THE ROLE OF SULPHATE IN THE RESISTANCE OF *PSEUDOMONAS AERUGINOSA* TO ANTIBACTERIAL AGENTS

A thesis submitted by

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TO MY FAMILY AND FRIENDS

THE UNIVERSITY OF ASTON IN BIRMINGHAM

The role of sulphate in the resistance of Pseudomonas aeruginosa to antibacterial agents.

by David William Miveld

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P. aeruginosa was grown in batch culture in a chemically defined medium and cells depleted of glucose were compared with cells depleted of sulphur in the presence and absence of glucose.

During sulphur depletion in the presence of glucose 260nm - absorbing compounds appeared in the medium, the amount being dependent on the sulphur source. The parallel increases in total and viable counts and the failure of potassium to accumulate outside the cell indicates that the appearance of 260nm - absorbing compounds was not due to cell damage. Inhibition by arsenate suggested that glucose was required as an energy source.

Sulphur-depleted cells in the presence of glucose are resistant to the action of EDTA whether measured by release of 260nm - absorbing compounds, death or lysis. Sulphur-depleted cells in the absence of glucose and glucose-depleted cells are sensitive to its action.

Sulphur-depleted cells in the presence of glucose, unlike glucose-depleted cells, are resistant to the lytic action of polymyxin. However, the polymyxin-induced release of 260nm - absorbing compounds and its bacterial action are increased by sulphur depletion. These latter effects are independent of glucose and, unlike its lytic action are increased by increasing the pH of the test menstruum.

Sulphur depletion was associated with increased amounts of outer membrane proteins E and G but no changes in divalent cations were identified. Other variations in outer membrane composition such as lipids and phophate would not appear to be sufficient to account for the varying sensitivities of these nutrient-depleted cells. The requirement of glucose for resistance to the lytic actions of EDTA and polymyxin and the partial restoration of sensitivity by arsenate, would suggest that in these cells energy is required to maintain an outer-membrane structure which prevents drug-induced lysis.

Key words: Pseudomonas aeruginosa, sulphur-depletion, outer membrane, EDTA, polymyxin.

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ABBREVIATIONS

CDM	chemically defined medium
CM	cytoplasmic membrane
DAP	2,5 - diaminopimelic acid
DPG	diphosphatidylglycerol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-bis-(β-aminoethylether)-N:N-tetracetic acid.
EMS	ethyl methane sulphonate
KDO	2-keto-3-deoxyoctonic acid
LPS	lipopolysaccharide
MOPS	3-N-morpholinopropane sulphonic acid
OM	outer membrane
PAGE	polyacrylamide gel electrophoresis
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PL	phospholipid
PB	polymyxin B
REL	readily extractable lipid
RELP	readily extractable lipid phosphate
SAM	S-adenosylmethionine
SDS	sodium dodecylsulphate
TCA	trichloroacetic acid
Tris	tris (hydoxymethyl)-aminomethane buffer
FA	Fatty acid

1. INTRODUCTION

1.1 The Gram-negative envelope

1.1.1 Introduction

The cell envelope of a Gram-negative bacterium is a unique and complex structure, through which a bacterium reacts with its environment. The envelope consists of a number of layers whose chemical composition and physical nature differ markedly. The envelope structure shown in Fig.1 described by Costerton and Cheng (1975), although somewhat outdated in view of recent advances (Nikaido and Nakae, 1979), still serves as a useful model for an appreciation of the basic structure of the envelope.

The cell envelope includes the cytoplasmic membrane (CM), the rigid peptidoglycan complex, the periplasmic space, the outer membrane (OM) and other layers external to the OM. These layers are discrete but there are several hundred zones of adhesion between the two membranes (Bayer, 1979).

The cell envelope is an extremely plastic structure and radical changes in its composition occur in response to changes in the environment (Brown, 1975).

1.1.2.1 Composition and structure of the cytoplasmic membrane

The CM is located between the cell wall and the cytoplasm. Osborn *et al.* (1972b) separated the CM from the OM and found its composition to be two-thirds protein and one-third phospholid (PL). Freeze-etching studies have shown that the frozen CM cleaves along a median hydrophobic zone (Nanninga, 1970). Particles 5-10nm in diameter are seen in one half of the PL bilayer and available evidence suggests they represent intramembranous proteins (Salton and Owen, 1976).

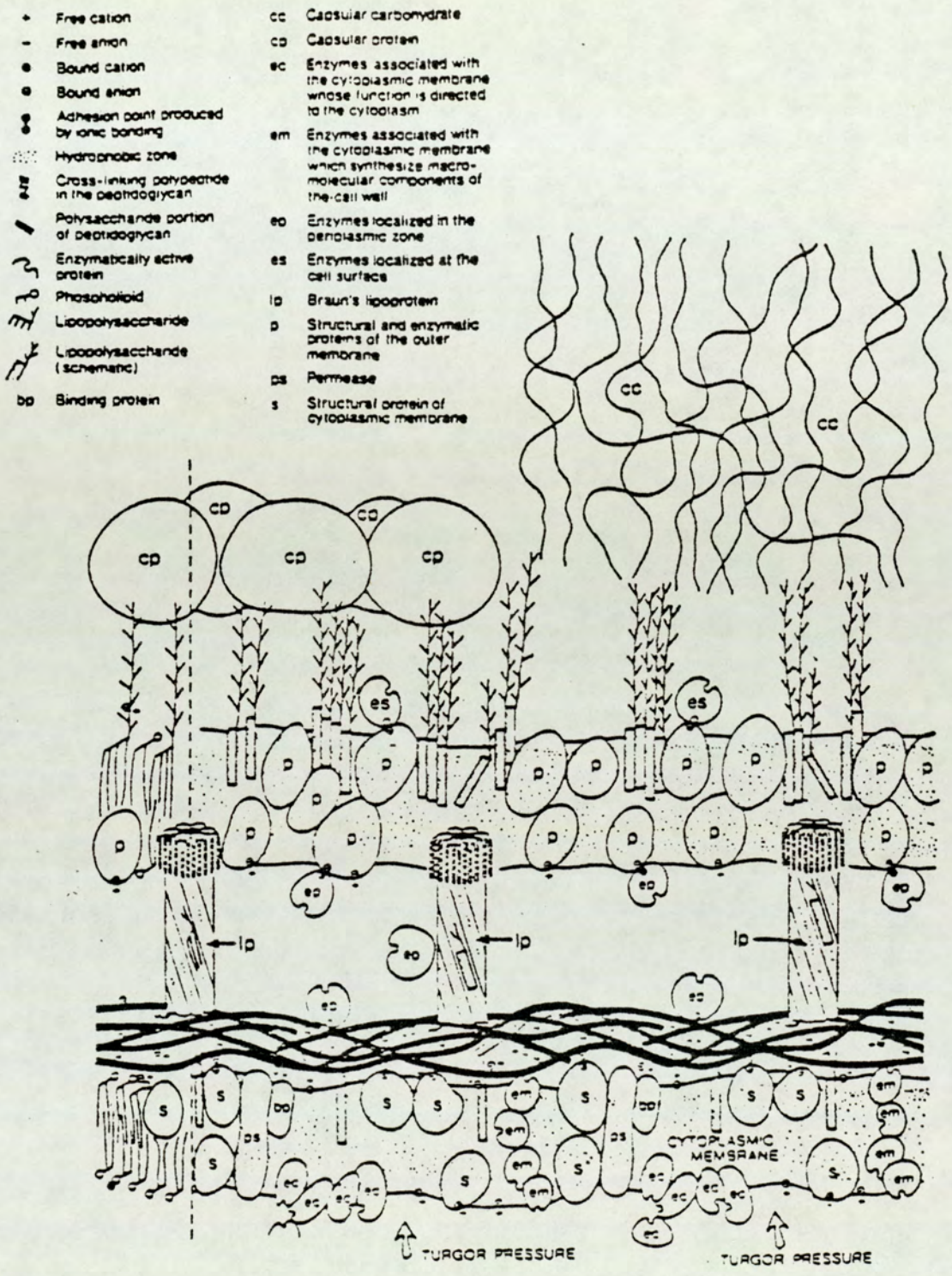


Fig. 1. The Gram-negative cell envelope (Costerton & Cheng, 1975)

The fluid mosaic model of membrane architecture is now accepted as being representative of the arrangement of PL and proteins in the CM (Singer, 1972; 1974; Singer and Nicolson, 1972), see Figs. 2 and 3.

The proteins in the CM have been classified into two types, peripheral and integral proteins (Singer, 1972). Peripheral proteins can be easily disassociated from the CM by mild treatment (eg. addition of chelating agents), which suggests they are only attached to the CM by rather weak non-covalent interactions. An example of a peripheral protein is cytochrome C (Singer, 1974).

Integral proteins constitute the major group, approximately 70% of the CM protein composition. Removal of integral proteins from the CM is more difficult and requires harsher treatment (eg detergents). Integral proteins are essential to the structural integrity of the membrane. The amphipathic integral proteins are arranged so that the ionic and highly polar groups protrude from the membrane into the aqueous phase and the non-polar regions are largely bound in the hydrophobic interior (Singer, 1972).

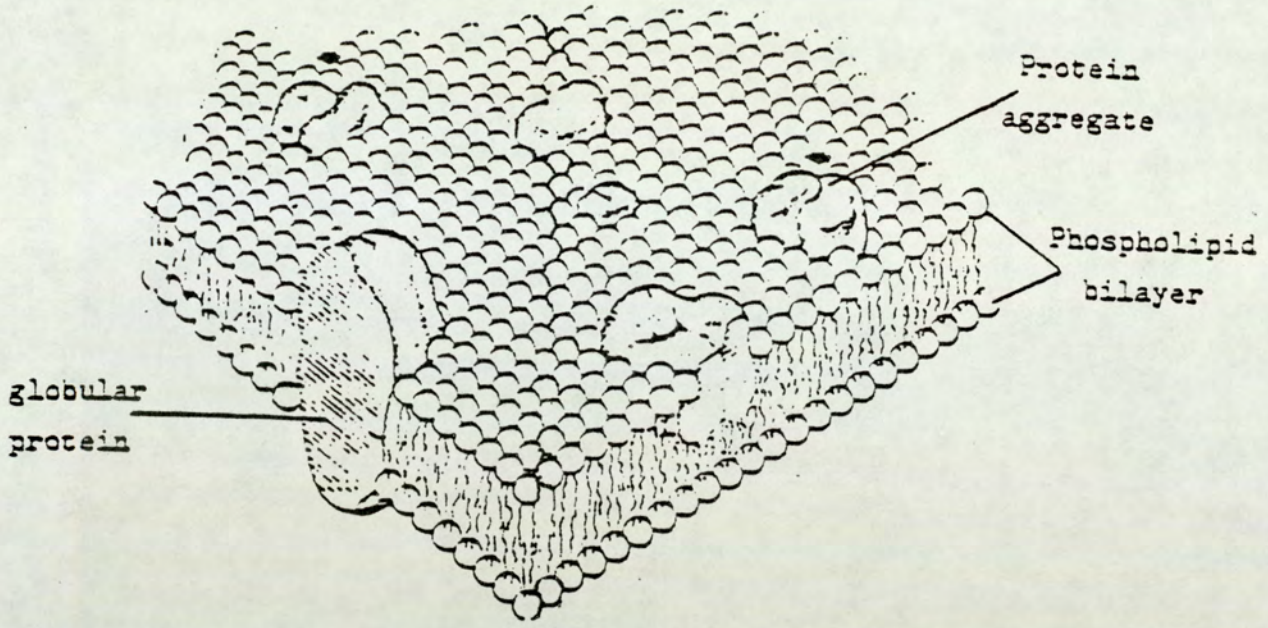


Fig. 2. A schematic representation of the three-dimensional organization of the cytoplasmic membrane (Singer, 1972)

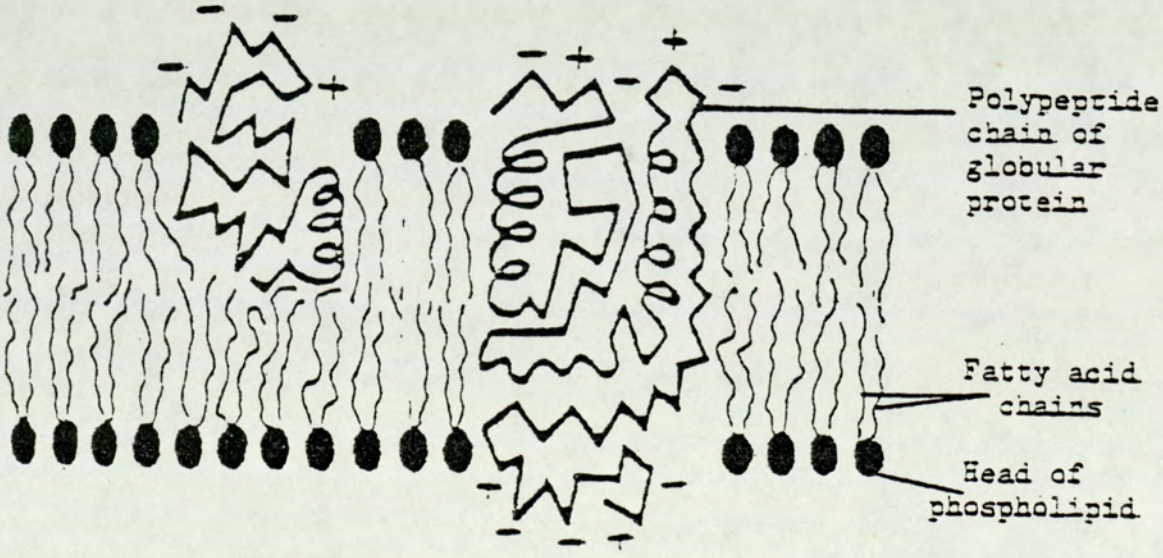


Fig. 3. A diagrammatic representation of a cross section of the cytoplasmic membrane (Singer,1972)

The major PL of the CM are phosphatidylglycerol (PG), diphosphatidylglycerol and phosphatidylethanolamine (PE) (Osborn et al., 1972b). The PL of the membrane is arranged in the form of a liquid hydrophobic bimolecular layer, in which hydrocarbon chains are arranged at right angles to the plane of the membrane, with the globular proteins embedded in the PL matrix and extended from it (Fig 3).

1.1.2.2 Function of the cytoplasmic membrane

The CM has many important functions. Its role in the electron transport chain and oxidative phosphorylation and in the transport of metabolites such as sugars and amino acids has been reviewed by Rogers et al. (1980a). It is also a vital interface for the cell envelope, because it is in this layer that the structural components of the cell wall are synthesised and assembled. The enzymes of PL synthesis, with the exception of phosphatidylserine (Raetz and Kennedy, 1972) are integral proteins of the CM (Cronan, 1979). The CM is also enriched for glycosyltransferase (involved in the biosynthesis of the core region of LPS), and O-antigen synthesis occurs exclusively in this region (Osborn et al., 1972a). The synthesis of OM proteins (Randall and Hardy, 1977) and the murein-lipoprotein complex (Braun, 1975) takes place in the CM.

1.1.2.3 Variation in the cytoplasmic membrane

The composition of the CM is not fixed in a stoichiometric way as enzyme systems, composition, structure and function can alter in order to adapt to the new environment. Gilleland et al (1974) have shown using freeze-etch studies that when Pseudomonas aeruginosa was depleted of magnesium, the appearance of the CM changed from an ordered net-like structure to one of disorder and large smooth areas

were observed. Physiological evidence suggests that sulphate-binding protein is associated with the CM (Pardee and Watanabe, 1968) and the amount of binding protein is known to vary with growth conditions (Pardee et al., 1966). A number of factors (eg. temperature) are known to affect the fatty acid composition of the CM. This adjustment in fatty acid composition is required for the membrane to function normally under different environmental conditions (Fox, 1972).

1.1.3.1 Peptidoglycan layer

The peptidoglycan layer is sandwiched between the OM and CM. It is probably a single or bimolecular layer, approximately 20-30A° thick (Mirelman, 1979), although estimates vary according to the method of staining (Glauert and Thornley, 1969). The amount of peptidoglycan varies in different species; for example in the marine pseudomonad B16 it comprises only 1-2% of the cell dry weight (Forsberg et al., 1972).

Heilmann(1972) analysed the peptidoglycan of P. aeruginosa and found the composition to be similar to that of other Gram-negative bacteria. The backbone consisted of repeating units of β-1, 4-N-acetylglucosaminyl-β-1,4-N acetylmuramic acid. The carboxyl groups of the peptidoglycan were the attachment sites of peptide side-chains. Approximately one out of four 2,5-diaminopimelic acid (DAP) residues (component of the side chains) were cross-linked to another unit. Metal ions seemed to play no role in the structure of the peptidoglycan. The peptide chains of P. aeruginosa were later shown to have the sequence NH_2 - (L-ala-D-glu)-DAP-D-ala-COOH(Heilmann, 1974). Extensive reviews have been written on the biosynthesis and growth of the peptidoglycan layer (Mirelman, 1979 , Rogers et al., 1980b).

Linked covalently to peptidoglycan is Braun's lipoprotein (Braun and Rehn, 1969; Braun, 1975); this and other peptidoglycan associated proteins will be considered in the section on the OM composition .

1.1.3.2 Peptidoglycan function

Although the OM is known to contribute to cellular rigidity (Cox and Eagon, 1968), peptidoglycan is considered to be the primary shape-determining component of the cell envelope. Forsberg et al. (1970) have shown that cells bounded by only the peptidoglycan layer maintain their shape; isolated peptidoglycan stuctures were found to be in the form of a bag-shaped macromolecule which has been referred to as a peptidoglycan sacculus (Forsberg et al., 1972).

Burman et al. (1972) consider that the covalently linked peptidoglycan-lipoprotein complex of the cell envelope acts as an inelastic foundation, helping to maintain the OM penetration barrier. When P. aeruginosa was grown in the presence of lysozyme or sublethal concentrations of ampicillin the permeability of the OM to antibacterial agents was increased. The peptidoglycan layer is not considered to comprise an effective barrier to the passage of small molecules because of its open structure (Costerton et al., 1974) although Burman et al. (1972) have shown that cholate is excluded by the intact peptidogylcan layer of Escherichia coli.

1.1.4 Periplasmic space

The periplasmic space occupies the same zone as the peptidoglycan-lipoprotein complex, between the CM and OM. The periplasm contains many enzymes; 5-nucleotidase, 3-nucleotidase, acid phosphatase, alkaline phosphatase and ribonuclease-1 have been found in the peri-

plasm of E. coli (Cerny and Teuber, 1972). Several of these enzymes are not exclusively periplasmic, but a large portion of their molecules are found in this area (Dvorak et al., 1970). Binding proteins are also situated in the periplasm (Pardee and Watanabe, 1968).

The position of the periplasmic space allows it to play an influential role in antibiotic resistance. Any drug entering the cytoplasm must first penetrate the barrier of the OM and then pass through the periplasm. The penicillin-degrading enzymes (β-lactamases) are located in the periplasmic space of P. aeruginosa (Brown, 1975), also the enzymes which inactivate kanamycin, neomycin and streptomycin (Doi et al., 1968)

The actual size of the periplasm in relation to the total cell volume is at the moment unclear. Stock et al. (1977) suggested a value of 20-40%, whilst Nikaido and Nakae (1979) found the volume to be up to 13% in the starved or stationary phase cell.

The structural integrity of the OM is important to the correct functioning of the periplasm. When the cell is damaged by membrane-active drugs eg polymyxin B (PB) (Cerny and Teuber, 1972) or undergoes osmotic shock (Neu and Chou, 1967) then very large amounts of the periplasmic contents are leaked into the menstruum. Genetic mutants with altered OM protein profiles also leak their periplasmic contents (Lazzaroni and Portalier, 1981).

1.1.5 Outer membrane

Only in the last 15 yrs have methods been developed to separate the OM and CM of the cell envelope. The original procedure of Miura and

Mizushima (1968) is based on buoyant density differences, the membrane being separated by equilibrium sucrose density gradient centrifugation. The OM banded at higher densities, presumably because it contains more lipopolysaccharide (LPS) and possibly more proteins than does the CM.

Other methods have been based on differences in electric charge (White et al., 1972) or selective solubilisation with detergents (Schnaitman, 1970).

The isolated OM has been shown to contain LPS, PL and proteins. Their structure, function and position in the OM will now be discussed.

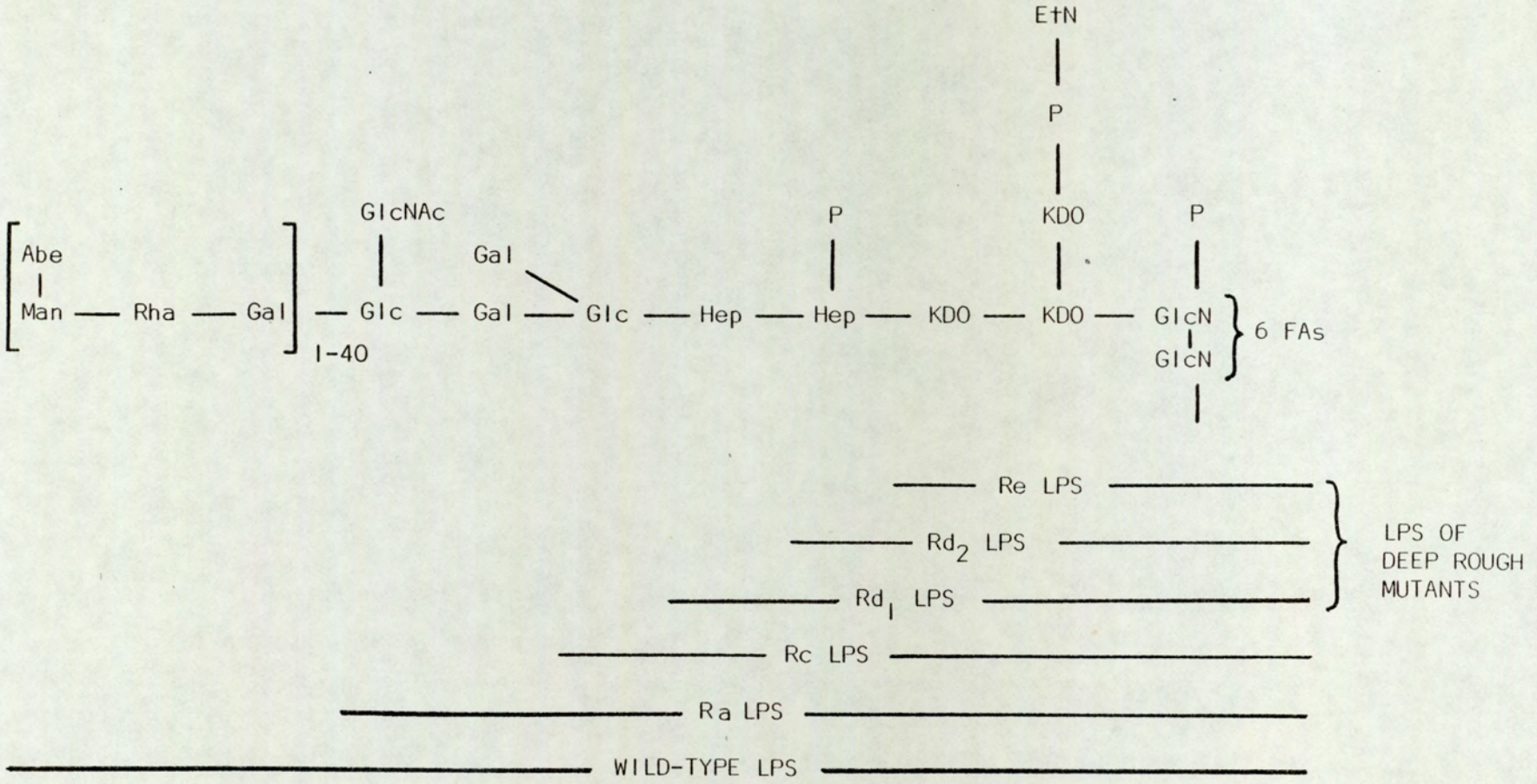
1.1.5.1 Lipopolysaccharide

The first LPS to be analysed in detail was that of Salmonella typhimurium. Fig. 4 shows the structure of the wild-type strain as well as various mutants. LPS consists of three moieties, namely lipid A, the 'core' and the 'O'-antigen (Nikaido and Nakae, 1979). LPS is an amphipathic molecule with a hydrophilic polysaccharide portion and a hydrophobic portion, lipid A. Although evidence would suggest that the general architecture is the same for all the enterobacteria and pseudomonads (Nikaido and Nakae, 1979) care should be taken in equating LPS of different genera.

Lipid A is generally a glycolipid with a D-glucosaminyl-B1→6-D-glucosamine backbone to which fatty acid (FA) residues are attached through amide and ester linkages (Nikaido and Nakae, 1979). The major fatty acids in P. aeruginosa are 2OH 12:O (2 hydroxydodecanoic acid), 3OH 12:O (3 hydroxydodecanoic acid), 12:O (lauric acid), 16:O (palmitic acid) and 3OH 10:O (3 hydroxydecanoic acid) (Wilkinson and Galbraith, 1975).

Fig. 4. Structure of lipopolysaccharides from strains of *S. typhimurium* (Nikaido & Nakae, 1979)

Abe	Abequose
Man	D-mannose
Rha	L-rhamnose
Gal	D-galactose
GlcNAc	N-acetyl-D-glucosamine
Glc	D-glucose
Hep	L-glycero-D-mannoheptose
P	phosphate
KDO	2-Keto-3-deoxyoctonic acid
EtN	ethanolamine
FA	fatty acid
Ra-Re	strains of *S. typhimurium*


EtN
P
KDO
P
GlcNAc
P
Abe
Gal
Man — Rha — Gal — Glc — Gal — Glc — Hep — Hep — KDO — KDO — GlcN
1-40
GlcN
6 FAs
Re LPS
Rd2 LPS
Rd1 LPS
LPS OF DEEP ROUGH MUTANTS
Rc LPS
Ra LPS
WILD-TYPE LPS

Early data suggested that LPS monomers were cross-linked (Osborn, 1969). However, Muhlradt et al. (1977), in studies with Salmonella, strongly support a non cross-linked monomeric structure and the presence of a hydroxyphosphoryl group at the reducing terminus of the disaccharide.

The 'core' region is usually linked to lipid A via 2-keto-3-deoxyoctonic acid (KDO) in P. aeruginosa (Wilkinson and Galbraith, 1975). The low-molecular weight (MWt) solutes released from the core of P. aeruginosa during mild hydrolysis include KDO, inorganic orthophosphates and pyrophosphates, ethanolamine mono, pyro and triphosphates. The same group of workers found the major identifiable components at the core to be: glucose, rhamnose, galactosamine, alanine, phosphorus and heptose (Wilkinson and Galbraith, 1975). The 'core' region and the charged groups of lipid A contain a very high density of charged groups and thus possess exceptional metal binding properties (Nikaido and Nakae, 1979). It seems likely that in P. aeruginosa as in other Gram-negatives there is a common core in all strains of a particular species (Meadow,1975).

The peripheral portion of LPS, the 'O' antigen consists of oligo-saccharide repeating units and shows extreme variability even within a single species. For example strains of S. typhimurium are known to make LPS molecules of many sizes (Munford et al.,1980). For this reason the 'O'-antigen has been used in fine serological typing (Nikaido and Nakae, 1979). The side-chains of P. aeruginosa have been chemically analysed by a number of workers (Kropinski et al., 1982; Wilkinson and Galbraith, 1975) and found to contain in various amounts (depending on the strain): fucosamine, quinovosamine, glucos-amine, galactosamine, an unidentified amino acid and a possible aminohexuronic acid.

An overall structure for P. aeruginosa has been proposed by Kropinski et al. (1979) (Fig. 5).

1.1.5.2 Lipids

Phospholipids (PL) are the major FA containing components of Gram-negatives. PL are complex lipids and their general structure is shown in Fig. 6, along with the various polar head groups of the major PL found in P. aeruginosa.

The PL of the enterobacteria and of other Gram-negative organisms are in general similar to those of E. coli (Goldfine, 1972). Osborn et al. (1972b) found that in S. typhimurium the OM is qualitatively similar to the CM but has quantitative differences, the OM having a higher concentration of PE but lower concentrations of PG and DPG. Lugtenberg and Peters (1976) found that PE in the OM contained more saturated FA than PE in the CM and overall the OM in comparison to the CM is enriched for saturated FAs.

Almost all the procedures used to separate the OM and CM depend on sonication or the use of the French pressure cell. Such treatments can cause the intermembraneous transfer of PL (Devor et al., 1976). Thus it is possible that the PL composition of the OM and CM may differ by a greater degree than is usually observed. Thus the more extreme values in the literature may more accurately reflect the 'in vivo' composition.

1.1.5.3 Proteins

Very rapid progress has been made in the identification and quantification of protein components of the OM, mainly owing to the technique

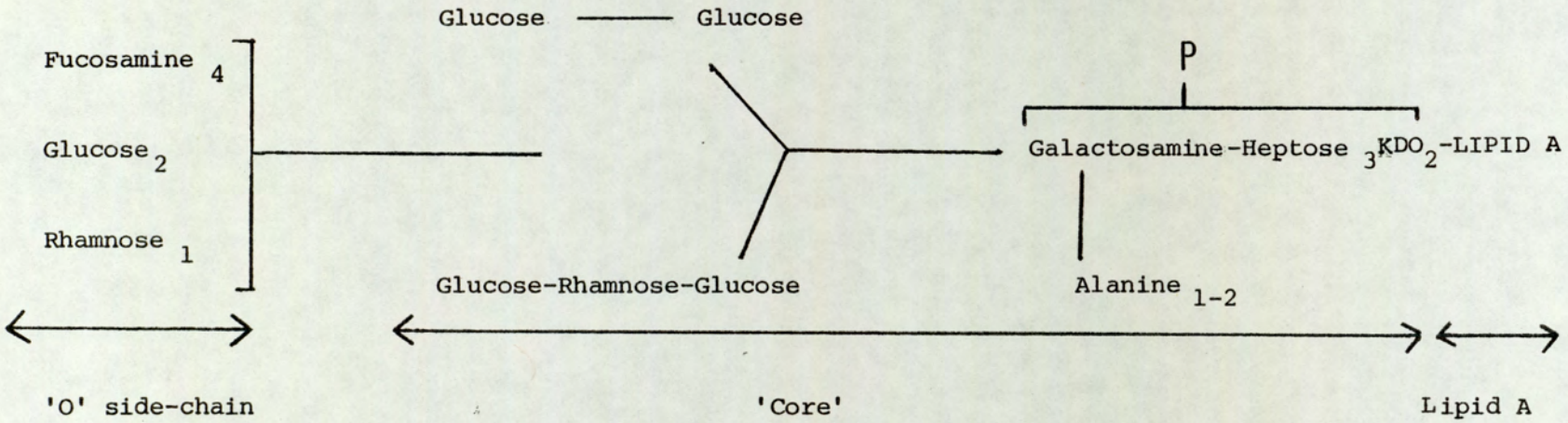


Fig. 5. Structure of P. aeruginosa lipoplysaccharide

(Kropinski et al., 1979)

Fig. 6. Structure of phospholipids

R1 and R2 - nonpolar hydrocarbon chain of fatty acid

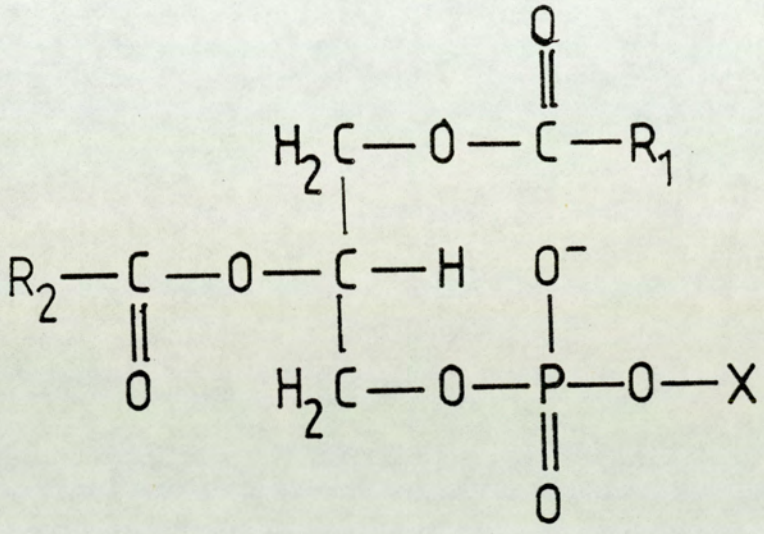
X - Polar head group

(1) = Phosphatidylethanolamine

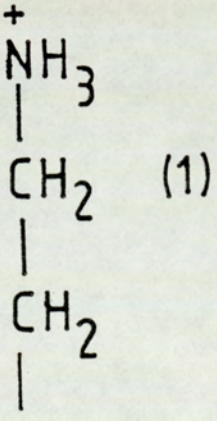
(2) = Phosphatidylglycerol

(3) = Diphosphatidylglycerol

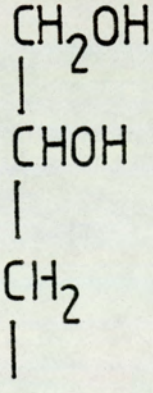
General structure



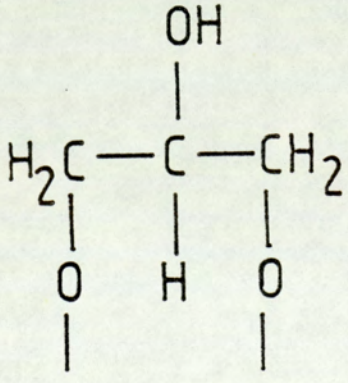
Polar head groups



(1)



(2)



(3)

of sodium dodecyl sulphate (SDS) - polyacrylamide gel electrophoresis (PAGE) (Schnaitman, 1970). Improved resolution has enabled a clearer identification of difficult-to-resolve protein components. Although P. aeruginosa OM proteins were investigated in the early seventies (Stinnett and Eagon, 1973) almost all our knowledge was initially accumulated about E. coli and S. typhimurium. This was probably a result of the greater difficulty in separating the OM and CM of P. aeruginosa, caused by the hypersensitivity of the organism to ethylenediaminetetraacetic acid (EDTA), a chelating agent frequently used in the preparation of the OM (Roberts et al., 1970). Early work on the OM proteins was carried out simultaneously by many laboratories using different nomenclature schemes. This has left the field in a somewhat confused state. It is now generally accepted that where possible a uniform rational nomenclature system be introduced, based on the structural genes in current linkage maps (Osborn and Wu, 1980). A useful summary of the various notation schemes is given in Table 1 for E. coli and S. typhimurium and Table 2 for P. aeruginosa.

1.1.5.3.1 Properties of proteins

Murein lipoprotein known also as Braun's lipoprotein, by whom it was discovered, has been characterised and reviewed in detail (Braun, 1975). In E. coli this small protein (7,000 daltons) consists of 58 amino acid residues and is linked by the ε-amino group of the C-terminal lysine to the carboxyl group of every 10-12th meso-DAP acid residue of peptidoglycan (Braun, 1975). Fig. 7 shows the lipoprotein-peptidoglycan complex of E. coli. The protein has an α-helical structure unlike the β-structure of other major OM proteins (Braun, 1975).

In E coli about one-third of the total lipoprotein is linked to

Table 1

Recommended genetic momenclature for major outer membrane proteins of E. coli and S. typhimurium (Osborn & Wu, 1980)

a Previous gene designations are parenthesised

b S=structural, R=regulatory ?=unknown

c no gene product corresponding to locus has been identified

d Tentative name, pending identification of structural gene locus

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

Table 2

Nomenclature of P. aeruginosa outer membrane proteins (Hancock & Carey, 1979)

a Using impure sodium dodecyl sulphate (SDS)

b Using ultrapure SDS

c Cited by Hancock and Carey (1979)

Name used by Hancock and Carey	Mol wt ($\times 10^3$)							
	Mizuno[c] and Kageyama	Hancock and Nikaido[c]			Matsushita et al.[c]		Booth and Curtiss[c]	Stinnett and Eagon[c]
	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 (49)		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)	9 (18)	V (16)	B (16.5)
				20.5				
I (=lipoprotein)	I (8)			9-12	9 (9.7)	9 (8.7)		

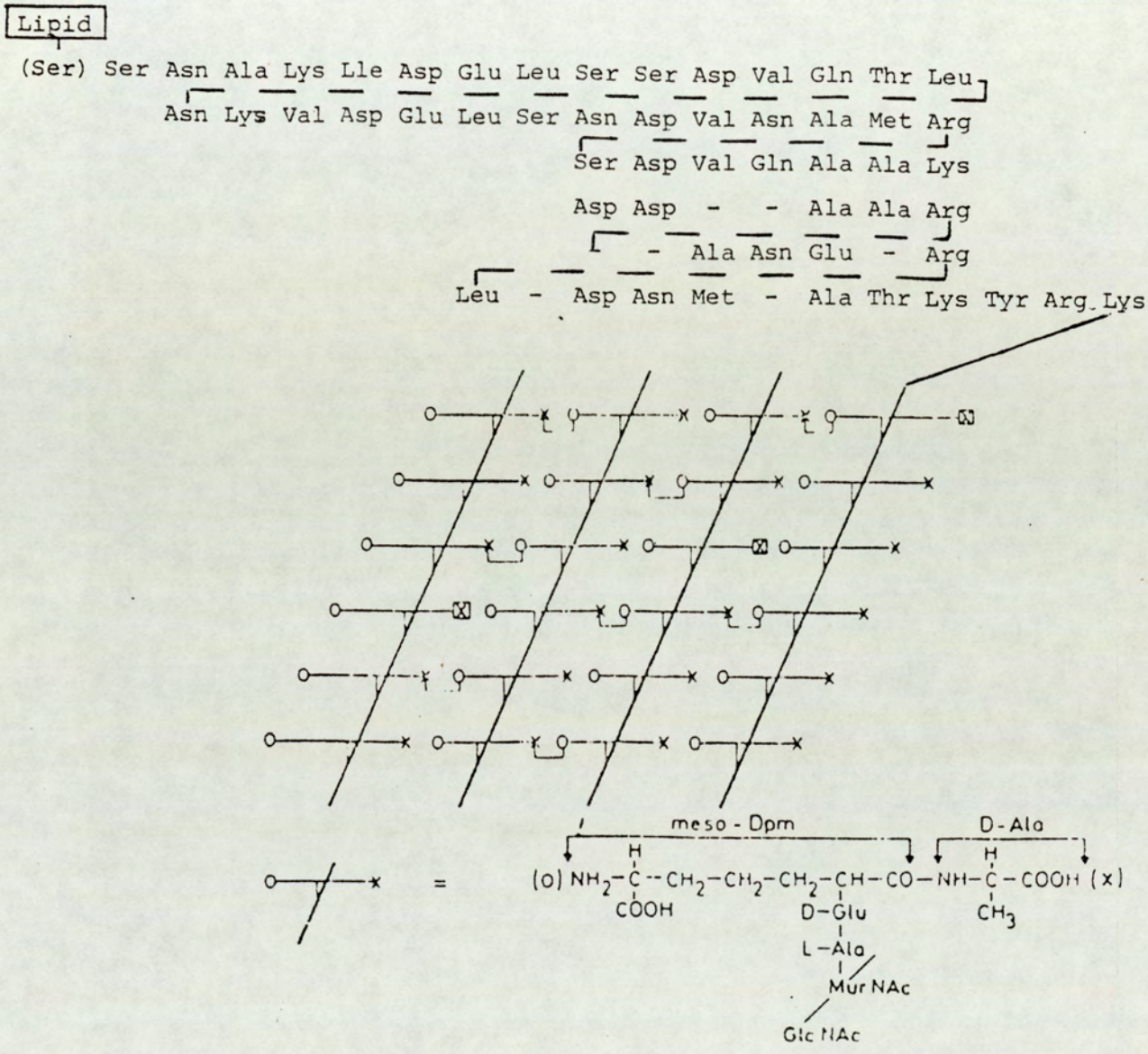


Fig 7 The lipoprotein-peptidoglycan complex of E. coli (Braun, 1973). The parallel heavy lines symbolize the polysaccharide chains. They are crossed-linked by the T-like peptide side chains which are drawn to allow a long-range covalent fixation of the peptidoglycan, which is a necessity in Gram-negative bacteria.

peptidoglycan (Braun and Hantke, 1974), whilst the remaining two-thirds occurs in a free form (Inouye et al., 1972). The free form is thought to be chemically indentical with the bound form (Braun et al., 1975).

Protein I of P. aeruginosa is thought to be analogous to the E. coli lipoprotein although considerable differences have been observed in the amino acid composition and FA content (Mizuno and Kageyama, 1979). Protein I also seems to exist in a form covalently linked to peptidoglycan, although probably in smaller amounts than in E. coli (Mizuno and Kageyama, 1979).

Heat-modifiable proteins were initially identified in E. coli and S. typhimurium as OM proteins with altered electrophoretic mobilities after heating in SDS prior to electrophoresis (Nakamura and Mizushima, 1976; Nikaido and Nakae, 1979). Hancock and Carey (1979) have identified 5 heat-modifiable proteins in P. aeruginosa: D1, D2, F, G and H1. Proteins D1, D2, G and H1 gave results similar to the heat-modifiable protein of E. coli K-12 (Schnaitman, 1973) in that heating of the proteins between 70-100°C in SDS caused a decrease in the mobility of the proteins (an increase in apparent M Wt) on SDS-polyacrylamide gels. The addition of LPS to these heat-modified proteins (except F) caused the modification to be reversed. Protein F seems to belong to a different class of heat-modifiable proteins, as it is unusually stable to boiling in SDS for long periods. Furthermore LPS treatment had no affect on it (Hancock and Carey, 1979).

Peptidoglycan-associated proteins are proteins which have a tendency to associate with the underlying peptidoglycan. Treatment of the cell

envelope with SDS at temperatures below 70°C solubilises all of the OM components except lipoprotein and peptidoglycan-associated proteins (Rosenbusch, 1974).

The successful resolution of proteins H1 and H2 by Hancock and Carey (1979) has shown they are in fact 2 quite distinct proteins. Protein H2 is peptidoglycan-associated unlike H1 (Hancock et al., 1981).

The proteins in the OM involved in the formation of hydrophilic pores are also peptidoglycan-associated (Nikaido, 1979). They are not noticeably hydrophilic and exist in large numbers in the bacterial cell (Rosenbusch, 1974). S. typhimurium produces 3 porins of apparent MWt 36,000, 25,000 and 34,000 daltons (Nakae, 1976). Protein F is the major pore-forming protein in P. aeruginosa (Benz and Hancock, 1981).

Porins of E. coli solubilised by SDS exist as trimers (Palva and Randall, 1979) and also seem to exist as trimers in the OM (Nikaido, 1979). Van Alphen et al, (1979) have shown that each individual pore contains only one porin species. When the cell envelope of E. coli is extracted with SDS at room-temperature, peptidoglycan sheets with hexagonally arranged oligomers of porin are left behind (Rosenbusch, 1974). As a result of this regular arrangement Steven et al. (1977) were able to use image-enhancement techniques in electron microscopy. The porins were clearly seen to exist as trimers, and a triplet of holes is produced by each monomeric unit. Ueki et al. (1979) examined the small-angle X-ray scattering produced by orientated layers of the intact OM of S. typhimurium. A most interesting feature was the presence of a central electron-transparent area or a 'hole' of 5-6nm in diameter. This could correspond to a single channel in the centre

of the porin trimer, but it is also possible that the triplet of holes observed by Steven et al. (1977) appeared as one large hole in this low-resolution study.

1.1.5.3.2 Functions of proteins

A possible role for lipoprotein in the cell envelope has been elucidated by use of an E. coli mutant having an altered peptidoglycan-lipoprotein structure (Yem & Wu, 1978). The mutant had an altered OM, increased sensitivity to chelating agents and leaked periplasmic enzymes. The authors concluded that lipoprotein plays an important role in the maintenance of the structural integrity of the OM.

Protein D1 is thought to form a glucose-permeable pore and be involved in a high affinity glucose transport system (Hancock and Carey 1980).

Protein H1 has been implicated in resistance to EDTA, polymyxin and gentamicin (Nicas and Hancock,1980) and will be discussed in the appropriate sections.

No clear roles have been assigned to proteins D2,E,G and H2.

The possibility that pores in the OM may exist arose from the logical assumption that separate pathways would probably exist for hydrophilic and hydrophobic molecules. Diffusion of hydrophilic compounds by the "hydrophobic pathway" would be extremly slow owing to their small partition coefficients (Nikaido, 1979). Early studies using oligo-saccharides and plasmolysed cells (Decad and Nikaido, 1976) with S. typhimurium and E. coli suggested the OM acted as a molecular sieve with an exclusion limit near 550-650 daltons. The ability to

purify the various components of the OM, make liposomes containing the various components and assess the permeability of a series of oligosaccharides enabled Nakae (1975) to propose that certain proteins played an essential role in the formation of pores. These proteins were named "porins" and rapid progress has been made in understanding their structure, genetic control and specificity (Osborn and Wu, 1980; Hall and Silhavy, 1981). The importance of pores in the transport of hydrophilic molecules has been illustrated by the isolation of an E. coli mutant deficient in a porin protein (Bavovil et al., 1977) which was found to have a transport-limited growth rate (Von Meyenburg, 1971).

Plasmolysed cells of P. aeruginosa were found to have a higher exclusion rate than E.coli or S. typhimurium. This was initially assumed to be a result of plasmolysis-induced cell wall damage (Decad and Nikaido, 1976). Hancock and Nikaido (1978) found that PL vesicles containing the OM proteins of P. aeruginosa only retained oligosaccharides of M Wt greater than 9,000 daltons. The authors suggested that the extremely large pores would be advantageous to P. aeruginosa. Proteins and in particular hydrophobic solutes surrounded by a "cage" of water molecules would be able to enter through these pores.

Benz and Hancock (1981) found the incorporation of protein F (from P. aeruginosa) into artificial lipid bilayers results in an increase of the membrane conductance by many orders of magnitude, the change in conductance being caused by the formation of large ion-permeable channels. The activity per unit-weight of purified protein F was about 100 times lower than porins from E. coli and S. typhimurium.

Possible inactivation during purification cannot be ruled out. The authors concluded that P. aeruginosa had very large pores but relatively few of these were open at any given time. Angus et al., (1982) compared the permeability of an antibiotic-susceptible mutant of P. aeruginosa with its wild-type to a wide range of antibiotics, including β-lactams. OM permeability to the antibiotics decreased as the antibiotic-resistance increased. Protein F did not appear to be altered in the mutant. The data was consistent with the idea of low OM permeability being caused by a low proportion of open functional pores.

A new major OM protein, designated protein P, has been shown to be induced in P. aeruginosa during phosphate depletion (Hancock et al., 1982). Protein P has been shown to form "channels" which are substantially smaller than all previously studied pores and highly specific for anions; little else is know about protein P.

A number of OM proteins are involved in the transport of nutrients. Iron transport in E. coli is dependent on a number of OM proteins (Hancock et al., 1976). An OM protein of E. coli is involved in nucleoside uptake and is a receptor protein for phage T6 and colicin K (Hantke, 1976).

1.1.5.4 Outer membrane structure

Nikaido and Nakae (1979) proposed an arrangement of the OM based on current knowledge of its properties (Fig 8.). They suggest that the outer half of the OM is almost exclusively occupied by protein and LPS, the PL molecules being mostly found in the inner half of the OM. Muhlradt et al. (1975), using electron microscopy, showed

Fig. 8. Outer membrane of Gram-negative bacteria (Brown et al., 1979) adapted from Nikaido & Nakae (1979)

LPS, lipopolysaccharide (the oligosaccharide chains extending from the LPS into the external environment are omitted for visual clarity).

TPP, Trimer of porin protein.

STP, Specific transport protein.

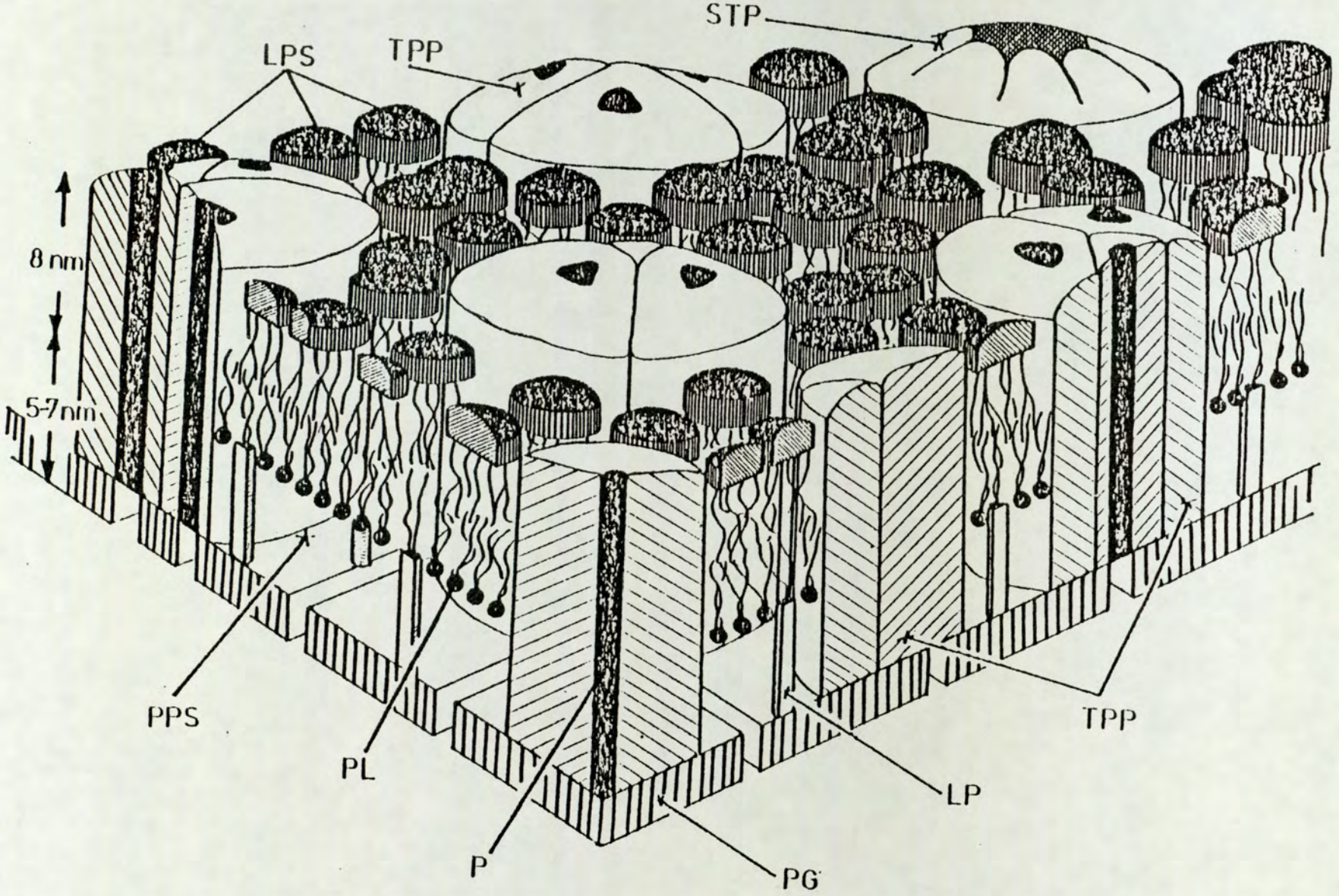
PPS, Periplamsic space.

