possible that released macromolecules might compete with molecules in the O.M. for P.B. resulting in the decreased action of P.B. especially if PB is used at low concentrations as in this study. It is also possible that differential release of macromolecules may occur dependent upon the growth medium composition.

It seemed unlikely that the variable PO_4^{3-} concentration found in the unwashed cell culture medium following growth affected PB sensitivity since the maximum PO_4^{3-} concentration studied was 0.85×10^{-4}M. The concentration of PO_4^{3-} added to the resuspension medium was constant at 2.5×10^{-4}M which was also unlikely to be inhibitory to PB since Table 27 shows that PO_4^{3-} concentrations to 0.52×10^{-1}M in the resuspension medium did not affect the lysis of P. aeruginosa.

The spent culture medium used for growth of cells at high concentrations of PO_4^{3-} (0.5×10^{-4}M) did show inhibitory action to 10 units ml^{-1} PB. If PB was added to the spent culture medium 1 hour prior to addition of cells and subsequent lysis the fall in E_{470} was dramatically reduced. Also addition of PB to spent culture medium resulted in less lysis than for addition to fresh resuspension medium, indicating that components of the spent culture medium are inhibitory to PB. The effect of spent culture medium upon PB lysis was less for cells grown at lower PO_4^{3-} concentrations (Table 31) presumably because of the altered chemical composition of envelope fragments released into the culture supernatant.

Chemical analyses of the lyophilised culture supernatants from cells grown in 0.1, 0.17 and 0.5×10^{-4}M PO_4^{3-} showed that there was little difference between the protein, REL'P' or KDO (LPS) content of cells grown in the two lower PO_4^{3-} containing media but that the supernatant from cells grown in the higher PO_4^{3-} concentration had a much reduced protein content and an increased KDO content (Table 32). Thus it appears that composition of the growth environment may alter the composition of macromolecules released during growth. It was surprising that no RELP-containing compounds were detected. The increased KDO content for cells grown in gross PO_4^{3-} excess is of interest and could indicate that some

inactivation of PB might result from interaction with released LPS thus making such cells appear more resistant to PB than they really are.

The results of this work on the effect of various PO_4^{3-} concentration in the growth medium of batch grown cells and their subsequent response to PB is in agreement with that of Boggis (1971) although the difficulty in producing the observed increase in sensitivity to PB at 40 units ml^{-1} for cells grown at low PO_4^{3-} concentrations is unclear. It may well reflect differences in the growth medium composition or the requirement of this strain for PO_4^{3-} to attain an E_{470} of 0.2. Also Boggis (1971) used doubly depleted cells (PO_4^{3-} and C). This double depletion may have resulted in an altered cell wall and an altered level of sensitivity to PB. Phosphate depletion and sensitivity to PB has not been widely studied. Dorrer and Teuber (1977) showed that P. fluorescens which was susceptible to PB (MIC 10µg ml^{-1}) when grown in normal medium became resistant to PB (MIC >128µg ml^{-1}) when grown in PO_4^{3-} limited media. Major cell envelope changes were seen and were related to decreased binding of PB by whole cells and O.M. fractions of P. fluorescens. It would seem reasonable to expect PO_4^{3-} depleted cells to exhibit changes in the phosphorus containing cell structures especially the wall PL and LPS (Minnikin and Abdolrahimzadeh, 1974). The changes shown in the cell walls of PO_4^{3-} depleted bacteria and the association of these changes with PB activity will be discussed in later sections (sections 4.4 and 4.5.1.).

4.2.4. Sensitivity to EDTA

The effect of EDTA upon P. aeruginosa grown in media containing various amounts of PO_4^{3-} was studied in a manner similar to that for PB. Cells grown in 0.1×10^{-4}M PO_4^{3-} showed resistance to EDTA up to a level

of 1570µg ml^{-1} (Fig. 59). Cells grown in an excess of PO_4^{3-} (1×10^{-4}M) showed increasing sensitivity as the EDTA concentration increased (Fig. 59). The effect on E_{470} of treatment with EDTA in the range zero to 1570µg ml^{-1} is shown in Fig. 58. The lysis curves were not log linear showing a reduction in the rate of lysis over the 180 minute period of study. The sensitivity of the P^+C^- cells increased linearly with EDTA concentration to 785µg ml^{-1} when cells exhibited approximately 25% lysis. In further studies 785µg ml^{-1} were used (Fig. 60). Other workers (Boggis, 1971 and Finch, 1976) used 350µg ml^{-1} presumably based on the work of Boggis (1971) who showed that this EDTA concentration was optimal for lysis of Mg^{2+} depleted and Mg^{2+} plentiful cells. The optimal EDTA concentration for use on PO_4^{3-} depleted and PO_4^{3-} plentiful cells was not determined by Boggis (1971).

P. aeruginosa 6750 showed increasing sensitivity to EDTA as the concentration of PO_4^{3-} in the growth medium was increased to 0.8×10^{-4}M (Fig. 61). Above this PO_4^{3-} concentration no further increase in sensitivity occurred. Unlike PB treated cells only a small decline in sensitivity was seen at higher PO_4^{3-} levels. Comparison of the lysis curves obtained in this study (Fig. 60) with those for C/P depleted cells (Boggis, 1971) failed to show the 'shoulder' between 0 and 60 minutes seen by the later worker. Little lysis was seen during the first 60 minute period following treatment with EDTA 350µg ml^{-1} but lysis proceeded at a linear rate for at least the next 120 minutes. Similar results were obtained for lysis by EDTA of Mg^{2+} plentiful cells studied by Kenward (1975), although his Mg^{2+} depleted cells showed rapid initial lysis followed by subsequent decrease in lysis rate, a pattern similar to that seen for cells in this study. Kenward (1975) suggests that Mg^{2+} depleted cell lysis curves suggest that cation removal from superficial sites occurs rapidly but that remaining cations

are more firmly bound in less readily accessible sites. Mg^{2+} is associated with LPS and PL in the cell envelope (Cox and Eagon, 1968), and also presumably with carboxyl groups of protein molecules. A reduction in PO_4^{3-} content of the medium results in an alteration and/or reduction in the various P-containing structures e.g. LPS, PL (see section 4.4), and a reduction in Mg^{2+} content. This reduction in Mg^{2+} may partly account for the lysis curves resembling more closely those of Mg^{2+} depleted cells. The higher EDTA concentration used in this study may have resulted in a more rapid removal of the cations.

The primary and secondary lysis rate constants were linearly dependent upon the PO_4^{3-} content of the medium used to grow the cells to PO_4^{3-} concentration levels of 0.8×10^{-4}M (Fig. 62). Above this level the rate constant was reduced and appeared to be independent of the medium PO_4^{3-} content of the medium.

The increasing resistance, as judged by E_{470} changes, of PO_4^{3-} depleted cells was also seen when colony count and total count parameters were studied (Fig. 64). As with PB treated cells close agreement between E_{470} and total count was noted, but significantly in this case good agreement with colony count was also seen possibly indicating that treated cells were less badly damaged by EDTA treatment. Maximum sensitivity to EDTA occurred at PO_4^{3-} levels of 0.8×10^{-4}M. Similar maximum sensitivity was demonstrated by Boggis (1971). This maximum sensitivity to EDTA occurs at a concentration some 5X that observed for PB treated cells.

The results in this batch work are in close agreement with those of Boggis (1971) who is the only worker to report on phosphate depletion and sensitivity of batch grown P. aeruginosa to EDTA.

4.3. The effect of medium phosphate content on the sensitivity of chemostat grown *P. aeruginosa* to Polymyxin B and EDTA

4.3.1. Growth characteristics of carbon limited and phosphate limited *P. aeruginosa* in the chemostat

The growth parameters E_{470}, dry weight, colony count and total count were determined for cells which had been grown in the chemostat for at least 5 volume changes at each dilution rate before measurements were made. The behaviour of *P. aeruginosa* under C-limited conditions is shown in Fig. 67. The curve has characteristics similar to those described by Tempest and Dicks (1967) for glycerol limited *A. aerogenes*. At low dilution rates the growth yield falls slightly since not all the C source added to the culture is utilised in cell structures, a proportion being lost as CO_2 due to oxidation of the C source to provide energy for growth and maintenance. There was good agreement over the range of dilution rates studied (0.05 - 0.625 hr^{-1}).

