THE EFFECT OF METAL CATIONS UPON CELL WALL CHEMISTRY, DRUG RESISTANCE AND SENSITIVITY TO COLD SHOCK OF *Pseudomonas aeruginosa*.

A thesis submitted by

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SUMMARY

1. The effect of graded concentrations of glucose and magnesium ions upon the growth of cultures of P.aeruginosa has been investigated.
2. Media were formulated that allowed a study of the effect of Mg^{2+}-depletion upon the lysis of P.aeruginosa by E.D.T.A., E.G.T.A. or polymyxin.
3. These media were also used to grow sufficient quantities of bacteria to study the chemistry of whole cells and cell walls.
4. Growth of P.aeruginosa in Mg^{2+}-depleted medium resulted in cultures that were resistant to lysis by E.D.T.A. or polymyxin and that possessed significantly different proportions of lipids (phospholipids) and cations in the cell walls when compared with Mg^{2+}-adequate cultures.
5. Supplementation of Mg^{2+}-depleted media with Ca^{2+} restored the sensitivity to lysis by E.D.T.A. or polymyxin but did not affect the phospholipid pattern of the cell walls.
6. Supplementation of Mg^{2+}-depleted media with Mn^{2+} resulted in only a slight loss of resistance to lysis by E.D.T.A. and polymyxin.
7. All Mg^{2+}-depleted cultures, irrespective of the supplementary cations present, were more sensitive to the action of E.G.T.A. than Mg^{2+}-adequate cultures.
8. Supplementation of Mg^{2+}-adequate media with Ca^{2+} and Mn^{2+} resulted in cultures that were significantly more sensitive to E.D.T.A., but not to polymyxin.
9. Cultures of P.aeruginosa containing the R-factor $RP1^{+}$ were resistant to lysis by carbenicillin and E.D.T.A.

and slightly less sensitive to polymyxin. The chemistry of the cell walls of P.aeruginosa $RP1^+$ was different from those of the parent strain, notably the lipopolysaccharide.

10. The resistance to carbenicillin was correlated with the production of β-lactamase. The resistance of P.aeruginosa $RP1^+$ to E.D.T.A. may be correlated with the retention of Mg^{2+} and Ca^{2+} during removal of readily extractable lipids from the cell walls.

11. Cold shock was less effective against Mg^{2+}-depleted cultures compared with Mg^{2+}-adequate. Cold shock of Mg^{2+}-depleted cultures also eliminated their resistance to E.D.T.A., E.G.T.A. and polymyxin.

12. All cultures of P.aeruginosa $RP1^+$ were significantly more sensitive to cold shock than the parent strain.

13. These results formed the basis for proposals upon the nature of the cell wall of P.aeruginosa and the action of E.D.T.A., E.G.T.A. polymyxin and cold shock upon this organism.

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ABBREVIATIONS.

L	litre/s
ml	millilitre/s
g	gramme/s
mg	milligramme/s
µg	microgramme/s
^{o}	degrees centigrade
cm	centimetre/s
µ	micrometre/s
nm	nanometre/s
$\mathring{A}$	Angstrom/s
hr	hour/s
min	minute/s
N	Normal solution
M	Molar solution
'g'	relative centrifugal force
U/ml	Units per millilitre
$O.D._{470}$	Optical density at 470 nm
O.M.	outer membrane
M.	murein-lipoprotein complex
P.	periplasmic space
C.M.	cytoplasmic membrane
L.P.S.	lipopolysaccharide
P.L.	phospholipid
P.S.	phosphatidyl-serine
P.C.	phosphatidyl-choline
P.G.	phosphatidyl-glycerol
D.P.G.	diphosphatidyl-glycerol
P.E.	phosphatidyl-ethanolamine
lys.P.G.	lysyl-phosphatidyl-glycerol

E.I.F.	ether insoluble fraction
F.A.N.	free fatty acids and neutral lipids
R.E.L.	readily extractable lipids
D.A.P.	diaminopimelic acid
K.D.O.	2,keto,3,deoxyoctonic acid
P.X.	polymyxin B sulphate
E.D.T.A.	ethylenediaminetetra-acetic acid
E.G.T.A.	ethyleneglycol-Bis-(β-aminoethylether)-N:N'-Tetra-acetic acid

ORGIN AND SCOPE OF THE WORK.

It has been found that Pseudomonas aeruginosa grown in chemically defined media (C.D.M.) ultimately depleted of magnesium (Mg^{2+}) and glucose became resistant to lysis by ethylenediaminetetra-acetic acid (E.D.T.A.) and polymyxin (Brown & Melling, 1969a,b). These authors also found that other divalent cations (notably calcium) partially restored sensitivity when they were included in the Mg^{2+}-depleted C.D.M. Boggis (1971) extended this work and studied the effect of a wide range of substitute divalent metal cations upon the sensitivity to lysis of P.aeruginosa by E.D.T.A., ethyleneglycol-Bis-(β-aminoethylether)-N:N'-Tetra-acetic acid (E.G.T.A.) or polymyxin. This author also assayed the cell walls of some of the cultures for certain chemical components.

It was decided to use a C.D.M. developed from that used by Boggis (1971) to grow up cultures of P.aeruginosa in Mg^{2+}-adequate and Mg^{2+}-depleted batches of this media supplemented with calcium or manganese, and to study in greater detail cell walls from these cultures. This media was also used for the preliminary growth studies and resistance studies as well as for preparation of the large batches (6 L) of cells required for cell wall analysis. A small number of growth experiments were performed using the large batch culture (6 L) apparatus to assertain that the predictions based on data from small scale (25 ml) experiments were valid in the 6 L system. In addition, it was decided to compare the cell wall chemistry of a strain of P.aeruginosa

harbouring the R-factor RP1 with the cell wall chemistry of the parent (no R-factor) strain.

During the course of the present work it was fortuitously observed that samples of P.aeruginosa kept in a refrigerator (4° for 60-min) prior to use in lysis experiments were apparently more sensitive to the anti-microbial chemicals than were samples not subjected to a temperature change. Furthermore, it was noted that this effect was not as marked in Mg^{2+}-depleted as in Mg^{2+}-adequate cultures. As a result of these observations it was decided to study a) the effect of Mg^{2+}-depletion upon the sensitivity of P.aeruginosa to cold shock and b) the effect of cold shock upon the sensitivity of this organism to lysis by E.D.T.A., E.G.T.A. or polymyxin.

INTRODUCTION

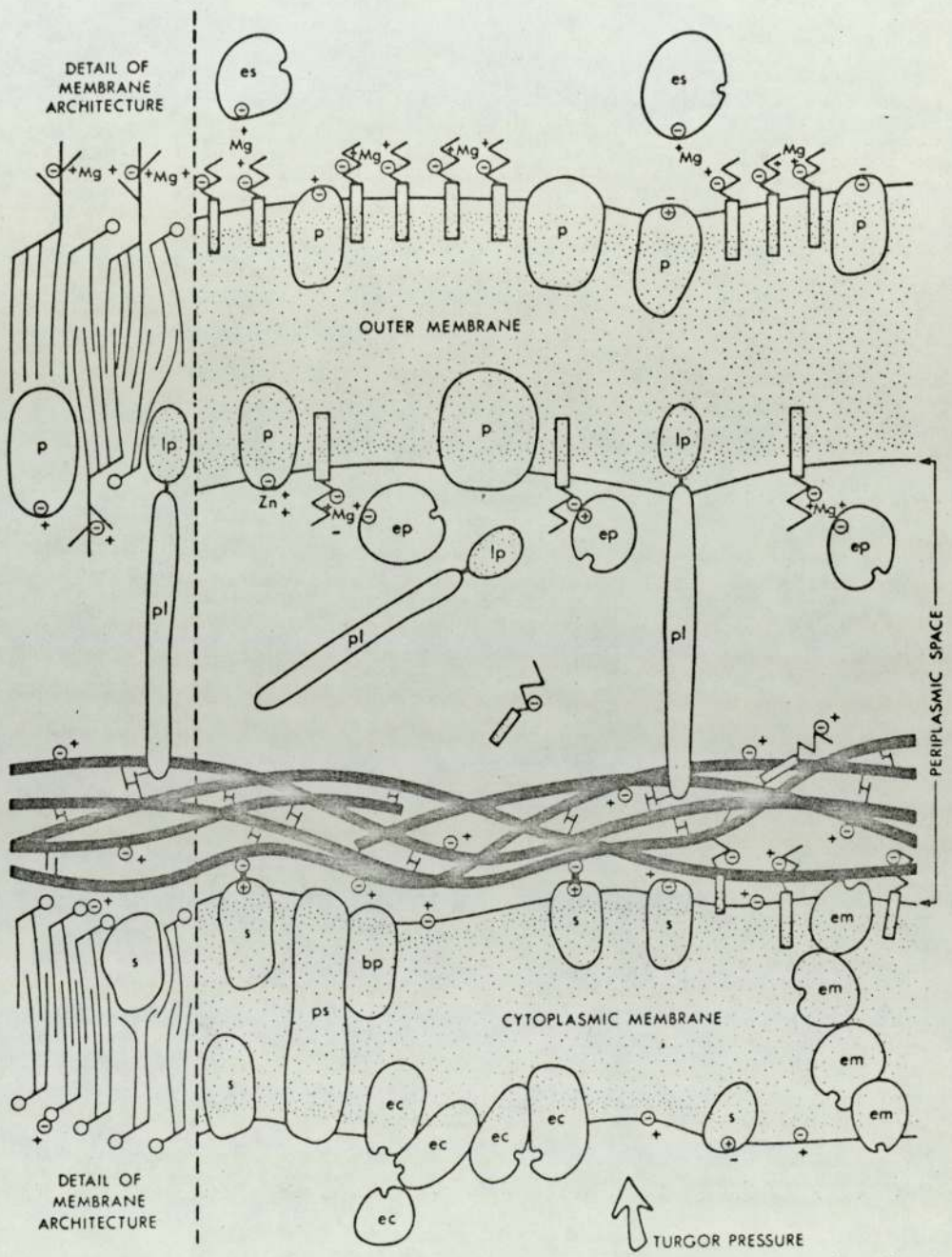
1. THE GRAM-NEGATIVE CELL ENVELOPE.

Introduction.

In recent years there have been several excellent reviews both of the chemistry (Salton, 1964; Martin, 1966; Rogers & Perkins, 1968) and of the structure and function (Glauert & Thornley, 1969; Martin, 1969; Reaveley & Burge, 1972; Braun, 1973; Leive, 1974; Costerton, Ingram & Cheng, 1974) of the Gram-negative cell envelope. Meadow (1974) has reviewed the envelope structure specifically of Pseudomonas aeruginosa and Brown (1975) has reviewed the role played in resistance by the envelope of P.aeruginosa.

The purpose of this section is briefly to describe a useful model of the cell envelope of Gram-negative organisms, and to point out the way in which various parts of the envelope of P.aeruginosa are thought to differ from such a model. The model described by Costerton et al. (1974) (see Fig. 1) will be used as being representative of a 'typical' Gram-negative cell envelope. Reference to Fig. 1 illustrates the four major regions present in the envelope: a) the cytoplasmic membrane (C.M.), b) the rigid murein-lipoprotein complex (M.), c) the periplasmic space (P.) and d) the outer membrane (O.M.). These structures all appear to be present in P.aeruginosa with one or two slight differences in composition, as will be described later in this section. In addition to the four regions just described, some bacteria, including strains of P.aeruginosa, possess a fifth region exterior to the lipopolysaccharide (L.P.S.) of the O.M.. This 'extra' layer usually consists of carbohydrate (slime or capsular material) or protein, and appears to be active

Figure 1. Cell envelope of Gram-negative bacteria (Costerton. et al, 1974).



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

in a protective manner, as for instance the outer coat observed in some rumen bacteria (Cheng & Costerton, 1973) or in aquatic microorganisms (Costerton et al., 1974).

The cytoplasmic membrane.

The C.M. is the interface between the cell contents and the cell wall. It has long been shown to have a 'double track' unit membrane structure similar to the cytoplasmic membranes found in all other cells examined. The chemistry, structure and function of the Gram-negative C.M. is comparable to the corresponding organelle in Gram-positive bacteria (Reaveley & Burge, 1972). The C.M. of P.aeruginosa appears to be characteristically similar to the membranes observed in the other Gram-negative species studied (Meadow, 1974). The synthesis of cell wall precursors and components (L.P.S.: Osborn, Gander, Parisi & Carson, 1972) and the active transport of solutes and substrates (sugar transport: Kundig & Roseman, 1971b) are located at the C.M.. Environmental conditions that affect the structure (and hence the function) of the C.M. would reasonably be expected to produce alterations in the composition of the cell wall with resulting changes in permeability control of the organism. Fiil & Branton (1969) have shown that there are numerous spherical structures arranged in a net-like pattern within the hydrophobic areas of the C.M. of Escherichia coli. This net-like arrangement was replaced by a paracrystalline arrangement when the cells were grown in magnesium-depleted medium in batch culture. It is conceivable that these spherical bodies are the protein-phospholipid (P.L.) complexes involved in transport and

synthesis. Similar spherical bodies and corresponding changes (with magnesium-depletion) have been observed in the C.M. of P.aeruginosa as well as in the O.M. (Gilleland, Stinnett & Eagon, 1974).

The murein-lipoprotein complex.

This corresponds to the 'R' layer reported by Weidel, Frank & Martin (1960). The major component appears to be the murein 'sacculus' which is comprised of polysaccharide chains that consist of alternating molecules of N-acetyl-glucosamine (N.A.G.) and N-acetylmuramic acid (N.A.M.). The polysaccharide chains are cross-linked by pentapeptide side chains on the N.A.M.. Not all the peptide side chains are involved in cross-linking, some act as sites for the covalent bonding of the lipoprotein molecules that anchor the rest of the cell wall to the murein. Braun (1973) has comprehensively described the murein-lipoprotein structure, and proposed a model for the attachment of the lipoprotein molecules. The lipoprotein molecules are covalently bound to the murein via the D-alanine residues of the peptide side chains and project towards the cell exterior where the lipid portion is embedded in, and bound to the O.M. by hydrophobic forces. The M. region seems to act as a point of anchorage for the O.M., and also as a 'containing net' to prevent osmotic rupture of the C.M.. The maintenance of the rigidity and the shape of the organism appears to be the major role of the M. region, but there is some evidence that it may also act as a barrier to some drugs (Burman, Norström & Bloom, 1972).

The murein-lipoprotein complex of P.aeruginosa appears to differ from that of other Gram-negative

organisms. The evidence reviewed by Meadow (1974) suggests that there might be less murein and less lipoprotein present in the cell wall of P.aeruginosa, and Braun, Rehn & Wolff (1970) were unable to find evidence of covalently bound protein in the murein of P.aeruginosa. It has been suggested that the reduced amount of lipoprotein in P.aeruginosa may result in a weaker attachment of the murein to the O.M. (Meadow, 1974). This hypothesis would partly explain the sensitivity of P.aeruginosa to ethylenediaminetetra-acetic acid (E.D.T.A.), since disruption of the O.M. would be facilitated if there was less lipoprotein present binding this structure to the murein layer. When the M. region of P.aeruginosa cell envelope was removed by the action of lysozyme, the rest of the cell wall (i.e. the O.M.) was able to maintain the bacillus shape of the cell (Carson & Eagon, 1966). When the O.M. was disrupted by E.D.T.A. the cells were osmotically fragile and lysed (Asbell & Eagon, 1966). This is compatible with the M. region having a weaker structure in P.aeruginosa compared with other species.

The periplasmic space.

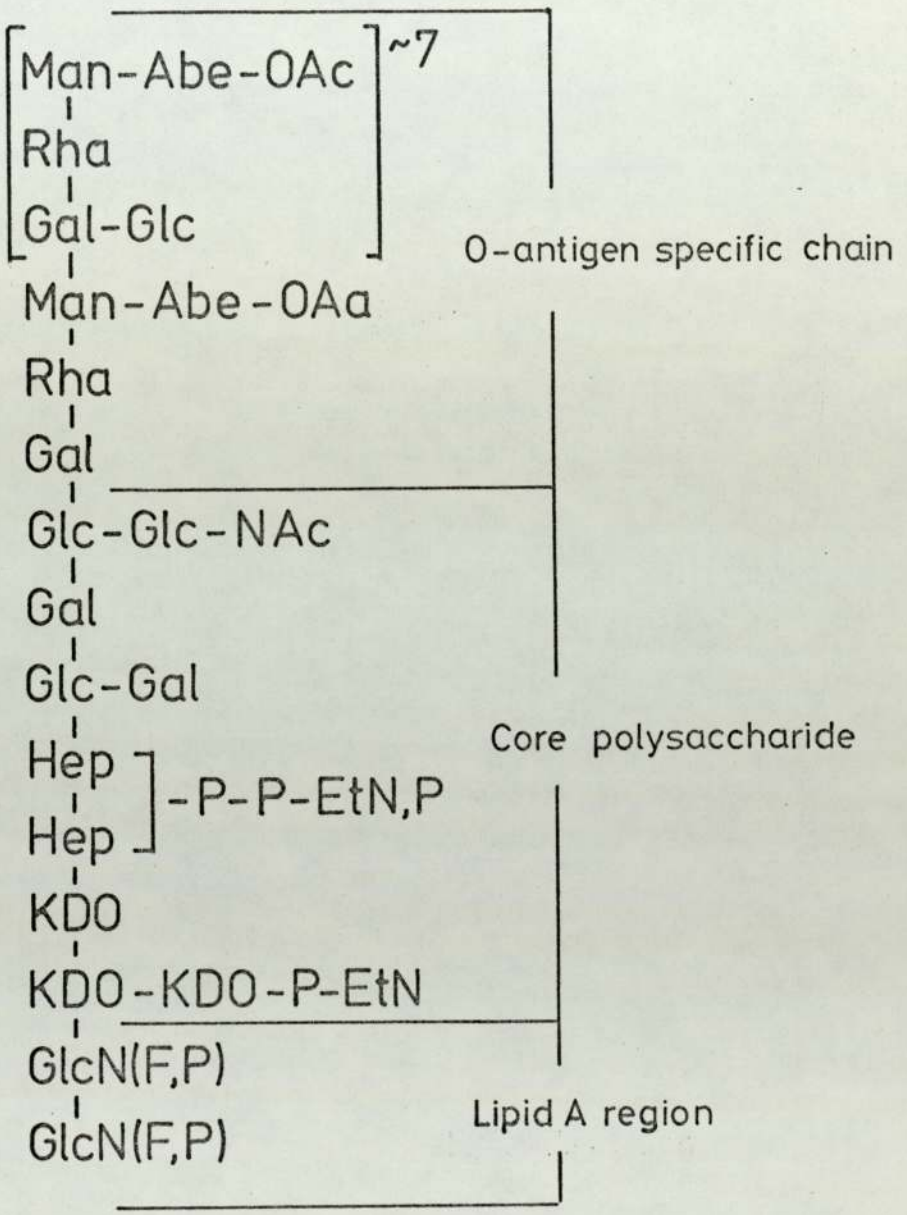
This region of the cell envelope corresponds to the electron transparent area, visible between the O.M. and the murein layer in ultra-thin sections of Gram-negative cell walls and whole cells (Glauert & Thornley, 1969). Certain enzymes (5' nucleotidase, 3' nucleotidase, acid phosphatase, alkaline phosphatase and ribonuclease 1) have been shown to be located in the P. region of E.coli (Cerny & Teuber, 1971) and several other Gram-negative bacteria including P.aeruginosa (Cheng, Ingram &

Costerton, 1970, 1971; Cerny & Teuber, 1972). It seems that in P.aeruginosa, as in other Gram-negative bacteria, penicillin degrading enzymes (β-lactamases) are located periplasmically (Brown, 1975; Richmond, 1975).

The outer membrane.

No structure comparable to the O.M. has been observed in the cell wall of Gram-positive bacteria, and it therefore acts as a major distinguishing factor of the cell walls of Gram-negative organisms. Examination of thin sections by electron microscopy have revealed that the O.M. has a typical 'double track' appearance of a cytoplasmic membrane (Glauert & Thornley, 1969). The O.M., unlike the C.M., contains a significant amount (30% of the cell wall weight) of L.P.S. (P.aeruginosa: Clarke, Gray & Reaveley, 1967a,b,c). The L.P.S. from the Gram-negative bacteria studied has been found to consist of three distinct regions: 1) the O-antigen specific polysaccharide chain, 2) the core polysaccharide and 3) the lipid A region (Fig. 2). Although the L.P.S. of the Gram-negative organisms has been found to follow this general pattern, the composition of these three regions varies between and within species (Wilkinson, Galbraith & Lightfoot, 1973; Meadow, 1974). Most of the L.P.S. appears to be bound to the external surface of the O.M. by hydrophobic bonds (lipid A is usually embedded in the hydrophobic region of the O.M.) and ionic binding (cation mediated cross-linking between the phosphate esters of the core polysaccharide and other negatively charged components of the O.M.). Electron microscope studies on freeze-etched preparations of a limited number of

Figure 2. Structure of lipopolysaccharide (Bader & Teuber, 1973).



Man - Mannose Abe - Abequose

Rha - Rhamnose Gal - Galactose

Glc - Glucose GlcN - Glucosamine

Hep = L-glycero-D-mannose

KDO = 2-keto-3-deoxyoctonic acid

EtN = Ethanolamine

P - esterified phosphate

F - fatty acid

Gram-negative bacteria suggests that the O.M. of P.aeruginosa may characteristically possess spherical subunits in the hydrophobic region of this structure (Costerton et al., 1974; Gilleland et al., 1974). These subunits are apparently a complex of protein and L.P.S. and are removed from the O.M. by the action of E.D.T.A.. When P.aeruginosa is grown in conditions of Magnesium-depletion (batch culture) not only is the amount of protein-L.P.S. complex increased, but it is also resistant to release by E.D.T.A.. Another difference between the O.M. of P.aeruginosa and other Gram-negative organisms is the phosphorus content of the L.P.S.. Wilkinson (1975) has shown that the L.P.S. of P.aeruginosa is much richer in polyphosphate groups and suggests that the stability of the O.M. structure relies upon cation-mediated cross-linking to a greater extent than in other Gram-negative species (Wilkinson & Galbraith, 1975). This hypothesis, coupled with the apparent small extent of murein-lipoprotein complex in P.aeruginosa is compatible with the marked sensitivity of this organism to E.D.T.A..

Slime

Mucoid strains of P.aeruginosa produce slime, mainly composed of polysaccharide, while P.aeruginosa 6750 will form slime if grown in chemically defined medium with gluconate as the carbon source instead of glucose (Brown & Scott-Foster, 1971). These authors found that the slime had a small, initial blocking effect upon the action of E.D.T.A. and polymyxin. Slime from P.aeruginosa has also been shown to antagonise phagocytosis (Schwarzmann & Boring, 1971) and may possibly inhibit other anti-

bacterial agents by acting as a diffusion barrier or drug binding site (Brown, 1975).

2. THE ROLE OF DIVALENT METAL CATIONS IN BACTERIA.

Introduction.

A detailed analysis of whole cell inorganic ion content has shown that magnesium (Mg^{2+}), calcium (Ca^{2+}), manganese (Mn^{2+}), iron (Fe^{2+}) and zinc (Zn^{2+}) were the divalent metal cations present in significant quantities in Gram-negative (Escherichia coli, Sphaerotilus natans) and Gram-positive (Micrococcus lysodeikticus, Bacillus cereus) organisms (Rouf, 1964). This author found that the Gram-positive organisms possessed a higher concentration of Mg^{2+} than Gram-negatives. Since the cells were only washed in distilled water, there is a possibility that cells from both staining types contained Mg^{2+} adsorbed onto the cell surface (Tempest & Strange, 1966). The higher levels of Mg^{2+} found in Gram-positives by Rouf (1964) most likely reflects the correspondingly higher level of teichoic acids found in Gram-positives, and that are thought to bind Mg^{2+} (Hughes, Stow, Hancock & Baddiley, 1971). Webb (1949) has demonstrated that Mg^{2+} is essential for the growth of bacteria in simple salts medium. The absolute requirement for Mg^{2+} is probably due to the specific requirement for ribosomal integrity (E.coli: McCarthy, 1962; Tissières, Watson, Schlessinger & Hollingworth, 1959), ribosomal and ribonucleic acid (RNA) synthesis (E.coli: Cohn & Ennis, 1967), membrane stability (E.coli: Lederberg, 1956), maintenance of permeability (Aerobacter aerogenes: Strange, 1964; E.coli: Leive, 1965b; Pseudomonas aeruginosa: Asbell & Eagon, 1966). The majority of enzymes depend upon the presence of Mg^{2+} for activity (Dixon & Webb, 1964); especially interesting are

those enzymes involved in the synthesis of cell wall components (fatty acids: Knivett & Cullen, 1967; peptidoglycan: Garrett, 1969; lipopolysaccharide: Edstrom & Heath, 1967; phospholipids: White, Albright, Lennarz & Schnaitman, 1971). Magnesium has been suggested as a structural component of the outer membrane of Gram-negative bacteria (Costerton, Ingram & Cheng, 1974), especially in P.aeruginosa (Asbell & Eagon, 1966; Brown & Melling, 1969a,b; Wilkinson & Galbraith, 1975). Calcium has been implicated in the structural organisation of the wall of Rhizobium trifolii (Humphrey & Vincent, 1962), as well as P.aeruginosa (Asbell & Eagon, 1966) and E.coli (Leive, 1968). Another role of the cations Mg^{2+}, Ca^{2+} and Zn^{2+} related to the organisation of the cell surface, is the cation dependent adsorption of bacteriophages to bacteria. The adsorption of T2 and T4 bacteriophages to E.coli was found to require the presence of Zn^{2+} in the cell wall (Kozloff & Lute, 1957). Bacteriophage H-SHPB(Ca) was able to adsorb onto Shigella dysenteriae strains provided that one of the following cations were present in the medium, Mg^{2+}, Ca^{2+}, strontium(Sr^{2+}) and barium (Ba^{2+}) (Beumer, Dirkx & Beumer-Jochmans, 1957), and Mg^{2+} was required for adsorption of bacteriophage to P.aeruginosa (Reese, Dimitiacopoulos & Bartell, 1974).

Divalent cations and ribosomes.

The requirement of Mg^{2+} for the stability of ribosome units has been demonstrated in systems isolated from rabbit reticulocytes (Edelman, Ts'o & Vinograd, 1960). These authors found that if the ribosomes were resuspended in buffer with a low K^+:Mg^{2+} ratio, their sedimentation

coefficients (S) were either 80S or 110S, the 110S units being dimers of the 80S units. If the K^+:Mg^{2+} ratio of the suspending buffer was high, then particles of 58S and 80S were observed. Since a low K^+:Mg^{2+} ratio effectively means a high relative concentration of Mg^{2+}, it appears that the Mg^{2+} stabilizes the aggregated subunits. Similar observations have been made by Tissières et al. (1959) with ribosomes prepared from E.coli, although the concentration of Mg^{2+} required to stabilize 70S particles was 0.005 M as opposed to 0.001 M for the rabbit ribosomes. Increasing the concentration of Mg^{2+} from 0.005 M to 0.01 M in a 70S ribosome preparation from E.coli, resulted in the appearance of 100S ribosomes, and decreasing the Mg^{2+} concentration of the 70S fraction to 0.00025 M caused the 70S ribosomes to disassociate into 30S and 50S fractions (Tissières et al., 1959). The 30S and 50S units could be reaggregated by increasing the Mg^{2+} concentration to 0.01 M; the 70S units so formed were however perfectly stable in 0.005 M Mg^{2+} even though the 30S and 50S units would not reassociate in this concentration. By studying the exchange properties of Mg^{2+} in E.coli ribosomal fractions, Rodgers (1964) found that approximately 35% of the Mg^{2+} bound in the 70S ribosomes, was involved in the binding of the 30S to the 50S ribosomes. The role of Mg^{2+} in maintaining the stability of ribosomes in vivo has been demonstrated by McCarthy (1962) using E.coli grown in chemically defined medium. It was found that if exponentially growing cells were harvested, washed in warm (37°) growth medium lacking Mg^{2+}, resuspended and maintained in this medium for 24-hr at 37°, the ribosome

content fell to 5% of the value for exponential cells. The ribosome content was followed by ultracentrifugation, and it was found that exponential phase E.coli possessed 100S, 70S, 50S and 30S ribosomes. Within 3-hr of resuspension, most of the 100S and some of the 70S ribosomes were lost. At 6-hr depletion only a small peak corresponding to 70S ribosomes was visible, and at 12-hr all that remained was a small peak corresponding to 30S ribosomes; after 24-hr no peaks could be observed at all. These results are compatible with those of Mendelsohn & Tissières (1959) who found that the ribosomes of washed exponential phase E.coli had virtually disappeared after incubation for 18-hr in 0.15 M phosphate or Tris buffer. The molarity of the buffer used by McCarthy (1962) in his media was only 0.066 M, but the total molarity of the salts in the Mg^{2+}-deficient medium was approximately 0.1 M, which compares well with the findings of Mendelsohn & Tissières (1959) that 0.15 M-NaCl in either 0.005 M phosphate or Tris buffer produced the same effect as the 0.15 M buffers. As Mg^{2+}-depletion caused whole cell ribosomes to disintegrate (releasing ultra-violet absorbing material into the menstruum, Mendelsohn & Tissières, 1959), so the addition of Mg^{2+} to 24-hr Mg^{2+}-depleted cells has been observed to initiate RNA synthesis (McCarthy, 1962; Morgan, Rosenkranz, Chan & Rose, 1966), protein synthesis (Cohn & Ennis, 1967) and cell growth, as measured by increase in turbidity at 650nm (McCarthy, 1962). On addition of Mg^{2+}, after a lag of 1-hr, Mg^{2+}-depleted E.coli cells started to synthetise ribosomes at an exponential rate equal to the exponential growth rate of 'normal' cells

in chemically defined medium containing Mg^{2+} (McCarthy, 1962). Ultracentrifugation bands were visible indicating large amounts of 30S and 50S ribosomes 2 - 3-hr after the addition of Mg^{2+}, but it was not until 6-hr that the ribosome content was nearly 'normal'. McCarthy (1962) also noted that although cells started to grow 1-hr after the addition of Mg^{2+}, the growth rate did not become exponential until 6-hr (i.e. when there were sufficient 70S ribosomes present). These results are compatible with the electron microscope observations (Morgan et al., 1966) of thin sections of Mg^{2+}-depleted E.coli (no ribosomes visible) and depleted E.coli 6-hr after the addition of Mg^{2+} (ribosomes visible, but smaller than non-depleted controls). Cohn & Ennis (1967) took 24-hr Mg^{2+}-depleted cells of E.coli, washed them and then suspended them in a simple salts medium containing Mg^{2+} and the nucleic-acid bases adenine (A), guanine (G), cytosine (C) and uracil (U), and followed the synthesis of RNA. Unlike McCarthy (1962) they found a fast initial rate of synthesis which decreased over the first hour to a value 20% - 40% of the initial rate. RNA synthesis proceded at this decreased rate for the next 3-hr, and then increased to the maximum initial rate. The observed drop in RNA synthesis could be avoided if a mixture of amino acids were included in the incubation mixture. Both McCarthy (1962) and Cohn & Ennis (1967) however found that the rate of protein synthesis was proportional to the ribosome content of the cells.

Webb (1968) has found that if E.coli was grown in a chemically defined medium deficient in Mg^{2+}, but

supplemented with Mn^{2+}, the cells and the ribosomes would incorporate the Mn^{2+}. Other workers using E.coli 30S and 50S ribosomes in vitro, have found that Mn^{2+} can replace 99% of the bound Mg^{2+} without any loss of synthetic ability, no change in sedimentation coefficients or ribonuclease sensitivity (Weiss, Kimes & Morris, 1973). However, when these authors tried this in vitro substitution of Mg^{2+} by Ca^{2+}, Sr^{2+} or Ba^{2+} some differences were observed. When Ca^{2+} was substituted in the 30S ribosomes the results were identical to those from Mn^{2+} treated ribosomes, but with the 50S ribosomes, Ca^{2+} substitution resulted in a 70% reduction in synthetic ability, a 2-fold increase in sensitivity to Ribonuclease 1 (RNase 1) and a drop from 50S to 48S. When Sr^{2+} or Ba^{2+} were substituted into the 30S ribosomes, the parameters observed (i.e. synthetic ability, sensititity to RNase 1 and sedimentation coefficient) were unaltered until the Mg^{2+}:RNA-protein (RNA-P) ratio was 0.09 and 0.01 respectively. When the Mg^{2+} of the 30S ribosomes was completely replaced by Sr^{2+} or Ba^{2+} the synthetic ability was decreased by 80% and 100% respectively, and the respective changes in sedimentation coefficients were from 30S to 27.5S and 30S to 28.5S. The 30S ribosomes inactivated by Sr^{2+} or Ba^{2+} could be reactivated after dialysis against Mg^{2+} under the conditions (dialysis followed by heating to 40° for 25-min) described by Weiss & Morris (1973). When Weiss et al. (1973) substituted Sr^{2+} or Ba^{2+} for Mg^{2+} in the 50S ribosomes, both cations produced the same effects. The parameters of ribosomal structure and function were not altered until the Mg^{2+}:RNA-P

ratio was lowered to approximately 0.075. The ribosomes were completely inactivated by the time either cation had lowered the Mg^{2+}:RNA-P ratio to 0.025. Concomitant with the inactivation of synthetic ability there was a 6 - 8-fold increase in RNase 1 sensitivity and the ribosomes disintegrated into particles with approximate sedimentation coefficients of 40S (major component), 30S and 20S. Synthetic activity was not restored by dialysis of substituted ribosomes against Mg^{2+}, and disc-gel electrophoresis studies revealed a loss of protein. Since the inactivation of 50S ribosomes by Sr^{2+} and Ba^{2+} resembles that produced by spermidine, a trivalent, organic cation, which caused the loss of protein from the substituted ribosomes after inactivation (Kimes & Morris, 1973), it was thought that the protein from Sr^{2+} and Ba^{2+} substituted ribosomes may have been lost after, and not during inactivation (Weiss et al., 1973).

When the growth rate of A.aerogenes in a chemostat was limited by the supply of Mg^{2+}, the bacterial concentration varied with the growth rate (Tempest, Hunter & Sykes, 1965). As the growth rate of the bacteria was increased, so the cellular concentration of both Mg^{2+} and RNA increased, whereas the RNA, expressed in g/ml of culture remained the same for all growth rates. These results indicate that the ribosome content of the bacteria may be directly governed by the amount of Mg^{2+} available in the cells; a hypothesis that is compatible with the observation that in Mg^{2+}-limited A.aerogenes the ratio of RNA to Mg^{2+} remains constant at several growth rates under conditions of Mg^{2+}-limitation (Tempest & Strange, 1966).

The Mg^{2+} content of Mg^{2+}-limited A.aerogenes cells was found to be half the value of carbon-limited cells, and washed suspensions of these cells increased in RNA content by 30% and 100% respectively when incubated in medium lacking Mg^{2+} (Tempest et al., 1965). The 50S ribosomes from Pseudomonas putida grown in a chemostat and limited by Mg^{2+}, were found to have a protein:RNA ratio of 0.45, whereas 50S units from carbon-limited cell grown at the same rate, had a protein:RNA ratio of 0.95 (Sykes & Tempest, 1965). The 50S units from the Mg^{2+}-limited culture however, disintegrated in vitro when suspended in Mg^{2+} concentrations that had no such effect on the carbon-limited ribosomes. The physiological conditions produced by Mg^{2+}-depletion in a batch culture are different from those produced by Mg^{2+}-limitation in a chemostat. However, both conditions result in the disintegration of ribosomes or the production of relatively unstable ribosomes (Mg^{2+}-depletion: McCarthy, 1962; Lusk, Williams & Kennedy, 1968; Webb, 1970a; Mg^{2+}-limitation: Sykes & Tempest, 1965). The results of in vivo and in vitro experiments are all compatible with the requirement for Mg^{2+}, Ca^{2+} or Mn^{2+} (Weiss et al., 1973) for the synthesis of ribosomes and their structural and functional integrity.

Divalent cations and the cell envelope.

The divalent cations in the envelope of bacteria are associated with maintaining the stability of the cytoplasmic membrane (Lederberg, 1956), maintenance of structural conformation and activity of membrane bound enzymes (Kundig & Roseman, 1971b; Endo & Rothfield, 1969) and as structural components providing cross-linkages

between the lipopolysaccharide component (Wilkinson & Galbraith, 1975) and the rest of the cell wall (Asbell & Eagon, 1966), probably involving the phospholipids in the outer membrane (Yu & Jordan, 1971; Costerton et al., 1974) or to the murein layer (Vincent & Humphrey, 1963).

Lederberg (1956) has shown that spheroplasts can be prepared from E.coli by incubating the log phase cells in the presence of sucrose (20%), penicillin (10^3 U/ml) and $MgSO_4$ (0.2%). If the Mg^{2+} was not included in this medium the spheroplasts formed tended to lyse, and the reversion to rods when the spheroplasts were removed from the presence of penicillin, dropped by about 66%. These results indicate that Mg^{2+} was required for cytoplasmic membrane stability. The lethal effects of cold shock are thought to be due largely to the loss of permeability control of the shocked bacteria (see section on Cold Shock). Strange (1964) found that permeability control of cold shocked A.aerogenes could be restored by the addition of Mg^{2+}, and the lethal effect of cold shock has been reduced by the inclusion of Mg^{2+}, Ca^{2+} or Mn^{2+} in the cold shock medium (Gorrill & McNeil, 1960; Sato & Takahashi, 1968b). Periplasmic enzymes have been released from E.coli by treating the cells with ethylenediaminetetra-acetic acid (EDTA) prior to cold shocking them in a hypotonic solution of Mg^{2+} (Heppel, 1967). This treatment, ('cold osmotic shock') did not have a lethal effect on P.aeruginosa (Patching & Rose, 1971), and it may be presumed that the Mg^{2+} ions protected or maintained the integrity of the cytoplasmic membrane. Since enzymes are removed from the periplasmic region it must be

assumed that the Mg^{2+} was unable to protect the permeability of the outer membrane, or rather, repair the damage done to this structure by the E.D.T.A.. The latter assumption is compatible with the observations of Leive (1968) that Mg^{2+} did not immediately restore the permeability of E.D.T.A.-treated cells of E.coli, although permeability was restored after approximately 45-min. From these results, Leive (1968) concluded that the Mg^{2+} removed by E.D.T.A. could only be replaced by a process involving energy, since deprivation of glucose or the presence of 2,4-dinitrophenol inhibited repair. In earlier experiments, Leive (1965c) found that E.D.T.A. released almost half of the lipopolysaccharide (L.P.S.) layer of E.coli. Costerton et al. (1974) have proposed a model of the Gram-negative cell envelope in which the L.P.S. components bound to the outer membrane was stabilized via cationic (Mg^{2+}) bridges. The time and energy requirement for the repair of E.D.T.A. damaged outer membrane observed by Leive (1968), could be explained by assuming that the damaged cells would have to a) synthesise L.P.S. to replace that lost during E.D.T.A. treatment, b) bind and stabilize the newly synthesised L.P.S. layer via cationic bridges to the outer membrane. Step a) would require an energy source and the presence of Mg^{2+}, while step b) would require Mg^{2+}. This theory is compatible with the observations that 1) Mg^{2+} was required by enzymes responsible for synthesising Salmonella typhimurium L.P.S. (Endo & Rothfield, 1969; Müller, Hinckley & Rothfield, 1972), 2) in S.typhimurium, L.P.S. has been shown to be synthesised at the cytoplasmic membrane (Osborn, Gander, Parisi & Carson, 1972), and

subsequently translocated to a small number (approximately 200/cell) of locations on the cell surface (Mülhradt, Menzel, Golecki & Speth, 1973), from which the L.P.S. was involved in a lateral movement which distributed it over the cell surface. The synthesis of L.P.S. observed by Mülhradt et al. (1973) was very rapid, the whole cell being covered within 2-min, whereas the delay in repair of the outer membrane of E.coli took approximately 45-min (Leive, 1968). This decrepancy may be due to a species difference and the difference in experimental conditions. Mülhradt et al. (1973) used a mutant of S.typhimurium (strain 1195) unable to synthesis complete wild type L.P.S. unless exogenous galactose was provided; synthesis was initiated by addition of galactose. In this system, the cell wall of the organism was not exposed to damaging conditions. The E.coli used by Leive (1968) was harvested and washed twice in 0.12 M Tris-HCl buffer at pH 8, before resuspension in the same buffer. The E.D.T.A. was added, and after 2-min vigorous shaking, the cells were diluted 10-fold into fresh growth medium. It took approximately 45-min before impermeability to actinomycin D was fully restored, presumably because damage to the outer membrane region of the cell walls had to be repaired in addition to the synthesis and incorporation of L.P.S.. The permeability of E.D.T.A.-treated E.coli to actinomycin D was not accompanied by any change in the active transport of carbohydrates or amino acids (Leive, 1968), indicating that the cytoplasmic membrane was not damaged. It is reasonable to assume that the removal of Mg^{2+} by E.D.T.A. would cause disruption or disorganisation of the outer

membrane and loss of L.P.S., allowing actinomycin D access to the cytoplasmic membrane, and thence into the cell. This theory is compatible with the observations of Sanderson, MacAllister, Costerton & Cheng (1974) that mutant strains of S.typhimurium possessing defective L.P.S. were more susceptible to actinomycin D (and other drugs attacking sites intracellular to the outer membrane) than were strains with wild type L.P.S.. E.D.T.A. has been shown to increase the sensitivity of P.aeruginosa resistant to quarternary ammonium compounds (MacGregor & Ellicker, 1958), although the results were slightly obscured by the sensitivity of the organism to E.D.T.A. alone. Repaske (1958) found that Azotobacter vinelandii, E.coli and P.aeruginosa could be made more sensitive to the action of lysozyme by pretreatment with E.D.T.A. or a cation exchange resin (Dowex 50 H^+), and suggested that metal cations present in the wall of untreated cells were responsible for preventing lysozyme action by steric hindrance. Hamilton-Miller (1965) found that the activity of penicillinase in A.aerogenes and Aerobacter ozoenae increased if the cells were studied in the presence of E.D.T.A.. Since cell-free preparations of the enzyme were not activated by E.D.T.A., he concluded that the E.D.T.A. altered the permeability of the cells to benzyl-penicillin, thereby effectively increasing the substrate concentration of the penicillinase. In later experiments (Hamilton-Miller, 1966), it was found that the change in permeability could be inhibited by Mg^{2+} or Ca^{2+}, and that Mg^{2+} or Ca^{2+} salts of E.D.T.A. had no effect, whereas the di-valent sodium salt was active. When P.aeruginosa is

treated with E.D.T.A. the cells lyse, an effect that has been observed by several workers (MacGregor & Ellicker, 1958; Brown & Richards, 1965; Eagon & Carson, 1965; Wilkinson, 1967; Brown & Melling, 1969b; Roberts, Gray & Wilkinson, 1970). Asbell & Eagon (1966) found that washed, stationary phase P.aeruginosa from a glucose salt medium containing yeast extract did not lyse when incubated with E.D.T.A. in 33 mM Tris-HCl buffer, pH 8, and 0.55 M sucrose. The cells retained their rod shape, but were found to lyse when washed and suspended in water. Osmotic stability could be restored to these 'osmoplasts' on addition of most divalent and trivalent metal cations. Only those cations normally present in the cell wall (Ca^{2+}, Mg^{2+} and Zn^{2+}) however permitted the restored cells to multiply. Osmoplasts to which monovalent cations were added were not restored to osmotic stability or viability. Treatment of cell walls of P.aeruginosa with E.D.T.A. was found to release Mg^{2+}, Ca^{2+} and Zn^{2+} (Eagon & Carson, 1965), and these cations have been found in 'ashed' cell walls by Eagon, Simmonds & Carson (1965). In addition to Mg^{2+}, Ca^{2+} and Zn^{2+}, P.aeruginosa cell walls have also been shown to contain significant amounts of K^{+}, Fe^{3+} and Na^{+}, and trace amounts of lead, cobalt, chromium, Mn^{2+} and Sr^{2+} (Eagon, 1969). Analysis of compounds released from P.aeruginosa by E.D.T.A. have shown that a protein-L.P.S. complex was released (Rogers, Gilleland & Eagon, 1969). When P.aeruginosa was observed by freeze-etching and electron microscopy it was found that the middle layer of the outer membrane region was packed with spherical units and rodlets, but E.D.T.A. treated cells (osmoplasts) contained considerably

less spherical units and rodlets in this region (Gilleland, Stinnett, Roth & Eagon, 1973). Electronmicroscopical examination of the material released by E.D.T.A. revealed that it was composed mainly of spherical units and rodlets of the same dimensions observed in the cell wall (Gilleland et al., 1973),and was reaggregated into the middle layer of the outer membrane by Mg^{2+} (which also restored osmotic stability). When P.aeruginosa, grown in a Mg^{2+}-depleted medium, was observed using similar techniques (Gilleland, Stinnett & Eagon, 1974), it was found that osmoplast cell walls did not differ in appearance from untreated Mg^{2+}-limited cells (i.e. no loss of spherical units was observed). The differences between untreated cells and osmoplasts of control cells (grown in Mg^{2+}-adequate medium) was observed to be similar to previous observations (Gilleland et al., 1973).

Humphrey & Vincent (1962) observed that cells of R.trifolii grown under conditions of Ca^{2+}-depletion, possessed weak cell walls and that Mg^{2+} would not replace Ca^{2+}. As a result of these and further studies (Vincent & Humphrey, 1963) the authors suggested that the Ca^{2+} was involved in the packing of the murein layer or in cross linking between the murein and L.P.S. layers. This theory is compatible with the results of studies on the electrophoretic mobility of Ca^{2+}-limited and non-limited cells (Humphrey, Marshall & Vincent, 1968). No change was observed between mobility of non-limited cells and those Ca^{2+}-limited cells in which Sr^{2+} had replaced Ca^{2+}, suggesting that the cations were involved at a site deep within the wall. This site may well be at the core

polysaccharide region of the L.P.S. in P.aeruginosa. The L.P.S. in most strains of P.aeruginosa has been found to be rich in phosphorous (Meadow, 1974; Wilkinson, 1975), mainly as the orthophosphate (85%), the rest being pyrophosphate and ethanolamine mono-, di- and triphosphates (Drewry, Gray & Wilkinson, 1971, 1972). The high phosphate ester content of the core polysaccharide confers a high potential for Mg^{2+} and/or Ca^{2+} binding by L.P.S. in P.aeruginosa, and it has been suggested that the outer membrane of this organism depends upon cationic cross linking between L.P.S. molecules and the other components for stability to a greater extent than in other Gram-negative species (Wilkinson & Galbraith, 1975).

The divalent cations Mg^{2+} and Ca^{2+} have been found to function as cofactors for specific enzymes involved in the synthesis of cell wall components and precursors. Knivett & Cullen (1965) observed that if the Mg^{2+} concentration of the chemically defined medium they were using to grow E.coli was halved (to 1 mM), then the concentration of unsaturated C_{16} and C_{18} fatty acids increased with a corresponding decrease in the level of C_{17} and C_{19} saturated cyclopropane fatty acids. If the Mg^{2+} concentration was decreased further (to less than 0.06 mM) the level of C_{16} unsaturated, cyclopropane fatty acids did not increase when the cells entered the stationary phase, although such an increase was observed in control (non-depleted) cells (Knivett & Cullen, 1967). Since the changes in fatty acid content occurred in the stationary phase, it would appear that they were due to the Mg^{2+} concentration and not the growth rate. It was observed

that Mg^{2+} stimulated the *in vitro* synthesis of phosphatidyl-glycerol (P.G.) but not phosphatidyl-ethanolamine (P.E.) or diphosphatidyl-glycerol (D.P.G.) by cell free extracts of *E.coli* (Benns & Proulx, 1974). By studying the increased incorporation of labelled ^{14}C glycero-3-phosphate into P.G. these authors found that most of the label was incorporated into the unacylated glycerol moiety, with little incorporation into the phosphatidyl residue. The activity of phospholipase 'C' from *E.coli* however, depends upon the presence of Ca^{2+}, although Mg^{2+} can replace Ca^{2+} to give a much lower activity (Proulx & Fung, 1969). It has been observed that the phospholipases are usually to be found in the outer membrane of *S.typhimurium* (Osborn *et al.*, 1972) and *E.coli* (Bell, Mavis, Osborn & Vagelos, 1971), whereas the phospholipid synthesising enzymes are found in the cytoplasmic membrane (*E.coli*: White *et al.*, 1971; Bell *et al.*, 1971; *S.typhimurium*: Bell *et al.*, 1971; Osborn *et al.*, 1972). These authors have found that Mg^{2+} was the most potent activator of the synthetase, whereas Ca^{2+} was the most potent cofactor for the lipase. These observations are compatible with the general observations that in most cellular systems (animal and bacteria) the distribution of Mg^{2+} and Ca^{2+} are such that most of the Mg^{2+} is intracellular, whilst most of the Ca^{2+} is extra-cellular, or on the cell surface, and is more likely to act in a structural role than Mg^{2+} (Williams & Wacker, 1967). Both Mg^{2+} and Ca^{2+} have been found to be involved in a multienzyme phosphotransferase sugar transport system isolated from *E.coli* (Kundig & Roseman, 1971a). The

isolated enzyme system consisted of three protein components, termed Enzyme 1, Enzyme 2 and a Histidine-protein (H-Pr.). It was found that Enzyme 1 catalysed the phosphorylation of H-Pr. and required Mg^{2+} for activity, whilst being inhibited by the presence of Ca^{2+}. Enzyme 1 and H-Pr. were found to be in the soluble enzyme fraction of E.coli cell homogenate, whilst Enzyme 2, which catalysed the transfer of phosphate from H-Pr. to the sugar, was located in the membrane fraction. When the membrane-bound Enzyme 2 was studied in more detail, it was found to be composed of two protein subunits and one lipid component (Kundig & Roseman, 1971b). The individual components were reaggregated most effectively by Ca^{2+} but Mg^{2+} was still required for enzyme activity. These workers proposed that Ca^{2+} was required to ensure that the protein and lipid components of Enzyme 2 were maintained in the correct configuration within or on the cytoplasmic membrane. The enzymes responsible for the synthesis of L.P.S. in S.typhimurium have also been shown to be located at the cytoplasmic membrane (Müller et al., 1972; Endo & Rothfield, 1969), and similar enzymes in E.coli have been shown to have a requirement for Mg^{2+} (Ghalambor & Heath, 1966; Edstrom & Heath, 1967). Similarly, the enzymes responsible for the synthesis of murein precursors have been isolated from cell cytoplasm (Rogers, 1970) and cell envelopes (Rogers, 1970; Garrett, 1969). These enzymes have been shown to require Mg^{2+} in preference to Ca^{2+} or Mn^{2+} for activity (Ito & Strominger, 1964). The addition of D-alanyl-D-alanine during the synthesis of the peptide in the uridine nucleotide murein precusor in

Staphylococcus aureus however was enhanced more by Mn^{2+} at low D-alanyl-D-alanine concentrations (Ito & Strominger, 1962). In vivo studies with Bacillus subtilis by Garrett (1968) revealed that under conditions of Mg^{2+}-limitation the N-acetyl hexosamine pool in the cells increased. If Mg^{2+} was added, the pool diminished, and it was assumed that envelope enzymes responsible for the final stages of murein synthesis were inactive due to the lack of Mg^{2+}. The lack of Mg^{2+} may, however, have caused a structural change in the envelope, preventing transfer of the precursors to the murein site.