PL, Phospholipid.

P, Hydrophilic pore.

PG, Peptidoglycan

LP, Braun's Lipoprotein


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

that ferritin -labelled antibody molecules against LPS became attached exclusively to the outer surface of the OM. Funahara and Nikaido (1980) studied the amount of LPS oxidised by galactose oxidase in intact cells and aqueous dispersions of LPS. The extent of oxidation was very similar, suggesting that nearly all LPS molecules in S. typhimurium are located in the outer leaflet of the OM.

In P. aeruginosa, particles seen on the concave-fracture face appear to be protein-LPS complexes, since EDTA treatment which results in the disappearance of such complexes from the cell wall (Rogers et al., 1969) results in the disappearance of a major fraction of these particles (Gilleland et al., 1973). Thus it seems likely that the outer half of the OM (corresponding to the concave-fracture face) is filled with protein-LPS complexes. Lambert and Booth (1982) investigated the exposure of the major OM proteins on the cell surface of P. aeruginosa with ^{125}I -lactoperoxidase, which catalyses iodination of tyrosine and histidine on the cell surface in the presence of hydrogen peroxide. Protein F was the major labelled protein whilst D1 and D2 were less heavily labelled. Since D1 and F both form pores in the OM it is likely that part of the polypeptide chains would be exposed on the cell surface.

The PL content of the OM in S.typhimurium is quite low and could not completely cover even one side of the membrane (Smit et al., 1975). Kamio and Nikaido (1976) used a mixture of phospholipase C and cyanogen bromide activated dextran which would covalently label any exposed PL on the outer leaflet of the OM. When whole cells of wild-type S. typhimurium were treated with this mixture, no labelled PE was found (PE is major PL of the OM). They therefore concluded that

the head groups of PL are not exposed on the outer surface of the OM. (Further experiments ruled out steric hindrance by polysaccharide chains).

Separate domains for PL and LPS have been corroborated by electron spin resonance studies, using spin-labelled probes. Artificial mixed bilayers of LPS and PL exhibit significantly lower fluidity than bilayers of PL alone. Yet PL hydrocarbon chains in the OM have a fluidity very similar to that in the CM, indicating that PL in the OM of S. typhimurium occupies a completely different region from LPS (Nikaido et al., 1977).

However, the observation by Gilleland et al. (1973) that in P. aeruginosa the outer half of the OM shows large areas which are smooth and particle-free, would suggest the presence of PL in the outer half of the OM.

1.1.5.5 Variation in OM composition

Variations in OM composition are known to occur in response to the presence or absence of nutrients in the growth media. Proteins D1 and D2 are induced in cells grown in a minimal media containing glucose as the carbon source, although when grown in a nutritionally rich medium D1 is not observed and D2 is only present at low levels (Hancock and Carey, 1979). OM proteins involved in iron transport in E. coli are overproduced in iron-deficient media (Hancock et al., 1976). Liproprotein in E. coli is known to increase in concentration during stationary phase (Wensink et al., 1982).

Alterations in one component of the OM are now thought to affect the function of other components. Kropinski et al., (1982) analysed the LPS from the wild-type and an antibiotic-susceptible mutant of

P. aeruginosa described by Angus et al. (1982). Quantitative analysis of the core oligosaccharides revealed that the mutant exhibited major deficiencies in both glucose and rhamnose. Evidence of alteration in the lipid A structure was also found, by acid hydrolysis and infra-red spectra techniques. On the basis of this data, Kropinski et al. (1982) suggested that the state of LPS can directly influence the number of open functional pores. Hancock and Carey (1979) have suggested purified porin protein F is usually associated with LPS. Day and Marceau-Day (1982) have shown that the actual chemical composition of LPS varies in accordance with environmental conditions.

1.1.6 Outer membrane transport

The passage of hydrophilic molecules has been discussed in Section 1.1.5.3.2. In summary the specificity and rate of transpore diffusion depends upon molecular size (Nikaido and Nakae, 1979), charge (Stock et al., 1977) and for molecules approaching the maximum size limit, lipophilicity (Nikaido, 1976). The transport of some materials may be facilitated by specific transport proteins located across the OM. Such proteins are analogous to those of the CM (Brown et al., 1979) and are higly specific in their action (Hantke, 1976).

Nikaido (1976) postulated that hydrophobic substances must penetrate by dissolving in the hydrocarbon interior of the OM. The OM of many wild-type bacteria is probably exceptional in that hydrophobic molecules are partly excluded by the surface hydrophilic polysaccharides, the close stacking of LPS (Brown et al., 1979) and the absence of PL in the outer surface (Kamio and Nikaido, 1976). A compound traversing the OM via the hydrophobic pathway would have to pass through aqueous and lipophilic layers. Somewhere between the two

extremes of a compound having a hydrophobic or hydrohilic nature, would the optimum balance for traversing the cell barriers occur (Brown et al., 1979).

1.1.7 Outer surface layers

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 layer and may be dispersed in liquid media (Liu, 1979). The production and composition of slime in P. aeruginosa may be affected by cultural conditions and have a small initial blocking effect upon the action of EDTA and PB (Brown and Scott-Foster, 1971). The main constituents of slime are a polysaccharide (50-60%), composed mainly of glucose with some mannose, DNA and RNA (20%) and hyaluronic acid (5%). Other minor components are protein, rhamnose and glucosamine (Brown et al., 1969b). It would seem reasonable, as Brown (1975) suggested, that a viscous slime layer could offer a penetration barrier to antibacterial agents. The proposal is contradicted by the work of Demko and Thomassen (1980) with clinical isolates of P. aeruginosa; in most cases the mucoid isolate was more susceptible to antibiotics than the non-mucoid isolate. Irvin et al. (1981a) have isolated mucoid strains of P. aeruginosa either hypersusceptible or resistant to antibiotics.

1.2 Action of EDTA on Gram-negative bacteria

1.2.1 Introduction

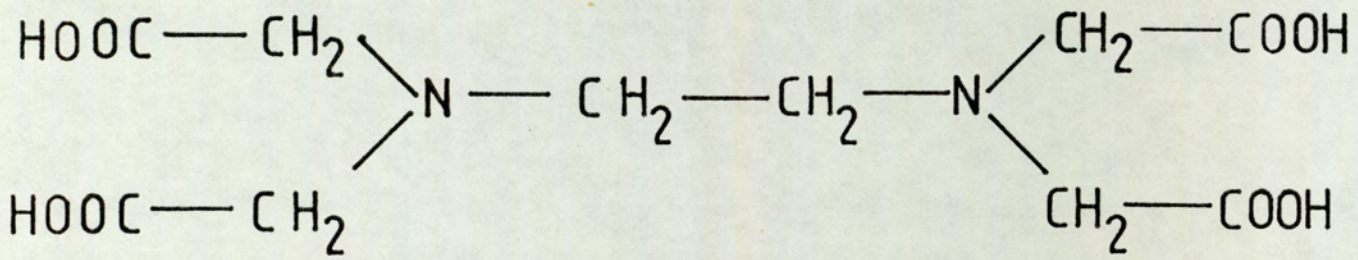
Ethylendiaminetetracetic acid (EDTA) is a tetrabasic molecule. Its structure is shown in Fig. 9. EDTA causes a relatively unspecific change in the permeability of Gram-negative bacteria, which results in sensitisation to a wide variety of antibacterial agents (Wilkinson, 1975) and will lyse *P. aeruginosa* (Brown and Melling, 1969a). EDTA is also used extensively in the preparation of OM (Miura and Mizushima, 1968). Its activity is thought to be due to its metal-ion chelating properties (Leive, 1974). Unfortunately the high concentration of divalent cations in body fluids would make most clinical applications impractical.

1.2.2 Chelation

EDTA is appreciably bound to bacterial cells over a wide range of concentrations (Garrard, 1971). A comparison of five different chelating agents indicated that the antibacterial activity of EDTA can be attributed to its chelating properties. The activities of the agents were measured by loss of viability, lysis and release of 260nm - absorbing compounds (Haque and Russell, 1974). Gray and Wilkinson (1965) also showed that compounds with chelating abilities significantly lower than that of EDTA were inactive against *P. aeruginosa*.

EDTA incubated with isolated cell walls of *P. aeruginosa* solubilises 26% Mg^{2+} and 18% Ca^{2+} (Cox and Eagon, 1968). Although this shows that EDTA can chelate such ions in wall material, it would not necessarily have such unrestricted access to ions in whole cells. If EDTA does chelate ions in the cell wall, then changes in pH would

Fig. 9. Structure of EDTA



be expected to affect its antibacterial activity. Shiveley and Hartsell (1964) have shown that increasing the pH from 6 to 8 increases the bactericidal activity of EDTA.

Mg^{2+} and Ca^{2+} are the predominant divalent cations in the cell wall of Gram-negative bacteria, with Mg^{2+} being more plentiful than Ca^{2+} in P. aeruginosa (Eagon, 1969). Roberts et al. (1970) studied the bactericidal ability of chelating agents with different affinities for Mg^{2+} and Ca^{2+} for P. aeruginosa. They concluded that Mg^{2+} was the most important cation for EDTA activity. This is confirmed by depletion of P. aeruginosa of Mg^{2+}; it becomes resistant to the lytic action of EDTA (Brown and Melling, 1969a). The addition of Mg^{2+}, unlike Ca^{2+}, to the growth medium restores sensitivity to EDTA after a number of cell divisions (Brown and Melling, 1969b). Ethyleneglycol-bis-β-aminoethylether-N:N'- tetracetic acid (EGTA) has a low affinity for Mg^{2+} and specifically chelates Ca^{2+} in the cell envelope. EGTA, unlike EDTA, has poor activity against P. aeruginosa. Kenward et al. (1979) suggested that EGTA cannot remove the 'external' Mg^{2+} in order to reach the deeper located Ca^{2+}.

1.2.3 Factors affecting activity

The response of an organism to EDTA is known to be dependent on the growth phase of the organism. Log phase cells are more sensitive to the lytic action of EDTA than stationary phase cells (Repaske, 1958). Early work on EDTA used poorly defined, complex media. The use of chemically defined media has illustrated the importance of characterising the media when assessing sensitivity to EDTA (Brown and Melling 1969a).

Resuspension procedures can also affect EDTA sensitivity. The bactericidal effect of EDTA in borate buffer on unwashed cells of P. aeruginosa was greater than on water-washed cells; buffer washed cells were even less sensitive (Gray and Wilkinson, 1965). The limited data available makes interpretation difficult, though Wilkinson (1975) considered it to be only a secondary effect.

The composition of the medium in which EDTA is tested is also known to affect activity. Tris-(hydroxymethyl)-amino-methane (Tris) buffer is widely used as a resuspending agent. Tris itself is known to chelate metal cations (Leive, 1974), and Goldschmidt and Wyss (1967) have proposed that Tris in conjunction with EDTA has a greater chelating ability than EDTA alone. Neu (1969) found that when Tris buffer was replaced by other amino buffer systems the action of EDTA and lysozyme was drastically reduced in E. coli. He concluded the other buffer systems had weaker chelating properties with EDTA. The release of 260nm - absorbing compounds from E. coli treated with EDTA is greater in the presence of Tris than in the presence of phosphate buffer at the same pH (Neu et al., 1967).

The lysis of bacteria by EDTA requires a hypotonic solution; osmotic protection against lysis can be provided by non-permeant molecules such as sucrose (Stinnett and Eagon, 1975).

1.2.4 Effect of EDTA on the outer membrane

Cox and Eagon (1968) found that in P. aeruginosa, EDTA/Tris solubilised approximately 30% of the total cell wall and 32% LPS from isolated cell walls. Muramic acid-containing material and PL were not solubilised. Leive et al., (1968) found that LPS release from whole cells of

E. coli was very rapid, with between 30-50% being removed, coupled with 5-10% protein and 5% PL within minutes of the addition of EDTA. The loss of components was not caused by cell lysis, as less than 0.1% release of nucleic acids was observed.

The quantitative and qualitative properties of the material released by EDTA will be dependent on a number of parameters; length of incubation and concentration of EDTA used, species of bacteria, type of medium and whether whole cells or isolated envelopes were used. Such material released from E. coli was found by Leive et al., (1968) to contain high M.Wt undegraded LPS and consisted of 2 fractions. F1 contained LPS associated with protein and PE, F2 consisted solely of LPS, with virtually no associated molecules. In addition, F1 contains at least two of the biosynthetic enzymes for LPS, galactosyl transferase (Levy and Leive, 1970) and glucosyl transferase (Leive, 1974). At $4^{\circ}C$, EDTA only releases F2 (Leive, 1974), although the permeability change in the OM does occur in E. coli (Leive, 1968). Voll and Leive (1970) found that mutants of E. coli resistant to EDTA-induced permeability changes released only 60-80% as much LPS as the parent strain, due to a decrease in the amount of F2. These results clearly indicate that in E. coli, loss of F2 is associated with permeability changes in the OM.

Levy and Leive (1968) found that in E. coli LPS was divided into releasable and non-releasable fractions, which appeared to be in equilibrium with each other. Leive (1974) proposed that the LPS which can be readily released is held in place by divalent cations and therefore the addition of Ca^{2+} or Mg^{2+} should shift the balance between the two fractions. This was confirmed using S. anatum grown in varying

Ca^{2+} concentrations. As the amount of Ca^{2+} was increased in the growth medium, the % of releasable LPS increased, although the amount of LPS synthesised did not vary between Ca^{2+} concentrations. The results show how much LPS is held in place in the OM by cations, and thus what fraction can be released by EDTA.

Stinnett et al., (1973) found that two OM proteins were released in the LPS - protein complex from isolated envelopes by EDTA. Using an improved technique, Hedstrom et al., (1981) identified OM proteins D1, D2, E, G and H1 released from whole cells of P. aeruginosa. Gilleland et al., (1974) used freeze-etching to study the effect of EDTA on the OM of P. aeruginosa. Prior to incubation with EDTA the concave cell wall (OM) contained spherical units (LPS-protein complexes). The addition of EDTA caused the removal of these units and the cell became osmotically fragile.

1.2.5 Mechanism of EDTA action on the outer membrane

The loss of LPS and an increase in OM permeability does not normally effect the viability of most Gram-negative bacteria, if the conditions of EDTA treatment are not too severe (Wilkinson, 1975). However, P. aeruginosa incubated with EDTA under such conditions will undergo lysis. Possible explanations of this phenomenon will now be considered.

Leive (1968) proposed the presence of an 'exceptional'autolysin. Warren et al., (1955) isolated an autolytic system which influenced the action of lysozyme on P. aeruginosa. However, no other workers have as yet isolated an 'exceptional' autolysin. If autolysis is

indeed relevant to the supersusceptibility of P. aeruginosa, structural failure in the cell wall would play an important role.

Separate attempts have been made to interpret the EDTA-sensitivity of P. aeruginosa in terms of OM composition. The cell wall of P. aeruginosa is unusually rich in phosphorus (Wilkinson, 1970) and this can be largely accounted for by the high phsophorus content of LPS (Wilkinson and Galbraith, 1975). The core region of LPS has a very high density of negatively-charged groups, and affords a specific affinity site for interaction with divalent cations (Schindler and Osborn, 1979). Wilkinson (1975) proposed that chelation of these cations by EDTA would remove the stabilising cations and create highly anionic regions in the cell. The consequent highly repulsive forces would open up the membrane and assist in the dissociative action of EDTA. Analyses of the cell walls of other pseudomonads (Wilkinson, 1970) suggested that EDTA sensitivity could be correlated with the amount, or the phosphorus content, of the LPS component.

The release of periplasmic enzymes by the addition of EDTA to P. aeruginosa has not been clearly demonstrated. Such enzymes are released from E. coli incubated with EDTA/Tris, although the cells used had previously undergone osmotic shock (Neu, 1969). Spirillum itersonii incubated with EDTA/Tris also releases periplasmic enzymes (Garrard, 1971).

Brown and Melling (1969b) found that glucose-depleted P. aeruginosa released 260nm - absorbing compounds when incubated with EDTA. Neu et al., (1967) also noted the release of such compounds from E. coli incubated with EDTA/Tris. Interpretation of the latter results is

difficult as Tris itself also causes the release of 260nm - absorbing compounds (Neu et al., 1967).

Although EDTA has only been shown to act on the OM, it should be remembered that La Porte et al., (1977) have shown that disruption of the OM by PB can affect CM function.

1.2.6 Resistance and repair mechanisms to EDTA

P. aeruginosa depleted of Mg^{2+} is resistant to the lytic action of EDTA (Brown and Melling, 1969a); this was correlated by Kenward et al. (1979) with a reduced Mg^{2+} content. Nicas and Hancock (1980) proposed that the OM protein H1 which is found in high concentrations in magnesium-depleted cells, replaces Mg^{2+} at its site on LPS and protects the LPS against attack by EDTA, possibly by steric hindrance.

LPS mutants of S. typhimurium with an altered lipid A region (Vaara et al., 1981) when grown in a medium containing a high Ca^{2+} concentration, released half as much as the wild-type strain when incubated with EDTA.

Gilleland et al., (1974) compared EDTA-sensitive and EDTA-resistant cell envelope of P. aeruginosa (grown in Mg^{2+}-plentiful and deficient media respectively). The EDTA-resistant cells were found to possess 18% less phosphorus, 16.4% more carbohydrate and 13.3% more KDO than the EDTA-sensitive cells. The OM of EDTA-resistant cells had increased numbers of compacted spherical units (LPS-protein complexes, Rogers et al., 1969); their appearance was unaltered by incubation with EDTA, while the sensitive-cell lost over half of these particles.

Incubation of P. aeruginosa in EDTA/Tris with sucrose (0.5M) causes the formation of osmotically-fragile rods termed 'osmoplasts'. These can be restored to osmotically-stable forms by the addition of divalent cations normally present in the cell envelope (Asbell and Eagon, 1966). The repair process is a purely physical process, as it is not inhibited by chloramphenicol or KCN (Stinnett and Eagon 1975). Osmoplasts of P. aeruginosa resuspended in a growth medium plus sucrose for 2 hrs are able to undergo self-repair and regain osmotic stability. This repair process is inhibited by chloramphenicol or KCN (Stinnett and Eagon, 1975).

Graham et al., (1979) found that in E. coli, 30% of the EDTA-released material could be reassociated with the OM in the presence of divalent cations, whilst only 10% would reassociate in their absence. Mg^{2+} specifically enhanced binding of F2(LPS) whilst Ca^{2+} enhanced binding of F1 and F2.

1.3 Action of polymyxin on Gram-negative bacteria

1.3.1 Introduction

The polymyxins are a group of related antibiotics obtained from a strain of Bacillus polymyxa. They are characterised by a heptapeptide ring containing a high % of 2,4-diaminobutyric acid and a fatty acid (FA) attached to the peptide through an amide bond (Figs 10 & 11). Their action has been reviewed by Newton (1956) and more recently by Storm et al., (1977)

The polymyxins are active against most Gram-negative bacteria although Proteus (Sud and Feingold, 1972) and Serratia (Suling and O'Leary, 1977) are generally resistant to the polymyxins. Nakajiima (1967) has reported a number of observations, that emphasise the importance of the cyclic peptide moiety and particularly its positive charge, for polymyxin activity. The length of the FA side-chain is also known to affect its activity (Storm et al., 1977).

1.3.2 Interaction with the outer membrane

Newton (1954) studied the competition between polymyxin and cations for sites on the cell. From a comparison of these affinities, coupled with the ability of cations to reverse charge on certain colloids, he concluded that the polymyxin-combining site on the cell maybe polyphosphates. Schindler and Osborn (1979) proposed that the KDO unit of LPS may afford a specific high affinity site for interaction with divalent cations. Bader and Teuber (1973) concluded, on the basis of comparable binding studies with various LPS derivatives, that the primary interaction between PB and LPS was with the KDO-lipid A region of LPS. It would also lend weight to the hypothesis of Storm et al., (1977) and Kenward et al., (1979) that the initial

Fig. 10 Structure of polymyxins (Storm et al.1977)

Polymyxin	R group	X	Y	Z
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

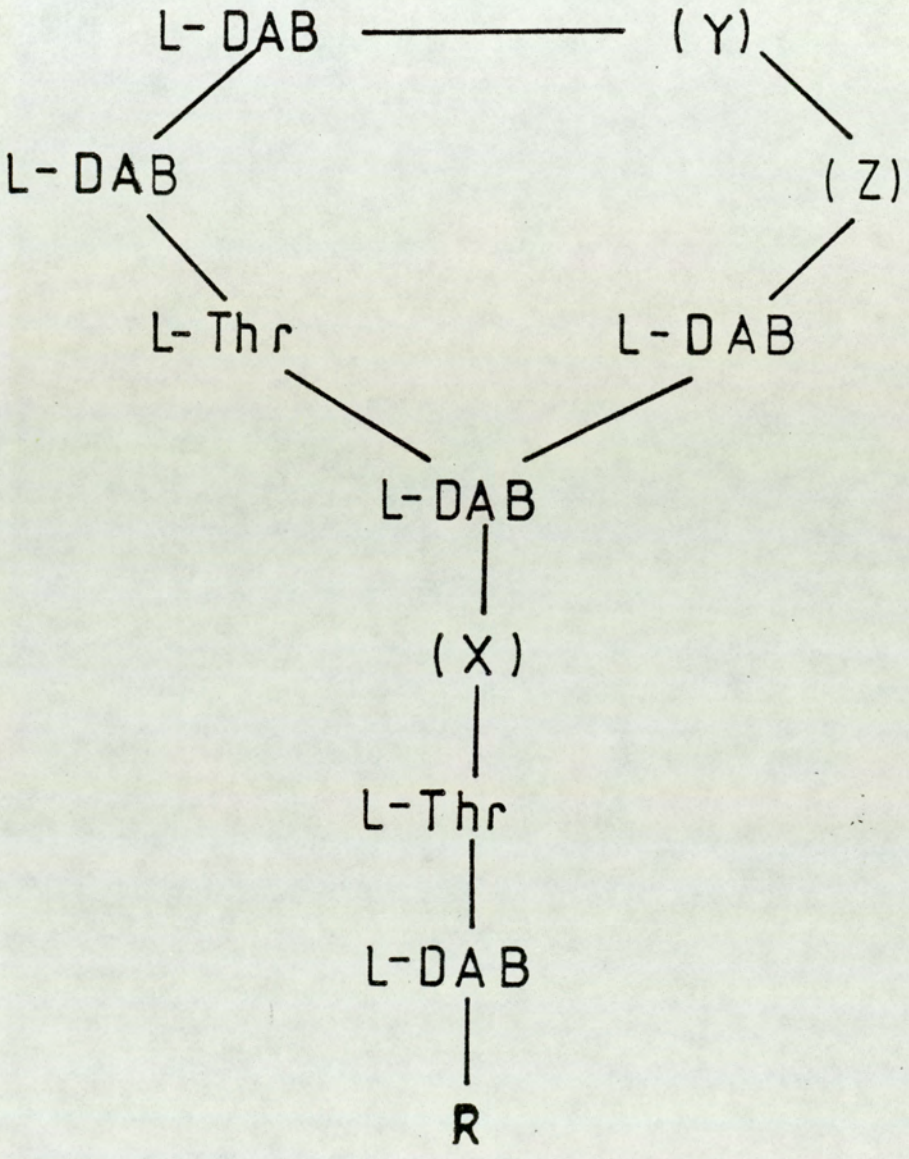
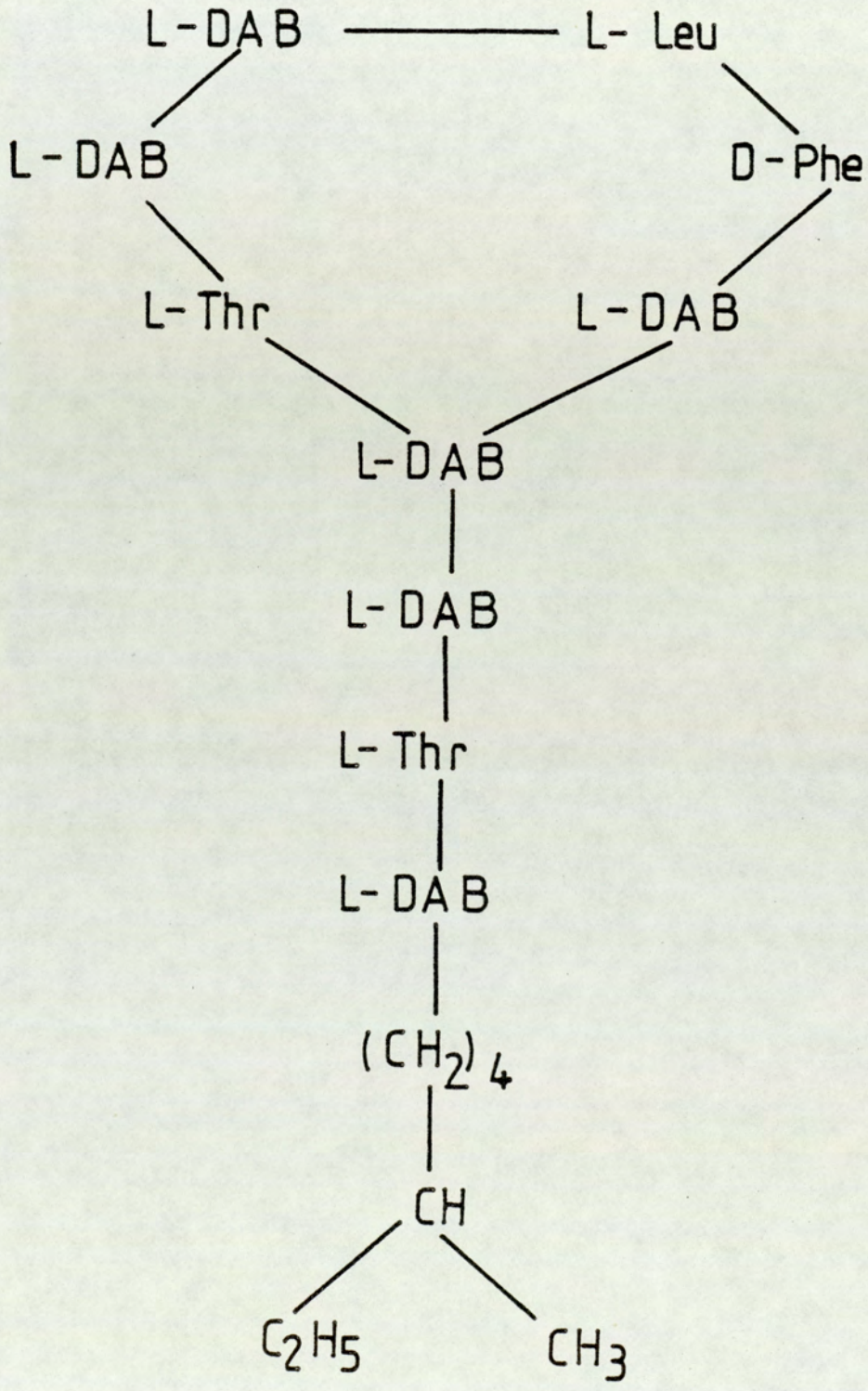

L-DAB
(Y)
L-DAB
(Z)
L-Thr
L-DAB
L-DAB
(X)
L-Thr
L-DAB
R

Fig. 11 Structure of polymyxin B



Legend as in Fig. 10

action of PB is displacement of Mg^{2+} and /or Ca^{2+} from the cell wall.

PB is known to inactivate LPS endotoxin activity in vaccines (Cooperstock and Riegle, 1981). Lopes and Innis (1969) isolated LPS from E. coli. When examined by electron microscopy it had a membraneous, ribbon like structure with periodic branching. The addition of PB resulted in breakdown of the structure. LPS from a PB-sensitive strain of Serratia marcescens was reduced from 1·1% to a negligible level after the addition of PB (Tsang et al., 1975).

A number of workers have used 'in vitro' techniques (monolayer, liposome studies) in an attempt to clarify the OM components with which the polymyxins interact, though these systems can be criticised on the grounds that they may not reflect the 'in vivo' state.

Hsu Chen and Feingold (1973) considered that for PB to interact with liposomes they required a certain concentration of phosphatidylethanolamine (PE). Their work suffers from the serious failing that the liposomes used contained 50% cholesterol, which is known to decrease sensitivity to PB (Imai et al., 1975). Teuber and Bader (1976a) fused lipid vesicles with cells of the PB - resistant, OM-lacking Acholeplasma laidlawii B. The association with the acidic PL, phosphatidylglycerol (PG) or diphosphatidylglycerol (DPG) produces a 10-30 fold increase in PB susceptibility. This increase was not obtained with PE or phosphatidylcholine (PC) vesicles. However, the CM may contain sterols, unlike those of Enterobacteria.

Imai et al., (1975) prepared liposomes from the purified PL of E. coli in varying ratios. Liposomes containing PE/DPG or PE/PG were extremely

sensitive to PB. The authors concluded that PE was not essential for the interaction with PB, but that a negatively - charged PL was required for sensitivity. Monolayer (Teuber and Miller, 1977; El Mashak and Tocanne, 1980) and fluorescent polarisation studies (Sixl and Galla, 1981) have also shown that PB interacts with acidic PL, whilst zwitterionic or positively-charged PL are unsuitable.

Teuber and Bader (1976b) used isolated CM and OM in conjunction with radioactive PB to calculate the theoretical binding capacities of LPS, PG, DPG and PE. The experimental answer yielded almost identical values if LPS, PG and DPG functioned as the actual binding sites for PB in isolated membranes. The authors concluded that this left little doubt that these components were the receptors for PB.

1.3.3 Effect on the outer membrane

The use of the fluorescent probe N-tolyl-α-napthylamine-8-sulphonic acid has shown that in the presence of polymyxin there is an immediate increase in the permeability of the OM of the sensitive bacterium (Newton, 1954). Periplasmic enzymes, which require an intact OM are therefore released into the menstruum (Cerny and Teuber, 1971). The OM which is normally insensitive to lysozyme, gentian violet and sodium deoxycholate becomes sensitive (Vaara and Vaara, 1981).

Electron microscopy has shown PB induced the formation of projections in the cell wall of *P. aeruginosa*. The number of projections decreased with a reduction in the concentration of PB (Koike *et al.*, 1969). Schindler and Teuber (1975) using spray freezing and freeze-etching techniques suggested the PB-induced blebs are projections of the outer layer of the OM. Koike and Iida (1971) found that previous

treatment of E. coli with PB, prevented LPS-specific phages from absorbing. A mutant of S. typhimurium deficient in LPS had smaller and fatter projections compared to that of the wild-type (Lounatmaa et al., 1976). Polymyxin E in S. typhimurium caused depressions in the outer concave fracture face and protrusions in the outer convex fracture face, indicating participation of both leaflets of the OM (Lounatmaa and Nanninga, 1976).

PB causes a decrease in both the total extractable lipid and the saturated FA in the OM of S. marcescens. The phospholipase activated was thought to be Al (Brown and Tsang, 1977). In P. aeruginosa there was activation of phospholipase C as well as free FA liberating phospholipases by PB (Kusano et al., 1977).

Although it is well documented that PB interacts with a number of components in the OM, little is known concerning the details of PB/membrane interaction. Hartman et al.,(1977) on the basis of a bilayer vesiclestudy proposed that the hydrocarbon chain of PB interacts with the hydrocarbon element of the membrane. The insertion of the hydrophobic tail into the hydrophobic interior is supported by the fact that deacylated polymyxins are much less active than the complete PB molecule against E. coli (Nakajiima, 1967). The peptide ring would lie flat upon the membrane surface in Hartman's model.

El Mashak and Tocanne (1980) on the basis of a monolayer lipid study suggested that the whole PB molecule penetrates the monolayer. The extent of film expansion, when PB interacts with the membrane, would make the penetration of the whole PB molecule a more likely alternative. In both cases, the insertion of the hydrophobic tail would lead to the

OM expanding, leading to disorganisation of the OM.

The importance of the OM as a barrier is well documented, but its importance in the normal functioning of Gram-negative bacteria has been illustrated by La Porte et al., (1977). PB was attached to agarose beads by stable covalent bonds, which precluded PB from penetrating beyond the OM. PB was found to inhibit the growth and respiration of E. coli solely by interacting with the OM. The authors suggested that pertubation of the OM eventually affected the selective permeability of the CM.

1.3.4 Effect on the cytoplasmic membrane and bacterial metabolism

Interactions between CM and PB have not been characterised to the same extent as for the OM. Teuber and Bader (1976b) found that PG and DPG from the CM bound PB, and therefore could be the receptors in the CM. Presumably, disruption would occur in a similar manner to that of the OM.

Koike et al., (1971) using electron microscopy found that after incubation with PB, the CM of P. aeruginosa became disorganised and eventually released cytoplasmic material through cracks in the CM. Schindler and Teuber (1975), on the basis of fluorescent microscopy studies, suggested that the fluorescent aggregates in the cytoplasm may be nucleic acid and/or ribosomes as a consequence of PB entering the cytoplasm. Wahn et al., (1968) had already reported a brightening of the nuclear area in PB -treated E. coli, followed by cytoplasm destruction and cell autolysis.

One of the earliest results of PB-pertubation of the CM is an increase in membrane permeability, which leads to the efflux of small M.Wt. compounds from the cytoplasm into the menstruum (Storm et al., 1977). With P. aeruginosa, pentoses, phosphate and compounds with an absorbance peak of 260nm are released from the cell (Newton, 1953). Salton (1951) observed a similar release following addition of cetyltrimethylammonium bromide; free purines and pyrimidines contributed to the absorbance maximum. Hugo and Bloomfield (1971) found that membrane-active drugs produce an initial rapid loss of 260nm-absorbing compounds, often accompanied by activation of autolytic enzymes, with a gradual breakdown of protein and nucleic acids to give soluble products which are also released from the cell. A similar effect is seen when PB acts on P. aeruginosa (Brown and Melling, 1969b).

PB depresses levels of adenosine triphosphate and inhibits respiration. The changes in respiration may be secondary to alterations in the CM, the efflux of metabolites used in electron-transport, or disorganisation of the electron transport chain (Storm et al., 1977).

Teuber (1974) found that at the minimum inhibitory concentration of PB in S. typhimurium there was optimal efflux of accumulated methyl-α-D-glucosepyranoside and complete inhibition of its uptake as well as DNA and RNA synthesis. Respiration and LPS, murein and protein synthesis needed a longer time or a higher concentration of PB before the inhibition occurred. The effects described will lead to cell death. The loss of the permeability barrier of the CM, the efflux of cellular metabolites, the entry of water with permeant ions (Klemperer et al., 1979) coupled with the damaged OM (Schindler and Teuber, 1975) will precipitate lysis.

1.3.5 Mechanism of resistance

Hamilton (1968) proposed that the mechanism of resistance to membrane-active drugs in Gram-negative bacteria was exclusion of the drug from the sensitive CM by the impermeable OM. When *Proteus mirabilis* which is normally resistant to PB is grown in the presence of penicillin G (which disrupts the permeability barrier of the OM) there is a 400-fold increase in PB sensitivity (Teuber, 1969). Gilleland and Farley (1982) compared the action of PB on the isolated OM of two strains of *P. aeruginosa*. The OM from the strain with adaptive resistance to PB was relatively unaffected by PB treatment, whilst that from the sensitive strain (from which the resistant strain was derived) showed extensive disruption. This was in direct contrast to the isolated CM of both strains which showed extensive disruption after PB treatment. The authors could find no evidence for a PB-inactivating enzyme in the periplasmic space of either strain.

The belief that the OM determines the level of PB resistance led workers to attempt to characterise its actual basis. In almost all cases, attempts have been made to find a sole reason for PB resistance. This assumption can be criticised on the grounds that as PB is known to have a number of effects in the OM, it is likely that a number of changes could contribute to resistance, and these effects may be additive.

Sud and Feingold (1970) compared the PL composition of a strain of *P. mirabilis* made sensitive to PB by growth in sulphadiazine with that of the insensitive wild-type from which it was derived. Their compositions were virtually identical. Suling and O'Leary (1977) compared the lipids of PB-resistant and susceptible strains of

Enterobacteriacae; a number of those resistant to PB had a lower ratio of PG/DPG. Such differences, however, were dependent upon a particular genus and species.

P. aeruginosa when grown on a wide variety of carbon sources has variable resistanct to PB (Conrad et al., 1979). Whole cell analysis of the readily-extractable lipids (REL) showed variation with the carbon source. In particular, growth on the acyl derivatives of branched-chain amino acids resulted in decreased amounts of unsaturated FA and concomitant increases in cyclopropane FA (Conrad et al., 1981).

The significance of the lipid studies mentioned is difficult to assess, as whole cells were used for the analysis. Changes in the PL composition of the OM may well be masked by alterations in the overall PL composition of the cell.

Gilleland and Conrad (1980) attempted to correlate the effect of the carbon source on PB resistance with KDO (LPS marker) and OM proteins. No correlation was found between PB resistance and loss of OM proteins and/or LPS from the cell envelope. Gilleland and Murray (1976) used freeze-etching to show P. aeruginosa (PB sensitive) had numerous particles in the outer half of the OM, unlike the PB-resistant strain (derived from the wild-type by growth in PB containing medium). The removal of PB from the growth medium caused the resistant strain to revert to the sensitive strain in appearance and sensitivity. Chemical analysis of the strains by Gilleland and Murray (1976) suggested PB resistance was associated with a loss of LPS and a number of OM proteins, one of these maybe involved in pore formation. Nikaido (1979) has tentatively linked a case of PB resistance in P. aeruginosa with drastically reduced levels of porin protein.

PB-resistant mutants of S. typhimurium bind less PB, as does the LPS extracted from them, than their corresponding wild-type (Vaara et al., 1979). Chemical analysis of the LPS revealed that the resistant strains had more ethanolamine containing compounds and 4-6 times more 4-amino-4-deoxy-L-arabinose as a substituent of the ester-linked lipid A phosphate than their sensitive parent strain (Vaara et al., 1981).

P. aeruginosa, when magnesium-depleted loses its sensitivity to PB, whilst glucose-depleted cells remain sensitive (Brown and Melling, 1969b). Differences in PL, REL and LPS could not be correlated with sensitivity to PB action, which appeared to be more dependent on OM cation content (Kenward et al., 1979). Nicas and Hancock (1980) found the concentration of OM protein H1 varied inversely with the cell envelope Mg^{2+} concentration. The authors proposed that H1 inhibited the action of PB by replacing Mg^{2+} at its site on LPS and protecting the site by steric hinderance. Gilleland and Beckhams (1982) PB-resistant strain of P. aeruginosa derived from growth in a PB-containing medium had reduced levels of porin protein and H1. The data shown makes it difficult to assess their resolution of H1 and H2, which is dependent on the denaturing conditions used for preparing the protein samples (Dr H. Anwar, Department of Pharmacy, University of Aston in Birmingham).

The possible mechanisms of P. aeruginosa resistance to PB are summarised below.

1) Reduced OM LPS
2) Reduced binding of PB to LPS
3) Reduced OM acidic PL

4) Reduced OM Mg^{2+} and Ca^{2+} content

5) Increased concentration of OM protein H1

6) Decreased concentration of porin protein

1.4 Sulphur metabolism in bacteria

1.4.1 Introduction

Sulphur is considered one of the ten major bio-elements, although compared to nitrogen or carbon it is present in the cell in quite small quantities. Sulphur is confined to a very restricted group of compounds in bacteria, predominantly the sulphur- containing amino acids, which constitute a relatively constant portion of the amino acids in total cellular protein (Jukes *et al.*, 1975).

P. aeruginosa possesses an extensive biochemical system for the utilisation of inorganic sulphur sources and is able to grow on sulphide, thiosulphate, tetrathionate, dithionite, metabisulphite, sulphate, sulphite but not on dithionate (Schook and Berk, 1978). It can also use 3-N-morpholinopropane sulphonic acid (MOPS) buffer as a source of sulphur (Noy, 1982). *Pseudomonas* C12B is capable of degrading sulphur-containing detergents for growth (Cloves *et al.*, 1980).

1.4.2 Sulphur uptake

The initial work on the sulphate, thiosulphate uptake system was with *S. typhimurium* by Dreyfuss (1964), who characterised the system as a rapid energy-and temperature-dependent system. An unusual feature of its regulation, is that linear uptake of sulphate is followed by a net loss of as much as 80% of the accumulated sulphate, followed by another smaller burst of uptake and loss. Dreyfuss and Pardee (1966) found the overshoot phenomenon occurred only when net sulphate uptake increased the internal sulphate concentration to about 10^{-4}M; lower amounts were taken up and retained. Although the entry process requires energy, the exit of sulphate and the exchange of intracellular

sulphate were relatively independent of the energy supply. The system appeared to be regulated by an early product of sulphate metabolism. A brief report has described a similar energy-dependent active uptake mechanism in E. coli (Springer and Huber, 1972).

When S. typhimurium is converted to spheroplasts or osmotically shocked the cells loose their ability to bind sulphate. The binding fraction lost from the cells was found in the menstruum, suggesting that the bacteria possess a highly specific sulphate-binding site near the cell surface (Pardee et al., 1966). Pardee (1966) isolated, purified and characterised a sulphate-binding protein from S. typhimurium. The protein had a M.Wt 32,000 and a typical amino-acid composition, except that it lacked sulphur-containing amino-acids; one sulphate ion was bound per sulphate-binding molecule. Ohta et al., (1971) found the concentration of the protein was decreased when grown in a medium containing added sulphate. The finding that mutants of S. typhimurium would not grow on sulphate or thiosulphate but had significant quantities of apparently normal sulphate-binding proteins, relegated it to a secondary role in sulphate transport (Ohta et al., 1971).

Kadner (1974) and Kadner and Watson (1974) have described a high and a low affinity transport system for methionine in E. coli; osmotic shock reduced transport by 50%. P. aeruginosa has a high affinity energy-requiring methionine transport system (Montie and Montie, 1979).

1.4.3 Sulphur metabolism

Sulphur metabolism has been extensively studied in E. coli (Smith, 1971), S. typhimurium (Qureshi et al., 1975) and Paracoccus denitrificans (Burnell and Whatley, 1980). P. aeruginosa is unusual in that it is

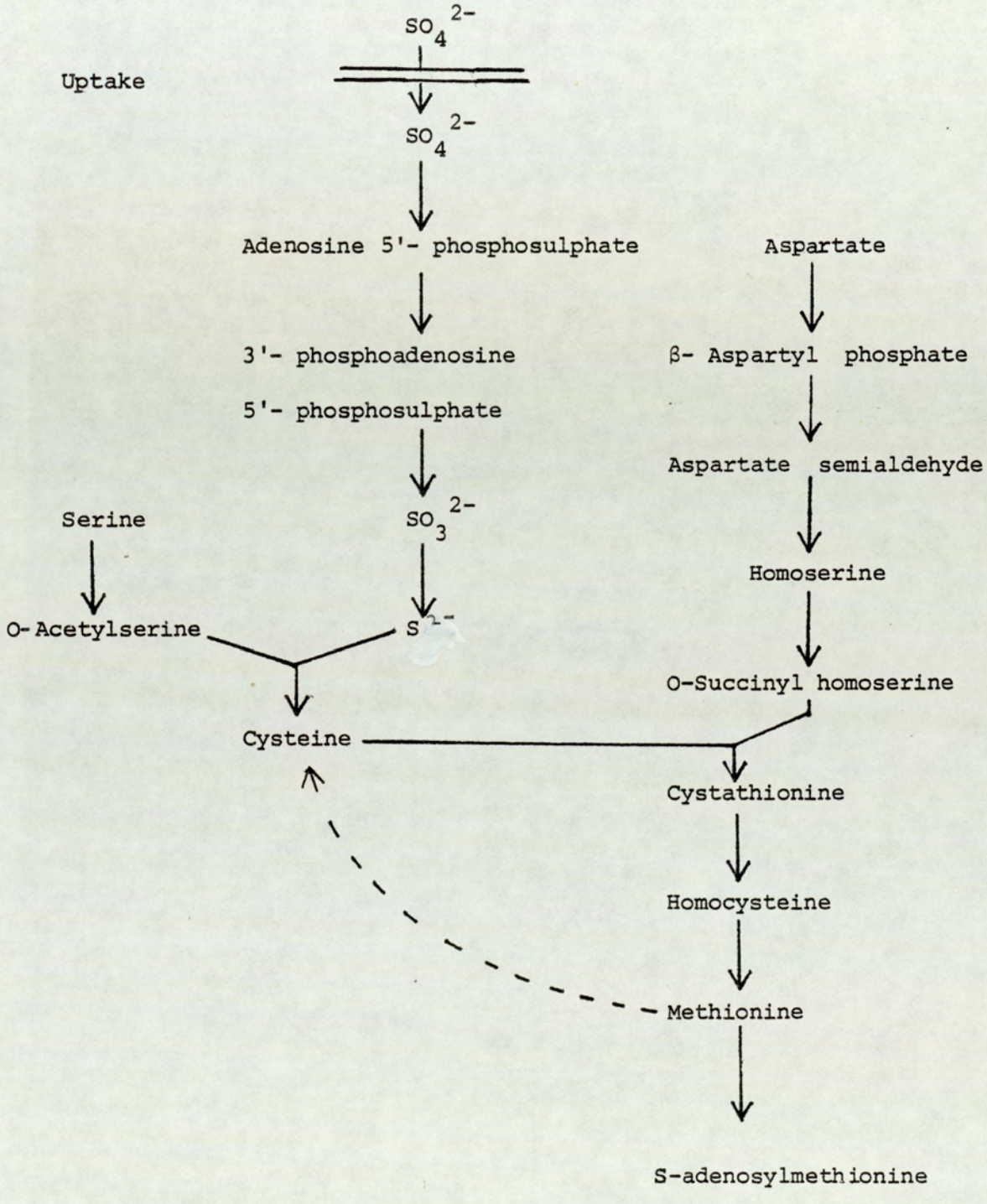
capable of synthesising cysteine via the sulphur assimilation pathway and also from methionine by the transulphuration pathway (Gunther et al., 1979). Fig. 12 shows the probable pathways of sulphur metabolism in P. aeruginosa and is based on the literature previously cited.

The bacterial cell needs to carefully regulate S-amino acid metabolism as above a certain concentration, cysteine is toxic, at least in E. coli (Nagy et al., 1969). In S. typhimurium, with the exception of serine transacetylase (which converts serine to O-acetylserine), the enzymes concerned with the biosynthesis of cysteine are subject to depression by sulphur depletion and to repression by sulphide and cysteine. O-acetyl serine, a direct precursor of cysteine, is necessary for derepression and is therefore an internal inducer, though sulphide and cysteine will block the effect of exogenous O-acetylserine (Kredich, 1971).

In E. coli methionine is known to repress the enzymes involved in the formation of homocysteine and methionine (Rowbury and Woods, 1964). The addition of methionine to the medium leads to a reduction in the specific activity of S-adenosylmethionine (SAM) synthetase. Thus this enzyme appears to be repressible rather than inducible (Holloway et al., 1970). Cysteine and methionine in P. denitrificans both stimulate the enzymes of their respective t-RNA synthetases (Burnell and Whatley, 1980). S-amino acid metabolism is undoubtedly complex; Burnell and Whatley (1980) concluded that although a large number of amino acids are involved in its regulation, methionine, O-acetylserine and particularly cysteine play an central role in P. denitrificans.

In P. halodurans approximately 15% of the total cellular sulphur during

Fig. 12. S-amino acid metabolism in P. aeruginosa



------ Reverse transulphuration

logarithmic growthwill be present as free low M Wt S-amino acids (Cuhel et al., 1981a). During sulphur depletion this figure is reduced rapidly (Cuhel et al., 1981b).

1.4.4 The role of sulphur in the cell

Protein synthesis in the bacterial cell is initiated by the binding of N-formylmethionyl-t-RNA to the initiation site of mRNA(Gottschalk, 1979). Protein function is dependent on the correct amino acid sequence and tertiary structure being maintained. Disulphide bonds, formed by the oxidation of two cysteine residues, play an important role in the maintenance of their structure.

The iron-sulphur proteins in which iron is bound to cysteine residues of the peptide chain and to sulfide, are electron carriers in the electron transport chain. Coenzyme A contains a terminal sulphydryl group, which is the reaction site of the molecule in biochemical reactions. Coenzyme A is a key intermediate in the metabolism of pyruvate, for example conversion of pyruvate to lipids and oxidation via the tricarboxylic acid cycle.

SAM is a methyl donor in a wide variety of biochemical reactions. The conversion of unsaturated FA to cyclopropane FA (Zalkin et al., 1963) and PE to phosphatidylcholine (Gottschalk, 1979) both involve methylation by SAM. Borek and Srinivasan (1965) have shown that methylation of nucleic acids occurs by SAM. The authors proposed that the introduction of a methyl group into nucleic acids would produce profound alterations in the structure of nucleic acids. The methyl group would affect the electron density distribution and possibly augment the strength of hydrogen bonding, or alter the formation to

proffer more sites for such bonding.

The synthesis of polyamines requires SAM as a methyl donor (Hafner et al., 1979). Polyamines have been implicated in ribosomal stabilisation (Turnock and Birch, 1973) and macromolecular synthesis (Abraham and Pihil, 1981). Wilkinson (1975) has suggested that in the event of magnesium-depletion, polyamines may replace Mg^{2+} in its role as a cationic bridge in the OM.

1.4.5 The effect of sulphur depletion

1.4.5.1 Cell growth

During the early stages of sulphate depletion *E. coli* continues to grow and divide and the increase in the absorbance of the growth medium is paralled by an increase in the number of viable cells. The DNA/viable cell ratio also remains constant (Bohinski and Mallette, 1967). During this period the cell probably uses up the pool of low M Wt S-amino acids, as in the case of *Pseudomonas halodurans* (Cuhel et al., 1981b).