The observed change in the 4 growth parameters for P-limited cultures showed considerable variation from the predicted curve (Fig. 68). With all limitation studied (Mg, P, K, S, N) except carbon Tempest and Dicks (1967) observed an increase in the dry weight of bacteria above the theoretically predicted curve at lower dilution rates. These results reflect a changing cell content of P which is lower as the growth rate decreases. As the value of dilution rate (D) was increased in this study the theoretical curve predicted from the Monod equation was not followed as it was by *A. aerogenes* (Tempest and Dicks, 1967). At lower dilution rates ($<0.2\ hr^{-1}$) the colony count was less than the total count possibly indicating that the cells were dead although the reason for this is uncertain. Above $D = 0.2\ hr^{-1}$ close agreement between all 4 parameters was evident.

The rapid fall in growth yield with increasing D caused practical problems in subsequent studies since at high D values the cultures from several chemostats had to be pooled following centrifugation and washing in order to achieve the E_{470} 0.2 required for drug treatment.

Investigation into the possible causes of the poor growth yield proved unsuccessful. Increasing the O_2 flow rate to the chemostat failed to increase the yield at any dilution rate suggesting that O_2 limitation was not a problem. Addition of trace elements as suggested by Evans et al (1970) to the chemostat feed vessel (Table 36) did not increase the yield at 3 dilution rates tested (Table 37). No further attempts to identify the cause of this problem was made and subsequent work was performed accepting the disadvantage imposed by this effect.

4.3.2. The effect of growth rate on the response of phosphate limited *P. aeruginosa* to Polymyxin B

The effect of increased growth rate (D = 0.05 - 0.33 hr^{-1}) on the PB sensitivity of P-limited cells is shown in Fig. 69. As D increased the cells became more sensitive to 10 units ml^{-1} PB. The lysis rate constant was linearly dependent upon D over the range 0.05 - 0.18 hr^{-1} (Fig. 70). As the growth rate increased similar changes were seen in the sensitivity of the cells as judged by colony and total count (Fig. 71). As before E_{470} and total count correlated better than E_{470} and colony count. The % change in E_{470} and total count was linear over the D values studied. Colony count was linear only over the range D = 0.05 - 0.2 hr^{-1} above this a decrease in % change was noted.

The effect of dilution rate on PB sensitivity was similar to that obtained by Dean et al (1976) for P-limited cells treated with 50 units ml^{-1} PB although their % survivors was less than in this study presumably due to the large amount of PB used. They showed however, an increase in

resistance as D was changed from 0.05 - 0.1 hr^{-1} but further increases in D resulted in increased sensitivity. These results suggest that more rapidly dividing cells are increasingly susceptible to PB. These results do not agree with those of Ombaka (1980) for PO_4^{3-} limited cells treated with 32.5 units PB. Ombaka (1980) was unable to correlate changes in D with changes in sensitivity. Large falls in colony count (3 - 5 log) were seen at all D values over the range 0.15 - 0.31 hr^{-1}. There was no obvious change in sensitivity with dilution rate change. Reasons for the discrepancy in results is not clear although the cells used by Ombaka (1980) appeared to have been very susceptible to change since her control cells treated with water were reported to have undergone approximately 50% lysis. This might indicate that instability of her cells was due to resuspension in a medium of very different composition to the growth medium. Such sensitivity to PB is thus not surprising considering the control sensitivity.

4.3.3. The effect of growth rate on the response of carbon limited *P. aeruginosa* to Polymyxin B

The effect of increasing dilution rate in the range 0.05 - 0.33 hr^{-1} demonstrated that the sensitivity of the cells to 10 units ml^{-1} PB was increased with increasing dilution rate i.e. the faster growing cells were more sensitive to PB at the concentration used (Fig. 72). Cultures showed a linear fall in E_{470} with time over the 60 minutes lysis period at all dilution rates. The lysis rate constant increased linearly with increasing dilution rate over the range tested (Fig. 73). The effect of dilution rate on total count and colony count paralleled the E_{470} results (Fig. 74). Good agreement was noted between the change in total count and E_{470} but again the % change seen for colony count following PB treatment was higher than that for E_{470} or total count.

There is disagreement between various workers on the effect of increasing growth rate upon the sensitivity of C-limited P. aeruginosa to PB. The results in this study are in general agreement with those of Dean et al (1976) who used 50 units ml^{-1} PB and studied the % survivors with increasing dilution rate. The % survivors fell from 62% to 0.8% as D increased from 0.05 - 0.5hr^{-1}. However, Finch and Brown (1975) could find no relationship between D (over the range 0.05 - 0.5 hr^{-1}) and ΔE_{470} for cells treated with 5, 15, 30 or 40 units ml^{-1} PB. Ombaka (1980) could also not correlate sensitivity changes to 32.5 units ml^{-1} PB as judged by viable count with changes in dilution rate.

The reasons for this discrepancy is unclear, although growth rate alterations produced by changes in dilution rate have been shown by other workers to affect the sensitivity of P. aeruginosa to 3 and 4 chlorophenol (Gilbert and Brown, 1978b). Faster growing C and Mg-limited chemostat grown cells were more sensitive to these agents than slower growing cells. Cell wall changes have also been noted in glycerol depleted A. aerogenes when cells were grown at different growth rates (Ellwood and Tempest, 1972). The KDO and P content increased with increasing growth rate. These changes suggest alteration in LPS and possibly other P containing envelope molecules such as PL. Since these molecules are receptors for PB it is possible that increasing the LPS and PL content of the cell envelope might result in increasing susceptibility to PB. It has been suggested that an increased LPS content may either hinder or aid drug penetration (Brown, 1975).

A direct comparison of the results of Ombaka (1980) and Finch (1975) with Dean et al (1976) and those of this study is difficult since different amounts of PB were used and also the individual dilution rates reported are

not similar although the ranges overlap. It would appear however that at approximately equivalent D values and PB concentrations the cells used by Finch (1976) and Ombaka (1980) were considerably more sensitive than those of Dean et al (1976). Those used by Finch (1976) and treated with 40 units PB at D = 0.05 gave 80% lysis whereas 48% of the cells survived treatment with 50 i.u. ml^{-1} at the same D value (Dean et al, 1976). Using 32.5 units ml^{-1} PB at all D values Ombaka (1980) obtained a 2 - 5 log fall in colony count. It is possible in view of previous results in this study that the unwashed cell media of Dean et al (1976) contained inhibitors for PB, although in this study washed cells were used. Finch (1976) and Ombaka (1980) used the same resuspension medium which differed considerably in content from their growth medium and may have contributed to the instability of their cells. The resuspension medium used in this study closely resembled the growth medium.

4.3.4. The effect of growth rate on the response of phosphate limited *P. aeruginosa* to EDTA

The cultures used in this study were from the same chemostats as used to study the effects of PB. The response to EDTA was similar to that observed in batch grown cells described previously (Fig. 75). A rapid lysis phase 0 - 30 minutes was followed by a slower phase 30 - 90 minutes Little change was noted between 90 and 180 minutes. The sensitivity to lysis by EDTA increased only slightly as the growth rate increased (Fig. 77). The increase in sensitivity seen over the range D = 0.07 - 0.6 hr^{-1} was less than that observed for PB treated cells. The % lysis increased only 3% (E_{470} values) over the range of D values 0.06 - 0.3 hr^{-1}. The lysis rate constants for the primary lysis phase (0-30 minutes) and the secondary lysis phase (30 - 90 minutes) were dependent upon the dilution rate (Fig. 76). The results for % change in colony count and

total count showed an increase with increased growth rate (Fig. 77). As demonstrated for batch grown cells treated with EDTA close agreement between E_{470} total and colony count was recorded. These results agree with those of Ombaka (1980). She showed only slight changes in sensitivity (as measured by colony count) to EDTA with increasing D value in the range 0.05 - 0.3 hr^{-1}. Increasing the growth rate of P-limited cultures of A. aerogenes from D = 0.1 to 0.7 resulted in a 3 fold increase in cell wall P although the KDO and presumably LPS content did not alter (Ellwood and Tempest, 1972). Although Mg^{2+} content of these cells was not measured, since it is associated with cell wall macromolecules it too might have increased but only slightly. Since EDTA susceptibility has been associated with the P content of the LPS, the structure of LPS and magnesium cation content it is likely that the results reported by Ellwood and Tempest (1972) would have resulted in an increase in sensitivity to EDTA with increasing dilution rate if any change occurred at all. Cell wall changes and sensitivity to EDTA will be discussed later (section 4.5.2.).