A role of divalent cations that also involves the structure of the cell wall is the adsorption and action of bacteriophages on the bacterial surface. Working with E.coli whole cells and cell walls, Kozloff & Lute (1957) have found that Zn^{2+} was required by T2 and T4 phages before lysis of cell wall material could be initiated. These workers observed that each cell wall of E.coli contained approximately 31,000 atoms of Zn^{2+}/cell and that cell walls washed with trichloroacetic acid contained no significant amount of Zn^{2+}. It was found that only the non-acid washed cell walls were lysed by T2 and T4 phages, although both cell wall samples allowed the adsorption of phage. It was suggested by Kozloff & Lute (1957) that the Zn^{2+} in the cell walls was responsible for exposing lytic enzymes in the phage tail by altering the configuration of the tail protein coat. It was observed that if this protein coat was altered in vitro by a cadmium/cyanide complex then the 'altered' phage lysed both non-acid washed and acid washed

cell walls. None of the cations Mg^{2+}, Ca^{2+}, Mn^{2+}, Fe^{2+}, molybdenum, cadmium or mercury would replace Zn^{2+} in sensitizing the cells or cell walls to the phages T2 and T4. In another study using E.coli and a different phage (R17), Paranchych (1966) found that although Mg^{2+} was not required for the adsorption of R17, this cation was essential for the invasion of the viral RNA. Furthermore, Mg^{2+} had to be present before phage adsorption, since the addition of the cation after adsorption did not lead to the invasion of the viral genetic material. It is interesting, and in contrast to Kozloff & Lute (1957), that Zn^{2+} (as well as Co^{2+} and Mn^{2+}) inhibited the invasion by R17. Presumably this discrepancy is due either to the difference in Zn^{2+} concentration or a different mode of invasion by the phage. Adsorption of phage 2 upon a slime producing strain of P.aeruginosa B1 was found to rely upon the presence of Na^{+}, Mg^{2+} or Ca^{2+} (Reese et al. (1974). These authors found that if the slime did not include a substrate specific for the depolymerase in the phage tail adsorption did not occur, regardless of the cations present. The pH spectrum of phage adsorption and depolymerase activity had a maxima at pH 7.5. The order of effectiveness of the cations was Na^{+} 89%, Mg^{2+} 80% and Ca^{2+} 69%. The role of the cations as enzyme activators can reasonably be dismissed since the action of Na^{+} is not compatible.

In summary, the roles of divalent metal cations are intimately linked with all aspects of the bacterial cell, either indirectly as an enzyme cofactor, or directly as a structural component of the cell envelope. Magnesium

emerges as the most important cation, long recognized as being essential for many enzyme functions and the structure and function of ribosomes, and intimately involved in the structure of P.aeruginosa cell walls. Consequently, depletion of Mg^{2+} supplied to the cell would be expected to produce gross changes in the structure and function of the cell.

3. EFFECTS UPON BACTERIA OF GROWTH LIMITATION DUE TO LACK OF MAGNESIUM IONS.

Introduction and definition of terms.

When bacteria are grown in chemically defined simple salts medium (C.D.M.) it has been found that magnesium (Mg^{2+}) is essential for growth (Webb, 1949). The effects on bacteria of growth limiting concentrations of Mg^{2+} in the medium vary between batch and continuous culture. The relationship between the growth rate of the culture of bacteria and the concentration of the limiting nutrient follows that of a rectangular hyperbola, and has been described by Monod (1949) in the following equation:

$$\mu = \mu_{max} \cdot \frac{C}{C_1 + C} \qquad 1$$

where μ is the growth rate at the nutrient concentration C, μ_{max} is the maximum growth rate and C_1 is the concentration of nutrient at which $\mu = \frac{1}{2}\mu_{max}$. Examination of equation 1 shows that if C_1 is relatively insignificant compared to C, then μ will closely approximate to μ_{max} and therefore a range of high concentrations of the 'limiting' nutrient will not alter the growth rate by a measurable amount. As the concentration of C falls however, and approaches near to C_1 so the growth rate will decrease in a hyperbolic manner (as described by equation 1). Thus, in batch culture conditions the cells will grow at a rate approximating to μ_{max}, until the value of C for the limiting nutrient is such that C_1 is no longer insignificant. Then the growth rate will decrease and continue decreasing until the nutrient is depleted and growth ceases. This 'de-acclerating' growth phase

(Monod, 1949) may be considered as a series of infinitely short exponential phases in which the value of C in each phase is less than that for the proceding short phase. The de-acclerating growth phase is relatively long for batch cultures limited by Mg^{2+} (McCarthy, 1962; Lusk, Williams & Kennedy, 1968; Gilleland, Stinnett & Eagon, 1974) although it is very short for a sole carbon and energy source such as glycerol (Herbert, 1961, Fig. 2b). In this thesis cells harvested from, or maintained in a medium in which growth has ceased due to the lack of a known, predetermined nutrient (or nutrients) will be termed 'nutrient-depleted cells'. For example, bacteria harvested from Mg^{2+}-lacking medium, or that are maintained in such a medium will be termed 'Mg^{2+}-depleted cells'.

The exponential growth rate can be maintained at any specific value between nearly μ_{max} and zero, provided that the limiting nutrient concentration can be held at the appropriate value as determined by equation 1 (Herbert, Elsworth & Telling, 1956). In conditions of continuous culture in a chemostat this situation is achieved by varying the rate at which the limiting nutrient is supplied to the growth vessel, taking into account the amount of nutrient that is consumed for energy, the amount actually used to increase bacterial mass and the rate at which the bacterial culture and any unused nutrient leaves the growth vessel. Under the conditions imposed in a chemostat, the growth rate is determined by the rate at which the limiting nutrient is supplied to the growth vessel. The cells in a chemostat culture vessel are still growing at an exponential rate but are not subjected to a complete

lack of the limiting nutrient. In this thesis, cells maintained in, or harvested from such conditions will be termed 'nutrient-limited cells', for example, 'Mg^{2+}-limited cells'. The conditions imposed by culturing bacteria in batch culture using a medium ultimately deficient in a specific nutrient will be termed 'nutrient-depletion', and similarly, the conditions imposed in a chemostat will be termed 'nutrient-limitation'.

The effect of Mg^{2+}-depletion and Mg^{2+}-limitation upon ribosomal structure and funtion was described in a previous section on the role of cations in bacteria. Lack of Mg^{2+} also affects the size of bacterial cells (Aerobacter aerogenes: Tempest & Ellwood, 1969), the permeability of the cell envelope (Escherichia coli: Brock, 1962; Pseudomonas putida: Sykes & Tempest, 1965), the rate of growth (E.coli: Lusk et al., 1968), the chemistry of the cell wall (Bacillus subtilis: Ellwood, Turner, Hunter & Moody, 1969; A.aerogenes: Tempest & Ellwood, 1969), phospholipid content (E.coli: Günther, Richter & Schmalbeck, 1975), the morphology of the cell envelope (E.coli: Morgan, Rosenkraz, Chan & Rose, 1966; Fiil & Branton, 1969; Pseudomonas aeruginosa: Gilleland et al., 1974), and the sensitivity to some antibacterial chemicals (ethylenediaminetetra-acetic acid: Brown & Melling, 1969a; Gilleland et al., 1974; polymyxin: Brown & Melling, 1969b, Brown, 1971, 1975).

Microscopical examination of bacterial cells from the exponential phase and stationary phase of batch culture, reveals that the former are larger than the latter. This difference in size can, in principle, be due either

to the difference in growth rate or the different physiological state of the cells. Although the physiological effect of growth rate cannot be discounted, growth rate is the more likely factor since Tempest & Ellwood (1969) have observed that the size of Mg^{2+}-limited A.aerogenes cells grown in a chemostat increased with increases in the growth rate. The increase in cell size noticed by these authors was accompanied by a decrease in the cell wall content of the cells (expressed as a percentage of whole cells) which would be expected due to the inverse relationship between the surface area of the cell and the cell volume. As the degree of Mg^{2+}-limitation was increased (i.e. by lowering the dilution rate) so cells of A.aerogenes synthesised cell walls with less total carbohydrate (Tempest & Ellwood, 1969), but with more lipopolysaccharide (L.P.S.) (as expressed by measurement of 2-keto,3-deoxyoctonic acid and heptose). The nature of the L.P.S. also changed with increasing Mg^{2+}-limitation since the ratio of heptose to 2-keto,3-deoxyoctonic acid (K.D.O.) decreased from 9.1 to 6.5. Ellwood et al. (1969) found that the cell walls of B.subtilis limited by Mg^{2+} in a chemostat possessed cell walls that contained 40% protein, 12% carbohydrate and 30% lipid, whereas mid-exponential phase cells from a batch culture in non-limiting medium, produced cell walls containing 20% protein, 23% carbohydrate and 5% lipid. Meers & Tempest (1970) observed that washed Mg^{2+}-limited cells of B.subtilis var. niger and A.aerogenes adsorbed Mg^{2+} to a greater degree than similarly treated phosphate-limited cells when suspended in 0.017% NaCl containing various concentrations

of $MgCl_2$. The amount of Mg^{2+} adsorbed was greater for cells of B.subtilis than for A.aerogenes and is probably due to the teichoic acids in the Gram-positive cell walls. The affinity of the B.subtilis cell walls for Mg^{2+} was lower than for the A.aerogenes cell walls, but was proportional to the amount of teichoic acid present. The chemistry of the cell envelopes from Mg^{2+}-depleted P.aeruginosa was determined by Gilleland et al. (1974) who found that the envelopes contained more carbohydrate, K.D.O. and protein, but less phosphorus than the cell envelopes from Mg^{2+}-sufficient cells. These authors also found that Mg^{2+}-depletion resulted in many more spherical units being packed into the middle layer of the outer membrane of the cell walls, and the presence of these extra units may explain the increase in carbohydrate, K.D.O. and protein since these units are presumed to be a protein-L.P.S. complex (Rogers, Gilleland & Eagon, 1969). Tempest & Strange (1966) found that the Mg^{2+} content of Mg^{2+}-limited A.aerogenes cells was 0.10% at a dilution rate of 0.1-hr^{-1}, whereas at a much higher dilution rate (0.8-hr^{-1}) the Mg^{2+} content was 0.26%. The concentration of Mg^{2+} in Mg^{2+}-limited A.aerogenes was found to be half that of glycerol-limited cells (Tempest, Hunter & Sykes, 1965). There is little evidence for the effect of lack of Mg^{2+} upon the Mg^{2+} content of cell walls. Fiil & Branton (1969) found no difference between the cell envelopes of Mg^{2+}-depleted E.coli and Mg^{2+}-sufficient cells, although the whole cells of E.coli showed a 2-fold difference between Mg^{2+}-depleted and Mg^{2+}-sufficient. This is compatible with the results of Tempest et al. (1965).

Studies on the cell walls of polymyxin resistant and sensitive strains of P.aeruginosa 6750 have shown that walls from resistant cells have a lower Mg^{2+} content than correspondingly sensitive strains (Brown & Watkins, 1970; Brown & Wood, 1972). The same strain of P.aeruginosa has been shown to aquire resistance to polymyxin when grown in conditions of Mg^{2+}-depletion (Brown & Melling, 1969b).

Lack of Mg^{2+} in bacteria leads to morphological changes as well as chemical changes. Morgan et al. (1966) found that thin sections of Mg^{2+}-depleted E.coli, observed by electron microscopy, showed apparent infoldings of the cytoplasmic membranes, although this effect was most pronounced in Mg^{2+}-depleted cells that were observed 6-hr after the addition of excess Mg^{2+} (this addition initiated growth). Similar infoldings were found when Fiil & Branton (1969) examined Mg^{2+}-depleted E.coli by freeze-etching and electron microscopy. These authors also found that the middle layer of the cytoplasmic membrane of Mg^{2+}-adequate cells (the control) contained spherical units 2 - 6 nm in diameter which were arranged in a net-like manner. These particles were present in apparently larger numbers in Mg^{2+}-depleted cells, but the net-like arrangement was no longer present; instead the spherical units in the Mg^{2+}-depleted cells were arranged in a paracrystalline fashion with large areas of the supporting membrane exposed. These results are very similar to those reported by Gilleland et al. (1974) who studied Mg^{2+}-depleted P.aeruginosa 6750 in a similar manner. The Mg^{2+}-depleted cells of P.aeruginosa were found to

have lost the net-like arrangement of the controls, but no paracrystalline arrangements were observed. Gilleland et al. (1974) also examined the middle layer of the outer membrane region of the cell walls, and found that the spherical units of the presumed protein-L.P.S. complex (Rogers et al., 1969) were present in larger numbers in the Mg^{2+}-depleted samples. Not only were the spherical units present in larger numbers, but they were not removed by the action of ethylenediaminetetra-acetic acid (E.D.T.A.) as was observed with the Mg^{2+}-adequate cells. Thus, Gilleland et al. (1974) were able to show both visually and chemically that resistance to E.D.T.A. brought about by Mg^{2+}-depletion (Brown & Melling, 1969b), was associated with an increase in the protein-L.P.S. complex of the cell wall. This is compatible with the findings of Sanderson, MacAlister, Costerton & Cheng (1974) that Salmonella typhimurium mutants that possessed incomplete L.P.S. (lacking heptose) were less resistant to antibiotics that act on intracellular targets than the wild type organism.

Magnesium-depletion has been observed to induce resistance to polymyxin as well as E.D.T.A. (Brown & Melling, 1969a,b), an observation that lead these authors to suggest a possible common initial site of action. This effect of Mg^{2+}-depletion on P.aeruginosa affecting the characteristic sensitivity to E.D.T.A. and to polymyxin has since been repeated, both in batch culture (Boggis, 1971) and in continuous culture (unpublished results, this laboratory). The inclusion of excess calcium (Ca^{2+}) in Mg^{2+}-lacking medium did not affect the amount of growth achieved with limiting amounts of Mg^{2+} in batch cultured

E.coli (Lusk et al., 1968). This indicates that the Ca^{2+} does not replace the Mg^{2+} in any growth rate limiting function, which seems likely since Wiess, Kimes & Morris (1973) found that in vitro Ca^{2+} could not completely replace the Mg^{2+} in the 50S ribosomes isolated from E.coli. However, it has been observed that P.aeruginosa grown in Mg^{2+}-lacking medium with excess Ca^{2+} are almost as sensitive to polymyxin and E.D.T.A. as control cells (Brown & Melling, 1969b; Boggis, 1971). This suggests that the Ca^{2+} may replace Mg^{2+} in a structural role in the cell wall but not in any metabolocally functional role. This is compatible with the findings of Eagon & Carson (1965) and Eagon (1969) that the cell walls of P.aeruginosa contained significant amounts of Ca^{2+}, and with the general view that Ca^{2+} is maintained extracellularly, or on the surface of the cell (Williams & Wacker, 1967).

As the evidence of Brown & Melling (1969a,b) and Gilleland et al. (1974) suggests, lack of Mg^{2+} alters the structural arrangement of the outer layers of the cell wall and this outer membrane plays an equally important role in cell permeability as the cytoplasmic membrane. The evidence of Brown & Melling (1969a,b) and Gilleland et al. (1974) has shown that these structural changes alter the sensitivity of the P.aeruginosa cell wall to polymyxin and E.D.T.A.. Brock (1962) observed that when E.coli ML35 was Mg^{2+}-depleted the cells lost impermeability to o-nitrophenyl-B-D-glactoside, and that Mg^{2+}-depleted cells behaved in a manner similar to control cells when exposed to novobiocin, an antibiotic which was shown by Brock (1962) to bind Mg^{2+}. The reverse effect upon

permeability was observed by Sykes & Tempest (1965) when they studied the oxidation of exogenous glucose by Mg^{2+}-limited P.putida cells grown in a chemostat. These authors found that the rate of oxidation was much slower in Mg^{2+}-limited cells than in control cells (glycerol-limited at the same growth rate). Over a period of 2 - 4-hr the Mg^{2+}-limited cells appeared to adapt to glucose oxidation until the rate was comparable with limited cells supplied with the added Mg^{2+}. Cell-free extracts prepared from Mg^{2+}-limited cells were observed to oxidise glucose immediately without any adaption, although added Mg^{2+} stimulated the rate to some extent. These results led Sykes & Tempest (1965) to suggest that the main effect of Mg^{2+}-limitation upon glucose oxidation was at the site of glucose entry into the cells. Such a theory is compatible with the effects of Mg^{2+}-depletion upon the cytoplasmic membrane (Fiil & Branton, 1969; Morgan etal., 1966), and the evidence of Kundig & Roseman (1971b) that the transport of sugars in E.coli was governed by a membrane bound Mg^{2+}-dependent enzyme system.

Günther et al. (1975) have found that when E.coli were Mg^{2+}-depleted, the total phospholipid (P.L.) content of washed whole cells increased, and that the pattern of the component P.L. also changed. Magnesium-depleted cells contained less phosphatidyl-ethanolamine (P.E.), phosphatidyl-glycerol (P.G.) and phosphatidyl-serine (P.S.), and more diphosphatidyl-glycerol (D.P.G.), on a percentage of the total P.L. basis. Despite these changes in the P.L. pattern, the overall content of the P.L. remained the same i.e. P.E.$\gg$D.P.G.$>$P.G.$>$P.S..

However, these workers did not distinguish between lipids from the outer membrane and the cytoplasmic membrane. Studies on whole cells of B.subtilis showed that cells batch cultured in C.D.M. possessed more lysyl-phosphatidyl-glycerol (lys.P.G.) and less P.G. than Mg^{2+}-limited and phosphate-limited whole cells grown in a chemostat (Minnikin & Abdolrahimzadeh, 1974). These authors found that the pH of the medium also affected the P.L. composition, independantly of the type of limitation imposed. Benns & Proulx (1974) used ^{14}C labelled glycero-3-phosphate (G-3-P) to study the effect of Mg^{2+} and adenosine triphosphate (ATP) on the synthesis of P.L. by E.coli preparations. When crude cell homogenates were incubated with G-3-P in the absence of Mg^{2+}, the label was distributed in P.E.> P.G.> D.P.G.. However, when the experiment was repeated in the presence of Mg^{2+} the distribution was P.G.≫ P.E.> D.P.G.. A similar effect was observed with particulate membrane fractions under the same conditions. Treatment of the labelled P.L. formed by these preparations with phospholipase C showed that most of the label was to be found in the glycerophosphate moiety of the molecule. Benns & Proulx (1974) suggested that the addition of Mg^{2+} and ATP stimulated the formation of an enlarged pool of endogenous phosphatidyl group precursors. It is reasonable to assume that Mg^{2+}-limitation or Mg^{2+}-depletion may result in a decrease in these precursors as well as an alteration in the ratio of P.L. in the wall.

One of the major effects due to lack of Mg^{2+} in bacteria, apart from the decrease in ribosome stability

and function, is the changes in the structure of the cell envelope (Fiil & Branton, 1969; Gilleland et al., 1974). These changes may reasonably be assumed to be due to alterations in the synthesis of envelope components (P.L., L.P.S. and protein) brought about by the lack of Mg^{2+}. Gram-positive bacteria, when Mg^{2+}-limited, have been observed to produce cells that are high in teichoic acid, thus increasing their efficiency in accumulating Mg^{2+} (Meers & Tempest, 1970; Hughes, Stow, Hancock & Baddiley, 1971). The Gram-negative bacterium A.aerogenes has been shown to be capable of adsorbing more Mg^{2+} when Mg^{2+}-limited than were carbon-limited control cells, although the component of the cell surface responsible was not reported (Meers & Tempest, 1970). Phospholipids from Mg^{2+}-limited and Mg^{2+}-depleted cells also show different patterns from control cells (Minnikin & Abdolrahimzadeh, 1974; Günther et al., 1975). Similar changes have been observed in the K.D.O. and carbohydrate content of the cell walls of A.aerogenes (Tempest & Ellwood, 1969) and P.aeruginosa (Gilleland et al., 1974). These changes, induced in the cell walls by the lack of Mg^{2+}, may well explain the observed changes in permeability to metabolic substrates (Sykes & Tempest, 1965) and to antibacterial agents (Brown & Melling, 1969a,b; Gilleland et al., 1974).

4. THE ACTION OF ETHYLENEDIAMINETETRA-ACETIC ACID AND POLYMYXIN B SULPHATE UPON GRAM-NEGATIVE BACTERIA.

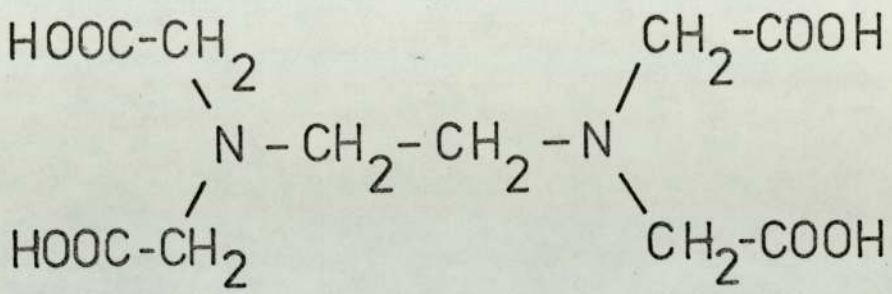
Introduction.

Many Gram-negative bacteria are inhibited by ethylenediaminetetra-acetic acid (E.D.T.A.) (Repaske, 1958; Leive, 1958; Wilkinson, 1975) and polymyxin (P.X.) (Newton, 1956; Garrod, Lambert & O'Grady, 1973; Bader & Teuber, 1973), but in relatively non-toxic concentrations these chemicals reduce drug resistance (E.D.T.A.: Leive, 1965a, b,c; Hamilton-Miller, 1965; P.X.: Warren, Grey & Yurchenco, 1957) or release periplasmic enzymes (E.D.T.A.: Garrard, 1971; Patching & Rose, 1971; P.X.: Cerny & Teuber, 1972). Both agents have an initial action on the outer membrane (E.D.T.A.: Eagon, Stinnett & Gilleland, 1975; Wilkinson, 1975; P.X.: Bader & Teuber, 1973) and a change in sensitivity to these agents may indicate a change in the outer membrane. Pseudomonas aeruginosa is characteristically sensitive to these compounds (Wilkinson, 1967; Brown, 1971) and consequently these drugs are useful 'markers' for monitoring changes in the outer membrane of this organism.

E.D.T.A. is a negatively charged organic molecule (see Fig. 3), that specifically chelates metal cations, while P.X. is a cationic,cyclic heptapeptide with a tri-peptide 'tail' terminating in a fatty acid residue (see Fig. 4), that binds hydrophobically and ionically to the lipid A region of cell wall lipopolysaccharide (L.P.S.). In addition to producing similar effects upon P.aeruginosa, the action of these two chemicals is antagonised by the presence of excess divalent metal cation in the test

Figure 3. Formula of EDTA and EGTA.

EDTA.



EGTA.

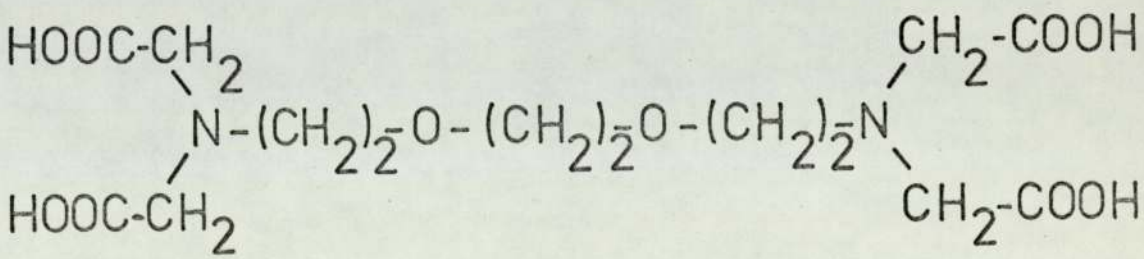
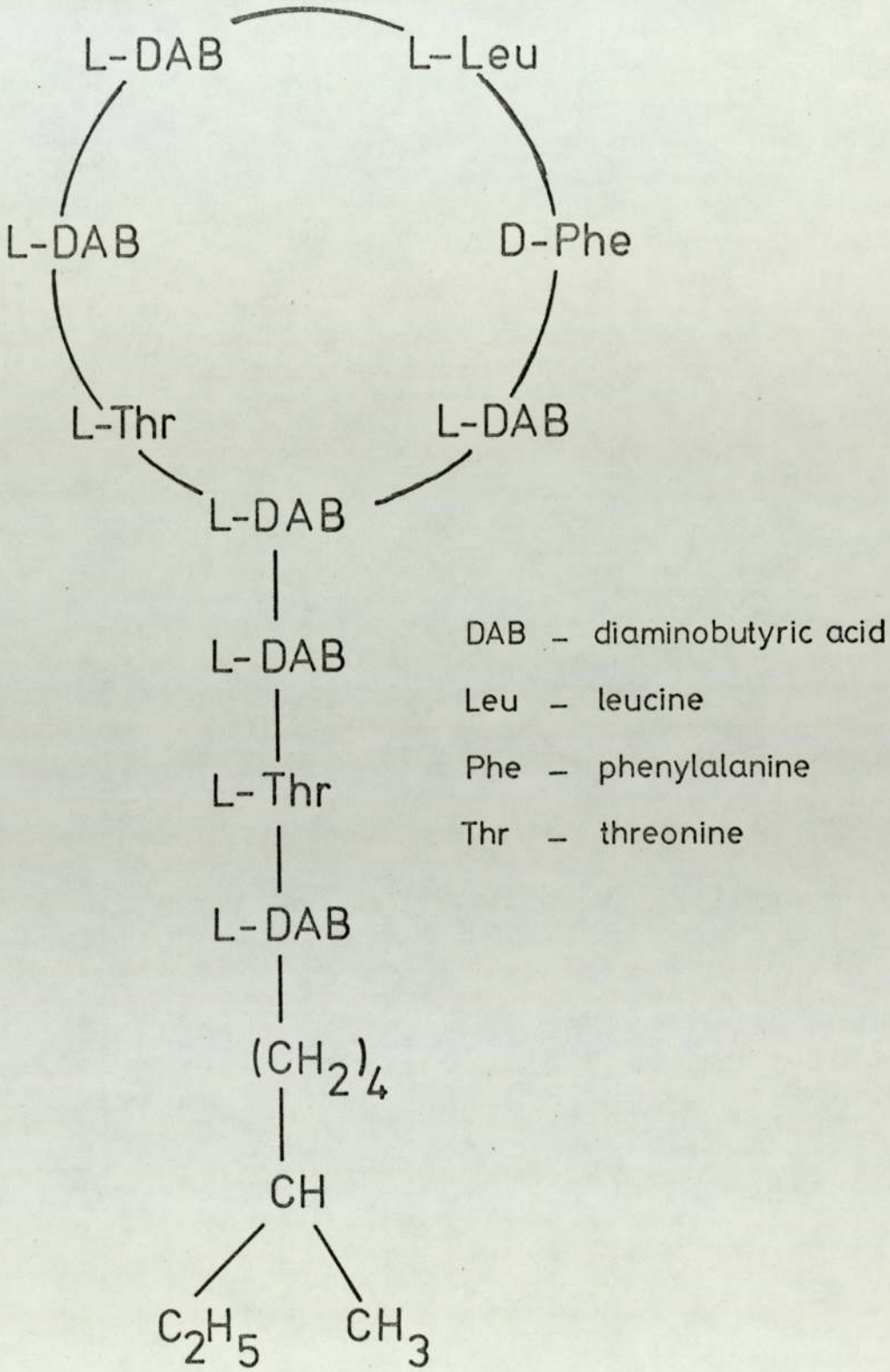


Figure 4. Structure of Polymyxin-B (Garrod et al, 1973).



medium (E.D.T.A.: Brown & Richards, 1965; Asbell & Eagon, 1966; Weiser, Asscher & Wimpenny, 1968; P.X.: Newton, 1954; Davis, Iannetta & Wedgwood, 1971). The antagonism of E.D.T.A. by divalent cations is clearly due to the chelation of the free cations in preferance to those bound in the cell wall. The mechanism of antagonism of P.X. is not so clear, but it is unlikely to be due to the formation of a metal ion-P.X. complex, since P.X. is a cationic molecule. Newton (1954) considered that the cations competed with the P.X. for sites in the cell envelope of P.aeruginosa. This site is most probably in the cell wall since Hsu-Chen & Feingold (1973) found that the phospholipids (P.L.) of the cytoplasmic membranes (C.M.) were not protected by calcium (Ca^{2+}) or magnesium (Mg^{2+}) against P.X..

In contrast to the antagonism of these drugs by excess cations, it has been found that lack of Mg^{2+} in the growth medium results in the resistance of P.aeruginosa to E.D.T.A. and P.X..(Brown & Melling, 1969a,b).

It is the purpose in this section to present briefly the evidence for the mode of action of E.D.T.A. and P.X. against Gram-negative organisms, stressing the sensitivity of P.aeruginosa, and discussing the peculiarities of the cell walls of this organism that may explain the observed sensitivity. The presentation of the evidence of E.D.T.A. action is dealt with in greater brevity than that for P.X. since the former has recently been the subject of two comprehensive reviews particularily emphasing its action upon P.aeruginosa (Eagon et al., 1975; Wilkinson, 1975).

Polymyxin.

The polymyxins are a group of peptide antibiotics, distinguished by a cyclic heptapeptide ring bearing a tripeptide side chain with a terminal fatty acid, 6-methyl-octanoic acid in the case of Polymyxin B sulphate (Garrod et al., 1973). Although the initial binding of P.X. to cells appears to be to the L.P.S. of the cell wall (Bader & Teuber, 1973), the primary target is the C.M. (Newton, 1956; Few, 1955; Pache, Chapman & Hillaby, 1972; Feingold, Hsu-Chen & Sud, 1974; Teuber, 1974). Gram-negative bacteria are generally more susceptible to P.X. than Gram-positive, although Proteus species are characteristically resistant among Gram-negative species (Sud & Feingold, 1970) and Bacillus megaterium and Micrococcus lysodeikticus are among the few Gram-positive species that are sensitive (Newton, 1956). It is perhaps significant that the major difference between the cell walls of Gram-positive and Gram-negative bacteria is the possession of an outer membrane (O.M.), composed of L.P.S., protein and P.L., by the Gram-negative organism (see previous section), and that the initial step in the action of P.X. is the binding to the L.P.S. and/or P.L. of the cell wall (Salmonella typhimurium: Bader & Teuber, 1973).

The binding of P.X. to the L.P.S. of the O.M. may well cause disruption of this structure, since it was found that periplasmic enzymes were released by the action of P.X. upon Escherichia coli, Pseudomonas fluorescens, S.typhimurium, Aerobacter aerogenes and Alcaligenes faecalis, but not from the resistant organisms Proteus mirabilis and Serratia marcescens (Cerny & Teuber, 1972).

These authors found little or no evidence for the release of cytoplasmic enzymes (i.e. damage to the C.M. under the conditions used; 1.5×10^{11} cells exposed to 2 mg P.X. at 37° for 1-min). Bader & Teuber (1973) investigated the site of P.X. attachment in the L.P.S. molecule by using L.P.S. isolated from mutants of S.typhimurium lacking various regions of the L.P.S. molecule (SL1135: wild type, complete L.P.S.; G30: L.P.S. lacking the 'O' antigen specific polysaccharide chain; SL1102: lacking both the 'O' antigen specific chain and the core polysaccharide). These authors found that the order of binding of P.X. to the L.P.S. was SL1102>G30>SL1135 (i.e. the more exposed the lipid A region the greater the P.X. binding capacity of the L.P.S.). Additional evidence for lipid A as the binding site was found when it was observed that lipid A fractions isolated from SL1135 and G30 possessed the same P.X. binding capacity. An earlier indication that the site of P.X. binding to L.P.S. may be at the proximal end of the molecule is to be found in the observations of McQuillen, presented by Newton (1956), that the electrophoretic mobility of several strains of P.aeruginosa was unaffected, even when P.X. was adsorbed by the cells to the extent of 300 µg/mg dry weight bacteria. One exception observed by McQuillen (1956) was P.aeruginosa var. Molyneux, which exhibited a decrease in electrophoretic mobility as the concentration of P.X. increased. Upon examination, this strain was found to be a 'rough' strain (i.e. possessing little or no'O' antigen specific side chain). The presence of the 'O' antigen specific side chain in the other strains used

may have effectively put the lipid A region approximately 100 Å below the cell 'surface' (Newton, 1956).

Direct microscopical evidence that P.X. disorganises the O.M. region of the cell wall of E.coli and P.aeruginosa has been presented by Kioke, Iida & Matsuo (1969). These workers observed that electron microphotographs of sections of P.X. treated cell depicted numerous projections on the surface of the cell walls. These projections became less apparent as the concentration of P.X. was decreased, or when the concentration of Mg^{2+} ions was increased in a system containing a fixed concentration of P.X. (25 µg/5 x 10^8 cells). In addition to the disorganisation of the L.P.S. in the O.M., Kioke et al. (1969) observed what they termed as 'cracks' in the C.M.. Whatever the nature of these C.M. lesions, they were sufficient to allow cytoplasmic material to leak from the cells. The nature of the components of the O.M. that produce these projections on addition of P.X. was further investigated by studying the uptake of L.P.S.-specific and lipoprotein-specific phages by E.coli and S.typhimurium (Kioke & Iida, 1971). These authors found that cells treated with P.X. were unable to adsorb L.P.S.-specific phages, while the adsorption of lipoprotein-specific phages was unaffected. Electron microphotographs of these cells revealed that the lipoprotein-specific phages were adsorbed to the region of the O.M. in between the P.X. induced projections. These results are compatible with the theory that P.X. binds to, and disrupts the L.P.S. region of the cell wall. Koike & Iida (1971) observed that cells previously exposed to

the L.P.S.-specific phage did not produce cell wall projections when treated with P.X.; presumably the phage bound irreversibly to the same site on the L.P.S.(the lipid A region), or masked this site from P.X.. Newton (1955), using a fluorescent derivative of P.X., 1-dimethyl-aminonaphthalene-5-sulphonyl chloride coupled to the γ-amino group of the α,γ-diaminobutyric acid in the P.X. molecule (D.A.N.S.P.), found that P.X. was bound equally well by both the cell wall and C.M. when P.aeruginosa was treated with the drug prior to disruption. This was in contrast to the results obtained when the experiment was repeated with B.megaterium; only 10% of the D.A.N.S.P. was taken up by the cell walls while the remaining 90% was taken up by the C.M.. Few (1955) studied the interaction of polymyxin E with lipid monolayers prepared from lecithin (P.C.), cardiolipin (D.P.G.), phosphatidyl-ethanolamine (P.E.) as well as total P.L. extracted from crude cell wall preparations of Pseudomonas denitrificans (sensitive to P.X.), and Staphylococcus aureus (resistant to P.X.). The P.X. formed strong complexes with the P.E. (contaminated with phosphatidyl-serine) and the lipid extracted from P.denitrificans, moderate complexes with D.P.G. and the lipids from Staph.aureus, whilst P.C. showed no evidence of complex formation. These results are in apparent contrast to the results of Teuber (1973) who found that a radio-labelled derivative of P.X. bound to phosphatidyl-glycerol (P.G.) and phosphatidic acid, but not to D.P.G. P.E. or phosphatidyl-serine (P.S.). Hsu-Chen & Feingold (1972) studied the action of P.X. upon erythrocytes, and liposomes prepared from erythrocyte P.L. and the P.L. from

E.coli. These authors found that neither the erythrocytes or the liposomes prepared from erythrocyte P.L. were lysed to any great extent by P.X., whereas the liposomes constructed from E.coli P.L. were extremely sensitive. Examination of the P.L. contents of erythrocyte membranes and E.coli P.L. revealed that the former contained P.C. and sphingomyelin (together forming about 60% of the total), P.E. and P.S.. The E.coli P.L. contained P.E. and P.G., 79% and 18% of the total respectively. This lead Hsu-Chen & Feingold (1972) to suggest that the P.E. in the C.M. was the target site. Teuber (1973) has found that P.X. binds specifically with P.G. in S.typhimurium. This phospholipid comprises 33% of the C.M. and 17% of the O.M. in S.typhimurium (Osborn, Gander, Parisi & Carson, 1972). It is conceivable that in the system studied by Hsu-Chen & Feingold (1972) the P.X. was lysing the liposomes by its action on the P.G. and not the P.E. in the lipids used. Since P.G. carries a negative charge and P.E. carries both a negative and a positive charge, it is reasonable to assume that P.X. could combine with both of these P.L., but that it might bind preferentially with P.G. in a mixture.

When P.aeruginosa is grown in chemically defined medium lacking Mg^{2+} in batch culture the Mg^{2+}-depleted cells have been found to be resistant to P.X. (Brown & Melling, 1969b). The cell walls of a P.X.-resistant strain of P.aeruginosa have been found to possess lower levels of Mg^{2+} than the parent strain (Brown & Watkins, 1970). From these two observations it appears that a lack of Mg^{2+} in the cell or cell wall of P.aeruginosa

may result in a change in the cell wall that restricts P.X. entry to the C.M.. It has been found that the whole cells and cell walls of the strain of P.aeruginosa trained to be P.X. resistant, bound less of the drug than the sensitive parent (Watkins, 1970). Thus it appears that the initial binding site of P.X. (lipid A region of L.P.S. and/or P.L. in the O.M.) may be masked by some conformational change. Alternatively, the cationic cross-links may be mediated by organic cations or polyamines (Wilkinson, 1975) in Mg^{2+}-depleted cells, and these cations may mask the binding site or restrict access by steric hindrance.

In conclusion, it appears that the action of P.X. may consist of the following sequence of events, described by Teuber (1974) using S.typhimurium. The P.X. initially binds to the lipid A region of the O.M. L.P.S. as the L.P.S. will be the first structure encountered by the drug. This binding appears to disrupt the L.P.S. organisation, resulting in the destruction of the permeability control of the O.M.. As large molecular species such as periplasmic enzymes are able to pass outwards through the damaged O.M. (Cerny & Teuber, 1972), it is reasonable to assume that the P.X. can pass through the disorganised O.M. and so bind with the P.G. and/or P.E. of both the inner-surface of the O.M. and the C.M.. The C.M. is disrupted by the P.X. and the cell starts to lose accumulated low molecular weight solutes and substrates. Active transport also ceases when the membrane is damaged, and at about the same time, ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) synthesis also ceases, presumably due to the precipitation of RNA-P.X. and DNA-P.X.

complexes (Nakajima & Kawamata, 1966). The synthesis of murein, L.P.S. and electron transport systems then slowly declines, leading eventually to cell death. It is interesting to note that this sequence of events described by Teuber (1974) as following the damage of S.typhimurium membrane by P.X., bears a marked resemblance to the sequence of events that lead to cell death when bacteria were cold shocked (see section 5). This similarity may be significant, since P.aeruginosa is characteristically sensitive to P.X. and to cold shock.

Ethylenediaminetetra-acetic acid.

Disodium ethylenediaminetetra-acetic acid chelates the divalent metal cations Mg^{2+}, Ca^{2+} and manganese (Mn^{2+}). The order of affinity is $Mn^{2+} > Ca^{2+} > Mg^{2+}$. The action of E.D.T.A. upon Gram-negative bacteria, and in particular the characteristic sensitivity of P.aeruginosa to this compound, have recently been comprehensively reviewed (Eagon et al., 1975; Wilkinson, 1975). The evidence presented by these authors suggests that E.D.T.A. acts primarily by removing divalent cations (probably Mg^{2+} and/or Ca^{2+}) from the cell wall. It is thought that Mg^{2+} plays a significant role in stabilizing the O.M. by cross-linking between the anionic components (protein, L.P.S. and P.L.); this cross-linking is thought to be especially important in P.aeruginosa (Asbell & Eagon, 1966; Wilkinson & Galbraith, 1975). The removal of this 'structural' Mg^{2+} would be expected to disorganise the O.M. and cause the partial loss or solubilization of O.M. components due to charge repulsion.

Lipopolysaccharide containing material has been

found to be released from P.aeruginosa treated with E.D.T.A. in an osmotically protective medium (Stinnett, Gilleland & Eagon, 1973; Gilleland, Stinnett & Eagon, 1974), and E.coli treated with E.D.T.A. has been shown to lose about 50% of the L.P.S. (Lieve, 1965c). The L.P.S. released by P.aeruginosa is complexed with lipoprotein. Although E.D.T.A. kills most bacteria only after prolonged exposure (Wilkinson, 1975), P.aeruginosa is killed rapidly by similar concentrations of E.D.T.A.. The osmoplasts (Asbell & Eagon, 1966) produced by the action of E.D.T.A. upon P.aeruginosa, lyse on dilution in water. If the E.D.T.A. is removed and excess Mg^{2+} or other divalent cations are added prior to the removal into water, then the osmoplasts were found to have regained osmotic stability. Recent work by Gilleland et al. (1974) has shown that this treatment with E.D.T.A. results in the loss of structural subunits from the hydrophobic region of the O.M.. Furthermore, the subunits were found to be a protein-L.P.S. complex and to be reaggregated in the O.M. when E.D.T.A. was removed and excess Mg^{2+} added, restoring osmotic stability. When P.aeruginosa was grown in Mg^{2+}-depleted medium (batch culture), the cells were found to be resistant to E.D.T.A. (Brown & Melling, 1969a,b), a finding recently reproduced by Gilleland et al. (1974) with the same organism. These authors also correlated the resistance to E.D.T.A. induced by Mg^{2+}-depletion with an increase in the number of protein-L.P.S. subunits in the O.M.. Treatment of the resistant cells with E.D.T.A. did not result in any of the subunits being removed from the O.M.. These results would tend to imply that the O.M. of

P.aeruginosa plays some role in maintaining the shape of the cell. Carson & Eagon (1966) have observed that P.aeruginosa incubated with lysozyme did not lyse; the remainder of the cell wall (i.e. O.M.) retained the bacillus shape of the cell except for slight 'ballooning' at the polar regions of the cell. Subsequent treatment with E.D.T.A. resulted in complete lysis. When viable counts were used to determine cell death, the death curve obtained with E.D.T.A. alone was identical to the curve obtained with E.D.T.A. and lysozyme in combination (Eagon & Carson, 1965). If optical density was used instead of viable count, there appeared to be less lysis with E.D.T.A. alone. This was found to be due to the light scattering properties of the large fragments of cell lysed by E.D.T.A.. These fragments were removed upon incubation with lysozyme. These results are compatible with a theory that the O.M. plays a major role in maintaining the shape of the cell in P.aeruginosa (Carson & Eagon, 1966) since the removal of the murein does not result in the disintegration of the cell, whereas the disruption of the O.M. (by E.D.T.A.) results in lysis. Such a theory is compatible with the low level (and probably weaker structure) of murein-lipoprotein in P.aeruginosa (Meadow, 1974).

Wilkinson (1975) has suggested that the marked sensitivity of P.aeruginosa may be due to the greater dependence of this organism upon intramolecular cationic cross-links between the L.P.S. molecules and other O.M. components for O.M. integrity. The L.P.S. from P.aeruginosa contain more phosphorus than L.P.S. from other Gram-negative species; it may therefore be expected

to have a greater metal ion binding potential (Wilkinson & Galbraith, 1975). If, as Carson & Eagon (1966) propose, this cation dependant O.M. is also a major structure of the cell wall responsible for the rigidity of the cell then the sensitivity of P.aeruginosa to E.D.T.A. is readily explained. The observed resistance of this organism to E.D.T.A. when grown in Mg^{2+}-depleted, chemically defined media (C.D.M.) (Brown & Melling, 1969a,b; Gilleland et al., 1974) may be due to the Mg^{2+}-depleted organism adapting organic cations or polyamines to mediate cross-linkages between the anionic components of the O.M. (Wilkinson, 1975). The polyamines spermidine and putrescine have been shown in vitro to replace about 50% of the Mg^{2+} in the 50S and 30S ribosomes prepared from E.coli without any loss in function (synthesis of the polypeptide polyphenylalanine) or increase in sensitivity to ribonuclease 1 (Kimes & Morris, 1973; Weiss & Morris, 1973).

Ethyleneglycol-Bis-(β-aminoethylether)-N:N'-Tetra-acetic acid.

Ethyleneglycol-Bis-(β-aminoethylether)-N:N'-Tetra-acetic acid (E.G.T.A.) (Fig. 3) is of interest since the log affinity constant for Mg^{2+} and Ca^{2+} are 4.7 and 10.4 respectively (Roberts, Grey & Wilkinson, 1970). Thus E.G.T.A. is a useful tool for studying the role of cations in the cell wall since it will chelate Ca^{2+} in preference to Mg^{2+}, even in the presence of free Mg^{2+}.

There is little published work upon the action of E.G.T.A. in relation to bacteria. Roberts et al. (1970) found that at pH 9.2, E.G.T.A. was 60% as effective as

E.D.T.A. in reducing the viability of P.aeruginosa. Boggis (1971) also found E.G.T.A. effective in lysing P.aeruginosa, but at pH 7.8.

In conclusion, all these agents appear to act initially by disrupting the outer membrane, either by binding with P.L. and/or L.P.S. molecults (P.X.) or by removing cross-linking cations (E.D.T.A. and E.G.T.A.). The resistance of P.aeruginosa to these drugs when the organism is cultivated in Mg^{2+}-depleted C.D.M. may well be reflected by chemical and/or conformational changes in the outer membrane.

5. COLD SHOCK

Introduction.

The length of this section is disproportionately long in relation to the importance of cold shock in the overall study of the cell wall. Although there are a great number of papers upon cold shock, the author was unable to find any comprehensive reviews upon the phenomenon. It was therefore thought relevant to include a detailed review of cold shock in this thesis.

Cold shock is the term used to describe the decrease in viability and loss of ultra-violet (U.V.) absorbing material observed when a culture of susceptible bacteria is subjected to a rapid drop in temperature. Sherman & Albus (1923) found that when Escherichia coli was rapidly cooled there was a decrease of about 95% in the viability of the culture. This effect of cold shock is very marked with cultures during the exponential phase of growth (E.coli: Sherman & Cameron, 1934; Hegarty & Weeks, 1940; Meynell, 1958; Sato & Takahashi, 1968b; Aerobacter aerogenes: Strange & Dark, 1962; Salmonella typhimurium: Gorrill & McNeil, 1960; Serratia marcescens: Strange & Ness, 1963; Bacillus subtilis: Sato, Suzuki & Takahashi, 1968; Pseudomonas fluorescens: Sato & Takahashi, 1968a; Pseudomonas aeruginosa: Gorrill & McNeil, 1960; Farrell & Rose, 1968). Stationary phase cells of some organisms are also susceptible to cold shock, but the effects are not so pronounced as in the exponential phase (A.aerogenes: Strange & Dark, 1962; B.subtilis: Smeaton & Elliot, 1967; Streptomyces hydrogenans: Ring, 1965a; P.aeruginosa:

MacKelvie, Gronlund & Campbell, 1967). It is difficult to decide if sensitivity to cold shock is a predominantly Gram-negative characteristic; certainly the majority of papers describe the effect in this type of organism, but this may be due only to the popularity of E.coli as a test organism. Sato & Takahashi (1969) observed that there was little or no difference between the percentage viability of exponential phase P.fluorescens and B.subtilis when cold shocked. Unfortunately the organisms used were grown in different media; a modified Schaeffer's medium (Sato et al., 1968) for B.subtilis and broth for P.fluorescens. Gorrill & McNeil (1960) have shown that Staphlococcus aureus grown under the same conditions as E.coli, S.typhimurium and P.aeruginosa (i.e. in broth), and subjected to conditions of cold shock in an identical manner to these organisms, was very resistant to cold shock. Without further study involving a range of Gram-negative and positive organisms grown and tested under identical conditions, no generalisation may be made as to whether one type of organism is more susceptible to cold shock than the other. The sensitivity of P.aeruginosa to rapid changes in temperature, pH and tonicity (Brown & Winsley, 1969) and to the compounds active against the cell envelope (polymyxin: Newton, 1953a,b; polysorbate 80: Brown & Winsley, 1969), make relevant a review of cold shock since it too affects the permeability of the cell envelope (Strange & Postgate, 1964; Farrell & Rose, 1967). Before proceding to the details of cold shock it may be beneficial to distinguish between this phenomenon and the related event of 'cold osmotic shock'.

Cold osmotic shock (C.O.S.), occurs when bacteria are suspended in a hypertonic, metabolically inert solute containing ethylenediaminetetra-acetic acid (E.D.T.A.), at the growth temperature, and are resuspended in a hypotonic solution of magnesium chloride at 0° (Patching & Rose, 1971). This treatment results in the loss of the ability to accumulate low molecular weight substrates (Neu & Heppel, 1965), and the loss of cell wall proteins (Piperno & Oxender, 1966) and enzymes (Heppel, 1967). Despite the loss of some permease activity, C.O.S., unlike cold shock, does not cause any decrease in viability (Patching & Rose, 1971).

Effects of cold shock.

The main effects of cold shock upon bacteria seem to vary little between species. The loss of viability is preceded by the leakage of U.V.-absorbing material in A.aerogenes (Strange & Dark, 1962) and P.aeruginosa (Farrell & Rose, 1967). Accumulated metabolic substrates (Ring, 1965a; Leder, 1972) as well as intracellular enzymes (MacKelvie et al., 1967) and proteins (Smeaton & Elliot, 1967) are also lost by the cell. Despite all this evidence of damage to the cell envelope, cold shock does not produce any change either in the total counts of the suspensions or the morphological appearance of the cells (Meynell, 1958; Gorrill & McNeil, 1960; Smeaton & Elliot, 1967). One exception to these observations was reported for a strain of P.aeruginosa in the late stationary phase; in this case not only were the cells susceptible to cold shock but they showed a loss of intracellular enzymes (catalase and glucose-6-phosphate dehydrogenase), and

lysed (MacKelvie et al., 1967).

The decrease in viability of cold shocked cells is very pronounced. For instance, Meynell (1958) found only 0.01% of exponential phase, broth grown E.coli cells survived such treatment. A similar effect was observed with P.aeruginosa grown in broth at 37° (Gorrill & McNeil, 1960), however Farrell & Rose (1968) using the same strain of P.aeruginosa grown at 30° in chemically defined media found a percentage survivors of about 20%. This may be due to the differences in growth temperature (37° as opposed to 30°), growth medium (broth as opposed to a glucose-salts medium) and the diluent used (30 mM-saline as opposed to distilled water. Using a chemically defined, nitrogen-limited medium, MacKelvie et al. (1967) showed that a culture of P.aeruginosa maintained at 37° in the stationary phase of growth for up to 48-hr were only 30% viabile when cold shocked. This is in agreement with the observations of Strange & Dark (1962) that stationary phase A.aerogenes harvested from a chemically defined medium and then suspended in buffered, aerated saline at 35° for 20-hr was as susceptible to cold shock as were exponential phase cells.