1.4.5.2 DNA and RNA synthesis

Mycobacterium bovis depleted of sulphate continues net synthesis of RNA without the continued net synthesis of protein or DNA (Spitznagel, 1961). A methionine-requiring mutant of *E. coli* has also been shown to accumulate large amounts of RNA without increasing its DNA or protein content (Borek et al., 1955). This mutant was also shown to release 260nm-absorbing compounds during log-phase and when depleted of methionine or glucose (Borek et al., 1956).

1.4.5.3 Protein synthesis

Little research has been completed on protein synthesis during sulphur depletion. Brunschede and Bremer (1971) studied the synthesis of proteins in a methionine - and proline-requiring mutant of E. coli. During amino-acid starvation the rate of protein synthesis drops within a few minutes by more than 90% and low M Wt proteins are preferentially synthesised. The authors suggested that the molecular weight distribution of proteins, is consistant with the idea that during starvation incomplete peptide chains are released prematurely from the ribosome. However, Subrahmanyam and Das (1976) consider that the smaller peptides produced are in fact proteins preferentially synthesised and completed. They point out that considerable amounts of complete and active enzymes are synthesised under such conditions. Cozzonne (1981) has suggested that degradation of existing proteins will act as a source of amino acids for protein synthesis. The reason for Klebsiella aerogenes excreting cysteine and methionine-containing proteins into the medium during sulphate limitation in continous culture is unclear (Neijssel and Tempest, 1975).

Robinson and Tempest (1973) studied the effects of sulphate-limitation on the OM proteins of K. aerogenes. The envelope from sulphate-limited cells contained one major protein, whereas those from glucose-limited cells contained three. However, this work was completed prior to recent improvements in the resolution of OM proteins (Cozens and Brown, 1981).

1.4.5.4 Energy metabolism

Poole and Haddock (1975) studied the effect of sulphate limitation on the electron transport chain and energy conservation in E. coli K_{12}.

They concluded that sulphate limitation results in the loss of the proton-translocating oxidoreduction segment of the electron-transport chain between NADH and the cytochromes (Site I). The authors suggested this could be a result of either the loss of iron-sulphur proteins in the NADH dehydrogenase region of the chain which leads to a 'short-circuit' of the proton-translocating oxidoreduction segment normally associated with this region, or to the synthesis of an additional respiration pathway which is characterised by a non-proton-translocating NADH dehydrogenase (i.e. a different enzyme) and extra cytochrome components (b 558 and d) which are synthesised during sulphate depletion. In the absence of further experimental evidence, both schemes must be considered tentative. Meijer et al. (1977) found that in sulphate-limited P. denitrificans Site I phosphorylation is also absent. The previous results are in direct contrast to Lawford (1977) working with P. denitrificans and Farmer and Jones (1976) with E. coli W; neither group could find loss of site I phosphorylation during sulphate limitation. The cause of this discrepancy in unclear.

E. coli W, when sulphate-limited, has a high maintenance energy; this is possibly a result of deliberate energy wastage by the cell (Farmer and Jones, 1976). Neijssel and Tempest (1975) suggested in the case of K. aerogenes, that a mechanism must exist for disposing of the excess of reducing equivalents that are generated as a consequence of overflow metabolism. Their results suggested that the cell maintains its internal redox balance by oxidising the reducing equivalents through to water. As a consequence of ATP generation at the expense of ADP, the energy charge within the cell will be disturbed. ATP-spilling reactions or the transfer of electrons to oxygen may therefore occur without concomitant oxidative phosphorylation.

1.4.5.5 Resistance to antibacterial agents

Relatively few reports have linked sulphate depletion or limitation with resistance to antibacterial agents. Goodel and Tomasz (1980) found that E. coli starved of methionine (induced by the addition of threonine to the growth medium) rapidly developed resistance to autolytic cell wall degradation by penicillins. The mechanism of resistance seemed to involve the production of a peptidoglycan relatively resistant to the hydrolytic action of crude peptidoglycan hydrolase extracts prepared from normally growing E. coli. Klemperer 1968) found that sulphate-depleted P. aeruginosa resistance to PB, (as measured by cell lysis and viable counts) was dependent on the presence of glucose.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Bacteria

The organism used for most of this study was Pseudomonas aeruginosa NCTC 6750, which was kindly supplied by Dr M Noy (Selly Oak Hospital, Birmingham). The organism was maintained on a nutrient agar slope at room temperature and subcultured at 2 weekly intervals. An auxotrophic mutant derived from P. aeruginosa 6750 (see Section 2.7) and designated PCM1 was maintained on nutrient agar, containing cysteine 2mM, at room temperature, and subcultured at 2 weekly intervals.

Prior to an experiment, a chemically defined medium (CDM) was inoculated from the relevant slope and incubated in an orbital shaker at 37°C for at least 12h. The medium was centrifuged (6,000g x 15min) and resuspended in CDM lacking the constituents which would be limiting in the ensuing experiments. The culture was then used as an inoculum for growth experiments. In a number of experiments where a very small inoculum was used, the spinning down and resuspension steps were omitted.

2.1.2 Media

Nutrient broth (Oxoid CM1), nutrient agar (Oxoid CM3) and drug sensitivity agar (Oxoid 261), Oxoid Ltd., Southwark, London were used. For CDM-agar, Lab M agar (Lab. M, Ford Lane, Salford) double strength was added to double strength CDM at 44°C.

The composition of the basic CDM used in this study is shown in Table 3. Ferrous chloride and glucose were sterilised by membrane filtration through a 0.22µm membrane. All other media were sterilised by autoclaving at 121°C for 20 min. Phosphate buffer was clarified by

Table 3

Basic chemically - defined medium

Ingredient	Final concentration
Glucose	2.0×10^{-2}
Na_2SO_4	2.6×10^{-4}
$(NH_4)_2HPO_4$	4.0×10^{-2}
$FeCl_2.4H_2O$	6.2×10^{-5}
$MgCl_2.6H_2O$	4.0×10^{-4}
KCl	5.0×10^{-4}
NH_4Cl	4.0×10^{-2}

Medium pH 7.5

membrane filtration through a 0.45 μm membrane before sterilisation.

2.1.3 Chemicals

The water used in the preparation of CDM was double distilled in glass stills and then distilled from alkaline potassium permanganate. The only exception to this was water used in the preparation of 8 L of CDM, for the eventual preparation of cell walls, which was double distilled. All chemicals used in the preparation of CDM and the organic solvents used in the chemical analysis were of Analar grade and were obtained from British Drug Houses Chemicals Ltd, Poole, Dorset or Fisons Scientific Apparatus Ltd., Loughborough, Leics.

Other chemicals were obtained as follows:- Trypsin, Ribonuclease 2-Keto-deoxyoctonic acid (KDO) and phospholipids (PL) (Diphosphatidylglycerol, Phosphatidylglycerol and Phosphatidyl-ethanolamine) were obtained from Sigma Chemical Company, London.
Lecithin and Ethylene diaminetetra-acetic acid (EDTA) (disodium salt): British Drug Houses Chemicals Ltd., Poole, Dorset.
Gas-liquid chromatography fatty acid standard Lot No. 4-5436: Supelco Inc., Bellefonte, Pennsylvania, U.S.A.
Gentamicin sulphate was kindly given by Schering Corporation, Kenilworth, New Jersey, U.S.A.
Polymyxin B sulphate was kindy given by Burroughs Wellcome & Co, London.

2.1.4 Apparatus

Spectrophotometric cuvettes: absorbance measurements were carried out in 1cm matched glass cuvettes (Helma, Westcliff-on-Sea, Essex). Absorbance measurements at 260nm were carried out in 1cm matched

quartz microcuvettes (Helma, Westcliff-on-Sea, Essex).

Spectrophotometers: absorbance measurements were carried out using a Cecil CE 373 or CE 242 (Cecil Instruments Ltd., Milton Industrial Estate, Cambridge). Atomic absorption spectroscopy was carried out using a Unicam S.P. 90A (Pye Unicam Instruments Ltd., Cambridge)

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

Orbital incubation: Gallenkamp Ltd., Loughborough, Leics.

IEC Centrifuge: International Equipment Co. Massachusetts, U.S.A

MSE Super Minor, MSE High Speed 18 and MSE Superspeed 50 centrifuges Measuring and Scientific Equipment Ltd, Crawley.

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

47mm 0.22µm Nuflo membrane: Oxoid Ltd., Southbank, London.

25mm 0.45µm type HA membrane: Millipore U.K Ltd., Wembley, Middlesex.

Before use all membranes were boiled in three changes of double distilled water to remove wetting agents and other chemicals (Brown et al., 1969a).

Automatic M.L.A pipettes 0.1ml and 0.25 ml tips: Frost Instruments Ltd., Wokingham, Berkshire.

Whirlimixer: Fisons Scientific Apparatus Ltd., Loughborough, Leics.

Potassium electrode G15-K and reference electrode R44/2-SD-1: Pye Unicam Instruments Ltd., Milton Industrial Estate, Cambridge.

pH meter Pye Model 290: Pye Unicam Instruments Ltd., Cambridge.

Colworth Droplette (viewer for viable counts): A J Seward, UAC House, Blackfriars Road, London.

'Wild' binocular phase - contrast microscope, Model M20: Micro Instruments (Oxford) Ltd., Oxford.

'Chromoscan 200' and Scan 201' and integrating equipment: Joyce, Loebel & Co Ltd., Gateshead.

Soniprobe type 7530A: Dawe Instruments, Concord Road, London W3.

Electrophoresis power supply: Bio Rad Laboratories, Watford.

Serroscribe (chart recorder): Smiths Industries, Instruments Co., Oxgate Lane, London NW2.

GLC chromatogram Pye Unicam 204: Pye Unicam Instruments Ltd., Cambridge.

2.1.5 Glassware

All glassware was Pyrex Brand (Corning Glass Ltd., Sunderland). For cleaning it was immersed in 5% Extran 300 (British Drug Houses, Poole Dorset.) at room temperature for at least 12 hrs. It was then rinsed once in distilled water 1% HCl, six times in distilled water and twice in double distilled water. After rinsing, the glassware was dried at 60°C, covered with aluminium foil and sterilised by dry heat at 160°C for 3h.

2.2 Basic Methods

2.2.1 Growth of CDM cultures

Bacterial growth was determined by measuring absorbance. The wavelength to measure absorbance is important and should satisfy the following conditions:

a) Medium should exhibit little or no absorption.

b) Metabolic products should exhibit little or no absorption.

c) The wavelength should import maximum sensitivity to changes in absorbance.

Light in the ultraviolet range is of little use as proteins and nucleic acids absorb in this range. Many workers use 420nm (Hodges, 1973) the most sensitive wavelength in the visible range. However, P. aeruginosa produces pigments that have a significant absorbance at this range (Watkins, 1970). The wavelength that satisfies the above requirements is 470nm. This wavelength has been used for the measurement of growth or lysis.

The relationship between the absorbance of light and growth or lysis can be explained by Beer-Lamberts Law (Monod, 1949). Readings of A_{470nm} above 0.3 have to be diluted to avoid artificially low absorbance values (Kenward, 1975).

2.2.2 Nutrient-depleted cultures

All experiments were performed using 25ml or 125ml of medium in 100ml or 500ml flasks. The flasks were maintained at 37°C in a Mickle reciprocal shaking water bath with a shaking rate of 120 x 5.4cm

throws min^{-1}.

To prepare nutrient-depleted cultures, CDM were designed to ensure that cells would cease growing exponentially at the required A_{470nm} due to the reduction in the concentration of the chosen nutrient. The initial A_{470nm} of the culture was varied according to the exponential A_{470nm} at which logarithmic growth was expected to cease. A_{470nm} readings were taken at 30 min intervals.

Glucose-depleted cells became stationary immediately, magnesium or sulphur-depleted cells continue to grow slowly. Table 4 shows the medium for small batch cultures for resistance studies, allowing growth of carbon-depleted cells to an A_{470nm} 0.25 and sulphur- or magnesium-depleted cells to an A_{470nm} 0.2-0.3 after 5 h of nutrient-depletion. The non-limiting nutrients are present in at least 5 times excess. Cells were depleted of sulphur or glucose for 5h before any further treatment, unless otherwise stated. Table 5 shows the notation used for the various depletions. The notation used for nutrient limitation is discussed in Section 3.6.

2.2.3 Resuspension studies

In a number of experiments the effect of resuspension was studied. The cells were centrifuged (6,000g x 15 min at room temperature) and then resuspended in pre-warmed CDM lacking the depleting constituents.

2.2.4 EDTA treatment

All cells were resuspended in the appropriate medium (Table 6) and incubated for 30 min. The pH was immediately adjusted to 7.3 using

Table 4

Composition of chemically defined medium used in small batch culture of P. aeruginosa

Ingredient (M)	Growth CDM							
	Glucose-depleted cells grown on:			Sulphur-depleted cells grown on:			Sulphur limited cells	Magnesium depleted cells
	Sulphate	Cysteine	Methionine	Sulphate	Cysteine	Methionine		
NH_4Cl	4.0×10^{-2}	4.0×10^{-2}	4.0×10^{-2}	4.0×10^{-2}	4.0×10^{-2}	4.0×10^{-2}	4.0×10^{-2}	4.0×10^{-2}
$FeCl_2 \cdot 4H_2O$	6.2×10^{-5}	6.2×10^{-5}	6.2×10^{-5}	6.2×10^{-5}	6.3×10^{-5}	6.2×10^{-5}	6.2×10^{-5}	6.2×10^{-5}
KCl	5.0×10^{-4}	5.0×10^{-4}	5.0×10^{-4}	5.0×10^{-4}	5.0×10^{-4}	5.0×10^{-4}	5.0×10^{-4}	5.0×10^{-4}
$(NH_4)_2HPO_4$	4.0×10^{-2}	4.0×10^{-2}	4.0×10^{-2}	4.0×10^{-2}	4.0×10^{-2}	4.0×10^{-2}	4.0×10^{-2}	4.0×10^{-2}
$MgCl_2.6H_2O$	4.0×10^{-4}	4.0×10^{-4}	4.0×10^{-4}	4.0×10^{-4}	4.0×10^{-4}	4.0×10^{-4}	4.0×10^{-2}	4.0×10^{-6}
Glucose	1.0×10^{-3}	1.0×10^{-3}	1.0×10^{-3}	2.0×10^{-2}	2.0×10^{-2}	2.0×10^{-2}	2.0×10^{-4}	2.0×10^{-2}
Na_2SO_4	2.6×10^{-4}	0	0	7.8×10^{-6}	0	0	0	2.6×10^{-4}
Cysteine*	0	2×10^{-4}	0	0	7.2×10^{-6}	0	0	0
Methionine*	0	0	1×10^{-4}	0	0	8.4×10^{-6}	1×10^{-4}	0

* sterilised by filtration through a 0.22µm membrane.

Table 5

Notation used for nutrient - depletion studies on P. aeruginosa

Sulphur source	Nutrients depleted	Notation
Sulphate	Glucose	$S^+ G^-$
Cysteine	Glucose	$Cys^+ G^-$
Sulphate	Sulphur	$S^- G^+$
Cysteine	Sulphur	$Cys^- G^+$
Methionine	Sulphur	$Met^- G^+$
Sulphate	Sulphur Glucose	$S^- G^-$

Table 6

Resuspension media for EDTA treatment

Ingredient (M)	Resuspending media		
	Glucose free	Sulphur free	Sulphur & glucose free
$(NH_4)_2 HPO_4$	4×10^{-2}	4×10^{-2}	4×10^{-2}
Glucose	0	4×10^{-3}	0

dilute HCl and EDTA was added, as a 0.1ml sample to each 24.9 ml of test suspension. The same volume of water was added to the control sample.

2.2.5 Polymyxin treatment

Unless otherwise stated, all experiments involving polymyxin B (PB) treatment were carried out either in the CDM in which the cells were grown (Table 4) or after resuspension (Section 2.2.3). When the effect of resuspension was studied cells were incubated for 30 min before the addition of PB. For studies on viability and release of 260nm - absorbing compounds, the pH of the CDM was adjusted to 7.3 using dilute HCl or NaOH before the addition of PB, which was added as a 0.1 ml sample to each 24.9 ml of the test suspension. The same volume of water was added to the control sample.

2.2.6 Total counts

Total counts were made using haemocytometer counting chambers. A suspension of P. aeruginosa grown in CDM was resuspended to an A_{470nm} 0.2. Five 0.1ml samples were each diluted in 0.9 ml of 0.9% NaCl with 2% formaldehyde to kill the bacteria. Counts were performed as described by Collins (1967). For an analysis of variance 5 counts were made using different counting chambers for each sample. The results in Table 7 were subjected to an analysis of variance as shown in Table 8.

The tabulated value of F for 4/20 degrees of freedom are 2.87 and 4.43 respectively. Thus variation between counts is not significantly greater than variation within counts. Using the means from Table 7 the coefficient of variation was found to be 1.4% within points and

Table 7

Total counts per slide for five replicate counts

Slide	Mean number cells/ small square					Means
	A	B	C	D	E	
1	6.26	6.26	6.61	6.90	6.36	6.48
2	6.70	6.51	6.71	6.35	6.95	6.64
3	6.46	6.25	6.49	6.65	6.55	6.48
4	6.45	6.40	6.70	6.26	6.53	6.47
5	6.21	6.29	6.68	6.34	6.45	6.39
Total (T)	32.08	31.71	33.19	32.5	32.84	-
Means	6.42	6.34	6.64	6.50	6.57	-

n = number of counts per slide = 5

m = number of slides = 5

n.m = total number of observations = 25

1) $\Sigma x^2 = 1054.915$

2) $\frac{\Sigma T^2}{n} = 1054.188$

3) $\frac{(\Sigma x)^2}{n.m} = 1053.911$

Table 8

Analysis of variance of five replicate counts

Source of variation	Sum of squares	Degrees of freedom	Mean squares	Variance ratio F
Between counts	0.277 (2-3)	4 m-1	0.0693	1.90
Within counts	0.727 (1-2)	20 nm-m	0.0364	

For n & m see Table 7

1.8% between points.

2.2.7 Viable counts

These were carried out by a droplette method. Several dilutions were made in nutrient broth except for the final one in nutrient agar at 44°C . 0.1ml drops were placed in Petri dishes, incubated overnight and the number of colonies counted with a viewer.

To check the accuracy of the method, 5 samples from a glucose-depleted culture were diluted and counted. The results in Table 9 were subjected to analysis in Table 10.

The tabulated values of F for 4/45 degrees of freedom at 5% and 1% significance are 2.58 and 3.76 respectively. Thus variation between counts is not significantly greater than variation within counts. Using the means from Table 9 the coefficient of variation is 3.9% between counts and 6.2% within counts.

2.2.8 Dry weights

These were performed by the method of Meynell & Meynell (1970a)

Table 9

Colony counts per plate for five replicate counts

	Counts					
Drops	A	B	C	D	E	Mean
1	28	20	25	24	17	22.8
2	18	23	11	27	19	19.6
3	20	22	18	23	25	21.6
4	27	15	29	17	22	22.0
5	23	31	22	22	19	23.4
6	16	20	25	33	22	23.2
7	23	26	20	25	18	22.4
8	18	22	25	17	21	20.6
9	24	14	17	19	26	19.8
10	22	22	17	25	26	22.4
Totals (T)	218	215	209	232	215	-
Means	21.8	21.5	20.9	23.2	21.5	-

n = number of counts per drop = 10

m = number of counts 5

n.m = total number of observations = 50

1) Σx^2 = 24666.00

2) $\Sigma T^2/n$ = 23747.90

3) $(\Sigma x)^2/n.m$ = 23718.42

Table 10

Analysis of variance of five replicate counts

Source of variation	Sum of squares	Degrees of freedom	Mean squares	Variance ratio F
Between counts	29.48 (2-3)	4 m-1	7.37	0.36
Within counts	918.1 (1-2)	45 nm-m	20.40	

For n and m see Table 9

2.3 Measurement of 260nm - absorbing compounds

2.3.1 Basic methods

2.3.1.1 Preparation of 260nm - absorbing compounds

To investigate the most reliable method for measuring 260nm - absorbing compounds, an overnight culture of P. aeruginosa in CDM was resuspended in 40mM $(NH_4)_2PO_4$, sonicated surrounded by an ice-bath for 6 x 1 min intervals and filtered through a 0.45 μm membrane. The preparation was stored at 4°C until required, in pyrex test tubes.

Appropriate dilutions were prepared in 40mM $(NH_4)_2HPO_4$ and their absorbance measured at 260nm. Fig 13 shows that the absorbance at 260nm is linearly related to concentration to an A_{260nm} of at least 0.9.

2.3.1.2 Effect of storage of sample

Samples of 260nm - absorbing compounds were stored in soda glass bottles at 4°C. The absorbance was measured at the time of storage and 24h later. Table 11 shows there is a slight increase in absorbance on storage. This could possibly be due to the dissolving of 260nm - absorbing compounds from the glass.

2.3.1.3 Effect of containers

5ml samples of freshly prepared $(NH_4)_2HPO_4$) were agitated violently in soda glass or pyrex test tubes, using a whirlimixer for 30 seconds. The absorbance of the solution was then measured. Table 12 shows that pyrex test tubes cause least contamination of samples by 260nm - absorbing compounds. In all further experiments pyrex test tubes were used to collect samples of 260nm - absorbing compounds.

Fig. 13. Relation between absorbance and concentration of 260nm-absorbing compounds

Results mean of 2 readings from 1 experiment.

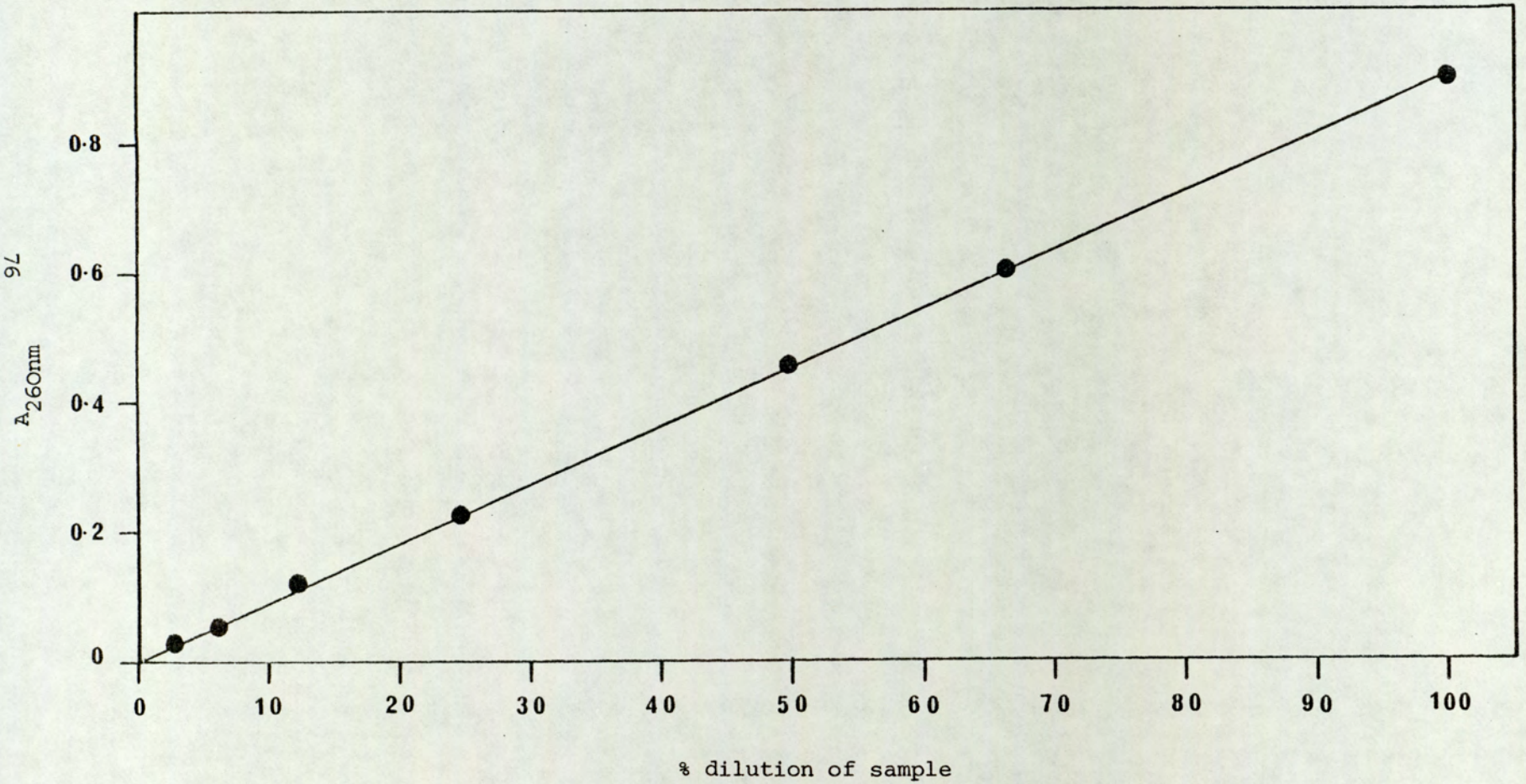

0·8
0·6
0·4
0·2
0
A_{260nm}
0
10
20
30
40
50
60
70
80
90
100
% dilution of sample

Table 11

Effect of storage on 260nm - absorbing compounds

Sample	A_{260nm}
Initial reading	0.822 ± 0.002
Value 24hrs later	0.836 ± 0.005

Mean of 3 readings ± SD from 1 experiment

Table 12

Effect of containers on 260nm - absorbing compounds

Sample	A_{260nm}
Pyrex test tube	0.0017 ± 0.0006
Soda glass test tube	0.0077 ± 0.0023

Mean of 3 readings ± SD from 1 experiment

2.3.1.4 Effect of pH on 260nm absorbance

1ml samples of 260nm - absorbing compounds were prepared in double distilled water and added to 9ml of ammonium phosphate buffer (4mM), from pH 5.9 - 7.7. Table 13 shows the absorbance of 260nm compounds is unaffected by pH between 5.9 and 7.7.

2.3.1.5 Adsorption of 260nm - absorbing compounds by membranes

Various concentrations of 260nm - absorbing compounds were prepared as in Section 2.3.1.1 and their A_{260nm} measured. Using a fresh membrane filter, approximately 1ml samples were passed sequentially through into a cuvette and the A_{260nm} again measured. Table 14 shows there is considerable difference between the reading before and after filtration, but by disregarding the first 2ml a constant reading can be obtained. Table 15 shows that at high concentrations of 260nm - absorbing compounds the membrane becomes saturated and the filtrate value approaches the pre-filtration value as the filtrate volume increases. Table 16 shows that the % loss is constant for each individual batch of membranes, but batches differ.

As a result of these experiments 5ml samples were removed from the test media and filtered through a 0.45 μm membrane filter. The first 2ml were discarded and the remaining 3ml stored in a pyrex tube, the A_{260nm} reading was taken shortly afterwards, within 1hr. This value does not represent an absolute value and the % loss is noted for each experiment.

2.3.2 Excretion of 260nm - absorbing compounds

2.3.2.1 Measurement of 260nm - absorbing compounds

A_{260nm} readings were taken when cells were in logarithmic phase or

Table 13

Effect of pH on absorbance by 260nm - absorbing compounds

pH	A_{260nm}
5.9	0.21
6.2	0.21
7.0	0.20
7.7	0.21

Mean of 2 readings from 1 experiment

Table 14

Adsorption of 260nm - absorbing compounds on to membrane filters

Type of reading		A_{260nm}
Pre-filtration		0.081 ± 0.001
Filtrate sample	1	0.087 ± 0.008
	2	0.072 ± 0.005
	3	0.066 ± 0.002
	4	0.067 ± 0.003
	5	0.067 ± 0.002

Mean of 3 readings ± SD from 1 experiment

Table 15

Effect of filtering high concentrations of 260nm - absorbing compounds

Type of reading		A_{260nm}
Pre-filtration		0.853 ± 0.002
Filtration sample	1	0.623 ± 0.022
	2	0.655 ± 0.005
	3	0.690 ± 0.007
	4	0.721 ± 0.004
	5	0.745 ± 0.005

Mean of 3 readings ± SD from 1 experiment

Table 16

Batch variation of adsorption of 260nm-absorbing compounds on to membranes

Batch of membranes	Pre-filtration	Filtrate	% loss
1	0.186	0.146	22 ± 1
1	0.077	0.060	22 ± 2
1	0.037	0.030	19 ± 1
2	0.188	0.130	31 ± 1
2	0.140	0.097	31 ± 2
2	0.066	0.048	27 ± 1
3	0.081	0.067	17 ± 2

Mean of 3 readings ± SD from 1 experiment

immediately after resuspension. This reading represents the background 260nm reading; all subsequent readings have this figure subtracted from them.

2.3.2.2 Effect of arsenate on excretion of 260nm - absorbing compounds

Cells were depleted of sulphur for 2.5h and resuspended in the following media at pH 7.30.

a) glycylglycine 40mM/glucose 4mM

b) glycylglycine 40mM/glucose 4mM/arsenate 10μM

c) glycylglycine 40mM/glucose 4mM/arsenate 100μM

A_{260nm} readings were taken at recorded intervals.

2.3.2.3 Distribution of 260nm - absorbing compounds

Depletion of cells of glucose or sulphur was begun at an A_{470nm} 0.5 (Table 17) and the excretion of 260nm - absorbing compounds into the CDM measured for 5h. The trichloracetic acid (TCA) extraction procedure of Gale & Foulkes (1953) was used to determine the distribution of 260nm - absorbing compounds in the cell at the end of 5h.

The cultures were resuspended in 5% w/v TCA (A_{470nm} 1.0) and 10ml quantities were held at 20°C for 2h. Cells were removed by centrifugation (10,000g x 10min). The extract (soluble pool) was filtered through a 0.45μm membrane. The cell pellets were resuspended in 50ml of 5% TCA. 10ml samples were heated at 98-100°C for 10 min, and centrifuged. The pellets were re-extracted in the same way another two times. The A_{260nm} of all extracts were measured against 5% w/v TCA blank. All results are expressed in relation to a bacterial suspension of A_{470nm} 0.5.

Table 17

Composition of chemically defined medium*

for batch culture of P. aeruginosa

Ingredient (M)	Sulphur depleted cells	Glucose depleted cells
Glucose	2.0×10^{-2}	2.0×10^{-3}
Na_2SO_4	2.6×10^{-5}	2.6×10^{-4}
$(NH_4)_2HPO_4$	4.0×10^{-2}	4.0×10^{-2}
$Fe\ Cl_2.4H_2O$	6.2×10^{-5}	6.2×10^{-5}
$Mg\ Cl_2.6H_2O$	4.0×10^{-4}	4.0×10^{-4}
KCl	5.0×10^{-4}	5.0×10^{-4}
NH_4Cl	4.0×10^{-2}	4.0×10^{-2}

*permits exponential growth to A_{470nm} 0.5

2.3.2.4 Characterisation of 260nm - absorbing compounds excreted during sulphur depletion.

Cells were depleted of sulphur at A_{470nm} 0.5 (Table 17) and centrifuged (10,000g x 10 min) after 5h. The supernatant was filtered through a 0.22µm membrane and then evaporated to dryness in a rotary evaporator. The residue was redissolved in 50ml of 90% butanol. The upper layer was removed (personal communication Dr A.V.Quirk, Applied Microbiology Research, Porton Down) evaporated to dryness in a rotary evaporator and stored at - 20°C.

The butanol-soluble residue was redissolved in 1ml of 95% butanol. Ascending thin-layer chromatography was used to fractionate it into its various components. Glass plates, 20 x 20cm, were spread with a 0.25mm layer of silica gel F254, type 60 (Merck Ltd). The slurry was prepared by mixing 60g gel with 120ml of water and the plates were air-dried overnight. Prior to use the plates were activated by heating at 70°C for 2h. The plates were loaded with the test sample and known standards. The plates were developed in a chromatographic tank containing ammonia: butanol: water (95:9:6) (Thomson, 1969). The compounds were visualised using an ultra-violet lamp.

2.3.3 Drug-induced release of 260nm - absorbing compounds

2.3.3.1 EDTA - induced release

The experiments were performed as described in section 2.2.4. Brown & Melling (1969a) proposed that EDTA forms a complex with some metabolic products of P. aeruginosa, and that this complex absorbs more strongly at 260nm than do its components. This non-additive increase in absorbtion did not occur with 260nm -released compounds. EDTA was added to the test medium and a sample was immediately removed

for the A_{260nm} readings. This figure represents the 'background' absorbance and all subsequent readings will have this figure subtracted from them.

2.3.3.2 Polymyxin - induced release

To determine the effect of pH, glucose-depleted cells were resuspended in ammonium phosphate buffer (4mM) between pH 5.5 and 7.5. Readings were taken from the test sample immediately before the addition of PB 20 uml^{-1} and after 60 mins incubation.

All subsequent experiments were performed as described in section 2.2.5. Readings taken from the control sample immediately before the beginning of an experiment represent the 'background' absorbance; all subsequent readings will have this figure subtracted from them.

2.4 Release of potassium

2.4.1 Measurement of potassium release

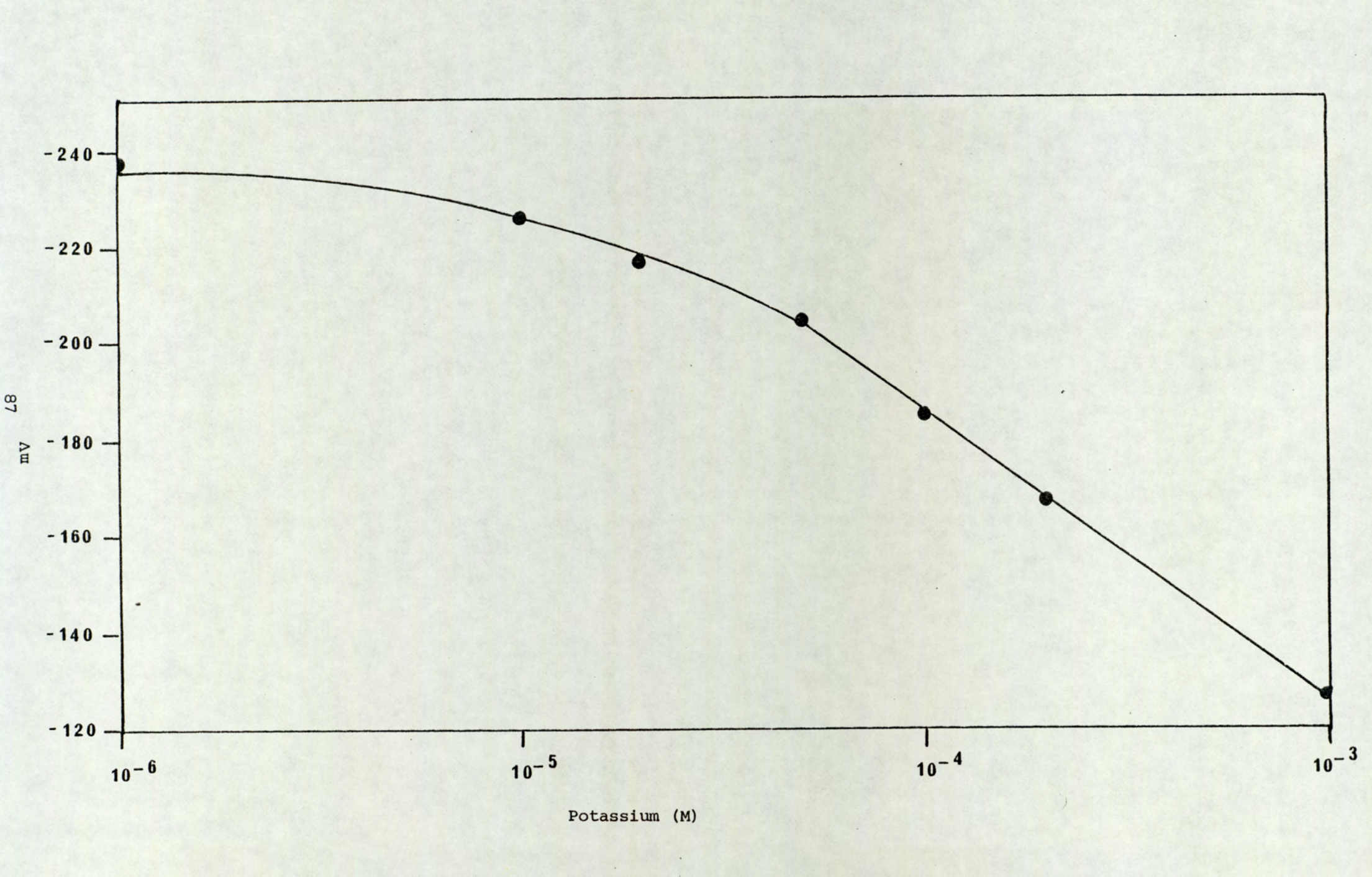
Cells were depleted of sulphur or glucose (Table 17) for 2.5h, centrifuged (8,000g x 10min) and resuspended in 100mM tris (hydroxymethyl)-aminomethane (Tris) buffer pH 7.3 ± glucose 20mM A_{470nm} 1.0. The concentration of potassium was estimated using a potassium electrode. Fig. 14 shows the potassium calibration curve obtained in 40mM Tris buffer pH 7.3. The concentration of potassium in the resuspension medium immediately after resuspension represents the 'background' potassium concentration. All subsequent readings have this figure subtracted from them.

2.4.2 Effect of glucose on the release of potassium

Cells were depleted of sulphur (Table 17) for 2.5h, centrifuged (8,000g x 10min) and resuspended in 100mM Tris pH 7.3 at A_{470nm} 1.0 and divided into 2 fractions. Potassium readings were taken at 30 min intervals. After 1h glucose 20mM was added to one of the fractions. Potassium readings were taken for both fractions for a further 2h.

Fig. 14. Potassium calibration curve

Results mean of 2 readings from 1 experiment


-240
-220
-200
-180
-160
-140
-120
mV
10^{-6}
10^{-5}
10^{-4}
10^{-3}
Potassium (M)

2.5 Measurement of lysis

2.5.1 Measurement of lysis

The change in A_{470nm} was used to estimate the extent of lysis. A reading was taken immediately before the addition of a lytic agent and at recorded intervals. All values are expressed as a % of the initial reading.

2.5.2 EDTA-associated lysis

Use of EDTA is described in section 2.2.4.

2.5.2.1. Effect of glucose on the activity of EDTA (800µM) on P. aeruginosa depleted of sulphur and glucose

Cells were depleted of sulphur for 2.5hrs, resuspended in a medium lacking sulphur and glucose (Table 6) and divided into 2 parts. Samples from the control flask were removed at 1h intervals and EDTA (800µM) added. The effect of EDTA was assessed after 30 min incubation. After 2h, glucose (4mM) was added to the second flask and a sample removed immediately and at 1h intervals and sensitivity to EDTA (800µM) assessed after 30 min incubation.

2.5.2.2 Effect of arsenate on the activity of EDTA (800µM) on nutrient-depleted P. aeruginosa.

Cells were depleted of sulphur for 5h and resuspended in the following media at a pH 7.30

a) glycylglycine 40mM

b) glycylglycine 40mM/glucose 4mM

c) glycylglycine 40mM/glucose 4mM/arsenate 100µM

After 30 mins incubation EDTA (800µM) was added. A_{470nm} readings were taken at 10 min intervals.

Previous experiments had shown that this concentration of arsenate had only a slight affect on the A_{470nm} and viability of P. aeruginosa depleted of sulphur in glycylglycine 40mM, pH 7.30 (Table 18).

2.5.2.3. Effect on sensitivity to EDTA of the sulphur depletion time

After 1h of sulphur depletion, samples were removed at 1h intervals and resuspended in $(NH_4)_2HPO_4$/glucose (Table 6). After 30 min incubation EDTA (800μM) was added and the sensitivity assessed after a further 30 min incubation.

2.5.2.4 Total counts

0.1ml samples were diluted in 1.2% NaCl/2% formaldehyde/1.6mM $MgCl_2$ to produce total counts of 400-600 bacteria per slide.

2.5.3 Polymyxin - associated lysis

To determine the effect of pH, glucose-depleted cells were resuspended in ammonium phosphate buffer (4mM) between pH 5.0 and 7.5. PB 20u ml^{-1} was added and the change in A_{470nm} estimated at 60 min.

As a result of this experiment, the pH of the test menstruum was not adjusted when studying lysis. Full details are in Section 2.2.5.

2.5.4 Preparation of culture filtrate

Cells were depleted of sulphate or glucose (Table 4) for 6h and centrifuged (25 min x 10,000g). Samples were filtered (0.45μm membrane) and concentrated by dialysis against polyethylene glycol at 4°C. Uninoculated complete CDM (Table 3) was also treated in the same way. Samples were stored after dialysis at -18°C.

Table 18

Effect of arsenate on P. aeruginosa depleted of sulphur after 90 mins treatment

Arsenate (μM)	% decrease in A_{470nm}	% decrease in survivors (cfu ml^{-1})
0	-7	-3
10	-6	10
100	0	15
1,000	5	12
10,000	9	21

Each result mean of 2 readings from 1 experiment

Prior to use, samples were vibrated in a sonic bath for 5 min and passed through a 0.45μm membrane filter to clarify them. They were then added to glucose-depleted cells to 4 times the concentration normally present after six hours of nutrient depletion. PB 20u ml^{-1} was added and the change in A_{470nm} estimated at 60 mins.

2.6. Measurement of percentage survivors

2.6.1 Measurement of viability

The initial viable number of bacteria was estimated from the control sample after the addition of water and at recorded intervals from control and test samples. All test values are expressed as % survivors of the initial value.

2.6.2 EDTA action

2.6.2.1 EDTA inactivation

0.25ml of all samples (including control) were initially diluted in 4.75ml of nutrient broth containing Mg Cl_2, which chelates any EDTA present (Roberts et al., 1970). Further dilutions were made in nutrient broth to produce counts of 20-40 cfu/drop (Section 2.2.7).

2.6.2.2 Effect of glucose on the activity of EDTA (800μM) on *P. aeruginosa* depleted of sulphur and glucose.

The protocol is as described in Section 2.5.2.1. The results are expressed as % survivors after 30 min incubation with EDTA.

2.6.3 Polymyxin action

2.6.3.1. Polymyxin inactivation

0.25ml of all samples (including control) were initially diluted in 4.75ml of lecithin broth (0.5% w/v lecithin, 4% w/v glycerin, nutrient broth) which neutralises any PB present (Kohn et al., 1963). Further dilutions were made in nutrient broth to produce counts of 20-40 cfu/drop (Section 2.2.7).

2.6.3.2 Polymyxin action

To determine the effect of pH, glucose-depleted cells were resuspended

in ammonium phosphate buffer (4mM) between pH 6.5 and 7.5. Viable counts were estimated immediately before the addition of PB 20u ml^{-1} and after 30 min incubation. As a result of this experiment all further experiments were carried out at pH 7.3 as described in Section 2.2.5. If necessary, unresuspended cells were initially diluted with CDM lacking any nutrients, to adjust the A_{470nm} to 0.20.

2.6.4 Gentamicin action

Gentamicin was added to drug sensitivity agar at 44°C to produce concentrations between 0.05 and 0.3µgml^{-1}. After setting, the plates were dried at 37°C for 60 min. Cells were depleted of sulphate or glucose and resuspended in buffer ± glucose (4mM) A_{470nm} 0.20 (Table 6) and incubated for 30 min. Dilutions were rapidly prepared in nutrient broth, such that a 0.1ml sample when spread on the surface produced approximately 300cfu on the control plate when incubated overnight at 37°C. All results for gentamicin activity are expressed as % survivors of this figure.

2.7 Preparation of auxotrophic mutants

The following techniques are based on the methods described by Meynell & Meynell (1970b).

2.7.1 Mutagenesis using Ethyl methane sulphonate (EMS)

20µl of EMS was added to 1ml $(NH_4)_2HPO_4$ (40mM) pH 7.4 and incubated at 37°C to dissolve the EMS. 0.1ml of an overnight culture of *P. aeruginosa* grown in CDM (Table 3) was added to the solution and incubated for 20 min.

2.7.2 Penicillin enrichment

0.1ml of an EMS - treated culture was added to 24.9ml CDM (Table 3) containing methionine (0.1mM) and cysteine (0.2mM) and incubated overnight at 37°C. The cells were centrifuged (6,000g x 10 min), resuspended in $(NH_4)_2HPO_4$ (40mM) pH 7.3 and used to inoculate a number of CDMs (Table 19) at A_{470nm} 0.05. The CDMs also contained sucrose (0.2M). When the cells had grown to A_{470nm} 0.04, carbenicillin 25,000µg ml^{-1} was added and the cells incubated for a further 3h. The cells were then spun down, resuspended in $(NH_4)_2HPO_4$ (40mM) pH 7.3 and incubated at room temperature for 30 min. They were then serially diluted in nutrient broth, so that a 0.1ml sample, when spread on the surface of a CDM agar plate containing methionine 0.1mM and cysteine 0.2mM, produced 20-40 cfu/plate, when incubated overnight.

2.7.3 Replica plating

The plates from the penicillin experiment were replica plated, using sterile velvet pads, onto CDM agar ± cysteine and/or methionine (Table 19) and incubated overnight. The plates prepared from unsupplemented CDM were compared with CDM agar supplemented with cysteine

Table 19

Composition of CDM for isolation of auxotrophic mutants of P. aeruginosa

Ingredient	Auxotrophic mutant failing to grow			
	Cys^-	Met^-	Cys^-	Met^-
CDM*	+	+	+	
Cysteine (0.2mM)	O	+	O	
Methionine (0.1mM)	+	O	O	

* CDM (Table 3)

+ present in medium

O absent from medium

and/or methionine. Auxotrophic mutants would fail to grow on unsupplemented agar.

3 auxotrophic mutants of P. aeruginosa were isolated designated PCM1, PCM2 and PCM3. All grew equally well on cysteine or methionine agar.

2.7.4 Alternative mutagenic agents

Further attempts were made to isolate mutants of P. aeruginosa auxotrophic only for cysteine or methionine, using PCM1 and the wild-type. A number of mutagenic agents were used: EMS and nitrosoguanidine (Meynell & Meynell, 1970b) and acriflavine and 9-aminoacridine (personal communication: Dr R.M.M.Klemperer, Department of Pharmacy, Univeristy of Aston in Birmingham). No further mutants were isolated.

2.8 Analysis of cell envelopes of P. aeruginosa

2.8.1 Preparation of outer and inner membranes

2.8.1.1 Preparation of cells for cell envelopes

Approximately 16 L cells A_{470nm} 1.0 were required to produce enough outer membranes for all the proposed analyses. Cells were grown in batches of 4 x 2 L of CDM in 5L flasks. The compositions of the various CDMs adjusted to obtain a large quantity of cells are shown in Table 20.

The pre-warmed CDM was inoculated with 5ml of log-phase culture grown in CDM (Table 3). The flasks were incubated in an orbital incubator at 150 revolutions min^{-1}. A_{470nm} readings were taken to calculate the time of depletion. Cells began to be depleted of sulphur at A_{470nm} 0.75 or glucose at A_{470nm} 1.0, and were depleted for a further 5h, before centrifugation. Log-phase cells were centrifuged at an A_{470nm} between 0.8 and 1.0. To deplete cells of sulphur and glucose, sulphur-depleted cells were centrifuged (6,000g x 10 min) and resuspended in CDM lacking sulphur and glucose (Table 20), incubated for 1h and then spun down again.

2.8.1.2 Preparation of outer and inner membranes

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

The centrifuged cells were washed with 200ml 0.9% NaCl and then centrifuged and resuspended in 30mls 20% sucrose in 0.05M MOPS (pH 7.8). The suspension was cooled to $0^{o}C$ and ribonuclease (2mg) and deoxyribonuclease (2mg) added. The cells were broken by passage through a French Pressure Press ($4^{o}C$, 15,000 lb sq in^{-1}). Following breakage,

Table 20

Chemically defined medium for 2L cultures of P. aeruginosa

Ingredient (M)	Growth CDM			Resuspension media
	Logarithmic phase cells	Glucose depleted cells	Sulphur depleted cells	Glucose and sulphur free
Glucose	2.0×10^{-2}	4.0×10^{-3}	2.0×10^{-2}	0
Na_2SO_4	2.6×10^{-4}	2.6×10^{-4}	3.9×10^{-5}	0
$(NH_4)_2HPO_4$	4.0×10^{-2}	4.0×10^{-2}	4.0×10^{-2}	4.0×10^{-2}
$FeCl_2.4H_2O$	6.2×10^{-5}	6.2×10^{-5}	6.2×10^{-5}	6.2×10^{-5}
$MgCl_2.6H_2O$	4.0×10^{-4}	4.0×10^{-4}	4.0×10^{-4}	4.0×10^{-4}
KCl	5.0×10^{-4}	5.0×10^{-4}	5.0×10^{-4}	5.0×10^{-4}
NH_4Cl	4.0×10^{-2}	4.0×10^{-2}	4.0×10^{-2}	4.0×10^{-2}

lysozyme (4mg) was added and enzyme digestion was allowed to proceed at 4°C for 30 min. The envelope fragments were diluted with an equal volume of double-distilled water and centrifuged (5,000g x 10 min) to remove unbroken cells. The supernatant was then centrifuged (38,000g x 60 min) and the membrane pellet resuspended in 15ml 20% sucrose in 0.05M MOPS (pH 7.8).

The membranes were prepared for density gradient centrifugation as described by Cozens & Brown (1981). They were layered into 20ml centrifuge tubes in the following order: 2ml membranes in 20% sucrose in 0.05M MOPS (pH 7.8), 9ml 60% sucrose in 0.05M MOPS and 9ml 70% sucrose in 0.05M MOPS. The membranes were centrifuged (100,000g) overnight in an MSE 20° angle rotor centrifuge. The 2 separate bands were removed from the centrifuge tubes using a syringe and needle. The 2 fractions were washed twice in double distilled water and centrifuged (38,000g x 60 min). The pellets were finally homogenised in water to give a dry weight of approximately 6mg ml^{-1} and stored at -20°C.

2.8.2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS - PAGE)

The method used for the 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 (B15).
Stock 2 (30% w/v acrylamide/0.8 % w/v B15).

Table 21 shows the composition of the running gel and stacking gel,

Table 21

Composition of gels for electrophoresis

Ingredient (mls)	Running gel	Stacking gel
Stock 1	18.5	0
Stock 2	0	5
10% APS	0.2	0.1
10% SDS	1.5	0.3
0.5M Tris (pH 6.8)	0	7.5
1.5M Tris (PH 8.8)	18.7	0
H_2O	34.0	16
TEMED	0.13	0.08

APS = Ammonium persulphate

TEMED = N N N^1 N^1 Tetramethylethylene diamine

Table 22 the sample buffer. 50µl of sample buffer was diluted with 50µl of sample and 10µl of bromophenol blue. The solution was boiled for 10 min; after cooling 20-80µl of solution was loaded onto the gel. The electrophoresis was performed at room-temperature, at a constant current (40 mA). The electrophoresis was discontinued when the tracking dye had moved approximately 12cm. Gels were stained with Brilliant blue R-250 overnight and destained with methanol 5% - acetic acid 10% solution.