4.3.5. The effect of growth rate on the response of carbon limited P. aeruginosa to EDTA

As the dilution rate of C-limited cultures was increased (D = 0.05 - 0.33 hr^{-1}) the cells showed an increased sensitivity to EDTA (Fig. 78). The lysis rate constant was linearly related to dilution rate in the range D = 0.07 - 0.3 (Fig. 79). Good agreement was obtained between E_{470} total and colony count (Fig. 80). The % change in growth parameter with increasing dilution rate was greater for C-limited cells than for the equivalent P-limited cells.

These results agree with those of Finch (1976) who also showed increasing sensitivity of C-limited P. aeruginosa to EDTA as the growth rate increased in the range 0.05 - 0.5 hr^{-1}. C-limited P. aeruginosa showed increased resistance to EDTA as the D value was increased from 0.05 - 0.1 hr^{-1} but a further increase in D value to 0.3 resulted in a sharp increase in sensitivity (Dean et al, 1976). Ombaka (1980) reported 100% survival of C-limited cells as D increased from D = 0.05 - 0.1. Further increases in D resulted in increasing sensitivity. It is likely that these changes may be the result of either an alteration in the Mg^{2+} content of the envelope of C-limited cells as the growth rate changes or a change in the accessibility of the cation to EDTA (Finch and Brown, 1975).

4.3.6. The effect of phosphate in the medium on sensitivity of chemostat grown P. aeruginosa to Polymyxin B

Cells growing in a chemostat may be stabilised at a particular growth rate and are subject to much less variation in cultural conditions than batch grown cells. The nature of chemostat growth, however, does not allow the double limitation of nutrients as used by Boggis (1971) and Brown and Melling (1969a,b,), to study the action of antimicrobial agents. Careful manipulation of the carbon and PO_4^{3-} concentrations allowed cells grown in various levels of PO_4^{3-} from P-limited to C-limited with a large excess of PO_4^{3-} to be studied. A standard dilution rate of 0.17 hr^{-1} was chosen since this gave an adequate yield of cells even under PO_4^{3-} limited conditions.

Fig. 81 shows the relationship between the PO_4^{3-} content of the medium and the % change in E_{470} total and colony count following treatment with 10 units ml^{-1} PB. As the PO_4^{3-} content of the medium increased the sensitivity to PB as measured by E_{470} and total count changes increased.

However the increase seen in colony count with increased PO_4^{3-} was followed by a fall in count at higher PO_4^{3-} concentrations.

The results confirm the findings using batch culture that PO_4^{3-} depletion resulted in an increase in resistance to PB as measured by E_{470} and total count. In the extreme conditions (P-limited or C-limited gross PO_4^{3-} excess) when colony count is the standard measure of sensitivity C-limited cells are more resistant to PB than P-limited cells. The difference in degree of resistance appears to be a reflection of the PO_4^{3-} excess in the medium as was seen in batch work. The results for C- and P-limited cells are in agreement with those of Dean et al (1976) and Ombaka (1980). However, when E_{470} or total count is used as a measure of sensitivity it would appear that P-limited cells are more resistant than C-limited ones.

4.3.7. The effect of phosphate in the medium on sensitivity of chemostat grown P. aeruginosa to EDTA

The sensitivity of cells grown at D = 0.17 hr^{-1} to EDTA increased as the PO_4^{3-} content of the medium was increased to 0.6 x 10^{-4}M. Above this PO_4^{3-} level little change in sensitivity was noted (Fig. 82). Good agreement between the 3 growth parameters was obtained. Thus, as shown with batch cultures increasing the PO_4^{3-} content of the medium resulted in increased sensitivity to EDTA. It is probable, as will be mentioned later, that the cells response to changes in environmental PO_4^{3-} is to alter the cell wall composition and structure. However, increased sensitivity would be expected to result from an increased Mg^{2+} and phosphorus content of the envelope constituents. Dean et al (1976) have shown that at dilution rate 0.1 hr^{-1} C-limited cell envelopes contain 2.6% Mg^{2+} compared with the 1.2% Mg content of P-limited envelopes. Similar changes although less dramatic were seen in this study.

4.4. Changes in cell wall chemical composition associated with changes in media phosphate content

4.4.1. General

The cell envelope chemistry of *P. aeruginosa* has been extensively investigated in terms of protein (Shinnett and Eagon, 1973), LPS (Wilkinson and Galbraith, 1975) and lipid (Hancock and Meadow, 1969) content.

Until recently little work has been done on the chemical nature of isolated inner and outer membranes of bacteria. The use of EDTA/lysozyme to prepare membrane fractions of *E. coli* (Miura and Mizushima, 1968) and *S. typhimurium* (Osborn et al, 1972) was found unsatisfactory for *P. aeruginosa* (Mizuno and Kageyama, 1978) probably due to hypersensitivity of *P. aeruginosa* to EDTA. Booth and Curtis (1977) have developed a membrane preparation technique involving EDTA and using this technique showed three bands (H, L and M). The heavy H band corresponded to the O.M. as judged by density (due to LPS content) and lacked I.M. marker enzyme activity. This preparative method was not entirely satisfactory since a large amount of unresolved membrane (M band) was obtained. Recently other techniques have been reported for the preparation of isolated I.M. and O.M. fractions from *P. aeruginosa* (Mizuno and Kageyama, 1978; Hancock and Nikaido, 1978 and Hancock and Carey, 1979).

The method of Hancock and Carey (1979) was used in this study and gave good separation of I.M. and O.M. fractions (Plate 1) with little contamination between membranes when the modifications to the density gradient conditions described by Cozens and Brown (1981) were adopted. The yield of membrane fractions especially I.M. from 8 litres of cells grown to an E_{470} 0.8 limited the number of tests and sometimes the accuracy of those tests although trends in chemical changes resulting from growth in various levels of PO_4^{3-} were noted.

4.4.2. Phosphorus changes in response to phosphate depletion

As expected reduction of medium PO_4^{3-} content resulted in a fall in O.M. P levels (Table 39). This fall reflects changes in the content of the major P-containing membrane components i.e. PL nd LPS or changes in the P content of LPS. PO_4^{3-} depletion of several Gram-positives, A. aerogenes (Ellwood and Tempest, 1972) and P. aeruginosa (Dean et al, 1976) resulted in a reduction of cell envelope P content.

The results in Table 39 show a significant fall of 45% in the total P content of the O.M. of batch grown cells as the PO_4^{3-} medium content was decreased. P-limited chemostat cells showed a fall of 42% in total P compared with the C-limited cells. The RELP content of the O.M. revealed a similar pattern of variation although more pronounced changes in the P content were seen (Table 39). A fall in RELP of 80% was seen for batch and 60% for chemostat grown cells. The RELP changes represent alteration in the PL content only since LPS lipid is not extracted under the conditions used. The extent of the fall in RELP suggests that the effects of PO_4^{3-} depletion were greatest upon the PL content of the O.M. This was confirmed by subsequent results. The amount of contaminating nucleic acid present in the O.M. was not determined; it has been assumed that if such contamination occurred the extent would be independent of growth conditions.

4.4.3. Lipid changes in response to phosphate depletion

In excess of 90% of the bacterial wall content of PL and glycolipids is found in the membranes (inner, mesosomal and outer membranes). The O.M. of wild type S. typhimurium had a protein:LPS : PL ratio of 1:1:0.3 (Osborn et al, 1972) and the PL:protein ratio is 1.5 - 2 x higher in the O.M. than the I.M. of P. aeruginosa (Mizuno and Kageyama, 1978). The

commonest Gram-negative PL are PE, PG and DPG, PC and PI being rare. The same PL are present in the O.M. and I.M. although the O.M. PG and DPG is lower and the PE content is increased (Lugtenberg and Peters, 1976). The fatty acid (FA) content of bacteria also varies although 15 - 19 carbon chain FA predominate (Rogers et al, 1980). The commonly REL extractable FA of P. aeruginosa are 16:0, 18:1, 16:1, 18:0, 17▽ and 19▽ (Wilkinson and Galbraith, 1975). The FA and PL composition will vary with growth conditions such as growth phase (Wilkinson and Galbraith, 1975) temperature (Marr and Ingraham, 1962) and medium composition (Minnikin and Abdolrahimzadeh, 1974).