As a result of studies on E.coli (Meynell, 1958) and A.aerogenes (Strange & Dark, 1962), it was suggested that the death of cold shocked cells was preceded by, and probably due to, changes in cell permeability. Strange & Dark (1962) analysed the U.V.-absorbing material from cold shocked, exponential phase A.aerogenes and found it to contain ninhydrin positive material, adenosine triphosphate (ATP) and nucleic acid bases. The ninhydrin positive material was found to be a mixture of mainly

free amino acids and peptides of low molecular weight. Using stationary phase B.subtilis, Smeaton & Elliot (1967) showed that the cold shocked cells released a protein with a molecular weight of approximately 12,000. They reported that this protein, an inhibitor of ribonuclease (RNase), was intracellular since it was only possible to obtain it from non-shocked cells by disintegration of the cells. It has been shown that cold shocked cultures also lose the ability to transport materials into the cell. Ring (1965a) observed that cold shocked Strep.hydrogenans were unable to maintain high intracellular levels of amino-isobutyric acid (A.B.A.); similarly, Leder (1972) has shown that E.coli was unable to maintain the high intracellular levels of thio-methylgalactoside (T.M.G.), valine and galactose. Piperno & Oxender (1966) have found that the loss of ability to transport leucine, isoleucine and valine in cold shocked and C.O.S. treated E.coli was correlated with the loss of a cell envelope protein that bound these amino acids. Further evidence that the permeability of the cell is affected was gained from the passage of external solutes into cold shocked cells. Strange (1964) has shown that when cold shocked A.aerogenes was suspended in 4 M-saline, little or no plasmolysis of the cells occurred. This finding was explained on the basis that cold shocked cells were unable to prevent passive entry of the saline due to lack of permeability control. In unshocked bacteria and most animal cells, the intracellular level of sodium ions is maintained at a low level (Williams & Wacker, 1967). The dye anilino naphthalene sulphonate (A.N.S.) fluoresces when conjugated with

protein. Addition of this dye to the cold shocked cultures of E.coli resulted in an increase in fluorescence compared with control cultures (Sato & Takahashi, 1968b). A similar effect has also been observed with an exponential phase culture of A.aerogenes (Strange & Postgate, 1964) and a stationary phase culture of B.subtilis (Smeaton & Elliot, 1967). These observations are of particular interest since Newton (1954) observed a similar pattern of fluorescence when P.aeruginosa, previously exposed to the action of polymyxin B sulphate was reacted with A.N.S.. P.aeruginosa was also found to produce fluorescence in the presence of a polymyxin derivative of the dye (Newton, 1955). The dye A.N.S. has also been shown to produce fluorescence when added to P.aeruginosa pretreated with the non-ionic detergent polysorbate 80 (Brown & Winsley, 1969). It is significant to mention here that Brown & Winsley (1969) found that treatment with polysorbate 80 did not result in a decrease in the optical density or total count of the P.aeruginosa cells, although there was significant leakage of U.V.-absorbing material. Similar effects have been observed as a result of cold shock in P.aeruginosa (Farrell & Rose, 1967). Brown & Winsley (1969) considered that since polysorbate 80 did not alter the optical properties of the cells, it was unlikely that the A.N.S. entered the cells. However, it may have bound to cell-envelope protein exposed by the action of the polysorbate 80 upon the cell surface. It is conceivable that the same explanation could be applied to the fluorescence of the A.N.S. treated cold shocked cells, although the observations of Strange & Postgate (1964)

that suggest the entry of RNase (mol.wt. approx. 14,000) into cold shocked A.aerogenes does not preclude the entry of A.N.S. into cold shocked bacterial cells. The evidence for the suggestion of Strange & Postgate (1964) consists of the observations that cold shocked cells release nucleic acid bases and lose viability upon rewarming (Strange & Dark, 1962), and that the rate of death is increased if RNase is added to the rewarmed cells. Since deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) are intracellular and have been found not to leak from cold shocked A.aerogenes (Strange & Dark, 1962) it was presumed that the RNase was able to enter cold shocked cells and that the activity of this enzyme, in part, was responsible for the lethal effects of cold shock. This hypothesis is supported by the findings that cold shocked B.subtilis released a protein inhibitor of RNase (Smeaton & Elliot, 1967) that is usually obtained upon disintegration of the cells. Sato & Takahashi (1969) also suggested that degradation of nuclear material may be an effect of cold shock, when they observed that nicotinamide adenine dinucleotide (NAD) and magnesium ions (Mg^{2+}) were required for the recovery of cold shocked E.coli. These authors noted that the NAD appeared to act as an energy source for the recovery process and cited the repair enzyme DNA ligase as having a similar requirement for Mg^{2+} and for NAD as an energy source. The role of DNA ligase in the recovery of cold shocked, exponential phase E.coli cells was confirmed by later experiments (Sato & Takahashi, 1970). There appeared to be no evidence for the auto-degradation of nucleic acids in P.aeruginosa, although

U.V.-absorbing material has been reported as leaking from cold shocked cells (Farrell & Rose, 1968). Strange & Postgate (1964) have shown that when cold shocked A.aerogenes was rewarmed in the presence of deoxyribonuclease (DNase, mol.wt. 61,500) and pepsin (mol.wt. 35,000) the death rate did not increase. This observation would indicate that molecules with a molecular weight significantly greater than 14,000 were unable to enter the cell. Exceptionally large molecules however, have been observed to leak from cold shocked, stationary phase cells of P.aeruginosa (MacKelvie et al., 1967). These workers were able to show the presence of significant amounts of catalase (mol. wt. 250,000) and glucose-6-phosphate dehydrogenase (mol.wt. 102,000) in the cell-exudate from the cold shocked cells. Since the enzymes are considered to be intracellular (Cerny & Teuber, 1972) considerable damage must have occurred to both the cytoplasmic membrane and the outer membrane during cold shock.

The change in permeability, loss of permease activity and intracellular material, and subsequently the death of cold shocked cells, do not significantly alter the total counts or morphology of the cells concerned. With exponential phase E.coli, Meynell (1958) found that the ratio of total counts of the cells before and after cold shock was within the range 0.89 - 1.08 (i.e. not significantly different from unity). Meynell (1958) also reported that the appearance of cold shocked E.coli when examined by dark-ground, phase-contrast and electron microscopy were indistinguishable from control cells. Very similar results were obtained by Gorrill & McNeil (1960).

with cultures of P.aeruginosa, and no morphological changes were apparent in cold shocked B.subtilis when examined by Gram-staining and electron microscopy (Smeaton & Elliot, 1967).

Mechanism of cold shock.

An early detectable effect of cold shock is a rapid increase in cell permeability (Strange & Dark, 1962) and loss of permease activity (Leder, 1972). A useful model for the Gram-negative cell wall is described by Costerton, Ingram & Cheng (1974). This model proposes two sites for permeability control, namely the cytoplasmic membrane and a similar structure on the cell wall surface refered to as the outer membrane. Costerton et al. (1974) and Leive (1974) have presented evidence to show that the two membranes consist of lipid-protein bilayers (see section on the cell wall). In this section any general effect on the cytoplasmic membrane, such as temperature or incorporation of fatty acids into membrane phospholipids (P.L.), will be assumed also to occur at the outer membrane in Gram-negative bacteria. Farrell & Rose (1967) suggested that 'freezing' of the fatty acid side chains in P.L.would in turn cause a distortion of the protein molecules in the membrane leading to inactivation of permease activity. They have also suggested that another effect of this membrane distortion may be the loss of low molecular weight solutes from the cell. If the hypothesis of Farrell & Rose (1967) is correct, then the effects of cold shock would not be apparent until the temperature of the diluent approached the transistion point of the major P.L. in the membranes, when a phase change of the P.L. from liquid to

liquid-crystalline might occur. Such an effect has been observed in studies on the loss of A.B.A. from Strep.hydrogenans at various temperatures (Ring, 1965a). When the loss of A.B.A. was plotted against the cold shock temperature a sigmoid curve was obtained. The 'turning point' on the curve (i.e. the temperature at which the loss of A.B.A. begins to increase rapidly) was about 4° - 5°. In a similar experiment with E.coli grown at 35°, Leder (1972) was able to show that no loss of accumulated T.M.G. occurred when cells were washed at 25°, 15° and 10°; cells washed at 5° lost about 50% of T.M.G., and cells washed at 0° lost 95% of T.M.G.. If these data are plotted in a similar manner to that described by Ring (1965a) a sigmoid curve is also obtained, again with a turning point of 4° - 6°. Gorrill & McNeil (1960) studied the effect of cold shock treatment on exponential phase P.aeruginosa, grown at 37°, using diluent at 37°, 18°, 4° and 0°. Using viable count, but not cell permeability, as a criterion for cold shock, they found that there was little or no effect at either 37° or 18°, but that viability was markedly decreased at 4° and decreased still further at 0°. These results would indicate a turning point between 18° and 4°, probably nearer to 4° than 18° (Gorrill & McNeil, 1960, Fig. 1). Smeaton & Elliot (1967) found that the turning point for the release of protein from cold shocked, stationary phase cells of B.subtilis was somewhat higher, at 14° - 16°. This may reflect the difference in fatty acid composition of the cytoplasmic membrane P.L.. A phase change of membrane lipids has been suggested by Van Steveninck & Ledeboer (1974) to explain their observations that active dried

yeast loses viability when reconstituted with cold (5°) water. The transition point was found to be approximately 14.5°. Farrell & Rose (1968) further supported their theory by arguing that since the transition temperature of P.L. decrease as the amount of unsaturated fatty acids in the side chains increase (Byrne & Chapman, 1964), then cells with higher unsaturated fatty acid content should appear less sensitive to cold shock. By growing P.aeruginosa at a low temperature (10°) they were able to increase the synthesis and incorporation of unsaturated fatty acids (Farrell & Rose, 1967, 1968). This increase in unsaturated fatty acids was subsequently correlated with an decrease in sensitivity to cold shock when a mesophilic strain of P.aeruginosa was grown at 10° instead of 30° (Farrell & Rose, 1968). Comparing a psychrophilic P.aeruginosa grown at 30°, with a mesophilic strain grown at 30°, Farrell & Rose (1968) were able to show that the decrease in viability for the psychrophilic strain was much less than for the mesophilic; this correlated with the slightly higher proportion of unsaturated fatty acid found in the psychrophile. Ring (1965b) has reported a shift to a lower temperature for the turning point for the release of A.B.A. from Strep.hydrogenans when this organism is grown at a lower temperature (22.5°); a result that is compatible with the theory of Farrell & Rose (1968). Increased synthesis of unsaturated fatty acids may also be achieved by growth in a nutritionally complex medium (glucose-salts medium, supplemented with casamino acids or yeast extract, Kates, 1964). E.coli, S.marcescens and A.aerogenes all exhibited resistance to cold shock when

grown in broth (Strange & Ness, 1963), although E.coli was shown by Meynell (1958) to be more sensitive when grown in broth rather than in chemically defined medium. Meynell (1958) was able to show that when an exponential phase of E.coli, cultured in chemically defined medium was supplemented with peptone (to a final concentration of 0.1 - 1.0% $^{w}/v$), the sensitivity increased concurrently with the observed increase in growth rate. The discrepancy between the results of Meynell (1958) and of Strange & Ness (1963) may be due to a difference between the number of organisms used. Meynell (1958) used a final concentration of E.coli of about 2×10^{7} cells/ml, whereas the concentration of cells used by Strange & Ness (1963) was about 1×10^{8}/ml. The unsaturated fatty acid content of bacteria may be manipulated by growing fatty acid auxotrophic bacteria in media supplemented with fatty acids of varying degrees of saturation. Using such a fatty acid auxotroph of E.coli, Overath & Trauble (1973) were able to correlate the turning point temperature for sugar transport with the transition temperature of the major P.L. components in the membrane. The role of membrane lipids is also indicated indirectly by the observation that cells of E.coli cold shocked in hypertonic diluent at 0° did not appear to lose accumulated T.M.G. (Leder, 1972). This author suggested that the hypertonic diluent dehydrated the cell membrane, and therefore effectively increased the proportion of total lipids in the membrane increasing the stability. Cold shock thus appears to cause a phase change in the P.L. of the cytoplasmic membrane, and presumably in the outer

membrane too in Gram-negatives, from the liquid to the liquid-crystalline phase. This results in distortion of the membrane and a subsequent increase in permeability and loss of transport ability, probably by loss of carrier proteins (Piperno & Oxender, 1966). The death of the cold shocked cells may be brought about by the combination of the loss of intracellular materials (Strange & Dark, 1962; Farrell & Rose, 1968), and the stimulation of autodegradation of nuclear material by RNase (Strange & Postgate, 1964). The initiation of RNase activity, probably due to the release of the inhibitor (Smeaton & Elliot, 1967), coupled with the inactivity of DNA ligase (Sato & Takahashi, 1970), would rapidly bring about death once cold shocked cells were rewarmed to the growth temperature.

Factors affecting cold shock.

Besides the factors already described in the previous section, other factors that affect cold shock are:- the concentration of the bacterial suspension (Strange & Dark, 1962; Strange & Ness, 1963), the length of time the bacteria are exposed to cold shock conditions (Sherman & Cameron, 1934; Gorrill & McNeil, 1960), the presence of leakage material from other chilled bacteria (Strange & Dark, 1962; Farrell & Rose, 1968), the growth phase of the cell (E.coli: Sherman & Albus, 1923; Hegarty & Weeks, 1940; Meynell, 1958; B.subtilis:Sato et al., 1968; P.fluorescens: Sato & Takahashi, 1968a), the rate at which the cells are cooled (Sherman & Cameron, 1934; Meynell, 1958), and the presence of divalent cations in the diluent (P.aeruginosa: Gorrill & McNeil, 1960; A.aerogenes:

Strange, 1964; E.coli: Sato & Takahashi, 1969).

Using exponential phase A.aerogenes Strange & Dark (1962) found that cell suspensions above 5 x 10^8 cells/ml showed an apparent resistance to cold shock. Leakage of U.V.-absorbing material from thick suspensions of A.aerogenes was found to occur despite the apparent resistance to cold shock and the pattern of leakage was similar to the pattern of cell death exhibited by suspensions containing lower concentrations of culture (approx. 10^7 cells/ml) (Strange & Ness, 1963). This lack of viability loss with thick suspensions was presumed to be due to the protection afforded by leakage products in the suspending medium.

If the bacteria are cold shocked and then immediately rewarmed to growth temperature, little or on loss of viability occurs. In experiments with P.aeruginosa (Gorrill & McNeil, 1960), A.aerogenes (Strange & Dark, 1962) and E.coli (Sato & Takahashi, 1969) it has been found that as the length of time before cells are rewarmed increases, so the viability decreases. This is compatible with the hypothesis that the loss of essential intracellular material causes death; consequently prompt removal from the cold stress reduced the drop in viavility.

The material leaked by cold shocked cells protects fresh cells from cold shock (Strange & Dark, 1962; Farrell & Rose, 1968). Of the leakage material from A.aerogenes, Strange & Dark (1962) found that it was the mixture of amino acids, added without the ATP or nucleic acid bases that provided significant protection against cold shock. This protection of leakage material has been

shown to be non-specific for two strains of P.aeruginosa studied by Farrell & Rose (1968). The leakage products from the mesophilic strain protected the psychrophlic strain against cold shock, and vice versa.

The physiological age of a bacterial culture determines its susceptibility to cold shock. Using broth grown E.coli in batch culture, Hegarty & Weeks (1940) found that cells in the lag phase were very resistant to cold shock and as the cells entered the exponential phase the sensitivity increased, reaching a maximum in the middle of this phase. As the cells entered the stationary phase, so the resistance increased again. This pattern has been repeated with E.coli (Meynell, 1958; Van Soestbergen, Steingruber, Weiss & Oles, 1968; Sato & Takahashi, 1968b) and also with other species (P.aeruginosa: Gorrill & McNeil, 1960; A.aerogenes: Strange & Dark, 1962; P.fluorescens: and B.subtilis: Sato & Takahashi, 1968a). Hegarty & Weeks (1940) observed that the growth rate of the E.coli they used was variable within the exponential phase of growth; indeed, the growth curved appeared characteristic of a partially synchronised culture. It was observed that during periods of slower growth (presumably 'lag' phase) in the exponential phase, that the cells were more resistant to cold shock. Strange & Dark (1962) found similar effects when they studied the effect of cold shock on a partially synchronised culture of A.aerogenes. In both these reported cases the resistance of the cells was maximal during the first period of cell division. The resistance then either increased slightly (E.coli: Hegarty & Weeks, 1940) or remained constant (A.aerogenes:

Strange & Dark, 1962) until the second cell division occurred when the decrease continued. These results indicate that bacteria are more susceptible to cold shock during septation and division and that 'newly formed' cells appear to be more susceptible than 'older' cells.

Evidence has been reported (see section on the mechanism of cold shock) that cold shock causes a phase change in the membrane P.L. and thus allows the leakage of essential substrates from the bacteria. Sherman & Cameron (1934) and Meynell (1958) have observed that if E.coli cells were slowly cooled (approx. 1°/min) to the cold shock temperaterature, then no loss of viability occurred, even in potentially lethal diluents like distilled water. These reports indicate that the cells have time to adapt to the lower temperature before the transition temperature is reached. The nature of this adaption is obscure and neither Sherman & Cameron (1934) or Meynell (1958) reported whether or not cell leakage occurred, so no comment can be made about the permeability of cells chilled in this manner.

The divalent cations Mg^{2+} and calcium (Ca^{2+}) have been reported to protect bacteria against the effects of cold shock (P.aeruginosa: Gorrill & McNeil, 1960; A.aerogenes: Strange & Dark, 1962; Strange & Postgate, 1964; B.subtilis; P.fluorescens and E.coli: Sato & Takahashi, 1969). In most cases Mg^{2+} on its own provided sufficient protection, but the recovery of B.subtilis also required the presence of acid-hydrolysed casein (Sato & Takahashi, 1969). The protective effect of Mg^{2+} and Ca^{2+} upon cold shocked A.aerogenes is interesting as

no trace of these cations was found in the leakage products of chilled cells (Strange & Dark, 1962), although Mg^{2+} has been observed to leak from cold shocked exponential and stationary phase E.coli (Sato & Takahashi, 1970). The protective action of Mg^{2+} ties in well with its property of stabilizing spheroplast membranes (Lederberg, 1956). Strange (1964) has shown that when Mg^{2+} was added to cold shocked A.aerogenes plasmolysis of the cells by 4 M-saline occurred, whereas no plasmolysis was observed if only 4 M-saline was added. It was suggested that the Mg^{2+} stabilized the membranes, thereby restricting entry of saline, resulting in plasmolysis. Strange & Postgate (1964) reported that Mg^{2+} protected cold shocked A.aerogenes against the action of RNase, presumably either by stabilizing the ribosomes or the envelope membranes. The protective action of Mg^{2+} ions is employed in the procedure of C.O.S. for extracting cell wall enzymes (Heppel, 1967) whilst retaining the viability of the cells concerned (Patching & Rose, 1971). Presumably the Mg^{2+} ion stabilizes the cytoplasmic membrane, but not the outer membrane, since no essential metabolites leave the cell, but the cell wall enzymes pass readily into the cell menstruum. This apparent specificity in the pretective action of the Mg^{2+} may reflect the quantitative difference between the P.L. and protein content of the two membranes (Costerton et al., 1974).

In conclusion, cold shock occurs when a relatively dilute (10^8 cells/ml or less) suspension of bacteria in the exponential phase of growth are cooled very rapidly to temperatures within the range 4° to -2° in a hypotonic

diluent or distilled water. The available evidence suggests that the primary effect is membrane damage involving transition of the P.L. in the cytoplasmic membrane (and probably the outer membrane in Gram-negative organisms) from the liquid to the liquid-crystalline phase. The result of this transition is a distortion of the membrane/s, resulting in the loss of low molecular weight metabolites, cell wall bound enzymes and transport proteins, some of which are responsible for active transport of substrates into the cells. There is some evidence that an inhibitor of RNase is released, and that the action of RNase, coupled with the apparent inactivity of DNA ligase and leakage of metabolites, leads to cell death.

Cold shock exerts its effect primarily by an alteration of the cell membrane/s. In this respect it is significant that P.aeruginosa is markedly sensitive to both cold shock and certain chemical agents that attack, and alter, the permeability of the cell envelope and the outer membrane (E.D.T.A.: MacGregor & Ellicker, 1958; Wilkinson, 1967; Brown & Melling, 1969a; Roberts, Gray & Wilkinson, 1970; polysorbate 80: Brown & Winsley, 1969; polymyxin: Newton, 1953a,b; Brown & Melling, 1969b). The possibility that these effects are related, presumably due to the nature of the Pseudomonad cell wall, has been suggested (Brown, 1971). As will be shown later in this thesis, growth of P.aeruginosa in Mg^{2+}-limited medium resulted in a decrease in the sensitivity of this organism to cold shock. These results are compatible with the observed decrease, under similar growth conditions, in the sensitivity of P.aeruginosa to E.D.T.A. and polymyxin

(Brown & Melling, 1969a,b), and provide further evidence for the hypothesis that the cell wall may be the common factor in the marked sensitivity of P.aeruginosa to certain membrane active drugs and cold shock.

EXPERIMENTAL

1. MATERIALS

Organism.

The organism used in this study was Pseudomonas aeruginosa N.C.T.C. 6750 (P.aeruginosa). The culture was obtained as a freeze dried sample from the National Collection of Type Cultures, Central Public Health Laboratory, Colindale Avenue, London NW9.5HT. On receipt, the organism was reconstituted according to the directions supplied with the sample, and cultured in nutrient broth at 37°for 24-hr. The homogeneity of the culture was checked by streaking onto nurtient agar (N.A.) and milk agar plates (Brown & Scott-Foster, 1970), to obtain single colonies. Isolated single colonies were used to prepare N.A. slopes which, after incubation at 37°for 24-hr, were stored at room temperature protected from light. Fresh agar slope subcultures were prepared by the above procedure at approximately three monthly intervals.

Using a wire loop a sample of culture from an agar slope was streaked onto a N.A. plate and incubated at 37°overnight. An isolated colony was removed with a wire loop and inoculated into chemically defined medium (C.D.M.), incubated, and then subcultured once more into C.D.M. before use as an inoculum for growth experiments. The inoculum was maintained by subculturing daily into fresh C.D.M., and used as an inoculum for each experiment for two weeks. At the end of two weeks this inoculum subculture was rejected and a fresh inoculum started as described.

Media.

Nutrient broth: Oxoid nutrient broth, C.M.1.

Nutrient agar: Oxoid nutrient agar, C.M.3.

Both the above media were obtained from Oxoid Ltd., Southwark Bridge Road, London S.E.1., and were prepared according to the manufacturers instructions. Both media were sterilized by autoclaving at 121° for 15-min.

Milk agar: This medium was prepared according to the method described by Brown & Scott-Foster (1970).

Chemically defined medium: The medium described below was used for routine subculturing of P.aeruginosa prior to experiments involving C.D.M.. This medium was modified for limitation and lysis experiments as described later.

Constituent	Final Concentration	
$(NH_4)_2HPO_4$	3.904×10^{-2} M	4.000×10^{-2} M
$NH_4H_2PO_4$	9.600×10^{-4} M	
$(NH_4)_2SO_4$	2.000×10^{-4} M	
NaCl	1.000×10^{-3} M	
KCl	1.000×10^{-3} M	
$MgSO_4.7H_2O$	1.645×10^{-4} M	
$FeSO_4.7H_2O$	8.950×10^{-6} M	
Glucose	2.000×10^{-2} M	

pH before inoculation 7.8.

The first five medium constituents were sterilized together by autoclaving at 121° for 15-min. The remaining constituents were sterilized separately, also by autoclaving. For subculturing, the first five medium constituents were made up as 2.5-fold and the last three as 5.0-fold stock solutions, and were aseptically added together and made up to volume with sterile water in the culture vessels. Medium constituents for 6 l batch

culture experiments were prepared as 30-fold (first five) and 60-fold (last three) stock solutions. In order to prevent the ferrous ion precipitating as ferric oxide, 0.1 ml hydrochloric acid (11.6 N) was added to every 100 ml of $FeSO_4.7H_2O$ stock solution, prior to sterilization.

Chemicals.

All the chemicals used in the preparation of C.D.M. were of AnalaR grade and were obtained from British Drug Houses Ltd., Poole, Dorset; Fisons Scientific Apparatus Ltd., Loughborough, Leicestershire; Hopkins & Williams Ltd., Chadwell Heath, Essex. All other chemicals were also of AnalaR grade unless stated to the contrary.

Trypsin (E.C. 3.4.4.4.) from beef pancreas, was crystallized, with an activity of 7,500 - 8,000 B.A.E.E. units/mg, and obtained from British Drug Houses Ltd..

Ribonuclease (E.C. 2.7.7.16.) from bovine pancreas, was four times crystallized, salt-free, and with an activity of approximately 50 Künitz units/mg; obtained from British Drug Houses Ltd..

Polymyxin B sulphate, B.P. was obtained as 'Aerosporin' from Burroughs Welcome & Company, London, in vials containing 500,000 International units.

Authentic 2-keto,3-deoxy octonic acid (K.D.O.), as the 1,4-lactone derivative, was gratefully received as a gift from Dr. B. A. Key, British Drug Houses Ltd..

Phospholipid standards were obtained from various suppliers: phosphatidyl glycerol - Lipid Products Ltd., Nutfield Nurseries, South Nutfield, Redhill, Surrey; cardiolipin (extracted from bovine heart) - Koch Light Laboratories Ltd., Colnbrook, Buckinghamshire; lecithin

(grade 1, extracted from egg) - British Drug Houses Ltd.; phosphatidyl-ethanolamine (extracted from egg) - Koch Light Laboratories Ltd..

Water was deionized prior to glass distillation, unless stated to the contrary.

Apparatus.

Spectrophotometers: Optical density measurements for growth curves and lysis experiments, and absorbance measurements of the coloured products of chemical assays, were made using a Unicam S.P. 600. Atomic absorption spectroscopy was carried out using a Unicam S.P. 90. For growth curves and lysis experiments 1 cm matched, glass cuvettes were used, but the chromophores and blanks from chemical assays were measured in matched, 1 cm fused quartz cuvettes. All spectrophotometers were obtained from Pye-Unicam Instruments Ltd., York Street, Cambridge.

Measurements of pH were made using the Pye model 290 pH meter, obtained from Pye-Unicam Instruments Ltd..

The Mickle reciprocating shaker bath and the Mickle tissue disintegrator were obtained through Camlab Ltd., Nuffield Road, Cambridge, CB4.1TH.

Centrifuges: Whole cells from 6 L batch cultures were harvested using a Sharples Super Centrifuge (rotating bowl type), obtained from Sharples Centrifuges Ltd., Camberley, Surrey. Cell wall preparations and fractions were centrifuged using either the International refrigerated centrifuge, model B20, manufactured by the International Equipment Company, 300 Second Avenue, Needham Heights, Massachusetts, U.S.A., or the M.S.E. Superspeed 18 refrigerated centrifuge, obtained from

Measuring & Scientific Equipment Company Ltd., Manor Royal, Crawley, Sussex.

Microscopical examinations of cultures were carried out using a 'Wild' model M20, binocular, phase contrast microscope, obtained from Micro Instruments (Oxford) Ltd., 7, Clarendon Street, Oxford, OX1.2PH.

The 'Chromoscan 200' and 'Scan 201' thin-layer chromatogram scanning and integrating equipment were manufactured by Joyce, Loebel & Company Ltd., Gateshead, NE11.0UJ, and supplied through Talbot Instruments, 6, Trafford Road, Alderley Edge, Cheshire, SK9.7NT.

Millipore membrane filtration apparatus was obtained from Millipore U.K. Ltd., Heron House, Wembley, Middlesex.

Automatic M.L.A. pipettes, 0.1 ml and 0.25 ml sizes, and tips, were obtained through Frost Instruments Ltd., Wokingham, Berkshire, RG11.1BZ.

Small (5 ml) hand operated tissue homogenisers and plungers of glass construction were obtained from F.T. Scientific Instruments Ltd., Rosebank Parade, Yeteley, Surrey.

Treatment of glassware.

Glassware was washed by total immersion in 5% v/v Decon 90 (British Drug Houses Ltd.,) for 30-min at 100°, or overnight at room temperature. On removal from the Decon 90, the glassware was rinsed once with distilled water, immersed in 1% v/v hydrochloric acid and then rinsed a further eight times in distilled water. After rinsing, the glassware was air dried at 60°, closed with aluminium foil and sterilized by heating at 160° for 3-hr.

Treatment of membrane filters.

Before use plain 0.2 μ and 0.45 μ pore size membranes were boiled in three changes of distilled water. Brown, Farwell & Rosenbluth (1969) found that this treatment removed wetting agents and other chemicals that affect the light absorption of filtered solutions.

The solvent resistant membranes used to remove residual cell walls from lipid extracts, were placed in the filter holders and washed by passing four x 2.5 ml volumes of the relevant solvent through them. This washing was necessary since it was observed that the first two washings were discoloured by materials from the filter. Washing could not be carried out by immersing the membranes prior to assembly, since in all solvents the membranes underwent lateral distortion and became too flaccid to handle and assemble without rupturing.

2. BASIC EXPERIMENTAL METHODS.

Colony counts.

A suspension of Pseudomonas aeruginosa grown in chemically defined medium (C.D.M.) to an optical density ($O.D._{470}$) of about 0.2 was sufficiently diluted with C.D.M. to allow a total count to be made. The result of the total count was used as the basis for making a series of dilutions of the culture such that 0.2 ml of the final dilution contained between 100 and 300 cells.

Five replicate dilutions of 4×10^5-fold were made using nutrient broth (N.B.) at room temperature as diluent. From each of the five final dilutions, five aliquots of 0.2 ml were spread on over-dried nutrient agar plates. The plates were incubated at 37° for 24-hr and the resulting colonies counted. Incubation for a further 24-hr showed no increase in colony count. The results are shown in Table 1.

TABLE 1

Colony counts per plate for five replicate counts.

Counts / Plates	A	B	C	D	E	Means
1	98	83	87	120	95	96.6
2	112	132	82	112	96	106.8
3	95	87	71	102	116	94.2
4	88	91	89	100	122	98.0
5	103	113	90	102	95	100.6
Totals T	496	506	419	536	524	-
Means	99.2	101.2	83.8	107.2	104.8	-

The results in Table 1 were subjected to an analysis of variance as shown in Table 2.

n = number of observations per count = 5

m = number of counts = 5

$n.m$ = total number of observations = 25

(1) $\sum x^2 = 251171.00$

(2) $\frac{\sum T^2}{n} = 247897.00$

(3) $\frac{(\sum x)^2}{n.m} = 246214.44$

TABLE 2

Analysis of variance of five replicate counts.

Source of variance	Sum of squares	Degrees of freedom	Mean squares	Variance ratio (F)
Between counts	(2) - (3) 1682.55	m - 1 4	420.64	2.55
Within counts	(1) - (2) 3274.00	n.m - m 20	163.70	

The tabulated values of F for $^4/_{20}$ degrees of freedom at 5% and 1% significance are 2.87 and 4.43 respectively. Therfore the variation between counts is not significantly greater than the variation within counts. Using the means from Table 1 the coefficient of variation (standard deviation/mean) is 9.24% between counts and 4.86% within counts.

Total counts.

Total counts were made using haemocytometer counting chambers having a chamber depth of 0.1 mm and 'Improved Neubauer' rulings. These chambers conformed to British Standard 748 (1963) and were obtained from Hawksley & Son Ltd., 12 Peter Road, Lancing, Sussex.

A suspension of P.aeruginosa grown in C.D.M. to an $O.D._{470}$ of about 1.2 was diluted 5-fold with C.D.M. containing 5% $^{v}/_{v}$ formaldehyde solution B.P. to kill the bacteria. This dilution was used to fill a counting chamber as follows. The cover slip was pressed down firmly over the counting grid until Newton's rings were visible at both areas of contact. Using a clean Pasteur pipette the dilution was allowed to run into the chamber. If the chamber contained air bubbles, or if the dilution had overflowed into the troughs at the side of the counting grid it was rejected. The chamber was viewed to check that each small square contained approximately 5 to 10 cells. To allow the bacteria to settle onto the grid, the chamber was set aside for 30-min before counting. To prevent drying out during this time the chamber was placed in a closed Petri dish containing wet filter paper. The chamber was viewed using a phase contrast microscope with a times 40 objective and a times 15 binocular eye piece. The bacteria in 80 preselected small squares were counted. Five similar counting chambers were each used as described to perform five replicate total counts. The results are shown in Table 3, and subjected to an analysis of variance as shown in Table 4.

TABLE 3

Total counts per slide for five replicate counts.

Slides / Replicates	A	B	C	D	E	Means
1	7.90	7.15	7.35	7.45	7.75	7.52
2	8.60	9.60	7.10	8.50	7.80	8.32
3	7.75	7.35	6.55	8.60	8.80	7.81
4	7.40	7.55	7.50	8.10	9.10	7.93
5	8.15	8.65	7.75	8.00	7.75	8.06
Totals T	39.80	40.30	36.25	40.65	41.20	-
Means	7.96	8.06	7.25	8.13	8.24	-

The tabulated figures are the mean number of cells per small square of the grid based upon 80 small squares in each count.

n = number of counts per slide = 5

m = number of slides = 5

$n.m$ = total number of observations = 25

(1) $\Sigma x^2 = 1582.9300$

(2) $\frac{\Sigma T^2}{n} = 1574.4110$

(3) $\frac{(\Sigma x)^2}{n.m} = 1571.3296$

TABLE 4

Analysis of variance of five replicate total counts.

Source of variance	Sum of squares	Degrees of freedom	Mean squares	Variance ratio (F)
Between slides	(2) - (3) 3.0814	m - 1 4	0.77035	1.81
Within slides	(1) - (2) 8.5190	n.m - m 20	0.42595	

The tabulated values of F for $^{4}/_{20}$ degrees of freedom at 5% and 1% significance are 2.87 and 4.43 respectively. Thus, the variation between counts is not significantly greater than the variation within counts. Using the means from Table 3 the coefficient of variation was found to be 3.74% within counts and 4.95% between counts.

Batch culture of P.aeruginosa in 6 L volumes.

The apparatus used was the same as described by Boggis (1971) which was similar to the system used by Harvey, Fewson & Holmes (1968). Medium (6 L), contained in a round, 10 L flat-bottomed flask, was stirred by a magnetic follower. The speed of the follower was adjusted such that the vortex produced extended down to the follower. As a result of the vortex touching the follower numerous small air bubbles were produced in the medium, thus providing a large surface area for gaseous exchange between the atmosphere and the culture. Air that had previously been passed through a sterile 0.45 μ membrane filter was blown into the culture vortex via an inlet tube positioned such that it was approximately 1 cm above the liquid level

when the follower was stationary. The issuing air was bubbled through 70% $^{v}/v$ ethanol to prevent the release of culture aerosol into the laboratory. The 10 L flasks used were calibrated to contain 5 and 6 L volumes. To prepare 6 L of medium, a clean empty flask containing a polytetrafluorethylene (P.T.F.E.) coated follower was filled with water to the 5 L calibration mark. The flask was closed with foil and autoclaved at 121° for 60-min and allowed to cool. Stock solutions previously sterilized were added aseptically and the volume made up to the 6 L mark with sterile water.

Harvesting of whole cells from 6 L cultures.

One hour after the culture had reached $O.D._{470}$ 1.0, the magnetic follower was switched off. The culture was siphoned, via a 200 cm glass coil immersed in crushed ice, into the bowl of a rotating bowl type continuous centrifuge. The bowl speed was approximately 22,000 rpm, equivalent to a relative centrifugal force of 12,000 x 'g'. The flow rate into the bowl was adjusted so that 6 L of culture was centrifuged in approximately 50-min, with 90% to 95% removal of bacterial cells. The harvested cells were scraped from the bowl into a 250 ml capacity centrifuge bucket containing 200 ml sterile water at 4°, and centrifuged at 7,500 x 'g' for 15-min in a refrigerated centrifuge. The cells were washed in two more changes of sterile water. The washed pellet was resuspended in approximately 30 ml water and the 'slurry' stored deep frozen until required for cell wall preparation.

The culture was cooled during harvesting to prevent any enzymic activity which may have caused lysis

of cells, and hence loss of cell wall material. The temperature of the culture emerging from the cooling coil was 15°. The continuous centrifuge rotor was precooled to 4° before use, but the temperature rose to approximately 15° by the end of the harvesting process. All subsequent centrifugation of whole cells and cell walls were performed in refrigerated centrifuges maintained between 0° and 5°.

Preparation of cell walls of P.aeruginosa.

The thawed whole cell suspension harvested as described above, was gently shaken to ensure disperal of cells in the water. Aliquots of 7 ml were placed into each of 4 tubes designed to be used with the Mickle tissue disintegrator. Into each tube 2 ml of water and 8 ml of 0.1 mm to 0.2 mm glass beads (Ballotini Glass Manufacturing Company, Pontefract Road, Barnsley, Yorkshire) were added, and the tubes stoppered with rubber bungs. The remaining whole cell suspension was accurately measured into a tared centrifuge tube and centrifuged at 7,500 x 'g' for 15-min. The pellet was dried over phosphorus pentoxide in vacuuo to a constant weight and the result used to determine the total dry weight of cells used for preparation of cell walls.

The glass beads and whole cell suspensions were violently agitated by the Mickle tissue disintegrator for 90-min at 4°. Over the period of agitation the temperature in the tubes rose from 0° to 20°. Residual whole cells, glass beads and small glass fragments were removed by centrifugation at 3,500 x 'g' for 20-min. The pellets were bulked, resuspended, and then disrupted for a further 45-min. The broken cell suspensions were bulked and

centrifuged at 13,000 x 'g' for 30-min. The resulting pellet consisted of three or four layers which were gently washed off one by one and examined microscopically. The bottom layer consisted of residual whole cells remaining from the first centrifugation. The upper layers contained large and small cell wall fragments, 'empty' cells and, in the top most layer, large membrane particles. The top layers were bulked and washed once more with water before enzyme treatment. The residual whole cells were discarded.

Crude cell walls were purified by enzyme digestion, using trypsin and ribonuclease as described by Gray & Wilkinson (1965). After enzyme digestion, the cell walls were washed twice with 0.9% saline, and four times with water. The final 'clean' pellet was resuspended in 25 ml water. An aliquot (12.5 ml) of this suspension was placed in tared centrifuge tubes and centrifuged at 13,000 x 'g' for 30-min. The pellet was dried over phosphorus pentoxide in vacuuo to constant weight. The remaining 12.5 ml of known dry weight/ml, was stored deep frozen (-15°).

Extraction of lipids from cell walls.

Dried cell walls, about 75 mg (accurately weighed), were suspended in 50 ml of chloroform/methanol solvent (2:1 by volume) containing 0.01%, by weight, β-hydroxytoluene as antioxidant (Brown & Watkins, 1970). The cell walls were extracted for 90-min at 37°, with shaking. The cell walls were removed by centrifugation, and re-extracted with a further 50 ml of fresh solvent for 90-min. The two extracts were pooled and the volume reduced to about 5 ml using a rotary evaporator, operating at a temperature

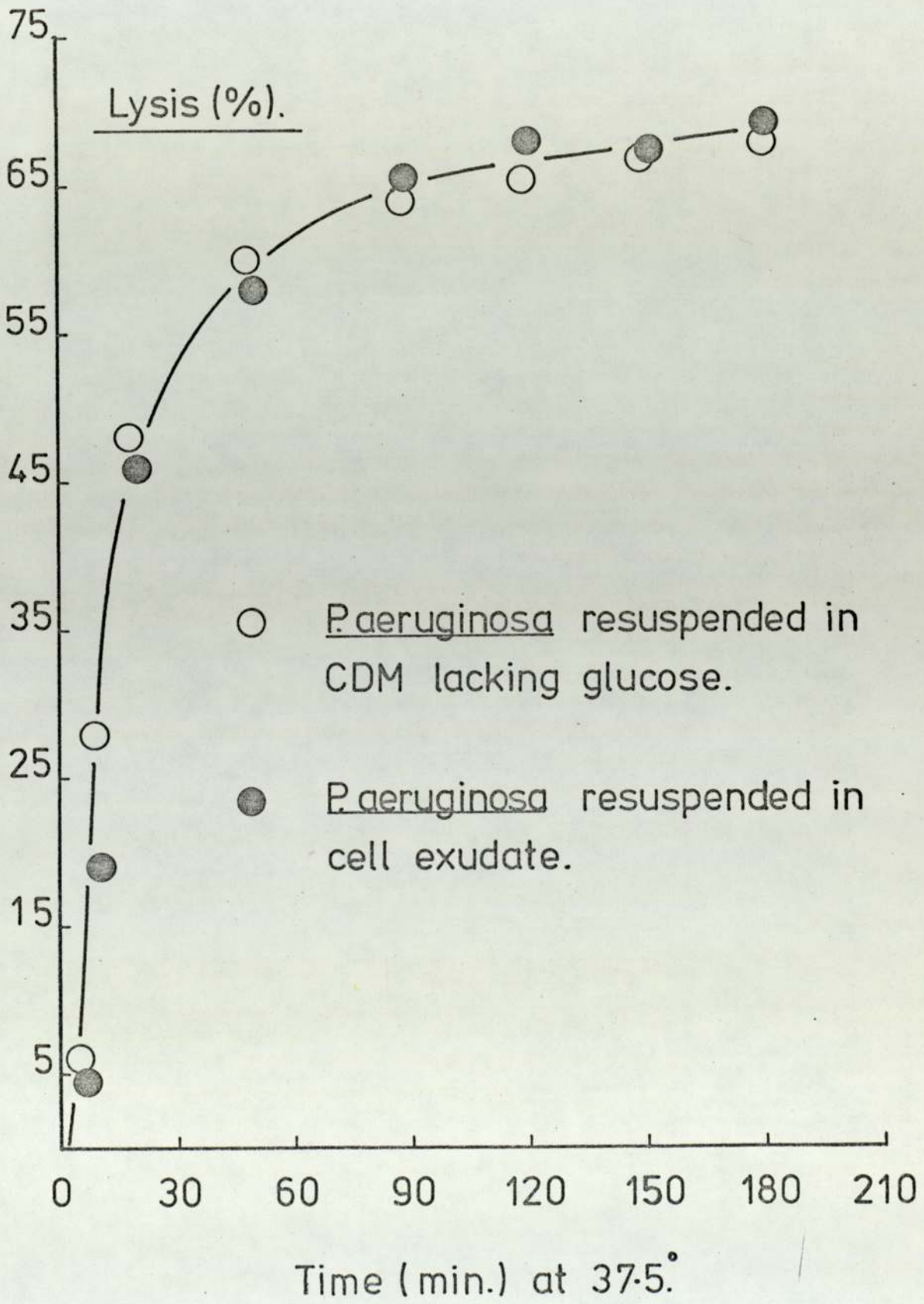
of 45°. The 5 ml of lipid extract was filtered through a 0.2 μ, solvent resistant membrane, to remove residual walls. The filter was washed with three 5 ml volumes of solvent, and the extract and washings bulked and evaporated to dryness. The readily extractible lipids (R.E.L.) and extracted walls were dried over phosphorus pentoxide in vacuuo to a constant weight. After the R.E.L. had been weighed it was taken up in a small volume (1 ml) of solvent and excess (30 ml) diethyl ether at -10° added to precipitate β-polyhydroxybutyrate (Brown & Watkins, 1970). The precipitated β-polyhydroxybutyrate (P.H.B.) was removed by centrifugation and the etheral extract evaporated to dryness. The ether soluble lipids were dried over phosphorus pentoxide in vacuuo to a constant weight. The P.H.B. fraction was retained. The ether soluble lipids (phospholipids, free fatty acids and neutral lipids) were weighed and taken up in 1 ml of solvent. Excess (30 ml) acetone at -10° was added to precipitate the phospholipids (P.L.). The P.L. were removed by centrifugation and the acetone evaporated to dryness. The acetone extract, which contained the free fatty acids and neutral lipids (F.A.N.), was dried to a constant weight over phosphorus pentoxide in vacuuo. The weights of P.H.B. and P.L. were estimated by the differences between R.E.L. and (P.L. + F.A.N.) for P.H.B., and (P.L. + F.A.N.) and F.A.N. for P.L.. The P.L. was further fractionated by thin layer chromatography using the solvent system (A) described by Minnikin & Abdolrahimzadeh (1971). The ratios of the standard P.L. in the sample were determined by densiometry using the Chromoscan 200 as described in section 4.

Procedure used to induce cold shock in cultures of P.aeruginosa.

Cultures of P.aeruginosa were grown to O.D.$_{470}$ 1.0 as previously described (section 2). The cells were washed twice by centrifugation (13,000 x 'g' for 1-min) with warm (37°) 0.04 M phosphate buffer, pH 7.8, containing ferrous sulphate, sodium chloride, potassium chloride and ammonium sulphate in the same concentrations as the growth medium described in section 1. The rotor was prewarmed to 37° and the short centrifugation used was found to be sufficient to produce a firm pellet. The temperature of the supernatent from the final washing was found to be within the temperature range 33° to 37°. The washed cells were resuspended in fresh washing medium to an O.D.$_{470}$ 1.0, and two 25 ml aliquots removed. One aliquot was added, with 'swirling', to 100 ml of the washing medium, precooled to 1°, and maintained at 1° on crushed ice for 60-min. This cold shocked culture was rewarmed slowly to 37° by leaving at room temperature for 30-min, then at 30° for 15-min and finally at 37° for 15-min. A control flask was set up at the same time in which 25 ml of washed cells were added to 100 ml of the washing medium maintained at 37°. When the cold shocked cells had been rewarmed, samples were removed from both the cold shocked and control cultures and viable counts prepared. The optical densities were read and the control culture diluted to a similar value as the cold shocked culture. The cultures were then used for lysis experiments as previously described. It was not possible to wash the cold shocked cells since the pellet obtained from the

first centrifugation could not be resuspended without the formation of 'clumps'. In order to determine whether the material released by the cold shocked cells interfered with the lysis by polymyxin, the following experiment was performed. A culture was cold shocked and the cells removed by centrifugation (7,500 x 'g' for 15-min). Cells from a control culture were washed and half the cells resuspended in the supernatent from the cold shocked cells. The remaining half were suspended in washing medium. The two resuspended cultures were then used for a lysis experiment involving polymyxin as the lytic agent. No enhancement or inhibition of polymyxin induced lysis was observed in the culture resuspended in the supernatent from cold shocked cells (see Fig. 5). Since polymyxin is antagonised by divalent cations it was assumed that there was not a sufficient concentration of cations in the exudate of cold shocked cells to antagonise the polymyxin concentration used.

Figure 5. Effect of cell exudate, from cold shocked P. aeruginosa, upon Polymyxin (50 U. ml^{-1}) mediated lysis of P. aeruginosa grown in Mg^{2+}-adequate CDM.



3. MEASUREMENT OF TURBIDITY.

Introduction.

When a parallel, monochromatic beam of light enters a suspension of cells, some of it will be diverted from the light path. This 'scattering' is due to reflection and refraction at the cell surface-medium interface, and produces the effect known as turbidity. This turbidity may be quantified by measuring the intensity of refracted light emerging from a culture, nephelometry, or by measuring the intensity of the undiviated light emerging from the culture, turbidimetry. The intensity of the emerging light may also be affected by absorption due to bacterial pigments, for example, pyocyanin in Pseudomonas aeruginosa, especially when working with very high concentrations of bacteria.

The incident light (I_o) and the emerging light (I) are related at relatively low bacterial concentrations by the Beer-Lambert Law:

$$I = I_o . 10^{-e.l.c}$$

where 'e' is the extinction coefficient, 'l' the distance the light travels through the culture, and 'c' is the concentration of bacteria. The extinction coefficient is unique, not only for each bacterial species, but also for differently treated cultures of the same species (Spaun, 1962). The more usual expression of the Beer-Lambert Law is:

$$\log_{10} (I_o/I) = e.l.c$$

The term $\log_{10} (I_o/I)$ is refered to as the optical density (O.D.).

Spectrophotometers that give readings in terms of $\log_{10}(I_o/I)$ are considered to be the most suitable for the measurements of turbidity (Monod, 1949) since they avoid confusion due to the use of arbitary units or 'galvanometer deflections'. The Unicam S.P. 600 spectrophotometer measures optical density over the wavelength range 350 to 1000 nm, and was used for all turbidity measurements during the course of this work.

Selection of wavelength.

When selcting a suitable wavelength to use in measuring turbidity of a bacterial culture, it is important to try and satisfy the following conditions:-

1) The medium constituents should exhibit little or no absorption.
2) Similarly metabolic products should also exhibit little or no absorption.
3) The wavelength used should be that which imparts maximum sensitivity to changes in optical density.

The total amount of light scattered, and therefore the intensity of undiviated light, is directly proportional to the ratio of cell size to the wavelength of the incident light (Koch, 1961). From this ratio it follows that for a given type of bacterium the shorter the wavelength, the more sensitive the instrument will to optical density changes. Reference to condition 2) above shows that light in the ultraviolet wavelengths will be of little use since proteins and nucleic acids absorb light in this range (<380 nm). Workers using nutrient broth cultures use wavelengths in the region 650 - 660 nm since there is little light absorption by broth at these wave-

lengths. When using non-pigmented organisms, other workers have found that the lowest wavelength to satisfy the three conditions mentioned above was 420 nm (Hodges, 1973; Handley, Quesnel & Sturgiss, 1974). P.aeruginosa however, produces a pigment, a pyocyanin, that exhibits an absorption maximum at 388 nm. Moreover, the absorption, although not maximal, is still significant at wavelengths below 460 nm (Watkins, 1970).

The culture filtrate from a 24-hr culture ($O.D._{470}$ approximately 2.0) grown in chemically defined media (C.D.M.), showed only a slight absorption at 470 nm. When culture filtrates from cultures of $O.D._{470}$ of 0.7 or less were observed, no absorption was detected. The wavelength used throughout this study for the measurement of growth or lysis was 470 nm.

Sampling of cultures for optical density measurements used in the construction of growth and lysis curves.

Growth limitation or lysis experiments were usually performed using 25 ml of medium in 100 ml conical flasks. The media for limitation studies involving small volumes (25 ml) were inoculated approximately 15-hr prior to the initial optical density reading ($O.D._{470}$). An inoculum culture was diluted 200-fold with C.D.M. lacking glucose and magnesium (Mg^{2+}), and 0.25 ml of this dilution used to inoculate 25 ml volumes of the limitation media. Using this procedure, it was empirically found that with an inoculum culture of $O.D._{470}$ 0.8 to 1.0, media inoculated at 17.00-hr on a given day would be at an $O.D._{470}$ 0.02 to 0.05 by 08.30-hr the following day.

When performing lysis experiments, a lysis medium

was prepared which contained all the constituents of the growth medium used to grow the culture to be lysed except the limiting nutrient, which was replaced by water. All cultures used in lysis experiments were limited by glucose at an $O.D._{470}$ of 1.0. 5 ml of the culture at $O.D._{470}$ 1.0 were placed into a 100 ml conical flask containing 20 ml of prewarmed (37.5°) lysis medium and the contents mixed. The optical density of the diluted culture was read immediately before the addition of the lytic agent, and assumed to be the optical density of the suspension at time zero.