2.8.3 Outer membrane composition

2.8.3.1 Assay of magnesium and calcium

Outer membrane (OM) preparations were assayed for calcium and magnesium by atomic absorption spectroscopy. 0.25ml of OM samples were digested in 2ml of concentrated nitric acid containing lanthanum ions (30µg ml^{-1} as lanthanum chloride). The samples were boiled to dryness. When cool, the ash was dissolved in 0.4ml of concentrated hydrochloric acid. The solution was made up to 10ml with a solution 1% lanthanum chloride in 1% hydrochloric acid. The lanthanum was added to overcome suppression by phosphate ions of absorbance due to calcium ions.

Standards containing calcium and magnesium were prepared in 1% lanthanum chloride in 1% hydrochloric acid. Fig. 15 shows the calibration curves obtained.

2.8.3.2 Total protein

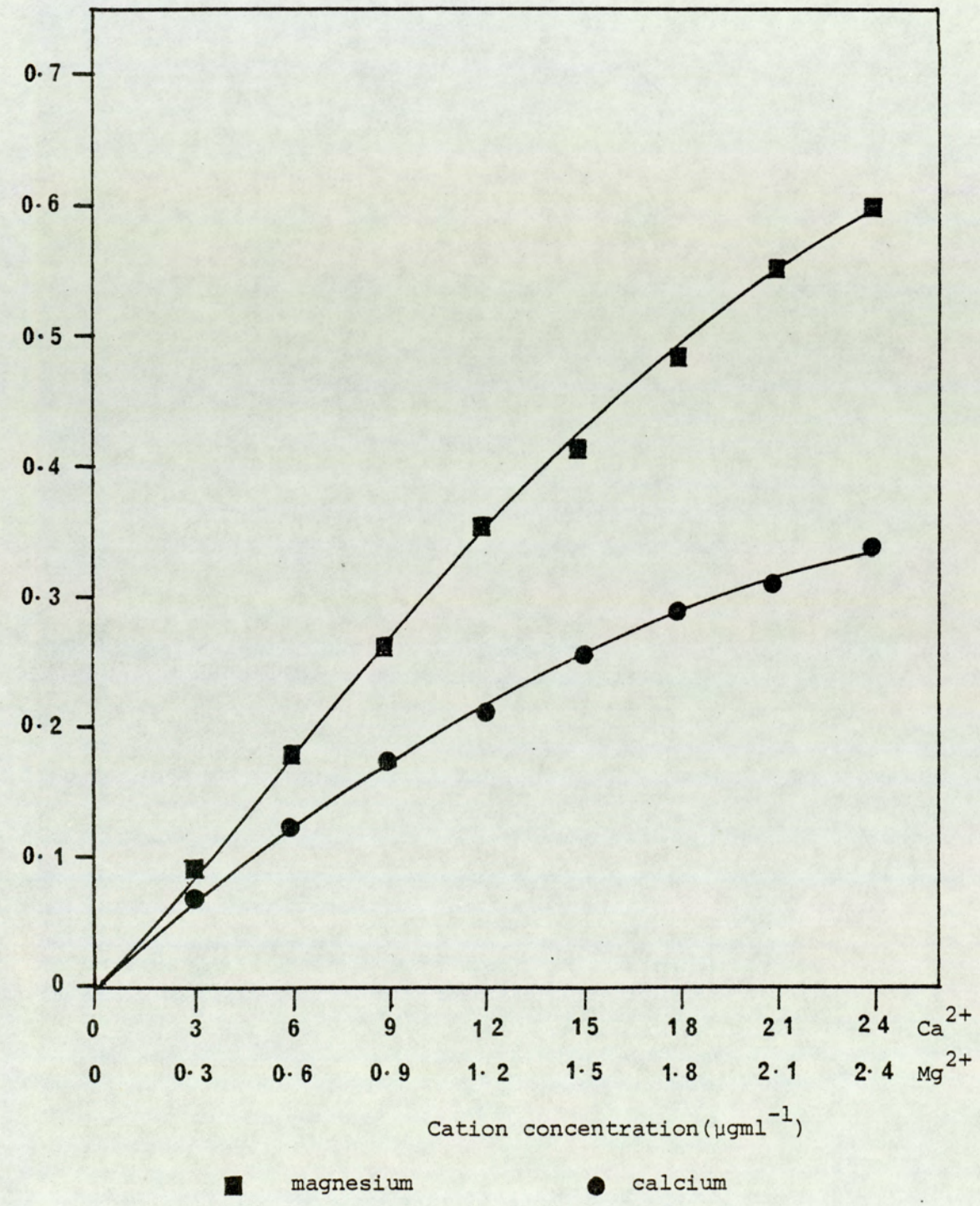
OM samples were initially diluted 30 times in double distilled water and then 0.5ml samples were assayed for total protein by the method of Lowry *et al.* (1951). Bovine serum albumin standards in the range 0-150µg ml^{-1} were prepared; Fig. 16 shows the calibration curve obtained.

Table 22

Composition of sample buffer

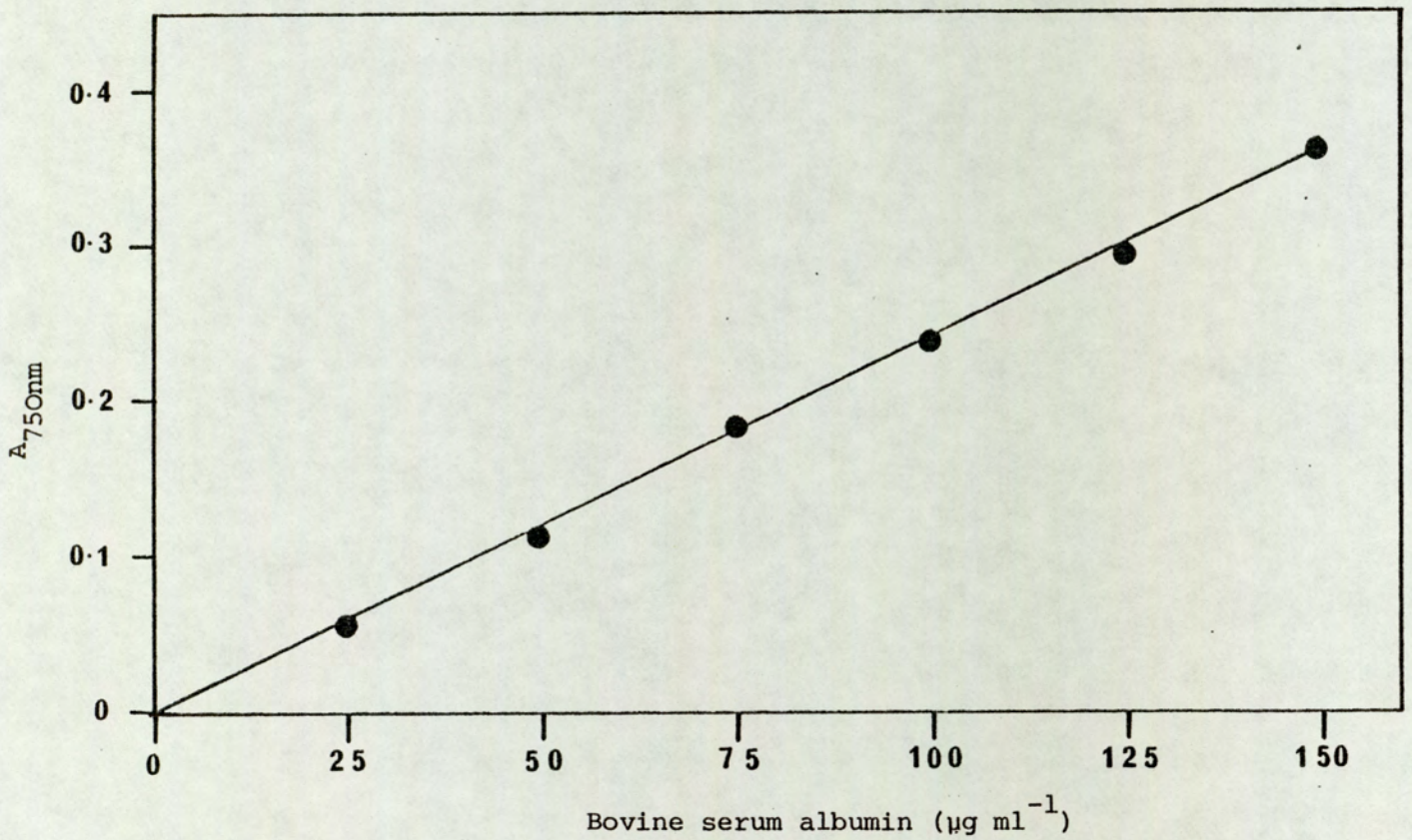
Ingredient	ml
0.5M Tris (pH 6.8)	5
10% SDS	10
Mercaptoethanol	0.5
Glycerol	5
H_2O	10

Fig. 15. Atomic absorption spectrophotometric assay of calcium and magnesium



Each point mean of 2 readings from 1 experiment

Fig. 16. Protein calibration curve



Each point mean of 3 readings from 1 experiment

2.8.3.3 2-Keto-3 deoxyoctonic acid (KDO)

0.1ml of OM samples were assayed for KDO by the method of Ellwood (1970) with the following modifications: samples were treated with periodic acid in H_2SO_4 for 20 min at 55°C personal communication: (Dr R M Cozens, Ciba-Geigy, Pharmaceutical Research, Basel, Switzerland);following the addition of thiobarbituric acid the solution was heated at 100°C for 20min (Osborn, 1963). KDO standards in the range 0-50µg ml^{-1} were prepared; Fig 17 shows the calibration curve obtained.

2.8.3.4 Total phosphorus

OM samples were initially diluted 5 times in double distilled water and then 0.1ml samples were digested to dryness in concentrated nitric acid containing anhydrous calcium carbonate (50 mg l^{-1}) (Baginski et al., 1967). The samples were redissolved in 0.1mls of conc H_2SO_4 and then assayed for phosphate by the method of Chen et al. (1956). Phosphate standards in the range 0-5 µg ml^{-1} were prepared; Fig 18 shows the calibration curve obtained.

2.8.3.5 Phospholipid phosphorus

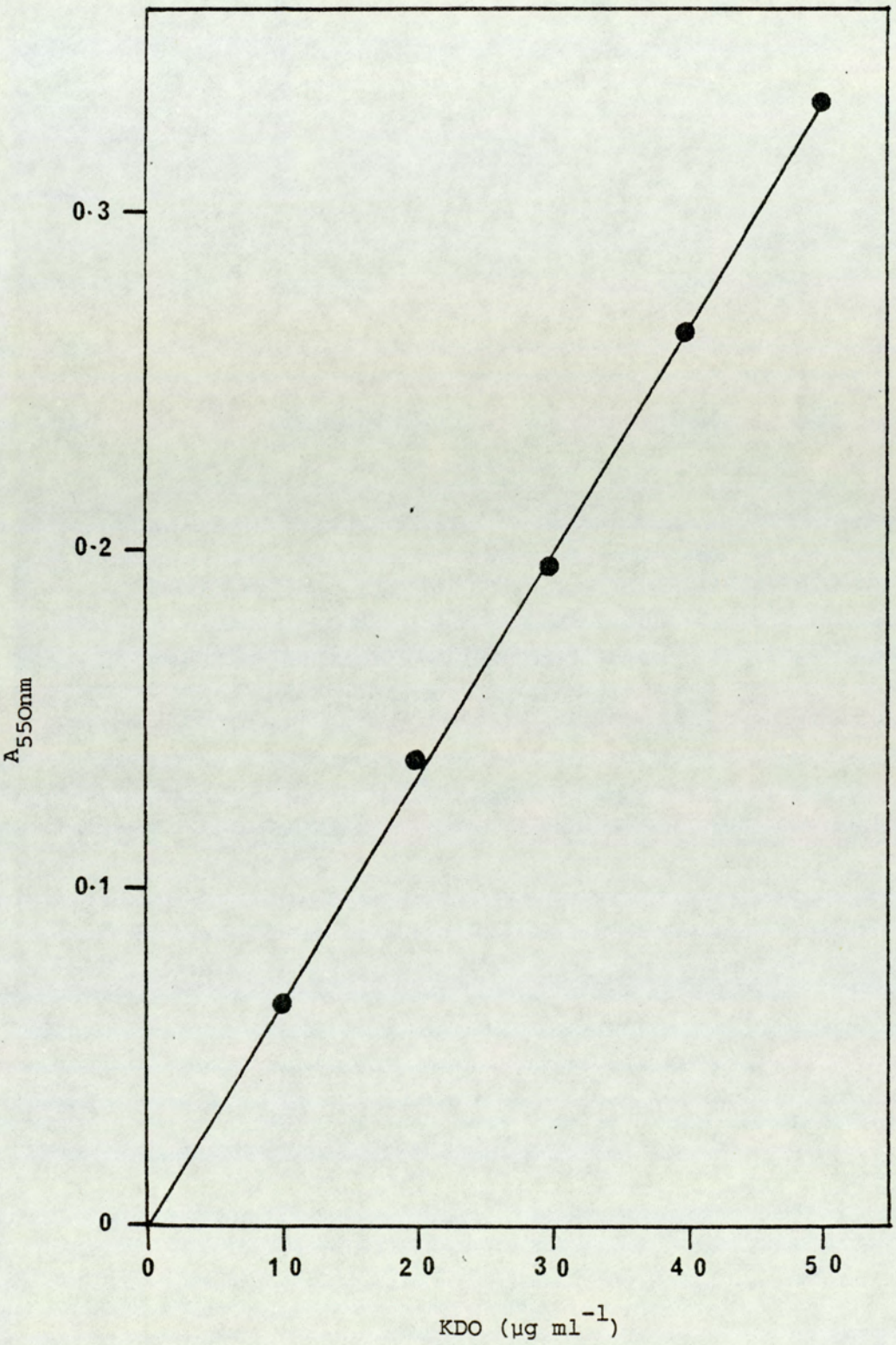
OM samples were initially diluted 5 times in double distilled water and 0.1ml samples shaken with 5ml absolute alcohol/ether (3:1)followed by centrifugation (18,000g x 10 mins). Samples were then assayed for phsophate as described in Section 2.8.3.4.

2.8.3.6 Phospholipid analysis

2.8.3.6.1 Extraction of lipids

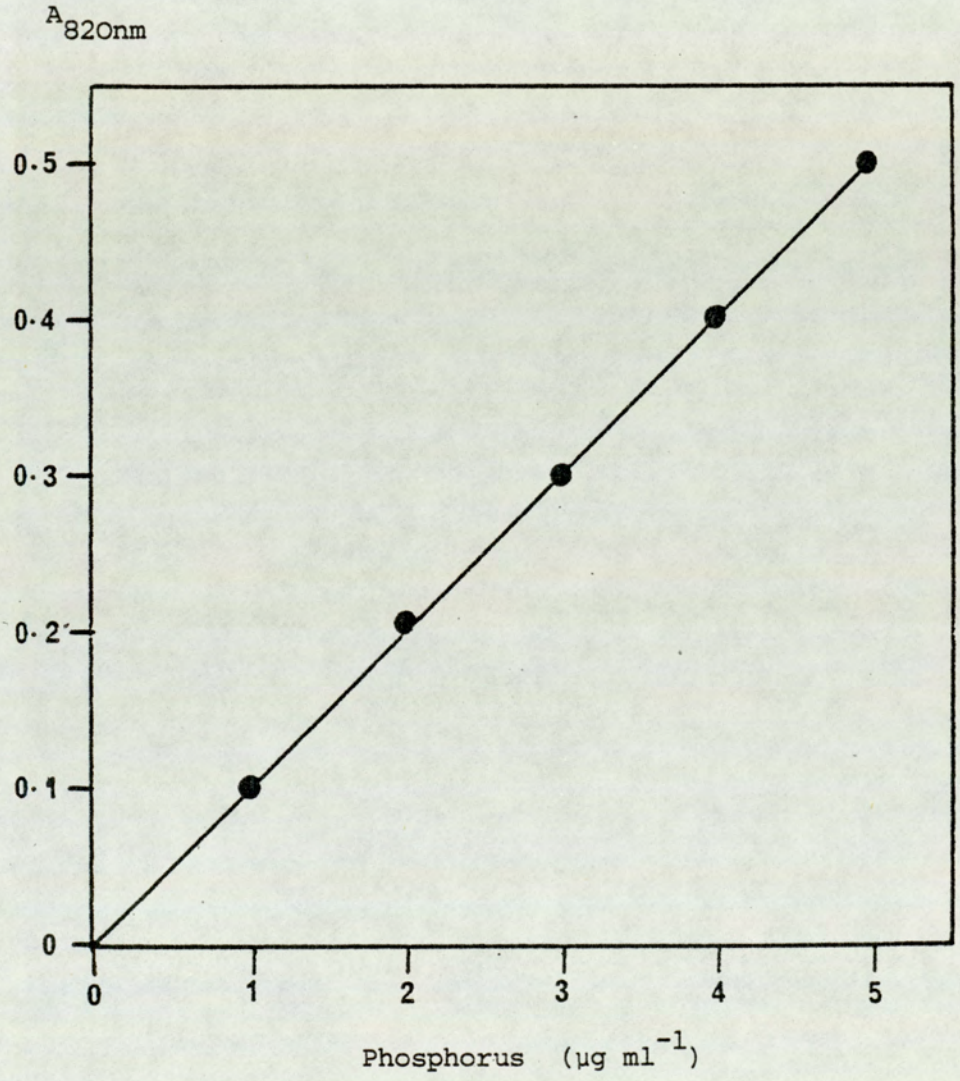
The protocol is based on the method of Ames (1968). 3ml of OM samples were mixed with 15ml of methanol/chloroform (2:1). A further 5ml of

Fig. 17. 2-Keto-3-deoxyoctonic acid calibration curve



Each point mean of 2 readings from 1 experiment

Fig. 18. Phosphorus calibration curve



Each point mean of 2 readings from 1 experiment

water was added and the mixture gently shaken. 5ml chloroform was added and the mixture again gently shaken and then allowed to stand for 2h. The lipids separate out in the chloroform fraction. The chloroform layer was filtered through phase separation paper (Whatman 1PS) into a tared tube. The samples were evaporated to dryness using a rotary evaporator and dried to constant weight in a vacuum desiccator over P_2O_5. The samples were stored at -20°C.

2.8.3.6.2 Phospholipid assay

The phospholipids (PL) were dissolved in 0.2ml chloroform/methanol (2:1). Ascending thin-layer chromatography was used to fractionate them into their component PL. Glass plates 20 x 20 cm were spread with a 0.25mm layer kiesel gel (Merck Ltd.). The slurry was prepared by mixing 60g kiesel gel with 120mls water and the plates were dried in air overnight. Prior to use the plates were activated by heating at 70°C for 2h. The plates were loaded with the membrane fractions and known standards. The plates were developed in a chromatography tank containing chloroform:methanol:water (65:25:4). The PL were visualised by spraying with phosphate spray (Ames, 1968).

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

2.8.3.7 Fatty acid analysis

2.8.3.7.1 Extraction of fatty acids

The method used was as described by Supelco (Bellefonte, Pensylvania, U.S.A). 0.5ml samples of OM were placed in pyrex hydrolysis tubes

with 5ml 5% sodium hydroxide in 50% aq. methanol. The tubes were heated at 100°C for 30 min in a water bath. After cooling, the pH was adjusted to 2.0 with conc HCl.5ml Boron trifluoride/methanol complex (14% BF w/v) was added and the mixture heated for 5 min at 80°C. The fatty acid (FA) methyl esters were then extracted from the cooled mixture with 10ml chloroform/petroleum ether (1:4). The upper layer was evaporated to dryness using a rotary evaporator. The samples were stored at -20°C.

2.8.3.7.2 Fatty acid analysis

The FA samples were taken up in 0.5ml petroleum ether and evaporated down to ca. 0.05ml. The samples were characterised by Gas-liquid chromatography, by comparing their retention times with known FA standards. The chromatogram conditions are listed below:

Column: 10ft x 2 mm ID x 1/4 in OD glass, packed with 3% SP-2100 DOH on 100/200 Supelcoport (Supelco Inc.)
Column temperature: 150°C - 223°C at 2°C min^{-1} increases.
Gas pressure: Hydrogen 14.5 psi
Air 6.5 psi
Sample size: 2 µl

Integration readings were calculated for each peak, and expressed as a % of the integration reading obtained for all the peaks in the sample.

3. EXPERIMENTAL AND RESULTS

3.1 Nutrient depletion studies

3.1.1 Carbon requirement

Fig. 19 shows the growth of P. aeruginosa in CDM containing various amounts of glucose. At low concentrations growth ceases abruptly, whilst at the highest concentrations tested the growth rate decreases gradually. Fig. 20 shows the linear relationship between the absorbance at the end of logarithmic growth and glucose concentration up to 4mM. Fig. 21 shows the biphasic relationship between the reciprocal of specific growth rate and the reciprocal of glucose concentration.

3.1.2 Sulphur requirement

Fig. 22 shows the growth of P. aeruginosa in CDM containing various amounts of sulphate. At all concentrations tested there is no abrupt cessation of logarithmic growth, rather a slowing of growth rate. Fig. 23 shows the linear relationship between the end of exponential growth and concentrations of added sulphate up to 52μM. Fig. 24 shows the biphasic relationship between the reciprocal of growth rate and the reciprocal of sulphate concentration.

3.1.3 Effect of nutrient depletion on A_{470nm}, total count, viable count and dry weight

Table 23 shows that absorbance increases slightly more than total or viable counts during sulphur depletion. The type of nutrient depletion has no effect on the relationship between the dry weight and absorbance (Table 24).

Fig. 19. The effect of glucose concentration on the growth of *P. aeruginosa*.

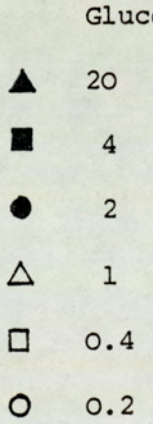


Fig 19. shows typical results for single flasks

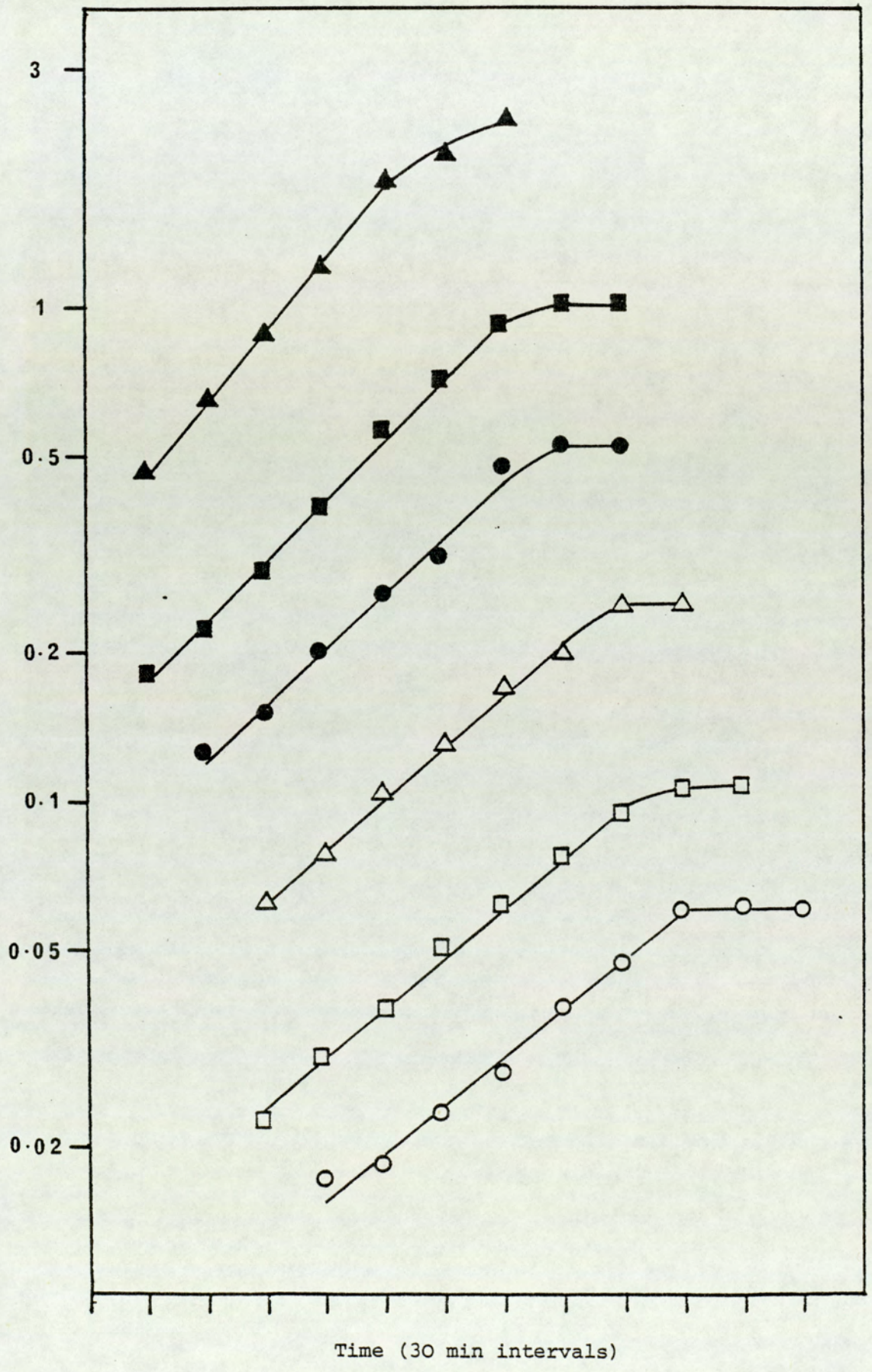

A470nm
3
1
0·5
0·2
0·1
0·05
0·02
Time (30 min intervals)

Fig. 20. The effect of glucose concentration on the maximum absorbance reached at the end of exponential growth for P. aeruginosa

Each point is the mean of 2 flasks from experiment in Fig. 19.

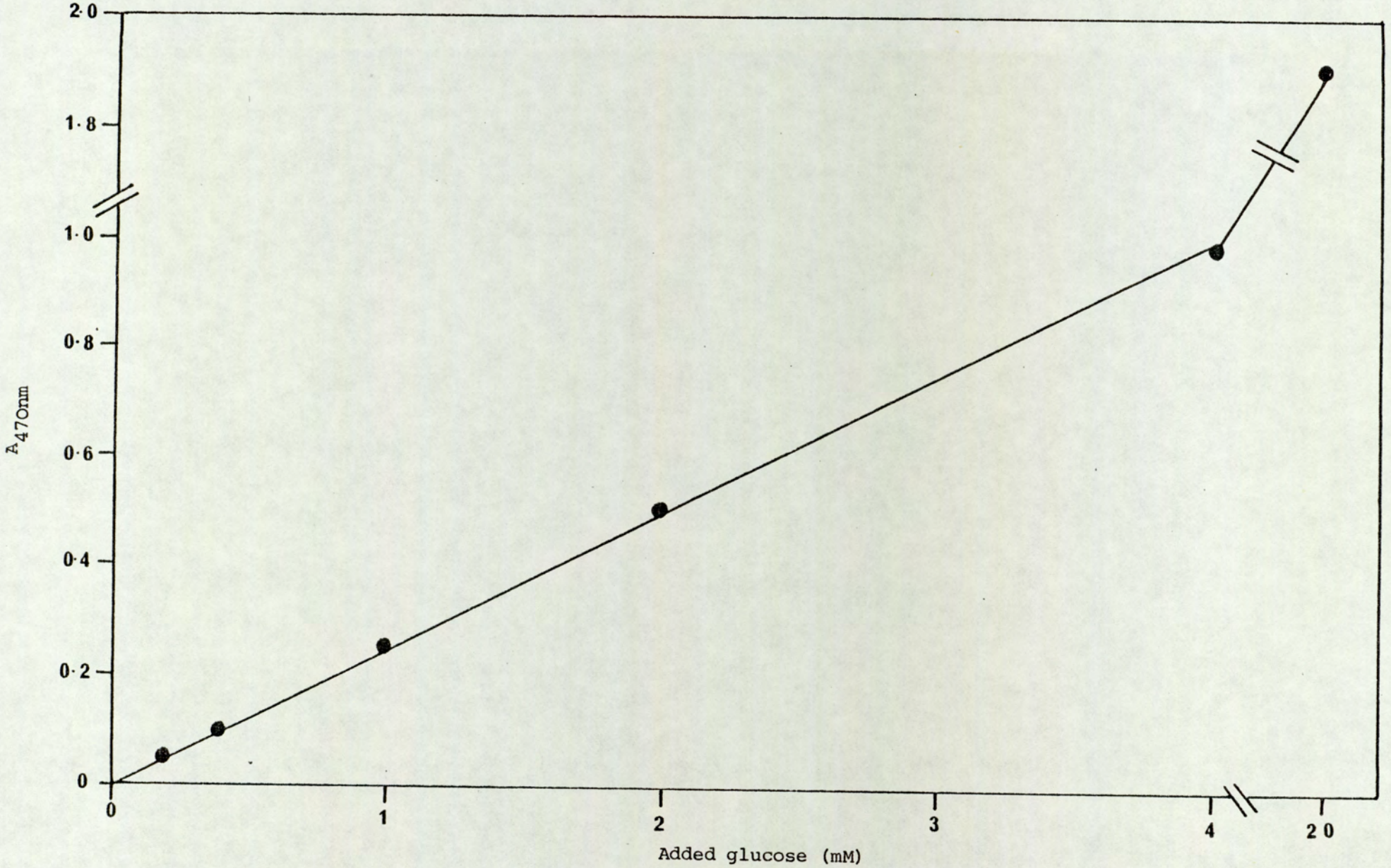

2·0
1·8
1·0
0·8
0·6
0·4
0·2
0
A_{470nm}
0
1
2
3
4
20
Added glucose (mM)

Fig. 21. Double reciprocal plot of P. aeruginosa specific growth rate versus medium glucose concentration

Each point is the mean of 2 flasks from experiment in Fig. 19.

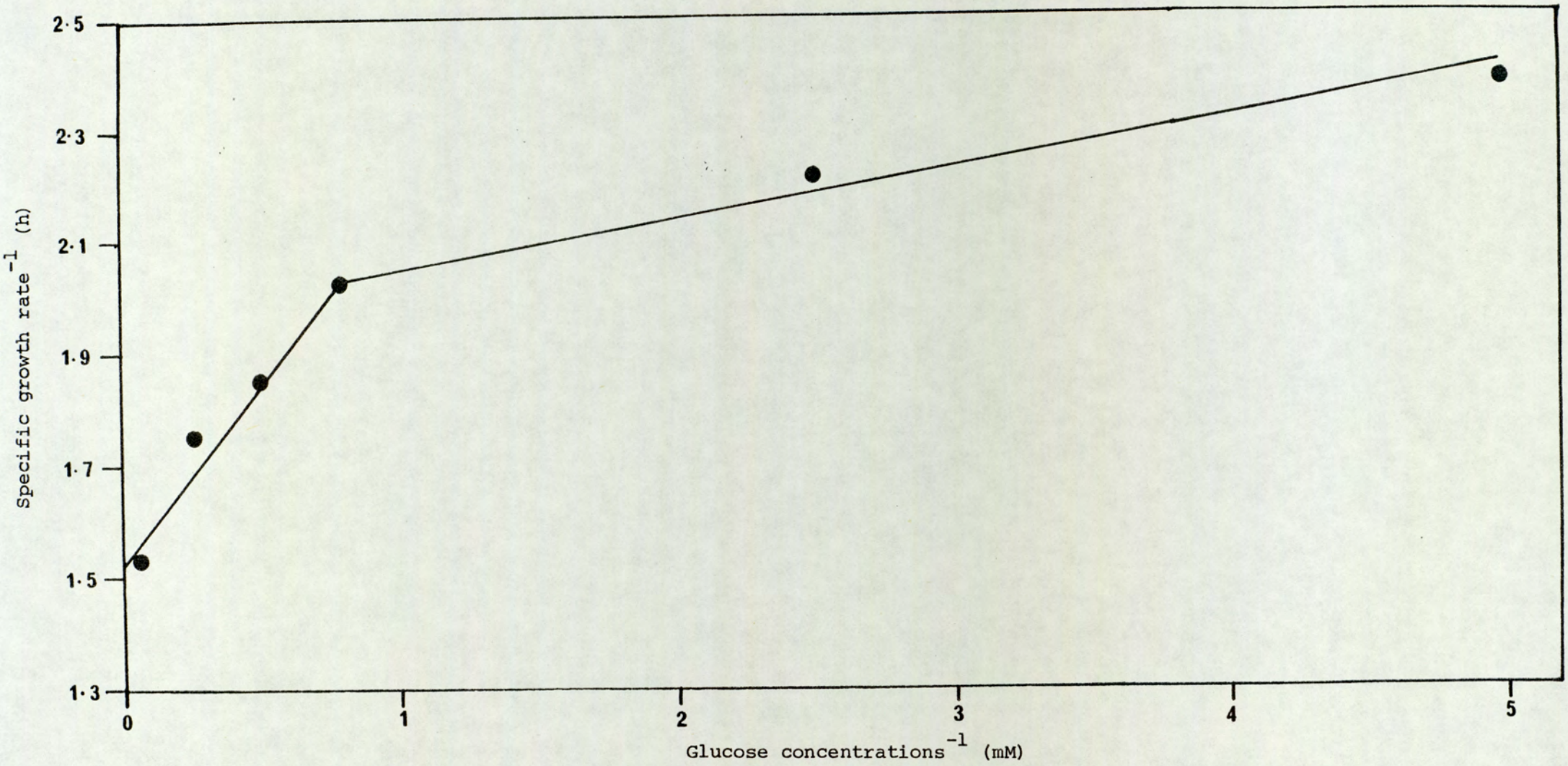

2·5
2·3
2·1
1·9
1·7
1·5
1·3
Specific growth rate^{-1} (h)
0
1
2
3
4
5
Glucose concentrations^{-1} (mM)

Fig. 22. The effect of sulphate concentration on the growth of *P. aeruginosa*

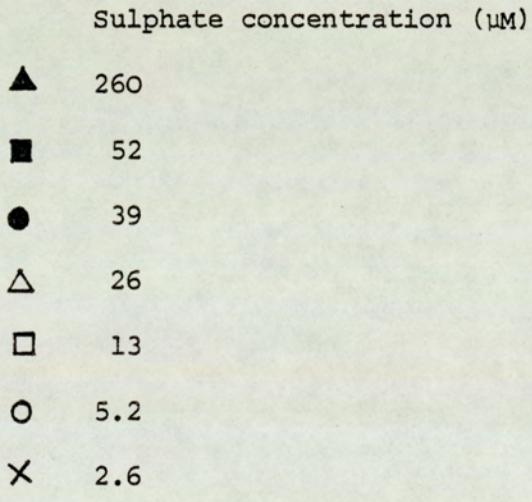


Fig. 22 shows typical results for single flasks

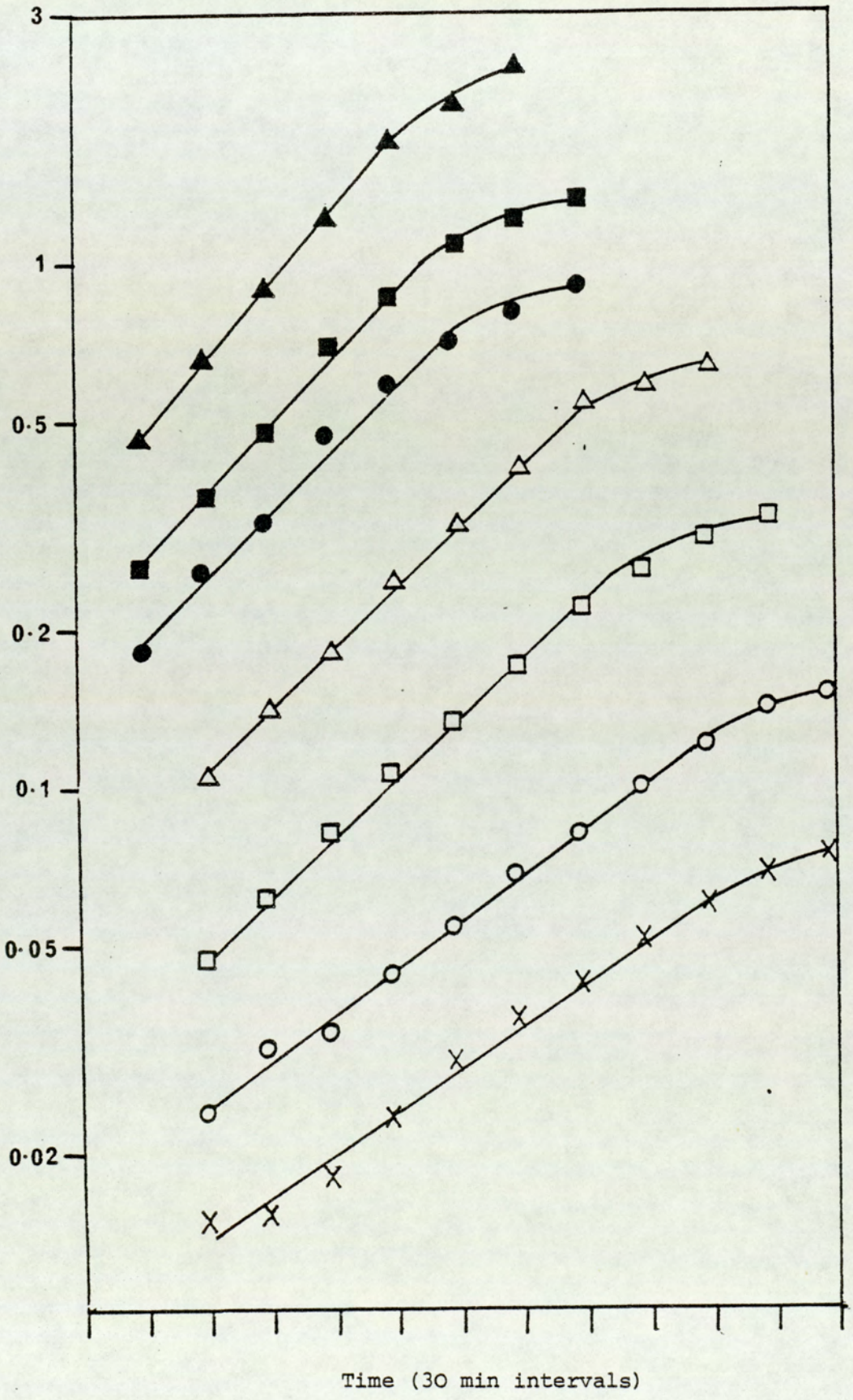

A_{470nm}
3
1
0·5
0·2
0·1
0·05
0·02
Time (30 min intervals)

Fig. 23. The effect of sulphate concentration on the maximum absorbance reached at the end of exponential growth for P. aeruginosa

Each point is the mean of 2 flasks from experiment in Fig. 22

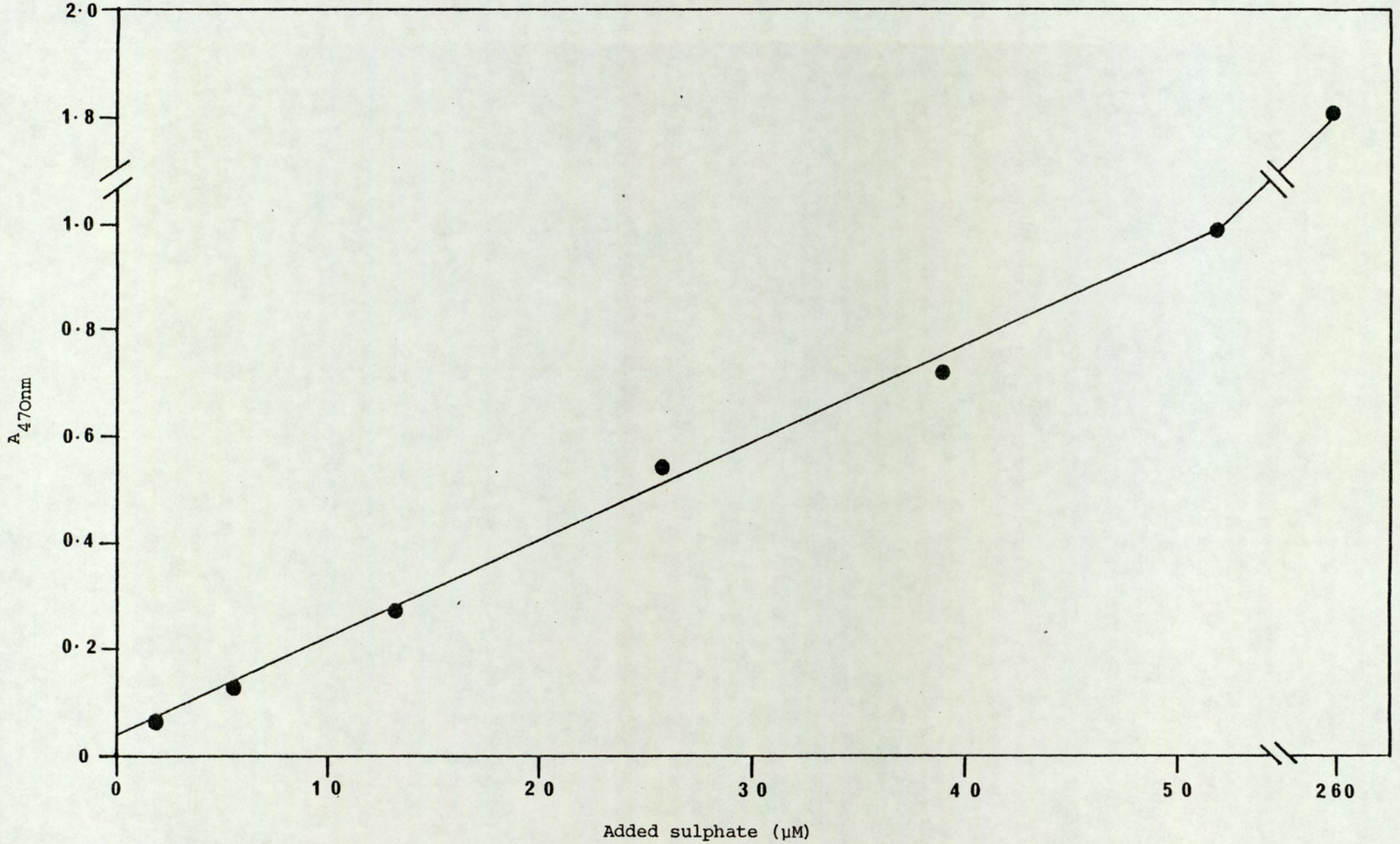

2·0
1·8
1·0
0·8
0·6
0·4
0·2
0
A_{470nm}
0
10
20
30
40
50
260
Added sulphate (μM)

Fig. 24. Double reciprocal plot of P. aeruginosa specific growth rate versus medium sulphate concentration

Each point is the mean of 2 flasks from experiment in Fig. 22.

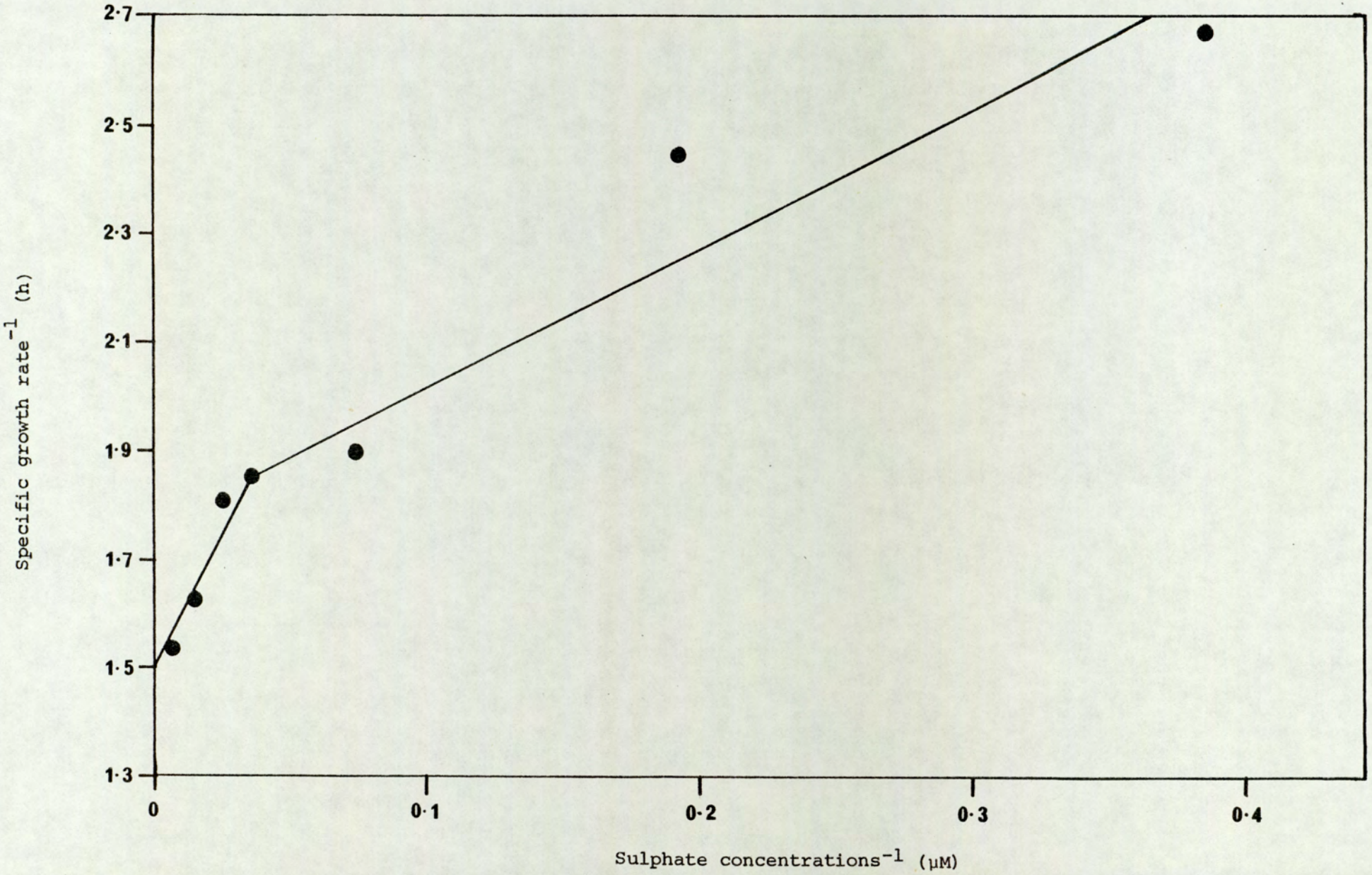

2·7
2·5
2·3
2·1
1·9
1·7
1·5
1·3
Specific growth rate^{-1} (h)
0
0·1
0·2
0·3
0·4
Sulphate concentrations^{-1} (μM)

Table 23

Effect of sulphur depletion on P. aeruginosa

Parameter	% increase after 5hrs nutrient depletion
A_{470nm}	29 ± 0
Total count	22 ± 2
Viable count	22 ± 2

Each result mean of 3 readings ± SD from 1 experiment (S^- G^+)

Table 24

Dry weight of whole cells of nutrient-depleted P. aeruginosa

Depletion	Dry weight $\mu g\ ml^{-1}$ at A_{470nm} 1.0
S^+ G^-	281
S^- G^+	298
Cys^- G^+	308
Met^- G^+	293

Each result mean of 2 readings from 1 experiment

3.2 Excretion of metabolites

3.2.1 Excretion of 260nm - absorbing compounds from nutrient depleted P. aeruginosa

3.2.1.1 When cells leave exponential growth and become depleted of sulphur, they leak 260nm - absorbing compounds (Fig. 25). The amount of 260nm - absorbing compounds excreted during sulphur-depletion depends on the sulphur source during exponential growth and is highest when sulphate is the source (Fig. 26).

3.2.1.2. The excretion of 260nm - absorbing compounds when exponential growth ceases is not a general property of P. aeruginosa cultures. It does not occur with glucose or magnesium depleted cells (Figs 27 & 28). The excretion of 260nm - absorbing compounds during sulphur-depletion requires the presence of glucose (Fig 28) and is partly inhibited in the presence of arsenate (Fig. 29).