PO_4^{3-} limitation produces profound changes in the PL of bacteria. Replacement of polar lipids by P-free lipids has been reported for many species (Johnson et al, 1973; Zywzina et al, 1979; Minnikin et al, 1974 and Minnikin and Abdolrahimszdeh, 1974). The later work is of interest since it reflects the ability of cells grown under limiting conditions to adapt to their environment by changing their polar lipid composition. Chemostat cultures of P. fluorescens showed reduced PE, PG and DPG levels and an increase in an ornithine amide lipid on changing from Mg to P-limited conditions. At a dilution rate of 0.2 hr^{-1} cells were devoid of PL and contained only the ornithine amide lipid and a trace of unidentified lipid. Such results support the hypothesis of interchangeability of certain polar lipids (Wilkinson, 1972 and Minnikin et al, 1972). In this case acidic lipids PG and DPG were replaced by traces of acidic glycolipid and the zwitterionic PE was replaced by the zwitterionic ornithine amide lipid. PO_4^{3-} depletion of P. fluorescens (Dorrer and Teuber, 1977) resulted in the appearance of an ornithine amide lipid and a decrease in PE, PG and DPG in isolated inner and outer membranes. These changes were accompanied by decreased binding of radioactively labelled PB to intact cells and

membrane fractions. The PL content of P B resistant P. aeruginosa was reduced compared to that of PB sensitive strains (Conrad and Gilleland, 1981) and decreases in PL/neutral lipid ratios were related to resistance to gentamicin (Pechey and James, 1974).

The methods used in this study to extract lipids from O.M. fractions were designed to distinguish PL, NL and FA, and polyhydroxybutyrate (PHB). The REL fraction should contain PL, NL and FA, and PHB (if present). Insoluble PHB may be removed from the REL fraction by addition by addition of ether (Brown and Watkins, 1970). NO PHB was detected in either I.M. or O.M. preparations from any limitations studied. Ether removal or subsequent solubilisation of the residue in acetone deposited the PL. Evaporation of the acetone soluble fraction yielded a brown oily residue of FA and neutral lipids which were not quantified or identified further.

The results of analysis of the O.M. show significant PL changes as the medium PO_4^{3-} content altered for both batch and chemostat grown cells (Table 39). Little change was evident in the REL content, however the PL content varied considerably, the pattern being similar to that observed for the REF P analyses (Table 39). Reduction of the medium PO_4^{3-} content resulted in a fall in O.M. PL from 24% for C^-P^+ membranes to 5% for P^-C^+ membranes. This fall was paralleled by an increase in the FA, NL content of the O.M. A similar trend was seen for chemostat grown cells.

The changes in the PL content of the I.M. closely resembled those seen in the O.M. although a smaller change was detected. It is probable that the differences seen between the I.M. batch and chemostat results (Tables 40 and 42) are related more to technical inaccuracies due to extraction losses and weighing problems associated with handling the small amount of I.M. material available for assay than from differences in PL content of the cells. The result of PO_4^{3-} depletion is a change in the PL content

of both the I.M. and O.M. of the cells, although the change in the I.M. was slightly smaller than that seen in the O.M.

The small amounts of material available, especially I.M. fractions, resulted in unsuccessful attempts to identify changes in the individual PL components resulting from growth in the different levels of PO_4^{3-}. Scans of TLC plates following resolution of the individual PL in chloroform: methanol:water (65:25:4) failed to yield accurately measurable traces, although by comparison with authentic standards PE, PG and DPG were identified in most samples. Visually it was apparent that as the PO_4^{3-} content of the medium was reduced the first PL to become undetectable on the TLC plate was PE.

There was insufficient material to determine whether the ornithine amide lipid was present. It is unlikely to have been present as a major lipid in the O.M. preparations studied since its Rf was similar to PE in in the 2 dimensional chromatograms of Minnikin and Abdolrahimzadeh (1974). The single dimensional chromatography used in this study resolved no component with an Rf similar to PE when stained with ninhydrin or phosphate detecting spray (Ames, 1968). Had the ornithine amide lipid been present it would have reacted with ninhydrin only. The samples did, however, contain an unidentified lipid which was associated with DPG. This may represent the lipid X described by Conrad and Gilleland (1981) in the REL of PB resistant P. aeruginosa.

Such changes as seen here in PL content with PO_4^{3-} depletion were to be expected and probably account substantially for the resistance to PB seen in PO_4^{3-} depleted cultures. Batch culture of E. coli in C and PO_4^{3-} depleted media showed similar lipid changes to those observed in this study (Gilbert and Brown 1978a) and in the studies of Minnikin and Abdolrahimzadeh

(1974). Gilbert and Brown (1978a) reported that PO_4^{3-} depleted E. coli whole cells had only 1/3 of the PL content of C-depleted cells. The PL decrease was related to a large fall in PE and DPG content, the levels of PG and PC remaining relatively constant.

4.4.4. Lipopolysaccharide changes in response to phosphate depletion

The LPS content of several strains of P. aeruginosa is similar (Wilkinson and Galbraith, 1975) and has a structure similar to other Gram-negatives, having lipid A and core polysaccharide linked via 2-keto-3-deoxyoctonic acid. LPS of several P. aeruginosa strains represented 17-30% of the whole walls (Wilkinson and Galbraith, 1975). The LPS of P. aeruginosa is unusually rich in P and a range of 3.1 - 5.6% P in LPS has been reported (Wilkinson and Galbraith, 1975). The FA content of various Pseudomonads differs slightly but 3OH myristic acid, which is characteristic of other Gram-negatives, is not found (Fensom and Gray, 1969). 2OH dodecanoic and 3OH dodecanoic acids are the most common FA in the LPS of P. aeruginosa (Wilkinson and Galbraith, 1975).

2 keto-3-deoxymannooctonic acid (KDO), a glycosidic component of the LPS of many Gram-negatives (Ellwood, 1970) has been widely used as a marker of LPS. The KDO content of the wall of P. aeruginosa ls was 0.6% (Ellwood, 1970), and KDO represented 2.3 - 3.4% of the LPS of several strains of P. aeruginosa (Wilkinson and Galbraith, 1975). P. aeruginosa 6750 contained 3.2% KDO in the LPS. Since other 2 keto-3-deoxy sugar acids (e.g. sialic acid) may react with thiobarbituric acid under the test conditions it is important that these acids are either absent from preparations or produce chromophores with negligeable absorbance otherwise falsely high KDO results may occur. For these reasons LPS estimations based on KDO measurements on whole cells were not recommended by Ellwood (1970) and were not used in this study. Cell envelopes and O.M. KDO contents only were measured.

Various factors such as nutrient limitation and growth rate may affect the KDO content of the cell (Dean et al, 1976; Gilbert and Brown, 1978 and Ellwood and Tempest, 1972). Changes in LPS content which may not affect the KDO assay have been shown in LPS mutants of E. coli by Pazoles and Kulpa (1977). PO_4^{3-} depleted P. aeruginosa (Dean et al, 1976) and E. coli K12 (Gilbert and Brown, 1978) had lower KDO levels than C depleted cells indicating a reduction in LPS content. Other changes in LPS core structure may occur since the change in the KDO content of the walls of A. aerogenes shown by Ellwood and Tempest (1972) was accompanied by changes in the heptose content of PO_4^{3-} limited cells. A similar increase in heptose content was shown by Dorrer and Teuber (1977) when P. fluorescens was grown in a PO_4^{3-} depleted medium.

The effect of PO_4^{3-} media changes on the KDO content of P. aeruginosa 6750 are shown in Table 39 for O.M. preparations and isolated cell walls. The results show an LPS content of approximately 20% (Table 39). This figure is similar to that for P. aeruginosa PAO1 (Hancock and Nikaido, 1978). Table 39 shows that the KDO content of the walls was 22 - 27%. It is unlikely that the KDO content in the cell wall should be higher on a weight for weight basis than that obtained for isolated O.M. since LPS resides solely in the O.M. (Smit et al, 1975). These results imply either a loss of LPS in preparation of the O.M. or that the wall fractions contained thiobarbituric acid reactive material. The figures obtained for these preparations are lower than those obtained with strain 6750 by Kenward et al (1979), and Gilbert and Brown (1978b). The LPS content of C-limited whole cells range from 17 - 40% depending on growth rate (Gilbert and Brown, 1978b) and represented 69% of the wall dry weight in the study of Kenward et al (1979). The difference in wall KDO value for C-depleted cells in this study and those of Kenward et al (1979) may reflect differences in wall preparative technique. Ballotini beads were used by Kenward et al

(1979) but the French pressure cell was used in this study. The subsequent washing procedure was similar. The wide variation in these results and those reported for O.M. preparations (Mizuno and Kageyama, 1978) indicate that further studies to elucidate the nature of thiobarbituric acid reactive material are indicated.