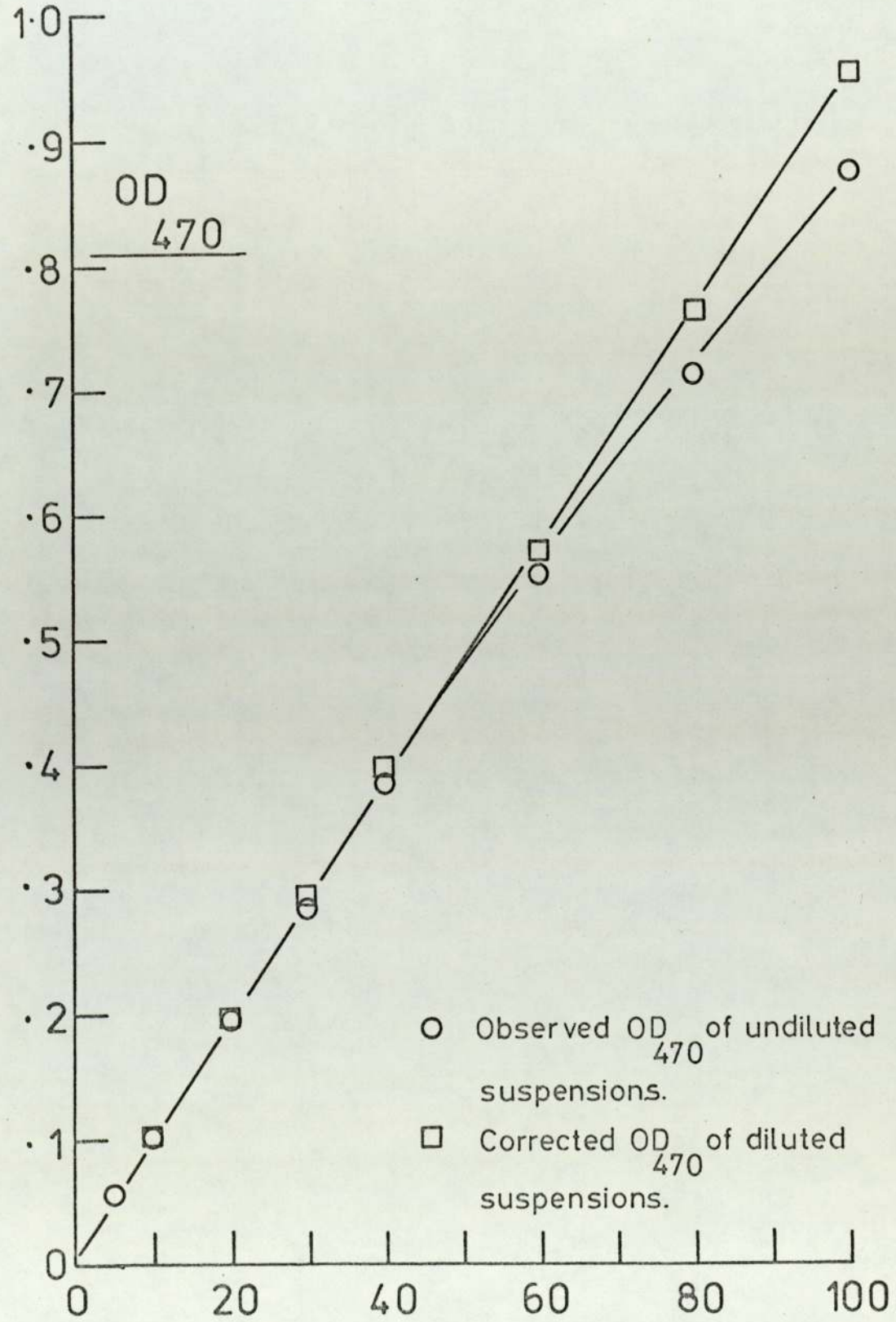
For both lysis and growth limitation experiments the 100 ml conical flasks were incubated at 37.5° in a Mickle reciprocal shaking water bath with a shaking rate of 120 x 5.4 cm throws per min. Samples were removed at timed intervals of about 30-min for growth experiments and about every 5-min at the start of lysis experiments. After the first 30-min, samples for lysis experiments were also taken at 30-min intervals. Using a Pasteur pipette enough culture to fill the cuvette to within 0.5 cm of the top (approximately 3 ml) was removed and placed in a 1 cm glass cuvette and the optical density measured. In view of the small volume of the culture used in an experiment (25 ml) the sample was returned to the flask after each reading, thus maintaining aeration conditions and allowing a large number of readings to be made. In the time taken for a reading to be made (approximately 1-min) the temperature of the sample fell to 30°, and on returning it to the flask the temperature in the flask dropped to 36°. The temperature returned

to 37.5° within 30-sec; no deleterious effects of this cooling were observed, neither was there any observable contamination of cultures during the course of an experiment. As the majority of experiments involved the use of several flasks containing different media or lytic agents each flask was supplied with a separate Pasteur pipette. After each reading had been taken and the sample returned to the flask the cuvette was rinsed with water and drained by touching the open end of the inverted cuvette onto absorbant paper.

Relation between optical density and cell concentration.

A series of dilutions of a stationary phase culture of P.aeruginosa were prepared using C.D.M. without glucose as diluent and the optical density of the suspensions measured. The relationship between optical density and cell concentration obeyed the Beer-Lambert Law up to an $O.D._{470}$ of 0.3, above which the optical density increased less than predicted in proportion to the increase in cell concentration (Fig. 6). When the same suspensions were diluted with the same diluent to give readings in the range $O.D._{470}$ 0.03 to 0.28 linearity was restored, at least up to a 'real' $O.D._{470}$ of 1.0. The decrease in the amount of light scattering exhibited by dense cultures may be due to secondary light scattering redirecting the refracted light towards the photcell in the spectrophotometer (Koch, 1961). Alternatively, or in addition, the cells closest to the light source may 'shield' cells further away from the light source, thus preventing their full contribution to the light scattering.

Figure 6. Relation between real and observed optical density of P. aeruginosa suspensions.



Correlation between optical density and viable counts of a culture of P.aeruginosa.

25 ml of C.D.M. were inoculated with 0.25 ml of a late log phase culture of P.aeruginosa and the growth monitored as described previously. Every 60-min, in addition to taking optical density measurements, suitable dilutions of 1 ml samples of the growing culture were made to prepare five replicate plates for a viable count determination. Growth curves were constructed by plotting log viable count and log $O.D._{470}$ against time (Fig. 7). Both curves exhibited similar characteristics and the exponential growth rate constants were the same (4.78×10^{-3}/min $\equiv$ to a doubling time of 63-min). The relationship between viable counts and optical density was observed to be linear (Fig. 8). The data used to construct Fig. 8 was also used to calculate the correlation coefficient 'r', using the formula:

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

where 'C' is the covariance, $\sum(x.y - \bar{x}.\bar{y})/n - 1$, '$S^2x$' the variance of the optical density readings, $\sum(x - \bar{x})^2/n - 1$, and '$S^2y$' the variance of the viable count determinations, $\sum(y - \bar{y})^2/n - 1$. From the data, C = 1.029, S^2x = 0.115 and S^2y = 9.440. The calculated value of 'r' is therefore 0.989. The tabulated values of 'r' for n - 2 degrees of freedom, at the 1% and 5% significance levels is 0.875 and 0.754 respectively. Thus, the correlation between optical density and viable counts was significant

Figure 7. Growth curves of P. aeruginosa constructed from optical density and viable count data.

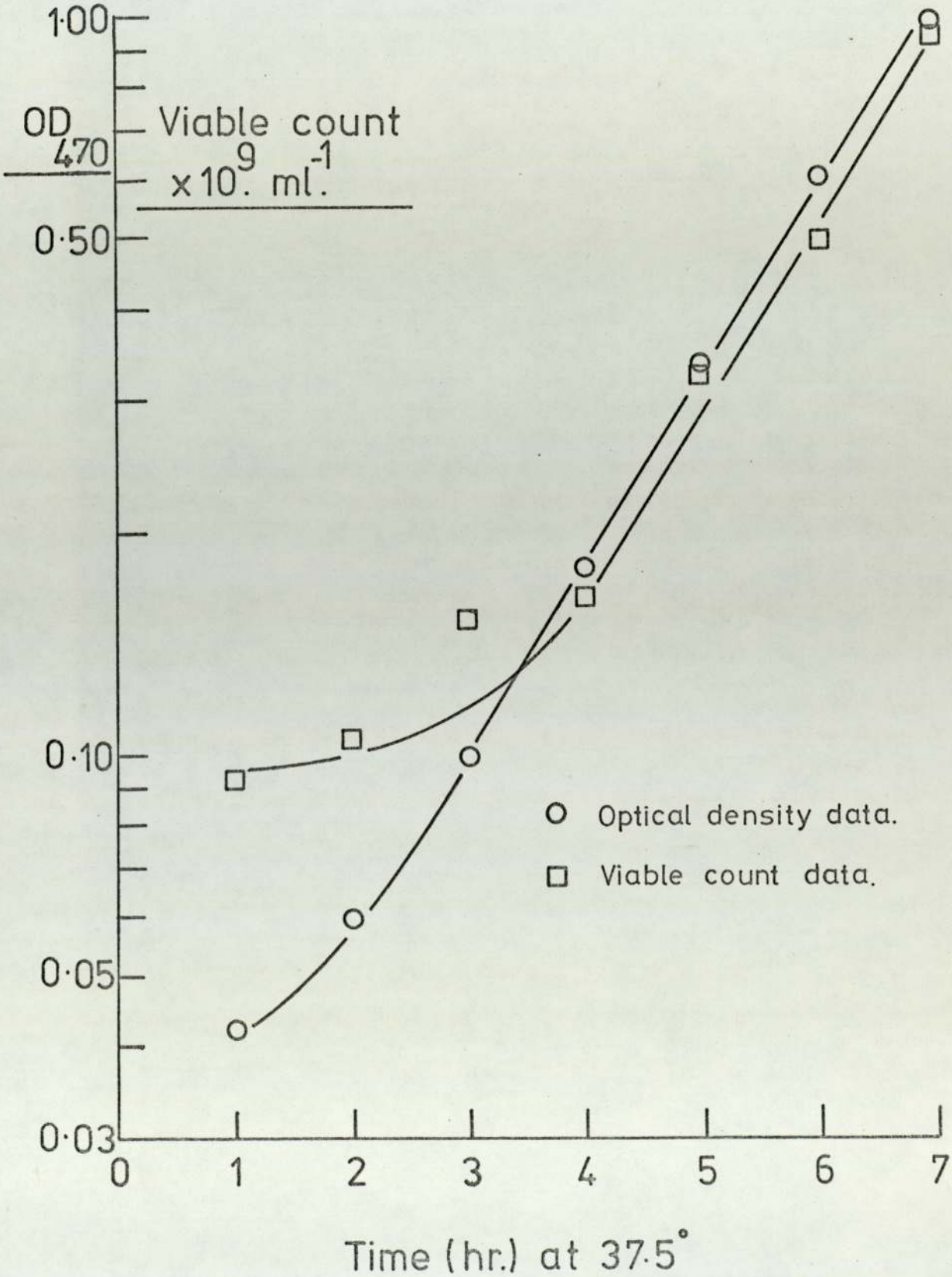
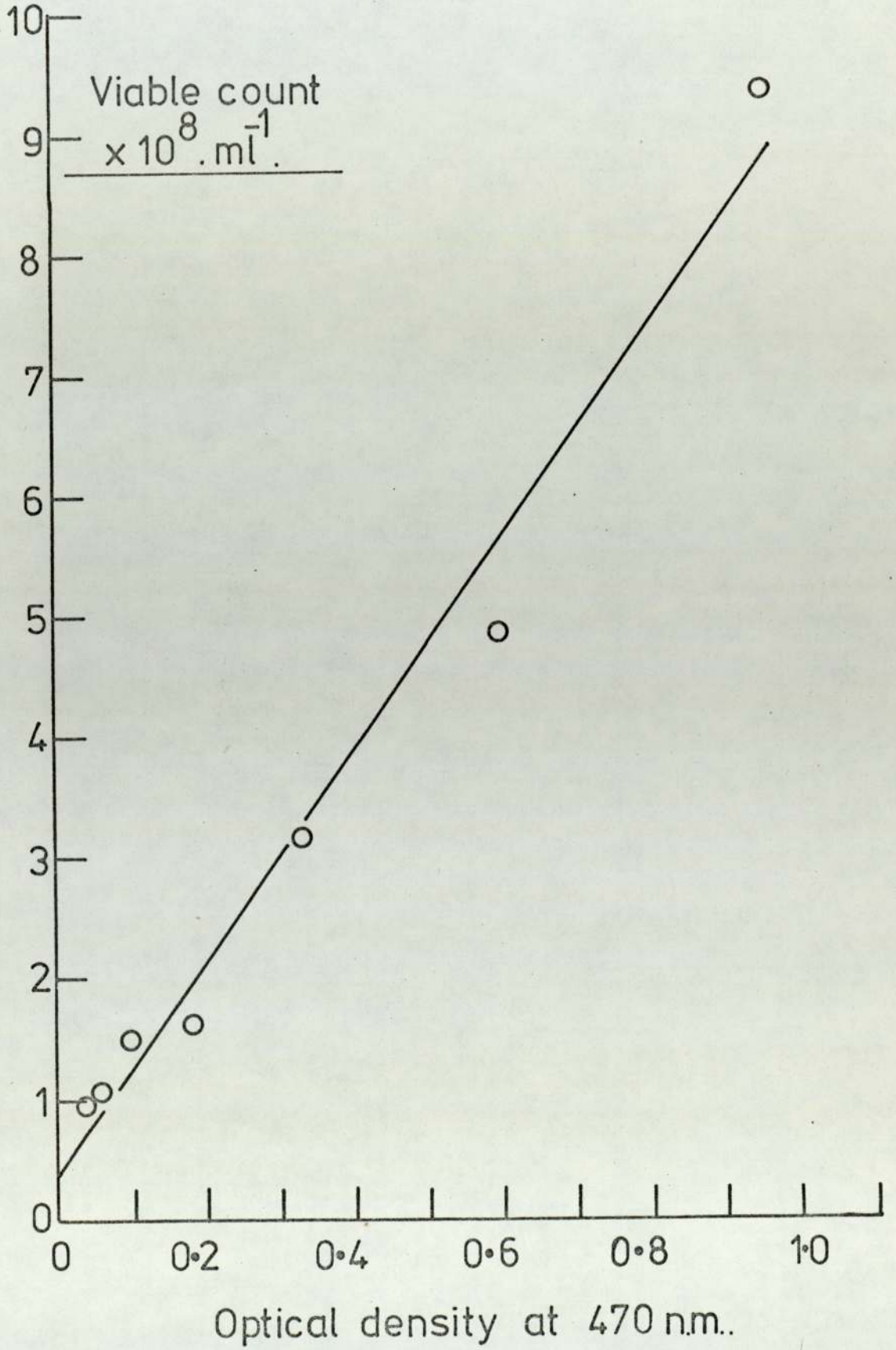


Figure 8. Relation between viable count and optical density of a log phase culture of P. aeruginosa.



within these levels.

Reproducibility of growth rate constants determined from P.aeruginosa cultures grown in chemically defined media on different days.

Six replicate cultures of P.aeruginosa were inoculated into C.D.M. on each of three days. Growth was followed by taking optical density readings at suitable time intervals in the manner previously described. Growth curves were constructed on three cycle semi-log paper by plotting $O.D._{470}$ on the log scale against the corresponding time interval on the linear scale. Using the linear part of the growth curve i.e. that part representing exponential growth, the growth rate constants 'K' can be determined from the following equation:

$$K = \frac{\log N_2 - \log N_1}{t_2 - t_1}$$

where 'N_2' and 'N_1' are the $O.D._{470}$ readings at times 't_2' and 't_1' respectively. The equation for K can also be written as follows:

$$K = \frac{\log(N_2/N_1)}{t_2 - t_1}$$

and in the case when the increase from N_1 to N_2 involves a doubling of the population, i.e. from N to 2N, the equation becomes:

$$K = \frac{\log 2}{t.d}$$

or using $\log_{10}$, the equation becomes:

$$K = \frac{0.301}{t.d}$$

where 't.d' is the doubling time of the culture. In this study, the growth rate constants were determined by dividing the doubling time, in minutes, into 0.301. The doubling times were read directly from the linear portion of the growth curves. The growth rate constants determined in this manner for each of the 18 replicate cultures are presented in Table 5, and the reproducibility was assessed by an analysis of variance (Table 6).

TABLE 5

Growth rate constants x 10^{-3} min^{-1} for replicate *P.aeruginosa* cultures grown on different days.

Flasks \ Days	1	2	3	Means
1	5.018	5.280	4.777	5.025
2	4.936	5.280	4.705	4.974
3	4.894	5.018	4.560	4.824
4	4.855	4.705	4.777	4.779
5	4.669	4.855	4.629	4.718
6	4.705	4.560	4.629	4.631
Totals T	29.077	29.698	28.077	-
Means	4.846	4.950	4.680	-

n = number of observations per day = 6

m = number of days = 3

n.m = total number of observations = 18

(1) $\Sigma x^2 = 419.86741$

(2) $\frac{\Sigma T^2}{n} = 419.29351$

(3) $\frac{(\Sigma x)^2}{n.m} = 419.07055$

TABLE 6

Analysis of variance of growth rate constants.

Source of variance	Sum of squares	Degrees of freedom	Mean squares	Variance ratio (F)
Between days	(2) - (3) 0.22296	m - 1 2	0.11148	2.9
Within days	(1) - (2) 0.57390	n.m - m 15	0.03826	

The tabulated values of 'F' for $^2/_{15}$ degrees of freedom at 1% and 5% significance is 6.36 and 3.70 respectively. Therefore the variation within days is not significantly greater than the variation between days within these levels. Using the means in Table 5, the coefficient of variation within days was calculated as 3.1%, and between days as 2.8%.

The total mean was 4.825×10^{-3}/min ≡ to a doubling time of 62.4-min.

Reproducibility of lysis values determined from replicate P.aeruginosa cultures grown in chemically defined media on different days.

Three replicate lysis experiments were performed (see section 3) on each of three days. Lysis curves

were constructed by plotting percent lysis against time. The percent lysis was determined from the optical density readings by the use of the following equation:

$$\text{percent lysis} = \frac{O.D._o - O.D._t}{O.D._o} \times 100$$

where '$O.D._o$' is the optical density at time zero, and '$O.D._t$' is the optical density at any given time 't'. For each group of three lysis flasks, a control flask in which water was added in place of the lytic agent (polymyxin, 50 U/ml) was also prepared. The percent lysis of the control was determined and subtracted from the experimental culture values to correct for endogenous lysis of the cultures. The results of lysis after 180-min are presented in Table 7, and an analysis of variance was performed on the data (Table 8).

TABLE 7

Percent lysis, after 180-min exposure to 50 U/ml polymyxin, of nine replicate P.aeruginosa cultures on different days.

Days / Replicates	1	2	3	Means
1	65.5	66.5	62.5	64.83
2	64.0	66.5	63.0	64.50
3	65.2	64.0	63.0	64.07
Totals T	194.7	197.0	188.5	-
Means	64.9	65.7	62.8	-

n = number of observations per day = 3

m = number of days = 3

n.m = total number of observations = 9

(1) $\sum x^2 = 37422.040$

(2) $\frac{\sum T^2}{n} = 37416.447$

(3) $\frac{(\sum x)^2}{n.m} = 37403.560$

TABLE 8

Analysis of variance of percent lysis values for twevle replicate cultures of P.aeruginosa.

Source of variance	Sum of squares	Degrees of freedom	Mean squares	Variance ratio (F)
Between days	(2) - (3) 12.887	m - 1 2	6.4435	6.9
Within days	(1) - (2) 5.593	n.m - m 6	0.9322	

The tabulated values of 'F' for $^2/_6$ degrees of freedom at 1% and 5% significance are 6.36 and 5.10 respectively. Therefore the variation between days was significantly greater than the variation within days at both these levels. This is explained by the very small within day variation. Using the means from Table 7, the coefficient of variation within days is 0.6%, whereas the variation between days is 2.3%. The 2.3% variation between days was considered acceptable.

4. CHEMICAL ASSAY PROCEDURES.

Assay of total protein.

Cell wall preparations were assayed for total protein using the method described by Lowry, Rosebrough, Farr & Randall (1951) as modified by Campbell & Sargent (1967).

A series of standard solutions of protein (bovine plasma albumin) were prepared with a concentration range 0 to 100 µg/ml. A calibration line was obtained from the mean results of three separate sets of determinations of the standards (Fig 9).

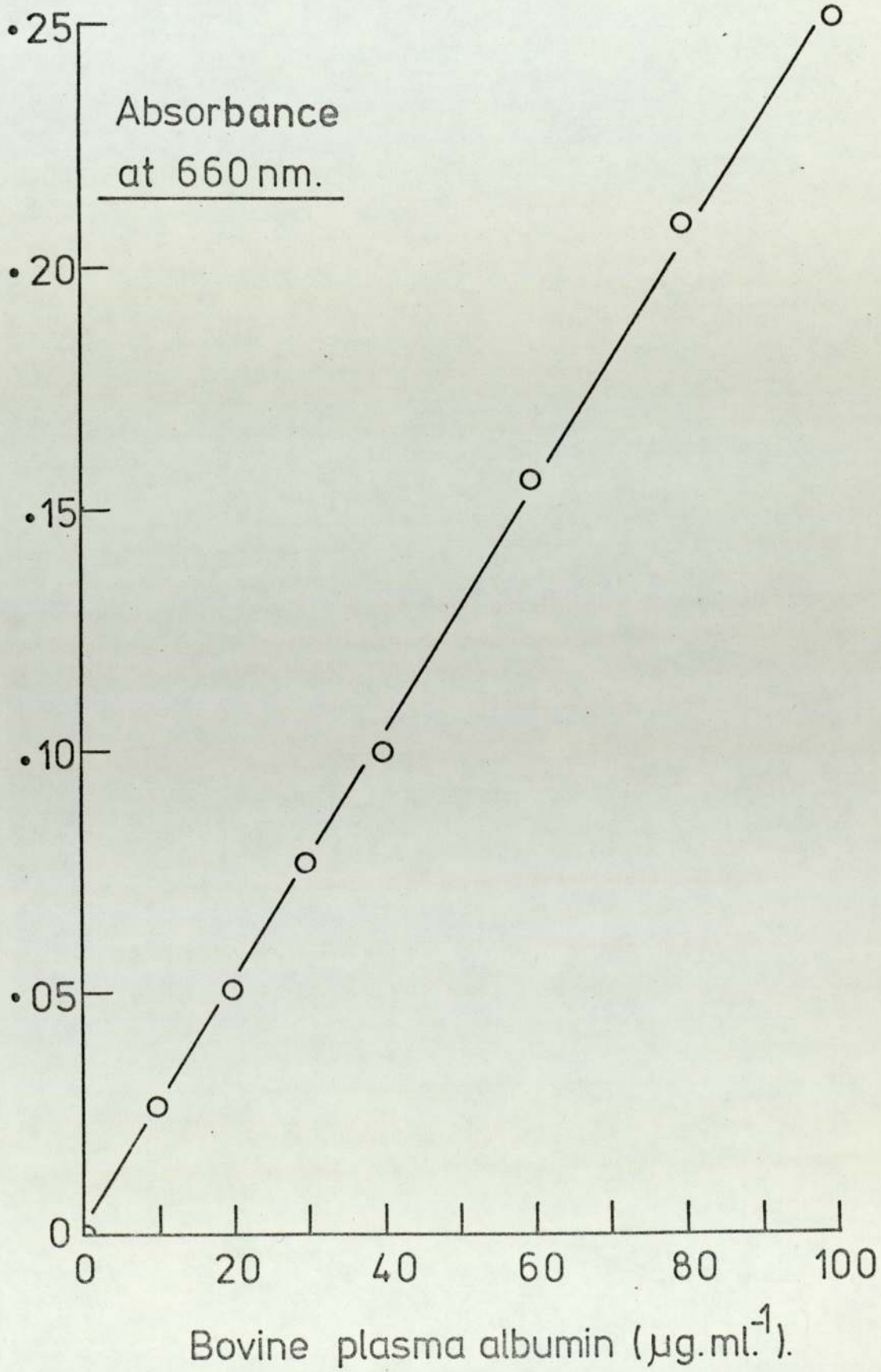
The reproducibility of the assay was assessed by determining the protein concentration of six replicate samples from a cell wall suspension containing an unknown weight of cell walls in an unknown volume of water. The results are shown in Table 9.

TABLE 9

Reproducibility of protein assay.

Replicates	Concentration of protein µg/ml
1	17.6
2	17.6
3	17.6
4	17.2
5	18.0
6	18.0
Mean	17.7
Coefficient of Variation	1.7%

Figure 9. Protein calibration curve (modification of Lowry et al., 1951).



Cell wall suspensions were diluted such that they contained 60 to 70 µg dry weight cell wall material/ml before they were assayed for protein. All determinations were made in triplicate.

Assay of total carbohydrate.

Cell walls were assayed for total carbohydrate using the method described by Dubois, Gillies, Hamilton, Rebers & Smith (1956).

A series of standard solutions of carbohydrate (glucose) were prepared with a concentration range from 0 to 120 µg/ml. A calibration line was obtained from the mean results of three sets of determinations of the standard solutions (Fig. 10).

The reproducibility of the assay was assessed by determining the concentration of carbohydrate, as glucose, in six replicate samples of a cell wall suspension containing an unknown weight of cell walls in an unknown volume of water. The results are presented in Table 10.

Cell wall suspensions were diluted to contain about 500 µg dry weight cell wall material/ml before they were assayed. All determinations were made in triplicate.

Assay of total phosphorus.

Cell wall preparations were assayed for total phosphorus (P) by a modification of the method described by Baginski, Foà & Zak (1967).

For each set of determinations, unknown solutions were compared with standard calibration solutions assayed at the same time. Standards and unknowns were assayed in triplicate and the mean value taken. The calibration line obtained is shown in Fig. 11.

Figure 10. Carbohydrate calibration curve (Dubois et al., 1956).

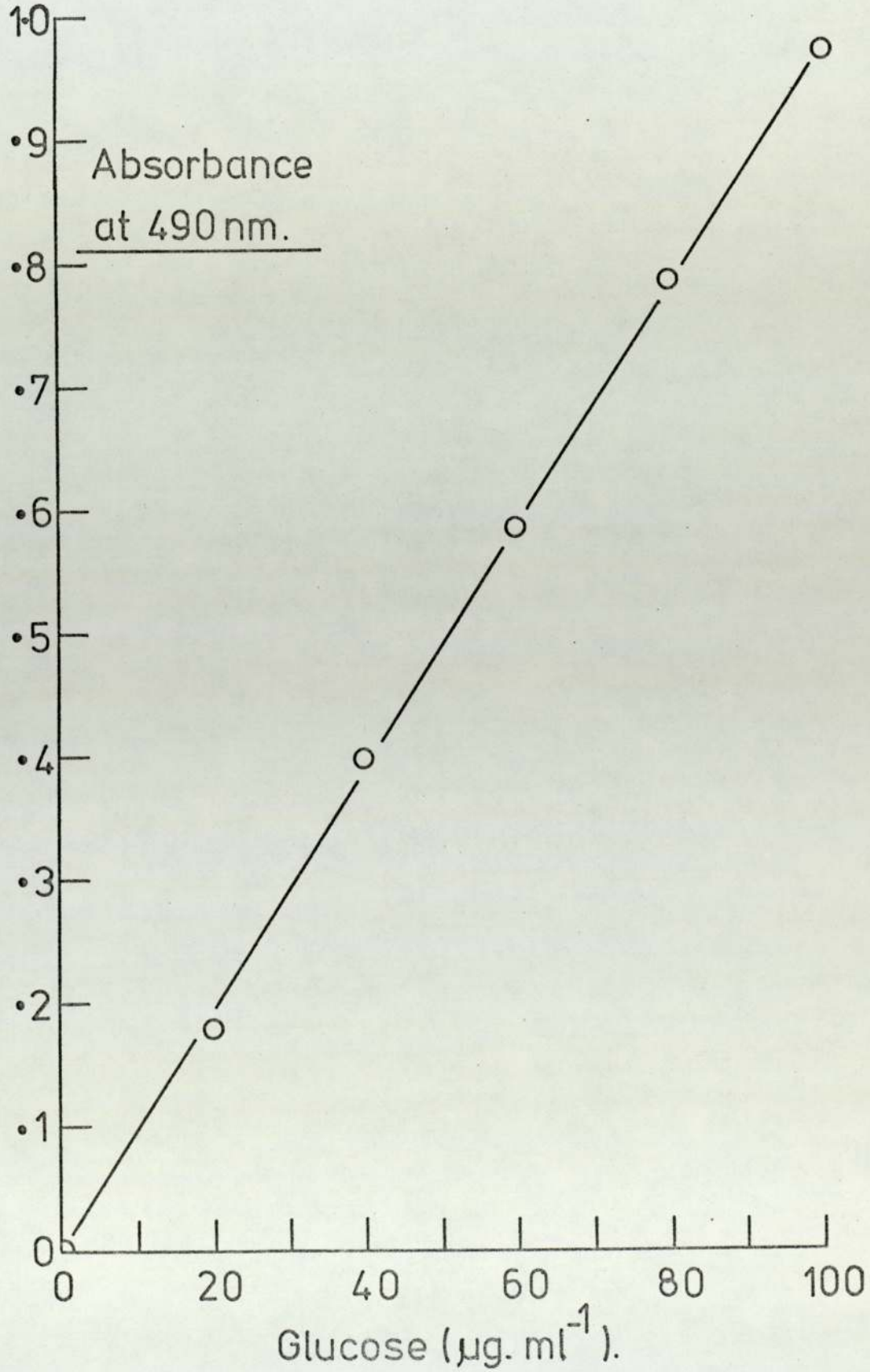


Figure 11. Phosphorus calibration curve (modification of Baginski et al., 1967).

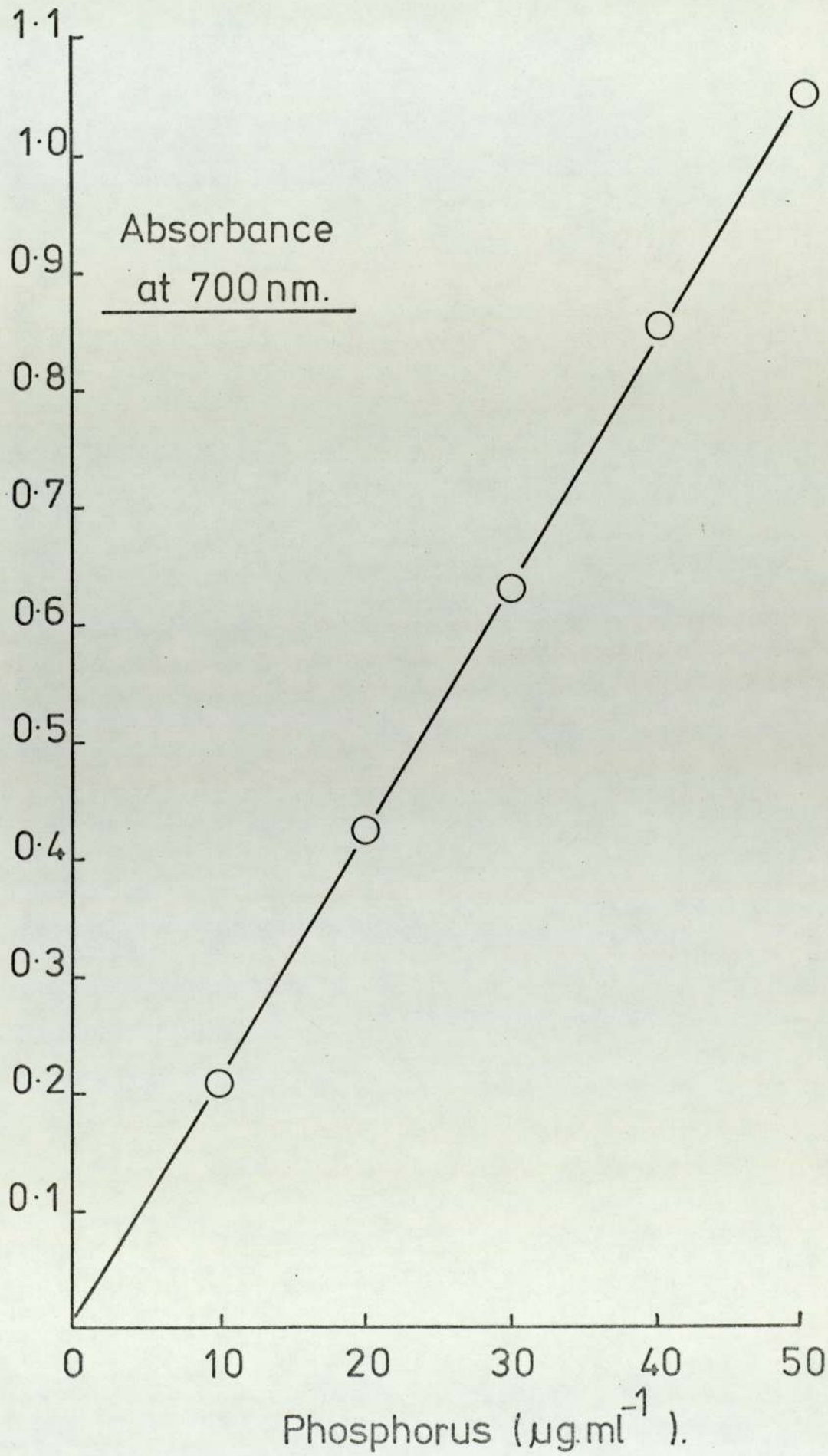


TABLE 10

Reproducibility of carbohydrate assay.

Replicates	Concentration of carbohydrate µg/ml
1	69.7
2	73.4
3	67.2
4	77.6
5	74.9
6	75.5
Mean	73.1
Coefficient of Variation	5.3%

The method described by Baginski et al. (1967) involves the incorporation of calcium (Ca^{2+}) in concentrated nitric acid to prevent loss of P from labile organic phosphates during the wet-ashing step. In this study it was intended to use the same wet-ashed sample for the assay of magnesium, manganese and Ca^{2+} as well as for P, and therefore lanthanum ions (La^{3+}) were used to replace the Ca^{2+} in the nitric acid.

In reducing conditions, P reacts with ammonium molybdate to produce a blue colour (Fiske & SubbaRow, 1925). As can be seen in Fig. 12 this blue colour continues to develop for at least two hour after the addition of the ammonium molybdate. The colour development is terminated by the addition of a sodium arsenite-sodium citrate reagent which complexes with the unused ammonium molybdate.

Reference to Fig. 12 shows that during the initial stages of colour development a small variation of the time at which the development is terminated would be expected to lead to a significant variation in the absorbance of the chromophore. To overcome this variation, the colour development was always terminated 60-min after the addition of the ammonium molybdate.

The reproducibility of this method was assessed by determining the concentration of P in twelve replicate samples of an unknown weight of cell walls suspended in an unknown volume of water. To six of these samples, 0.1 ml of a standard solution of 10 µg P/ml was added, and to the remaining six, 0.1 ml of water. The results are shown in Table 11.

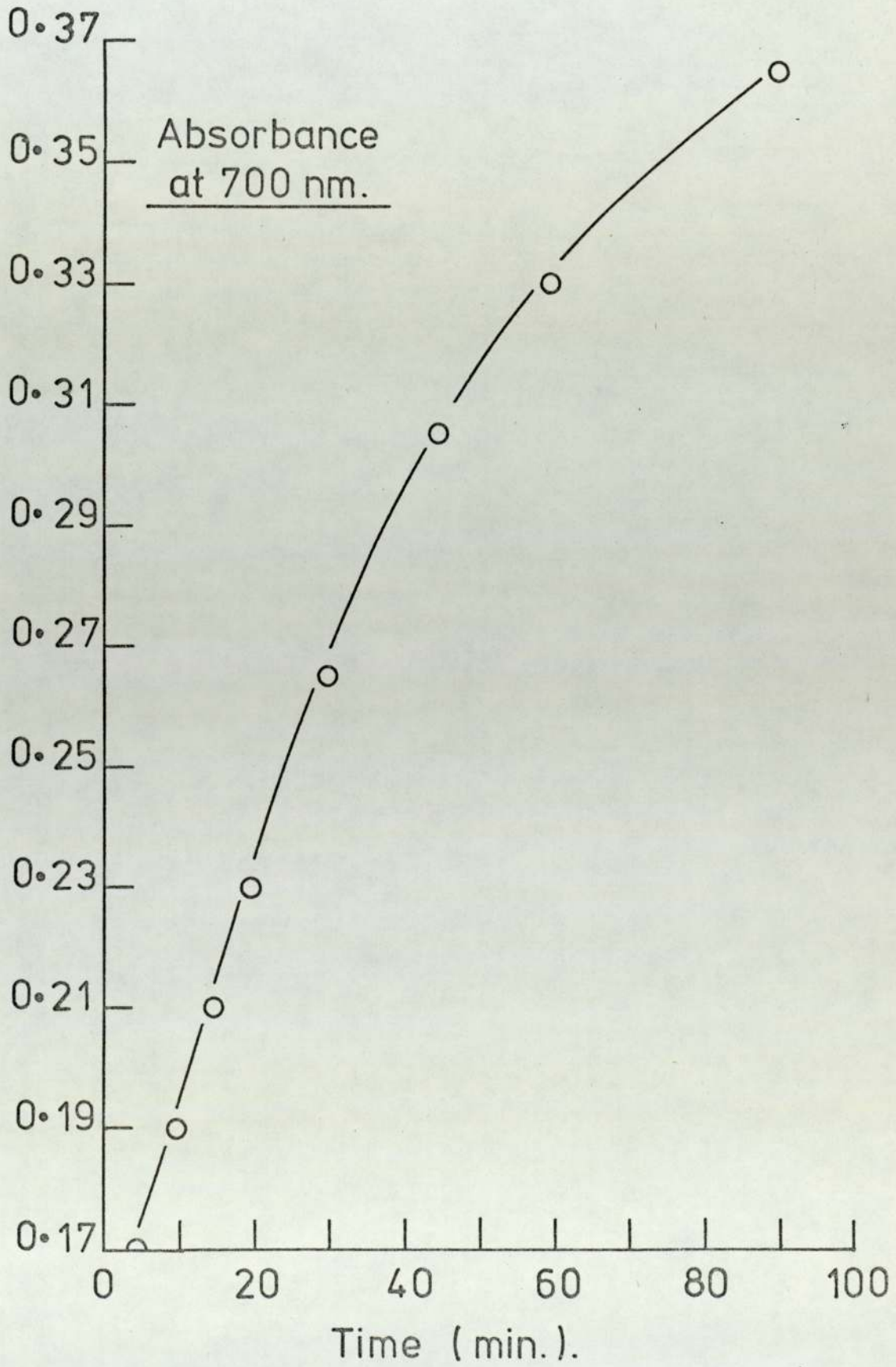
TABLE 11

Reproducibility of total phosphorus assay.

Replicates	Concentration of P µg/ml	
	Sample	Sample + P
1	22.3	33.8
2	24.1	30.8
3	21.4	35.9
4	26.2	32.9
5	25.0	35.8
6	25.2	33.9
Means	24.0	33.9
Coefficient of Variation	7.6%	5.6%

The difference between the mean values is 9.82 µg P/ml,

Figure 12. Phosphorus assay: Effect of time upon colour development.



a recovery of 98.2%.

The cell wall suspensions were diluted to contain approximately 1,200 µg dry weight cell wall material/ml prior to analysis.

Assay of 2-keto,3-deoxyoctonic acid.

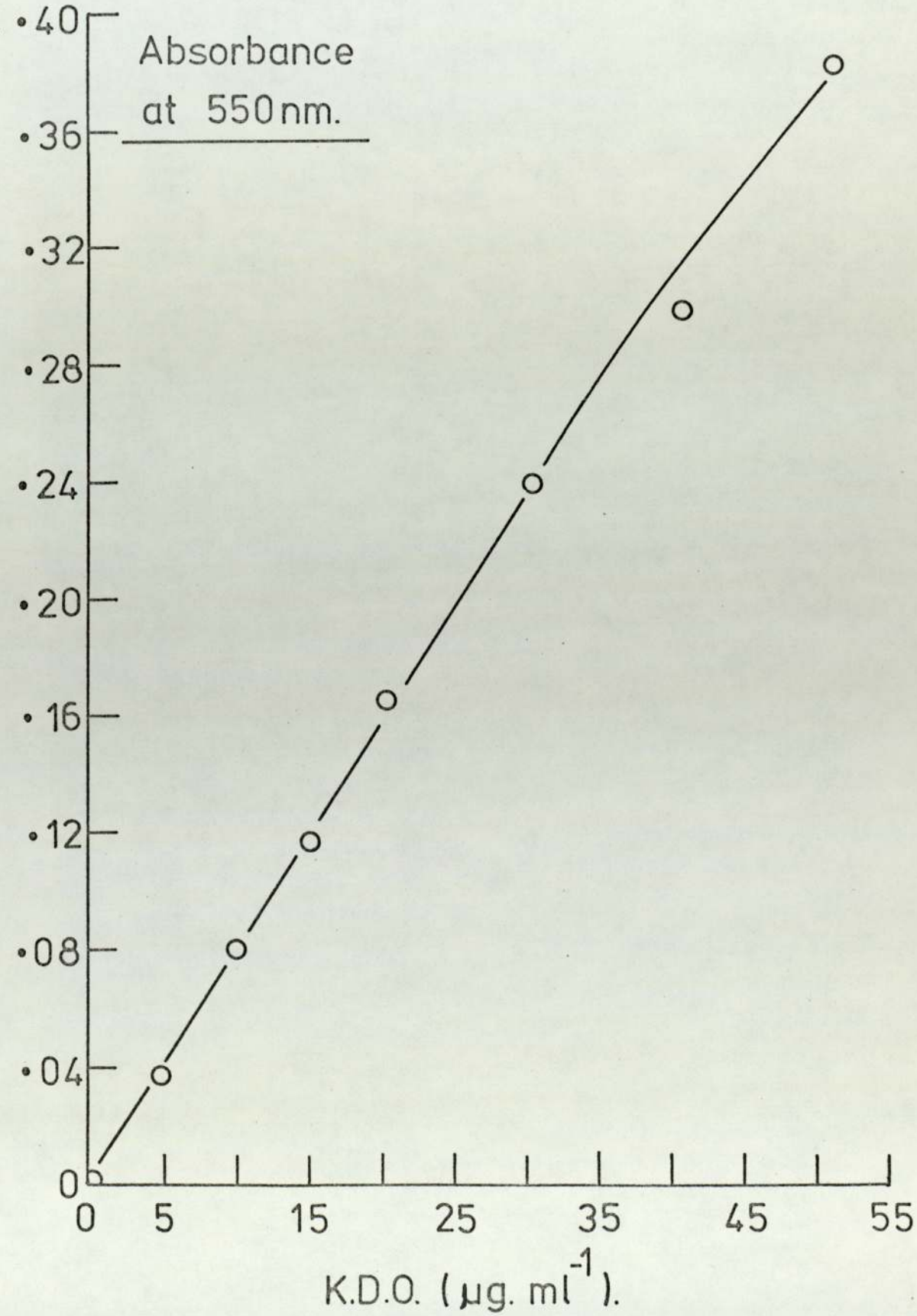
The 2-keto,3-deoxyoctonic acid (K.D.O.) content of cell walls was assayed using the method described by Weissbach & Hurwitz (1959).

A series of standard solutions of K.D.O. (as the 1,4-lactone derivative) were prepared in 0.1 N-sulphuric acid, with concentrations ranging from 0 to 51 µg/ml, and a calibration line obtained (Fig. 13). The calibration line became non-linear at K.D.O. concentrations greater than 30.6 µg/ml.

The cell walls were hydrolysed prior to analysis as follows; 0.5 ml of cell wall suspensions were added to powder ampoules containing 0.5 ml of 1 N-sulphuric acid and 4 ml of water. The ampoules were sealed and placed in a boiling water bath for 30-min. The cooled hydrolysates were filtered through 0.22 µ millipore membrane filters, and 0.1 ml aliquots assayed according to the conditions described by Weissbach & Hurwitz (1959).

Since the reaction with thiobarbituric acid is positive for all 2-keto,3-deoxy-sugar acids (Ellwood, 1970), all cell wall samples were assayed in the presence of an internal standard (0.1 ml of 20.4 µg/ml) and again in the presence of 0.1 ml of water. The acidic butanol extraction of the chromophore described by Ellwood (1970) was not used since little or none of the cloudiness he noted was observed at the concentrations used.

Figure 13. 2,keto-3,deoxy-octonic acid (K.D.O.) calibration curve (Weissbach & Hurwitz, 1959).



The reproducibility of the assay was assessed by determining the K.D.O. concentration of six replicate samples from an acid hydrolysate of an unknown weight of cell walls. The results are shown in Table 12.

TABLE 12

Reproducibility of K.D.O. assay.

Replicates	Concentration of K.D.O. µg/ml
1	21.0
2	21.9
3	20.8
4	21.9
5	22.6
6	20.1
Mean	21.4
Coefficient of Variation	4.3%

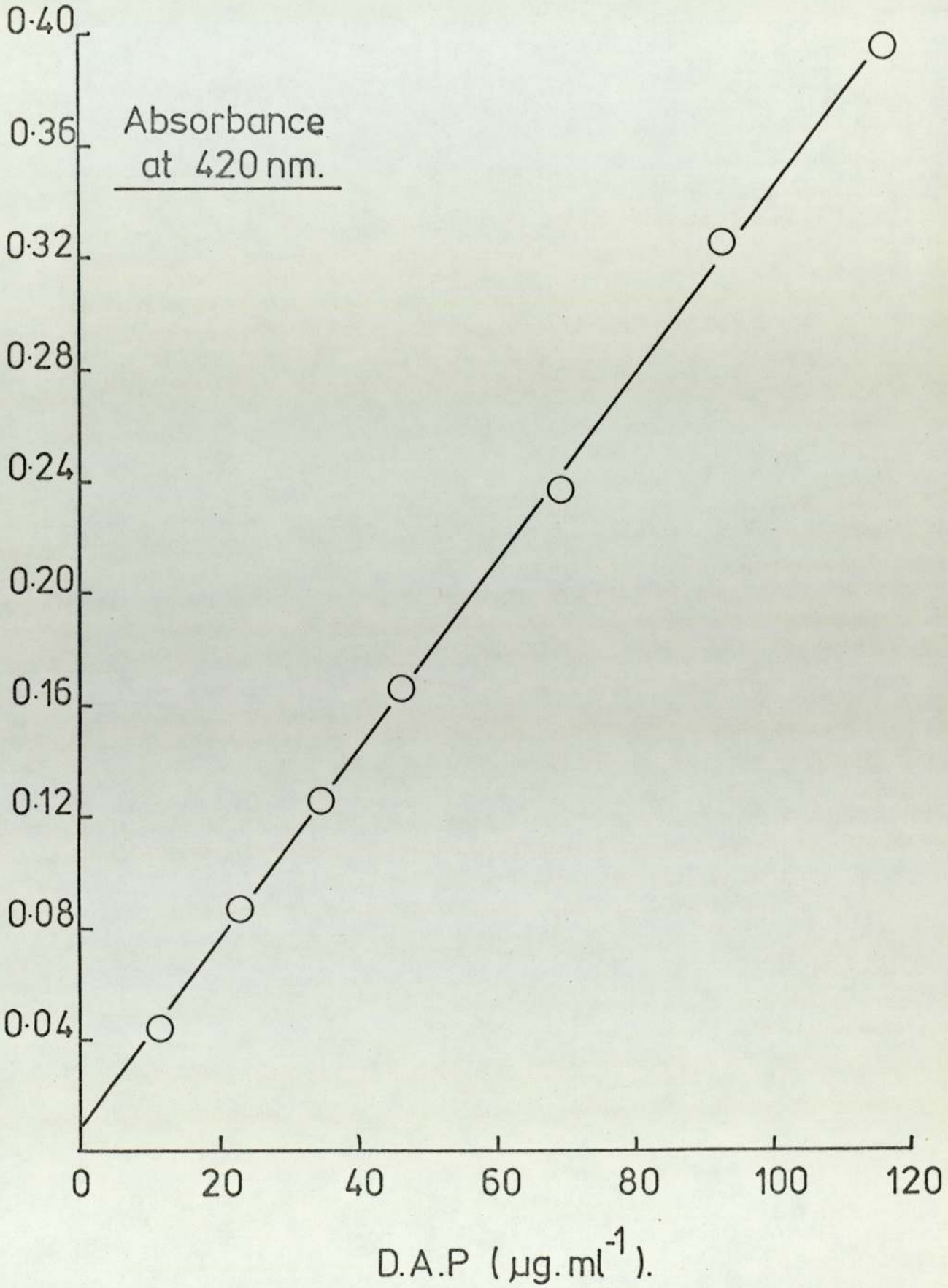
The cell wall hydrolysates were assayed directly as described without any prior dilution of the sample.

Assay of meso,2,6-diaminopimelic acid.

Cell wall preparations were assayed for meso,2,6-diaminopimelic acid (D.A.P.) using the colourimetric method described by Gilvarg (1958).

A series of standard solutions of D.A.P. were prepared with a concentration range 0 to 116 µg/ml. A calibration line was obtained from the results of three replicate determinations of the standards (Fig. 14).

Figure 14. 2,6,meso-diaminopimelic (D.A.P.) calibration curve (Gilvarg, 1956).



The method described by Gilvarg (1958) is a modification of the method used by Chinard (1952). Gilvarg (1958) heated samples in acid ninhydrin for 20-min at 100°. However, reference to Fig. 15 shows that colour development was not complete until 30-min has elapsed. In this study therefore, the reaction at 100° was allowed to continue for 35-min. The volumes used by Gilvarg (1958) were found to be inconvenient if accuracy was to be achieved using 1 ml pipettes. It was decided to keep the ratios of the volumes the same. Using a sample size of 0.2 ml, the volume of concentrated hydrochloric acid, ninhydrin reagent and the propanol:water mixture were therefore changed to 0.11, 0.62 and 6.76 ml respectively.

Cell wall suspensions were hydrolysed in 6.1 N hydrochloric acid at 105 for 4-hr to release amino acids (Gray & Wilkinson, 1965). The hydrolysates were neutralized by the addition of excess (2.5 ml) 3 M-sodium carbonate to 2.32 ml of hydrolysate. The neutralized hydrolysates were filtered through 0.22 μ millipore membrane filters. Since there may be some interferance from other amino acids (Gilvarg, 1958) samples were assayed in the presence of an internal standard.

The reproducibility of the assay was assessed by determining the concentration of D.A.P. in six replicate samples of an unknown weight of cell wall suspended in an unknown volume of water. The results are presented in Table 13.

The cell wall hydrolysates were assayed in triplicate and the mean values taken.

Figure 15. D.A.P. assay: Effect of time upon colour development.