Fig. 25. Excretion of 260nm - absorbing compounds from *P. aeruginosa*

● $S^- G^+ A_{470nm}$ 0.18 at beginning of nutrient depletion

◑ $S^+ G^- A_{470nm}$ 0.20 at beginning of nutrient depletion

↓ Time at which cells leave exponential growth and become nutrient depleted

Each point mean of 3 flasks from 1 experiment

Mean loss of 260nm - absorbing compounds on filtering 19%

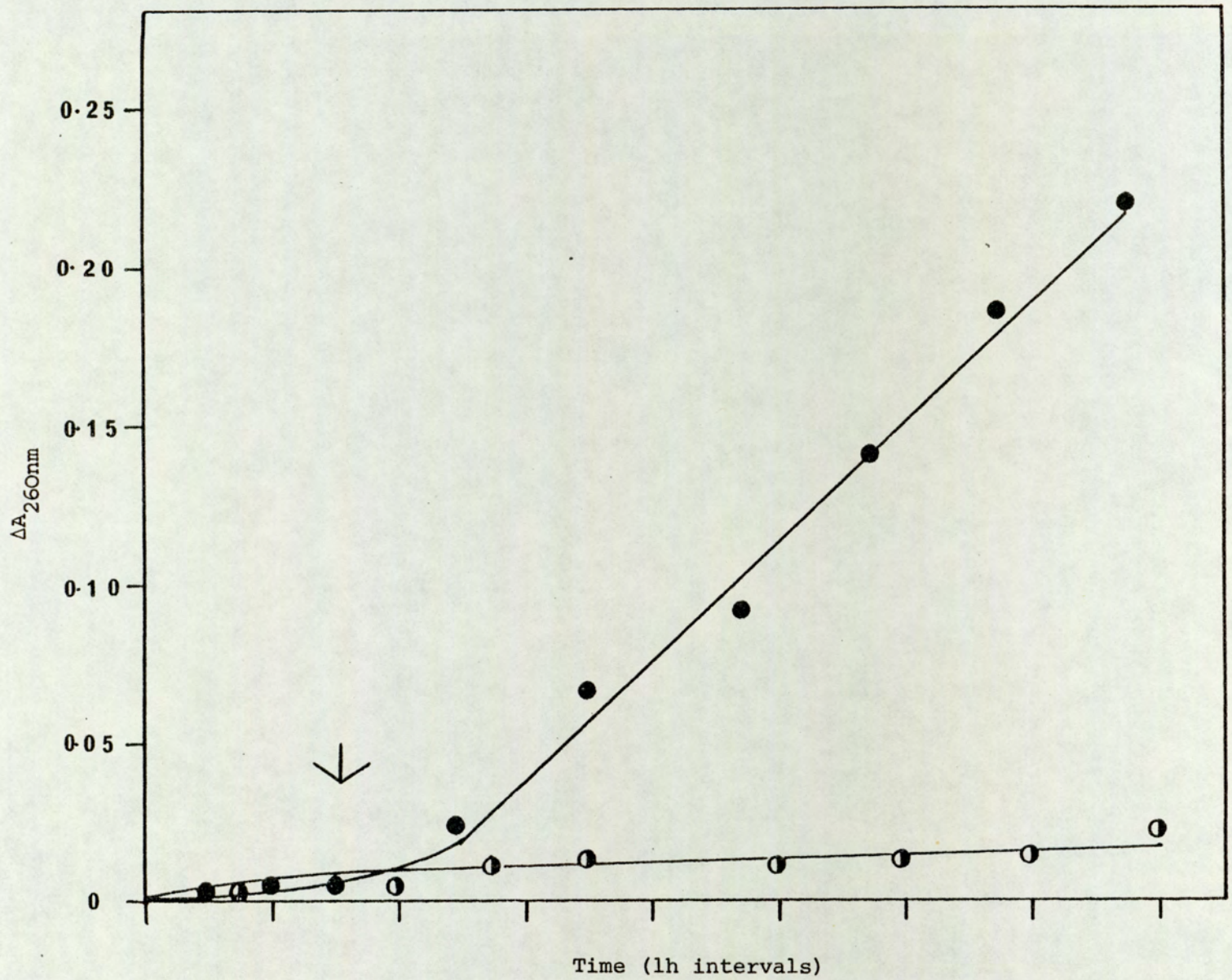

0·25
0·20
0·15
0·10
0·05
0
ΔA_{260nm}
Time (1h intervals)

Fig. 26. Effect of sulphur source on the excretion of 260nm - absorbing compounds during nutrient depletion of *P. aeruginosa*

● $S^- G^+ A_{470nm}$ 0.20 at beginning of nutrient depletion

■ $Cys^- G^+ A_{470nm}$ 0.20 at beginning of nutrient depletion

▲ $Met^- G^+ A_{470nm}$ 0.20 at beginning of nutrient depletion

Each point mean of 1 flask from 3 experiments

Mean loss of 260nm - absorbing compounds on filtering 24%

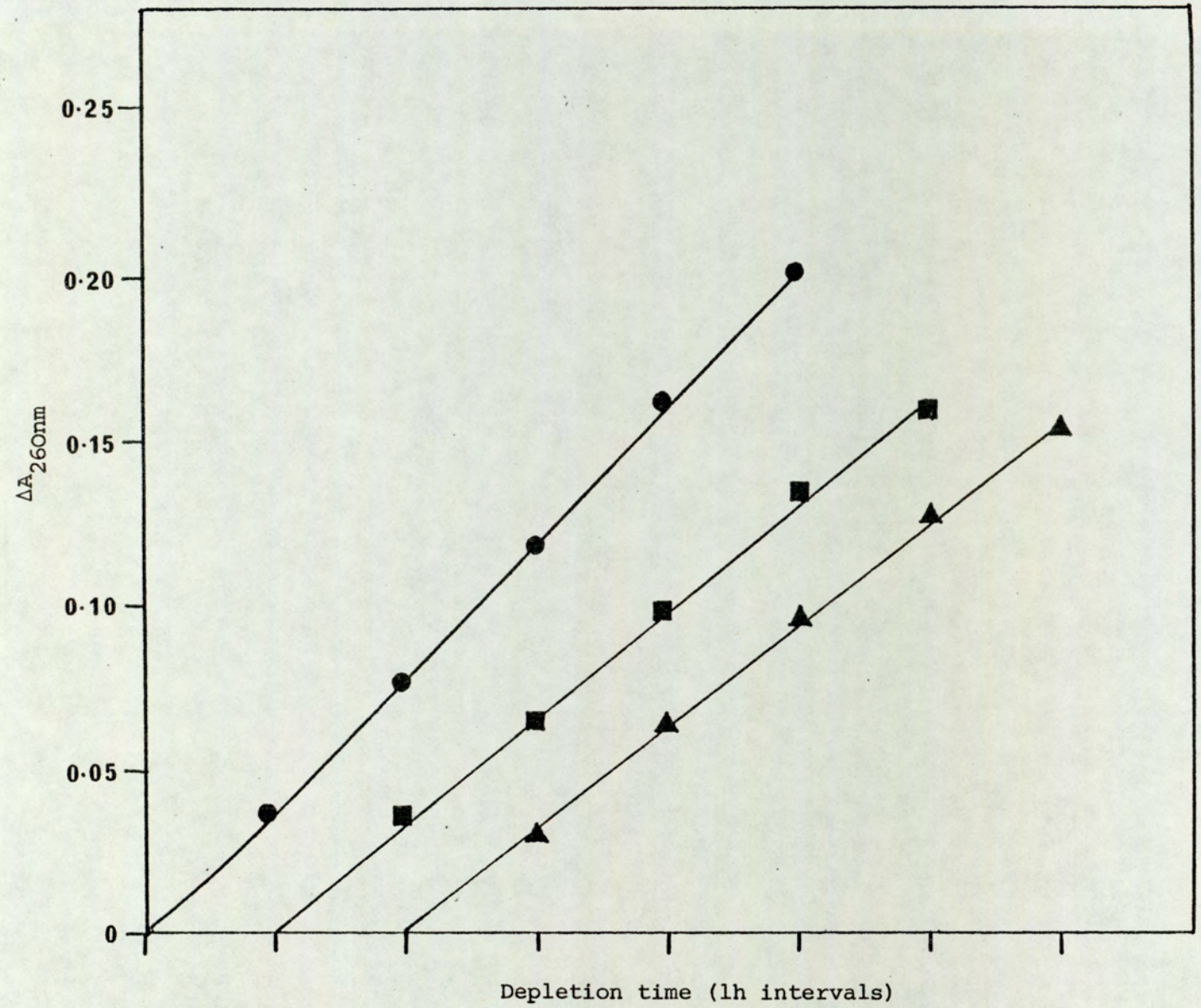

0·25
0·20
0·15
0·10
0·05
0
ΔA_{260nm}
Depletion time (1h intervals)

Fig. 27. Excretion of 260nm - absorbing compounds during nutrient depletion of P. aeruginosa

● $S^- G^+ A_{470nm}$ 0.19 at beginning of nutrient depletion

× $Mg^- G^+ A_{470nm}$ 0.22 at beginning of nutrient depletion

Each point mean of 1 flask from 2 experiments

Mean loss of 260nm - absorbing compounds on filtering 23%

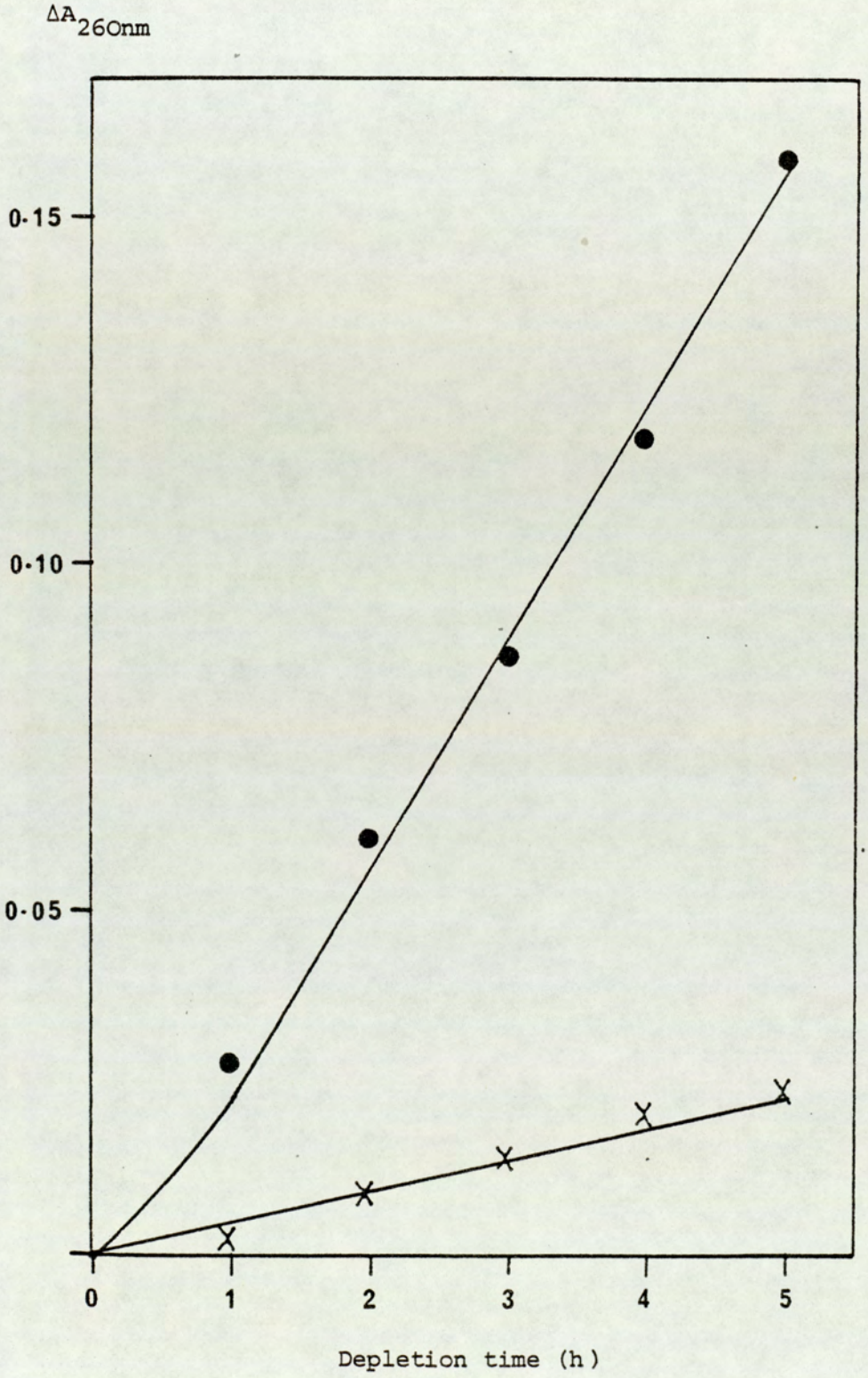

ΔA260nm
0·15
0·10
0·05
0
1
2
3
4
5
Depletion time (h)

Fig. 28. Excretion of 260nm - absorbing compounds during nutrient depletion of P. aeruginosa

● S^- G^+ A_{470nm} 0.25 on resuspension

○ S^- G^- A_{470nm} 0.22 on resuspension

◑ S^+ G^- A_{470nm} 0.20 on resuspension

Each point mean of 3 flasks from 1 experiment

Mean loss of 260nm - absorbing compounds on filtering 18%

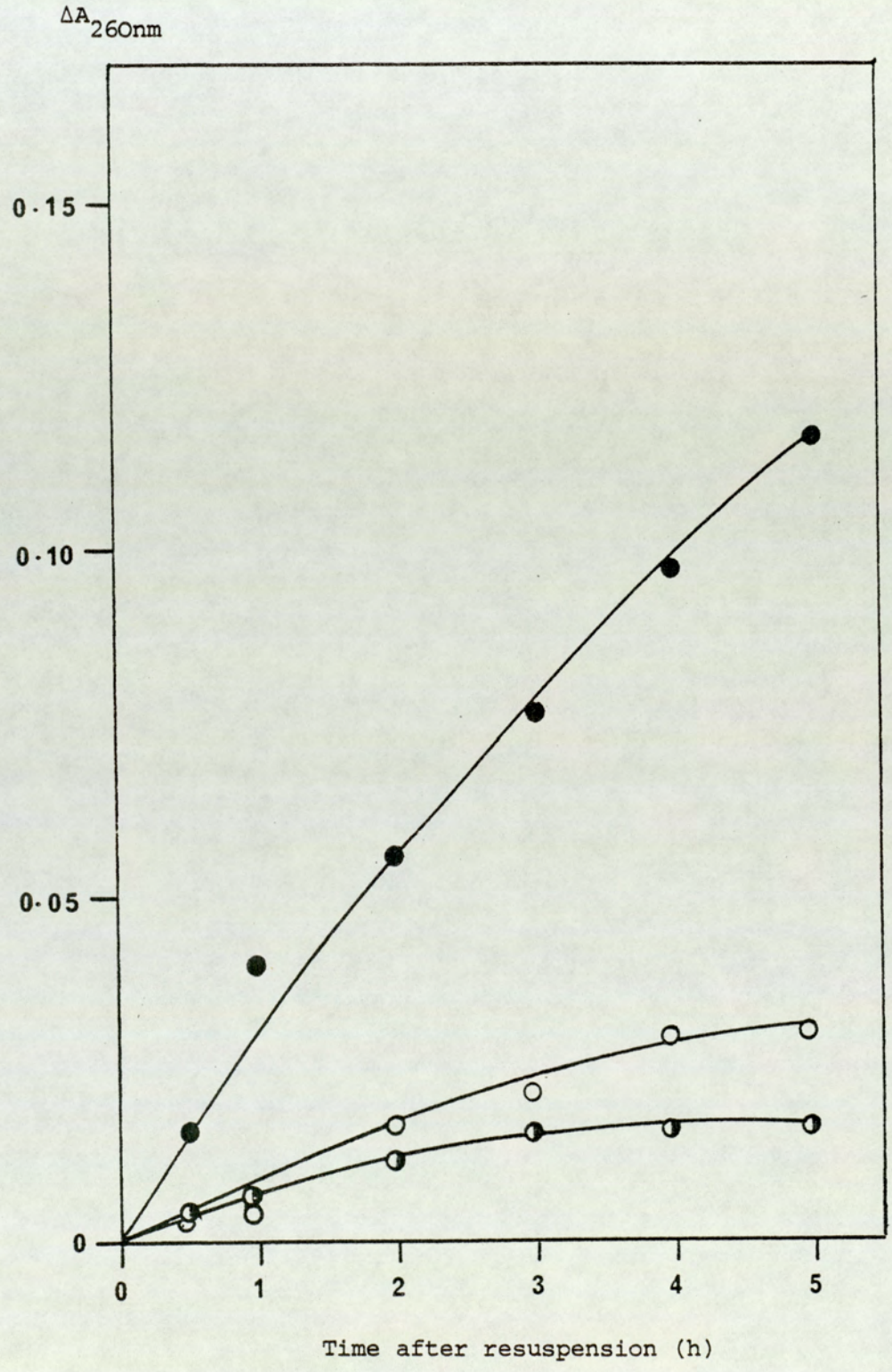

ΔA260nm
0·15
0·10
0·05
0
0
1
2
3
4
5
Time after resuspension (h)

Fig. 29. Effect of arsenate on excretion of 260nm - absorbing compounds from P. aeruginosa depleted of sulphur

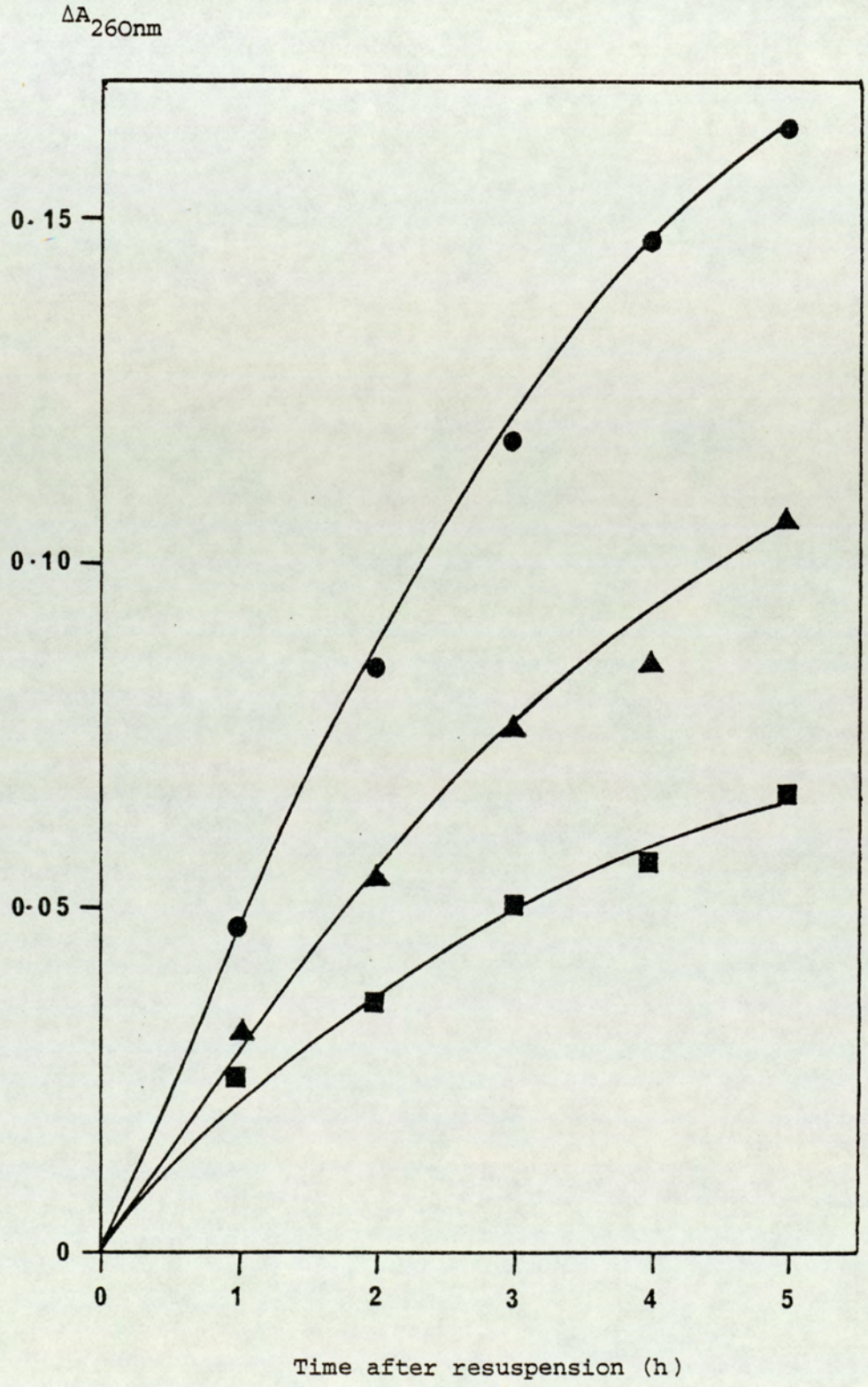
● $S^- G^+ A_{470nm}$ 0.20 at t = o

▲ $S^- G^+ A_{470nm}$ 0.20, before the addition of arsenate 10μM at t = o

■ $S^- G^+ A_{470nm}$ 0.20, before the addition of arsenate 100μM at t = o

Each point mean of 2 flasks from 2 experiments

Mean loss of 260nm - absorbing compounds on filtering 17%


ΔA260nm
0·15
0·10
0·05
0
0
1
2
3
4
5
Time after resuspension (h)

3.2.2 Characterisation of 260nm - absorbing compounds

3.2.2.1 Table 25 shows the distribution of 260nm - absorbing compounds 5h after the onset of nutrient depletion, in comparison with the amount excreted into the medium. The most noticeable feature, besides the large amount of 260nm - absorbing compounds excreted into the medium by S^- G^+ cells, is the size of the pool material in the S^- G^+ cells compared to S^+ G^- cells.

3.2.2.2 Cytosine, uracil and possibly cAMP were tentatively identified as 3 of the 5 compounds excreted from the cell during sulphur depletion (Table 26).

3.2.3 Effect of resuspending agents

Although release of 260nm - absorbing compounds by S^+ G^- cells is relatively low in CDM its extent depends on the medium and is increased considerably by resuspension in Tris buffer (Fig. 30).

Table 25

Distribution of 260nm - absorbing compounds in nutrient-depleted *P. aeruginosa*

Sample Examined	Source of absorbing compounds	Absorbance 260nm		Absorbance 280nm	
		$S^- G^+$ cells	$S^+ G^-$ cells	$S^- G^+$ cells	$S^+ G^-$ cells
Culture supernatant	Excretion of small molecules from cells	0.505	0.048	0.162	0.025
Cold TCA extract	Pool compounds	0.147	0.076	-	-
Hot TCA extract	Structural compounds	0.803	0.855	-	-

Each result mean of 1 reading from 2 experiments.

Table 26

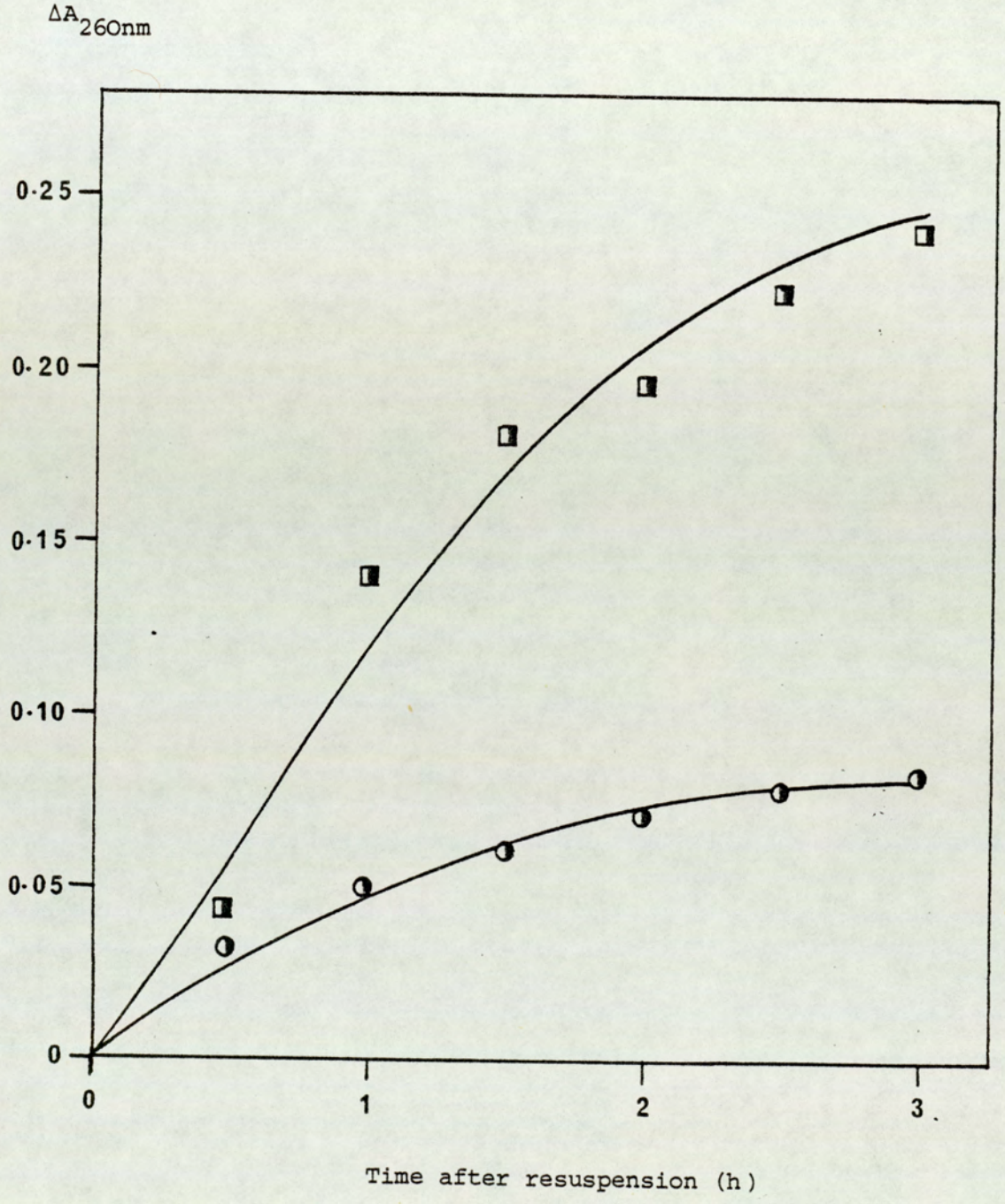
Characterisation of 260nm - absorbing compounds excreted from P. aeruginosa depleted of sulphur*

Standard	Presence in supernatant
Adenine	N.D.
Cytosine	+
Guanine	N.D.
Thymine	N.D.
Uracil	+
cAMP	+

+ = co-chromatographed with standard

N.D. = not detectable

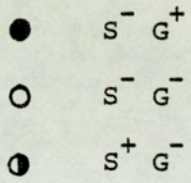
* = Also presence of 2 uncharacterised compounds in S^- G^+ supernatant


ΔA 260nm
0·25
0·20
0·15
0·10
0·05
0
0
1
2
3
Time after resuspension (h)

3.2.4 Potassium release following nutrient-depletion

After resuspension in Tris buffer, both sulphur-and glucose-depleted cells release potassium into the medium in the absence of glucose, whilst the S^- G^+ cells take up potassium (Fig. 31). The addition of glucose to S^- G^+ cells inhibits the release of potassium and after a short lag promotes the uptake of potassium (Fig. 32).

Fig. 31. Release of potassium from nutrient - depleted *P. aeruginosa*



A_{470nm} 1.0 at t = 0

Each point mean of 1 flask from 2 experiments

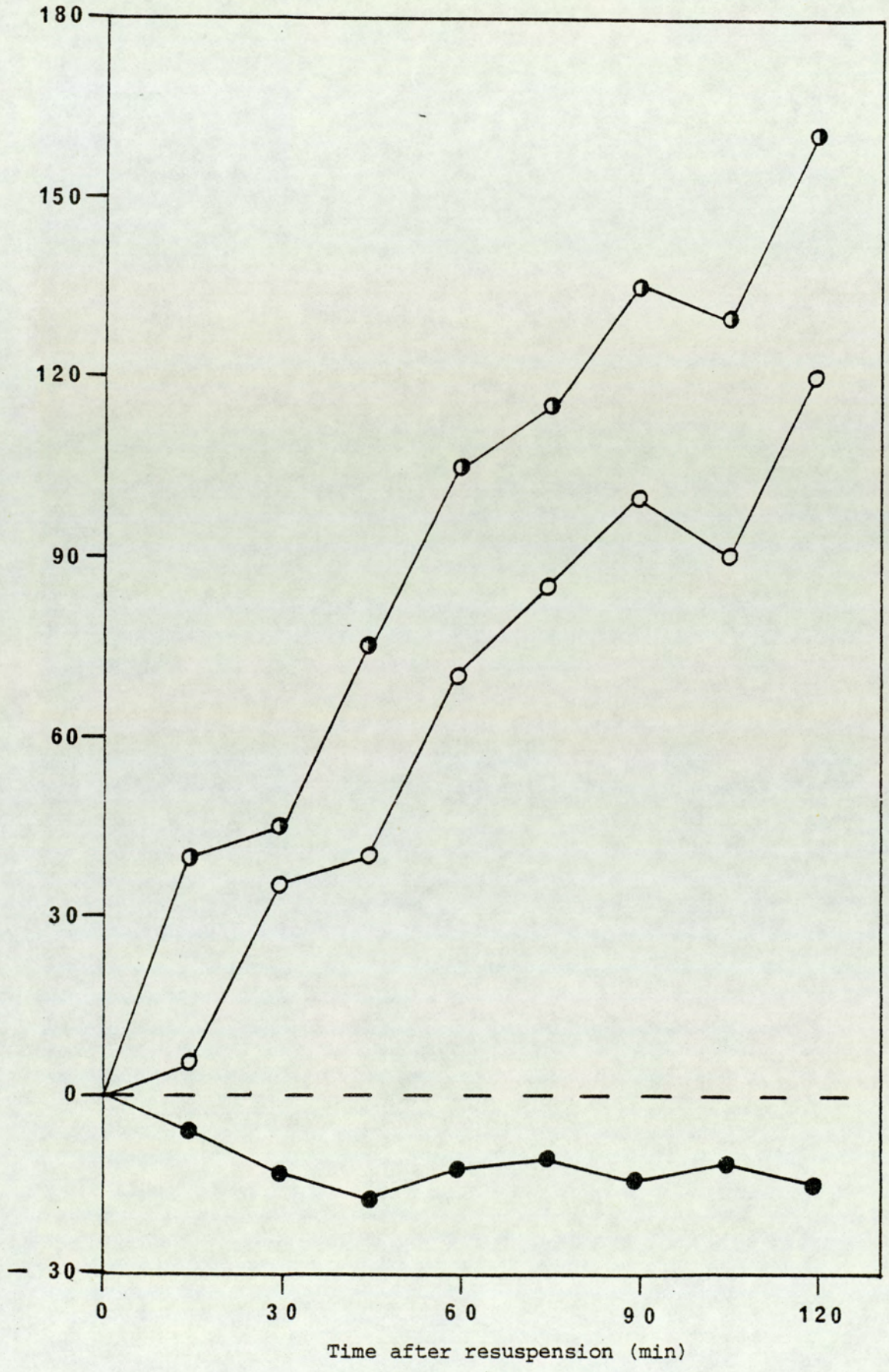

Change in potassium concentration
(μM) in medium
180
150
120
90
60
30
0
30
0
30
60
90
120
Time after resuspension (min)

Fig. 32. Effect of glucose on the release of potassium from *P. aeruginosa* depleted of sulphur

○ $S^- G^-$

↓ glucose (20mM) added to 1 flask

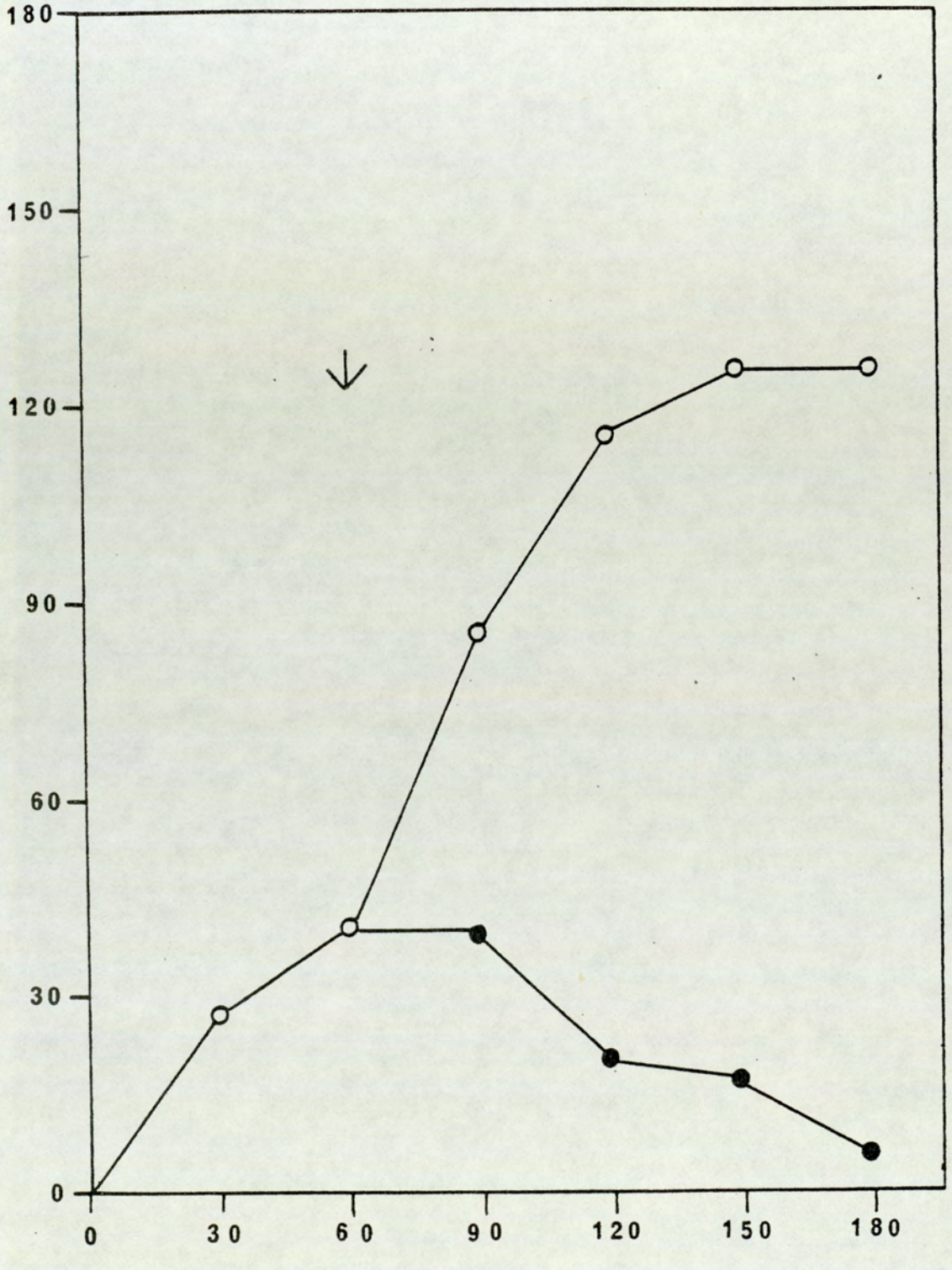
● flask after addition of glucose

A_{470nm} 1.0 at t = 0

Each point mean of 1 flask from 2 experiments

Change in potassium concentration

(μM) in medium



3.3 Action of EDTA on nutrient - depleted cells

3.3.1 Cell lysis

3.3.1.1 S^- G^+ cells are resistant to the lytic action of EDTA, as shown by the slight increase in A_{470nm}, whilst S^+ G^- cells are sensitive to its action, as shown by the decrease in A_{470nm} (Fig. 33). The resistance of sulphur-depleted cells is dependent on the presence of glucose (Fig 34).

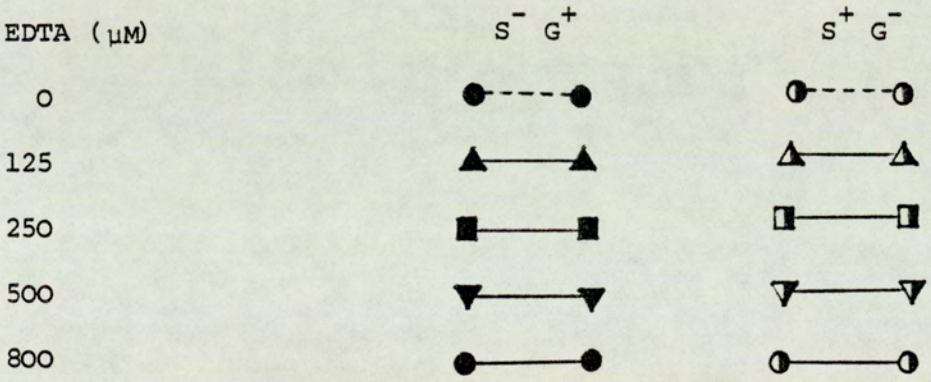
3.3.1.2 The addition of glucose to S^- G^- cells increases resistance to EDTA immediately and completely restores it in 1h (Fig. 35).

3.3.1.3 The addition of arsenate to S^- G^+ cells reduces the effect of glucose in maintaining the resistance of sulphur-depleted cells (Fig. 36)

3.3.1.4 There is no increase in extent of resistance to EDTA when sulphur depletion is increased from 1 to 4h (Fig. 37).

3.3.1.5 Table 27 shows that changes in A_{470nm} after treatment with EDTA are paralleled by changes in the total count.

Fig. 33. Action of EDTA on P. aeruginosa depleted of either glucose or sulphur



A_{470nm} 0.20 before the addition of EDTA

Each point mean of 1 flask from 2 experiments

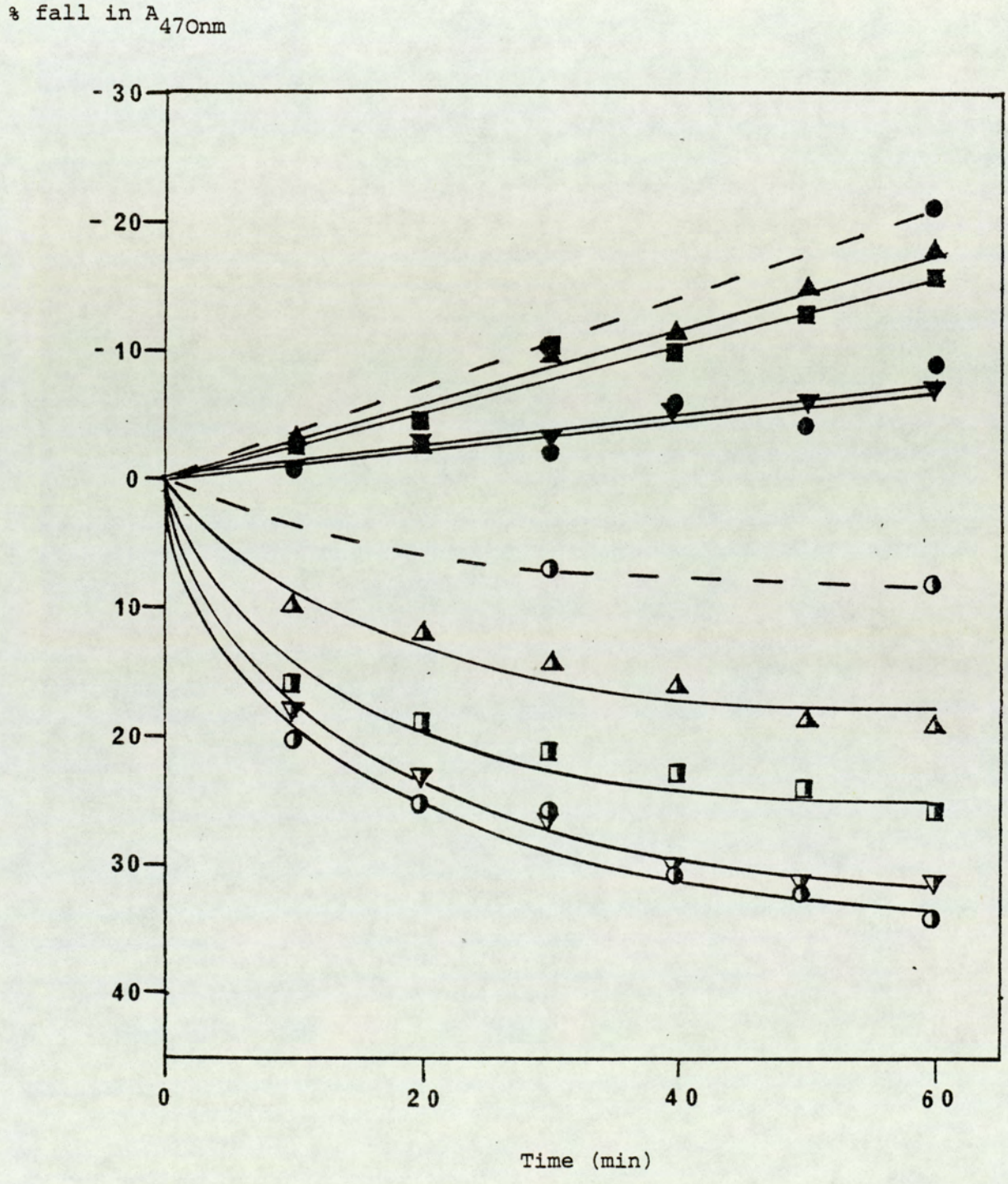
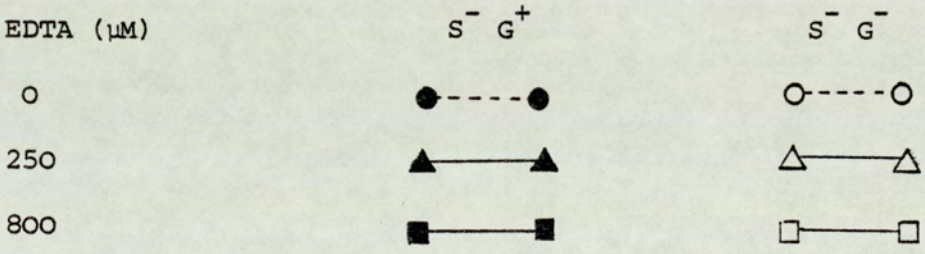

% fall in A_{470nm}
- 30
- 20
- 10
0
10
20
30
40
0
20
40
60
Time (min)

Fig. 34. Effect of glucose on the activity of EDTA on *P. aeruginosa* depleted of sulphur



A_{470nm} 0.20 before the addition of EDTA

Each point mean of 1 flask from 2 experiments

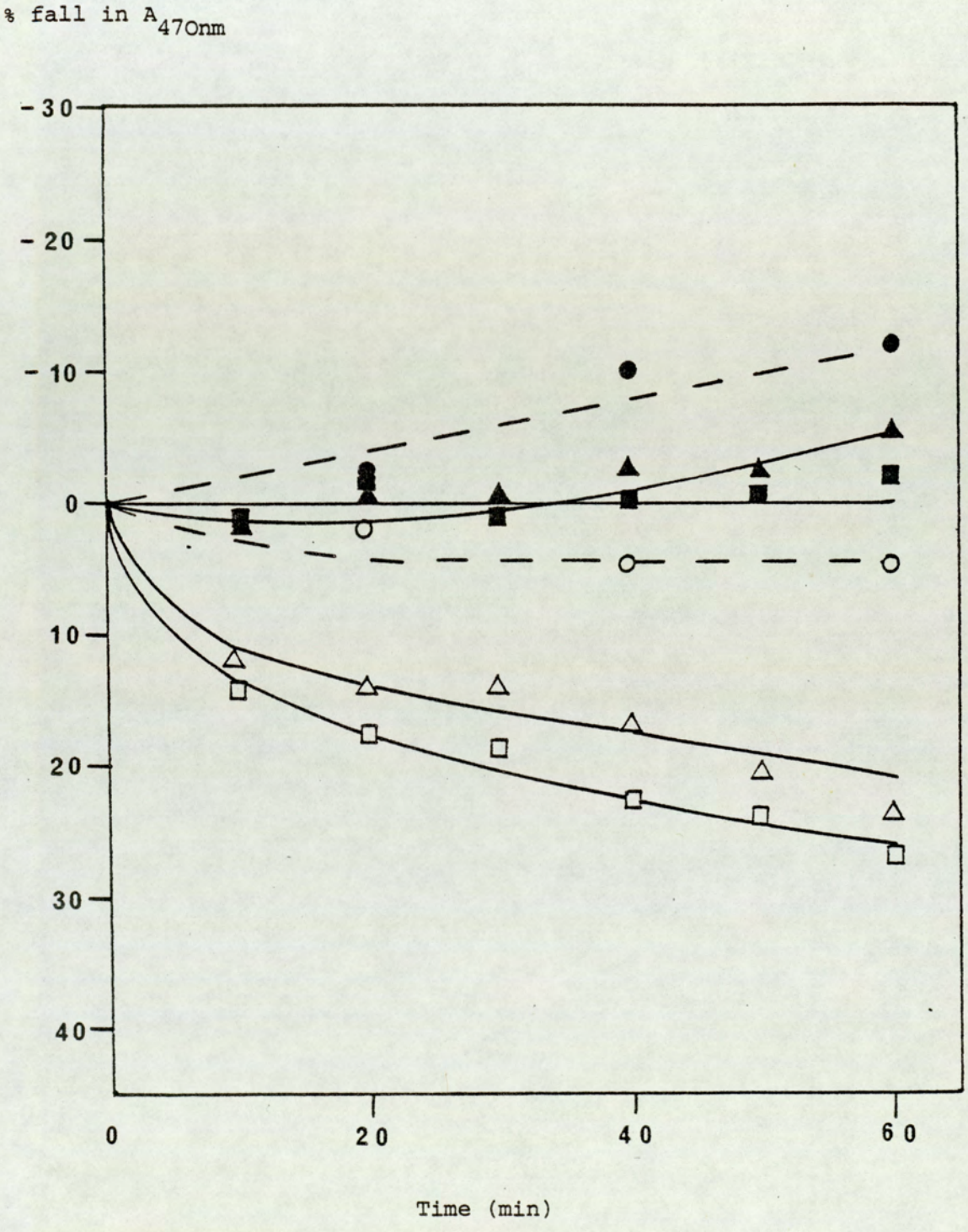

% fall in A_{470nm}
-30
-20
-10
0
10
20
30
40
0
20
40
60
Time (min)

Fig. 35. Effect of glucose on the activity of EDTA (800µM) on *P. aeruginosa* depleted of sulphur and glucose

○ $S^- G^-$

↓ glucose (4mM) added to 1 flask

● flask after addition of glucose

A_{470nm} 0.20 before the addition of EDTA

Each point mean of 1 flask from 2 experiments

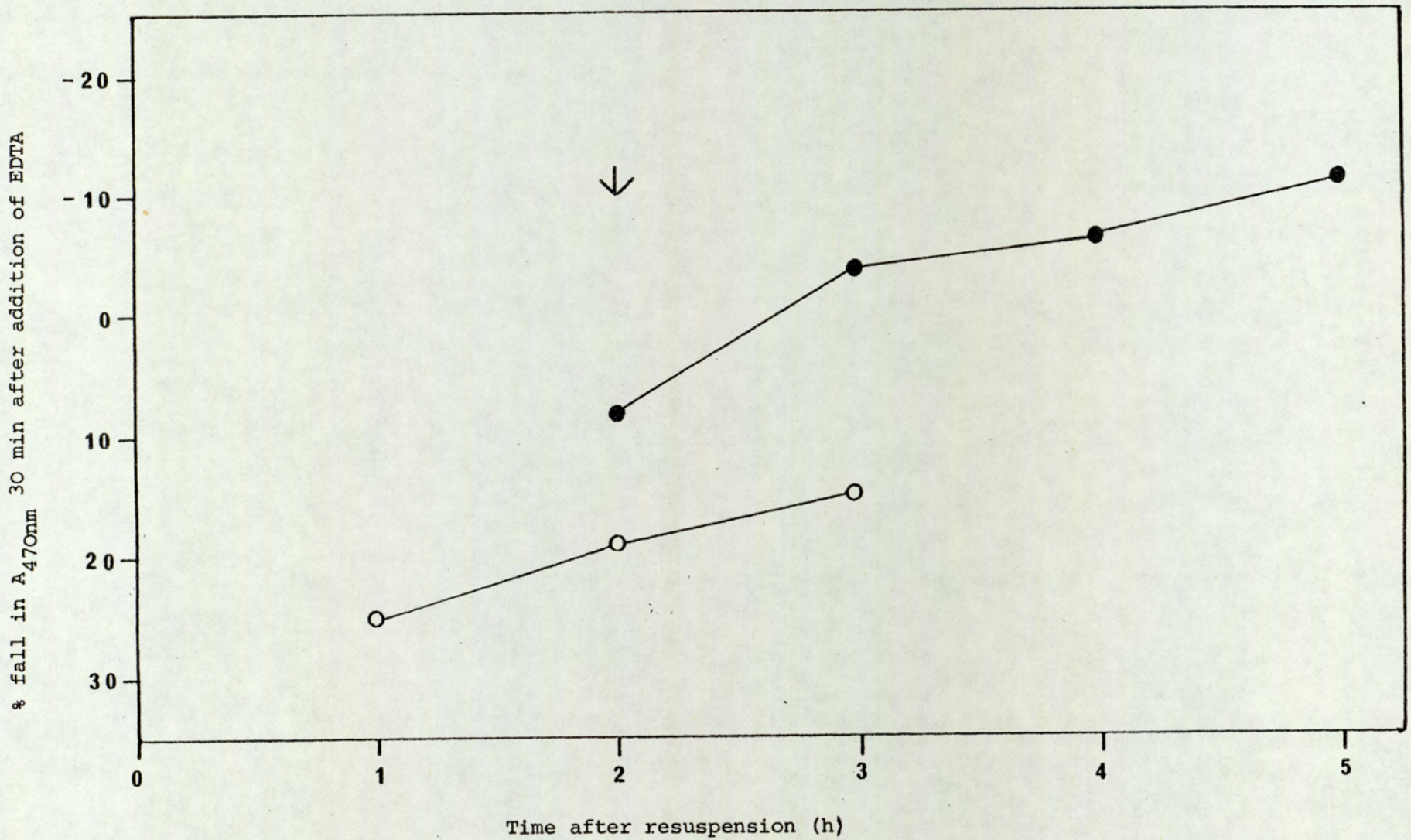
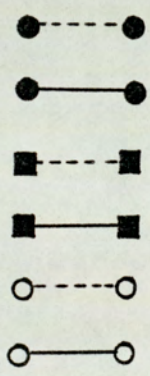

% fall in A_{470nm} 30 min after addition of EDTA
-20
-10
0
10
20
30
0
1
2
3
4
5
Time after resuspension (h)

Fig. 36. Effect of arsenate on the activity of EDTA (800µM) on nutrient - depleted P. aeruginosa



	EDTA (µM)	Arsenate (µM)
$S^- G^+$	0	0
$S^- G^+$	800	0
$S^- G^+$	0	100
$S^- G^+$	800	100
$S^- G^-$	0	0
$S^- G^-$	800	0