The effect of medium PO_4^{3-} reduction here was a loss in LPS as judged by the fall in KDO content of both batch and chemostat grown cells (Tables 39 and 41). No attempt was made to determine whether PO_4^{3-} changes resulted in a fall in the LPS P content or any other core changes.

Variation in LPS content has been used to explain both exclusion and penetration of drugs (Brown, 1975). PB resistant P. aeruginosa showed reduced KDO and carbohydrate content (Gilleland and Lyle, 1979) although other changes were noted which may be as important in PB resistance (Conrad and Gilleland, 1981). Differences in the LPS content including sugar deficiencies in the core oligosaccharides and change in the FA composition of P. aeruginosa K799 and its antibiotic hypersusceptible mutant Z61 have been reported (Angus et al, 1982, Kropinski et al, 1982). Such LPS changes may influence the number of open functional protein F pores in the O.M. and antibiotic susceptibility possibly ionically through charged PO_4^{3-} groups or via hydrophobic interactions with acyl chains on lipid A residues or both.

4.4.5. Divalent cation changes in response to phosphate depletion

Calcium and magnesium are major components of the walls of P. aeruginosa (Eagon, 1962) and are important in maintaining envelope stability by association with anionic cell wall components particularly the PO_4^{3-} group of LPS and PL (Costerton et al, 1974, Cox and Eagon, 1968). Removal

of cations is important in the action of EDTA (Wilkinson, 1975) and a reduction in wall Mg^{2+} content gave increased resistance to PB and EDTA (Brown and Melling, 1969a,b) and aminoglycosides (Nicas and Hancock, 1980).

Little attention has been paid to the effect of PO_4^{3-} depletion on Mg^{2+} and Ca^{2+} levels in the cell wall, although in view of the relationship between cations and P containing cell wall macromolecules some inter-relationship with P content is to be expected. The extent of Mg^{2+} binding to cell wall components will vary with the PO_4^{3-} content of the LPS, with the total amount of PL and the quantitative relationships of the acidic PL (e.g. PG and DPG). The amount of protein H1 in the O.M. may also affect the binding capacity for Mg^{2+} (Nicas and Hancock, 1980). Dean et al (1976) showed that the Mg^{2+} content of P. aeruginosa cell envelope fell 54% on changing from C to P limitation. This accompanied a fall in envelope P content of 58%.

Cell walls of batch grown P. aeruginosa 6750 contained between 0.27 and 0.37% Mg^{2+} (Table 39) which is higher than corresponding figures quoted by Cox and Eagon (1968) but lower than those for C-depleted cells reported by Kenward et al (1979). Mg^{2+} may be loosely bound to cell walls and therefore walls must be washed before estimation of the cell wall Mg^{2+} content. It is unlikely that the slightly higher Mg^{2+} content of the walls in this study was related to failure to remove loosely bound Mg^{2+} since a more intensive wash procedure was used here than that described by Cox and Eagon (1968). It is more likely that differences in media Mg^{2+} composition are responsible for the results.

Cell wall Ca^{2+} analyses (Table 39) revealed a cell wall content of 0.24 - 0.36% for batch grown cells depending upon the PO_4^{3-} excess in the growth medium. These figures agree with those for C-depleted P. aeruginosa reported by Kenward et al (1979).

The reduction in the medium PO_4^{3-} content resulted in a decrease of 23% and 6% for Mg^{2+} and Ca^{2+} respectively in batch grown cells (Table 39). P-limited chemostat cultures showed a lower cell wall Mg^{2+} level than C-limited cells and the observed change of Mg^{2+} was lower than that for batch grown cells (Table 41). P^-C^+ cells contained 6% less Ca^{2+} than C^-P^+ cells (Table 39). A similar difference in wall Ca^{2+} level was seen between P- and C-limited chemostat grown cells (Table 41).

Although the wall Ca^{2+} and Mg^{2+} content fell with PO_4^{3-} reduction in the media the loss of Mg^{2+} was always greater than that of Ca^{2+}. Such PO_4^{3-} changes have in this study been shown to lead to increased resistance to PB and EDTA. Interestingly Brown and Watkins (1970) reported that P. aeruginosa trained to grow in 50 u PB showed a decreased wall Mg^{2+} content and P content although the Ca^{2+} content remained constant compared with sensitive strains. Cells made resistant to PB by growth under Mg^{2+}-limiting conditions showed the expected fall in Mg^{2+} but the wall Ca^{2+} level altered only slightly. Major changes in cation content, however, have been reported by Conrad and Gilleland (1981) when PB resistant and sensitive strains were compared. Increases in the Ca^{2+} and Mg^{2+} content of cells was associated with an increase in the P content (Boggis, 1971). However, as the Ca^{2+} and Mg^{2+} levels increased the ratio of P:total cations decreased suggesting no strict inter-relationship between P and the divalent cation content of the cell wall. PO_4^{3-} depletion may result in production of a cell wall where cross linking by divalent cations is less necessary for stability than in walls of cells grown in P-rich media. The greater reduction in Mg^{2+} than Ca^{2+} resulting from P-depletion may imply stronger binding of Ca^{2+} to the wall components.

4.4.6. Protein changes in response to phosphate depletion

The I.M. and O.M. of P. aeruginosa are rich in protein. The I.M. of P. aeruginosa contained more protein than the O.M. (Booth and Curtis, 1977; Hancock and Nikaido, 1978) although the reverse relationship was shown by Mizuno and Kageyama (1978). The protein content of each membrane was approximately 50% in these studies and the I.M. and O.M. fractions in this study also contained approximately 50% protein (Tables 39,40,41 and 42).

The O.M. of P. aeruginosa contains at least 8 major proteins (D1, D2, E, F, G, H1, H2 and I), although the number will vary depending upon growth conditions, protein solubilisation conditions and the gel system used (Hancock and Carey, 1979). SDS PAGE reveals a large number of protein bands in the I.M. (Mizuno and Kageyama, 1978). Since growth, preparative and test conditions greatly affect the characterisation of these proteins it is not surprising that workers have resolved different numbers of O.M. proteins and ascribed to them differing molecular weights. Using 14% acrylamide gel Hancock and Carey (1979) resolved proteins D and H described by Mizuno and Kageyama (1978) into D1 and D2 and H1 and H2. A summary of the composition and apparent molecular weights of O.M. proteins as described by 5 research groups has been given by Hancock and Carey (1979) and is shown in Table 2. Several O.M. minor proteins are inducible and under inducing conditions proteins not normally considered major proteins may attain major proportions.

Several O.M. proteins of m.wt. 30K - 40K are "heat modifiable" (Di Rienzo et al, 1978; Hancock and Carey, 1979). Thus the temperature of protein solubilisation by SDS affects the number of protein bands and their positions on SDS electrophoretograms. Protein F (33K) was converted to protein F^{*} (37K) either by boiling for long periods in SDS or trichloroacetic acid treatment (TCA) (Mizuno and Kageyama, 1978). Heat treatment

of O.M. or purified protein F at 88°C or less for 10 minutes resulted in all the porin remaining in the F form (Hancock and Carey, 1979) but treatment at 100°C for 50-60 minutes resulted in conversion of most of the protein to the F^* form. P. aeruginosa proteins D1, D2, H2 and G are also heat modifiable (Hancock and Carey, 1979). In the present study the recommendations of Hancock and Carey, (1979), were closely followed. Membrane fractions were solubilised in SDS mercaptoethanol for 10 minutes at 100°C and run on 14% acrylamide gels.

Proteins F, H2 and I are noncovalently bound to peptidoglycan (Hancock et al, 1981). Protein I (9-12K) of P. aeruginosa is probably equivalent to the Braun lipoprotein of E. coli (m.wt. 7.2K) (Mizumo and Kageyama, 1978 and Hancock et al, 1981a).

Variation in growth conditions will affect the protein composition of the O.M. Although growth temperature (30 - 42°C) had no effect on the protein pattern of P. aeruginosa (Mizumo and Kageyama, 1978) it did affect the amount of protein b and c produced by E. coli (Bassford et al, 1977). The relative amounts of protein 1a and 1b of E. coli K12 was dependent upon the growth medium (Bassford et al, 1977). Osmolarity of the growth medium also affects the relative amounts of proteins produced (Van Alpen and Lugtenberg, 1977).