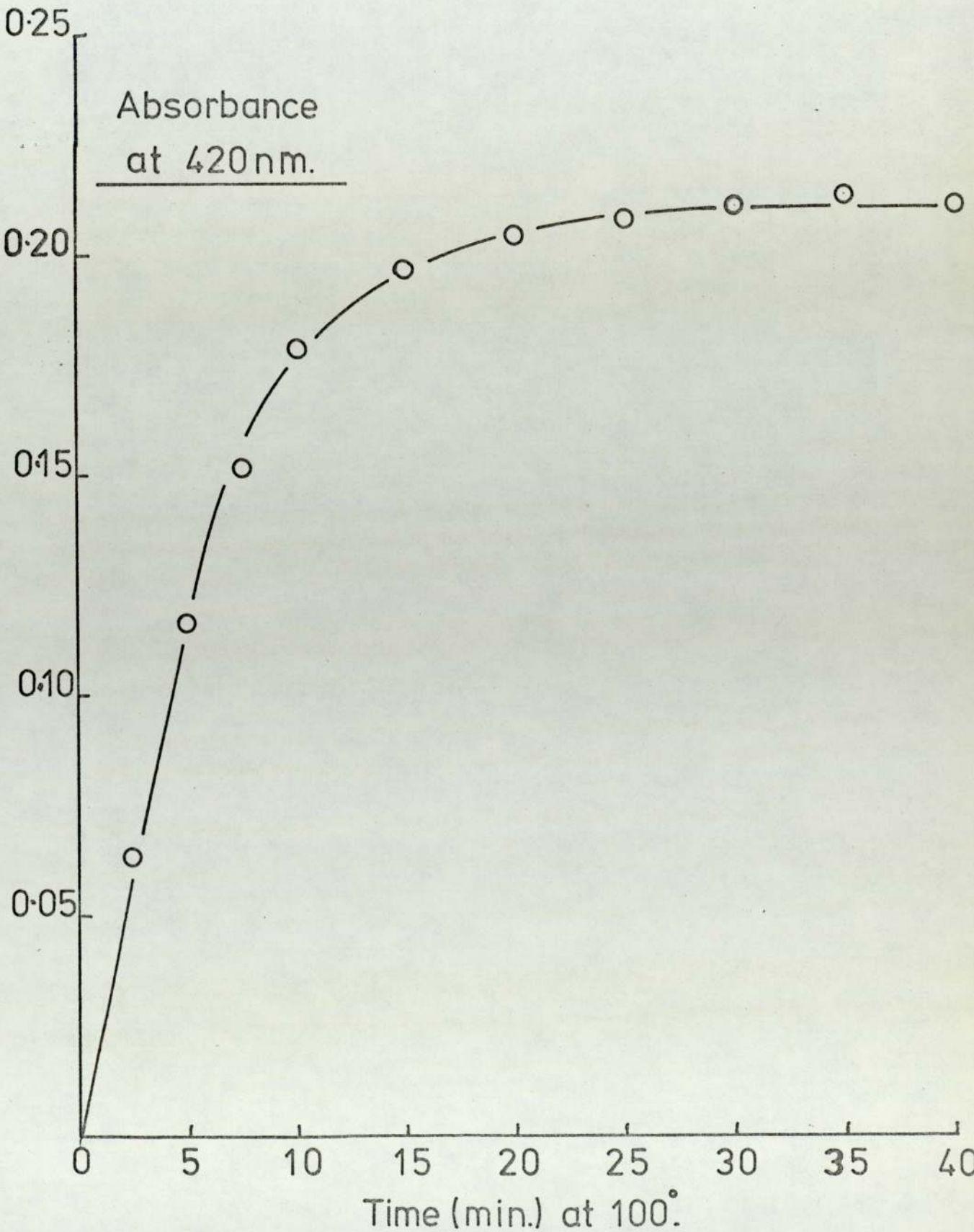


TABLE 13

Reproducibility of D.A.P. assay.

Replicates	Concentration of D.A.P. µg/ml
1	89.5
2	84.8
3	89.8
4	83.9
5	89.8
6	90.1
Mean	88.0
Coefficient of Variation	3.2%

Assay of amino sugars.

Cell wall suspensions were assayed for total hexosamine using the method described by Good & Bessman (1964).

Standard solutions of glucosamine hydrochloride were prepared with the concentrations ranging from 0 to 120 µg/ml. The calibration line obtained from three separate determinations is shown in Fig. 16.

The absorbance of the chromophore was measured at 585 nm, the wavelength of maximum absorption (see Fig. 17). Boggis (1971) found the peak absorbance to occur at 585 nm, but Good & Bessman (1964) used a wavelength of 570 nm.

Cell walls were hydrolysed in 6.1 N-hydrochloric acid at 105° for 4-hr (Gray & Wilkinson, 1965) prior to

Figure 16. Hexosamine calibration curve (Good & Bessman, 1964).

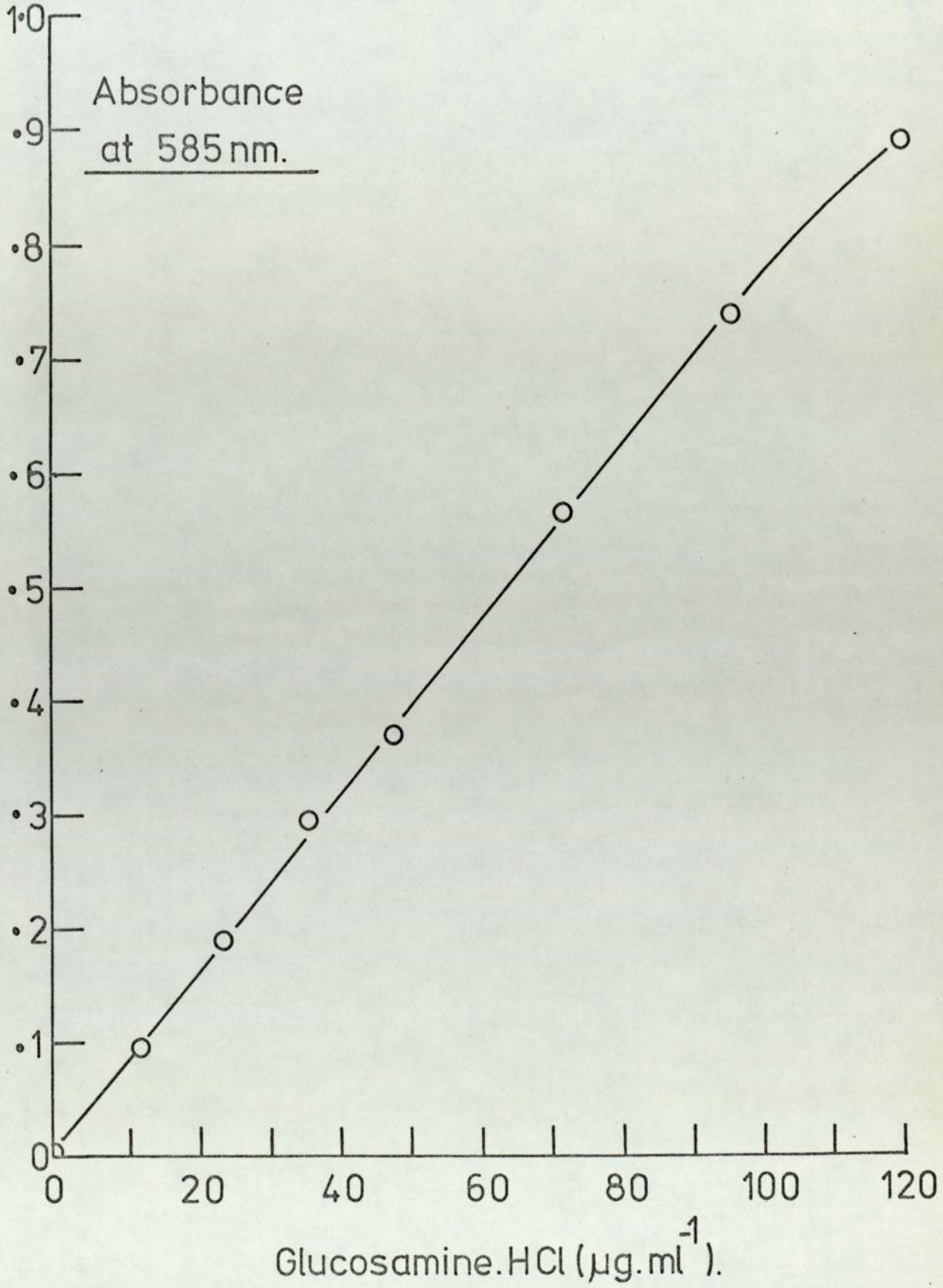
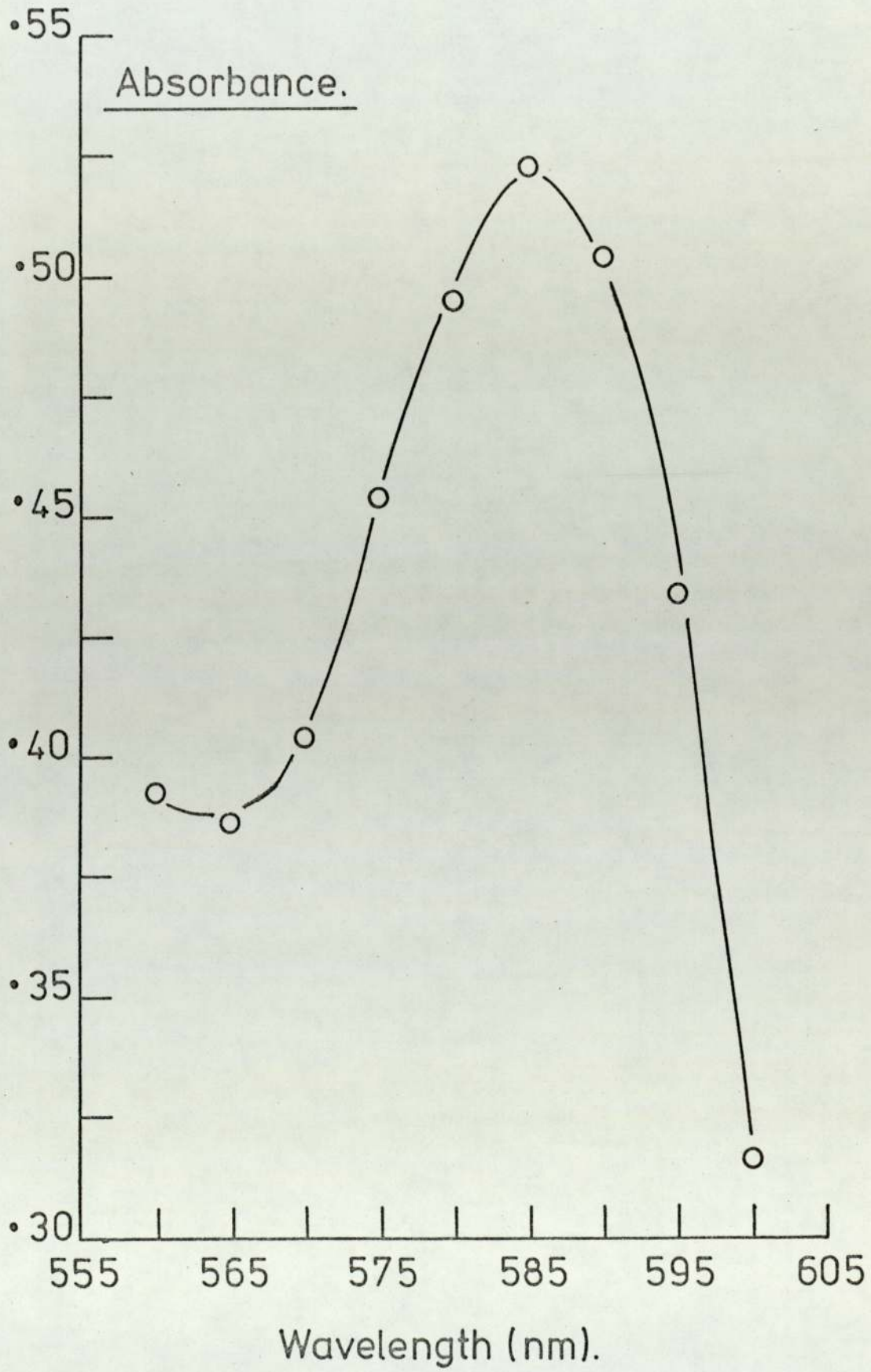


Figure 17. Absorption spectrum of glucosamine.HCl (67.5 ug. ml^{-1}) determined by the method of Good & Bessman (1964).



analysis. The hydrolysates were neutralized by careful addition of excess (i.e. 0.75 g to 2.32 ml hydrolysate) solid sodium carbonate. The neutralized hydrolysates were filtered through 0.22 μ millipore membrane filters to remove excess suspended sodium carbonate and residual cell wall material. Aliquots of 0.5 ml were assayed using an internal standard of 24 μg hexosamine in 0.5 ml of water.

The reproducibility of the assay was assessed by determining the concentration of hexosamine in six replicate samples from an unknown weight of cell walls suspended in an unknown volume of water. The results are presented in Table 14.

TABLE 14

Reproducibility of hexosamine assay.

Replicates	Concentration of hexosamine μg/ml
1	38.4
2	38.5
3	39.3
4	39.2
5	39.2
6	39.2
Mean	39.0
Coefficient of Variation	1.0%

Assay of magnesium, calcium and manganese.

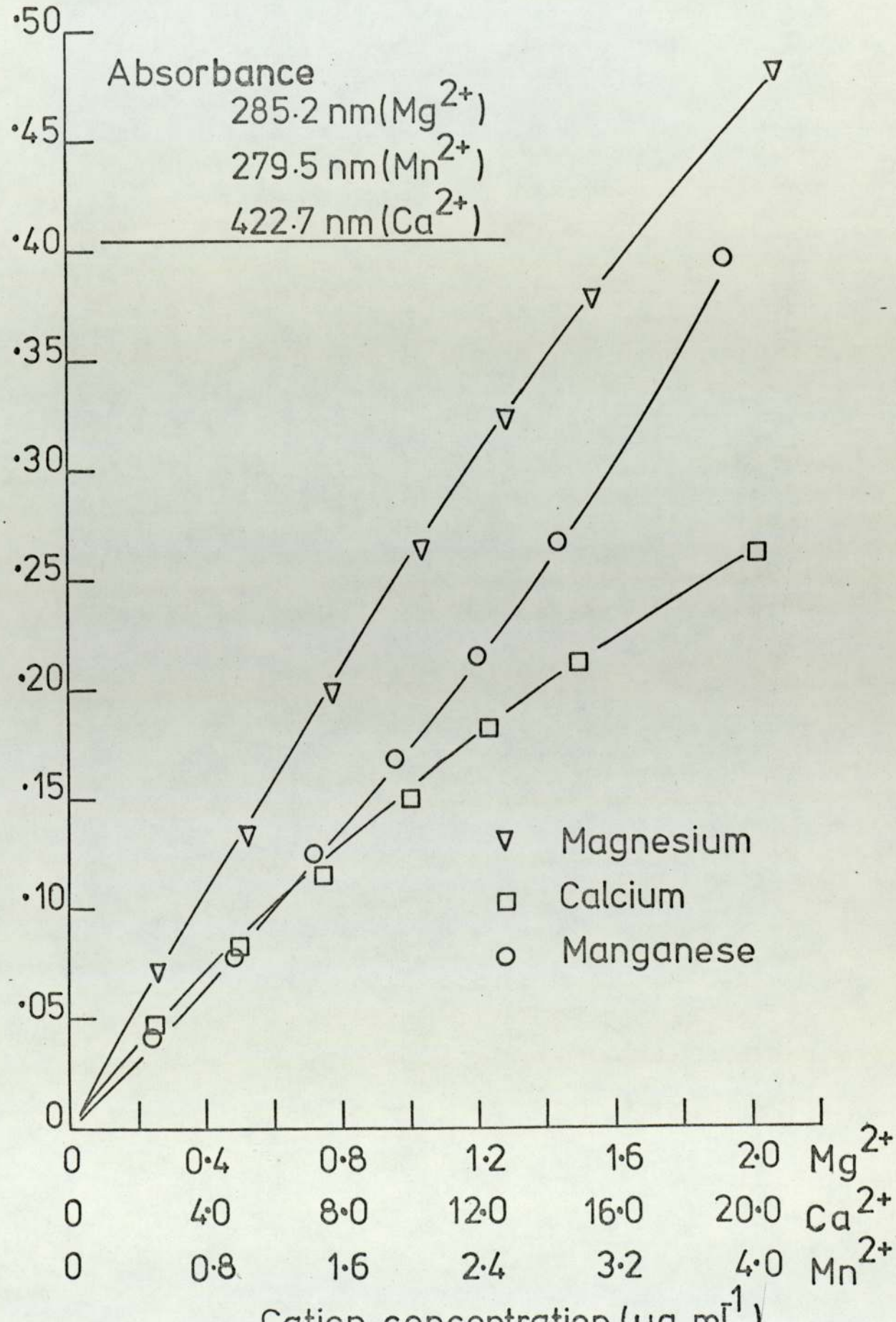
Cell wall preparations were assayed for magnesium (Mg^{2+}), calcium (Ca^{2+}) and manganese (Mn^{2+}) by atomic absorption spectroscopy. Each cation was assayed using the instrumental conditions described in 'Unicam Atomic Absorption Methods' (Pye-Unicam Instruments Ltd., York Street, Cambridge).

Standard solutions were prepared in a 1% $^{v}/v$ solution of hydrochloric acid containing 1% by weight lanthanum (La^{3+}) ions. The standard solutions were assayed before and after the cell wall preparations for each set of determinations and a set of calibration lines obtained (Fig. 18). Both the standard and unknown solutions were each read three times and the mean values taken.

The La^{3+} ions were added to cell wall preparations to overcome suppression of absorbance of Ca^{2+} ions due to phosphate ions (Willis, 1961). Since the same preparation was used to assay all three cations, the standard solutions for each cation were also made up in 1% La^{3+}.

Cell walls were prepared for analysis in the following manner; 0.5 ml of cell wall suspension, containing between 4 and 8 mg dry weight of walls/ml, was digested by boiling in 2 ml of concentrated nitric acid. The samples were heated to dryness and on cooling the 'ash' was dissolved in 0.4 ml of concentrated hydrochloric acid. The acid was transfered to a 10 ml volumetric flask and the digestion vessel washed with four, 2 ml volumes of 1% La^{3+} ions in 1% hydrochloric acid solution. The washings were also added to the

Figure 18. Atomic absorption spectrophotometric assay of magnesium, calcium and manganese.



volumetric flask, and the volume made up to 10 ml. This solution was then assayed either directly or after suitable dilution. Each cell wall sample was treated as described in duplicate. Lipid fractions had the solvent (chloroform/methanol, 2:1) removed under a stream of nitrogen before they were treated as described.

The reproducibility of the assay was determined by measuring the cation content of six replicate samples of a cell wall suspension containing an unknown weight of cell walls in an unknown volume of water. The results are presented in Table 15.

TABLE 15

Reproducibility of the assay for magnesium, calcium and manganese.

Replicates	Magnesium µg/ml	Calcium µg/ml	Manganese µg/ml
1	0.54	3.9	1.12
2	0.53	3.6	1.10
3	0.56	3.7	1.12
4	0.53	3.6	1.12
5	0.56	3.9	1.11
6	0.54	3.7	1.11
Mean	0.54	3.7	1.11
Coefficient of Variation	2.51%	3.7%	0.73%

Assay of phospholipids.

The phospholipids (P.L.) were isolated from whole

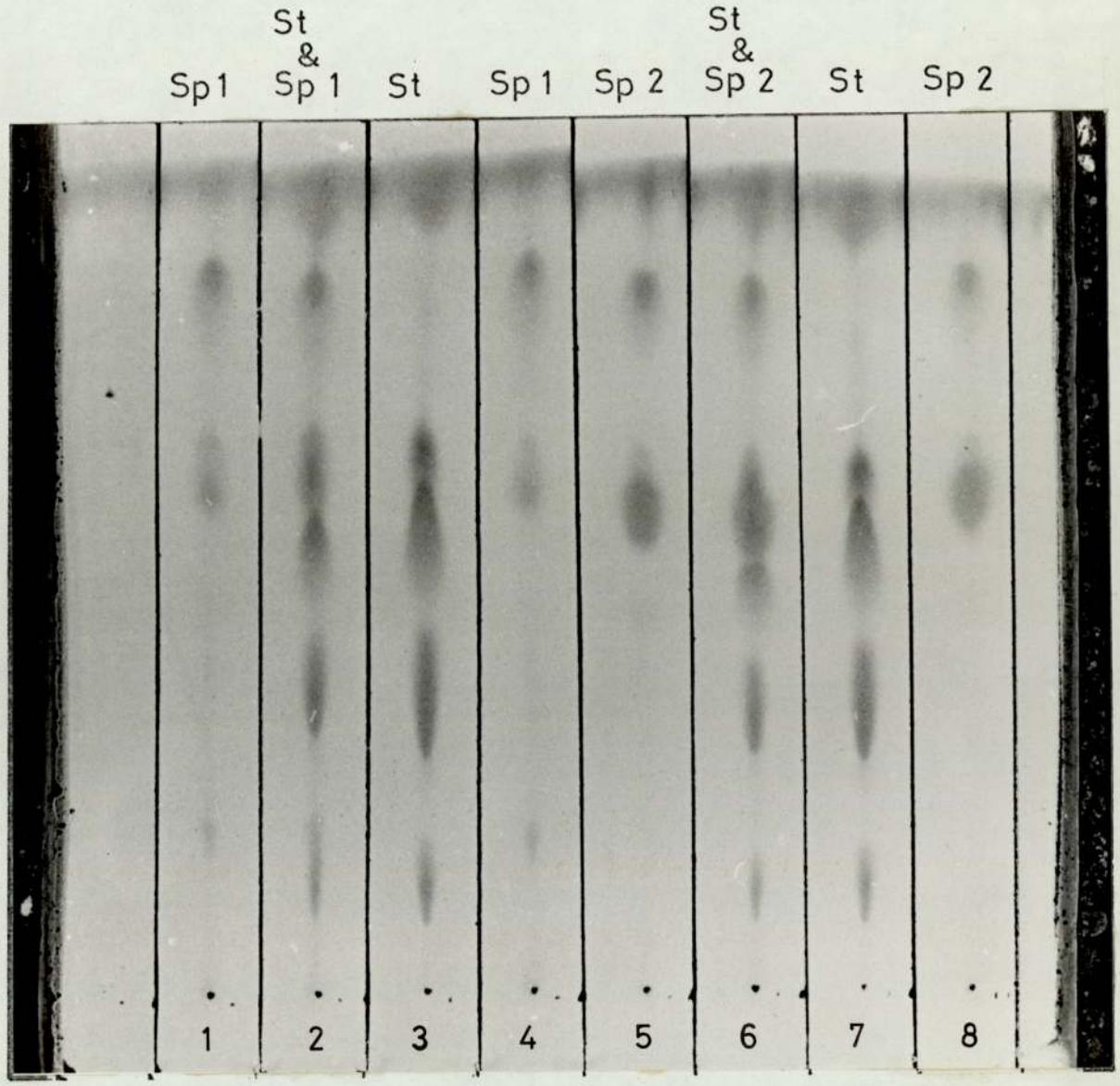
cells and cell walls as described in section 2. The P.L. fractions were dried under nitrogen and stored at -15° until required.

The total P.L. fractions were fractionated into their component P.L. by ascending thin layer chromatography (T.L.C.). Glass plates, 20 x 20 cm, were spread with a 0.25 mm layer of Silica Gel G/UV_{254} (Macherey, Nagel & Co., D-516, Duren, West Germany). The slurry was prepared by mixing 30 g of silica gel with 60 ml of aqueous sodium acetate (0.2%), and the plates air-dried overnight (Minnikin & Abdolrahimzadeh, 1971), and activated by heating at 70° for 90-min. The plates were ruled into eight 20 x 2 cm channels, and each channel loaded with a sample and/or standard P.L. mixture. The loading pattern for each plate was as follows (see Plate 1);

Channel 1	Sample 1
Channel 2	Sample 1 + Standards
Channel 3	Standards
Channel 4	Sample 1
Channel 5	Sample 2
Channel 6	Sample 2 + Standards
Channel 7	Standards
Channel 8	Sample 2

The plates were developed in a lined chromatography tank using the following solvent:- chloroform - methanol - water (65:25:4 by volume), containing 0.01% butylated hydroxytoluene as antioxidant (Brown & Watkins, 1970). After development, the plates were air-dried, sprayed with 15% ($^{W}/v$) ammonium sulphate, and the spots visualized by charring at 160° for 3-hr. Densitometry measurements of

Plate 1. Phospholipid assay: Developed chromatogram of sample and standard phospholipids.



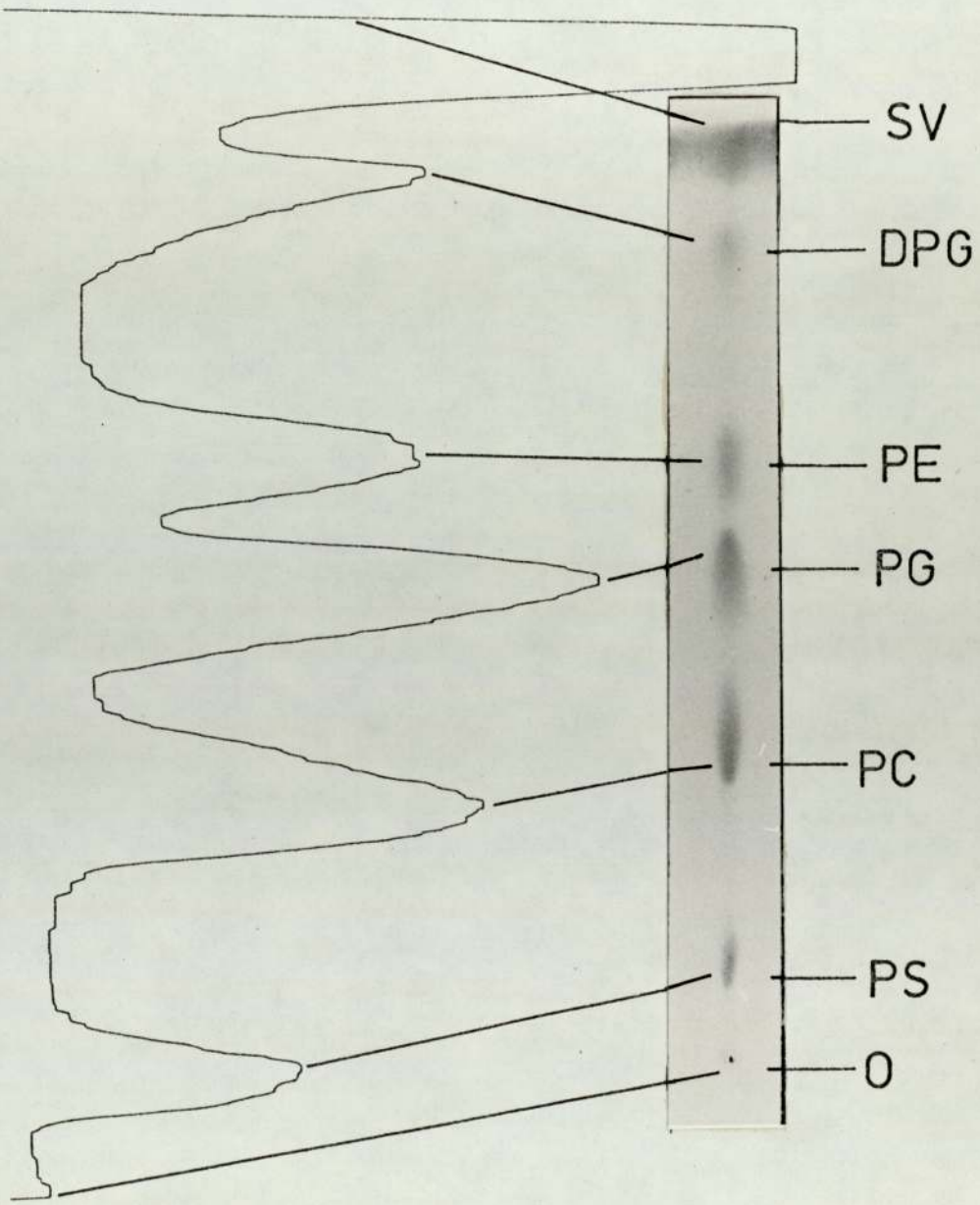
Sp 1 = Sample 1

Sp 2 = Sample 2 St = Standards

the chromatograms were made using a 'TLA 201' scanning attachment coupled with a 'Chromoscan 200' densitometer following the instrumental conditions described in the manufacturers manual (Joyce-Loebel Ltd., Gateshead, Newcastle, NE11.0UJ). The Rf values for individual lipids and standards were determined from the Chromoscan traces (see Fig. 19), the apex of a peak being taken as the middle of the corresponding spot. The proportion of an individual lipid present (as a percentage of the total P.L.) was determined using the integration readings for the corresponding peak, and expressing them as a percentage of the integration reading obtained for all the peaks in a sample. The values quoted are the means of the duplicates for each sample.

Reproducibility of fifteen replicate separations of a standard P.L. mixture were determined as follows. Fifteen T.L.C. plates were prepared as described; three of these were spotted in duplicate with a mixture of phosphatidyl-serine (P.S.), phosphatidyl-choline (P.C.), phosphatidyl-ethanolamine (P.E.) and phosphatidyl-glycerol (P.G.). The remaining twelve plates were spotted with the same standard mixture to which diphosphatidyl-glycerol (D.P.G.) had been added. The plates were developed, visualized and scanned as described. The means of the duplicate Rf values of each standard on the fifteen plates were used to calculate the standard deviation, coefficient of variation, the overall mean and the range, based on the mean plus or minus twice the standard deviation. These results are shown in Table 16. The values marked with an asterisk were outside the ranges and the results were

Figure 19. Comparison of densitometer chart recording with the thin layer chromatogram of phospholipid standards.



SV = Solvent front DPG = diphosphatidyl glycerol
PE = phosphatidyl ethanolamine
PG = phosphatidyl glycerol
PC = phosphatidyl choline
PS = phosphatidyl serine O = Origin

TABLE 16

Reproducibility of replicate Rf determinations of standard phospholipids.

Statistical Parameter	P.S.	P.C.	P.G.	P.E.	D.P.G.
	0.10	0.28	0.49	0.58	0.83
	0.12	0.30	0.48	0.57	0.80
	0.09	0.25	0.48	0.58	0.84
	0.12	0.30	0.49	0.59	0.80
	0.10	0.29	0.50	0.61	0.83
	0.09	0.25	0.44	0.55	0.86
	0.09	0.28	0.46	0.57	0.83
	0.10	0.25	0.47	0.57	0.85
	0.08	0.26	0.42	0.53	0.83
	0.09	0.27	0.43	0.52	0.84
	0.09	0.28	0.45	0.58	0.84
	0.09	0.27	0.47	0.60	0.81
	0.09	0.28	0.49	0.57	-
	0.10	0.26	0.45	0.59	-
	0.12	0.34 *	0.54 *	0.65 *	-
Mean	0.10	0.27	0.47	0.58	0.83
Standard Deviation	0.01	0.02	0.03	0.03	0.02
Coefficient of Variation %	12.2	8.4	6.3	5.5	2.5
Range From	0.07	0.23	0.41	0.51	0.79
Range To	0.12	0.32	0.53	0.64	0.87

recalculated omitting these values from the data. The recalculated values are presented in Table 17.

TABLE 17

Statistical parameters recalculated from data in Table 16.

Statistical Parameter		P.S.	P.C.	P.G.	P.E.	D.P.G.
Mean		0.01	0.27	0.46	0.57	0.83
Standard Deviation		0.01	0.02	0.02	0.03	0.02
Coefficient of Variation %		12.2	6.0	5.1	4.4	2.5
Range	From	0.07	0.24	0.42	0.52	0.79
	To	0.12	0.30	0.51	0.62	0.87

The coefficients of variation were considered acceptable (even the 12.2% obtained for P.S.) in view of the many variables that affect T.L.C. (i.e. uniformity of layer thickness, uniformity in preparing batches of the silica gel slurry, the temperature of the laboratory and hence the temperature in the T.L.C. tank).

5. NUTRIENT DEPLETION STUDIES ON P.aeruginosa GROWN IN CHEMICALLY DEFINED MEDIA.

Introduction.

Boggis (1971) investigated the limitation of growth of Pseudomonas aeruginosa by magnesium (Mg^{2+}), iron, glucose, nitrogen, phosphate, sulphate and potassium, and found that, within limits, there was a linear relation between the maximum population density (determined by optical density, $O.D._{470}$ max.) and the initial concentration of the limiting nutrient in the medium. These results are compatible with those previously obtained for Mg^{2+}, iron and glucose (Melling, 1968; Watkins, 1970). On the basis of these results, Boggis (1971), formulated a chemically defined medium (C.D.M.) that would enable large volumes (8 L) of Mg^{2+}-adequate and Mg^{2+}-depleted P.aeruginosa cultures to be grown in batch culture to $O.D._{470}$ 2.0. Both the Mg^{2+}-adequate and the Mg^{2+}-depleted media contained sufficient glucose to maintain growth only to $O.D._{470}$ 2.0. These media contained twice the molar concentrations of the non-limiting nutrients compared with the media used by Boggis (1971) in his limitation and lysis studies. Boggis (1971) found that exponential growth was only maintained to an $O.D._{470}$ of approximately 1.2, and that thereafter the growth rate steadily decreased, probably due to lack of adequate aeration, until lack of glucose stopped growth at $O.D._{470}$ 2.0.

The object of these series of Mg^{2+} and glucose depletion studies was to obtain data that would indicate if the use of the 'double strength' medium designed for 8 L batch culture (Boggis, 1971) affected the relationship

between nutrient concentration and the parameters used to measure the effect of nutrient depletion. In addition, it was desirable to see if the characteristics of nutrient depletion determined in small scale experiments (25 ml) could be reproduced in the large scale (6 L) batch culture apparatus. It was also the object to use the data obtained from such studies to formulate a medium that would be suitable for preparing cultures of P.aeruginosa to a pre-determined $O.D._{470}$ at which the growth of the culture would cease due to the lack of glucose or of glucose and Mg^{2+}.

Experimental.

The media used in the nutrient depletion studies are summarised in Table 18, and details of sampling procedure for growth curves have been described previously (section 3). In experiments with large volume cultures (6 L) the media were inoculated with 25 ml of a glucose-depleted ($O.D._{470}$ approximately 1.0) culture, and readings were taken after 3-hr to allow for the lag period. In both systems (25 ml and 6 L batches) readings below $O.D._{470}$ 0.02 were considered inaccurate as they occur at the extreme end of the optical density scale of the Pye-Unicam S.P. 600. 'Carry over' of the limiting nutrient in the inoculum was considered insignificant in both large and small scale experiments. In the small scale depletion studies the inoculum was effectively diluted 20,000-fold (0.25 ml of the 200-fold dilution into 25 ml medium), and in the 6 L studies the dilution was 240-fold. A 240-fold dilution of the highest inoculum Mg^{2+} concentration (8.226×10^{-5} M) would give a value of 3.290×10^{-7} M, significantly lower than the lowest Mg^{2+} concentration used in 6 L (4.113×10^{-6} M).

TABLE 18

Chemically defined media used in nutrient depletion studies upon P.aeruginosa.

Medium component \ Depleted nutrient	Magnesium	Glucose
$(NH_4)_2HPO_4$	3.904×10^{-2}	3.904×10^{-2}
$NH_4H_2PO_4$	9.600×10^{-4}	9.600×10^{-4}
$(NH_4)_2SO_4$	3.645×10^{-4}	2.000×10^{-4}
NaCl	1.000×10^{-3}	1.000×10^{-3}
KCl	1.000×10^{-3}	1.000×10^{-3}
$FeSO_4$	8.950×10^{-6}	8.950×10^{-6}
$MgSO_4.7H_2O$	*	1.645×10^{-4}
Glucose	2.000×10^{-2}	*

pH 7.8. All concentrations are molar.

* Nutrient concentration as described in the text.

Since the inocula for the large scale experiments were glucose-depleted ('Clinistix' glucose strips gave negative results), 'carry over' of glucose was considered insignificant.

Glucose depletion in 25 ml volumes.

The growth curves for glucose-depleted cultures of P.aeruginosa are shown in Fig. 20. The exponential phase of the cultures containing the two lowest initial glucose concentrations (5.0×10^{-4} M and 1.0×10^{-3} M) appeared to exhibit slightly lower growth rates than the other cultures. All the cultures exhibited a very rapid cessation of growth, followed by a low rate of lysis. Fig. 21 shows that the relation between the initial glucose concentration

Figure 20. Growth of P aeruginosa in CDM containing graded concentrations of glucose.

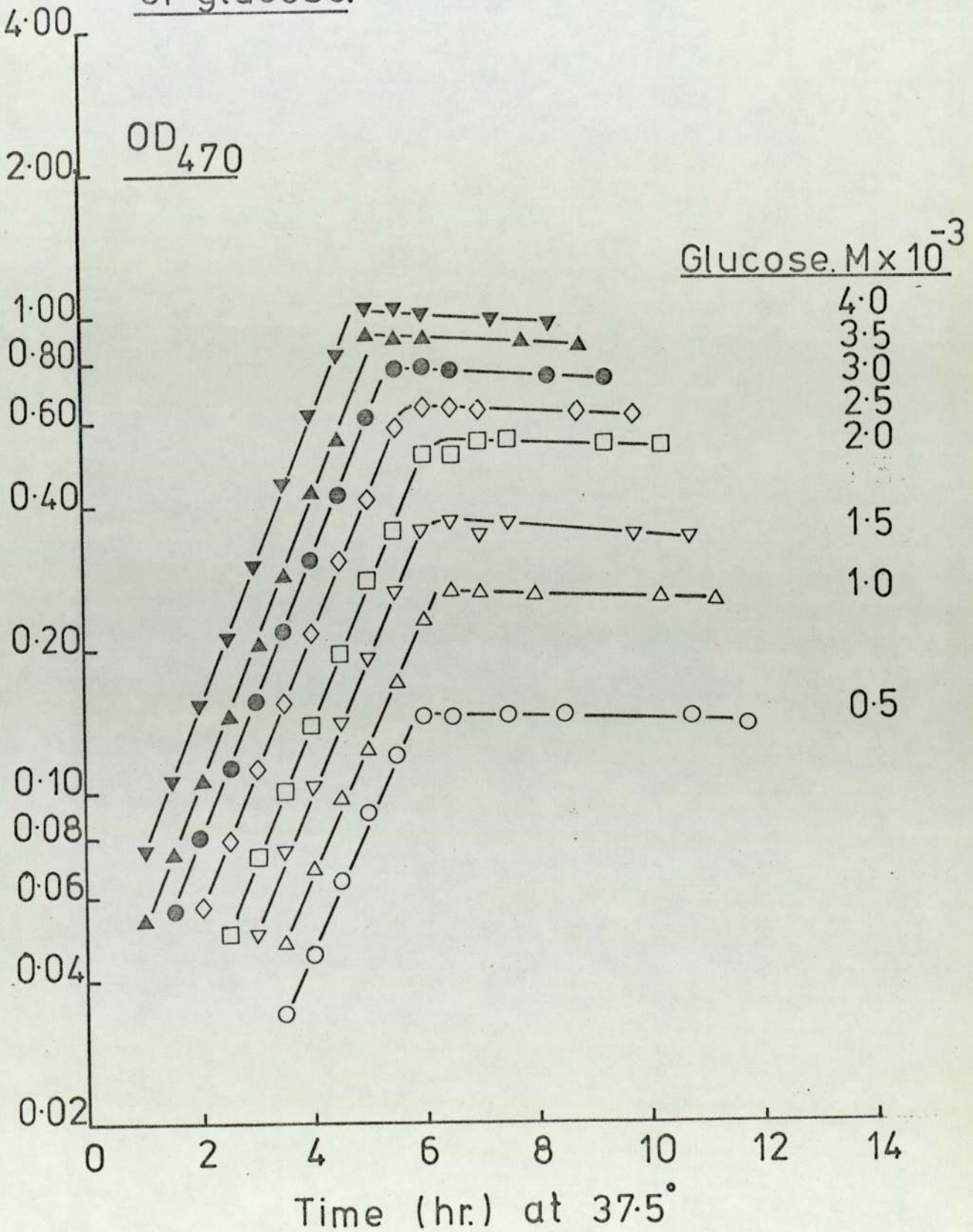
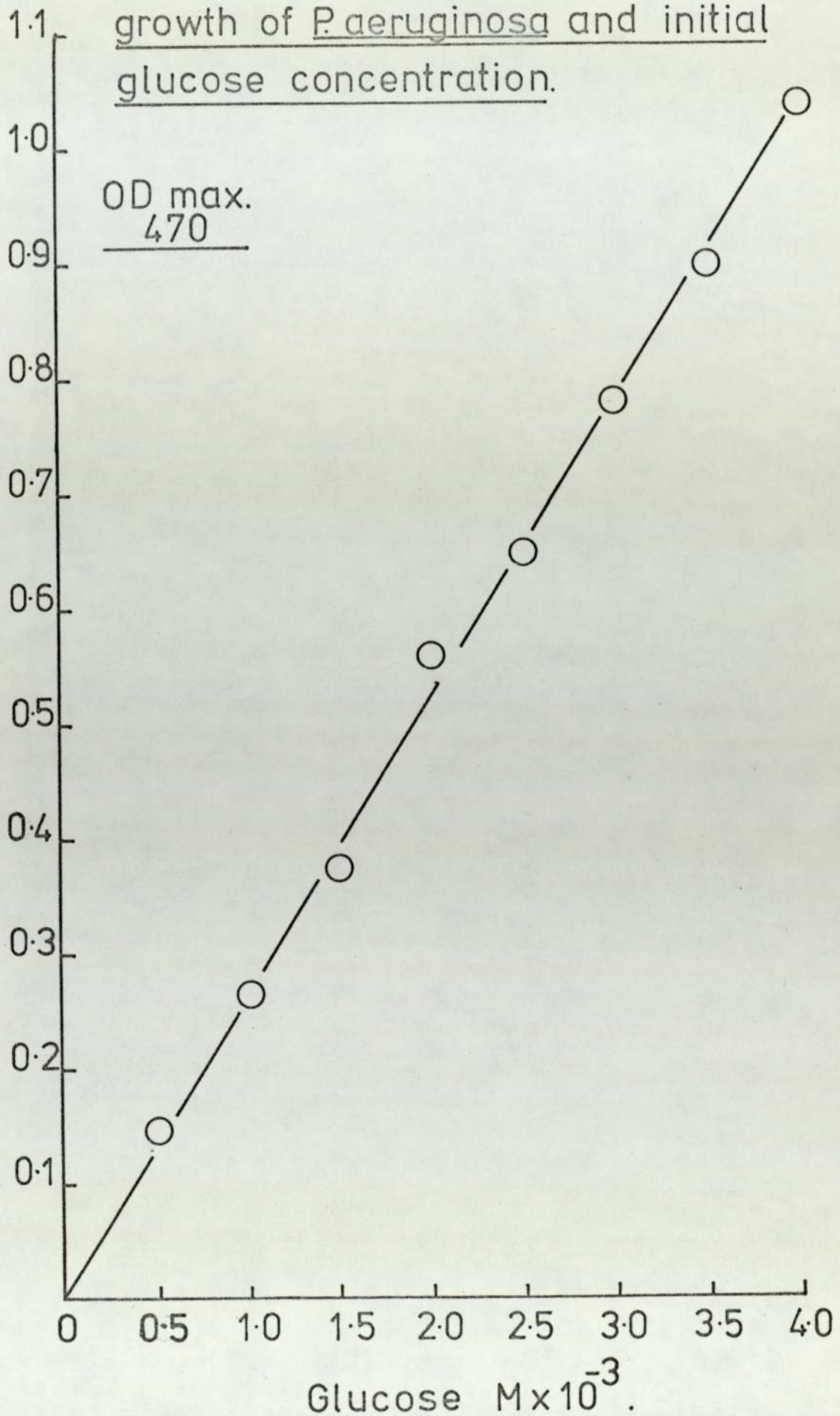


Figure 21. Relation between maximum growth of P. aeruginosa and initial glucose concentration.



and the maximum growth ($O.D._{470}$) was linear within the concentration range used, and that an initial concentration of 3.8×10^{-3} M was required for growth to cease at $O.D._{470}$ 1.0.

Magnesium-depletion in 25 ml volumes.

The growth curves for Mg^{2+}-depleted cultures are shown in Fig. 22 and 24. The first set of curves illustrate the growth curves for Mg^{2+} concentrations ranging from 1.645×10^{-7} M to 3.29×10^{-6} M, and show that the exponential growth rate is dependant upon the initial Mg^{2+} concentration below 2.47×10^{-6} M. The growth rates after exponential growth had ceased gradually decreased, but did not decrease to zero. As an index of Mg^{2+}-depletion, the $O.D._{470}$ 3-hr after the growth rate became non-exponential ($O.D._{470}$ max.) was plotted against the initial concentration of Mg^{2+}. The relation of $O.D._{470}$ max. to the initial Mg^{2+} concentration is seen to be linear between 1.645×10^{-7} M and 3.290×10^{-6} M (Fig. 23). The second set of growth curves for Mg^{2+}-depleted cultures (Fig. 24) are for Mg^{2+} concentrations from 2.060×10^{-6} M to 4.110×10^{-5} M. All the growth curves are similar and show no variation in exponential growth rate with the exception of the 2.060×10^{-6} M culture. The relation between $O.D._{470}$ max. and initial concentration is shown in Fig. 25 and is linear up to a Mg^{2+} concentration of 1.650×10^{-5} M. Fig. 25 shows that Mg^{2+} appears to be the major growth limiting nutrient at an $O.D._{470}$ 1.0.

Glucose-depletion in 6 L volumes.

An inoculum (25 ml) of glucose-depleted P.aeruginosa at 37.5° was added to 5,965 ml of C.D.M. at 37.5°. This addition

Figure 22. Growth of P.aeruginosa in GDM containing graded concentrations of Mg^{2+}.

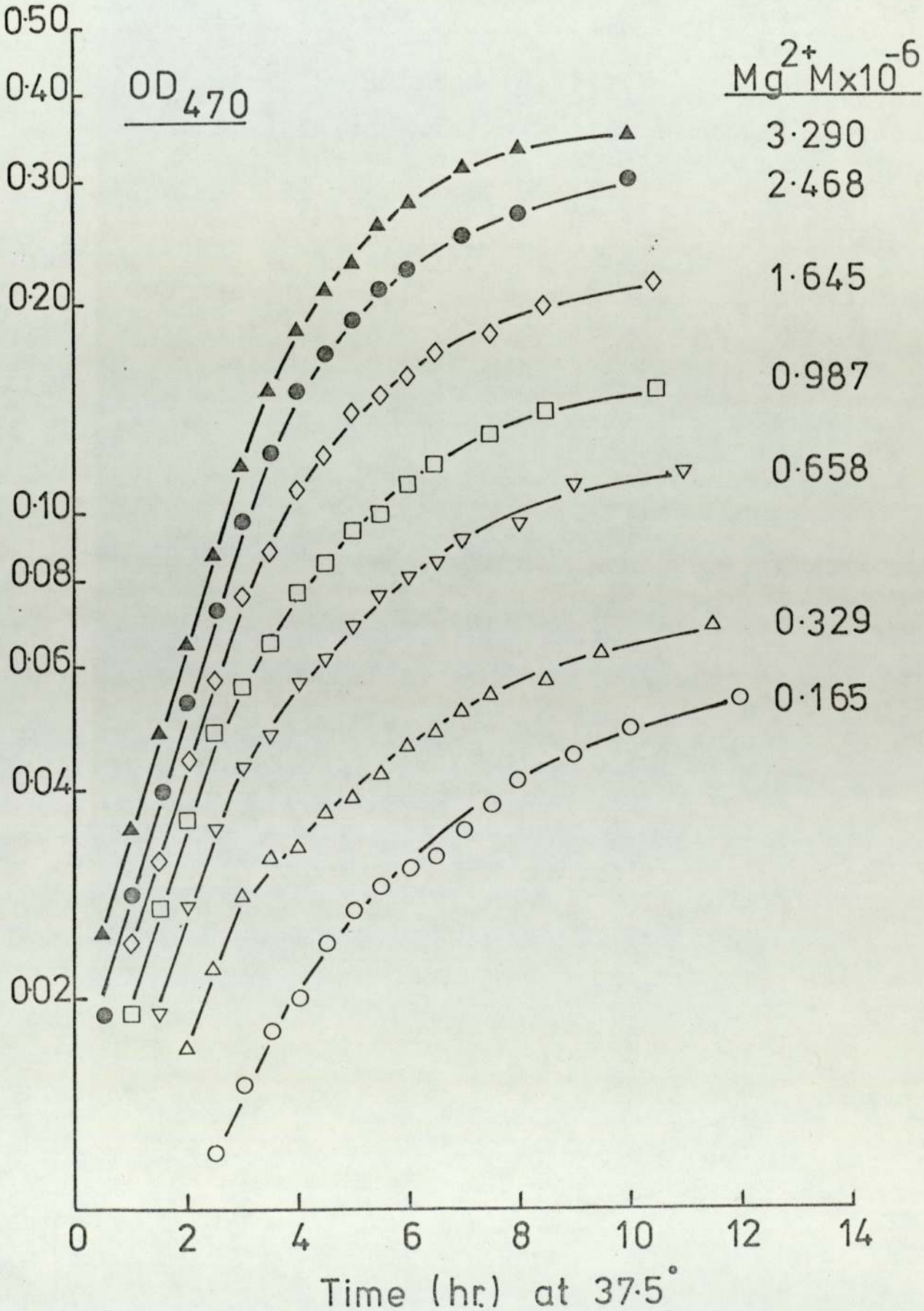


Figure 23. Relation between maximum growth of P.aeruginosa and initial magnesium concentration.

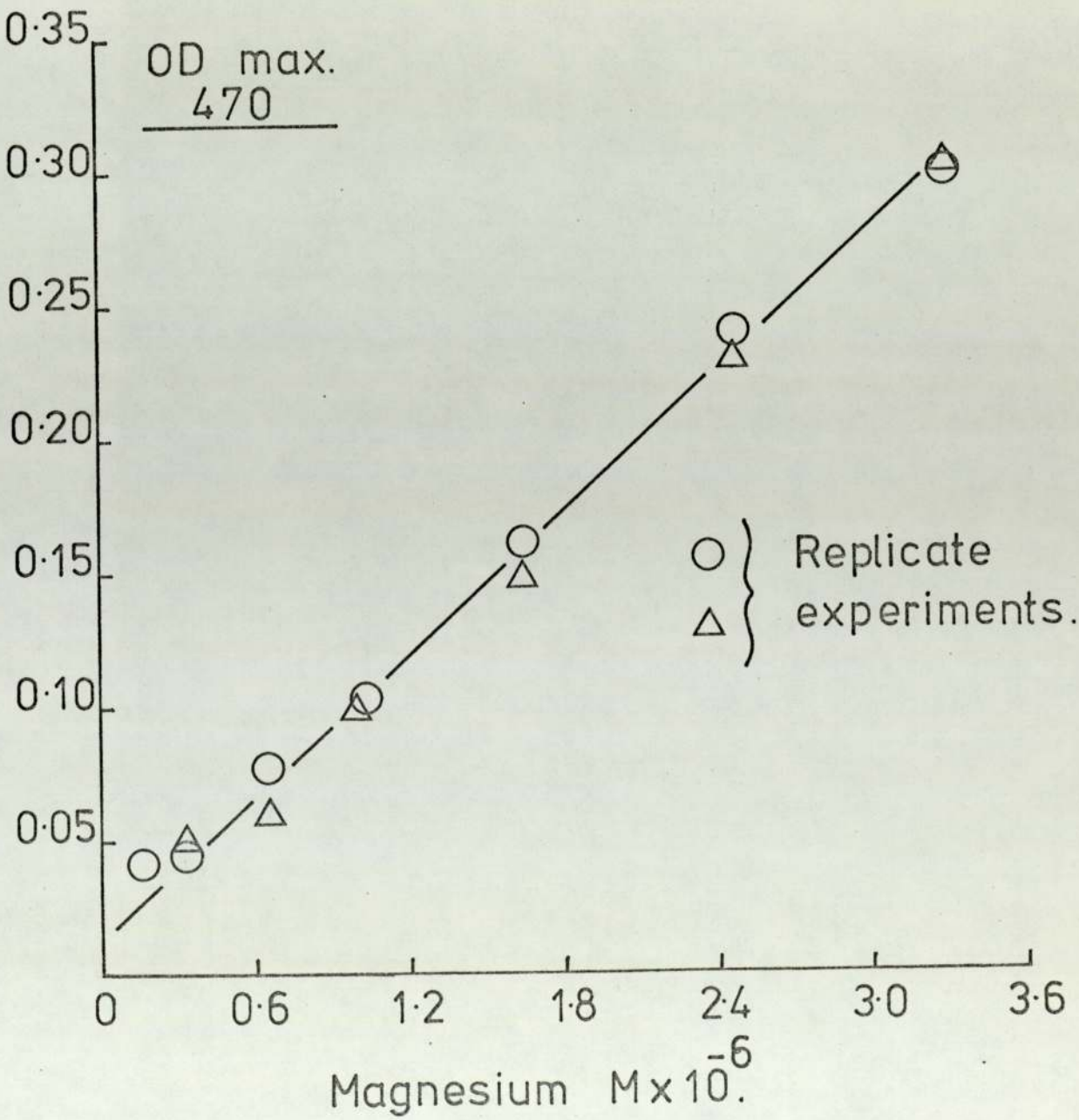


Figure 24. Growth of P. aeruginosa in CDM containing graded concentrations of Mg^{2+}.