A_{470nm} 0.20 before the addition of EDTA

Each point mean of 1 flask from 2 experiments

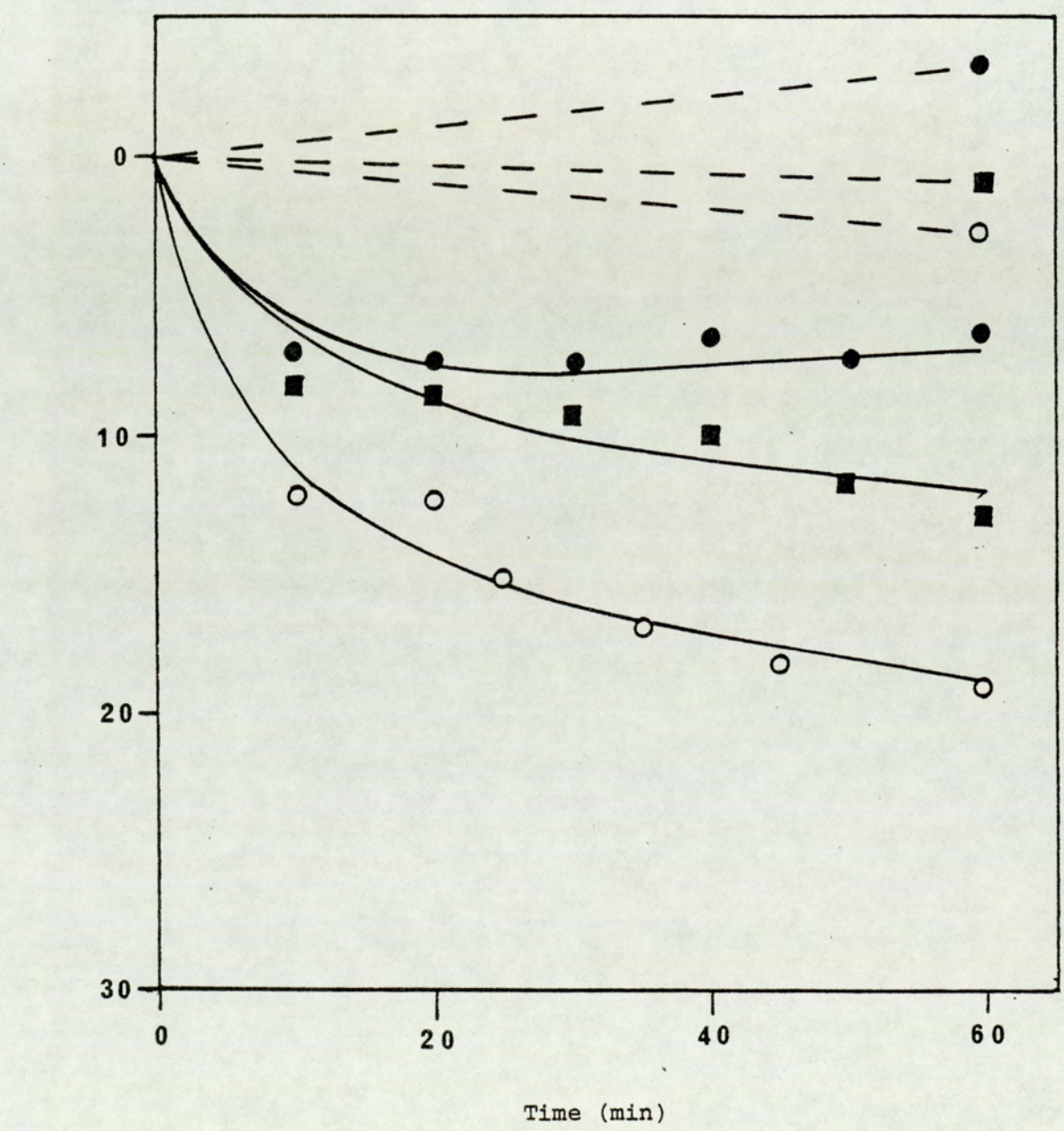

% fall in A_{470nm}
0
10
20
30
0
20
40
60
Time (min)

Fig. 37. Effect of sulphur - depletion time on the activity of EDTA (800μM) on P. aeruginosa

● S^{-} G^{+} A_{470nm} 0.20 before the addition of EDTA

Each point mean of 1 flask from 2 experiments

% fall in A_{470nm} 30 min

after addition of EDTA

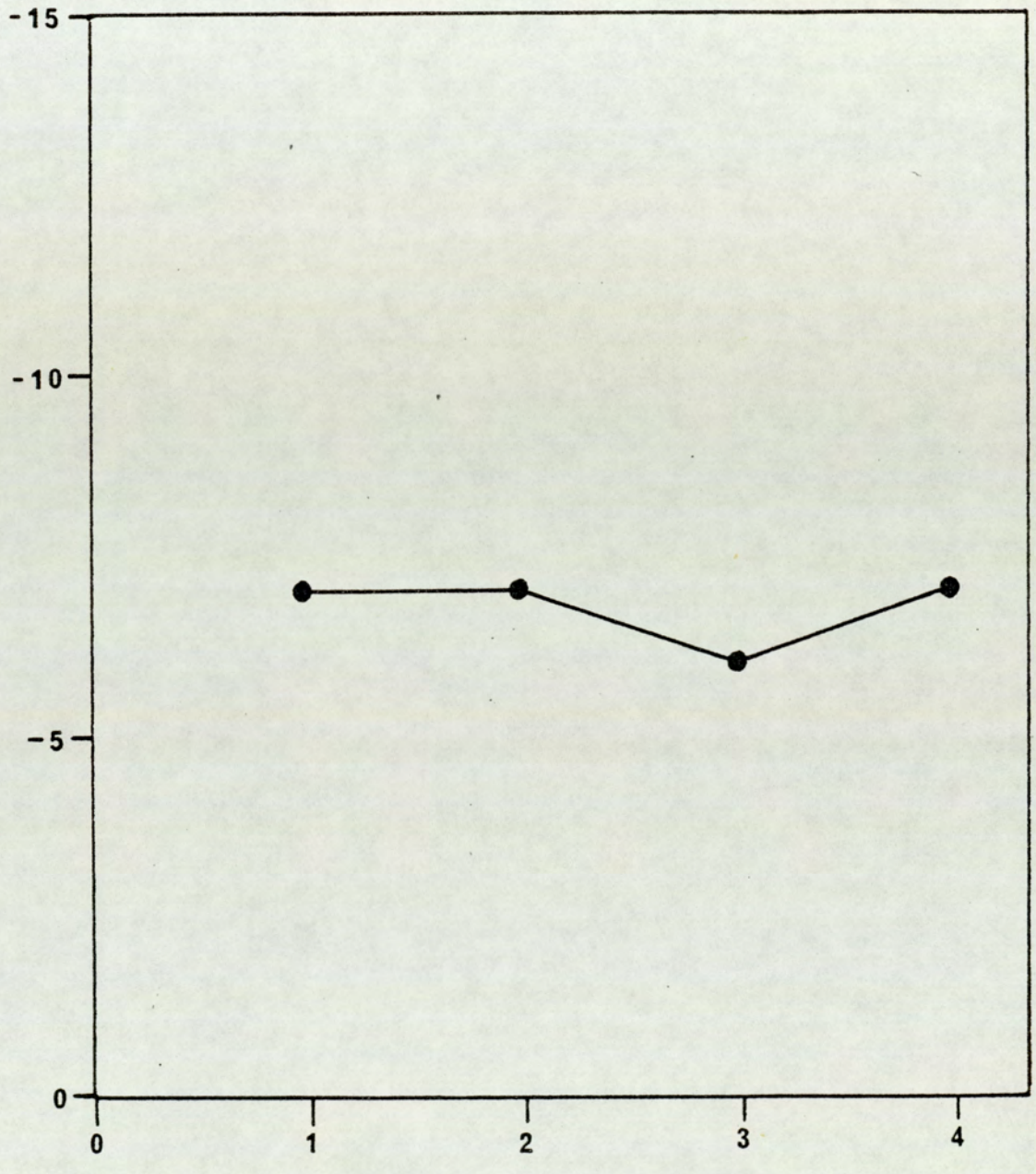


Table 27

Relationship between fall in total count and in absorbance of *P. aeruginosa* after treatment with EDTA (800μm)

Depletion	Time after addition of EDTA (mins)	% decrease in A_{470nm}	% decrease in total count
$S^+ G^-$	20	17	22
$S^+ G^-$	40	22	19
$S^+ G^-$	60	27	32
$S^- G^+$	20	0	-7
$S^- G^+$	40	-4	-1
$S^- G^+$	60	-9	-11

Results 1 flask each from 1 experiment

3.3.2 Bactericidal action

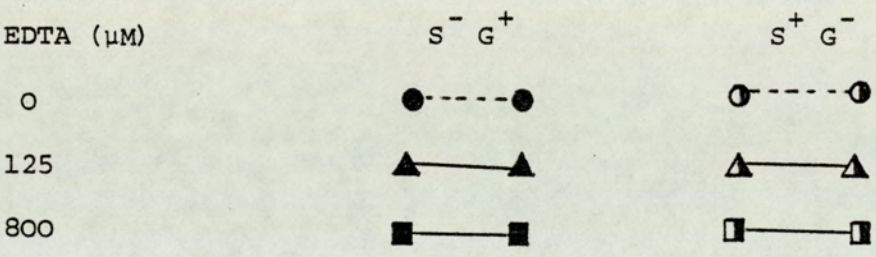
3.3.2.1 S^- G^+ cells are more resistant to the bactericidal action of EDTA than S^+ G^- cells (Fig. 38).

3.3.2.2. S^- G^- cells are also relatively sensitive to the bactericidal action of EDTA; the addition of glucose increases their resistance to EDTA (Fig m 39).

3.3.3. Release of 260nm - absorbing compounds

The resistance of the sulphur-depleted cells to EDTA-induced release of 260nm -absorbing compounds is dependent on the presence of glucose (Fig. 40).

Fig. 38. Action of EDTA on nutrient - depleted P. aeruginosa



A_{470nm} 0.20 before the addition of EDTA

Each point mean of 1 flask from 2 experiments

% survivors

(cfu ml^{-1})

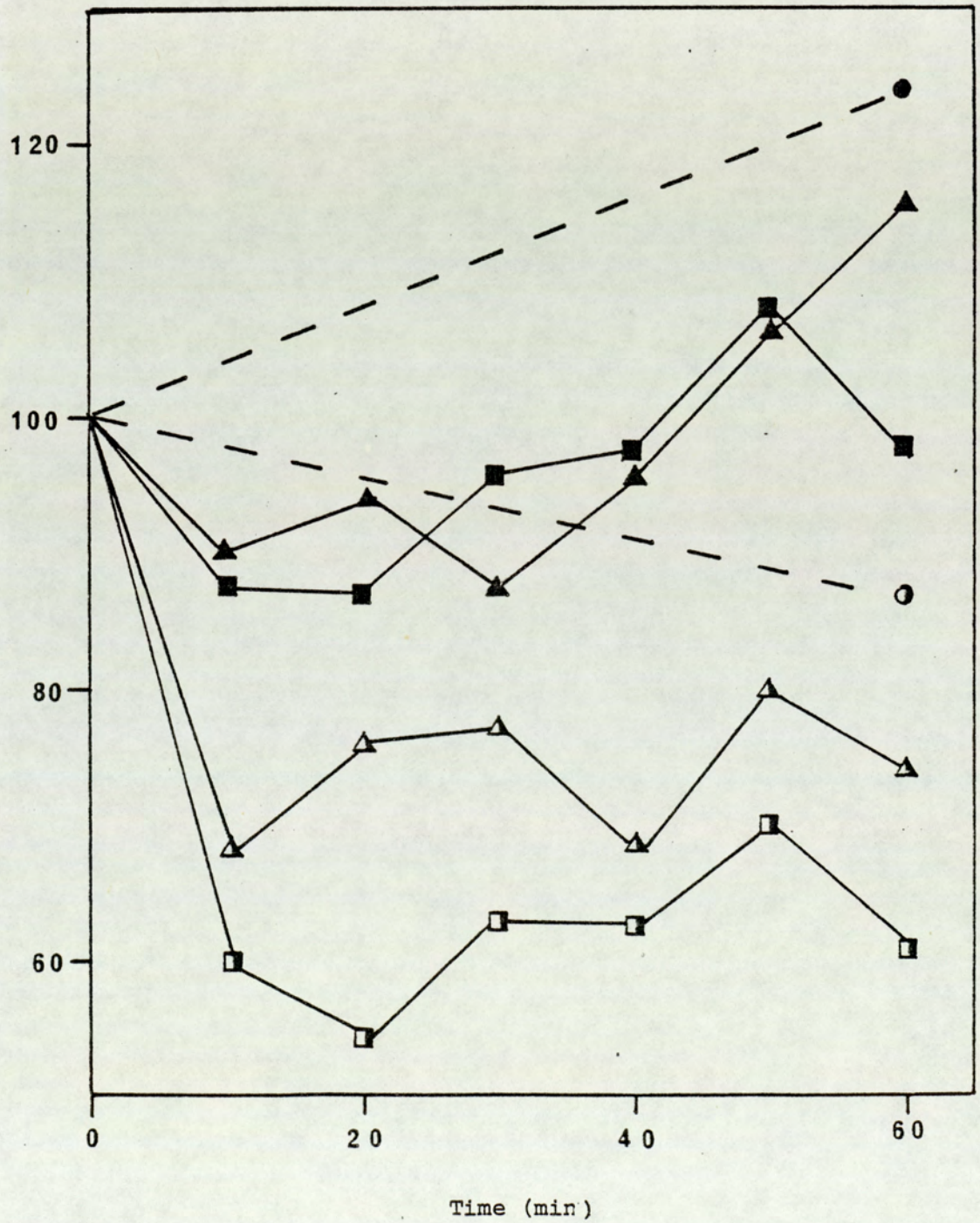


Fig. 39. Effect of glucose on the activity of EDTA (800µM) on P. aeruginosa depleted of sulphur and glucose

○ $S^- G^-$

↓ glucose (20 mM) added to 1 flask

● flask after addition of glucose

A_{470nm} 0.20 before the addition of EDTA

Each point mean of 1 flask from 2 experiments

Survivors at 30 mins calculated from survival curve

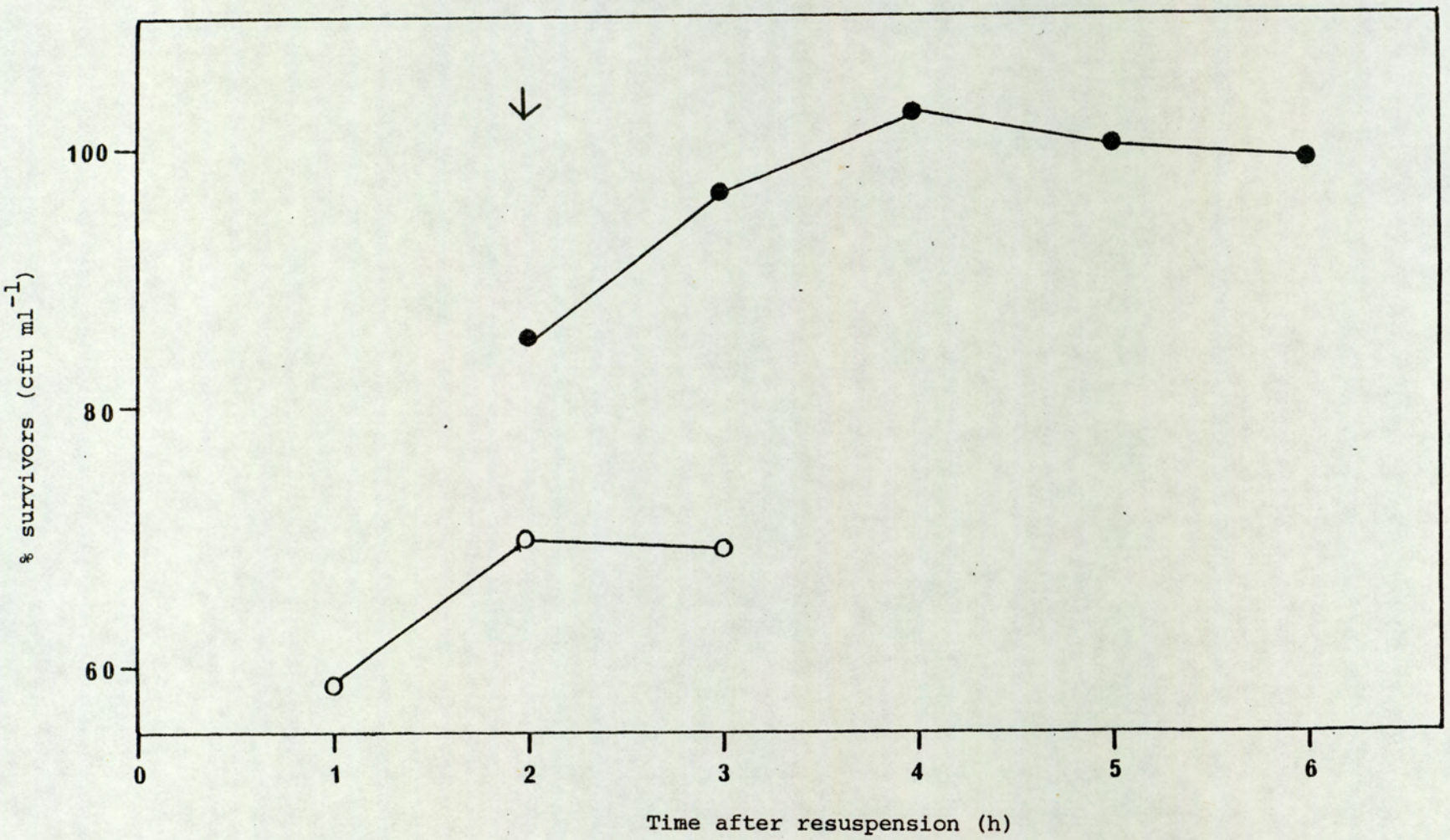
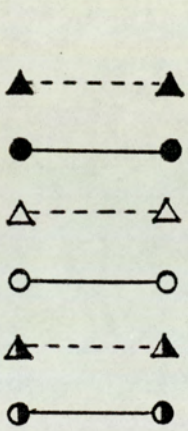

100
80
60
0
1
2
3
4
5
6
% survivors (cfu ml^{-1})
Time after resuspension (h)

Fig. 40. Action of EDTA on nutrient - depleted P. aeruginosa

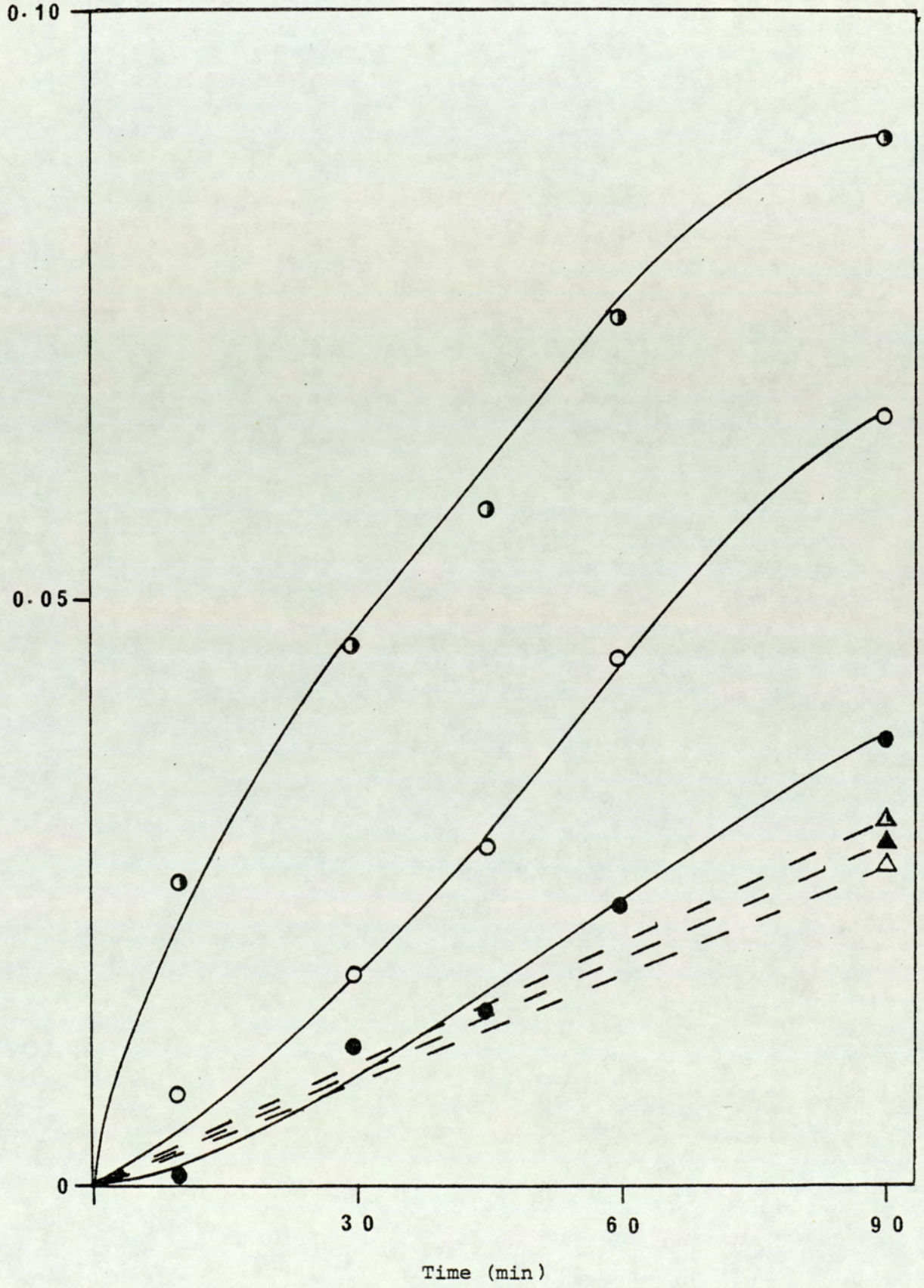


	EDTA (μM)
$S^- G^+$	0
$S^- G^+$	800
$S^- G^-$	0
$S^- G^-$	800
$S^+ G^-$	0
$S^+ G^-$	800

A_{470nm} 0.20 at t = 0

Each point mean of 1 flask from 2 experiments

Mean loss of 260nm - absorbing compounds on filtering 17%


ΔA_{260nm}
0·10
0·05
0
30
60
90
Time (min)

3.4 Action of polymyxim on nutrient - depleted cells

3.4.1 Cell lysis

3.4.1.1 The lytic action of polymyxin (PB) is unaffected by the pH of the CDM between the pH range 5.5 - 7.5 (Table 28). Table 29 shows the pH of the CDM after 5h of nutrient depletion.

3.4.1.2 S^- G^+ cells are relatively resistant to the lytic action of PB compared to S^+ G^- cells (Fig. 41). The slight daily variation for the lysis of S^+ G^- cells is shown in Fig. 42. The resistance of sulphur - depleted cells is dependent on the presence of glucose, which is demonstrated by using resuspended cells (Fig. 43).

3.4.1.3 The resuspension of S^+ G^- cells in fresh CDM causes a slight increase in the extent of lysis (Fig. 44). The addition of various concentrated culture filtrates to S^+ G^- cells protects them against the action of PB (Table 30).

Table 28

Effect of pH on the activity of polymyxin (20u ml^{-1}) on P. aeruginosa depleted of glucose

pH	% fall in A_{470nm} at 60 mins
5.5	45
6.5	45
7.5	46

Each result 1 flask from 1 experiment

Table 29

pH of the CDM after 5hrs of nutrient depletion of P. aeruginosa

Nutrient Depletion	A_{470nm}	pH
$S^- G^+$	0.22	7.20
$S^+ G^-$	0.20	7.40

Each result mean of 1 flask from 2 experiments

Fig. 41. Action of polymyxin ($20u\,ml^{-1}$) on P. aeruginosa depleted of either glucose or sulphur for four hours

● $S^- G^+ A_{470nm}$ 0.25 before the addition of PB

Each point mean of 4 flasks from 1 experiment

◑ $S^+ G^- A_{470nm}$ 0.22 before the addition of PB

Each point mean of 2 flasks from 1 experiment

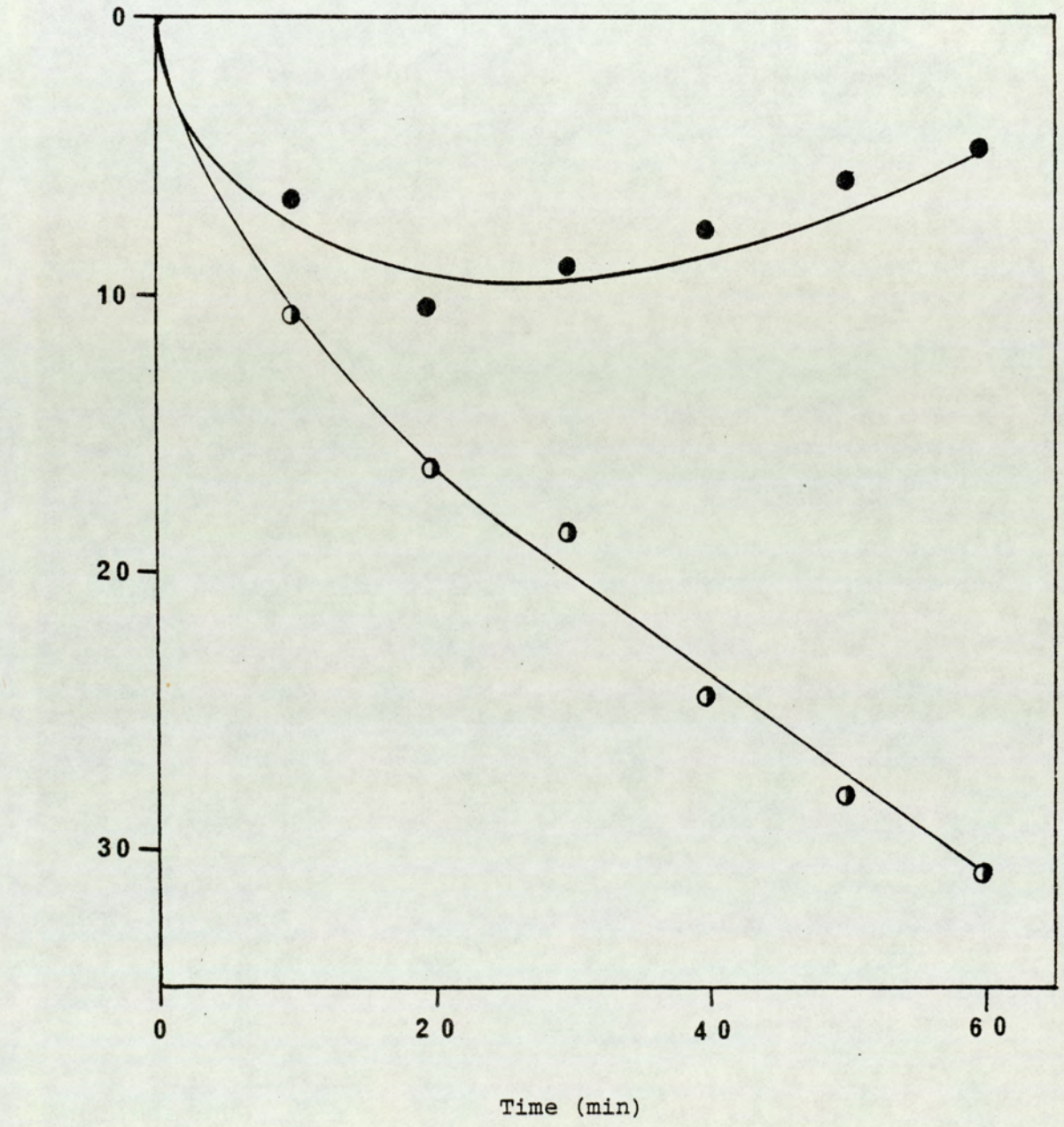

% fall in A_{470nm}
0
10
20
30
0
20
40
60
Time (min)

Fig. 42. Action of polymyxin (20u ml^{-1}) on *P. aeruginosa* depleted of glucose for four hours showing variation between days

◑ ◧ S^+ G^- tested on different days

A_{470nm} 0.20 before the addition of PB

Each point mean of 2 flasks from 1 experiment

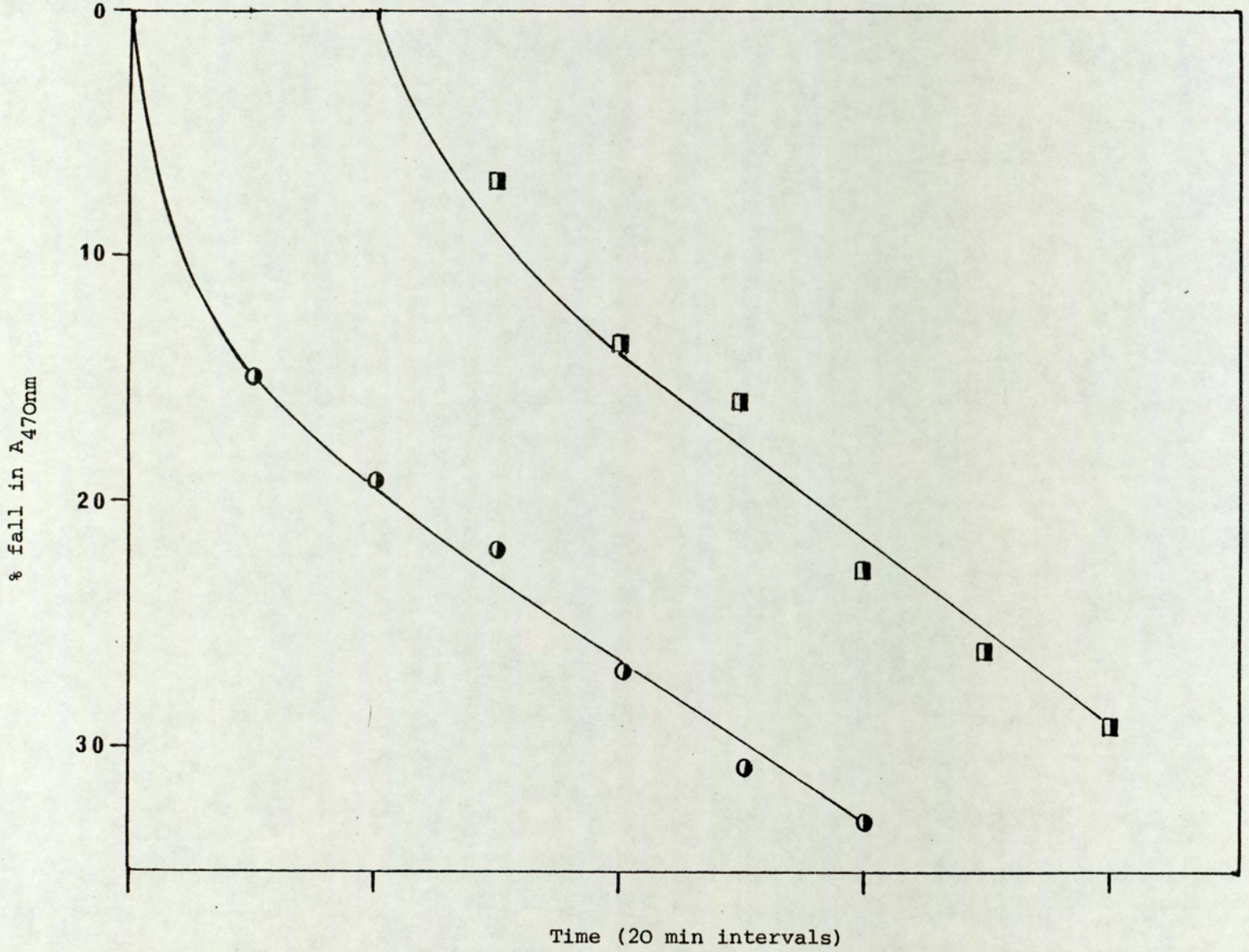

0
10
20
30
% fall in A_{470nm}
Time (20 min intervals)

Fig. 43. Effect of glucose on the activity of polymyxin (20u ml^{-1}) on P. aeruginosa depleted of sulphur for seven hours

● S^- G^+ unresuspended; A_{470nm} 0.26 before the addition of PB

▲ S^- G^+ resuspended; A_{470nm} 0.23 before the addition of PB

○ S^- G^- resuspended; A_{470nm} 0.25 before the addition of PB

Each point mean of 1 flask from 2 experiments

% fall A_{470nm}
0
10
20
30
Time (20 min intervals)

Fig. 44. Effect of resuspension on the activity of polymyxin ($20u\,ml^{-1}$) on P. aeruginosa depleted of glucose for four hours

◑ $S^+ G^-$ unresuspended; A_{470nm} 0.20 before the addition of PB

▲ $S^+ G^-$ resuspended; A_{470nm} 0.20 before the addition of PB

Each point mean of 2 flasks from 1 experiment

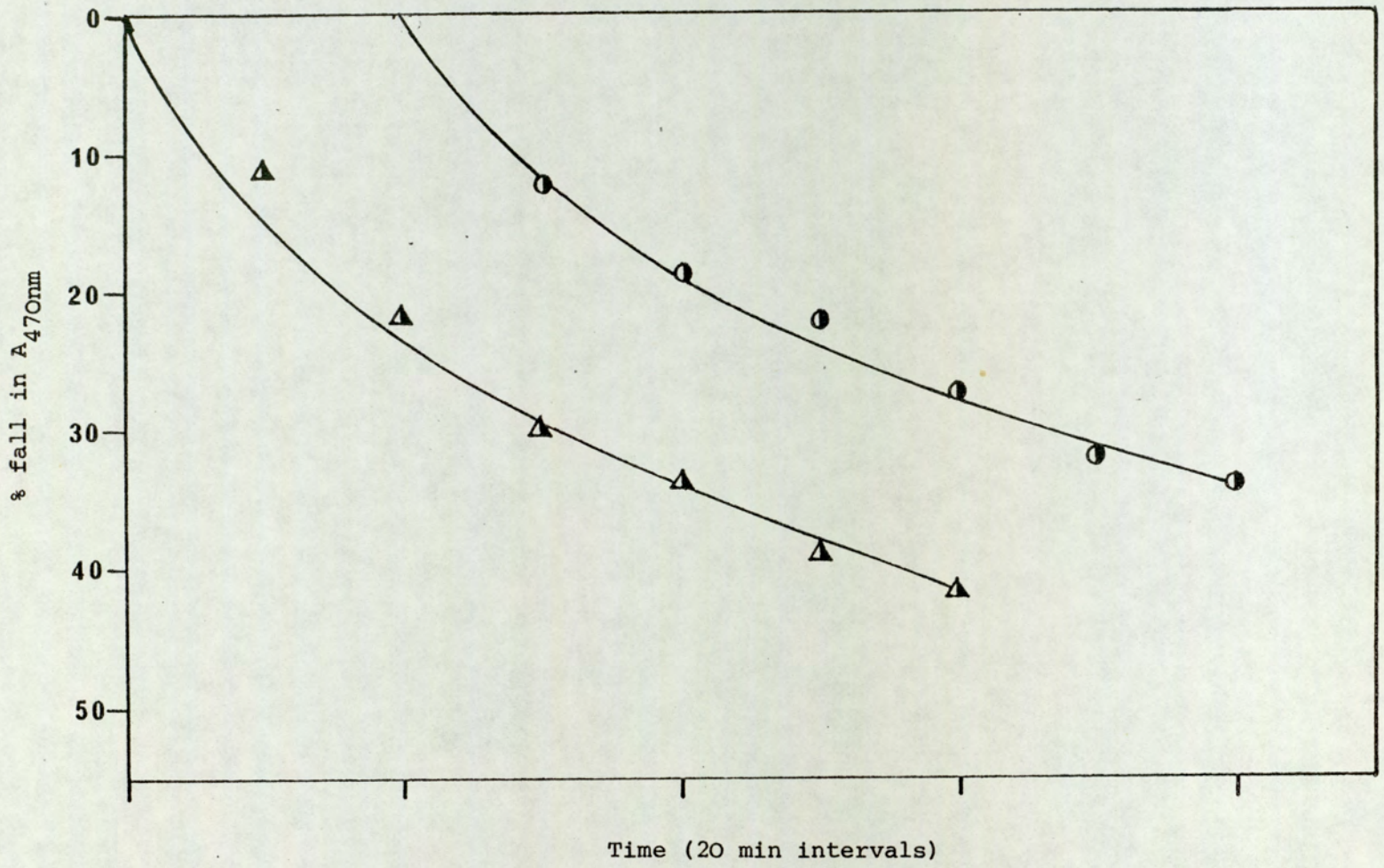

0
10
20
30
40
50
% fall in A_{470nm}
Time (20 min intervals)

Table 30

Effect of culture filtrate on the activity of polymyxin (20u ml^{-1}) on P. aeruginosa depleted of glucose

Component added	% fall in A_{470nm} at 60 mins
H_2O[1]	34
CDM[2]	17
S^-G^+ culture filtrate[2]	14
S^+G^- culture filtrate[2]	7

1. Result mean of 1 flask from 2 experiments.
2. Each result mean of 2 flasks from 2 experiments.

3.4.2 Bactericidal action

3.4.2.1 The bactericidal action of PB is increased with increasing pH of the CDM (Table 31).

3.4.2.2 Fig. 45 shows the action of varying concentrations of PB on nutrient - depleted *P. aeruginosa*. Using $10u\,ml^{-1}$ PB, there was no significant differences in the number of survivors from $S^- G^+$ or $S^+ G^-$ cultures. With increasing concentrations of PB the $S^- G^+$ culture was found to be more sensitive to the action of PB (Fig. 45 & Table 32). Fig. 46 summarises the relationship between the % survivors and the PB concentration for $S^- G^+$ and $S^+ G^-$ cells. The presence of glucose in the test medium has no effect on the action of PB on sulphur-depleted cells (Fig 47).

3.4.2.3 Both glucose - depleted and sulphur - depleted cells are more sensitive to PB after resuspension (Figs 48 & 49).

Table 31

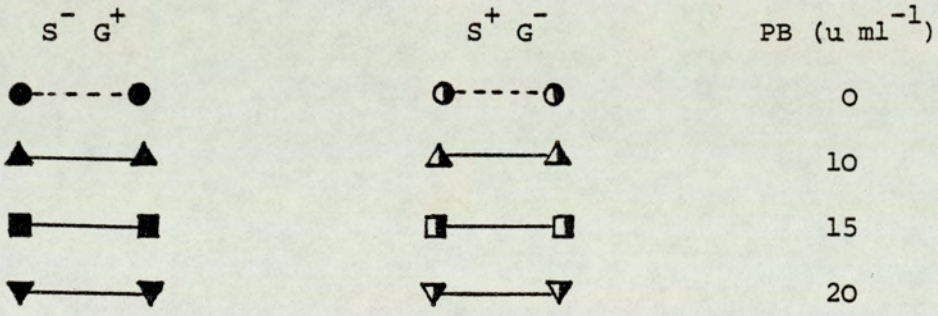
Effect of pH on the activity of polymyxin (20u ml^{-1}) on P. aeruginosa depleted of glucose

pH of CDM	% survivors (cfu ml^{-1}) at 30 mins
6.55	50.4
7.00	56.1
7.25	39.1
7.52	3.2

Each result mean of 1 flask from 2 experiments.

Fig. 45. Action of polymyxin on nutrient - depleted

P. aeruginosa

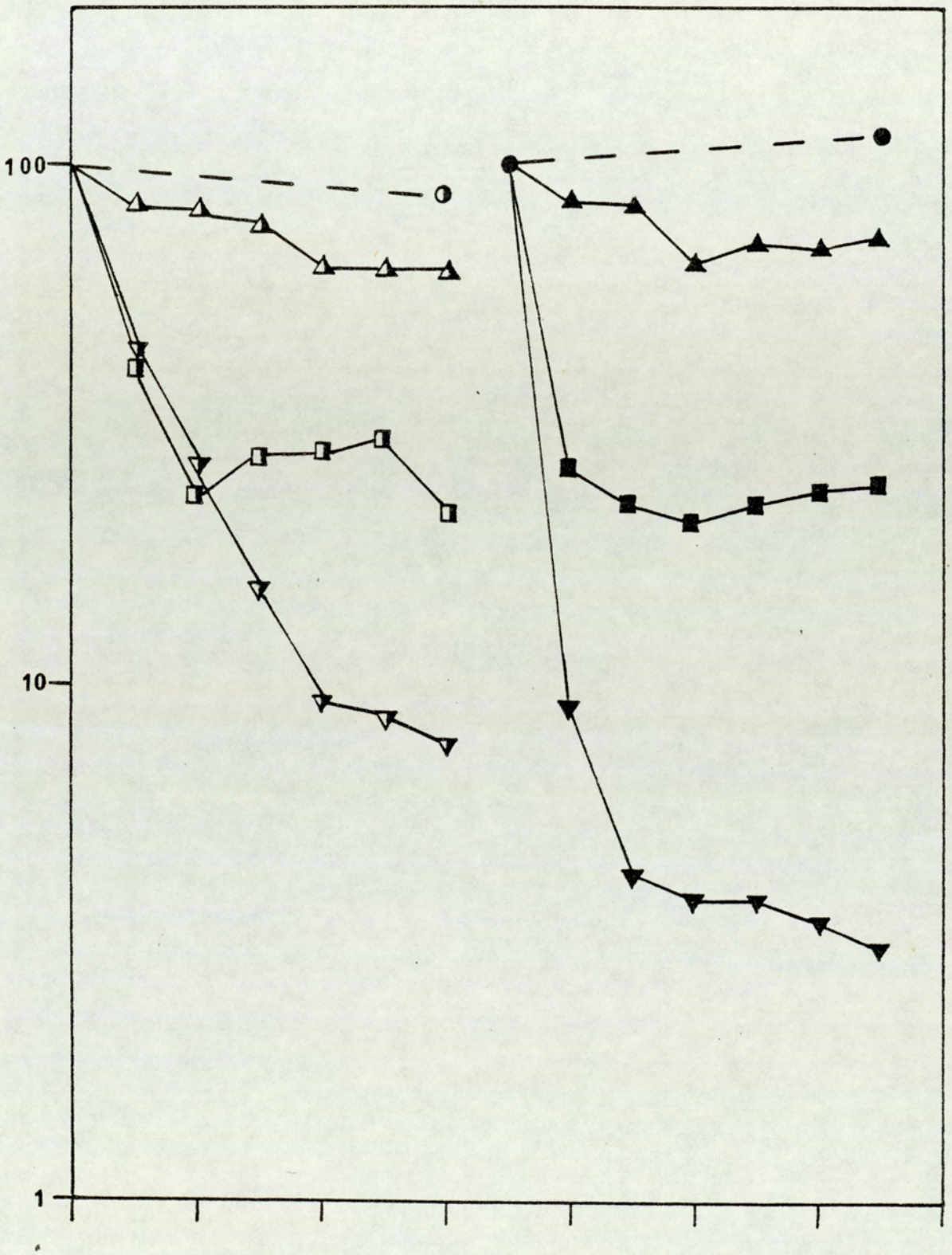


A_{470nm} 0.19 before the addition of PB to unresuspended cells

Each point mean of 1 flask from 2 experiments

% survivors

(cfu ml^{-1})



Time (20 min intervals)

Table 32

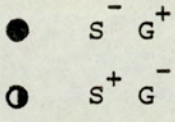
Action of polymyxin on nutrient-depleted *P. aeruginosa*

Polymyxin ($u\ ml^{-1}$)	% Survivors			
	5 mins		10 mins	
	$S^+\ G^-$	$S^-\ G^+$	$S^+\ G^-$	$S^-\ G^+$
40	4.8	$<4x10^{-4}$	$<4x10^{-4}$	-
60	$<4x10^{-4}$	$<4x10^{-4}$	-	-

A_{470nm} 0.19 before the addition of PB to unresuspended cells.

Each point mean of 1 flask from 2 experiments.

Fig. 46. % Survivors at 60 min vs polymyxin concentration



Data for Fig. 46 compiled from Fig. 45.

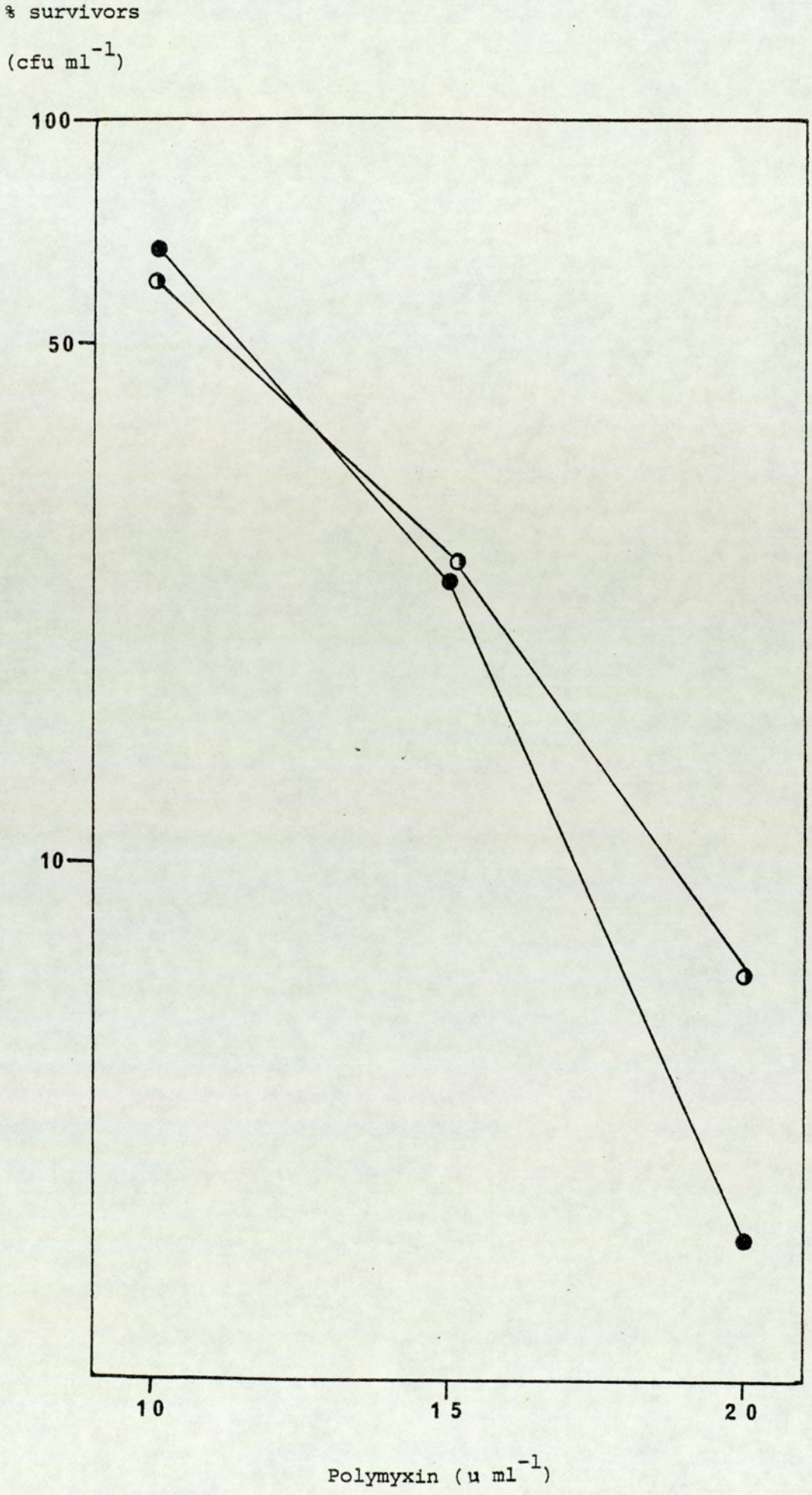
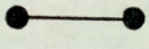
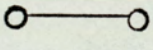

% survivors
(cfu ml^{-1})
100
50
10
10
15
20
Polymyxin (u ml^{-1})

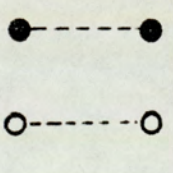
Fig. 47. Effect of glucose on the activity of polymyxin (15u ml^{-1}) on P. aeruginosa depleted of sulphur



$S^- G^+$ resuspended; A_{470nm} 0.20 before the addition of PB



$S^- G^-$ resuspended; A_{470nm} 0.20 before the addition of PB



controls, no PB added

Each point mean of 1 flask from 2 experiments

% survivors

(cfu ml^{-1})

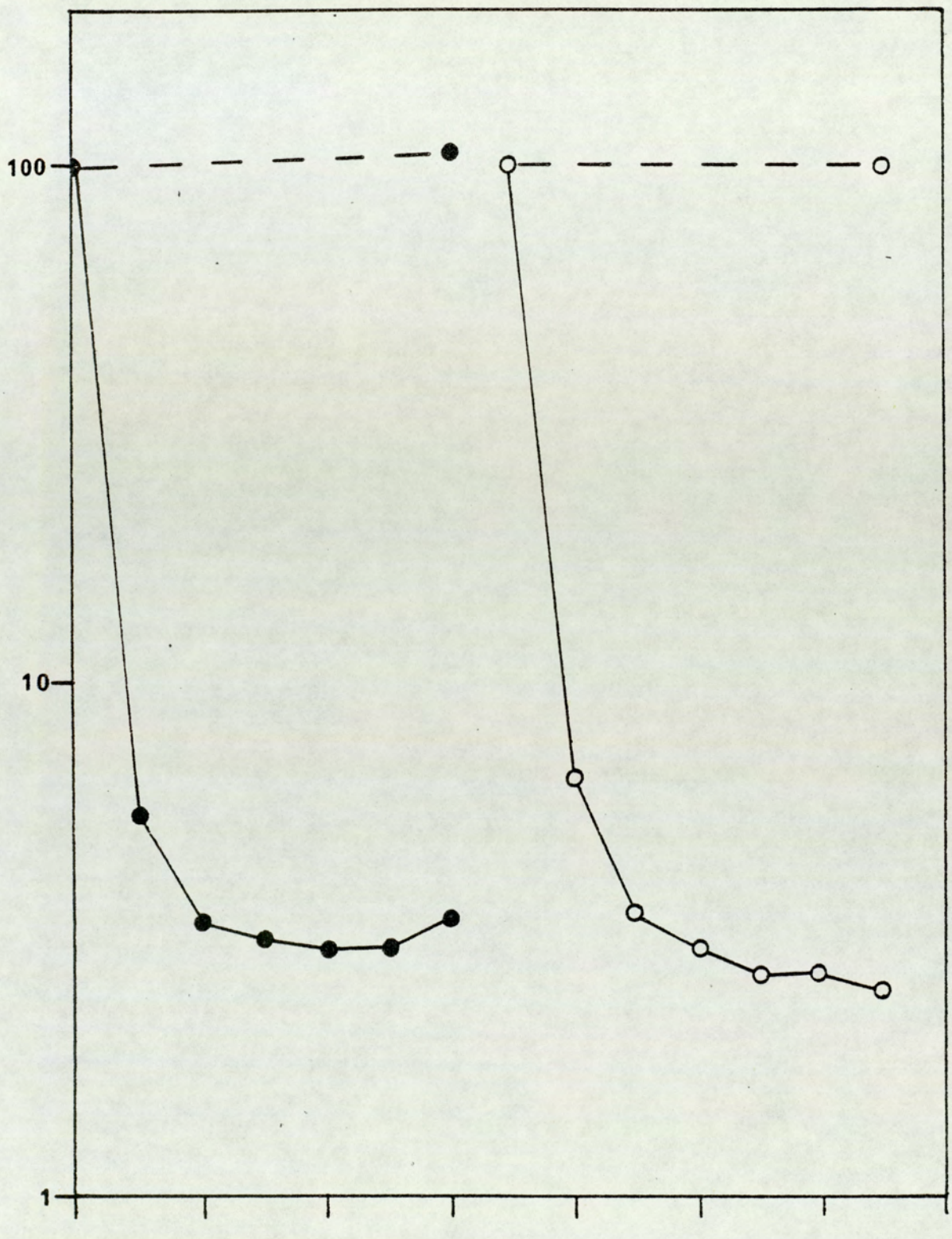
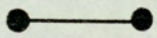
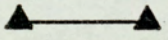