Growth of P. aeruginosa PAO1 on glucose minimal media induced the production of proteins D1, D2 and E (Hancock and Carey, 1979). Iron limitation resulted in induction of high m.wt. (70-80K) iron binding proteins in P. aeruginosa (Mizuno and Kageyama, 1978). Mg^{2+} was replaced by protein H1 under Mg^{2+} limiting conditions and resulted in increased resistance to EDTA, PB and gentamicin (Nicas and Hancock, 1980). Under P-limiting conditions E. coli K12 showed induction of the peptidoglycan associated protein e (Overbeeke and Lugtenberg, 1980). This pore is not

specific for PO_4^{3-} but is probably synthesised as a PO_4^{3-} scavenging mechanism under conditions of reduced PO_4^{3-}. Hancock et al (1982) reported that under conditions of low PO_4^{3-} P. aeruginosa produces an O.M. protein P which functions as a pore for inorganic anions and PO_4^{3-}.

In this study a standard method of preparation of the membranes for gel electrophoresis was used and for comparative purposes molecular weight standards and an O.M. preparation from P. aeruginosa PAO1 was included on the gels (Plate 2). P. aeruginosa PAO1 has a well characterised O.M. protein pattern but to date the O.M. protein pattern for strain 6750 has not been reported.

Electrophoresis of I.M. preparations (not shown) from cells grown in different PO_4^{3-} conditions gave similar results showing a large number of protein bands with slight evidence in some preparations of characteristic O.M. proteins contaminating the specimens.

In O.M. preparations (Plate 2) proteins with electrophoretic mobilities equivalent to those designated D1, D2, E, F and I in strain PAO1 (Hancock and Carey, 1979) were present in strain 6750 under all growth conditions. However, G, H1 and H2 were barely detectable in chemostat grown cells under P-limitation. In chemostat grown cells under C-limitation G and H2 were also virtually undetectable whereas H1 was present in relatively large amounts. Proteins G, H1 and H2 were detected in batch grown cells at all PO_4^{3-} concentrations used.

A cluster of high molecular weight proteins (65-80K) were present under all conditions but in varying amounts. These proteins were present in lowest amounts in P-limited chemostat grown cells and in highest amounts in C-limited chemostat cells. Although the molecular weights of this protein cluster are in the same range as the iron binding proteins (Mizuno and

Kageyama, 1978) it seems unlikely that they all represent iron binding proteins since iron was present in excess in all media and cells were grown to the same E_{470} under all growth limiting conditions. Their precise nature and function in the cell remains obscure. It would seem that proteins H2 and G were not synthesised under certain growth conditions and are presumably not essential under these conditions.

The amount of protein F did not vary greatly under the different growth conditions used in this study. Protein D1 was present in high concentration under all growth conditions. Since this protein can act as a pore for glucose its presence is to be expected in cells grown in glucose containing CDM. Proteins D1 and D2 were resolved into separate bands under the conditions used. No protein of equivalent molecular weight (48K) to protein P of P. aeruginosa (Hancock et al, 1982) or e of E. coli (Overbeeke and Lugtenberg, 1980) were detected under any of the nutritional conditions used in this study.

Although no clear changes in O.M. protein composition could be related to the amount of PO_4^{3-} in the medium variation in the amounts of certain proteins especially the reduction in G, H1 and H2 under P-limited chemostat growth is of interest. The relationship between individual proteins can vary under different nutrient conditions whilst the total amount of protein in the O.M. remains essentially constant.

4.5. Correlation of alteration in cell envelope chemistry produced by growth in media containing various amounts of phosphate and resistance to Polymyxin B and EDTA

4.5.1. Resistance to Polymyxin B

The polymyxins interact initially with the O.M. of Gram-negatives and bind to O.M. components (Storm et al, 1977). It is likely that the receptor function of LPS is the lipid A moiety (Bader and Teuber, 1973). Although there is evidence that PL may be important in uptake of PB there is disagreement whether a single PL and if so which one, or a combination of PL are involved (Teuber, 1973, Hsu Chen and Feingold, 1973).

The method of penetration of PB remains uncertain. Two hypotheses have been put forward. Hartmann et al (1978) and Sixl and Galla (1979a) propose insertion of the hydrophobic tail of PB into the hydrophobic region of the O.M. whilst the charged hydrophilic moiety lies on the O.M. surface. Conrad and Gilleland (1981) suggest that subsequent uptake may then be via porin proteins although no mechanism was suggested by these authors. If such a mechanism was functional in PB sensitive organisms it would presumably involve penetration of the hydrophilic PB moiety into the porin channel. This would only occur when the hydrophobic interaction between the PB tail and the O.M. had been overcome. Alternatively El Mashek and Tocanne (1980) suggest the whole of the PB molecule penetrates the lipid bilayer causing disorganisation of the membrane and further penetration.

Interaction of PB with the PL of the I.M. (Storm et al, 1977 and Teuber and Bader, 1976b) and subsequent disruption of the osmotic equilibrium and leakage of cell contents (Newton, 1956; Teuber, 1974) has been assumed to account for the antimicrobial action of PB. However,

PB linked to agarose beads (Storm et al, 1977) cannot penetrate the O.M. but nevertheless had antimicrobial action against cells, implying that the binding of PB to the O.M. may be sufficient to cause disruption of the O.M. and loss of osmotic stability and leakage of cellular contents.

Resistance of PB appears to be due to an exclusion mechanism, the drug being excluded from the I.M. by the O.M. (Brown, 1975). This has stimulated much chemical (Brown and Watkins, 1970; Gilleland and Lyle, 1979; and Conrad and Gilleland, 1981) and ultrastructural (Gilleland and Murray, 1976) work into differences between resistant and sensitive cells. Comparison between PB sensitive wild types and PB resistant mutants have been made (Vaara et al, 1979) as well as comparisons between P. aeruginosa and naturally occurring P B resistant strains including Proteus spp (Brown and Wood, 1972). Strains made resistant by nutrient depletion (Kenward et al, 1979) as well as those trained to grow in high PB concentrations and resistant clinical isolates have also been studied (Conrad and Gilleland, 1981).

It must not be forgotten, however, when comparing O.M. changes in P.B. resistant organisms that the bacterial O.M. is a dynamic structure and depletion or loss of one chemical component may result in widespread compensatory changes in other components which do not necessarily imply drug resistance but merely an adaptation of the organism to survive in its surroundings. Conrad and Gilleland (1981) point out that care should be taken when extrapolating results from enteric organisms to P. aeruginosa since P. aeruginosa has been shown to have larger pore exclusion limits than other enteric organisms (Hancock et al, 1979) and also has more PL exposed on the outer surface (Nikaido and Nakae, 1979).

Although there is little evidence to support the role of proteins in PB reception or uptake there is conflicting evidence about the role of proteins in resistance. PB has a low molecular weight and if it is taken up by a porin mechanism then changes in O.M. protein composition may be important. Gilleland and Conrad (1980) were unable to correlate the degree of resistance to PB of P. aeruginosa 015 with total protein content or loss of individual O.M. proteins and they suggested lipid changes were more important. Resistance to PB due to loss of O.M. protein is supported by the ultrastructural and chemical studies of Gilleland and Murray (1976) and Gilleland and Lyle (1979). Freeze etching revealed structural difference in O.M. particle numbers and arrangement when cells trained to grow in 6000 units PB ml^{-1} and cells made resistant by growth in Mg^{2+} depleted media were studied (Gilleland and Murray, 1976 and Gilleland, 1977). Polymyxin resistance was associated with the loss of 3OM proteins of m.wt. 24K, 36.5K and 47K (Gilleland and Lyle, 1979). It is interesting to note that a 36K protein in P. aeruginosa is a major porin protein (Hancock et al, 1979). These strains with loss of protein were slower growing and more resistant to antibiotics (characteristics expected of porin deficient strains) than strains not showing protein loss (Conrad and Gilleland, 1981). The production of protein H1 by mutants of P. aeruginosa or cells grown under Mg^{2+} conditions showed increased resistance to antibiotics including PB (Nicas and Hancock, 1980).

No changes in protein pattern associated with PO_4^{3-} depletion were seen in this study which could clearly be correlated with altered resistance to PB.