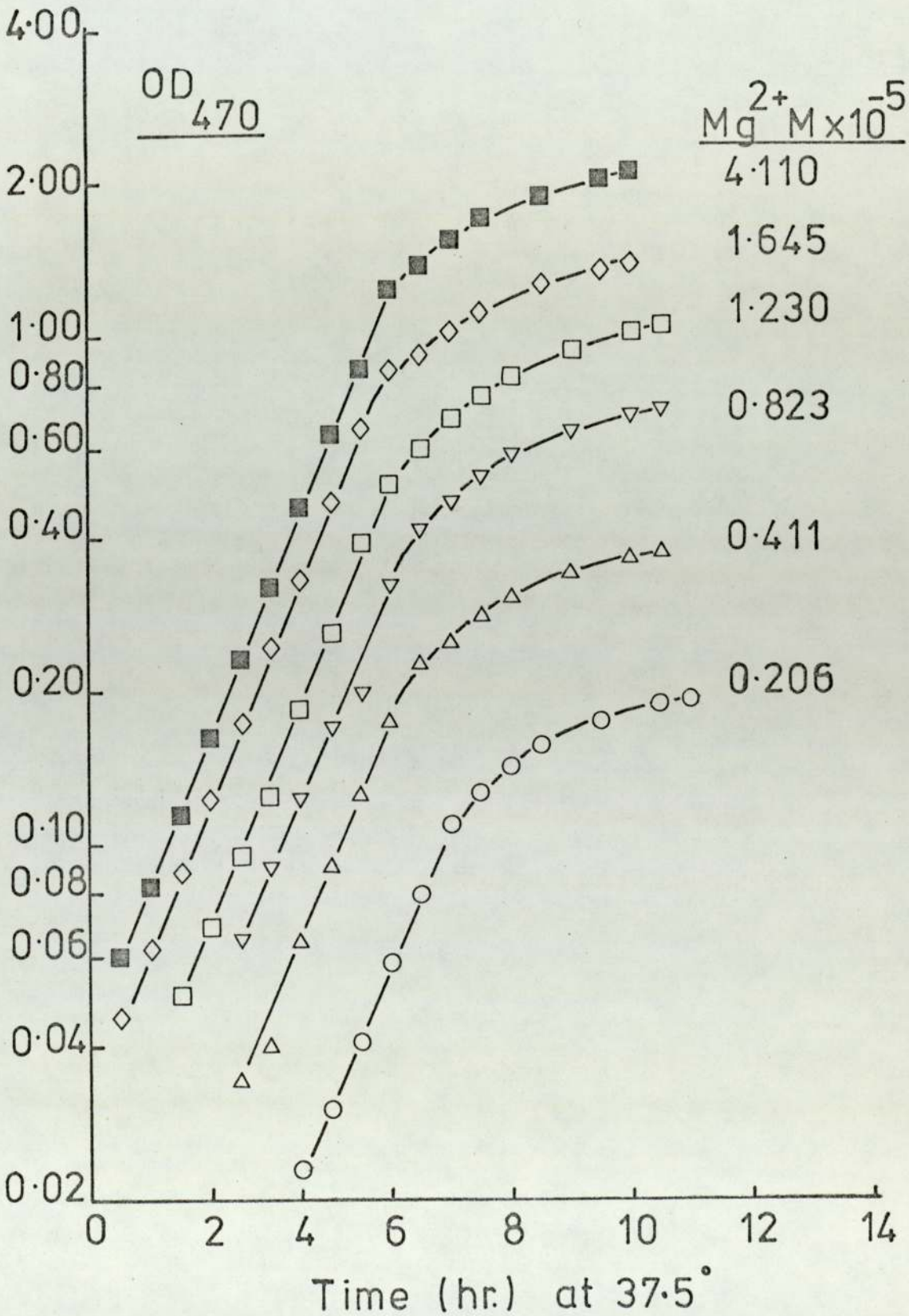
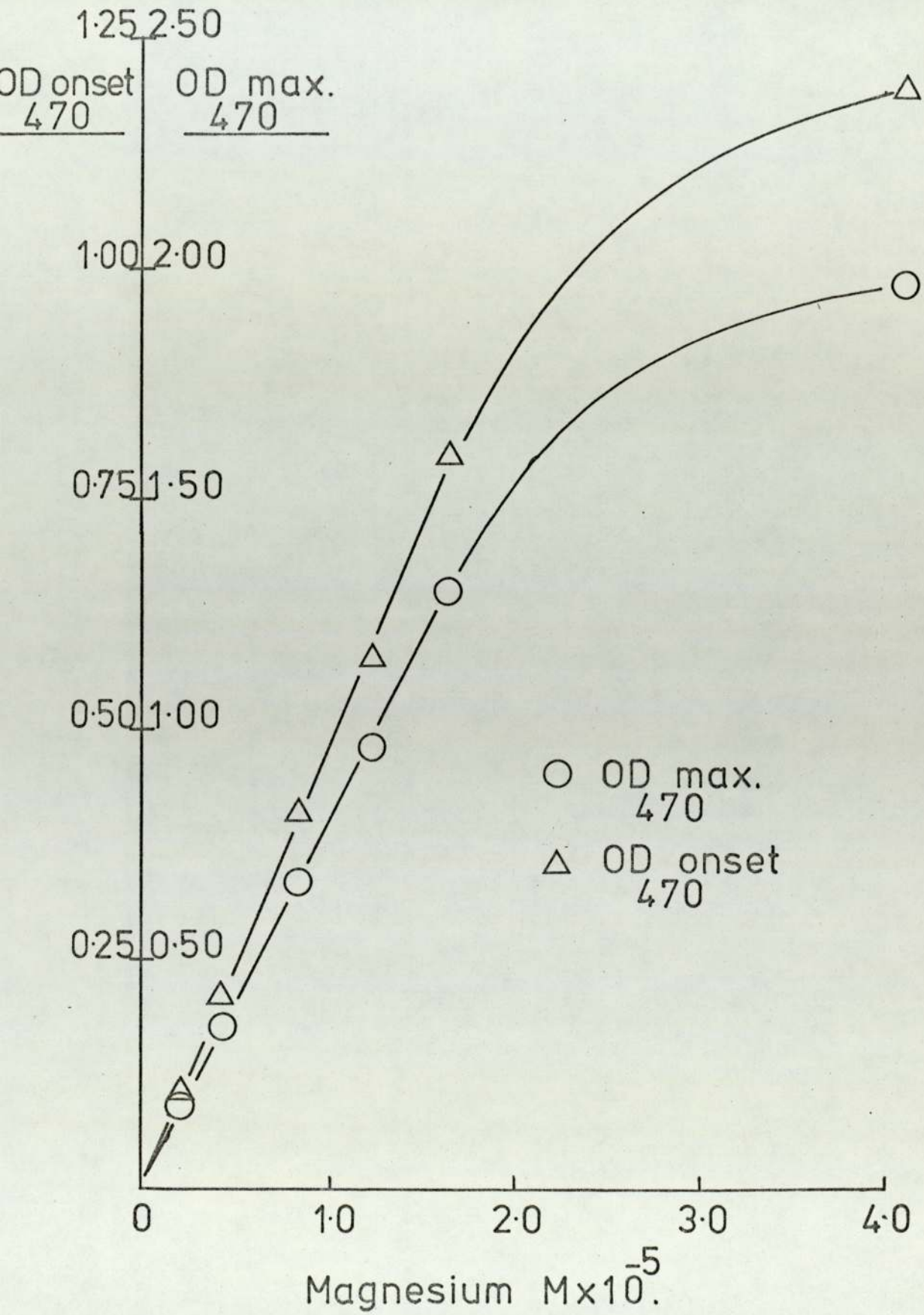


Figure 25. Relation between initial Mg^{2+} concentration and onset of growth rate limitation or maximum growth of P. aeruginosa.



was immediately followed by the addition of 2 ml of stock glucose (0.62 M) to give a final glucose concentration of 2.06×10^{-4} M. Optical density measurements were made at 15-min intervals, until the growth had ceased. At this point 1 ml of the stock glucose was added and measurements again taken until growth ceased. This procedure was repeated, adding variable amounts of glucose (see Fig. 26), until a total of 11 ml of glucose had been added, and growth had ceased. At this point the volume of the medium was 6,001 ml, and the total glucose added was equivalent to an initial concentration of 11.4×10^{-4} M. The growth curve is presented in Fig. 26 and shows that exponential growth proceded at each addition without any appreciable lag period. The growth rates of the five exponential curves appeared not to differ significantly. The relation between the $O.D._{470}$ max. and the cumulative concentration of glucose added is shown in Fig. 28 and is linear over the concentrations used. The molarities were calculated for each volume between 5,990 and 6,001 ml. The other nutrients were present in the concentrations shown in Table 18 at a culture volume of 6,000 ml. Thus they were present in a slight excess of the stated concentration at the start of the experiment; this was considered preferable to starting with 6,000 ml and increasing the volume to 6,011 ml resulting in a slight lowering of the stated concentrations.

The preceding experiment was repeated using a range of glucose concentrations from 11.4×10^{-4} M to 3.8×10^{-3} M. The growth curves are shown in Fig. 27. The relation between the cumulative concentrations of

Figure 26. Effect of added glucose upon the growth of P. aeruginosa in a 6L. batch of CDM.

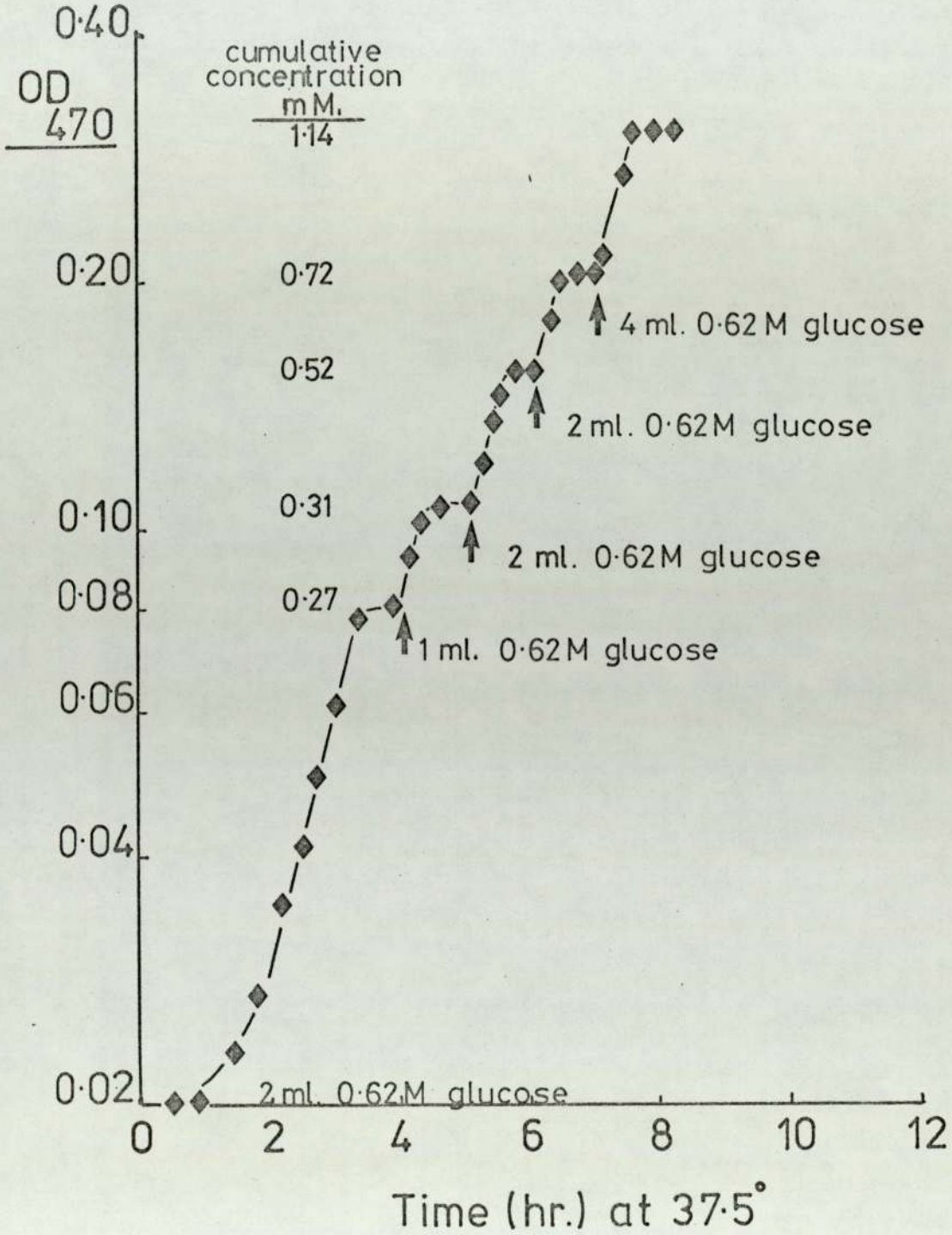


Figure 27 Effect of added glucose on the growth of P. aeruginosa in a 6L. batch of CDM.

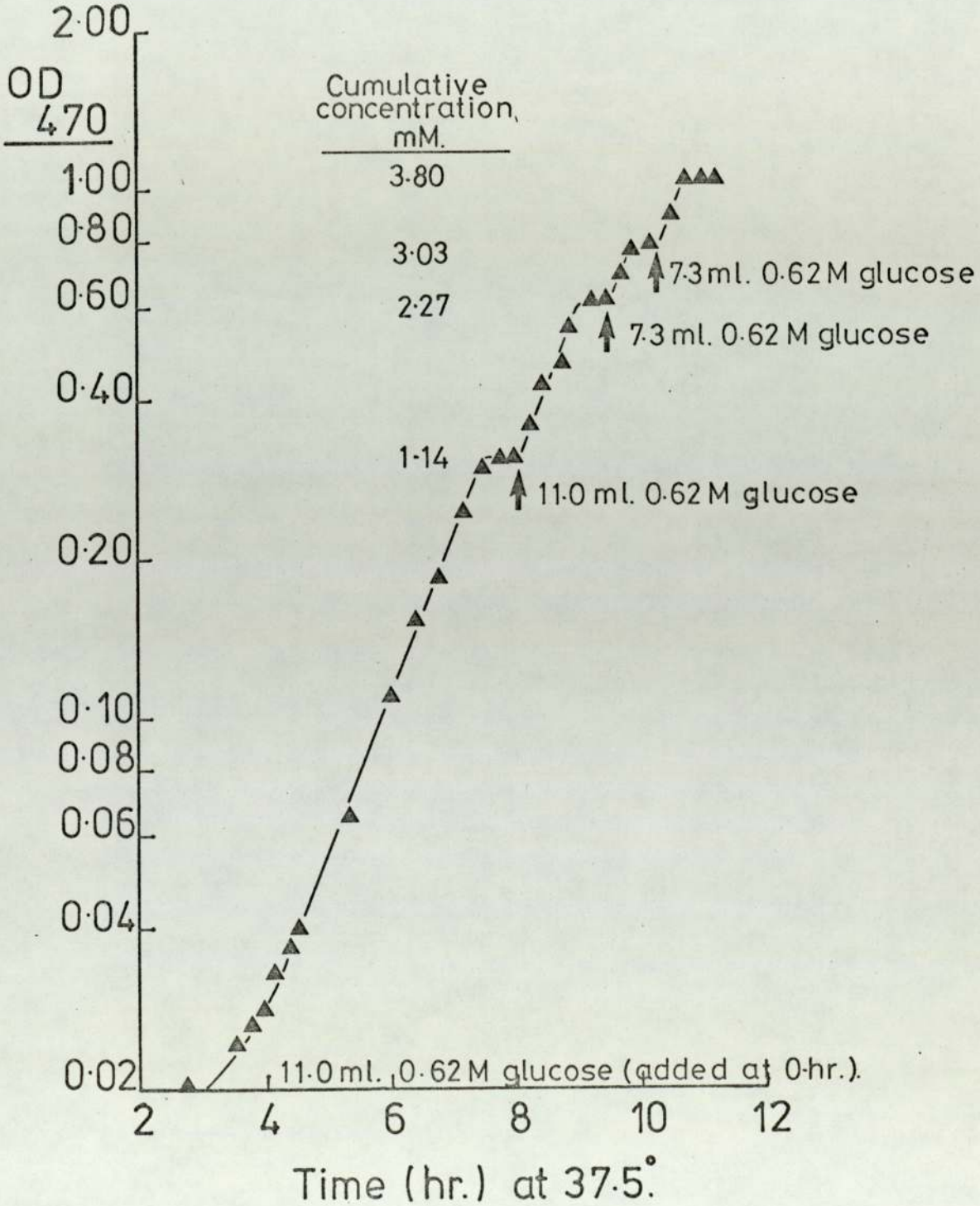
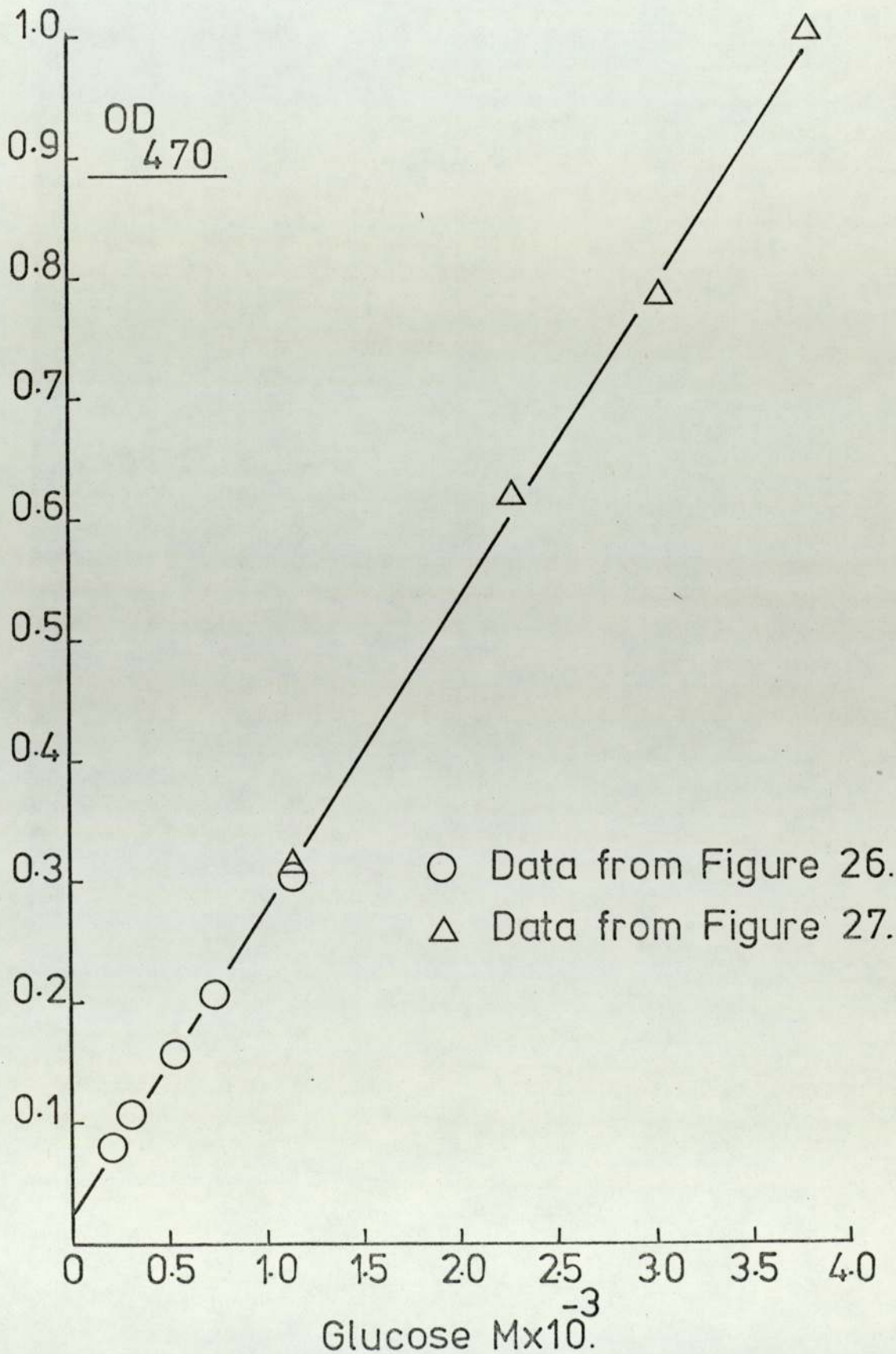


Figure 28. Relation between maximum growth of P. aeruginosa and cumulative concentration of glucose in 6L. batches of CDM.



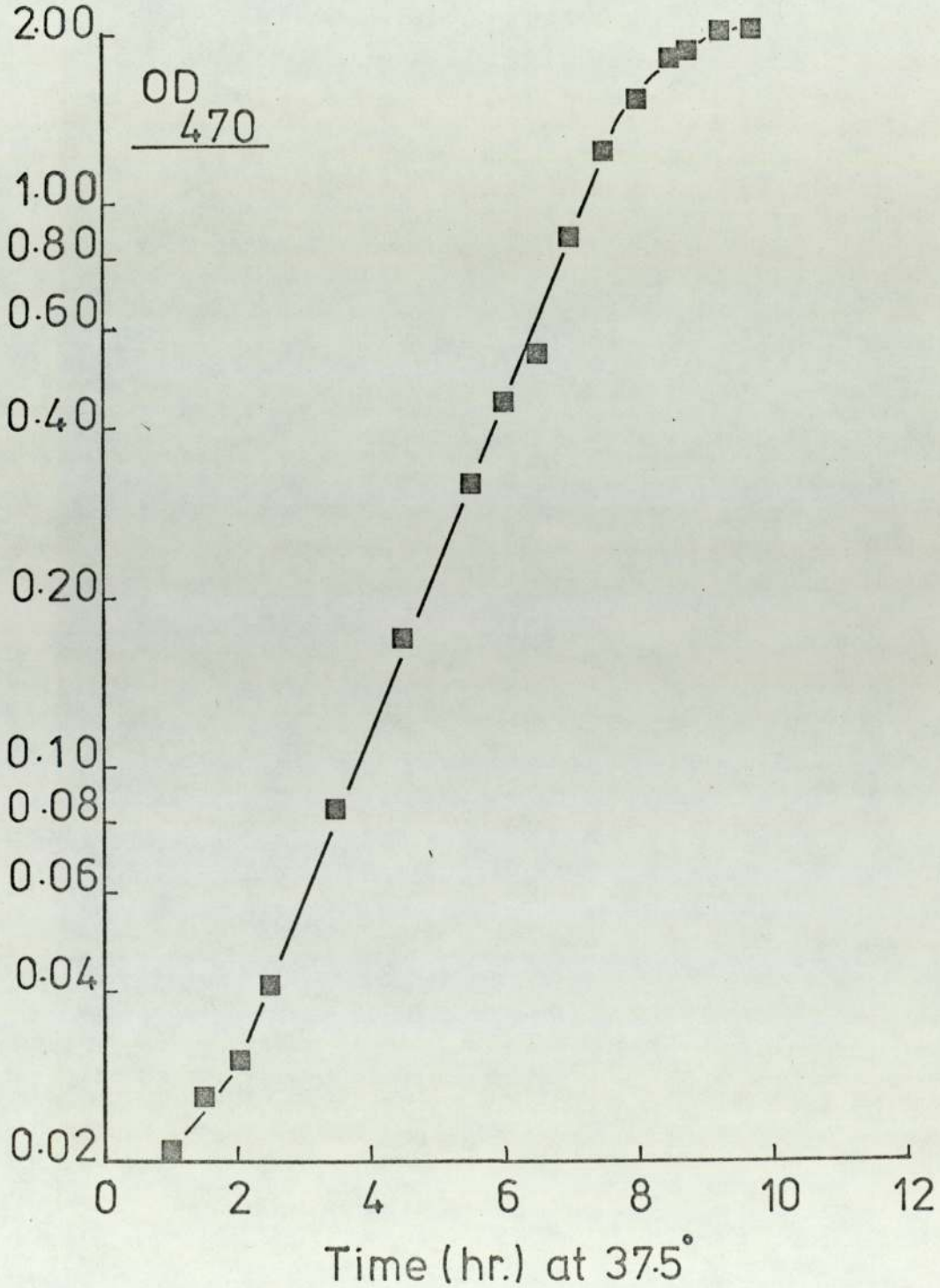
glucose and the O.D.$_{470}$ max. is linear over the range of concentrations used (Fig. 28). The relations from both experiments are compatible with one another.

To check the growth rate up to an O.D.$_{470}$ 1.0, 6 L of media containing sufficient glucose (1.5×10^{-2} M) to support unlimited growth to O.D.$_{470}$ 4.0 was inoculated with 25 ml of a stationary phase culture. Optical density measurements were made every hour up to O.D.$_{470}$ 0.3, then half hourly, and finally at 15-min intervals until growth ceased. The growth curve (Fig. 29) was plotted as the measurements became available. As soon as it was apparent that the growth was no longer exponential the readings were taken every 15-min. As can be seen in Fig. 29, a reduction in the exponential growth rate did not take place until an O.D.$_{470}$ of 1.4 or greater. This reduction was probably due to lack of oxygen. It was decided on the basis of this experiment to limit the 6 L culture at O.D.$_{470}$ 1.0 when preparing cells for analysis and resistance studies.

Magnesium-depletion in 6 L volumes.

The experiments were performed in a similar manner to that used for glucose-depletion studies. The medium, without Mg^{2+}, was made up to 5,971 ml and inoculated with 25 ml of culture grown in C.D.M. containing 8.23×10^{-5} M-Mg^{2+}. 'Carry over' of Mg^{2+} in the inoculum was considered insignificant since the final concentration of Mg^{2+} in the inoculum would be 3.29×10^{-7} M whereas the lowest Mg^{2+} concentration in the experiment was 4.11×10^{-6} M-Mg^{2+}. Magnesium was added to the growth medium in 1 ml volumes from a sterile stock solution

Figure 29. Growth of P. aeruginosa in 6L. CDM containing plentiful Mg^{2+} (1·645 mM.) and glucose (8·1 mM.).



containing 2.47×10^{-2} M. The cumulative concentration of Mg^{2+} achieved was 4.11×10^{-6} M, 8.22×10^{-6} M, 1.23×10^{-5} M and 1.65×10^{-5} M. The parameter used to measure lack of Mg^{2+} in this experiment was the onset of growth rate limitation. Although the determination of the exact point at which onset occurs is open to some variation in interpretation (see Fig. 30), the relation of the estimated onset to Mg^{2+} concentration was linear and compatible with the results from 25 ml volume experiments (Fig. 31). As with the 6 L glucose experiment, the addition of Mg^{2+} to Mg^{2+}-depleted cultures resulted in growth without any appreciable lag, an effect that has been observed before (Brown & Melling, 1969b).

On the basis of this and preceding experiments it was decided to prepare Mg^{2+}-depleted cultures by growth in a medium containing 8.23×10^{-6} M-Mg^{2+}, up to an $O.D._{470}$ 1.0 at which point growth would cease due to the lack of glucose. In order to determine how much glucose would be required by an ultimately Mg^{2+}-depleted and glucose-depleted culture to grow to $O.D._{470}$ 1.0 the following experiment was performed. To 6 L of C.D.M. containing 8.23×10^{-6} M-Mg^{2+} and 6.00×10^{-3} M-glucose (sufficient to support maximum growth rate to $O.D._{470}$ 1.5) 0.3 ml of a stationary phase culture were added. Readings of optical density were commenced 15-hr after inoculation (compare 25 ml volume limitation studies) and taken at 30-min intervals until the $O.D._{470}$ was 0.4, at which point readings were made every 15-min. Growth ceased at $O.D._{470}$ 0.68. At this stage, 30 ml of stock glucose was added and optical density measurements resumed until

Figure 30. Effect of added magnesium on the growth of P.aeruginosa in a 6L. batch of CDM.

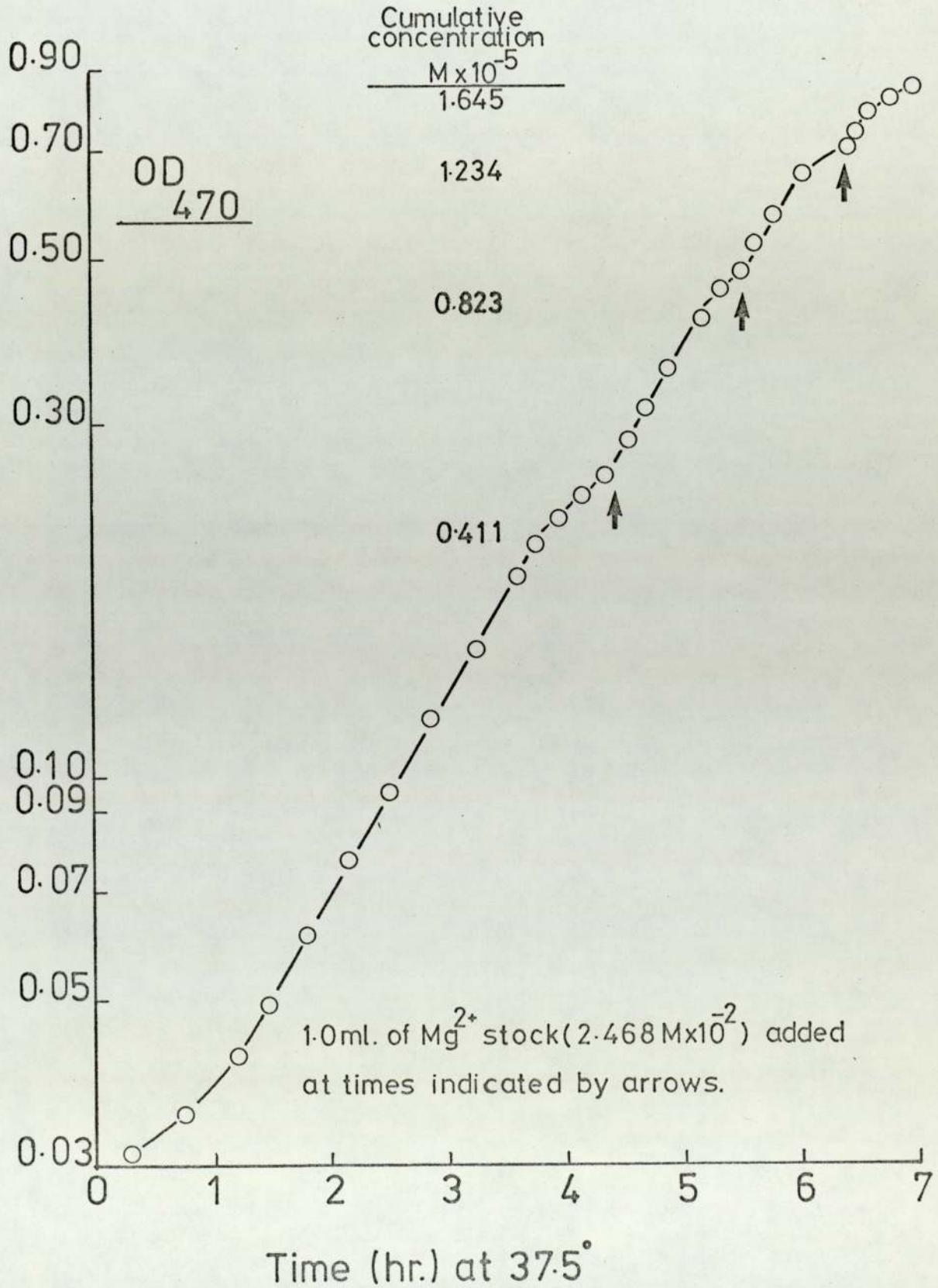
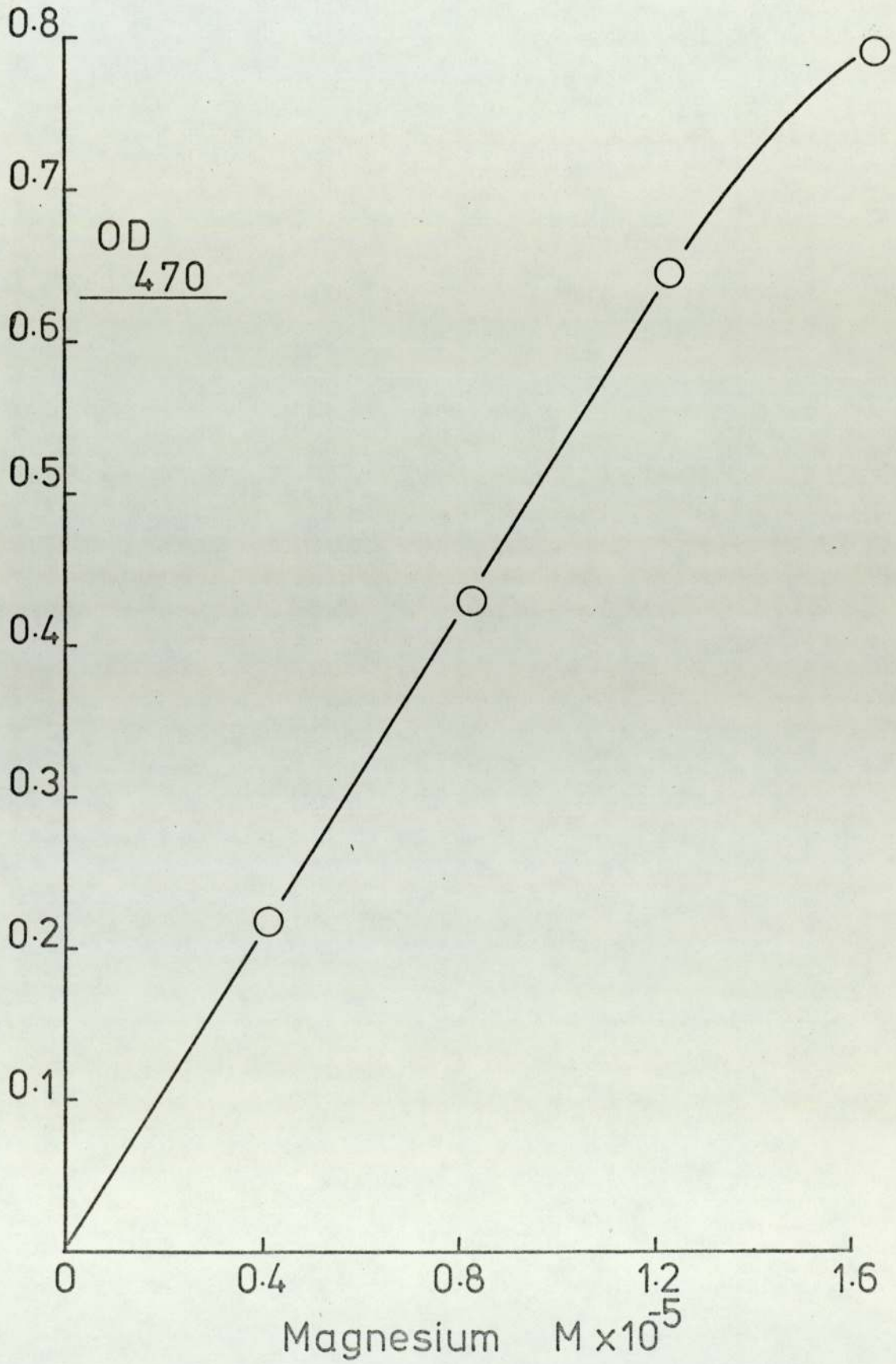


Figure 31. Relation between cumulative concentration of Mg^{2+} and onset of growth rate limitation of P.aeruginosa in a 6L. batch of CDM.



growth ceased again, this time at $O.D._{470}$ 0.84. A further addition (30 ml) of glucose was made and growth followed until it ceased, this time at $O.D._{470}$ 0.99. No further additions of glucose were made, but one final reading was made 39-hr after inoculation at which time the $O.D._{470}$ was 1.05. This experiment was repeated and found to give reproducible results. In this experiment it was necessary to start with a culture volume of exactly 6 L so that the growth rate would decrease due to Mg^{2+}-depletion at an $O.D._{470}$ value one would expect to occur for 8.23×10^{-6} M-Mg^{2+} (i.e. $O.D._{470}$ 0.4). Therefore, the growth of the culture after the addition of glucose was in media containing other nutrients at a slightly lower concentration (0.5 - 1.0% lower) than depicted in Table 18 however, since the orginal concentrations were 3 to 4-fold in excess of that required to reach $O.D._{470}$ 1.0 this was considered insignificant. On the basis of this experiment it was decided that the C.D.M. containing 8.23×10^{-6} M-Mg^{2+} should contain 1.20×10^{-2} M-glucose to support growth to an $O.D._{470}$ 1.0. A 6 L batch of this medium was prepared, inoculated, and growth was followed by optical density measurements through the latter stages of growth. The growth of the culture ceased at an $O.D._{470}$ 1.20, 30-hr after inoculation. 'Clinistix' tests for glucose were negative.

Thus the following concentrations of glucose and magnesium were used to prepare cultures of Mg^{2+}-adequate and Mg^{2+}-depleted P.aeruginosa:-

Mg^{2+}-adequate culture - 1.65×10^{-4} M-Mg^{2+}

3.80×10^{-3} M-glucose

Mg^{2+}-depleted culture - 8.23×10^{-6} M-Mg^{2+}

1.20×10^{-2} M-glucose

Effect of added calcium or manganese upon the growth of Mg^{2+}-adequate and Mg^{2+}-depleted cultures of P.aeruginosa.

The addition of calcium (Ca^{2+}) to P.aeruginosa growing in conditions of Mg^{2+}-depletion has been shown partially to restore the sensitivity of this organism to ethylenediaminetetra-acetic acid (E.D.T.A.) and polymyxin (P.X.) (Brown & Melling, 1969b), and significantly alter the maximum growth of the culture (Boggis, 1971). Boggis (1971) also found that manganese (Mn^{2+}) had a similar effect, although not to such an extent as Ca^{2+}.

The media used for the growth experiments of Mg^{2+}-depleted cultures is detailed in Table 19. Four 25 ml batches of C.D.M. containing the cations Mg^{2+}; Mg^{2+} and Ca^{2+}; Mg^{2+} and Mn^{2+}; Mg^{2+}, Ca^{2+} and Mn^{2+}, were prepared and inoculated. Growth curves were constructed from $O.D._{470}$ measurements and are presented in Fig. 32. The presence of Ca^{2+} and/or Mn^{2+} appeared to have no effect upon the exponential growth rate, the onset of limitation due to lack of Mg^{2+}, nor upon the $O.D._{470}$ max. These results indicated that the added cations do not require the concentration of the carbon source of the medium to be altered from 0.012 M, since they did not significantly alter the growth rate prior to, or after Mg^{2+}-depletion onset or the final $O.D._{470}$.

The media used for the growth experiments of Mg^{2+}-adequate cultures is detailed in Table 20. Four 25 ml batches of C.D.M. containing the cations Mg^{2+}; Mg^{2+} and Ca^{2+}; Mg^{2+} and Mn^{2+}; Mg^{2+}, Ca^{2+} and Mn^{2+} were prepared

TABLE 19

Chemically defined media used to prepare cultures of Mg^{2+}-depleted P.aeruginosa grown in the presence of excess Ca^{2+} or Mn^{2+}.

Medium component \ Cation	none	Ca^{2+}	Mn^{2+}	Ca^{2+} + Mn^{2+}
$(NH_4)_2HPO_4$	3.90×10^{-2}	3.90×10^{-2}	3.90×10^{-2}	3.90×10^{-2}
$NH_4H_2PO_4$	9.60×10^{-4}	9.60×10^{-4}	9.60×10^{-4}	9.60×10^{-4}
$(NH_4)_2SO_4$	3.56×10^{-4}	3.56×10^{-4}	3.36×10^{-4}	3.36×10^{-4}
NaCl	1.00×10^{-3}	8.35×10^{-4}	1.00×10^{-3}	8.35×10^{-4}
KCl	1.00×10^{-3}	1.00×10^{-3}	1.00×10^{-3}	1.00×10^{-3}
$FeSO_4.7H_2O$	8.95×10^{-6}	8.95×10^{-6}	8.95×10^{-6}	8.95×10^{-6}
$MgSO_4.7H_2O$	8.23×10^{-6}	8.23×10^{-6}	8.23×10^{-6}	8.23×10^{-6}
$CaCl_2.6H_2O$	-	1.65×10^{-4}	-	1.65×10^{-4}
$MnSO_4$	-	-	2.00×10^{-5}	2.00×10^{-5}
Glucose	1.20×10^{-2}	1.20×10^{-2}	1.20×10^{-2}	1.20×10^{-2}

inoculated and growth curves constructed as before. Fig. 33 shows that the presence of Ca^{2+} or Mn^{2+} has no significant effect upon the exponential growth rate or the point at which growth rate ceases to be exponential.

TABLE 20

Chemically defined media used to prepare cultures of Mg^{2+}-adequate P.aeruginosa grown in the presence of excess Ca^{2+} or Mn^{2+}.

Medium component \ Cation	none	Ca^{2+}	Mn^{2+}	Ca^{2+} + Mn^{2+}
$(NH_4)_2HPO_4$	3.90×10^{-2}	3.90×10^{-2}	3.90×10^{-2}	3.90×10^{-2}
$NH_4H_2PO_4$	9.60×10^{-4}	9.60×10^{-4}	9.60×10^{-4}	9.60×10^{-4}
$(NH_4)_2SO_4$	2.00×10^{-4}	2.00×10^{-4}	1.80×10^{-4}	1.80×10^{-4}
NaCl	1.00×10^{-3}	8.38×10^{-4}	1.00×10^{-3}	8.36×10^{-4}
KCl	1.00×10^{-3}	1.00×10^{-3}	1.00×10^{-3}	1.00×10^{-3}
$FeSO_4 \cdot 7H_2O$	8.95×10^{-6}	8.95×10^{-6}	8.95×10^{-6}	8.95×10^{-6}
$MgSO_4 \cdot 7H_2O$	1.65×10^{-4}	1.65×10^{-4}	1.65×10^{-4}	1.65×10^{-4}
$CaCl_2 \cdot 6H_2O$	-	1.65×10^{-4}	-	1.65×10^{-4}
$MnSO_4$	-	-	2.00×10^{-5}	2.00×10^{-5}
Glucose	1.50×10^{-2}	1.50×10^{-2}	1.50×10^{-2}	1.50×10^{-2}

Figure 32. Growth of P.aeruginosa in Mg^{2+}-depleted CDM supplemented with Ca^{2+} or Mn^{2+}

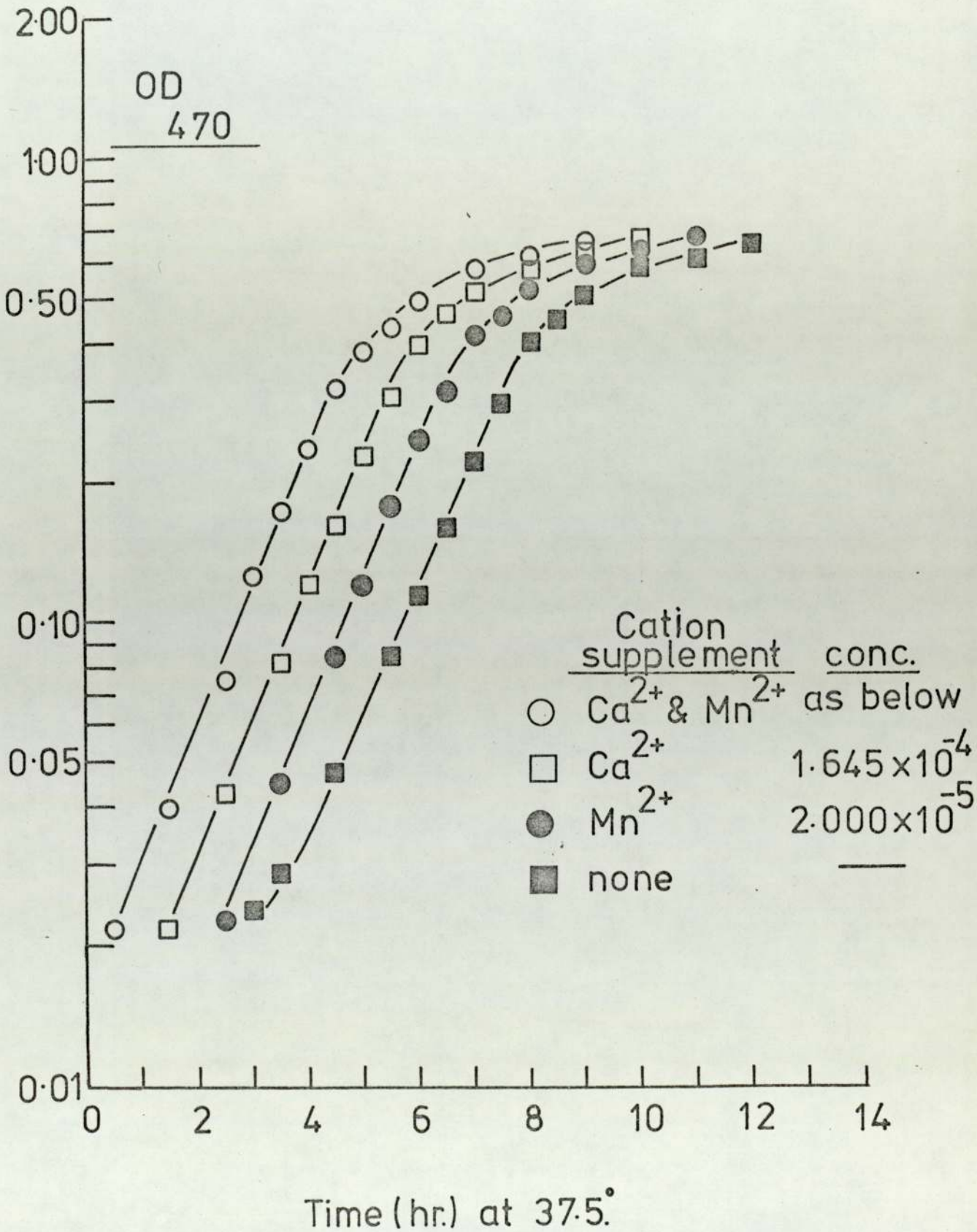
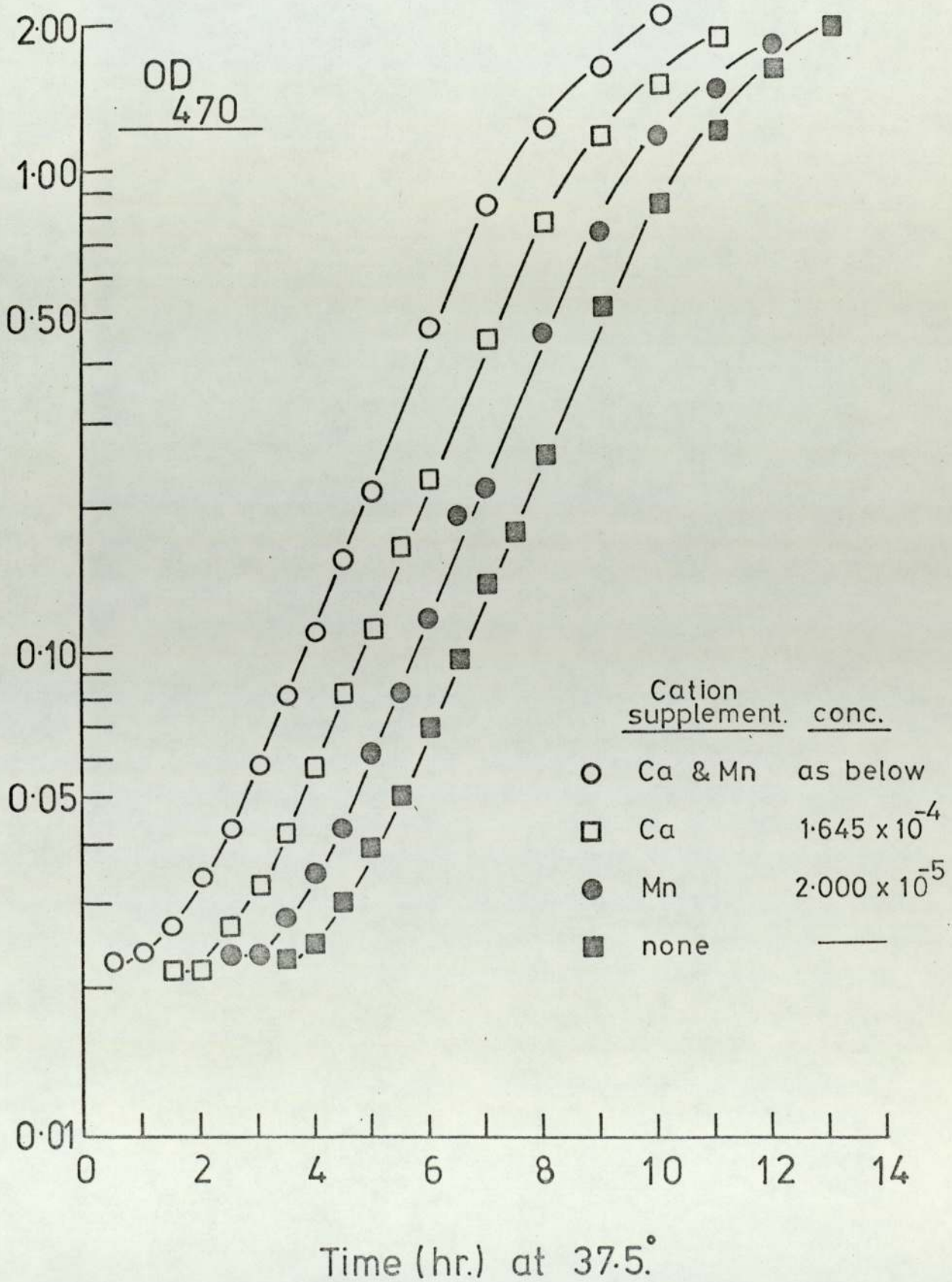


Figure 33. Growth of P.aeruginosa in Mg^{2+}-adequate CDM supplemented with Ca^{2+} or Mn^{2+}



6. RESISTANCE STUDIES ON NUTRIENT DEPLETED CULTURES OF *P.aeruginosa* GROWN IN MAGNESIUM-ADEQUATE AND MAGNESIUM-DEPLETED CHEMICALLY DEFINED MEDIA SUPPLEMENTED WITH CALCIUM OR MANGANESE.