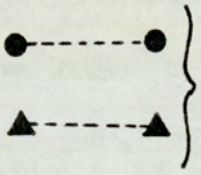
Fig. 48. Effect of resuspension on the activity of polymyxin ($15u\ ml^{-1}$) on P. aeruginosa depleted of sulphur



$S^-\ G^+$ unresuspended; A_{470nm} 0.19 before the addition of PB



$S^-\ G^+$ resuspended; A_{470nm} 0.20 before the addition of PB



controls, no PB added

Each point mean of 1 flask from 2 experiments

% survivors

(cfu ml^{-1})

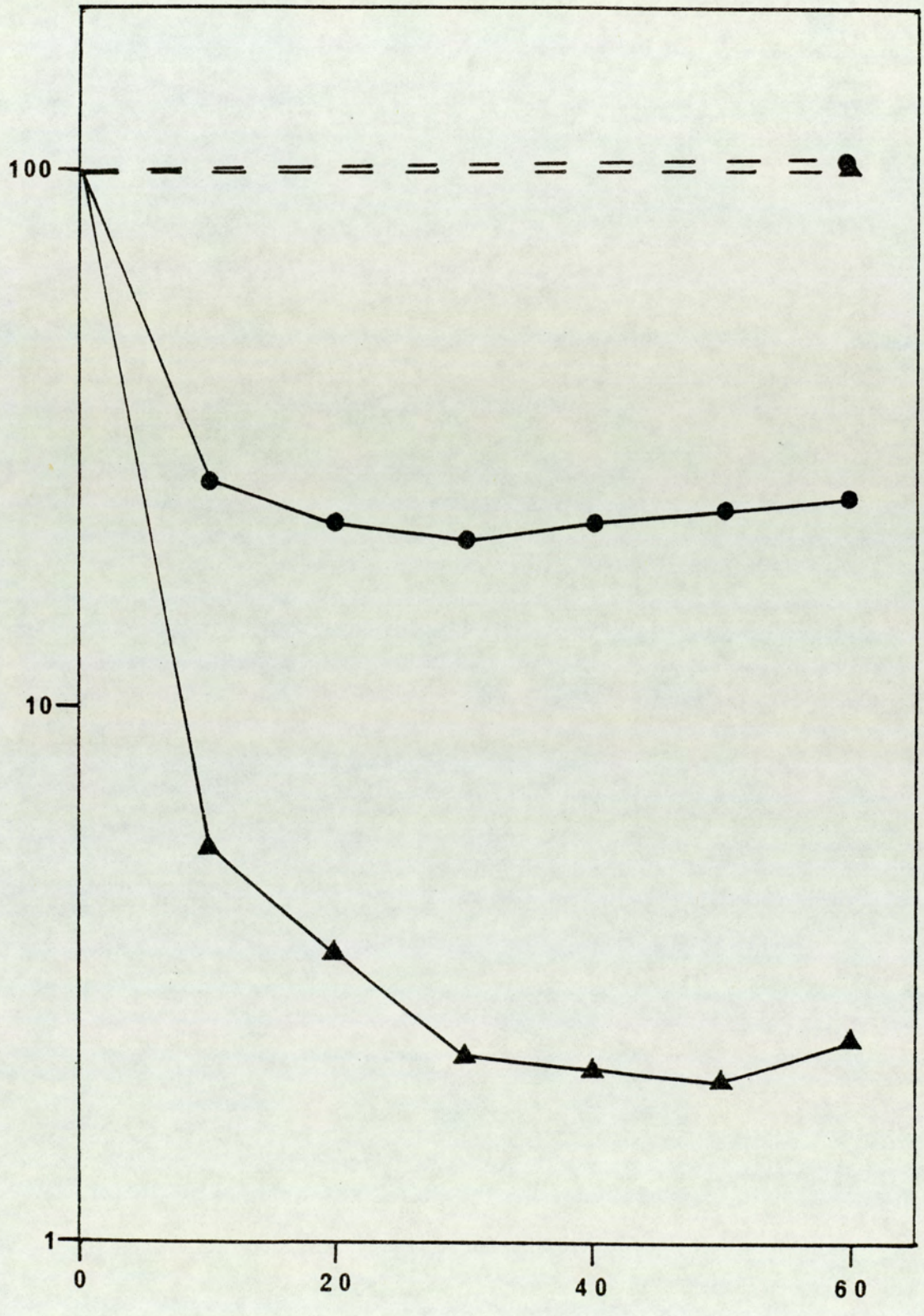
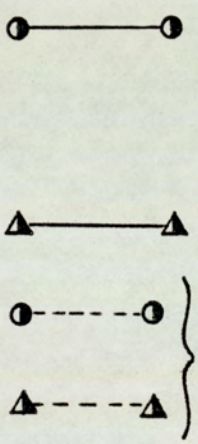


Fig. 49. Effect of resuspension on the activity of polymyxin ($15u\ ml^{-1}$) on P. aeruginosa depleted of glucose



S^+ G^- unresuspended; A_{470nm} 0.19 before the addition of PB

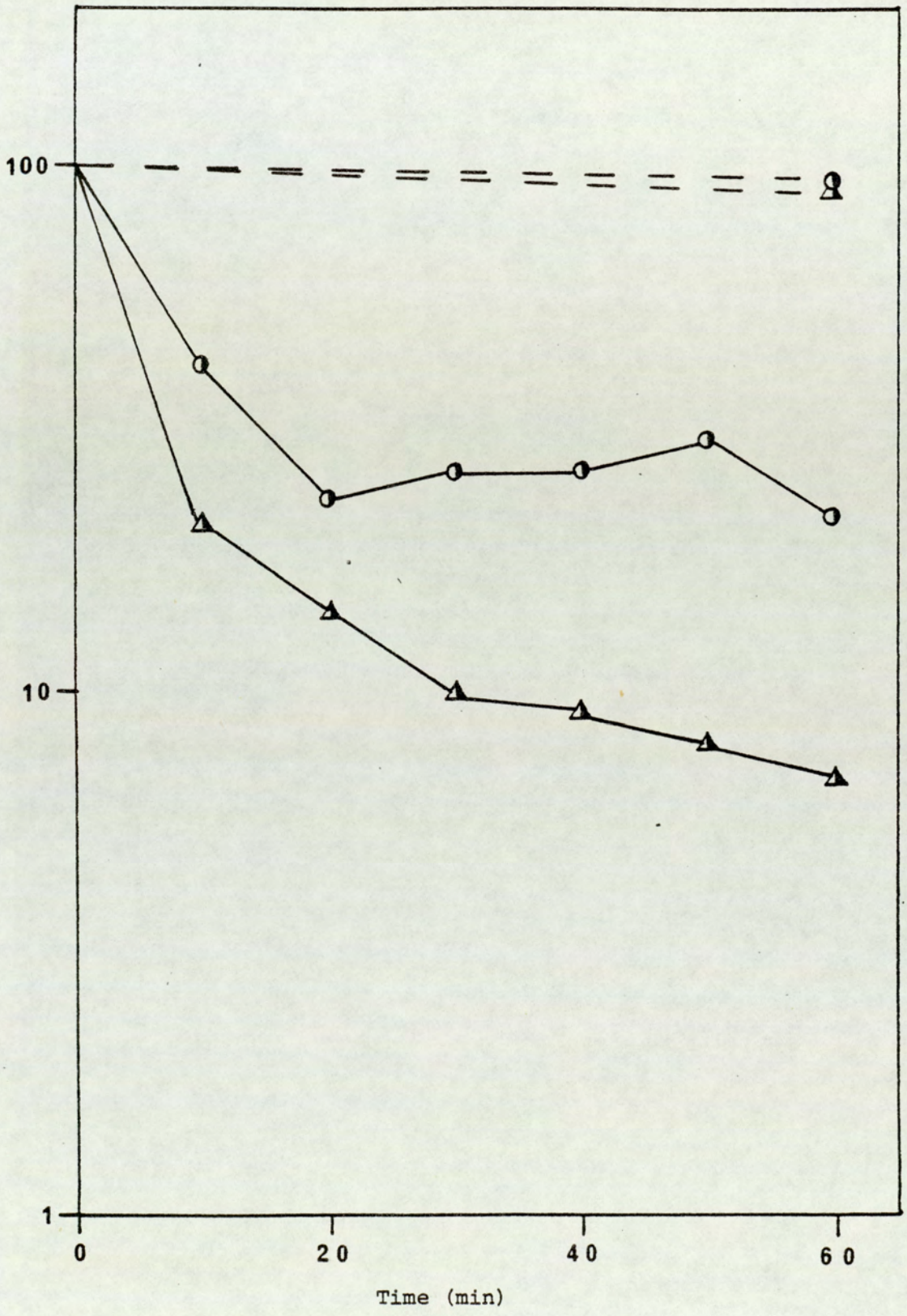
S^+ G^- resuspended; A_{470nm} 0.19 before the addition of PB

controls, no PB added

Each point mean of 1 flask from 2 experiments

% survivors

(cfu ml^{-1})



3.4.3 Release of 260nm - absorbing compounds

3.4.3.1 PB - induced release of 260nm - absorbing compounds increases with increasing pH of the CDM (Table 33)

3.4.3.2 PB induced release of 260nm - absorbing compounds from all cells tested (Figs. 50, 51 & 52). As the concentration of PB increased, release of 260nm - absorbing compounds reaches a maximum and then declines (Fig. 53). The most resistant cells are $S^+ G^-$ and maximum release occurs at 20u ml^{-1} PB; $S^- G^+$ cells are slightly more sensitive at low concentrations and reach a maximum at 40 u ml^{-1}. Higher concentrations of PB were only tested on resuspended cells (Figs. 54 & 55, summary Fig. 56). Resuspension seems to increase the sensitivity of cells (as seen by comparing Figs. 50 & 54), but in no case was release of 260nm - absorbing compounds increased by PB concentrations greater than 60 uml^{-1}.

Table 33

Effect of pH on the activity of polymyxin ($20u\ ml^{-1}$) on P. aeruginosa depleted of glucose

pH	ΔA_{260nm} (60-0mins)
6.0	0.029
6.3	0.033
7.0	0.057
7.5	0.188

Mean loss of 260nm-absorbing compounds on filtering 15%

Fig. 50. Action of varying concentrations of polymyxin ($u\ ml^{-1}$) on *P. aeruginosa* depleted of sulphur

A_{470nm} 0.19 before the addition of PB to unresuspended cells (S^- G^+)

Each point mean of 1 flask from 2 experiments

Mean loss of 260nm - absorbing compounds on filtering 20%

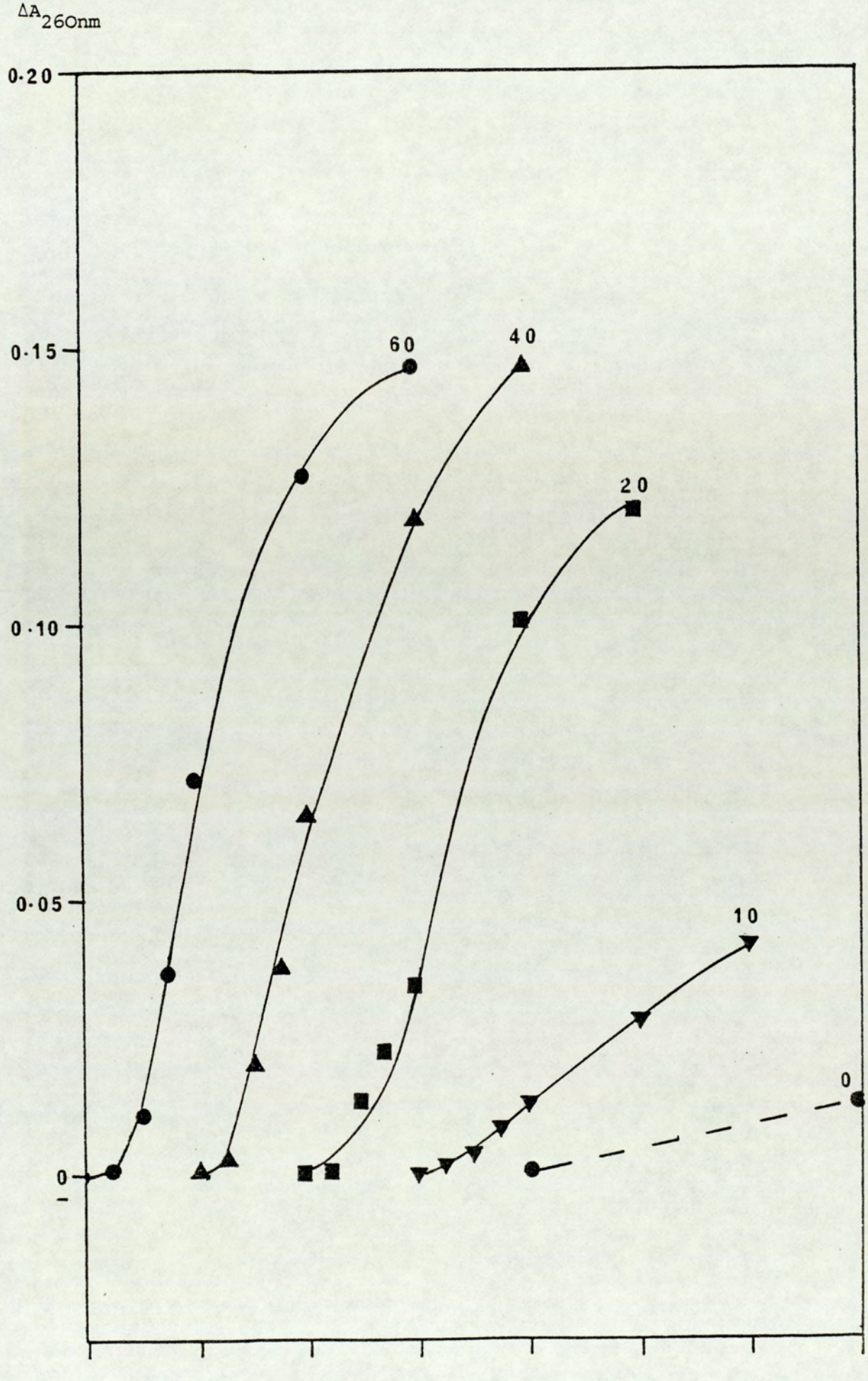

ΔA260nm
0·20
0·15
0·10
0·05
0
60
40
20
10
0
Time (20 min intervals)

Fig. 51. Action of varying concentrations of polymyxin (u ml^{-1}) on *P. aeruginosa* depleted of sulphur and glucose

A_{470nm} 0.19 before the addition of PB to resuspended cells (S^- G^-)

Each point mean of 1 flask from 2 experiments

Mean loss of 260nm - absorbing compounds of filtering 24%

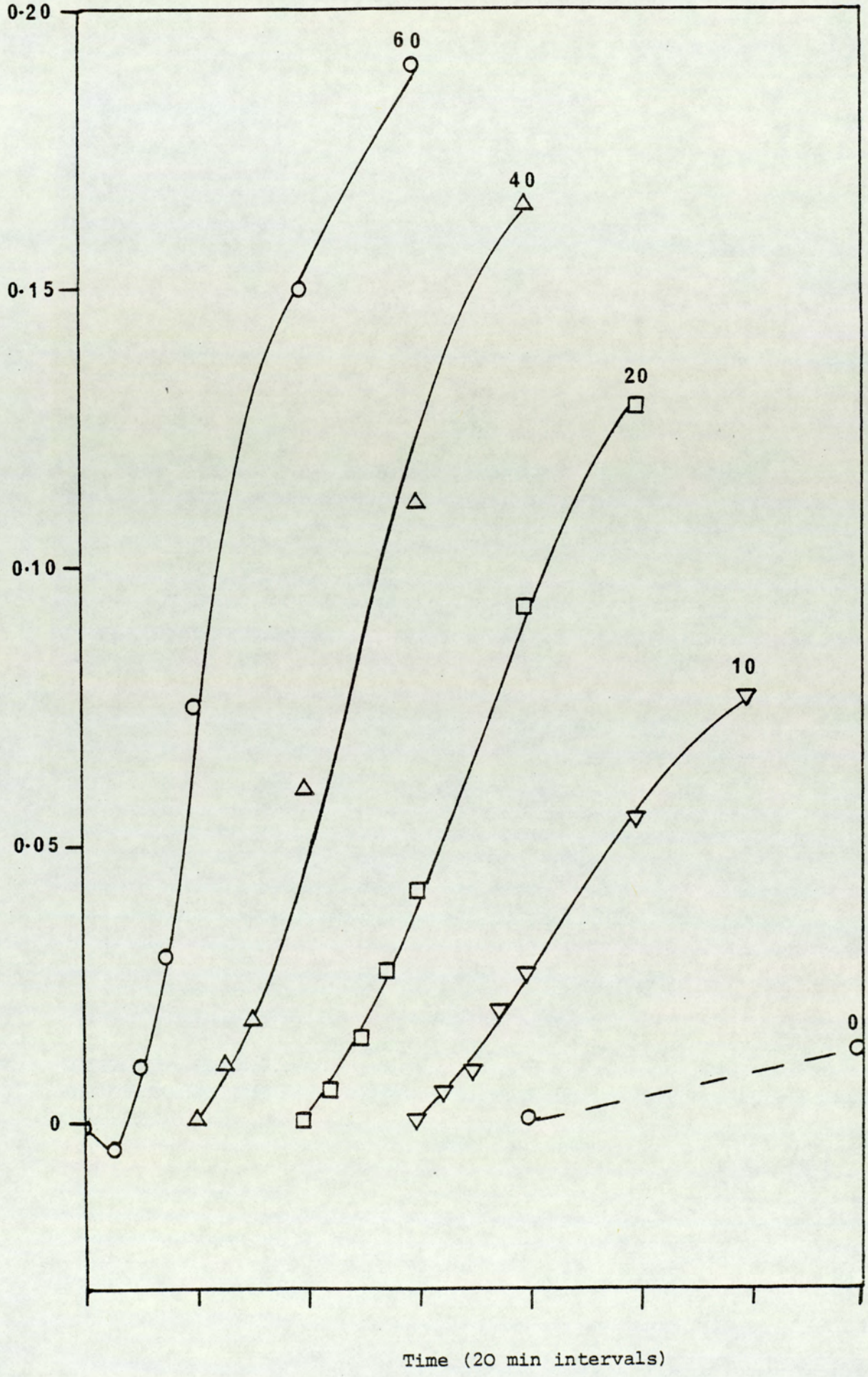

ΔA260nm
0·20
0·15
0·10
0·05
0
60
40
20
10
0
Time (20 min intervals)

Fig. 52. Action of varying concentrations of polymyxin ($u\ ml^{-1}$) on P. aeruginosa depleted of glucose

A_{470nm} 0.19 before the addition of PB to unresuspended cells ($S^+ G^-$)

Each point mean of 1 flask from 2 experiments

Mean loss of 260nm - absorbing compounds on filtering 20%

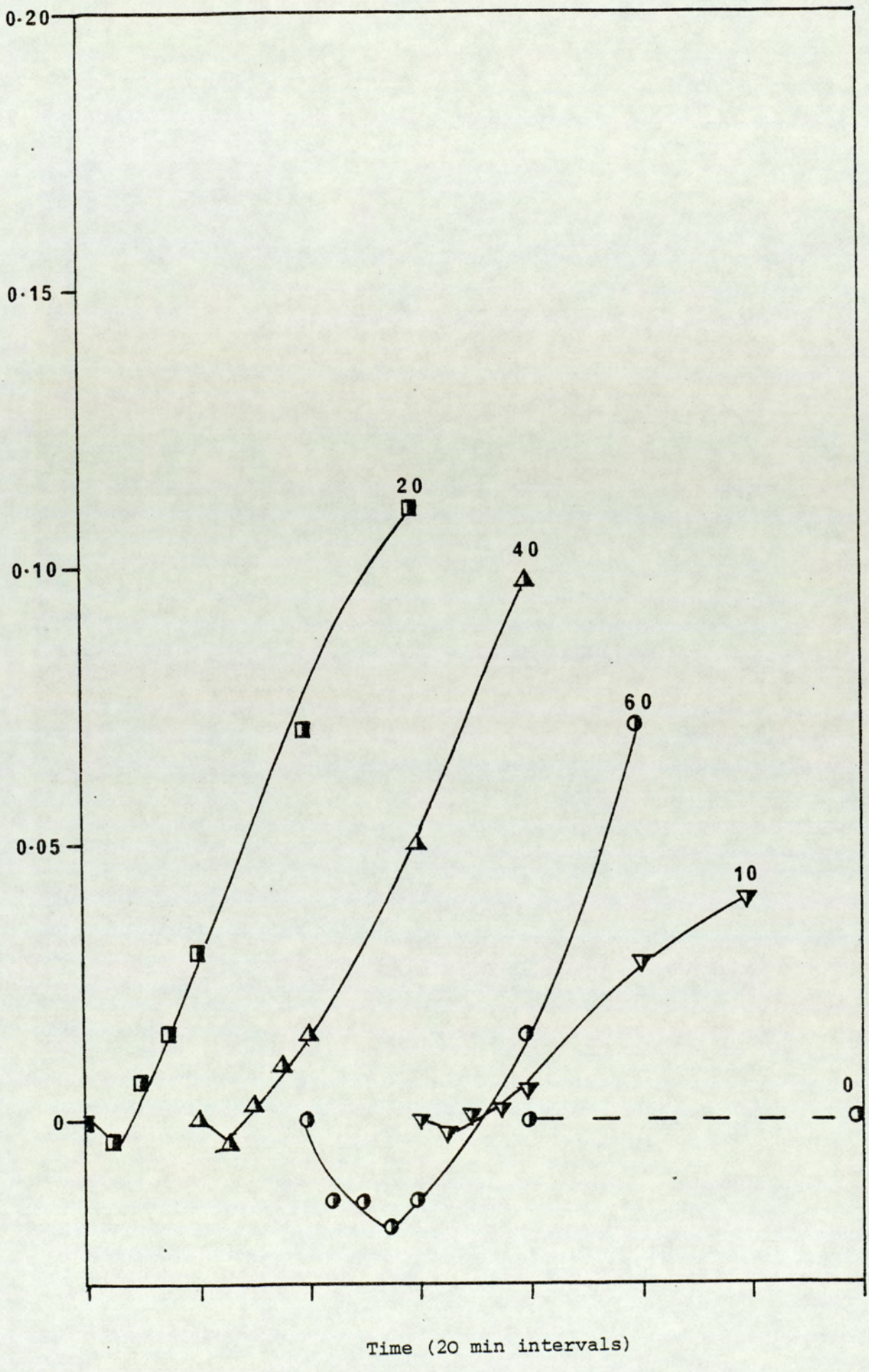

ΔA260nm
0·20
0·15
0·10
0·05
0
20
40
60
10
0
Time (20 min intervals)

Fig. 53. Release of 260nm - absorbing compounds 60 min after addition of polymyxin to nutrient - depleted P. aeruginosa

● S^- G^+ unresuspended

○ S^- G^- resuspended

◑ S^+ G^- unresuspended

The results for this figure were compiled from Figs. 50, 51 & 52.

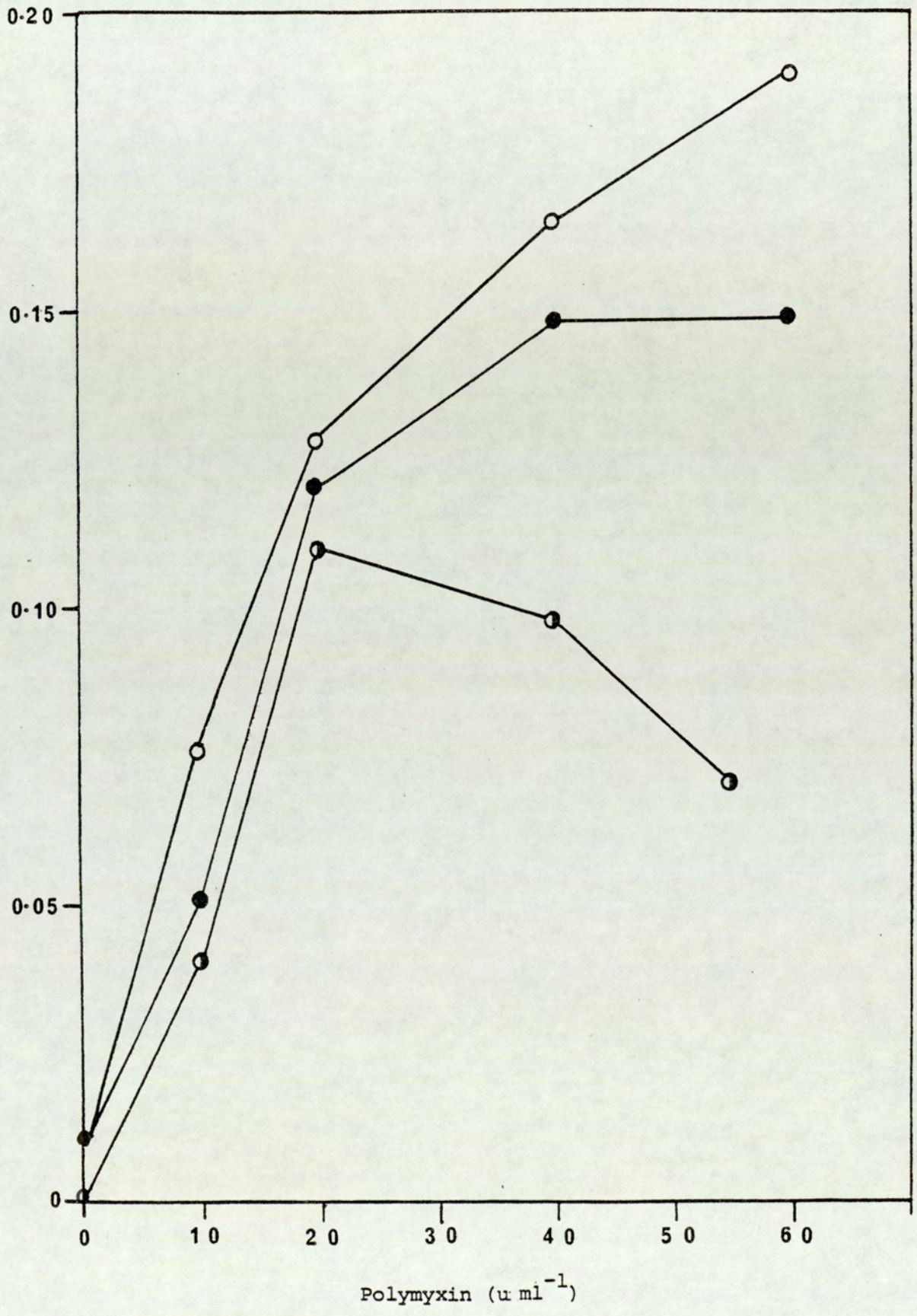

A_{260nm}
0·20
0·15
0·10
0·05
0
0
10
20
30
40
50
60
Polymyxin (u ml^{-1})

Fig. 54. Action of varying concentrations of polymyxin (u ml^{-1}) on *P. aeruginosa* depleted of sulphur

A_{470nm} 0.20 before the addition of PB to resuspended cells (S^- G^+)

Each point mean of 1 flask from 2 experiments

Mean loss of 260nm - absorbing compounds on filtering 11%.

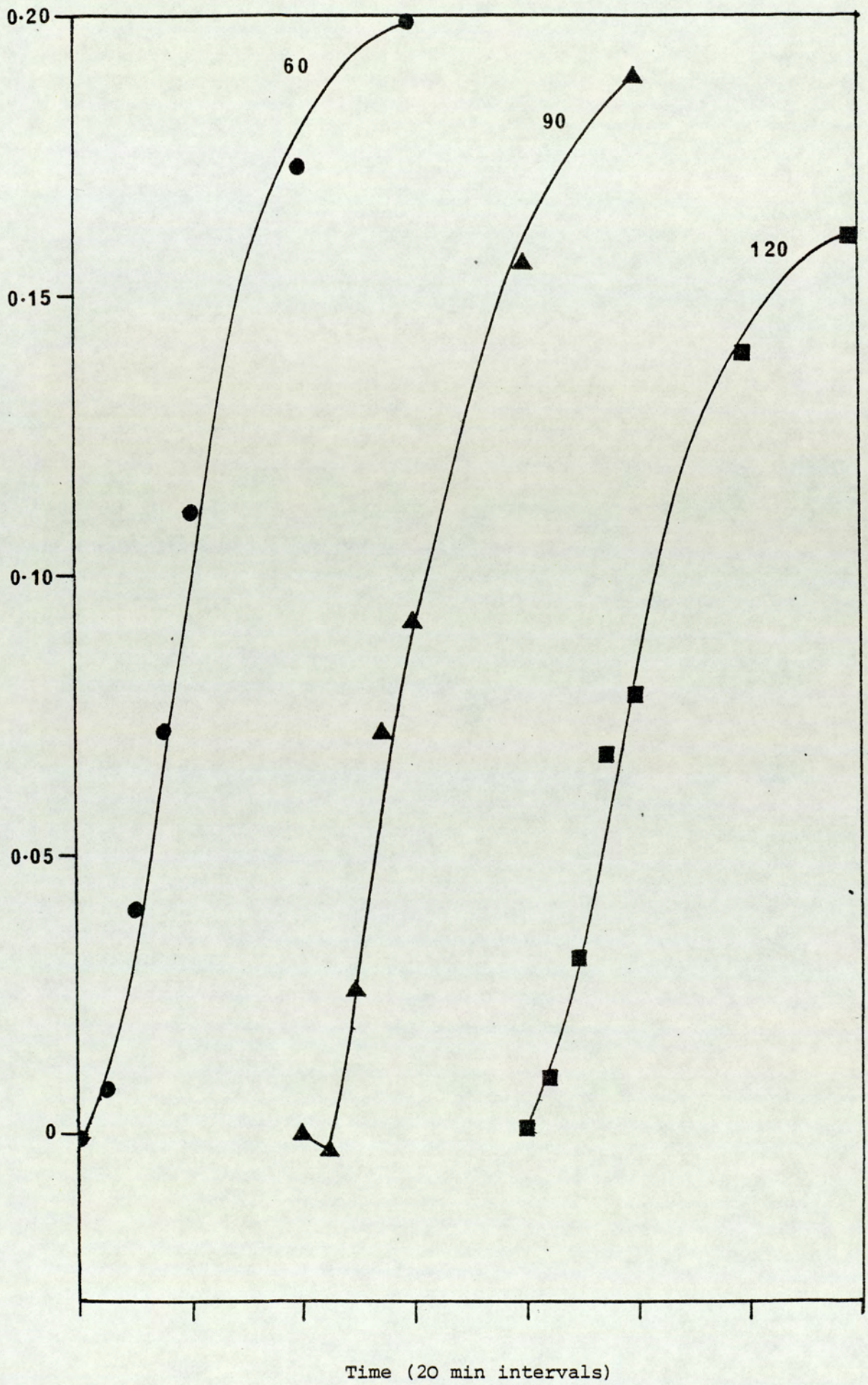

ΔA260nm
0·20
0·15
0·10
0·05
0
60
90
120
Time (20 min intervals)

Fig. 55. Action of varying concentrations of polymyxin (u ml^{-1}) on *P. aeruginosa* depleted of sulphur and glucose

A_{470nm} 0.20 before the addition of PB to resuspended cells (S^- G^-)

Each point mean of 1 flask from 2 experiments

Mean loss of 260nm - absorbing compounds on filtering 13%

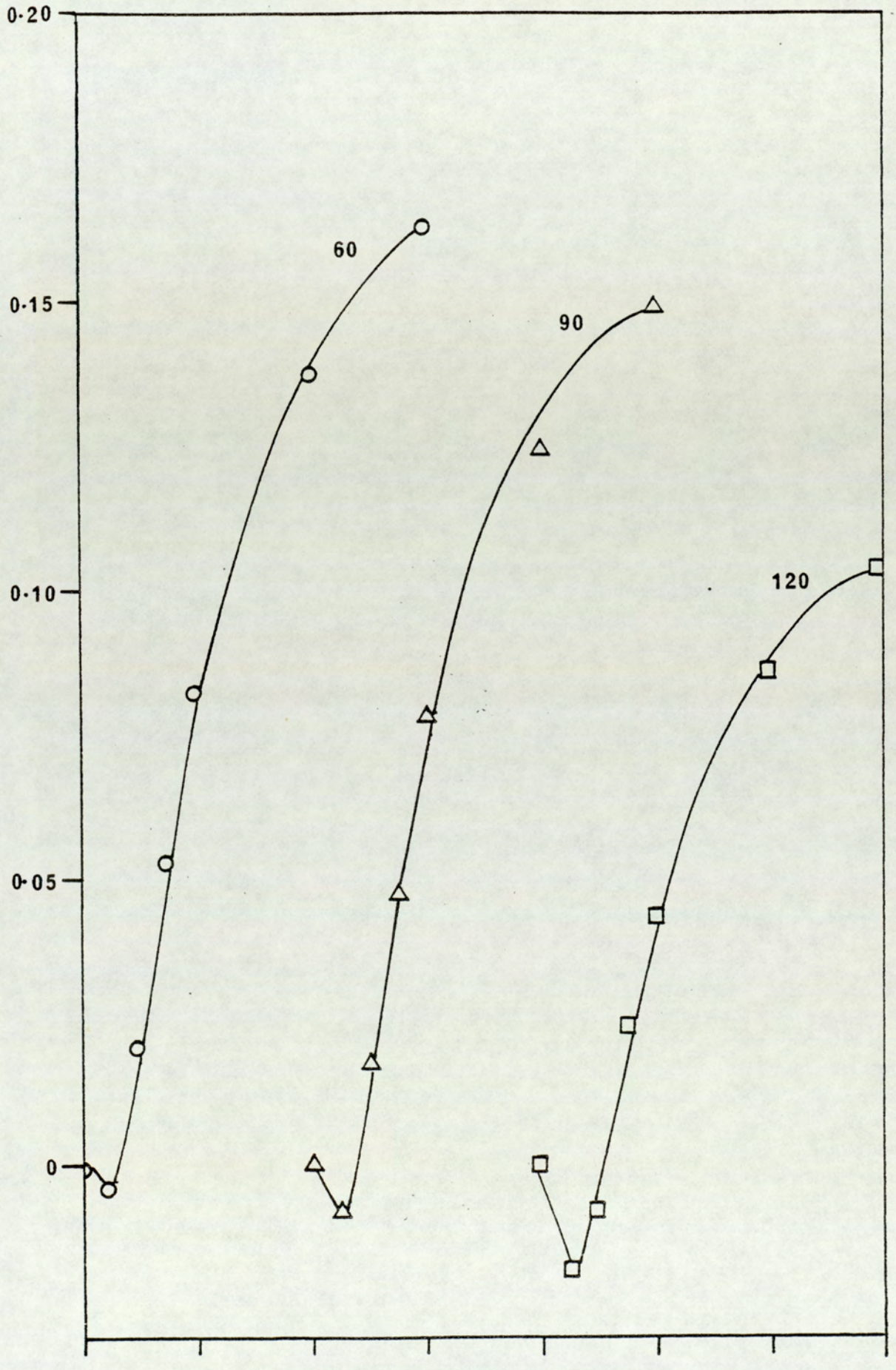
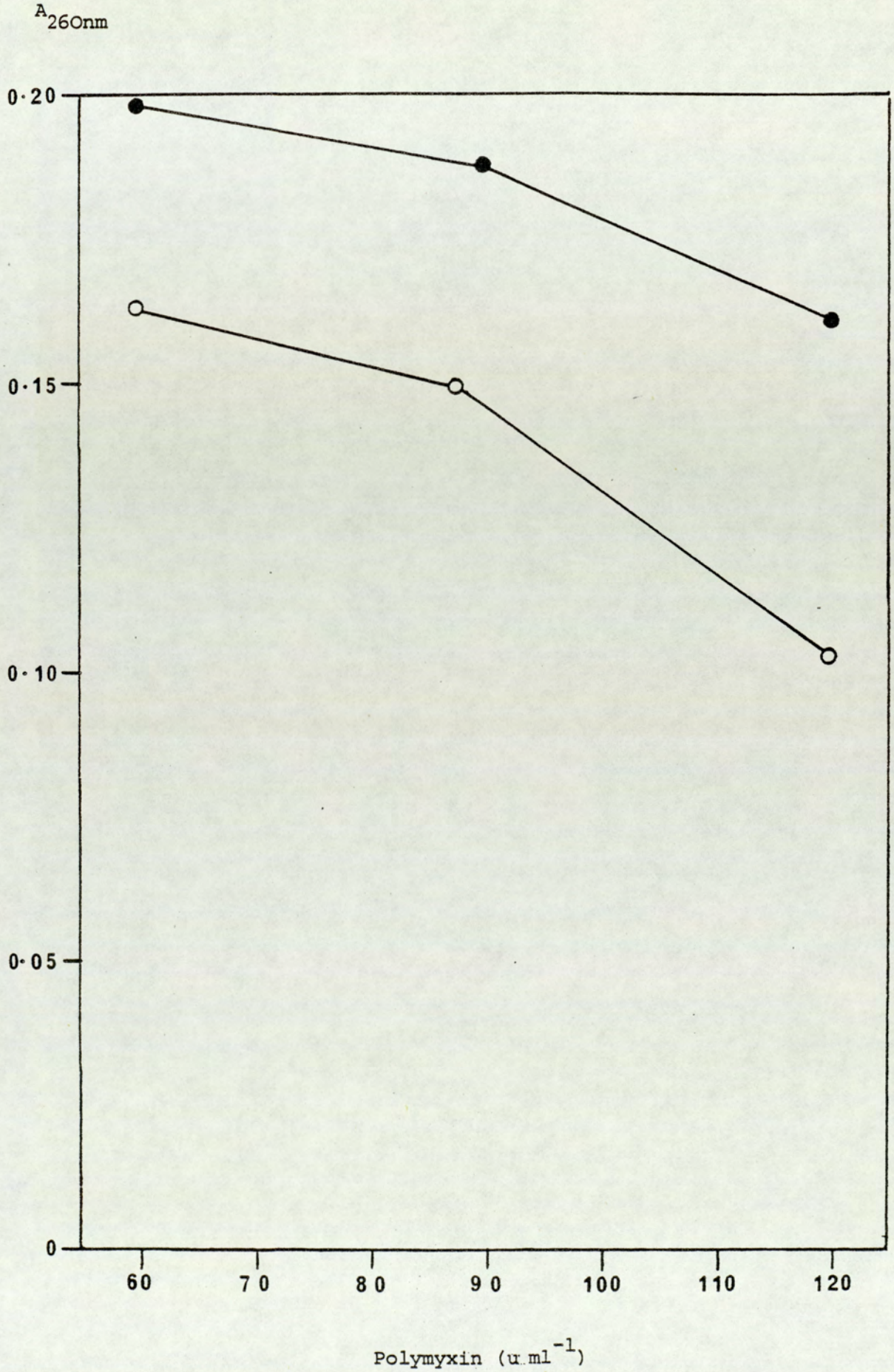

ΔA260nm
0·20
0·15
0·10
0·05
0
60
90
120
Time (20 min intervals)

Fig. 56. Release of 260nm - absorbing compounds 60 mins after addition of polymyxin to nutrient - depleted *P. aeruginosa*

● $S^- G^+$ resuspended

○ $S^- G^-$ resuspended

The results for this figure are compiled from Figs. 54 & 55


A_{260nm}
0·20
0·15
0·10
0·05
0
60
70
80
90
100
110
120
Polymyxin (u.ml^{-1})

3.4.3.3 The sulphur source, on which glucose-depleted cells are grown, does not affect PB - induced release of 260nm - absorbing compounds (Fig. 57). However, it does affect the release of 260nm - absorbing compounds following sulphate, methionine or cysteine depletion. (Fig. 58).

Fig. 57. Action of polymyxin (60 u ml^{-1}) on *P. aeruginosa* depleted of glucose

● $S^+ G^- A_{470nm}$ 0.20 before the addition of PB to unresuspended cells.

◧ $Cys^+ G^- A_{470nm}$ 0.20 before the addition of PB to unresuspended cells.

Each point mean of 1 flask from 2 experiments

Mean loss of 260nm - absorbing compounds on filtering 14%

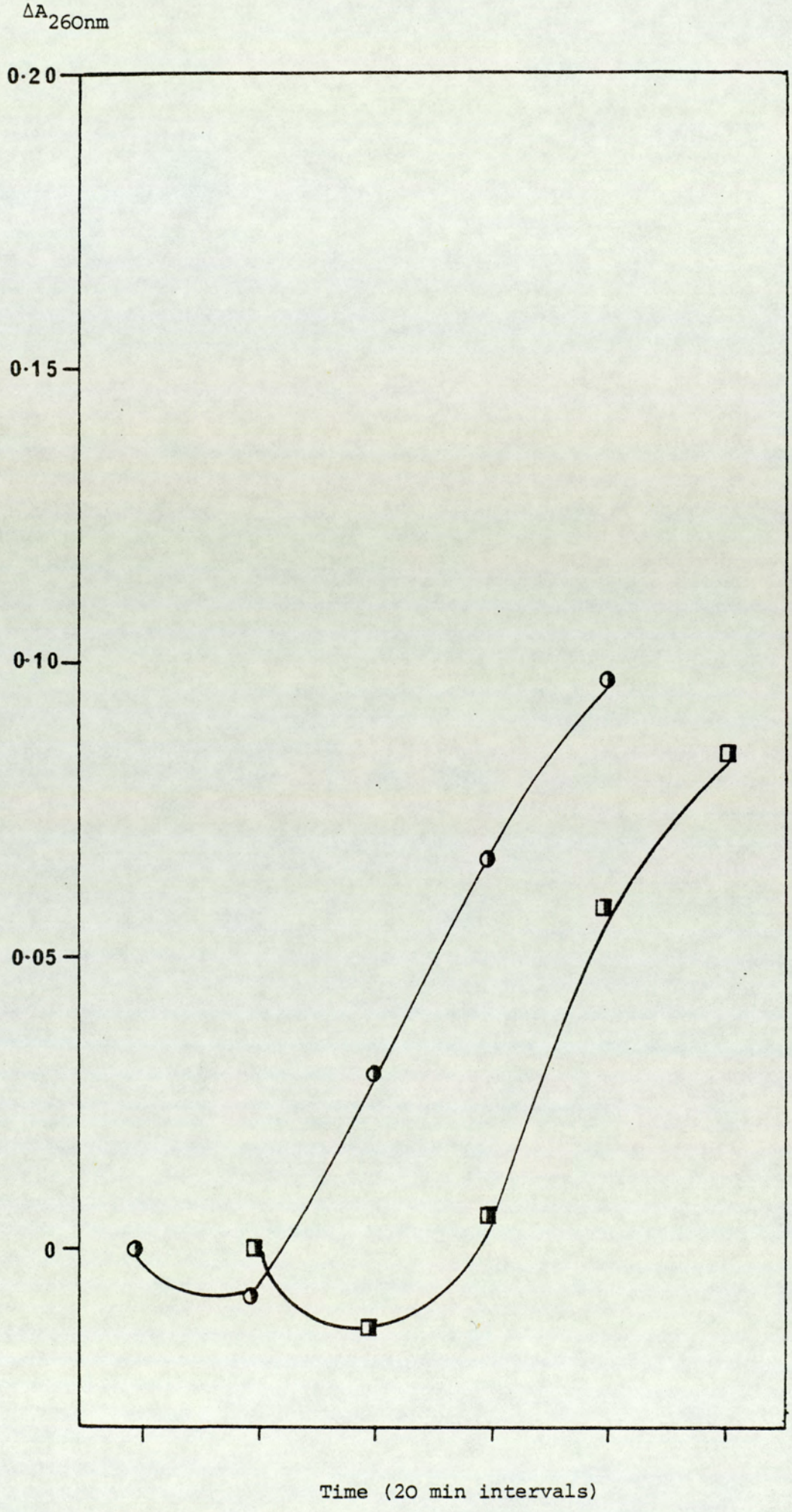

ΔA260nm
0·20
0·15
0·10
0·05
0
Time (20 min intervals)

Fig. 58. Action of polymyxin (60 u ml^{-1}) on P. aeruginosa depleted of sulphur

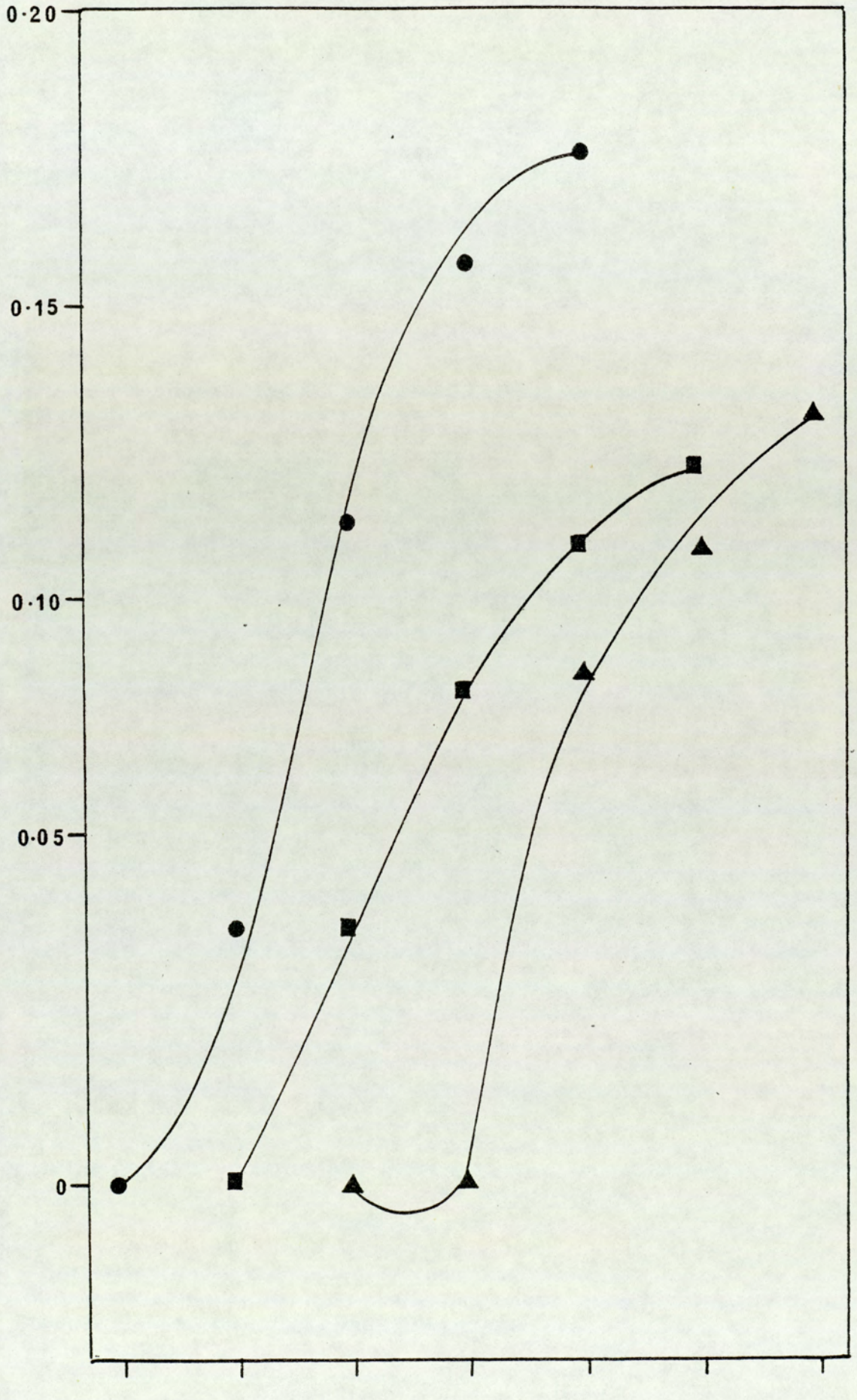
● $S^- G^+ A_{470nm}$ 0.20 before the addition of PB

■ $Cys^- G^+ A_{470nm}$ 0.20 before the addition of PB

▲ $Met^- G^+ A_{470nm}$ 0.20 before the addition of PB

Each point mean of 1 flask from 2 experiments

Mean loss of 260nm - absorbing compounds on filtering 8%


ΔA_{260nm}
0·20
0·15
0·10
0·05
0
Time (20 min intervals)

3.5 Action of gentamicin on nutrient-depleted cells

Table 34 shows the slightly greater resistance of S^- G^+ to gentamicin compared to S^+ G^-. The resistance of sulphur-depleted cells is not dependent on the presence of glucose (Table 35).

Table 34

Survival of nutrient-depleted *P. aeruginosa* grown on drug sensitivity agar containing gentamicin

Concentration of gentamicin ($\mu g\ ml^{-1}$)	% survivors	
	$S^+ G^-$	$S^- G^+$
0.05	102	105
0.10	60	102
0.15	31	100
0.20	9	73
0.25	3	11
0.30	<1	<1

Each result mean of 1 sample from 2 experiments.

Table 35

Effect of glucose on the sensitivity of P. aeruginosa depleted of sulphur to gentamicin

Concentration of gentamicin ($\mu g\ ml^{-1}$)	% survivors $S^- G^-$	$S^- G^+$
0.05	91	106
0.10	97	93
0.15	75	82
0.20	38	38
0.25	19	13
0.30	<1	<1

Each result mean of 1 sample from 2 experiments.

3.6 Drug-sensitivities of auxotrophic mutant

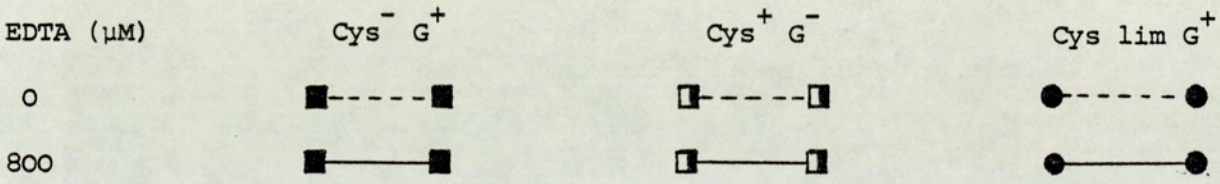
3.6.1 Lytic action of EDTA

P. aeruginosa PCM1 Cys^- G^+ cells are resistant to the lytic action of EDTA, as shown by the slight increase in A_{470nm}, whilst Cys^+ G^- cells are sensitive to its action, as shown by the decrease in A_{470nm} (Fig. 59). When PCM1 is grown in CDM + methionine 0.2mM (Table 4) it has initially a long doubling time (7hrs). When PCM1 is treated with EDTA during this period designated Cys lim G^+ it produced an identical response to that of the Cys^- G^+ cells.