Storm et al (1977) have suggested that the initial action of PB is the displacement of Mg^{2+} and/or Ca^{2+} from cell walls, although PB does not chelate these cations (Newton, 1956), and reduction in cell envelope

cations may be important in resistance to PB. C/Mg depleted P. aeruginosa were more PB resistant than C/Mg plentiful cells and the former cells showed a decreased cation content Kenward et al (1979). PB resistant Pr. vulgaris had lower Ca^{2+} and Mg^{2+} contents than P. aeruginosa although the wall cation content correlated less well for PB sensitive and resistant K. aerogenes strains studied (Brown and Wood, 1972). The envelopes of PB resistant P. aeruginosa contained 60-80% less Mg^{2+} and 40-69% less Ca^{2+} than those of PB sensitive strains (Conrad and Gilleland, 1981). Reversion of PB resistant strain to sensitivity was accompanied by almost complete reversion to normal cation levels. In this study a reduction of Mg^{2+} and to a lesser extent Ca^{2+} was detected following PO_4^{3-} depletion. Since PO_4^{3-} depletion was associated with increased resistance to PB these cation changes may play a part (although probably minor) in this increased resistance.

Lipid alterations may affect the sensitivity of Gram-negatives especially P. aeruginosa to PB, although Sud and Feingold (1970) reported that the lipid composition of PB sensitive and resistant strains of Pr. mirabilis were similar. However, they did not distinguish between wall and cytoplasmic membrane lipids. PB resistant Pr. vulgaris and K. aerogenes had low wall lipid contents (Brown and Wood, 1972) and replacement of I.M. and O.M. PL byornithine amide lipids resulted in resistance of PO_4^{3-} depleted P. fluorescens to PB (Dorrer and Teuber, 1977).

The REL content of resistant P. aeruginosa was increased although the PL content was decreased (Conrad and Gilleland, 1981). These authors also reported that the REL contained increased unsaturated FA and decreased cyclopropane FA compared to sensitive strains. This increased REL content may, however, by compensatory for losses of LPS and protein from the O.M.

Such compensatory changes were reported for S. typhimurium by Smit et al (1975). The PB resistant strains examined by Conrad and Gilleland (1981) had reduced PG and PE levels and this would be expected to reduce the ability of the O.M. to bind PB. Similar changes were reported by Dorrer and Teuber (1977). Liposome preparations containing PE were sensitive to PB (Hsu Chen and Feingold, 1973) and Teuber (1973) demonstrated stable complex formation between PB and PG but not with PE.

In this study major changes in O.M. PL content were seen on reducing the PO_4^{3-} content of the medium for both batch and chemostat grown cells, although no major change in REL was noted. Although no quantitative analysis of the individual O.M. PL was possible the results suggested that the PL replacement was accompanied by a preferential loss of PE. It is likely that the major changes in PL content seen in this study can account for the increased resistance to PB seen as the medium PO_4^{3-} content was reduced.

Analysis of cell walls of PB resistant P. aeruginosa by Brown and Watkins (1970) showed that the lower P content of the cell walls of resistant strains following extraction of REL with chloroform/methanol was associated with low LPS content. 25% of the LPS content of the walls of P. aeruginosa was lost in association with acquisition of PB resistance (Gilleland and Lyle, 1979). Since the LPS especially the lipid A moiety has been shown to be important in binding of PB such losses or changes in the structure of the core lipid A as reported by Vaara et al (1981) for S. typhimurium and Kropenski et al (1982) for P.aeruginosa resulted in altered sensitivity to PB.

In this study a reduction in LPS (judged by KDO changes) occurred in both wall and O.M. preparations as the PO_4^{3-} content of the medium was reduced. It is possible that the accompanying fall in P content may have resulted in an altered LPS structure and such changes would be compatible with increasing PB resistance.

4.5.2. Resistance to EDTA

The mode of action of EDTA upon the O.M. of Gram-negatives has been reviewed by Brown (1975), Wilkinson (1975) and Leive (1974). The initial action of EDTA appears to be the chelation of divalent ions and the work of Brown and Melling (1969a,b) suggests that Mg^{2+} may well be the most important cation involved. Cation extraction is rapidly followed by partial solubilisation of the cell envelopes of sensitive bacteria. In P. aeruginosa this solubilised complex contains approximately 60% protein 30% LPS and 10% loosely bound lipid as demonstrated by ultra-structural and chemical methods (Gilleland et al, 1973; Rogers et al, 1969 and Roberts et al, 1967). Analysis of the proteins released in this complex showed it to contain proteins D1, D2, E, G and H1 (Hedstrom et al, 1981). These results do not agree with those of Hancock et al (1981) who could detect little protein except E. This result may reflect the use of a 10X higher EDTA concentration by Hancock et al (1981) which may have caused extensive cellular damage and a dilution of the proteins by I.M. and cytoplasmic proteins. Solubilisation does not involve peptidoglycan and usually the permeability of the I.M. remains unimpaired (Wilkinson, 1975).

The dissociation of the O.M. may be explained if the extracted cations are not considered merely as counter ions to anionic components but as structural component stabilising cross bridges between protein, lipid, LPS and other molecules. A possible secondary event in dissociation

of the O.M. is the activation by EDTA of phospholipase enzymes (Hardaway and Buller, 1979) which will further breakdown membrane phospholipids.

The reasons for the increased susceptibility to EDTA of P. aeruginosa have been reviewed by Wilkinson (1975). In general P. aeruginosa contains less lipoprotein than other less sensitive Gram-negatives (Martin et al, 1972) suggesting other molecules may assume increased importance in membrane stabilisation of P. aeruginosa. The release of a Pr LPS PL complex also suggests that the specificity of EDTA may depend upon other components. Metal cations are associated with membrane proteins and lipids (Razin, 1972) and Mg^{2+} is associated with PL in the envelope (Gordon and Macleod, 1966 and Brown and Watkins, 1970). However, under non limiting conditions the lipid content of P. aeruginosa is not greatly different from other Gram-negatives (Hancock and Meadow, 1969). Thus PL is probably not a key molecule in the action of EDTA. The LPS of Pseudomonas aeruginosa is especially rich in P (Gray and Wilkinson, 1965b). The P is present in both lipid A and polysaccharide portions of LPS. P. aeruginosa and P. alcaligenes were more sensitive to EDTA than other Pseudomonas spp and contained more P and more easily EDTA extractable P than the later species (Wilkinson, 1970). The presence in the core polysaccharide of polyphosphate residues (Drewry et al, 1973) with strong metal binding capacity would also support the role of Mg^{2+} and PO_4^{3-} binding being important in stabilisation of the O.M. and also explain the increased susceptibility of P. aeruginosa to EDTA. The replacement of Mg^{2+} at its binding site on the LPS by either protein H1 (Nicas and Hancock, 1980) or polyamines (Drewry et al, 1972) may also explain the resistance of Mg^{2+} depleted cultures to EDTA. The importance of LPS lipid A changes and resistance to EDTA has been shown in S. typhimurium by Vaara et al (1981). The S. typhimurium pmr A mutation does not affect the diffusion of hydrophobic

or hydrophilic molecules through the O.M. However, the pmr A mutants were more resistant to EDTA induced permeability changes than wild type strains. Replacement of the PO_4^{3-} by 4 amino 4 deoxy Larabinose (4 ARAN) to approximately 60-70% in the pmr A mutants (Vaara et al, 1981) may mean that the involvement of Mg^{2+} in the stabilisation of the O.M. of these mutants was reduced resulting in increased EDTA resistance.

It is unlikely that all the changes associated with growth in media of decreased PO_4^{3-} content in this study can be related to the resistance seen to EDTA. Reduction in O.M. P content was demonstrated as the medium PO_4^{3-} was reduced (Table 39). Analysis of the RELP and PL changes (Table 39) suggest that the major response to PO_4^{3-} reduction was a decrease in PL although a fall in LPS content was also indicated. Although PL have not been shown to be important directly in the action of EDTA changes in the total PL content and the ratios of the individual PL will result in charge alterations and altered binding properties of the components of the O.M. particularly cations. The reduction in LPS content of the O.M. seen in this study may have been accompanied by changes in the structure of the LPS in response to PO_4^{3-} depletion. Such changes would be expected to result in increased resistance to EDTA.

Accompanying the loss of P and other changes in the O.M. P containing macromolecules was a reduction in the Mg^{2+} wall content. A reduction in wall Mg^{2+} has been shown to be associated with an increased resistance to EDTA (Kenward et al, 1979) and whilst the Mg^{2+} content did not fall in PO_4^{3-} depleted cultures studied here as much as it did in the Mg^{2+} depleted cultures studied by Kenward et al (1979) it is likely that the 23% fall in Mg^{2+} content was partly responsible for the resistance to EDTA noted here.