Introduction.

The resistance was assessed by the rate and extent of lysis (see Tables 36 & 37) of these cultures by the following agents: ethylenediaminetetra-acetic acid (E.D.T.A.), ethyleneglycol-Bis-(β-aminoethylether)-N:N'-Tetra-acetic acid (E.G.T.A.) and polymyxin B sulphate (P.X.) Both E.D.T.A. and E.G.T.A. were used at a final concentration of 350 µg/ml, while P.X. was used at a final concentration of 50 U/ml. All the drugs were added in 0.25 ml volumes of stock solutions to 25 ml of culture. Details of the procedure used for lysis experiments was described in section 3.

E.D.T.A. mediated lysis.

The lysis curves for the E.D.T.A. mediated lysis of *P.aeruginosa* cultures grown in Mg^{2+}-adequate medium supplemented with calcium (Ca^{2+}) or manganese (Mn^{2+}) are presented in Fig. 34. All the curves show a similar pattern of lysis viz: a slow initial rate of lysis followed by a faster, apparently linear rate of lysis which decreases after 160-min contact time. The presence of Ca^{2+} and/or Mn^{2+} in the growth medium appears to have increased the sensitivity of the cultures slightly. The extent of lysis after 180-min showed that the order of sensitivity of the cultures was Mg^{2+}, Ca^{2+}, $Mn^{2+} > Mg^{2+}$, $Ca^{2+} > Mg^{2+}$, $Mn^{2+} > Mg^{2+}$.

The lysis curves for the E.D.T.A. mediated lysis

of P.aeruginosa cultures grown in Mg^{2+}-depleted medium supplemented with Ca^{2+} or Mn^{2+} exhibited a similar pattern of lysis, but the rates and extent of lysis for the individual cultures was markedly different (Fig. 35). The control Mg^{2+}-depleted culture (not supplemented with Ca^{2+} or Mn^{2+}) shows a fast initial rate, which soon decreases to approximately zero. The culture supplemented with Mn^{2+} appeared to have a very short period of fast initial lysis which decreased, and there after the rate remained constant. The extent of lysis after 180-min was similar for both the control and the Mn^{2+}-supplemented culture. The secondary rate of lysis for the Ca^{2+}-supplemented culture was greater than for the preceding cultures, and also appeared linear to the end of the experiment. The culture supplemented with both Ca^{2+} and Mn^{2+} lysed at the fastest rate, but was still linear to the end of the experiment. Only the culture supplemented with both cations was restored to the sensitivity of the Mg^{2+}-adequate control, while the other cultures were more or less resistant, depending upon the presence of Ca^{2+} and Mn^{2+} respectively. Supplementation by Ca^{2+} only restored the sensitivity of the Mg^{2+}-depleted culture to half the value of the Mg^{2+}-adequate control culture. In those Mg^{2+}-depleted cultures in which sensitivity to E.D.T.A. was restored by Ca^{2+} or Ca^{2+} and Mn^{2+} in the growth medium, the action of E.D.T.A. appeared to differ from the action upon Mg^{2+}-adequate cultures. In the latter, the lysis was at first slow, and then the rate increased, whilst in the former the opposite occurred.

Figure 34. EDTA mediated lysis of *P. aeruginosa* grown in Mg^{2+}-adequate CDM supplemented with Ca^{2+} or Mn^{2+}.

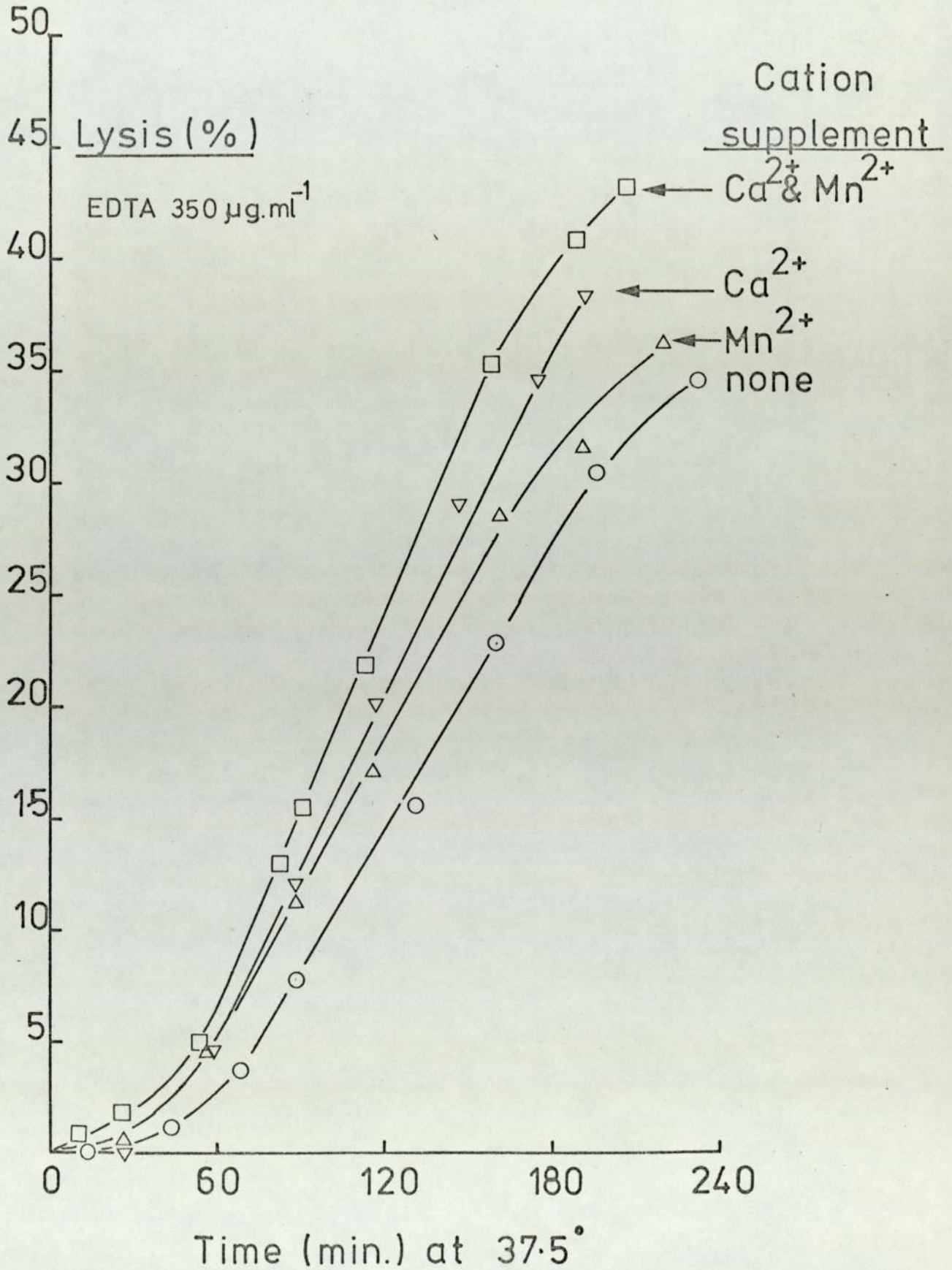
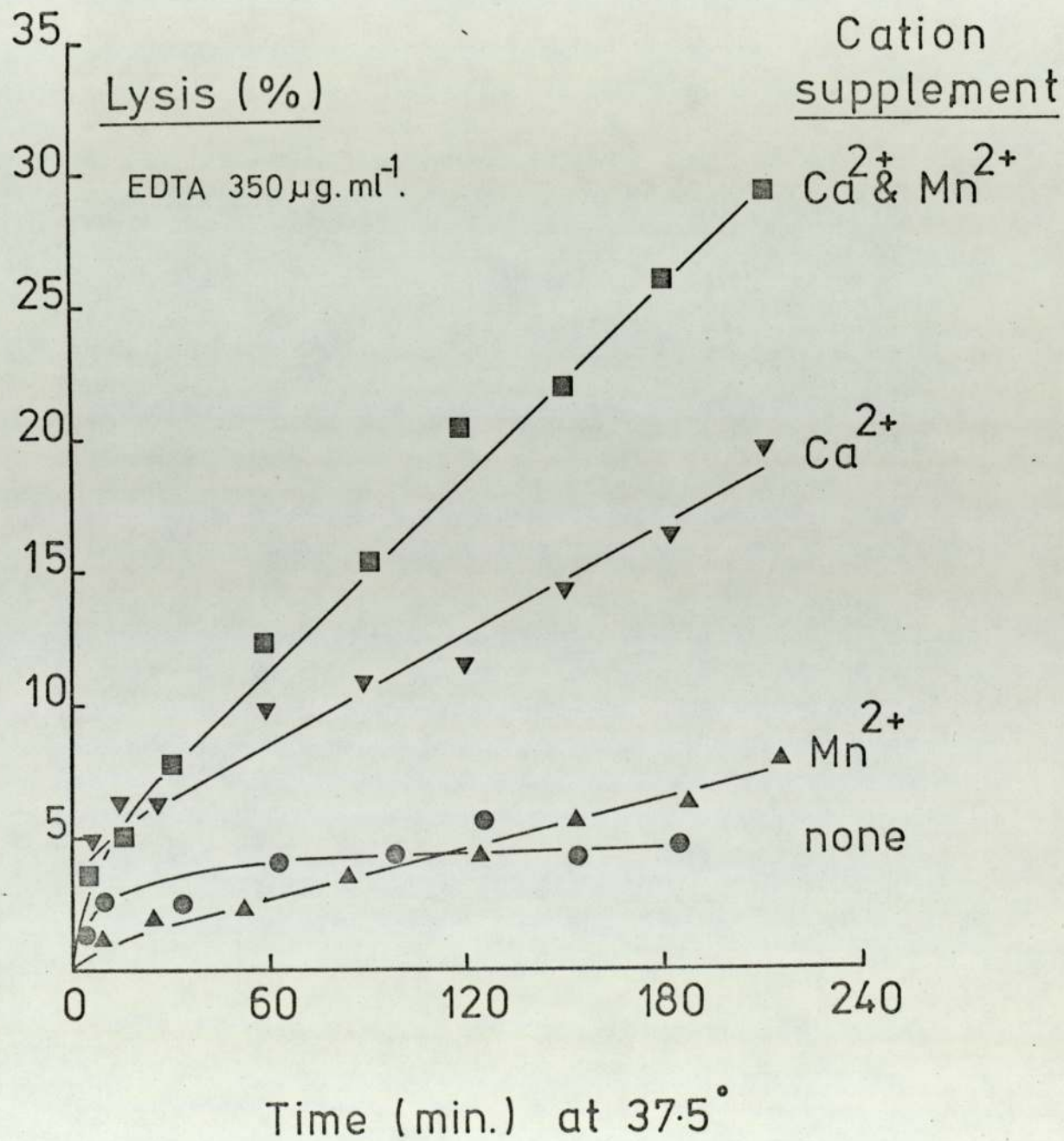


Figure 35. EDTA mediated lysis of *P. aeruginosa* grown in Mg^{2+}-depleted CDM supplemented with Ca^{2+} or Mn^{2+}



E.G.T.A. mediated lysis.

All the P.aeruginosa cultures grown in Mg^{2+}-adequate medium supplemented with Ca^{2+} or Mn^{2+} appeared to be relatively resistant to the action of this chelating agent. Fig. 36 shows that the maximum extent of lysis was 7% after 210-min contact time. The least resistant culture was the one supplemented with both Ca^{2+} and Mn^{2+}. The most resistant were the control culture and the Ca^{2+}-supplemented one. The culture supplemented with Mn^{2+} exhibited a resistance mid-way between these two. The initial rate of lysis in all the cultures was low, and only rose after approximately 90-min exposure (50-min for the Ca^{2+}, Mn^{2+} supplemented culture) to the drug.

All the Mg^{2+}-depleted cultures exhibited a fast initial rate of lysis followed by a lower, but constant rate of lysis (Fig. 37). The two cultures supplemented with Ca^{2+} exhibited a faster initial rate of lysis than the control culture or the Mn^{2+} supplemented culture. The rate of lysis of the control culture decreased to approximately zero. The rate of lysis of the cultures supplemented with Ca^{2+} or Mn^{2+} both had similar secondary lysis rates, while the culture supplemented with both Ca^{2+} and Mn^{2+} exhibited the fastest secondary lysis rate. The control culture appeared to be as resistant to E.G.T.A. as the Mg^{2+}-adequate control. The supplemented cultures were all less resistant to E.G.T.A. than the control, the extent of lysis after 180-min for the Ca^{2+}, Mn^{2+}; Ca^{2+}; Mn^{2+}-supplemented cultures being 17.2%; 14.0%; and 9.7% respectively. Supplementing the Mg^{2+}-depleted growth medium with Ca^{2+} and/or Mn^{2+} appeared to reverse

Figure 36. EGTA mediated lysis of *P. aeruginosa* grown in Mg^{2+}-adequate CDM supplemented with Ca^{2+} or Mg^{2+}.

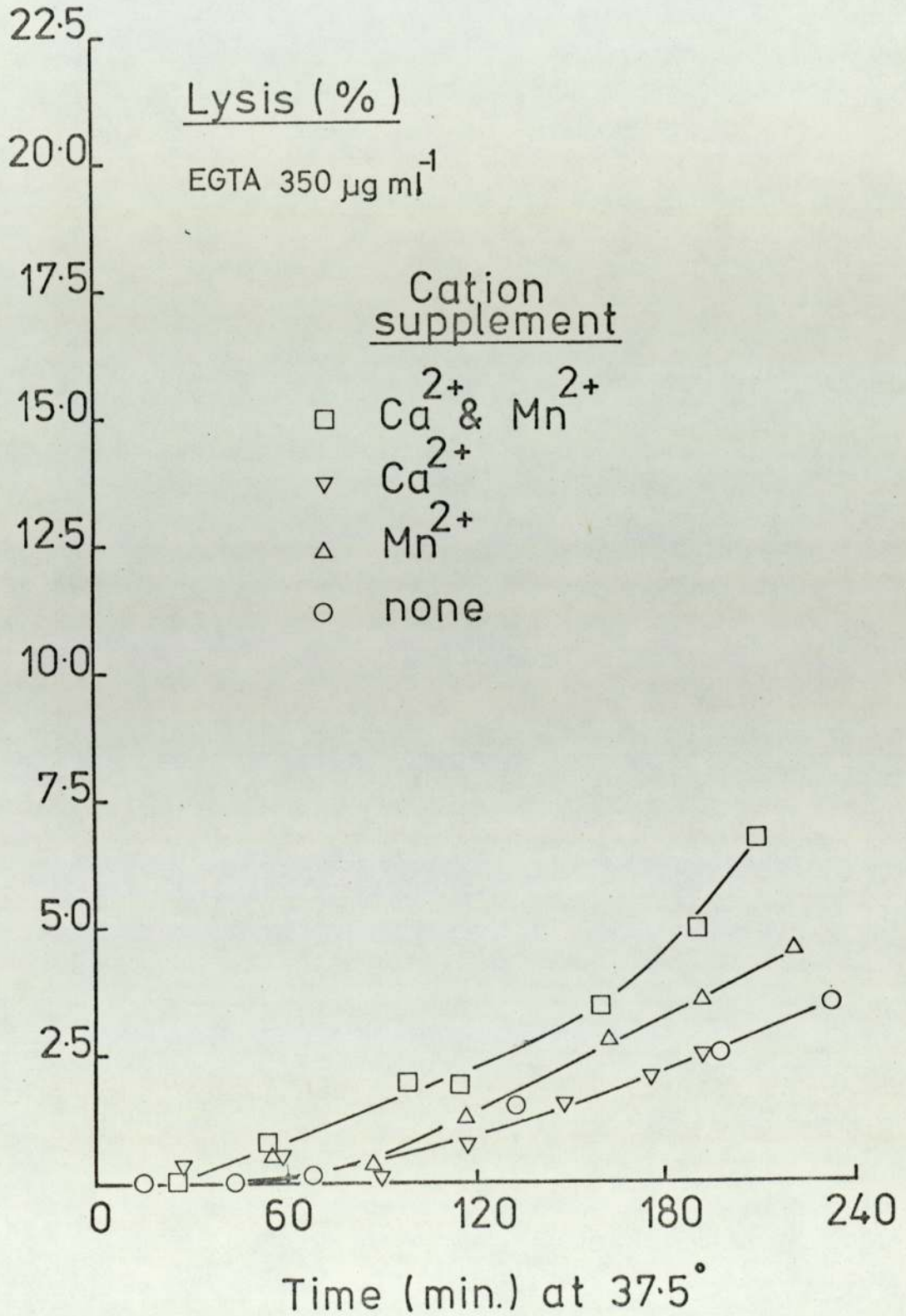
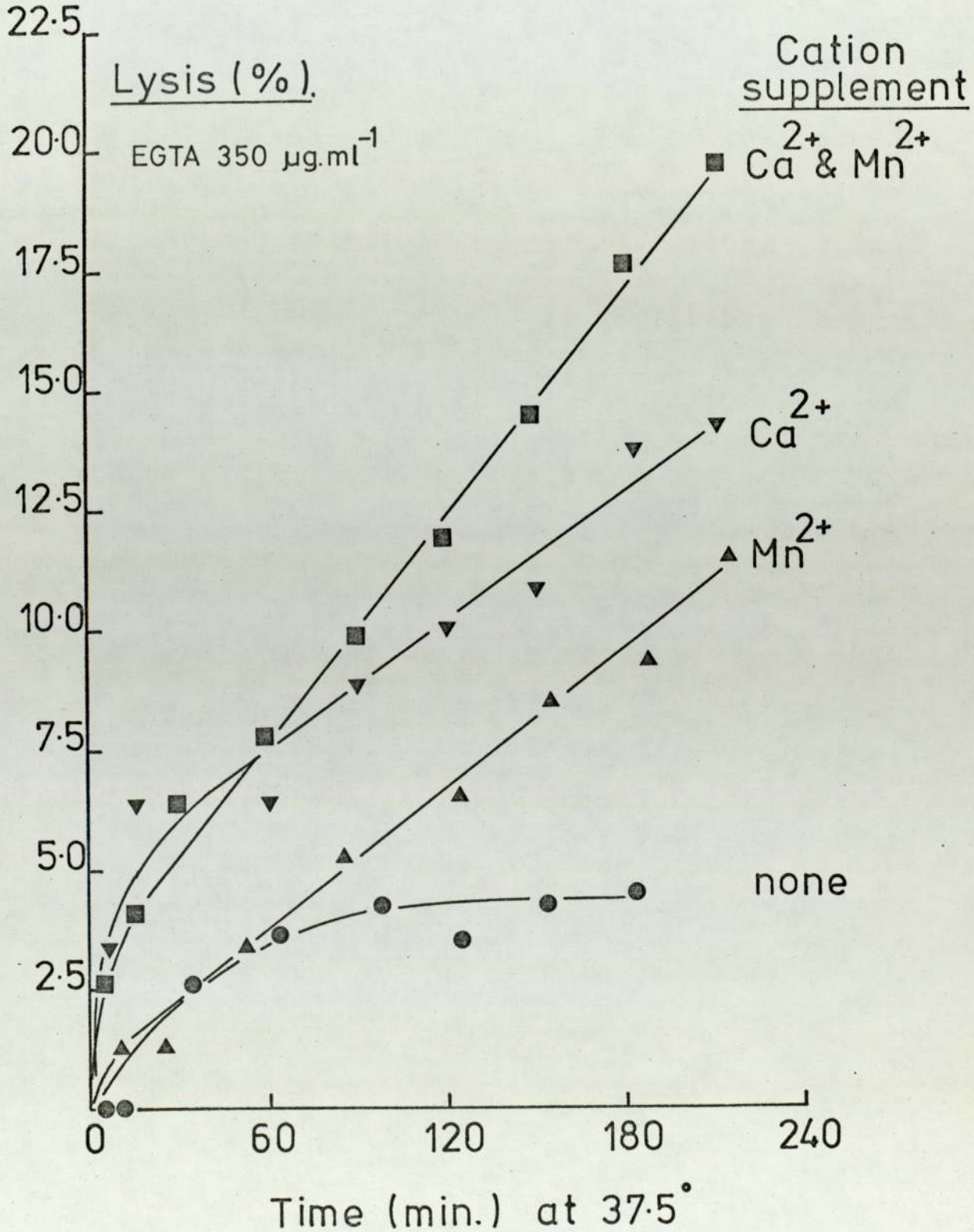


Figure 37. EGTA mediated lysis of P.aeruginosa grown in Mg^{2+}-depleted CDM supplemented with Ca^{2+} or Mn^{2+}.



the resistance of similarly supplemented Mg^{2+}-adequate cultures.

P.X. mediated lysis.

Fig 38 shows that all the P.aeruginosa Mg^{2+}-adequate cultures supplemented with Ca^{2+} or Mn^{2+} exhibited a similar pattern of lysis; a very fast initial rate of lysis which gradually decreased until it approached zero after about 160-min. The control culture, and the culture supplemented with Mn^{2+} exhibited very similar rates and extents of lysis. The cultures supplemented with Ca^{2+} (with or without Mn^{2+}) showed a significantly lower initial rate of lysis, but the extent of lysis of these two cultures were compatible with the control after 180-min. It would appear that the presence of Ca^{2+} or Ca^{2+} and Mn^{2+} inhibited the initial action of P.X. upon cultures of P.aeruginosa but this may be due to competition for active sites rather than an effect of the cell structure.

The lysis curves in Fig. 39 illustrate the P.X. mediated lysis of P.aeruginosa cultures grown in Mg^{2+}-depleted medium supplemented with Ca^{2+} or Mn^{2+}. The control Mg^{2+}-depleted culture (no supplement) appeared to be resistant to the action of P.X.. After a slow rate of lysis during the first hour of contact time, the rate decreased until it was approximately zero. The extent of lysis after 180-min was 6%. The Mn^{2+}-supplemented culture was not resistant to the same extent as the control, and the lysis rate appeared to be constant over the time of the experiment. The extent of lysis after 180-min, although significantly greater than the control (17.5% compared to 6%), was very much lower than that observed

Figure 38. Polymyxin mediated lysis of P.aeruginosa grown in Mg^{2+}-adequate CDM supplemented with Ca^{2+} or Mn^{2+}.

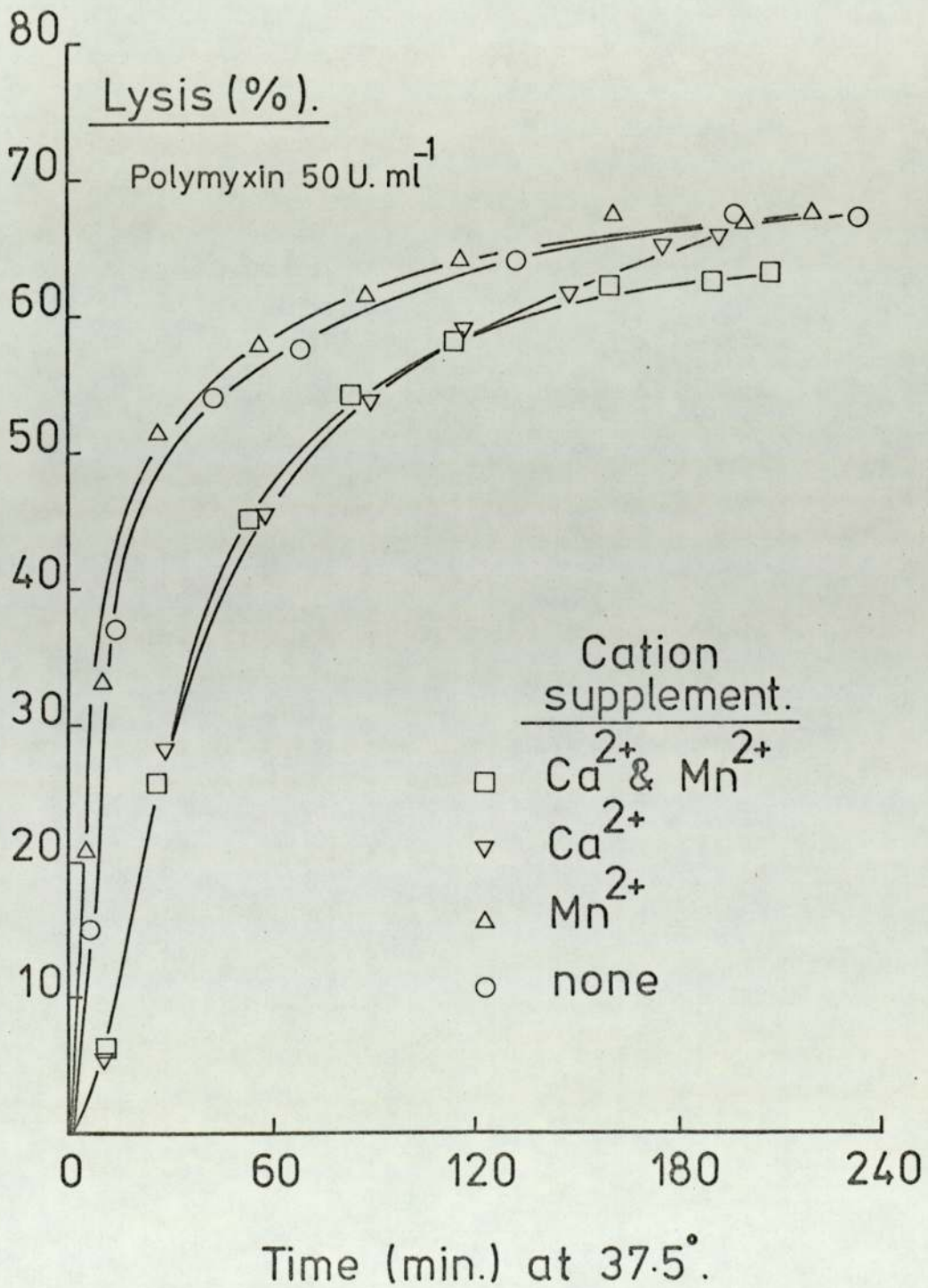
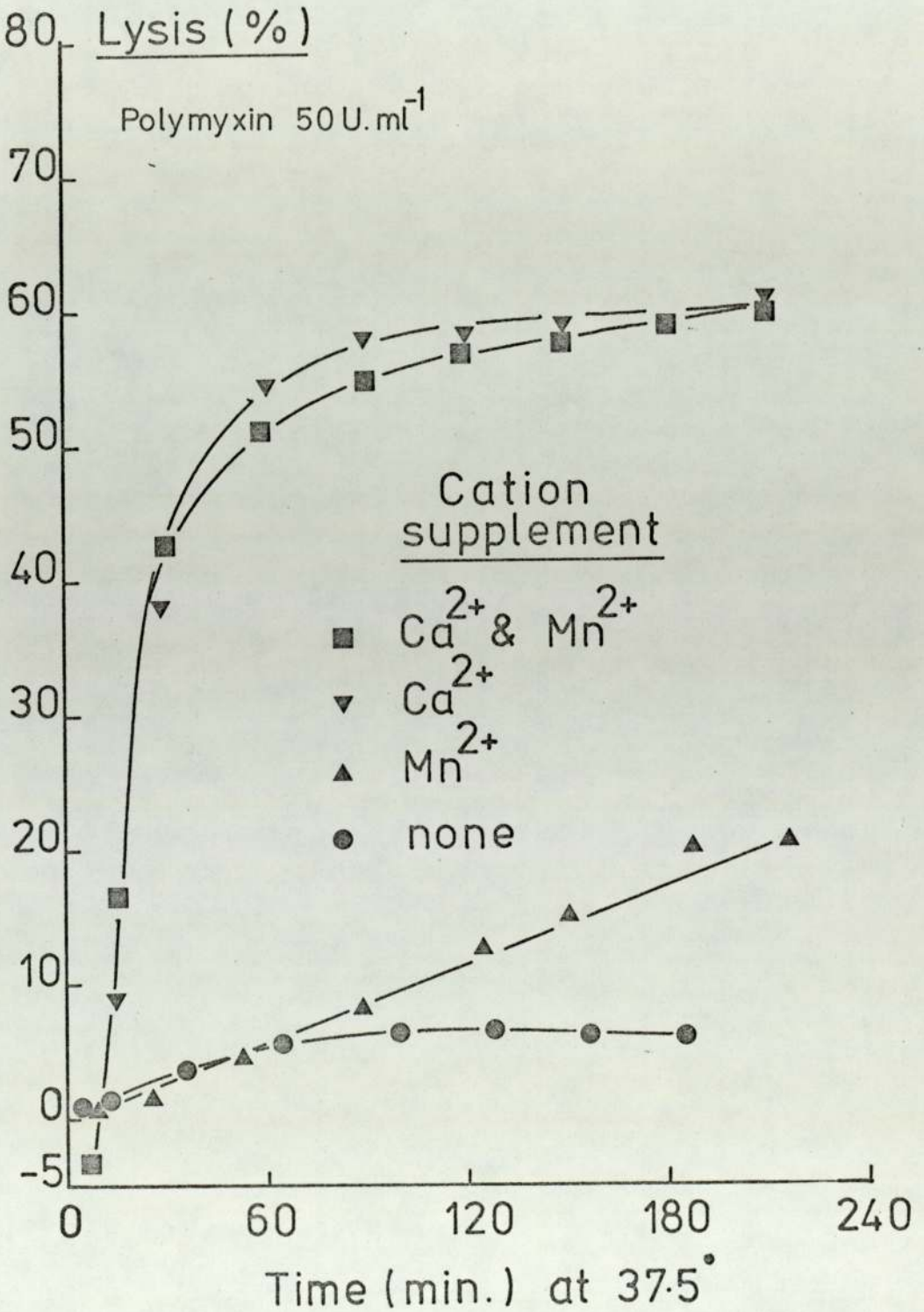


Figure 39. Polymyxin mediated lysis of P. aeruginosa grown in Mg^{2+}-depleted CDM supplemented with Ca^{2+} or Mn^{2+}.



for the Mn^{2+}-supplemented, Mg^{2+}-adequate culture. The two cultures supplemented with Ca^{2+} showed very similar lysis curves. The pattern of lysis was similar to that of the control Mg^{2+}-adequate culture. At the start of the experiment there appeared to be an increase in the optical density. This may be due to the previously reported optical effects of drug binding to the cell surface (Hugo & Longworth, 1964). The rate, and extent of lysis shown by the Ca^{2+}-supplemented cultures was similar to the control, Mg^{2+}-adequate cultures. It would appear that supplementation of Mg^{2+}-depleted growth media with Ca^{2+} (with or without Mn^{2+}) almost completely restored P.X. sensitivity to the cultures. Supplementation with Mn^{2+} also appeared to restore some sensitivity, but the kinetics of this lysis is different (i.e. linear).

7. RESISTANCE STUDIES ON COLD SHOCKED CULTURES OF *P.aeruginosa* GROWN IN MAGNESIUM-ADEQUATE AND MAGNESIUM-DEPLETED CHEMICALLY DEFINED MEDIA SUPPLEMENTED WITH CALCIUM OR MANGANESE.

Introduction.

The procedure used to induce cold shock prior to lysis experiments has been described in a previous section (see section 2). It was observed that cold shocked cultures underwent autolysis when they were rewarmed to 37.5°. This autolysis was particularly marked in the Mg^{2+}-adequate cultures, as was the decrease in viability counts (Tables 21 & 22). When an attempt was made to correct the lysis values obtained from Mg^{2+}-adequate cultures treated with the drug, the resulting lysis curves gave no useful indication of what was happening (see Fig. 40). The lysis curves presented to illustrate the effects of Ethylenediaminetetra-acetic acid (E.D.T.A.), ethyleneglycol-Bis-(β-aminoethylether)-N:N'-Tetra-acetic acid (E.G.T.A.) and polymyxin (P.X.) have not therefore been corrected for the control lysis. The lysis curves for the Mg^{2+}-depleted cultures were corrected for control lysis, and a series of these corrected curves are also presented to facilitate comparision with non-cold shocked, Mg^{2+}-depleted cultures.

Autolysis of cold shocked *P.aeruginosa* cultures grown in Mg^{2+}-adequate media supplemented with Ca^{2+} or Mn^{2+}.

Fig. 41 shows that all the Mg^{2+}-adequate cultures exhibited significant lysis (55 - 70%) upon rewarming to 37.5°. The cultures did not all lyse to the same extent however. The control, Mg^{2+}-adequate culture (no supplement)

TABLE 21

Sensitivity to cold shock of cultures of P.aeruginosa grown in Mg^{2+}-adequate chemically defined media supplemented with Ca^{2+} or Mn^{2+}.

Culture	1	3	5	7
* Percent survivors	0.35	0.19	0.60	0.28

These number signify the type of cation supplement and are as described in Tables 23 - 30 inclusive.

$$* \frac{\text{Viable counts of cold shocked cells}}{\text{Viable counts of control}} \times 100$$

TABLE 22

Sensitivity to cold shock of cultures of P.aeruginosa grown in Mg^{2+}-depleted chemically defined media supplemented with Ca^{2+} or Mn^{2+}.

Culture	2	4	6	8
* Percent survivors	3.20	1.88	5.01	0.20

These number signify the type of cation supplement and are as described in Tables 23 - 30 inclusive.

$$* \frac{\text{Viable counts of cold shocked cells}}{\text{Viable counts of control}} \times 100$$

Figure 40. Effect of correcting EDTA mediated lysis of cold shocked, Mg^{2+}-adequate *P. aeruginosa* for lysis exhibited by the control culture.

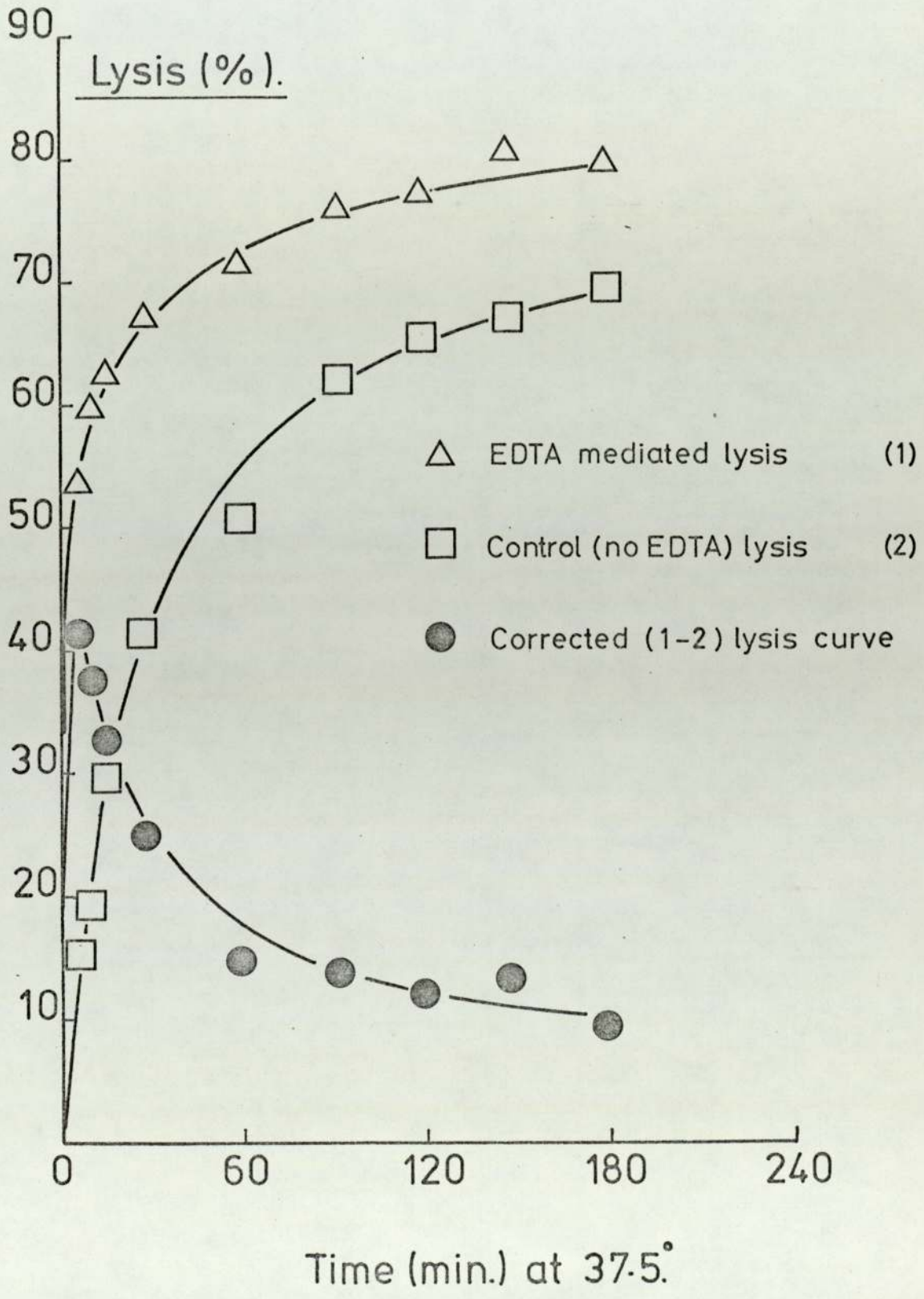


Figure 41. Cold shock induced lysis of *P. aeruginosa* grown in Mg^{2+}-adequate CDM supplemented with Ca^{2+} or Mn^{2+}.

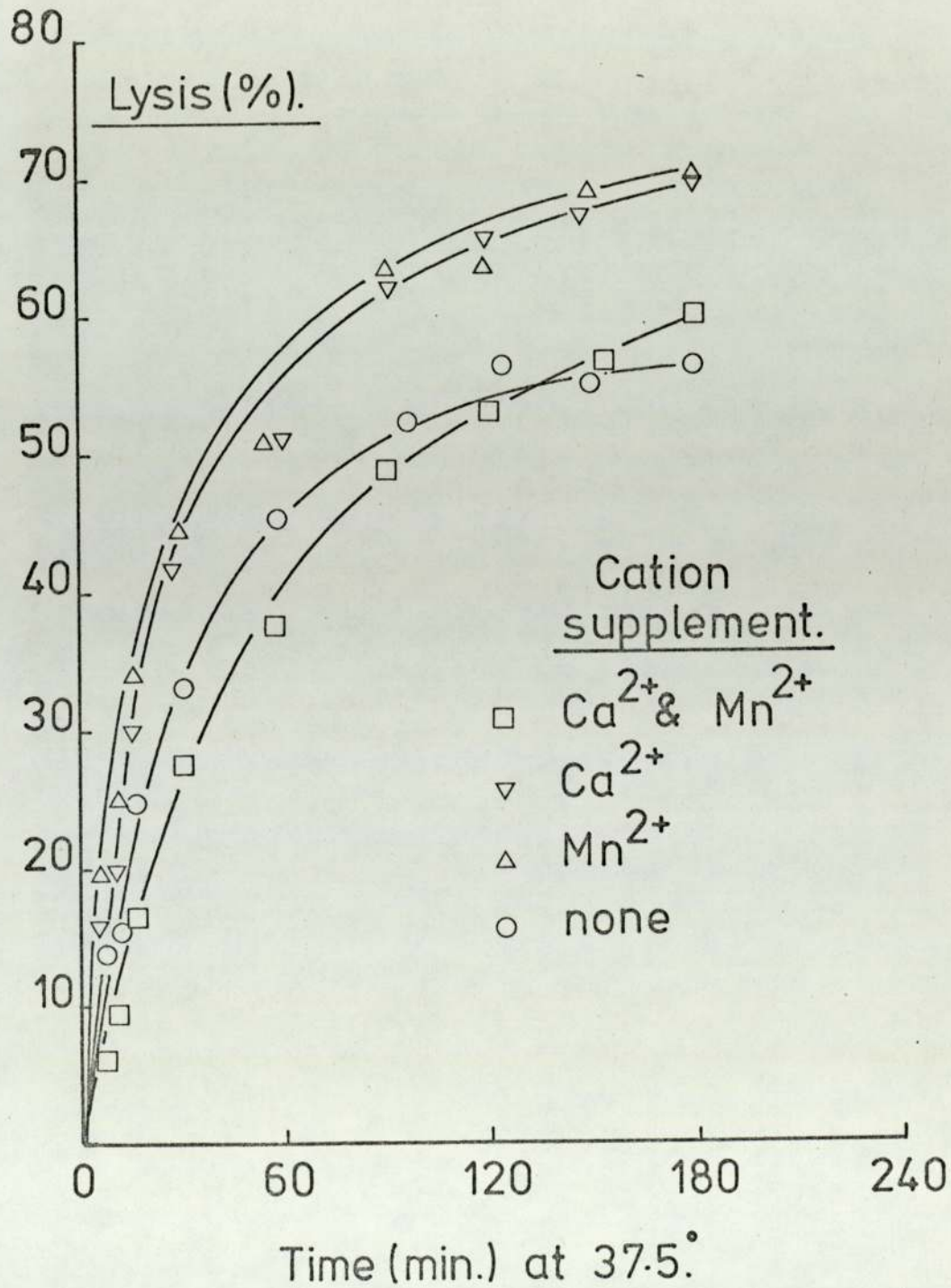
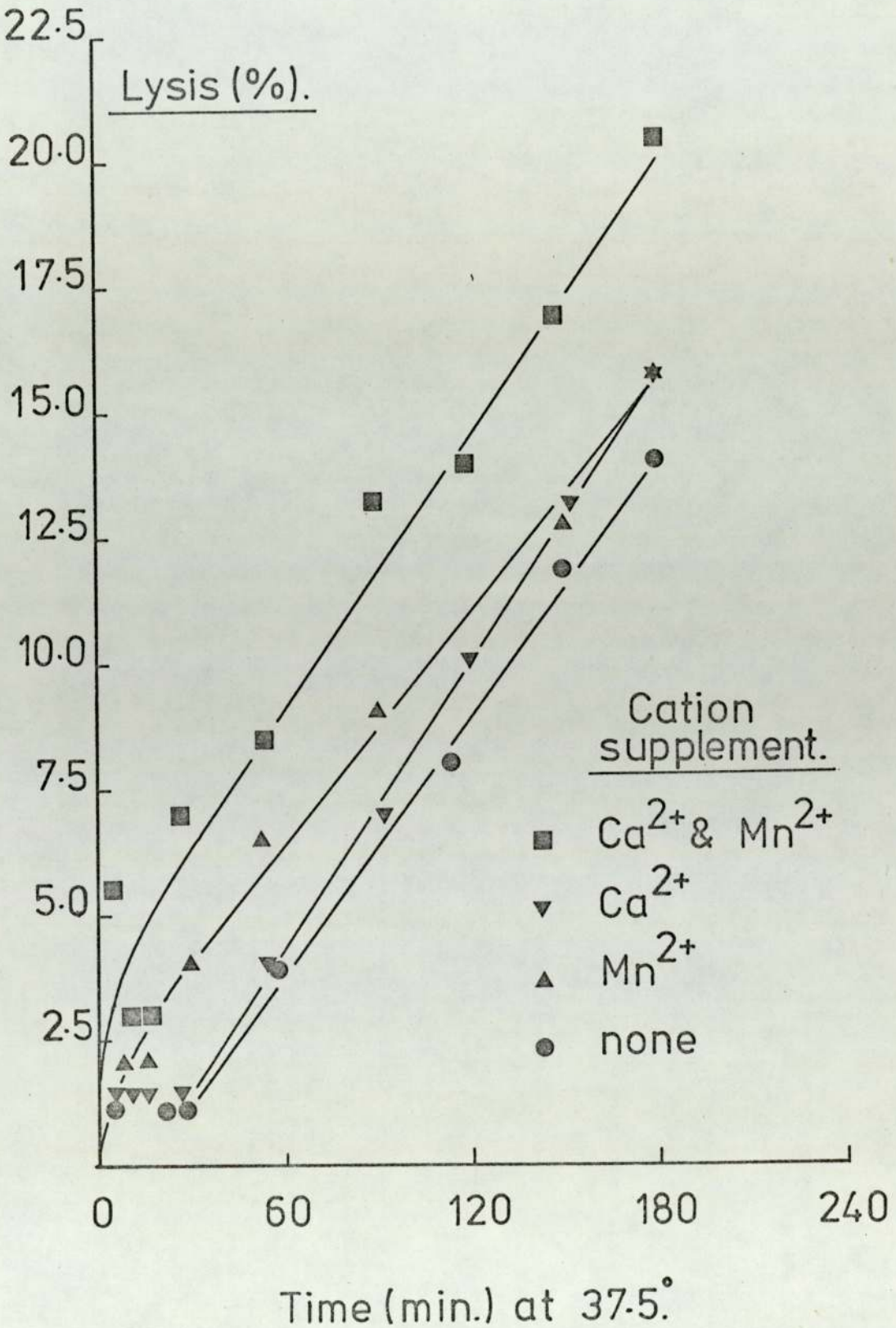


Figure 42. Cold shock induced lysis of *P. aeruginosa* grown in Mg^{2+}-depleted CDM supplemented with Ca^{2+} or Mn^{2+}.



and the Mn^{2+} and Ca^{2+} supplemented culture lysed to a lesser extent than the two cultures supplemented with Ca^{2+} and Mn^{2+} respectively. It appears that the addition of Mn^{2+} and Ca^{2+} singly, but not in combination, to Mg^{2+}-adequate cultures rendered those cultures more susceptible to post cold shock autolysis.

Autolysis of cold shocked P.aeruginosa cultures grown in Mg^{2+}-depleted media supplemented with Ca^{2+} or Mn^{2+}.

All the cultures exhibited a significant amount of autolysis upon rewarming to 37.5°, but the extent was markedly less than for the corresponding Mg^{2+}-adequate cultures. The kinetics of lysis by Mg^{2+}-depleted cells was different from the Mg^{2+}-adequate cultures, and in all cases the lysis was linear with time (see Fig. 42). The control culture (no supplement) exhibited the lowest extent of lysis over 180-min, but not the lowest rate. The Mn^{2+}-supplemented culture appeared to have the lowest rate, but like the Ca^{2+} and Mn^{2+} supplemented culture also had a high initial rate of lysis. The Ca^{2+} and Ca^{2+} and Mn^{2+} supplemented cultures appeared to possess similar secondary rates of lysis. Both the control culture and the Ca^{2+}-supplemented culture appeared to have a 'lag' of 30-min during which there was little or no lysis.

E.D.T.A. mediated lysis of cold shocked P.aeruginosa cultures grown in Mg^{2+}-adequate media supplemented with Ca^{2+} or Mn^{2+}.

The lysis curves presented in Fig. 43 show that all the cultures exhibited extensive lysis upon rewarming and treatment with E.D.T.A.. The lysis in all cultures was initially very rapid, and the rate decreased gradually

throughout the experiment, approaching zero at 180-min. The control culture and the Ca^{2+} and Mn^{2+} supplemented culture both appeared less sensitive to the action of E.D.T.A. than did the cultures supplemented with Ca^{2+} and Mn^{2+} respectively. This is comparible with the lysis of the control cultures (no drug), although the extent of lysis was greater with E.D.T.A.. The extent of lysis, and the kinetics of the lysis of cold shocked cells was markedly different from non-cold shocked cultures, and resembles the P.X. mediated lysis of non-cold shocked Mg^{2+}-adequate cultures.

E.D.T.A. mediated lysis of cold shocked P.aeruginosa cultures grown in Mg^{2+}-depleted media supplemented with Ca^{2+} or Mn^{2+}.

Fig. 44 illustrates the different patterns of lysis shown by the various cultures. The control culture (no supplement) showed a rate of lysis that decreased gradually as the experiment proceded. The extent of lysis was significant, but much less than the corresponding Mg^{2+}-adequate culture. The Mn^{2+} supplemented culture exhibited an apparently linear rate of lysis for about 60-min, the rate then decreased, until at about 180-min it approached zero. The extent of lysis of this culture was significantly greater than the control value, and approached that exhibited by the $Mn^{2+}$and Ca^{2+}supplemented culture and the Ca^{2+} supplemented culture respectively. The latter culture exhibited a lysis curve very similar to that obtained from the corresponding Mg^{2+}-adequate cultures, although the initial rate of lysis was lower, as was the extent of lysis at 180-min. It appears that Ca^{2+} and

Figure 43. EDTA mediated lysis of cold shocked P.aeruginosa grown in Mg^{2+}-adequate CDM supplemented with Ca^{2+} or Mn^{2+}.