3.6.2 Release of 260nm - absorbing compounds by polymyxin

Results using PCM1 were similar to those using the wild-type. Sulphur-depleted cells were more sensitive to PB-induced release of 260nm - absorbing compounds than glucose - depleted cells. There was no difference in the sensitivity of methionine or cysteine depleted cells (Fig. 60).

Fig. 59. Action of EDTA on nutrient - depleted P. aeruginosa PCM1



A_{470nm} 0.20 before the addition of EDTA

Each point mean of 1 flask from 2 experiments

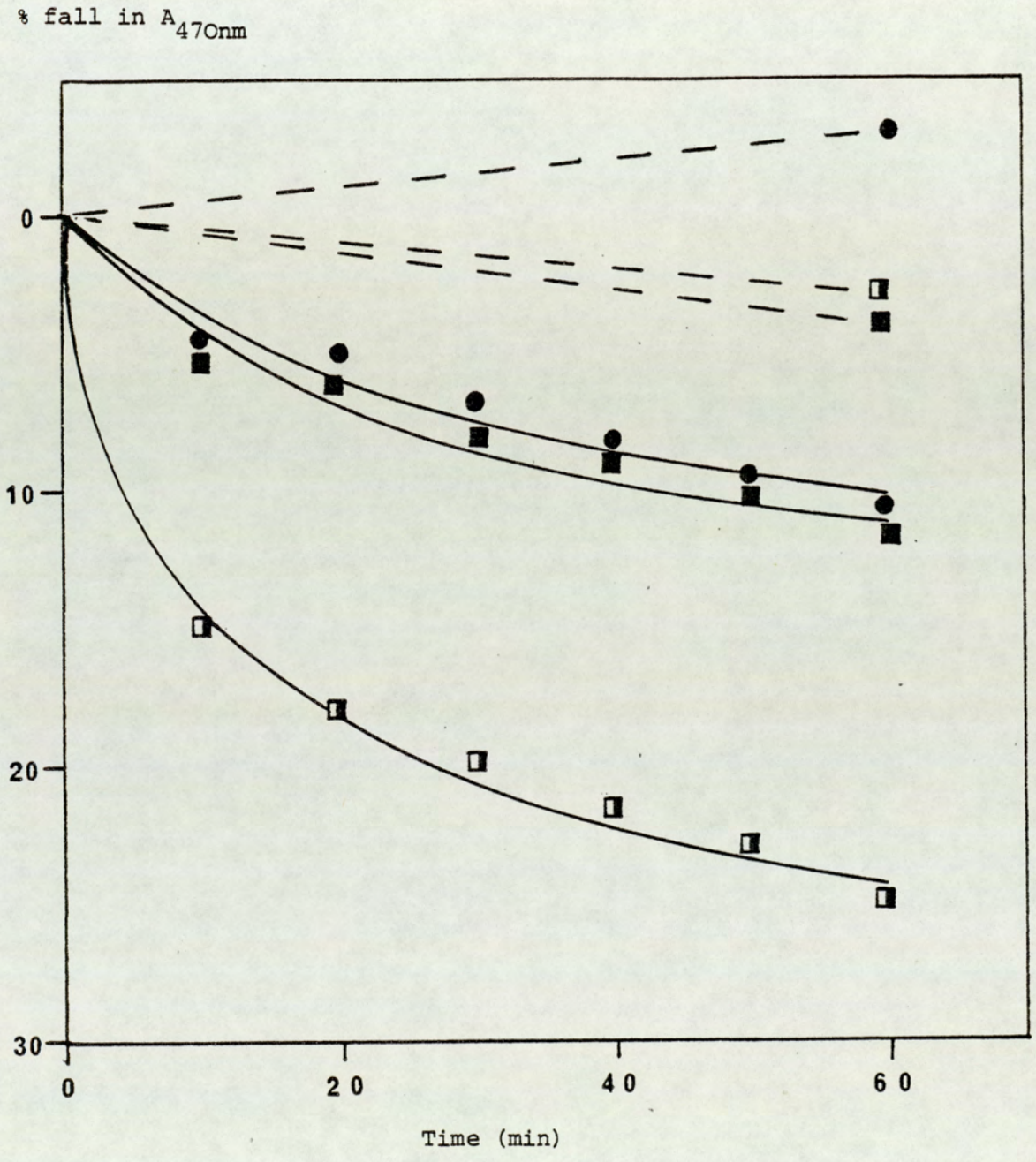

% fall in A_{470nm}
0
10
20
30
0
20
40
60
Time (min)

Fig. 60. Action of polymyxin (60u ml^{-1}) on nutrient - depleted *P. aeruginosa* PCM1

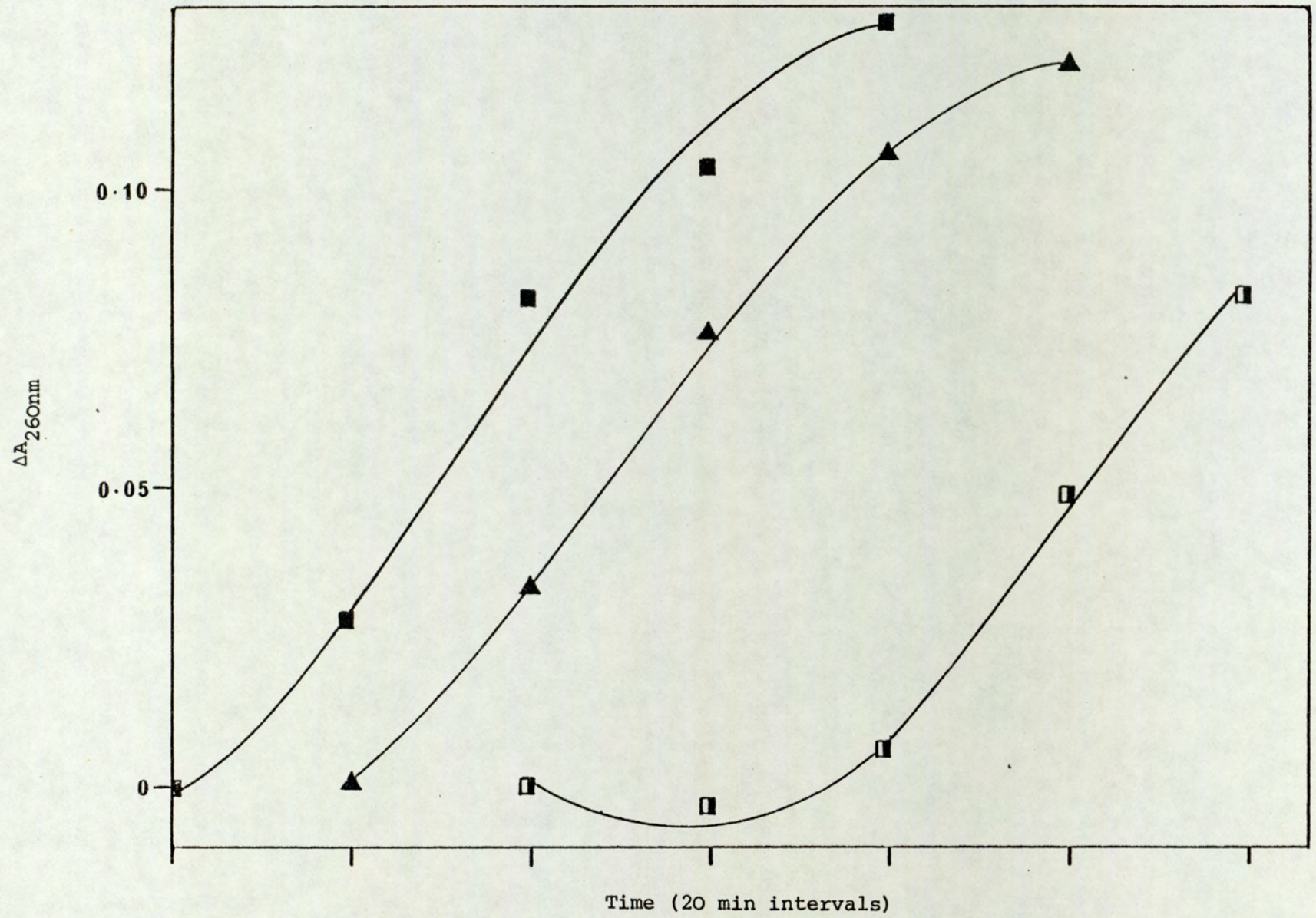
■ Cys^{-} G^{+} A_{470nm} 0.20 before the addition of PB to unresuspended cells.

▲ Met^{-} G^{+} A_{470nm} 0.20 before the addition of PB to unresuspended cells.

◨ Cys^{+} G^{-} A_{470nm} 0.20 before the addition of PB to unresuspended cells.

Each point mean of 1 flask from 2 experiments

Mean loss of 260nm - absorbing compounds on filtering 11%


0·10
0·05
0
ΔA_{260nm}
Time (20 min intervals)

3.7 Chemistry of cell envelopes

3.7.1 Protein profiles

Plate 1 shows the protein profiles of P. aeruginosa in a number of cells. The salient feature of all the CM fractions examined is the lack of any major proteins, but rather the presence of a wide variety of minor proteins. The log phase cells appear to lack a low molecular weight protein present in the depleted cells and all types of cells examined have distinct profiles in the region equivalent to D-F in the OM.

There is no apparent difference in the OM proteins of S^- G^- and S^- G^+ cells. Sulphur depletion induces an increase in the amount of proteins E and G, and a high molecular weight protein which has not been classified.

3.7.2 Outer membrane composition

Table 36 summarises the extensive chemical analyses completed on the OM fractions. The only major difference is the reduced level of KDO in logarithmic phase.

3.7.3 Outer membrane phospholipids

Fig. 61 shows a typical densitometer recording. Table 37 shows the phospholipid (PL) analysis of the various cells. Diphosphatidyl-glycerol (DPG) is drastically reduced during logarithmic phase.

3.7.4 Outer membrane fatty acids

Fig. 62 shows a typical gas-liquid-chromatogram recording. Table 38 shows the individual fatty acids (FA) present in P. aeruginosa OM. Table 39 categorises the FA into various types; log, S^- G^- and S^- G^+ OM

have particularly low levels of cyclopropane FA compared to S^+ G^- OM.

Plate 1

Protein profiles of P. aeruginosa

	Type	Cells
1	CM	S^- G^-
2	CM	S^- G^+
3	CM	Log
4	CM	S^+ G^-
5	OM	S^- G^-
6	OM	S^- G^+
7	OM	Log
8	OM	S^+ G^-

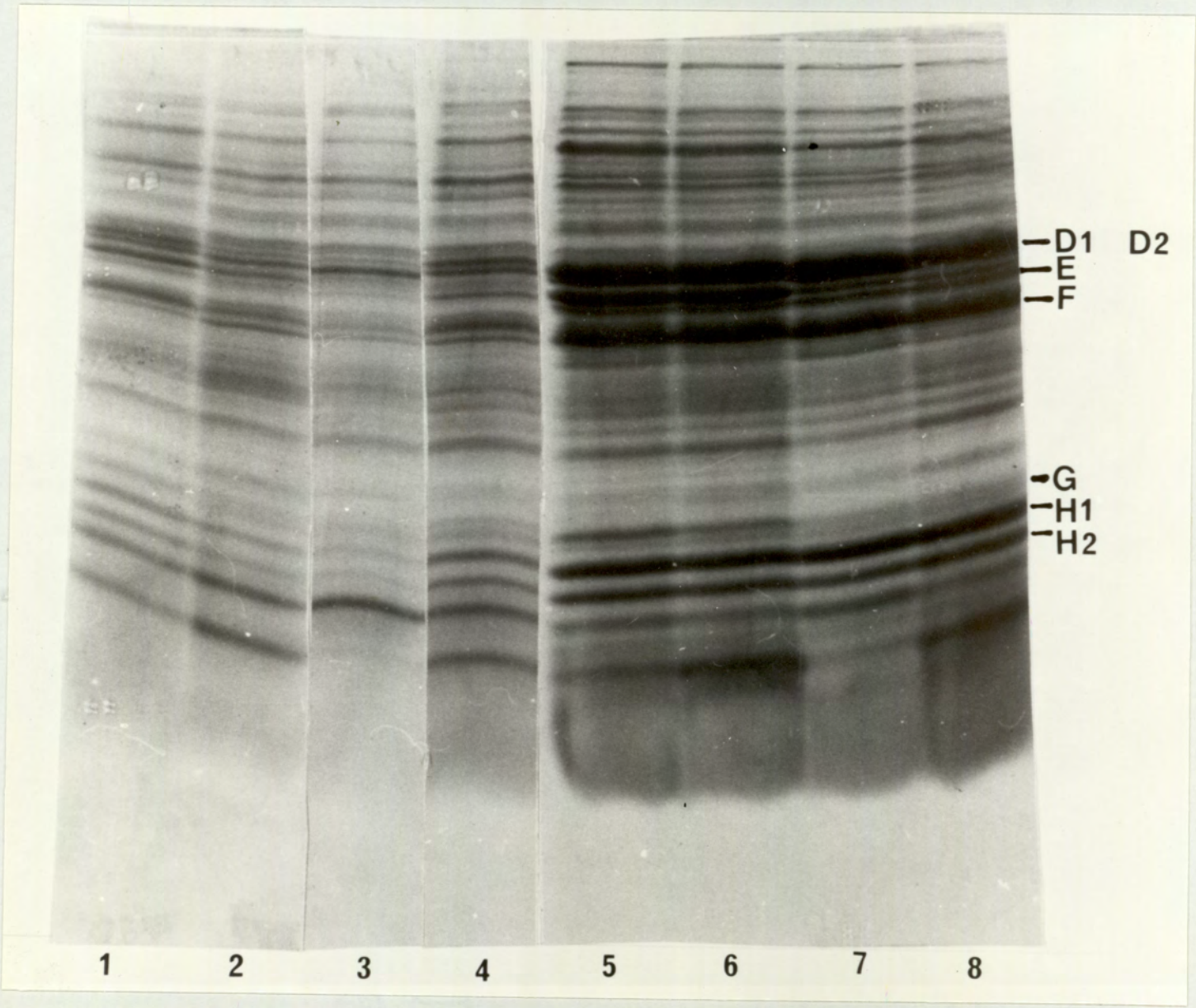

D1
D2
E
F
G
H1
H2
1
2
3
4
5
6
7
8

Table 36

Outer membrane composition of P. aeruginosa

Assay	% dry weight of outer membrane			
	Cells			
	$S^- G^-$	$S^- G^+$	Log	$S^+ G^-$
Protein[1]	54 ± 3	57 ± 4	57 ± 4	55 ± 1
REL[2]	28	30	26	28
KDO[1]	1.36 ± .03	1.37 ± .09	1.17 ± .04	1.36 ± .04
Mg^{2+2}	0.96	0.93	0.84	0.84
Ca^{2+2}	0.43	0.37	0.30	0.38
P[2] (including RELP)	2.70	2.97	3.02	2.67
RELP[2]	0.78	0.72	0.73	0.68

1. 3 readings from 1 sample, mean and SD.

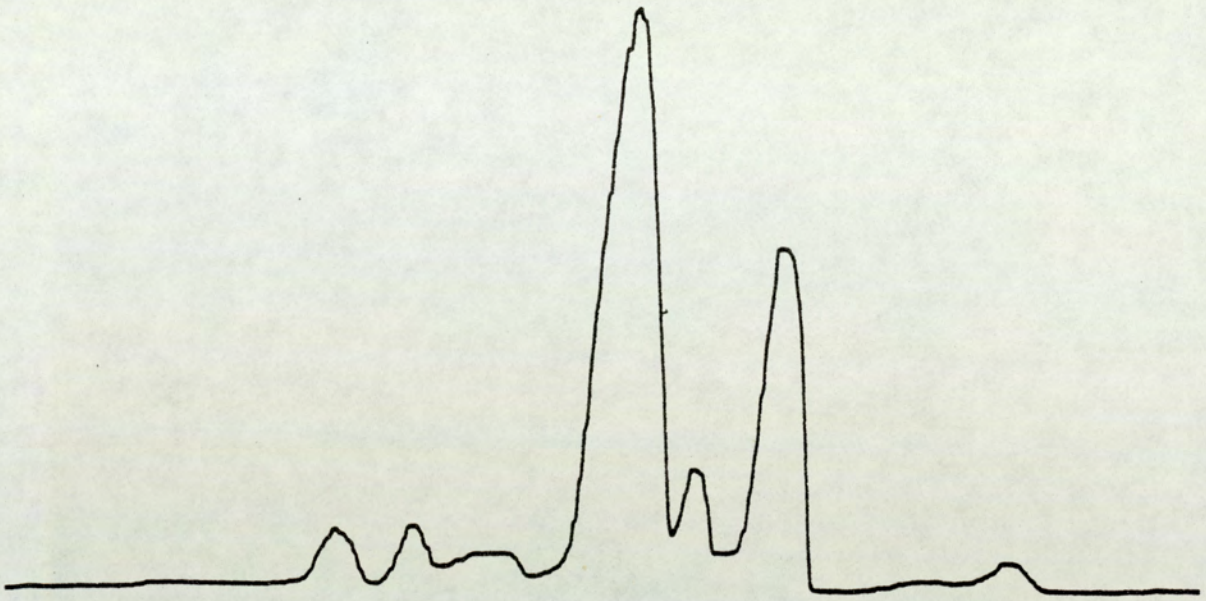
2. 2 readings from 1 sample.

Fig. 61. Densitometer recording of phospholipid sample

Fig. 61 shows typical results, after fractionation by TLC in Chloroform/methanol.

Fig. 62. Gas liquid chromatogram of fatty acid sample

Fig. 62 shows typical results after fractionation by GLC



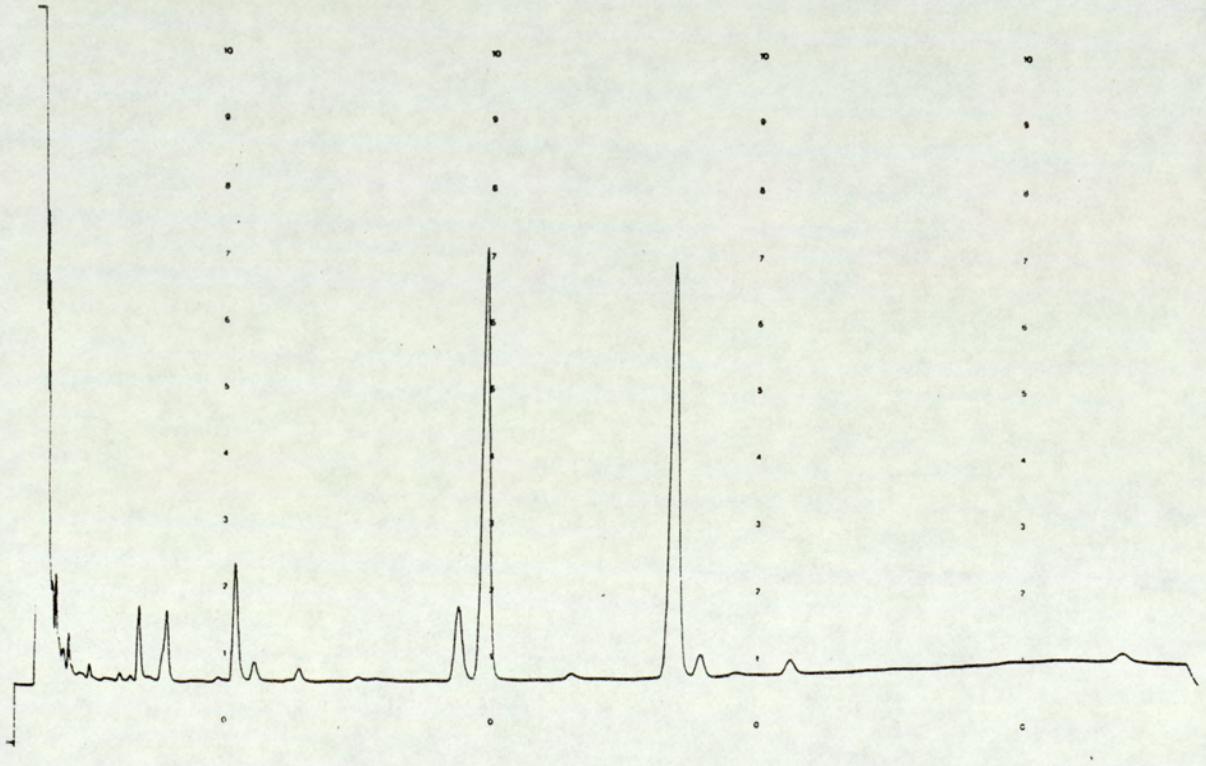


Table 37

Outer membrane phospholipids of P. aeruginosa

% Phospholipid	Cells			
	S^- G^-	S^- G^+	Log	S^+ G^-
PG	10.7	17.9	23.8	16.1
PE	58.0	54.2	60.4	54.0
DPG	24.4	18.6	8.6	23.6
X	3.6	5.6	7.2	2.5
Y	3.3	3.7	N.D	3.8

Results are from sample

N.D. = not detectable

X and Y = uncharacterised phospholipids

Table 38

Outer membrane fatty acids of P. aeruginosa

	Cells			
% F.A	S^- G^-	S^- G^+	Log	S^+ G^-
3.OH 10:0	4.0	3.0	2.8	2.6
12:0	5.5	4.2	2.5	5.0
2.OH 12:0	7.8	5.1	5.0	5.7
3.OH 12:0	1.4	0.6	0.9	0.9
14:0	0.3	0.5	0.5	0.4
16:1	2.5	3.2	6.0	4.2
16:0	41.3	40.5	40.4	39.5
17.0Δ	0.7	0.6	0.3	1.3
18:1	32.5	39.1	39.0	31.6
18:0	0.6	0.9	1.5	1.2
19:0Δ	3.4	2.3	1.1	6.6

Results are from 1 sample

Table 39

Outer membrane fatty acid types of P. aeruginosa

Type of fatty acid	% total fatty acids			
	Cells			
	$S^- G^-$	$S^- G^+$	Log	$S^+ G^-$
Unsaturated	35.0	42.3	45.0	35.8
Saturated	60.9	54.8	53.6	56.3
Cyclopropane	4.1	2.9	1.4	7.9

4. DISCUSSION

4.1 Nutrient depletion studies

4.1.1 Introduction

The quantitative nutrient requirements of P. aeruginosa for aerobic growth have been determined by Noy (1982). The medium used is based on this, calculating the concentration of all ingredients which would theoretically permit growth to reach A_{470nm} of at least 5.0. As the investigation involved comparing glucose and sulphur depleted cells, the requirements were determined with the medium used in Noy's study.

4.1.2 Glucose

Glucose was supplied as the energy source and carbon source. Carbon is the basic structural component of all organic compounds and is therefore used by microbes in large quantities. The linear relationship between cessation of logarithmic growth and glucose concentration up to 4mM (Fig. 20), indicates that when glucose was present at a concentration of 20mM the progressive decline in growth rate (Fig. 19) was not a result of glucose depletion. Progressive decline in growth rate at high glucose concentrations is likely to be due to lack of oxygen. The concentration of dissolved oxygen in the medium drops rapidly above A_{470nm} 1.6 (Finch, 1976). Extrapolation of the curve relating maximum growth to substrate concentration (Fig. 20) through the origin indicates that there was no other carbon source present in the medium which could be used by P. aeruginosa.

Fig. 21 shows the biphasic relationship between the reciprocal of growth rate and the reciprocal of glucose concentration, confirming the presence of low and high affinity systems for glucose utilisation, as described by Dawes et al. (1976). They found that when glucose

is in excess, the low affinity system operates and the periplasmic enzymes, glucose dehydrogenase and gluconate dehydrogenase, metabolise glucose via an extracellular pathway. Gluconate and 2-oxogluconate accumulate in the medium, since they are produced faster than they can be transported into the cell. Under glucose-restricted conditions, the extracellular pathway enzymes are repressed and there is an increase in the high affinity enzymes; hexokinase and glucose-6-P-dehydrogenase are produced. This ensures that glucose is taken up as rapidly as possible.

4.1.3 Sulphur

The progressive decrease in the growth rate when exponential growth ceases due to insufficient sulphur (Fig. 22) is probably due to the decreased synthesis of the large number of sulphur-containing components in the bacterial cell (as discussed in section 1.4.4). The appearance of 260nm-absorbing compounds in the medium at this time suggests that unbalanced growth is the result (section 4.2). The linear relationship between cessation of logarithmic growth and sulphate concentration up to 52μM (Fig. 23) indicates that when sulphate was added to the CDM at 360μM a gross excess was present. The extrapolation of this line for maximum exponential growth back to the abscissa (Fig. 23) shows that there was a slight sulphate contamination, presumably from other CDM constituents equal to 1.6×10^{-6}M of sulphate. This is rather less than concentrations reported by other workers (eg Noy, 1982 reported 8.0×10^{-6}M contamination). The redistillation of double distilled water from alkaline potassium permanganate was used to keep the contamination of sulphate from this source to a minimum.

Fig 24 shows the biphasic relationship between the reciprocal of growth rate and the reciprocal of sulphate concentration, suggesting a high and low affinity uptake system, depending upon the external sulphate concentration. The initial uptake mechanism may be binding of sulphate to a specific periplasmic protein, as in S. typhimurium (Pardee, 166, section 1.4.2). In the latter species its formation is decreased when cells are grown in a medium to which sulphate has been added (Ohta et al., 1971).

4.2 Excretion of 260nm-absorbing compounds during sulphur depletion

The release of 260nm-absorbing compounds from bacteria has been used by a number of workers (Newton, 1953; Hugo and Bloomfield, 1971) as an indicator of cellular damage. After treatment with polymyxin B (PB) it is related to loss of viability (Brown and Melling, 1969b). However, Table 23 shows that both viable and total counts increased by comparable amounts during sulphur depletion in the presence of glucose, although 260nm-absorbing compounds accumulated in the medium at the same time (Fig 25). Little accumulation of 260nm-absorbing compounds occurs in the absence of glucose (Fig 28).

Potassium release from the cell has also been used as a parameter of membrane damage. Lambert and Hammond(1973) found that during cellular damage following treatment with quaternary-ammonium compounds potassium release preceded release of 260nm-absorbing compounds. Therefore, it would be expected that if the accumulation of 260nm-absorbing compounds in the medium was due to cellular damage, potassium would be released into the medium. Fig. 31 shows the results are the opposite of this; sulphur-depleted cells in the presence of glucose take up potassium, whilst S^- G^- and S^+ G^- cells excrete potassium into the medium. The excretion of potassium by S^- G^- and S^+ G^- cells is probably due to the damaging effects of Tris buffer on the OM (Irvin et al., 1981b). This is also reflected in the excessive excretion of 260nm-absorbing compounds from S^+ G^- cells resuspended in Tris buffer (Fig 30). The addition of glucose to the S^- G^- cell prevents excretion of potassium and after a short lag-phase promotes its uptake (Fig 32.).

Although S^- G^+ cells excrete considerable amounts of 260nm-absorbing

compounds into the medium, the total amount of 260nm-absorbing compounds within S^- G^+ and S^+ G^- cells is approximately equal after 5hrs of nutrient depletion (Table 25); also this excretion is inhibited by arsenate. These results confirm that this excretion is due to the energy-dependent synthesis of 260nm-absorbing compounds, rather than autolytic degradation of cellular contents. The higher value for pool material in S^- G^+ cells (Table 25) may reflect the maximum amount of 260nm-absorbing compounds possible in cells, before it is excreted into the medium.

Two of the 260nm-absorbing compounds excreted into the medium have been tentatively identified by TLC as cytosine and uracil, both pyrimidines. The identification of cAMP is more doubtful, as although it chromatographed with the standard, the test compound, unlike the standard, fluoresced under the u.v lamp.

The excretion of nucleic acid fragments from methionine-depleted E. coli described by Borek et al. (1955) would appear to be a different process as excretion also occurred in glucose-depleted and log-phase cells. The cause of this excretion remains unclear, as when cells are depleted of amino acids a large number of cellular activities, including synthesis of nucleotides, are inhibited (Cozzone, 1981). The excretion of 260nm-absorbing compounds by S^- G^+ cells maybe a possible mechanism for the conversion of unwanted metabolites into a form which can be efficiently removed from the cell. Alternatively it may be that S^- G^+ cells are incapable of reducing synthesis of 260nm-absorbing compounds to the level required by the slow growing cells.

4.3 Changes in cell envelopes of P. aeruginosa

4.3.1 Introduction

As discussed in section 1.3.5, resistance may be due to exclusion by the OM. This can be achieved in several ways, one possible mechanism being the presence of an inactivating enzyme in the periplasm, although none has ever been isolated for polymyxin B (PB) (Gilleland and Farley, 1982). Alternatively the outer membrane (OM) might be able to absorb all the membrane-active drug and thus prevent any reaching the CM. This would seem an unlikely explanation of resistance to PB, as La Porte et al. (1977) have shown it can damage the cell simply by interacting with its outer surface. The most likely methods of resistance are that the target molecules are altered in such a manner that no interaction between PB and the membranes can take place, or the target molecules may be no longer present at the cell surface or they can be protected through steric hindrance by other components of the OM.

EDTA is known to act specifically on the OM (Wilkinson, 1975); alterations in the OM will thus have a significant effect upon its action.

4.3.2 Outer membrane composition

It has been shown in this study that cells depleted in various ways vary in their resistance to EDTA (Figs 33 & 34) and PB (Figs 41 & 43). Qualitative and quantitative variations in the OM are known to affect the sensitivity of P. aeruginosa to EDTA and PB (Kenward et al., 1979) and they are thought to have a common site of action in the OM (Brown and Melling, 1969b). The results of the OM analysis will be discussed mainly in relation to resistance to these agents.

4.3.2.1 Proteins

Hancock and Carey (1979) found that proteins D1, D2 and E are induced when cells were grown in a minimal medium in the presence of glucose. However Plate 1 shows that a band relating to unresolved D1 and D2 was induced in log and stationary cells whether depleted of sulphate or glucose, possibly therefore being induced during log-phase and persisting after depletion. Proteins E and G were only overproduced in sulphur-depleted cells. Protein D1' is a pore-forming protein (Hancock and Carey, 1980), but no role has been assigned to proteins D2, E and G.

The uncharacterised high molecular-weight protein induced by sulphur depletion was unexpected. Brunschede and Bremer (1971) suggested that the probability of a polypeptide being completed during amino-acid starvation would decrease exponentially with increases in its molecular weight. Cozzone (1981) has suggested that degradation of existing proteins in the cell could provide a source of amino-acids for protein synthesis.

No differences in the level of OM protein H1 was found between cells (Plate 1). Magnesium-depletion of P. aeruginosa results in increased resistance to EDTA and PB (Brown and Melling, 1969b) and over-production of H1 (Nicas and Hancock, 1980) as discussed in section 1.3.5. Drastically reduced levels of protein F (porin protein) have been implicated in PB resistance (Nikaido, 1979), presumably preventing PB entering the cell as a result of a reduced number of pores. No variation in the levels of protein F were found in the cells tested (Plate 1).

4.3.2.2 Divalent cations

As discussed in sections 1.2.6 and 1.3.5 reduced levels of divalent cations have been implicated in resistance to EDTA and PB (Kenward, et al., 1979). However, no significant differences in the concentration of Mg^{2+} and Ca^{2+} were found between these nutrient-depleted cells (Table 36).

4.3.2.3 Lipopolysaccharide

Lipopolysaccharide (LPS) was estimated here using the 2-keto-3-deoxyoctonic acid (KDO) assay, which gives an indication of the amount of core polysaccharide present in the cell.

As discussed in section 1.2.6, EDTA resistance has been associated with a reduced phosphorus content through a raised KDO level in the cell envelope (Gilleland et al., 1974), whilst PB resistance has been associated with a reduced KDO content (Gilleland and Lyle, 1979) (Section 1.3.5). Alterations in the chemical composition of LPS have also been implicated in sensitivity to these agents (Vaara, 1981; Vaara et al., 1981). No significant differences in phosphorus or KDO content were found between nutrient-depleted cells (Table 36). Chemical analysis of LPS was not done in this study, and alterations would not be reflected in the KDO assay.

Teuber (1974) has correlated the length of the O-antigen chain of S. typhimurium with resistance and binding to PB. The possibility that LPS maybe involved in PB resistance is relevant when considering the proposal by Kropinski et al. (1981) that variations in the chemical composition of LPS may affect the number of open functional pores in P. aeruginosa. However, virtually all studies (section 1.3.2)

and the work of Nikaido (1976) with LPS mutants of S. typhimurium suggest that PB enters the cell through interactions with the OM rather than through pores.

4.3.2.4 Lipids

Logarithmic cells of P. aeruginosa have a low level of diphosphatidylglycerol (DPG) in the OM compared to nutrient-depleted cells(Table 37). This is in agreement with the work of Randle et al. (1969) who found that DPG rises during the transition between logarithmic and stationary phase, and reaches a maximum during the stationary phase of most Gram-negative bacteria. The major phospholipid (PL) in the OM of all the cells studied was phosphatidylethanolamine (PE) (Table 37) which confirms the work of Brown and Watkins (1970) on the cell wall PL of P. aeruginosa.

Consideration of PB action (Section 1.3.2) and resistance (1.3.5) indicates a reduction in the PL, DPG and phosphatidylglycerol (PG) maybe involved in resistance to PB. S^- G^- cells have a low level of PG compared to S^- G^+ and S^+ G^- cells, although the combined total of PG and DPG is constant at approximately 35% of all the cells tested (Table 37).

Brown and Watkins (1970) found that PB resistance in P. aeruginosa was associated with a drastic reduction in the total PL content of the cell wall, although qualitatively the PL compositions were similar. In this study readily extractable lipid phosphate (RELP) was used to assay the total PL in the OM. There was no large scale differences in RELP or readily extractable lipid (REL) between any of the cells tested (Table 36).

4.3.2.5 Fatty acids

The fatty acid (FA) composition of the cell is drastically affected by the growth medium (Conrad et al., 1981) and incubation temperature (Marr and Ingraham, 1962), and significant differences also occur between strains of P. aeruginosa (Wilkinson and Galbraith, 1975). Therefore unless all these factors are constant, comparisons of FA composition will be of little relevance.

The FA analysis of the OM (Table 38) will include any free FA, FA attached to PL and four of the six FA attached to the lipid A portion of LPS. The remaining two FA are attached through amide linkage and will not be removed by alkaline hydrolysis as used in this study (Hancock et al., 1970).

The proportion of cyclopropane FA varies between cells tested (Table 39); logarithmic cells have the lowest level. Jungkind and Wood (1974) found that in Streptococcus faecalis,the proportion of cyclopropane FA was also lowest in logarithmic growth. Cyclopropane FA are synthesised by methylation of the comparable unsaturated FA by S-adenosyl methionine (SAM) (O'Leary, 1965); this may explain the lower proportions in sulphur-depleted cells compared to glucose-depleted cells (Table 39). Jungkind and Wood (1974) also found cyclopropane FA decreased in methionine-limited cells. However, O'Leary's(1965) proposal,'that cyclopropane FA have some major and indispensable function', as yet still unidentified, would seem more applicable in Klebsiella, where proportions of cyclopropane FA rise as high as 37% (Dunnick and O'Leary, 1970) than the maximum 7.9% proportion in this study (Table 39).

Storm et al. (1977) considered the insertion of the FA chain of PB in a PL bilayer might lead to pertubation of the normal FA packing, since the FA chain of PB is shorter than the average FA chain of PL. This is in agreement with the observation that longer FA chains attached to PB actually diminish its activity. Presumably the insertion of the FA chain into the hydrophobic interior of the OM coupled with the displacement of Mg^{2+} and Ca^{2+} from their sites in the OM would lead to OM disorganisation.

The fluid character of the membrane is largely determined by the relative proportions of FA, increasing concentrations of unsaturated and cyclopropane FA increasing membrane fluidity. Nikaido (1979) suggested that the low fluidity of LPS FA will hinder the dissolution of hydrophobic molecules into the membrane interior. PB resistance in Klebsiella has been correlated with a drastic reduction in total cyclopropane FA composition. In this study the variations in cyclopropane FA (Table 39) would not appear to be of significant magnitude to explain the varying sensitivities of nutrient-depleted cells.

4.3.2.6 Discussion

The preparation of OM samples is complex (section 2.8), prone to transferance losses and possible contamination of OM and CM samples (Devor et al., 1976). Differences in OM composition in this study do not appear to correlate with resistance to EDTA and PB, and many of the factors implicated may well be coincidental rather than causal. In the following section, resistance will not only take into account OM composition but include thermodynamic considerations of OM structure.

4.4 Action of EDTA on nutrient-depleted cells

4.4.1 Cell lysis

Extreme sensitivity to EDTA has been postulated by Leive (1968) to be due to the presence of an 'exceptional' autolysin. The inhibition of the autolysin (which has yet to be isolated) does not seem the most likely role of glucose in the sulphur-depleted cell (Fig 34) for preventing EDTA-induced lysis.

Nikaido and Nakae (1979) have speculated that the asymmetric structure of the OM, makes it inherently unstable and energy is constantly required to maintain the thermodynamically unfavourable structure. Nieva-Gomez and Gennis (1977) have shown that binding of fluorescent probes to intact E. coli is strongly affected by the energy state of the cell. The probes appeared to monitor structural changes in the cell envelope which accompanied the energisation and de-energisation of the cell. Farmer and Jones (1976) found that in E. coli the energy requirement for maintenance in a continuous culture varied with differing nutrient limitations. This may partly reflect the differing energy requirements of the varying OM. It is therefore possible that sulphur-depleted cells of P. aeruginosa in the presence of glucose maintain a thermodynamically unfavourable structure of the OM, a structure nevertheless necessary for resistance to EDTA. This maybe achieved by inhibiting the EDTA-induced release of OM components (section 1.2.4) required for the cell's stability (section 1.2.5). Alternatively glucose may prevent the cascade of events resulting in lysis after the loss of OM components from sulphur-depleted cells. The partial restoration of sensitivity to EDTA by arsenate (Fig 36.) is likely to be due to the inhibition of energy production from glucose (Mahler and Cordes, 1971). Nieva-Gomez and Gennis (1977) found

that energy-dependent changes in the cell envelope were reversible; this is in agreement with the finding that sulphur-depleted cells in the presence of glucose are resistant to EDTA (Fig 33), become sensitive in its absence (Fig 34) and become resistant once more when glucose is added to S^- G^- cells (Fig 35).

A number of repair processes have been described in P. aeruginosa after EDTA treatment (section 1.2.6) which required the addition of divalent cations to the media (Asbell and Eagon, 1966) or incubation in a hypertonic growth medium (Stinnett and Eagon, 1975), and allowed cells to regain osmotic stability. Neither of these conditions were present in this study. However, EDTA-treated cells of E. coli when resuspended in a growth medium lacking divalent cations could bind 10% of the released material (Graham et al. 1979).

4.4.2 Bactericidal action

The effect of EDTA on the viability of nutrient-depleted P. aeruginosa (Fig 38) closely parallels the results for lysis (Fig 33), the presence of glucose being required for the resistance of the sulpur-depleted cell (Fig 39). Leive and Kollin (1967) found that prolonged exposure to EDTA is toxic to E. coli. They proposed that this was a result of membrane disorganisation allowing EDTA to enter the cell and chelate divalent cations required for ribosomal integrity and enzyme function, which would eventually result in RNA breakdown. This, coupled with the osmotic fragility of the cell, leads to cell death and lysis. The results in this study show that sulphur-depleted cells in the presence of glucose, can prevent the proposed sequence of events occurring.

4.4.3 Release of 260nm-absorbing compounds

Release of 260nm-absorbing compounds from sulphur depleted cells in the presence of glucose is minimal, whilst S^- G^- and S^+ G^- release significantly greater amounts (Fig 40). These results are in agreement with the lysis (Fig 33) and viability results (Fig 38) and reflect in the former case the inability of EDTA to penetrate into the cell.

4.4.4 Cell lysis of P. aeruginosa PCM1

S^- G^+ cells of PCM1 are slightly more sensitive to the action of EDTA (Fig 59) compared to the wild-type (Fig 33), though different sulphur sources were used, and this may explain the differing responses.

The initial slow growth of PCM1 in CDM + methionine (Table 4) suggests that during this period, growth is limited by the conversion of methionine to cysteine. On the basis of this assumption these cells were designated as cysteine limited (Cys lim). The Cys lim G^+ cells produce the same response to EDTA as the Cys- G^+ cells, supporting this assumption.

4.5 Action of polymyxin on nutrient-depleted cells

4.5.1 Effect of pH

The lytic action of PB is unaffected between the pH range 5.5-7.5 (Table 28), which is in agreement with Noy (1982). However, between the pH range 7.0-7.5 the action of PB is drastically increased as measured by % survivors (Table 31) and release of 260nm-absorbing compounds (Table 33). Few and Schulman (1953) found that increases in pH increase the amount of polymyxin E bound to B. subtilis and its release of 260nm-absorbing compounds. The magnitude of the effect reported would not be a sufficient explanation of the results presented here, although the response of a Gram-negative might be different. The effect of pH on surface charge, which maybe implicated in an initial electrostatic interaction between PB and the cell surface can probably be ruled out as the total surface charge as measured by electrophoretic mobility in E. coli is unchanged between the pH range 5-9 (Neihof and Echols, 1973). The slight differences in the amount of ionised PB compared to the unionised form over the pH range tested (Table 40) indicates lysis is dependent on the ionised form of PB.

The % survivors is unexpectedly high at lower pH (Table 31). Table 40 shows that between the pH range 7.0-7.5, there is a three-fold increase in the unionised form of PB. It may well be that this is the active form of PB which disrupts the CM causing cell death. An unionised drug would also be able to penetrate the hydrophobic OM more easily and reach the sensitive CM.

260nm-absorbing compounds present in the medium will consist of released pool material, nucleic acids degraded in non-lysed cells and nucleic

Table 40

Effect of pH on the % ionisation of polymyxin B

pH	Ionised	Unionised
5.50	99.96	0.04
6.50	99.60	0.40
7.00	98.76	1.24
7.25	97.81	2.19
7.50	96.17	3.83

acids degraded after release into the CDM along with autolytic enzymes. Nuclease content is known to vary between species (Wade and Robinson, 1963), but optimal activity in E. coli occurs in alkaline conditions (Robertson et al., 1968). The large increase in the release of 260nm-absorbing compounds as the pH increases above 7.0 (Table 33) maybe a reflection of this phenomenon.

The experiments were completed to assess the effect of slight differences in the pH of the test media (Table 29), and show how important it is to standardise the test system.

4.5.2 Effect of resuspension

S. typhimurium, during growth, sheds OM particles which contain protein, LPS and PL (Munford et al., 1980). The rate at which OM particles are shed from E. coli and S. typhimurium is greatest during stationary phase. Since PB is known to interact with these components (section 1.3.2) the amount of PB available to interact with the cell will be reduced. The slight increase in the lytic action of PB when $S^+ G^-$ cells are resuspended (Fig 44) is a reflection of the phenomenon. Furthermore, the addition of various culture filtrates to $S^+ G^-$ cells protects against the lytic action of PB (Table 30). The effect of resuspension on the action of PB on $S^- G^+$ and $S^+ G^-$ cells, can most clearly be seen when measured by% survivors (Figs 48 and 49). The culture filtrate from $S^+ G^-$ cells appears to have a more protective effect than from $S^- G^+$ cells (Table 30), although such differences are not apparent when assessed by % survivors (Figs 48 and 49). The method used to prepare the culture filtrate is complex, prolonged (section 2.5.4) and prone to transferance losses; this may explain the apparently contradictory results.

4.5.3 Cell lysis

As discussed in section 1.4.5.5, E. coli when starved of methionine becomes resistant to autolytic cell wall degradation, as a result of chemical alterations in peptidoglycan (Goodell and Tomasz, 1980). The fact the sulphur-depleted cells required the presence of glucose for increased resistance to PB (Fig 41) and that the cells immediately became sensitive on resuspension without glucose (Fig 43), would seem to eliminate this as the primary mechanism of their resistance to PB. Goodell and Tomasz (1980) found resistance developed rapidly after methionine depletion. S^- G^- cells tested here were previously sulphur depleted for at least 4h, during which time alterations in peptidoglycan structure could have taken place.

As discussed in section 4.4.1 glucose may be required to maintain a thermodynamically unfavourable structure for resistance to the lytic action of PB.

4.5.4 Bactericidal action

As discussed in section 1.3.4 PB causes extensive disruption of the CM with concomitant loss of cytoplasmic contents leading to cell death. Increasing concentrations of PB reduce the number of survivors of all cells tested (Fig 46), but there is a higher initial rate of killing in the S^- G^+ cell compared to the S^+ G^- cell (Fig 45). This may reflect differing CM compositions. Plate 1 shows the CM protein profiles of the different cells tested. The large number of proteins present make analysis difficult, though individual proteins do vary in concentration. Poole and Haddock (1975) found in E. coli that sulphur-limitation possibly results in the loss of the iron-sulphur proteins of the electron-transport drain. The rate of killing in

E. coli by PB decreases rapidly with temperature and reflects the liquid-crystalline state of the cell membrane (Hodate and Bito, 1982).

Glucose has no affect on the % survivors of sulphur-depleted cells after PB treatment (Fig 47), suggesting that the lytic and bactericidal effects of PB are separate actions. Klemperer (1976) also found the $S^- G^+$ cell of P. aeruginosa more resistant to the lytic action of PB compared to its bactericidal action, though the % survivors is slightly higher in $S^- G^+$ cells compared to $S^+ G^-$ cells. This can be explained by the lower pH of the $S^- G^+$ test medium (personal communication: Dr R M.M.Klemperer, Department of Pharmacy, University of Aston in Birmingham) and the effect of pH on the bactericidal action of PB (Table 31).

The lecithinbroth used in this study inactivates PB present in the test medium (Kohn et al., 1963), but it is unlikely to inhibit PB once inside the cell. The method used to assess the % survivors after incubation with PB, will detect cells which are capable of replicating but will not detect cells which are viable but incapable of replicating. Teuber (1974) showed that PB inhibited the ability of S. typhimurium to replicate before inhibition of respiration and LPS, murein and protein synthesis.

4.5.5 Release of 260nm-absorbing compounds

As discussed in section 1.3.4 membrane-active agents cause the release of purines and pyrimidines from the cell and autolytic degradation of nucleic acids. The initial release of 260nm-absorbing compounds is higher from sulphur-depleted cells compared to glucose-depleted cells (Figs 50, 51 and 52) and reflects the larger pool content of sulphur-

depleted cells (Table 25).

There is an optimum concentration of PB for both the initial rate of release of 260nm-absorbing compounds and the total amount released. For unresuspended S^+ G^- cells this is 20u ml^{-1} and for sulphur-depleted cells when resuspended 60u ml^{-1} (Figs 53 and 56). Brown and Melling (1969b) also reported that above an optimum concentration of PB there is a decrease in the amount of released 260nm-absorbing compounds. If the decrease is due to inhibition of autolytic enzymes, it would suggest that the sulphur-depleted cells had higher concentrations of autolysins present in the cell, requiring a higher concentration of PB for their inhibition. Alternatively sulphur-depleted cells may have a different autolytic enzymic system. Both of these proposals would be a possible explanation of the release of 260nm-absorbing compounds from sulphur-depleted cells being dependent on the sulphur source (Fig 58).

At high concentrations of PB there is an apparent uptake of 260nm-absorbing compounds by all nutrient-depleted cells tested and it is most noticeable in S^+ G^- cells (Fgis 52, 54 and 55). Salton (1951) found a similar effect with CTAB and suggested that it might be due to the adsorption of 260nm-absorbing compounds present in the medium on to the cell. This would seem to be a possible explanation of the effect seen with PB.

4.5.6 Release of 260nm-absorbing compounds from P. aeruginosa PCM1

The release of 260nm-absorbing compounds from nutrient-depleted PCM1 (Fig 60) is broadly similar to that of the wild-type (Figs 57 and 58). An exception is Met^- G^+ cells. The cause of the difference is unclear,

although it may suggest differences in sulphur metabolism between the wild-type and PCM1.

4.6 Action of gentamicin on nutrient-depleted cells

Nicas and Hancock (1981) correlated resistance to EDTA, PB and gentamicin in *P. aeruginosa* with an increase in the OM protein H1. Gentamicin resistance was therefore investigated. However, there was no overproduction of H1 in any nutrient-depletions (Plate 1) although S^- G^+ cells were more resistant to killing by gentamicin than S^+ G^- cells (Table 34). Glucose has no effect (Table 35).

Angus *et al.*,(1982) compared a wild-type strain of *P. aeruginosa* with a mutant. They correlated hypersusceptibility to gentamicin and PB with an increase in OM permeability. The data was consistent with the idea of a low proportion of open functional pores affecting permeability. Plate 1 however, shows the amount of porin protein (F) is constant for the nutrient depletions, although it does not indicate the number of open pores. Nakae and Nakae (1982) compared aminoglycoside sensitivity in a mutant of *E. coli* producing reduced amounts of porin protein compared with the wild-type. The results indicated that entry through pores was not a rate-limiting step.

Day (1980) on the basis of a comparison of a core-defective mutant of *P. aeruginosa* and the wild-type, proposed that changes in the core region were responsible for differences in binding of gentamicin to LPS, as has been proposed for PB (Vaara *et al.*, 1981). However, the nature of the interaction appeared to be related to the amount of organic phosphate associated with LPS, which as previously stated does not vary between nutrient-depletions (Table 36).

Aminoglycosides, like PB, are cations. Abdel-Sayed *et al.*, (1982) found that magnesium or spermine retarded the diffusion of

dihydrostreptomycin through the OM in P. aeruginosa. Mg^{2+} concentration in this study does not vary significantly between nutrient-depletions (Table 36). Since the synthesis of spermine requires S-adenosylmethionine (Schlenk, 1965) it would be unlikely to be increased in sulphur-depleted cells.

Resistance to gentamicin has been correlated with alterations in the electron-transport chain (Bryan et al., 1980; Bryan and Kwan, 1981). Their studies indicated a selective coupling of gentamicin uptake with terminal electron-transport. Poole et al. (1975) found that sulphate limited E. coli has a drastically altered electron-transport chain; this maybe the cause of the resistance of sulphur-depleted P. aeruginosa.

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