No protein changes which could be associated with altered sensitivity to EDTA were seen in this study.

4.6. Concluding Discussion

The aim of this study was to produce a chemically defined medium for growth of P. aeruginosa which would permit limitation of any medium constituent whilst all other nutrients could be present in a controlled excess. Such limited cells could then be used for a variety of studies including the testing of their susceptibility to antimicrobials. Since the work of Boggis (1971) several workers (Kenward, 1975; Finch, 1976 and Ombaka, 1980) have studied limitation of one or more of the individual constituents of the medium but until the present study limitation of all the individual constituents in a complete medium had not been studied. Boggis (1971) varied the composition of his medium slightly depending upon the nutrient limitation under study and also did not include Fe^{2+} in many of his limitation studies thus iron limitation may have intervened in several of the other limitations he studied. The relationship between E_{470} and nutrient concentration was often only studied to low E_{470} values and the maximum linear E_{470} onset or max value was not determined nor was the maximum yield of cells (as judged by E_{470}) for any particular nutrient determined by Boggis (1971).

The aim of formulation of a single medium to study all limitations was thwarted by the utilisation of MOPS buffer as a source of S and this made reformulation of the medium necessary for study of SO_4^{2-} depletion. Acid ammonium phosphate buffer was used to replace MOPS when SO_4^{2-} depletion was investigated.

The quantification of the N, P, K, Mg, Fe, C, SO_4 requirements of P. aeruginosa 6750 in this study enabled the effect of PO_4^{3-} depletion upon sensitivity to PB and EDTA to be investigated. Such sensitivity was determined for PB by two methods i.e. that of drug treatment in the

original growth medium and drug treatment following harvesting, washing and resuspension in CDM lacking glucose. Experiments showed that various factors such as the number of centrifugations, the growth medium pH over the range 6.6 - 7.4 and the pH of the resuspension medium over the range 6.75 - 7.5 had little effect on the stability of the cells or the amount of subsequent lysis following treatment with PB. However, the resuspension medium PO_4^{3-} composition and the amount of excess nutrients present had considerable effect on the stability of the cells following washing and harvesting. Cells grown in PO_4^{3-} rich medium became very unstable during the equilibration period prior to lysis and were more susceptible. to lysis if washed and resuspended in CDM lacking glucose and PO_4^{3-}. The use of 2.5×10^{-4}M PO_4^{3-} in the washing and resuspension medium resulted in stabilisation of the washed cells. The instability of cells caused by changed in medium seen in this study and changes in the test situation as described by others (Farwell and Brown, 1971), reinforce the decision to use a resuspension medium of formulation as close as possible to that of the growth medium such that cell stressing was minimal. The minimisation of such stress factors was considered to outweight the criticism of the use of a complex resuspension medium containing divalent cations which might influence the subsequent results of lysis by PB and EDTA (Newton, 1956 and Wilkinson, 1975).

The effect of a reduction in the PO_4^{3-} content of the growth medium upon subsequent resistance to PB was shown to be dependent not only upon the PO_4^{3-} content of the medium but on the PB concentration used. The effect of PB concentration upon the sensitivity pattern of limited cells had not been thoroughly investigated before this study. The study showed that the optimum concentration of PB used should be determined for each limitation and it should not be assumed that the concentration optimum for lysis of Mg^{2+} depleted cells will be optimal for PO_4^{3-} depleted cells.

The results showed that for concentrations of PB up to 40 units ml^{-1} cells became more sensitive to PB and the growth medium PO_4^{3-} content was increased to $0.16 \times 10^{-4}M$. For PB concentrations above 40 units ml^{-1} the amount of PO_4^{3-} necessary to detect such resistance changes was too low to permit growth to an acceptable E_{470} prior to drug treatment. Above a PO_4^{3-} level of $0.16 \times 10^{-4}M$ cells apparently became more resistant to PB. This resistance was apparent when E_{470} total and colony count was used as the parameter of sensitivity. This apparent resistance was not dependent upon the presence of excess PO_4^{3-} in either the growth medium or the washing and resuspension medium, but may have been related to macromolecules released into the growth medium or O.M. protein changes. The inactivation of PB by macromolecules released at high media PO_4^{3-} concentrations was possibly responsible when 10 units ml^{-1} of PB were used but was not considered to be relevant at higher PB concentrations. Cells grown in high PO_4^{3-} concentrations lost significantly more LPS (as judged by KDO content) into the growth medium than cells grown in lower PO_4^{3-} concentrations. Since PB is known to bind to LPS it is possible that competition between medium LPS and O.M. LPS for PB occurs, further reducing PB activity. This was supported by two experimental findings (1) that when PB was incubated with spent medium prior to addition of cells and subsequent lysis the observed lysis was less than without preincubation and (2) when clean resuspension medium was used the increase in resistance with increasing PO_4^{3-} concentration was less than that observed in unwashed cells.

As the PO_4^{3-} content of the growth medium was increased the resistance of cells to EDTA decreased although unlike PB treated cells no increase in EDTA resistance was seen at even higher PO_4^{3-} concentrations signifying that if cell wall site(s) changes associated with the higher PO_4^{3-}

concentration did occur they were of less importance in resistance to EDTA than PB.

The growth characteristics of C and P-limited chemostat grown cells were determined over a range of growth rates. Whilst C-limited cultures followed closely the curves obtained by Ellwood and Tempest (1967) the P-limited cells showed poor correlation with their curves for P-limited A. aerogenes. No reason for this behaviour was found but they were not O_2 or trace element limited suggesting the results are probably satisfactory.

Phosphate limited chemostat cultures showed increasing sensitivity to PB as the growth rate increased, a finding in general agreement with that of Dean et al (1976) but in disagreement with Ombaka (1980). However, in the latter case control cells were extremely sensitive suggesting that the extreme sensitivity to PB was in part related to the instability of untreated cultures possibly arising from washing and harvesting procedures. Carbon limited chemostat cultures showed increasing sensitivity to PB as the growth rate increased. This was not in agreement with the work of Finch (1976) who failed to show any change in sensitivity to PB with increasing growth rate.

Little change in sensitivity to EDTA was noted as the growth rate of P-limited cells was increased. Increasing sensitivity was seen when the growth rate of C-limited chemostat grown cells was increased, a finding also shown by Finch (1976).

The effect of increasing PO_4^{3-} concentration in the chemostat growth medium was to increase the cells sensitivity to PB when E_{470}, total and colony count was used as sensitivity parameter. However at a PO_4^{3-} level of 0.16×10^{-4}M the sensitivity to PB decreased when the colony count was

the parameter used but not when E_{470} or total count was used, suggesting that the cells were dead but not lysed. The reason for this behaviour remains unclear. Increased sensitivity to EDTA was also noted with increasing PO_4^{3-} content in the medium.

The effect of growth in decreasing amounts of PO_4^{3-} in both batch and chemostat experiments was to produce a reduction in the P content, the RELP the PL and KDO content of the O.M. Cell wall Mg^{2+} and the KDO content also fell but wall Ca^{2+} content fell only slightly. Quantitatively O.M. and I.M. proteins remained constant. PO_4^{3-} depletion also resulted in a lower I.M. PL content, although the change in PL content was slightly lower than that observed in the O.M. Some or all of these changes may have been responsible for the increased resistance to EDTA and PB as the PO_4^{3-} content of the growth medium was reduced. The major changes in O.M. and I.M. PL content associated with PO_4^{3-} depletion in the study are similar to those reported in E. coli by Gilbert and Brown (1978). The reduction in PL content was accompanied by an increase in the FA and neutral lipid content of the O.M. Insufficient material was available for determination of changes in individual PL although the first to disappear on the TLC plates was PE. These changes in PL and LPS content or composition were possibly responsible for increased resistance to PB. Changes in Mg^{2+} and LPS content and possibly LPS P content associated with reduced medium PO_4^{3-} levels may have been responsible for the observed EDTA resistance.

Changes in sensitivity to PB and to EDTA of batch and chemostat grown cells were associated with changes in the PO_4^{3-} content of the growth medium. These changes were accompanied by chemical changes in the isolated membranes or cell wall fractions. Some of these changes are likely to have resulted in changes in drug sensitivity.

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