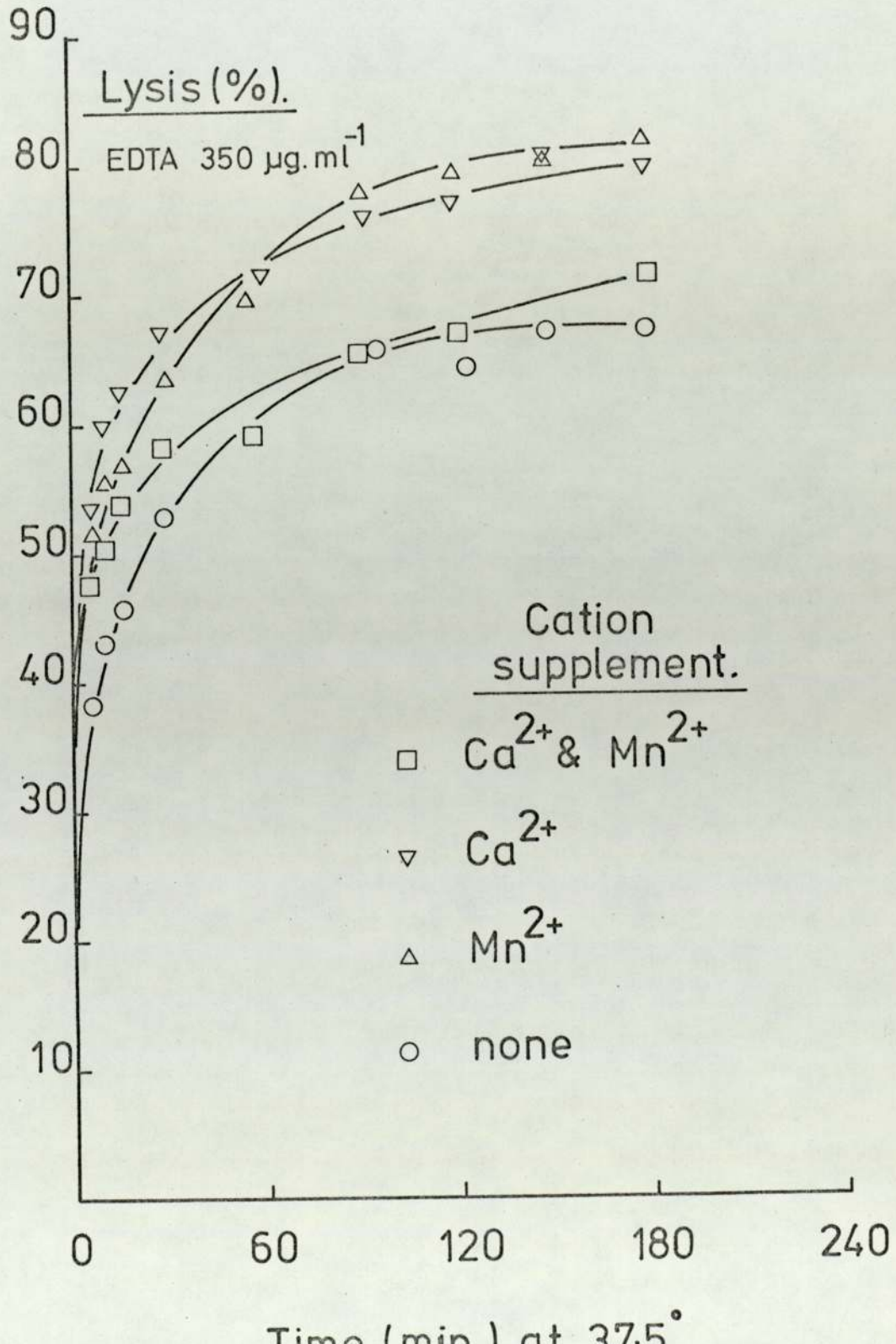
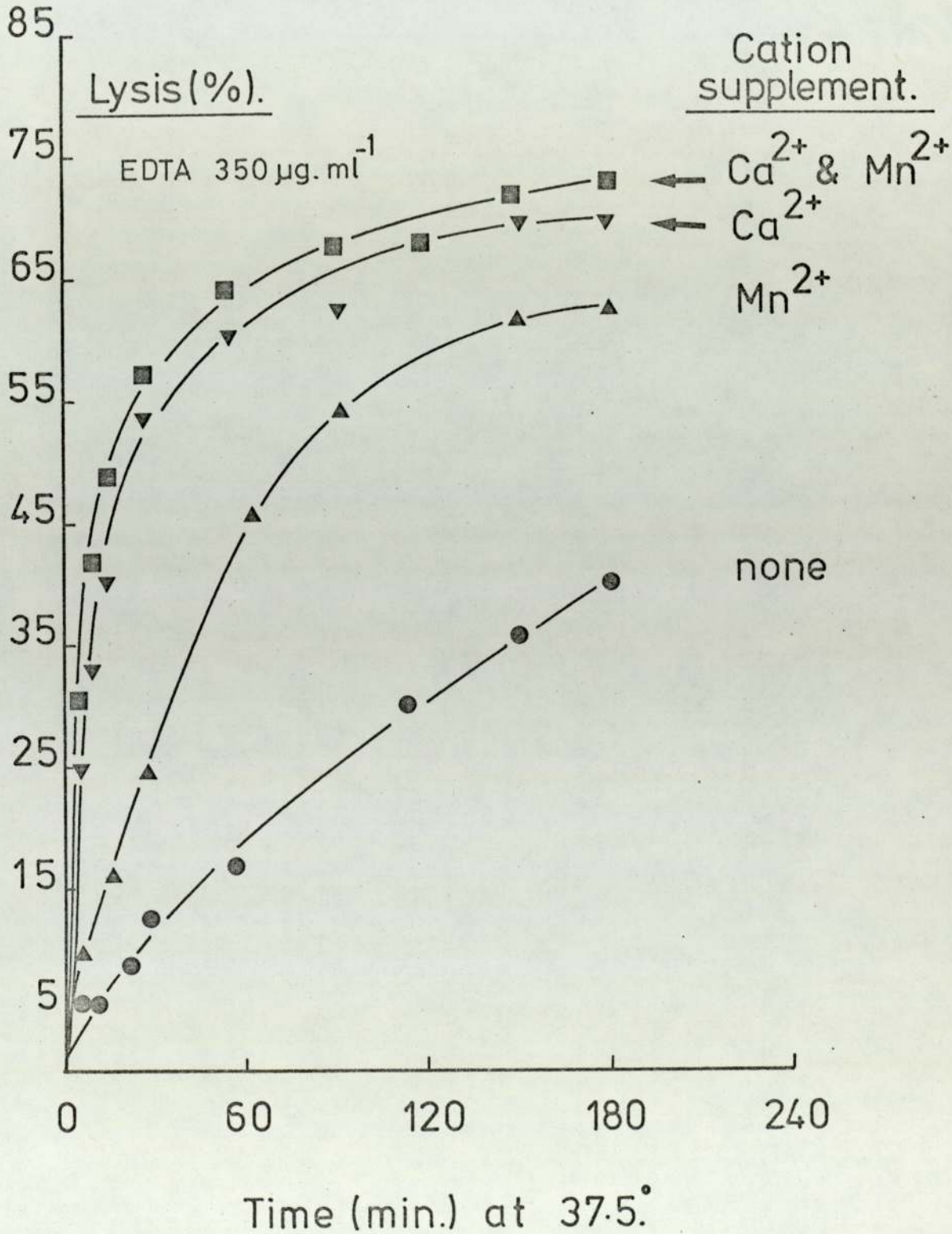


Figure 44. EDTA mediated lysis of cold shocked P.aeruginosa grown in Mg^{2+}-depleted CDM supplemented with Ca^{2+} or Mn^{2+}.

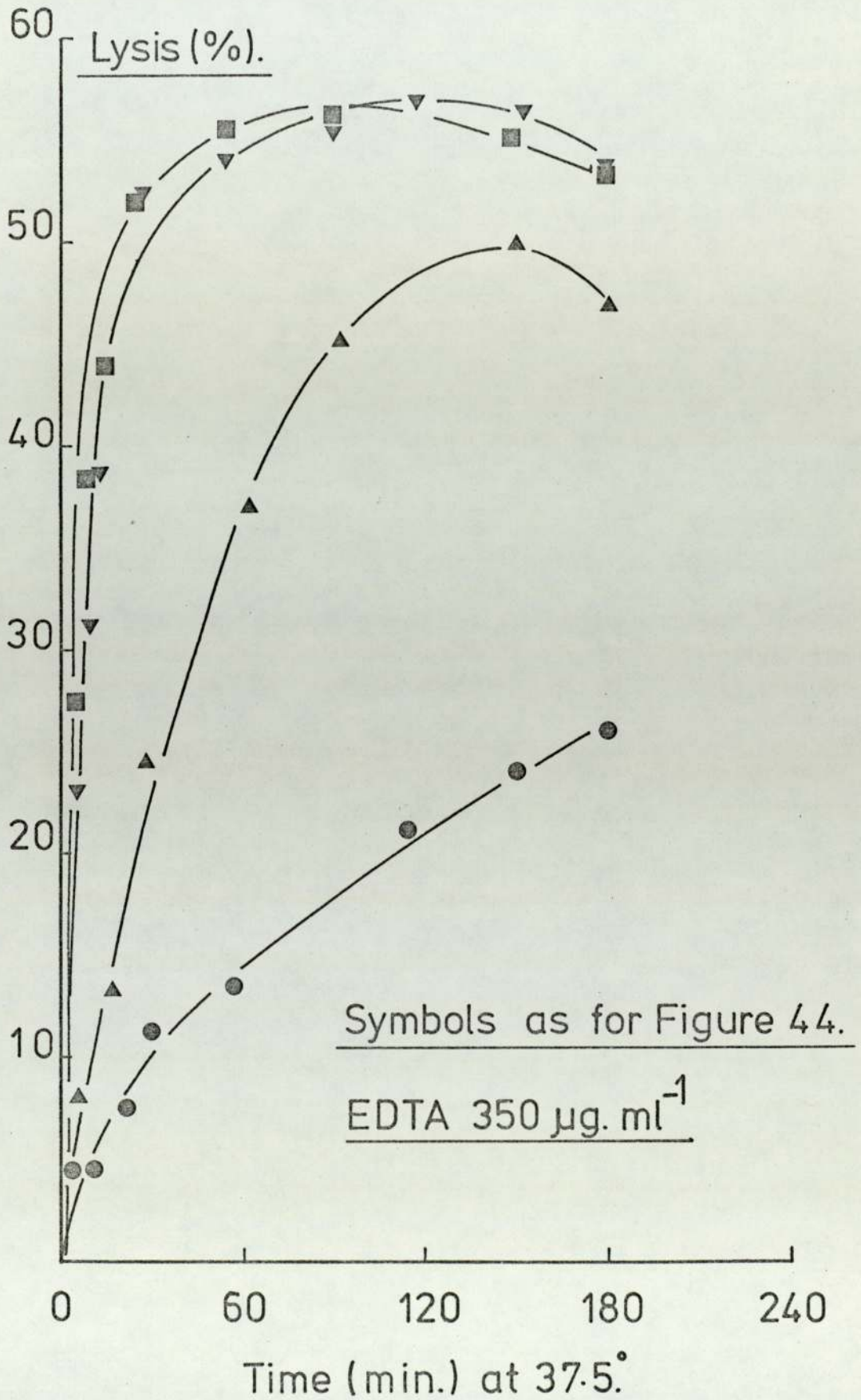


Ca^{2+} and Mn^{2+} supplementation of the Mg^{2+}-depleted medium restored the sensitivity to E.D.T.A. of Mg^{2+}-depleted cultures. Cultures supplemented with Mn^{2+} only were a little more resistant to the action of E.D.T.A. and cold shock. The lysis curves for E.D.T.A. treated, cold shocked Mg^{2+}-depleted cultures, corrected for control (no drug) lysis, are shown in Fig. 45. The pattern of lysis is similar to that observed in the uncorrected data, but the extent of lysis was obviously lower. Comparison of Fig. 45 with Fig. 35 (E.D.T.A. mediated lysis of non-cold shocked Mg^{2+}-depleted cultures) show that both the kinetics, and extent of E.D.T.A. mediated lysis were altered by cold shock. The Ca^{2+} supplemented cold shocked cultures exhibited twice the percent lysis in 180-min compared with the non-cold shocked cultures. The kinetics of cold shocked cultures all gave a fast initial rate decreasing to zero (the control being the exception), whereas in the non-cold shocked cultures the fast initial rate was succeeded by a slower, constant rate. Thus it appears that cold shock enhanced the action of E.D.T.A. upon Mg^{2+}-depleted cultures, especially when the Mg^{2+}-depleted media were supplemented with Ca^{2+} or Mn^{2+}.

E.G.T.A. mediated lysis of cold shocked cultures of P.aeruginosa grown in Mg^{2+}-adequate media supplemented with Ca^{2+} or Mn^{2+}.

All the cultures exhibited extensive lysis of a similar pattern in the presence of E.G.T.A.. The kinetics and extent of lysis resembled that of the control (no drug) cultures. As can be seen in Fig. 46, the Ca^{2+} and Mn^{2+} supplemented culture and the control culture (no supplement)

Figure 45. EDTA mediated lysis of cold shocked *P. aeruginosa* grown in Mg^{2+}-depleted CDM supplemented with Ca^{2+} or Mn^{2+}: Data corrected for lysis of contols (Fig. 42).



behaved in a similar manner, but this differed (lower rates and percent lysis) from the Ca^{2+} and Mn^{2+} supplemented cultures. This difference was apparent in both the control (no drug) and the E.G.T.A. treated cultures. After an initial fast rate, the lysis then proceeded at a decreasing rate until, at the end of the experiment the rate approached zero.

<u>E.G.T.A. mediated lysis of cold shocked cultures of P.aeruginosa grown in Mg^{2+}-depleted media supplemented with Ca^{2+} or Mn^{2+}.</u>

The lysis curves are presented in Fig. 47. Both the cultures supplemented with Ca^{2+} or Ca^{2+} and Mn^{2+} exhibited marked sensitivity to E.G.T.A.. The culture supplemented with Mn^{2+} lysed at a decreasing rate for 20-min, after which the rate remained constant. This appears to be the case for the control culture too, although the rates were somewhat lower. Supplementation of Mg^{2+}-depleted cultures with Ca^{2+} reversed the partial resistance to E.G.T.A., whereas supplementation with Mn^{2+} did not produce as great an effect, although the culture was nevertheless significantly less resistant than the control. Fig. 48 shows the lysis curves for cold shocked Mg^{2+}-depleted cultures corrected for control (no drug) lysis. The shapes are similar to the uncorrected curves, but they differ from the corresponding non-cold shocked cultures (Fig. 37). The Ca^{2+} and Ca^{2+} and Mn^{2+} supplemented cold shocked cultures were markedly more sensitive to E.G.T.A. than non-cold shocked cells. After a rapid initial rate the cold shocked cells lysed at a decreasing rate which approached zero towards the end of the experiment. The

Figure 46. EGTA mediated lysis of cold shocked *P.aeruginosa* grown in Mg^{2+}-adequate CDM supplemented with Ca^{2+} or Mn^{2+}.

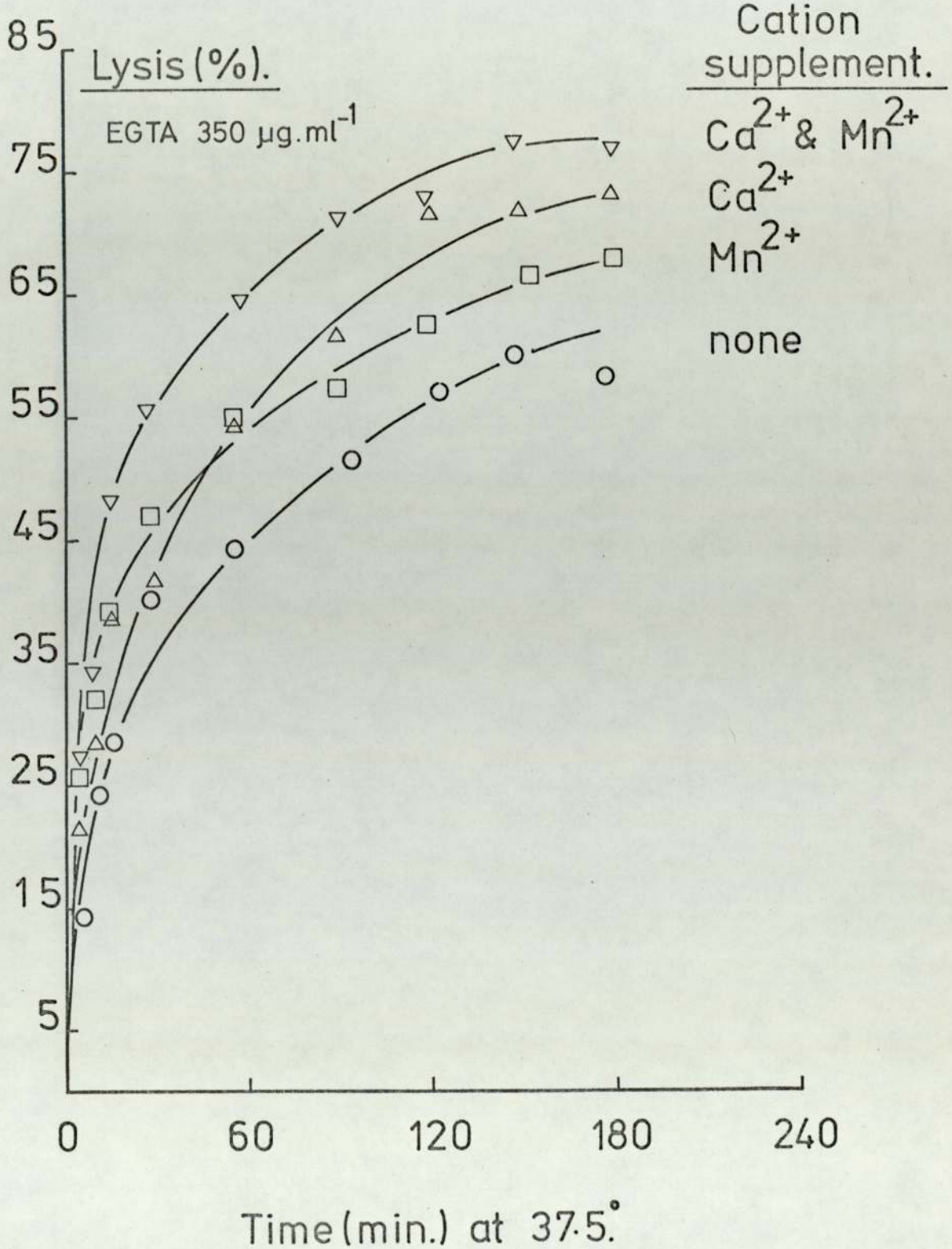


Figure 47. EGTA mediated lysis of cold shocked P.aeruginosa grown in Mg^{2+}-depleted CDM supplemented with Ca^{2+} or Mn^{2+}.

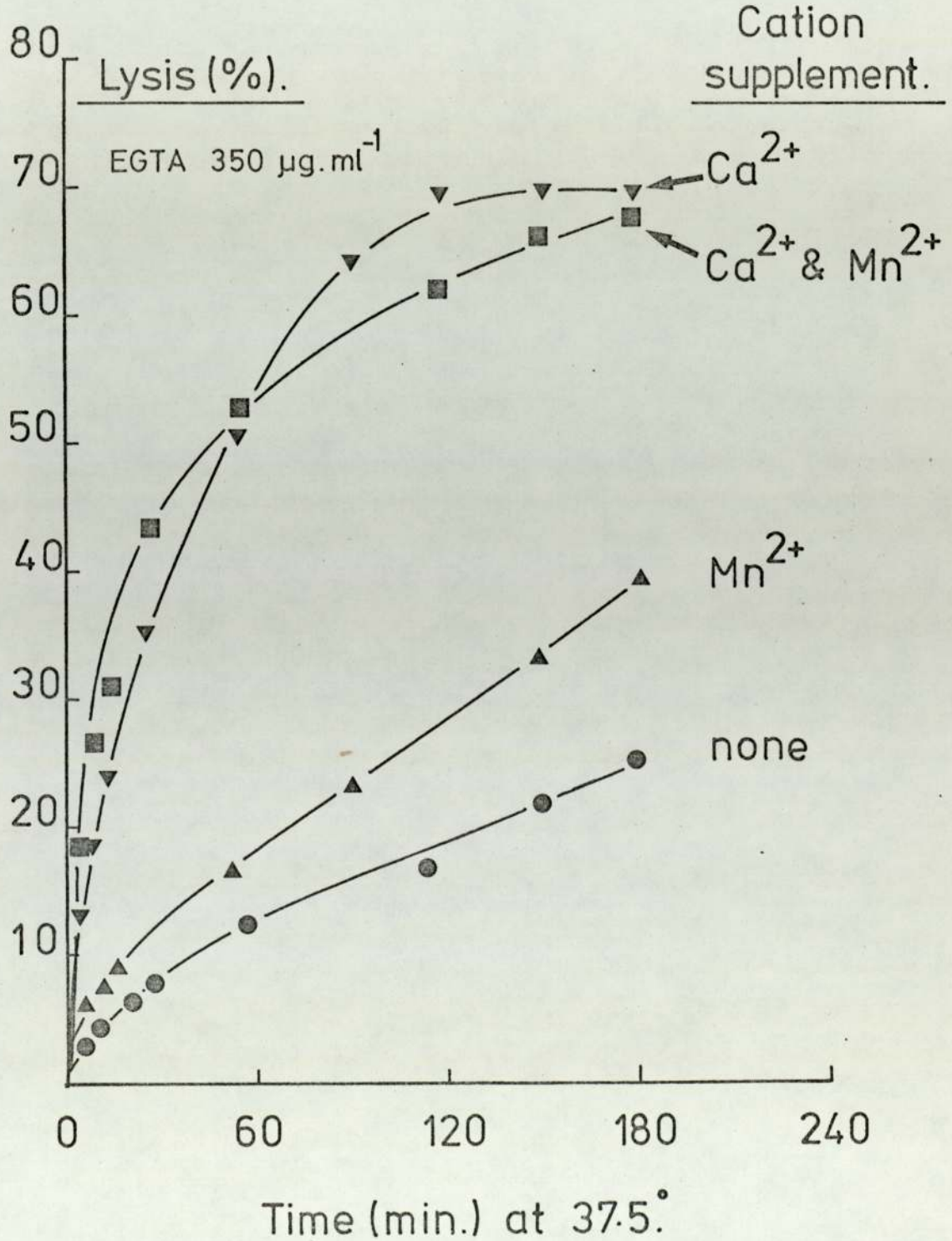
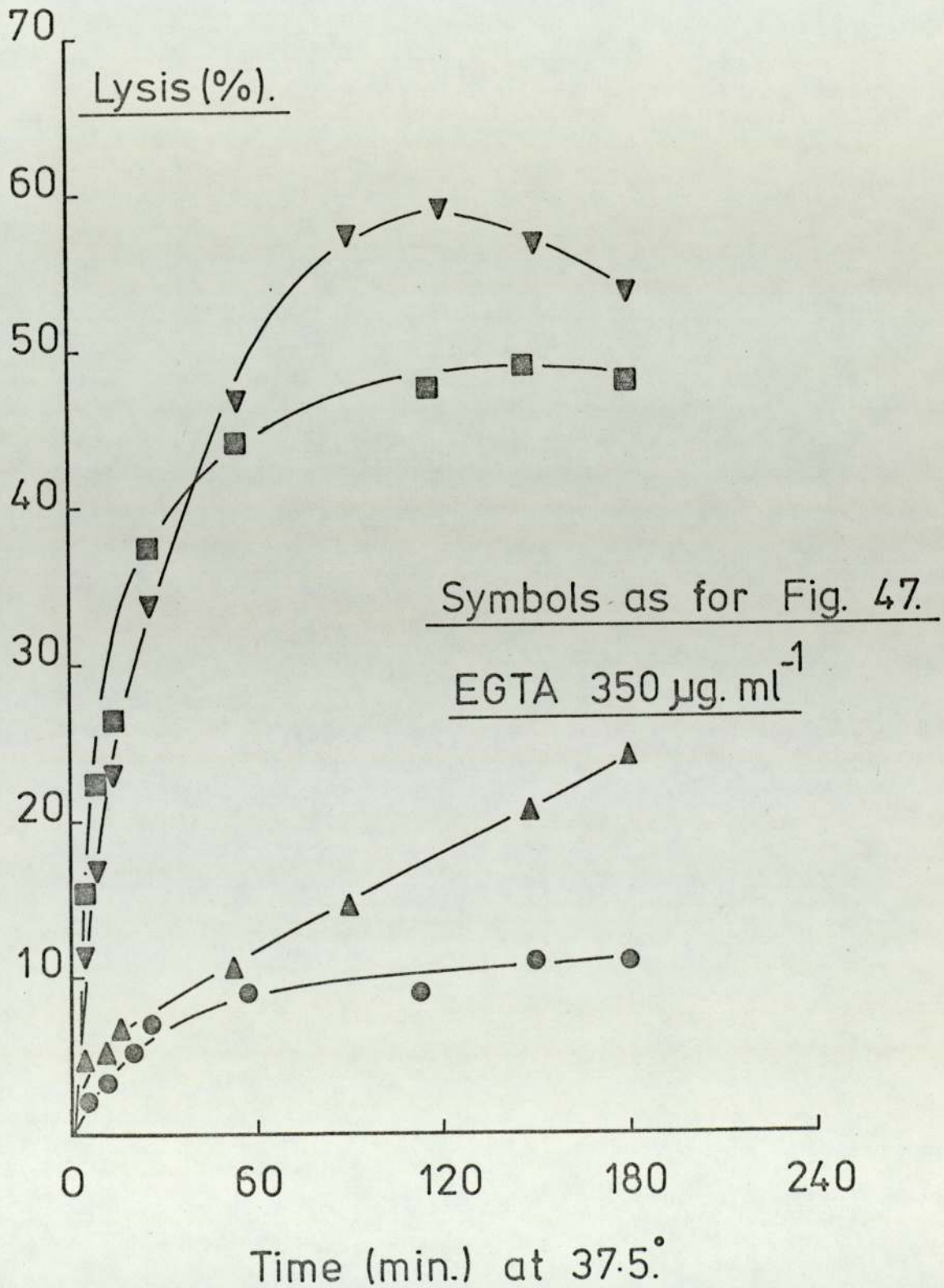


Figure 48. EGTA mediated lysis of cold shocked P.aeruginosa grown in Mg^{2+}-depleted CDM supplemented with Ca^{2+} or Mn^{2+}. Data corrected for lysis of controls (Fig. 42).



Mn^{2+} supplemented and control cold shocked cultures, although lysing to a greater extent than non-cold shocked cultures, did so in a similar manner. The lysis was linear with time in both cases and the control cultures both approached zero rate.

Polymyxin mediated lysis of cold shocked cultures of P.aeruginosa grown in Mg^{2+}-adequate media supplemented with Ca^{2+} or Mn^{2+}.

Fig. 49 shows that all the cultures lysed in a similar manner, and that with the exception of the Ca^{2+} and Mn^{2+} supplemented culture, they lysed to a similar extent. The lysis exhibited by the cultures was similar to the lysis observed in the control cultures (no drug), but was not as extensive. Cultures supplemented with Ca^{2+} or Mn^{2+} appeared slightly more sensitive to post cold shock lysis with P.X., whereas the culture supplemented with Ca^{2+} and Mn^{2+} appeared to be less sensitive. The observation that P.X. treated cells lysed to a lesser extent than the control (no drug) cultures suggests that P.X. has a protective effect in terms of reducing the observed drop in optical density.

Polymyxin mediated lysis of cold shocked cultures of P.aeruginosa grown in Mg^{2+}-depleted media supplemented with Ca^{2+} or Mn^{2+}.

All the cultures except the control (no supplement) showed similar lysis patterns, and all the cultures lysed to the same extent over the period of the experiment (Fig. 50). The lysis rate of the control culture was significantly lower than the supplemented cultures. The lysis of all the cultures was in marked contrast to the

Figure 49. Polymyxin mediated lysis of cold shocked *P. aeruginosa* grown in Mg^{2+}-adequate CDM supplemented with Ca^{2+} or Mn^{2+}.

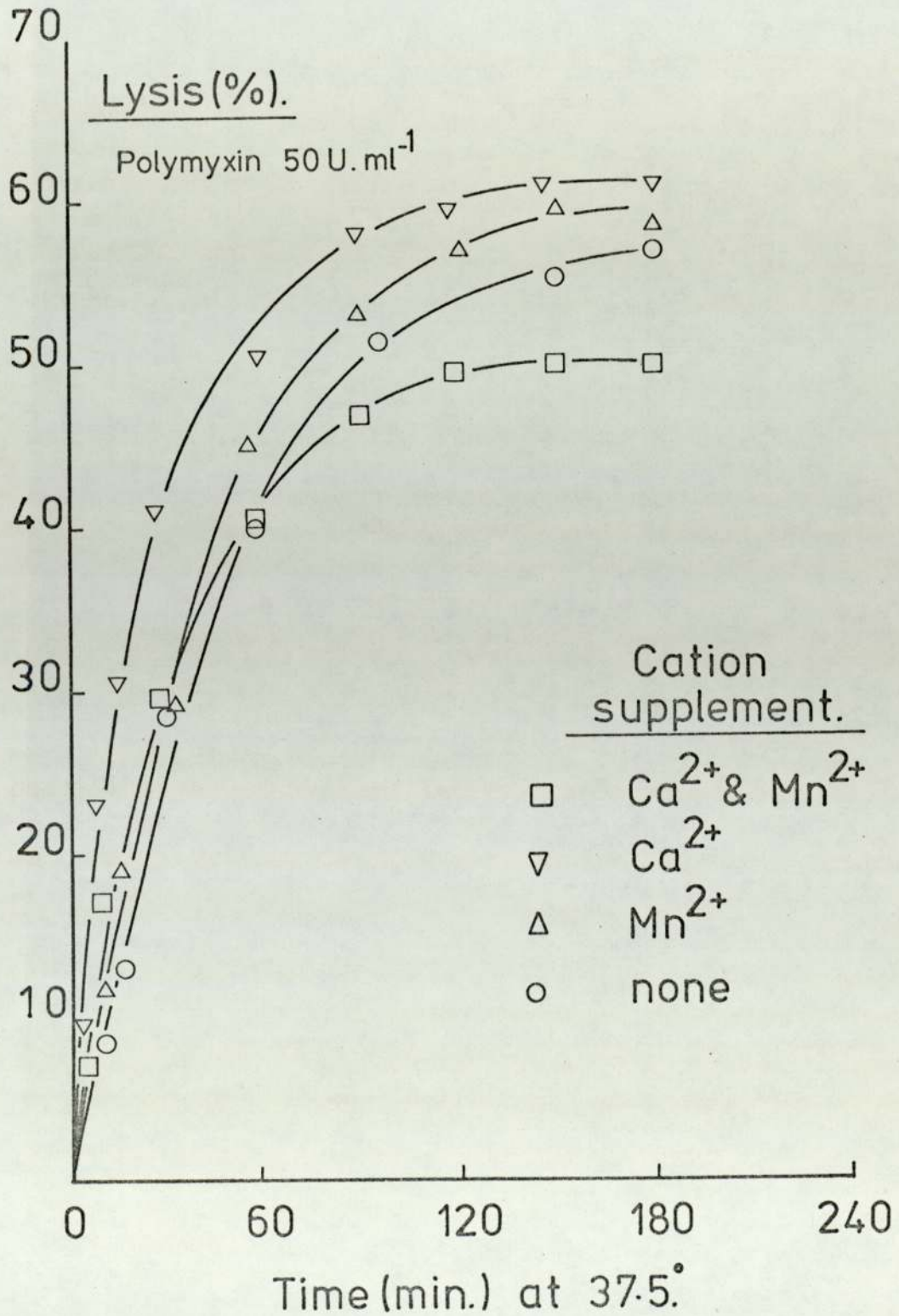


Figure 50. Polymyxin mediated lysis of cold shocked P.aeruginosa grown in Mg^{2+}-depleted CDM supplemented with Ca^{2+} or Mn^{2+}.

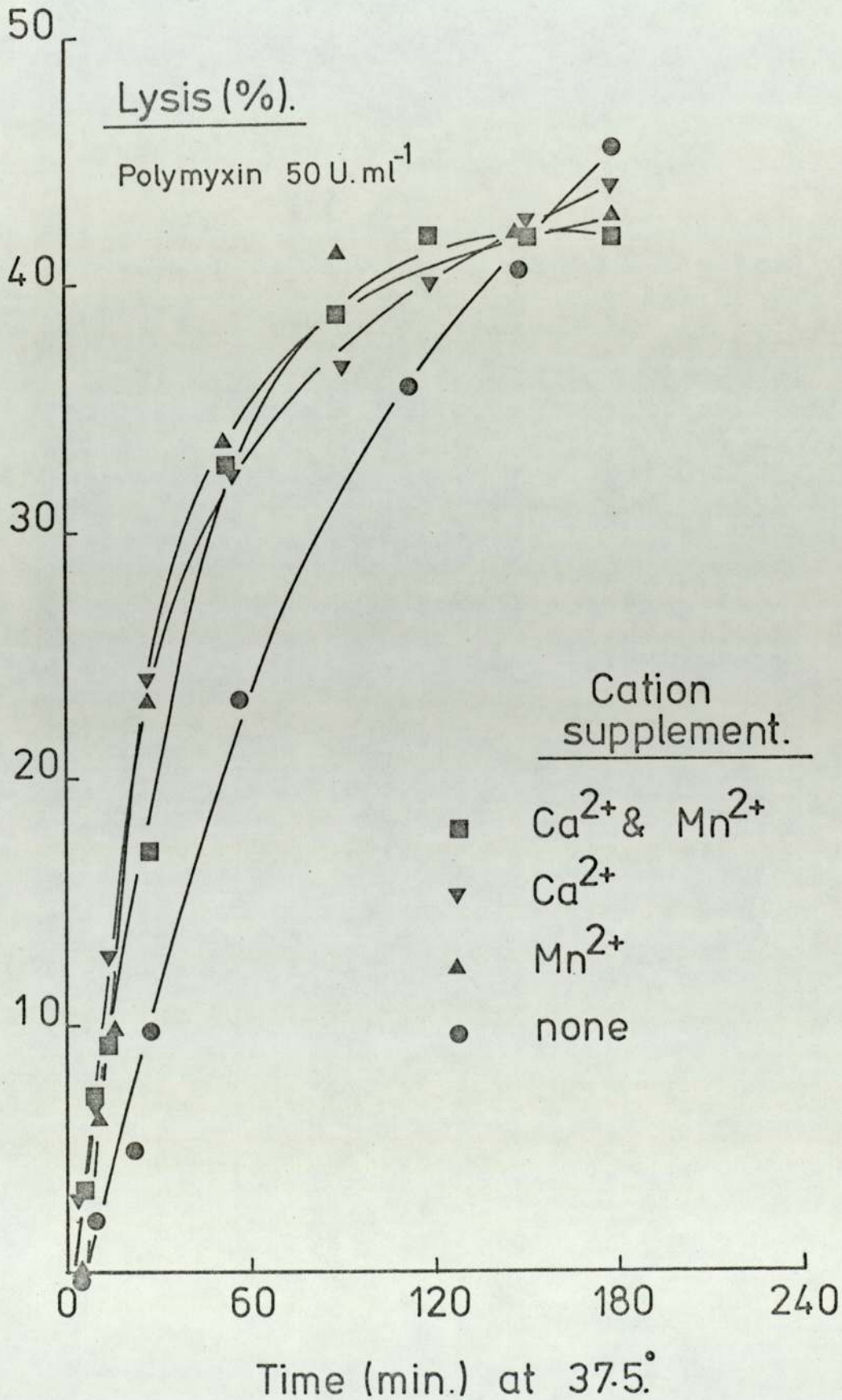
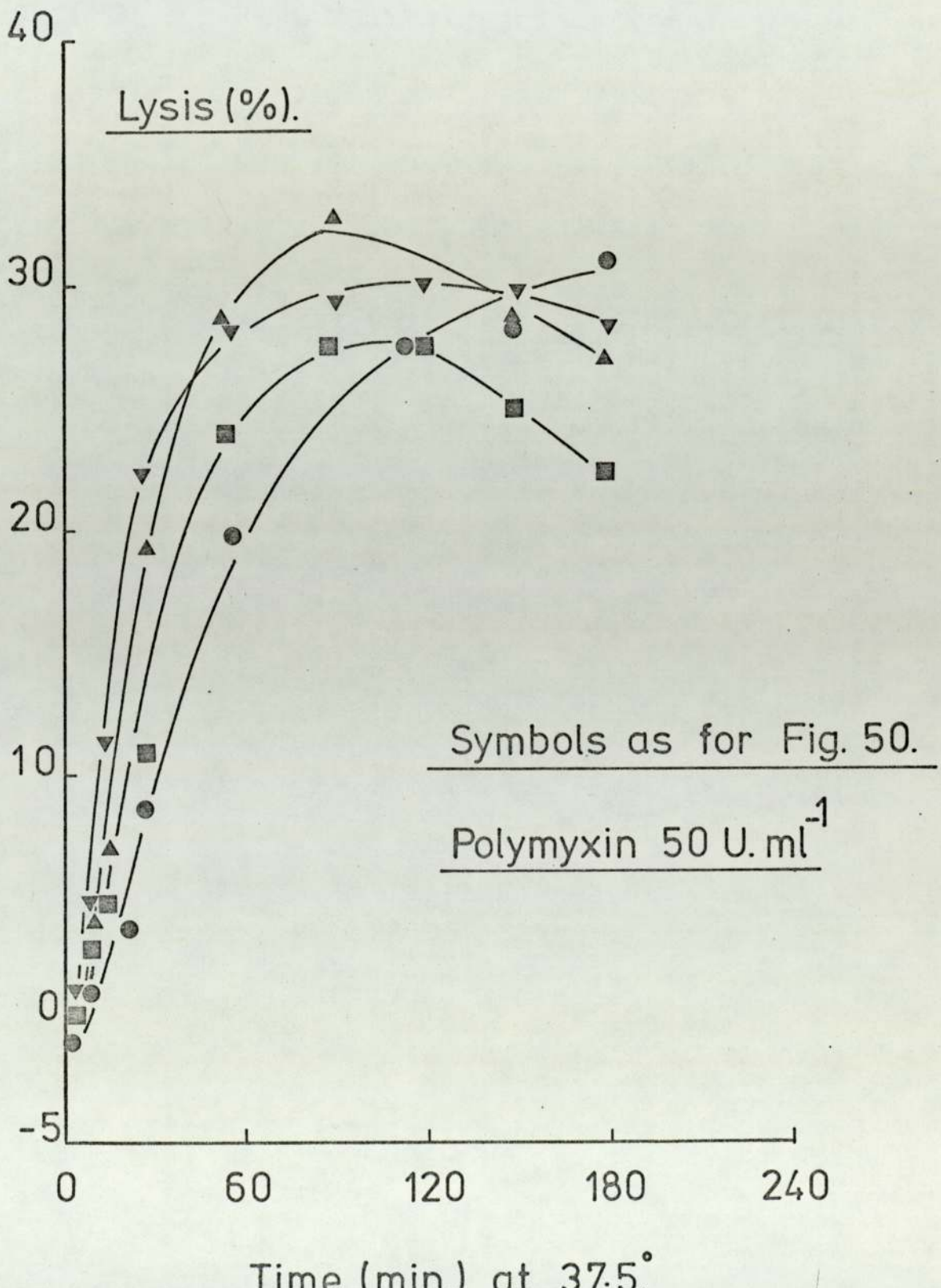


Figure 51. Polymyxin mediated lysis of cold shocked P. aeruginosa grown in Mg^{2+}-depleted CDM supplemented with Ca^{2+} or Mn^{2+}: Data corrected for lysis of controls (Fig. 42).



lysis exhibited by the control (no drug) culture (Fig. 39). The corrected lysis curves (Fig. 51) show that apart from the initial rate of lysis, there was little difference between the cultures. Cold shock rendered the control and Mn^{2+}-supplemented cultures almost as sensitive to P.X. as the Ca^{2+} and Ca^{2+} and Mn^{2+}-supplemented cultures. Comparison of Fig. 52 with Fig. 38 shows that cold shock appeared to render the Ca^{2+} and Ca^{2+} and Mn^{2+}-supplemented cultures less sensitive to P.X. mediated lysis than similar non-cold shocked cultures, and at the same time to decrease the resistance of the control and Mn^{2+} supplemented cultures.

8. CHEMISTRY OF WHOLE CELLS AND CELL WALLS PREPARED FROM CULTURES OF *P.aeruginosa* GROWN IN MAGNESIUM-ADEQUATE AND MAGNESIUM-DEPLETED CHEMICALLY DEFINED MEDIA SUPPLEMENTED WITH CALCIUM OR MANGANESE.

The preparation of the whole cells, the cell walls, the fractionation of the lipids and the chemical assay methods used have been described in sections 3 and 4. Wherever possible the assays were performed in triplicate, or in duplicate if the amount of sample was limited. The results of each assay are usually presented as a percent of the dry weight of the sample assayed. The results of the assays for the cations Mg^{2+}, Ca^{2+} and Mn^{2+} are presented as nanomoles cation/100 µg sample. For convenience the cultures were allocated the numbers 1 - 8 as follows:-

Culture 1	Mg^{2+}-adequate, no supplement
Culture 2	Mg^{2+}-depleted, no supplement
Culture 3	Mg^{2+}-adequate, supplemented with Ca^{2+}
Culture 4	Mg^{2+}-depleted, supplemented with Ca^{2+}
Culture 5	Mg^{2+}-adequate, supplemented with Mn^{2+}
Culture 6	Mg^{2+}-depleted, supplemented with Mn^{2+}
Culture 7	Mg^{2+}-adequate, supplemented with Ca^{2+} and Mn^{2+}
Culture 8	Mg^{2+}-depleted, supplemented with Ca^{2+} and Mn^{2+}

These numbers were used in the following Tables of results; the a and b subscript denote replicate cultures.

TABLE 23

Chemistry of whole cells from cultures of P.aeruginosa grown in Mg^{2+}-adequate chemically defined media supplemented with Ca^{2+} or Mn^{2+}.

Culture / Assay	1		3		5		7	
	a	b	a	b	a	b	a	b
Cell wall	18.2	18.3	12.9	13.9	14.4	18.5	14.5	15.2
Carbohydrate	-	7.8	-	6.8	-	6.2	-	7.3
P	-	2.7	-	1.6	-	2.1	-	3.4
†Mg^{2+}	-	10.3	-	8.6	-	8.6	-	8.6
†Ca^{2+}	-	3.0	-	9.2	-	5.0	-	11.2
†Mn^{2+}	-	ND	-	ND	-	1.6	-	2.0
R.E.L.	-	11.6	-	9.4	-	9.4	-	10.4
P.L.	-	-	-	3.4	-	2.5	-	5.5
*P.L.	-	-	-	36.5	-	26.9	-	52.7
*F.A.N.	-	-	-	48.6	-	47.6	-	40.1
*E.I.F.	-	-	-	14.8	-	25.5	-	7.2
**P.S.	-	ND	-	ND	-	ND	-	ND
**P.C.	-	ND	-	ND	-	ND	-	1.3
**lys.P.G.	-	ND	-	ND	-	ND	-	ND
**P.G.	-	ND	-	3.4	-	2.3	-	10.3
**P.E.	-	25.4	-	51.4	-	45.7	-	50.5
**D.P.G.	-	52.4	-	39.4	-	45.4	-	44.1

- not assayed

† expressed as nmoles cation/100 µg cell weight

ND not detectable

* expressed as percentage of R.E.L.

** expressed as percentage of P.L.

Tr trace

TABLE 24

Chemistry of whole cells from cultures of P.aeruginosa grown in Mg^{2+}-depleted chemically defined media supplemented with Ca^{2+} or Mn^{2+}.

Culture / Assays	2		4		6		8	
	a	b	a	b	a	b	a	b
cell walls	17.9	19.3	20.2	19.7	18.6	20.2	20.3	24.6
carbohydrate	-	7.9	-	5.5	-	6.6	-	6.7
P	-	1.7	-	1.1	-	1.5	-	1.5
†Mg^{2+}	-	Tr	-	Tr	-	Tr	-	Tr
†Ca^{2+}	-	3.7	-	4.2	-	ND	-	6.7
†Mn^{2+}	-	ND	-	ND	-	3.6	-	1.3
R.E.L.	-	21.1	-	15.7	-	14.6	-	15.4
P.L.	-	3.1	-	Tr	-	Tr	-	1.9
*P.L.	-	14.9	-	Tr	-	Tr	-	12.7
*F.A.N.	-	45.5	-	47.3	-	63.2	-	42.0
*E.I.F.	-	40.0	-	52.7	-	36.8	-	46.7
**P.S.	-	ND	-	ND	-	ND	-	ND
**P.C.	-	ND	-	ND	-	ND	-	ND
**lys.P.G.	-	12.2	-	6.7	-	27.8	-	19.1
**P.G.	-	3.2	-	4.2	-	7.3	-	16.1
**P.E.	-	11.4	-	24.0	-	22.3	-	12.0
**D.P.G.	-	61.4	-	51.3	-	37.2	-	52.9

TABLE 25

Chemistry of cell walls from cultures of P.aeruginosa grown in Mg^{2+}-adequate chemically defined media supplemented with Ca^{2+} or Mn^{2+}.

Culture / Assay	1		3		5		7	
	a	b	a	b	a	b	a	b
P	2.5	2.7	2.5	2.6	2.9	2.5	2.9	3.2
†Mg^{2+}	16.4	16.0	16.9	9.9	9.9	11.1	9.5	10.3
†Ca^{2+}	9.2	9.2	23.7	16.7	7.7	4.7	17.2	21.0
†Mn^{2+}	0.2	0.2	0.1	0.2	2.9	3.8	4.4	4.6
K.D.O.	2.0	2.1	1.6	2.0	1.5	2.2	1.1	2.1
Hexosamine	-	5.3	-	4.7	-	7.0	-	6.7
Carbohydrate	11.0	10.5	12.2	9.8	9.7	13.3	10.0	12.2
Protein	39.3	46.5	37.7	44.2	40.0	50.7	41.8	56.1
D.A.P.	-	1.8	-	2.7	-	3.9	-	3.3
R.E.L.	16.8	21.0	11.1	19.5	20.2	16.0	19.9	18.6
P.L.	8.3	3.8	4.7	2.8	-	2.3	-	6.6
*P.L.	-	17.9	-	14.2	-	14.6	-	35.1
*E.I.F.	-	ND	-	43.5	-	56.3	-	20.2
*F.A.N.	-	82.0	-	42.3	-	29.1	-	44.6
**P.S.	-	9.0	-	5.6	-	12.5	-	6.3
**P.C.	-	1.6	-	6.0	-	ND	-	6.5
**lys.P.G.	-	1.6	-	1.9	-	1.4	-	3.6
**P.G.	-	ND	-	7.8	-	ND	-	ND
**P.E.	-	18.3	-	44.9	-	38.5	-	38.3
**D.P.G.	-	61.3	-	41.3	-	45.4	-	35.5

TABLE 26

Chemistry of cell walls from cultures of P.aeruginosa grown in Mg^{2+}-depleted chemically defined media supplemented with Ca^{2+} or Mn^{2+}.

Culture / Assay	2		4		6		8	
	a	b	a	b	a	b	a	b
P	-	1.9	1.9	2.1	2.2	2.2	1.7	2.1
†Mg^{2+}	-	2.1	4.9	4.1	3.3	2.5	5.3	3.3
†Ca^{2+}	-	3.7	20.7	17.7	6.2	5.2	15.7	18.5
†Mn^{2+}	-	0.1	0.1	0.1	4.2	7.3	3.8	4.9
K.D.O.	-	1.7	1.6	1.6	1.6	2.1	1.5	2.0
Hexosamine	-	5.7	-	7.3	-	7.1	-	8.5
Carbohydrate	-	13.8	10.9	12.5	10.5	13.0	10.0	13.7
Protein	-	43.9	43.0	48.4	41.5	49.7	53.9	49.6
D.A.P.	-	2.5	-	3.3	-	3.1	-	2.6
R.E.L.	22.9	22.5	23.3	19.8	15.3	19.0	21.5	20.5
P.L.	6.0	4.2	8.9	3.6	-	6.2	-	3.8
*P.L.	-	18.8	-	18.4	-	32.7	-	18.3
*E.I.F.	-	43.8	-	43.8	-	45.7	-	45.7
*F.A.N.	-	36.6	-	37.8	-	22.3	-	37.1
**P.S.	-	8.1	-	9.8	-	14.5	-	5.7
**P.C.	-	ND	-	ND	-	6.8	-	3.0
**lys.P.G.	-	22.8	-	56.9	-	32.2	-	42.1
**P.G.	-	5.3	-	5.3	-	11.4	-	11.6
**P.E.	-	6.3	-	8.1	-	10.9	-	5.4
**D.P.G.	-	45.7	-	15.0	-	30.3	-	27.1

TABLE 27

Chemistry of Mg^{2+}-adequate cell walls of P.aeruginosa after lipid extraction.

Culture / Assay	1		3		5		7	
	a	b	a	b	a	b	a	b
P	1.6	2.0	3.0	1.7	3.8	2.9	3.3	3.4
Mg^{2+}	4.7	2.5	2.8	2.1	2.2	3.9	1.7	7.2
Ca^{2+}	3.8	4.5	6.5	4.6	2.5	2.5	4.6	4.6
Mn^{2+}	.05	ND	.07	ND	.09	.09	.10	.11
D.A.P.	6.1	-	8.0	-	7.3	-	8.5	-
K.D.O.	2.5	-	-	-	2.5	-	4.2	-
Carbohydrate	2.4	-	3.3	-	2.7	-	2.5	-

TABLE 28

Chemistry of Mg^{2+}-depleted cell walls of P.aeruginosa after lipid extraction.

Culture / Assay	2		4		6		8	
	a	b	a	b	a	b	a	b
P	1.6	1.8	2.1	1.9	2.4	1.7	2.4	3.0
Mg^{2+}	1.4	0.6	1.4	1.2	0.9	0.9	1.2	0.8
Ca^{2+}	3.4	1.3	5.6	4.8	2.7	4.4	5.3	2.3
Mn^{2+}	ND	ND	ND	ND	1.1	0.9	0.2	.07
D.A.P.	2.4	-	4.6	-	4.8	-	3.4	-
K.D.O.	-	-	2.5	-	2.7	-	1.7	-
Carbohydrate	7.9	-	5.5	-	6.6	-	6.7	-

TABLE 29

Lipids from Mg^{2+}-adequate cell walls of P.aeruginosa.

Culture / Assay	1		3		5		7	
	a	b	a	b	a	b	a	b
P (% R.E.L.)	4.2	-	4.8	-	4.7	-	3.4	-
P (% P.L.)	-	-	1.4	-	1.4	-	2.2	-
†Mg^{2+}	0.7	-	5.2	-	2.1	-	2.3	-
†Ca^{2+}	1.0	-	6.3	-	1.3	-	8.7	-
†Mn^{2+}	0.1	-	0.2	-	0.3	-	0.3	-
**P.S.	-	9.0	-	5.6	-	12.5	-	6.3
**P.C.	-	1.6	-	6.0	-	ND	-	6.5
**lys.P.G.	-	1.6	-	1.9	-	1.4	-	3.6
**P.G.	-	ND	-	7.8	-	ND	-	ND
**P.E.	-	18.3	-	44.9	-	38.5	-	38.3
**D.P.G.	-	61.3	-	41.3	-	45.4	-	35.5

TABLE 30

Lipids from Mg^{2+}-depleted cell walls of P.aeruginosa.

Culture / Assay	2		4		6		8	
	a	b	a	b	a	b	a	b
P (% R.E.L.)	2.2	-	1.7	-	2.8	-	1.2	-
P (% P.L.)	-	-	0.4	-	1.2	-	0.4	-
Mg^{2+}	0.7	-	0.8	-	1.9	-	0.6	-
Ca^{2+}	1.9	-	2.8	-	0.8	-	1.2	-
Mn^{2+}	ND	-	ND	-	0.3	-	0.2	-
**P.S.	-	8.1	-	9.8	-	14.5	-	5.7
**P.C.	-	ND	-	ND	-	6.8	-	3.0
**lys.P.G.	-	22.8	-	56.9	-	32.2	-	42.1
**P.G.	-	5.3	-	5.3	-	11.4	-	11.6
**P.E.	-	6.3	-	8.1	-	10.9	-	5.4
**D.P.G.	-	45.7	-	15.0	-	30.3	-	27.1

9. RESISTANCE STUDIES ON CULTURES OF *P.aeruginosa* POSSESSING THE R-FACTOR RP1.

Introduction.

The R-factor was kindly donated by Dr. E. Lowbury (Medical Research Council, Burns Unit, General Accident Hospital, Birmingham) and transfered to *P.aeruginosa* 6750 via *Escherichia coli* K12. Organisms harbouring the R-factor were designated *P.aeruginosa* $RP1^+$, and the parent strain without the R-factor, *P.aeruginosa* $RP1^-$. Cultures of *P.aeruginosa* $RP1^+$ were grown in Mg^{2+}-adequate chemically defined media, without supplementary Ca^{2+} or Mn^{2+}, and harvested in the manner previously described (section 2). The preparation of cell walls and lysis experiments were performed as in sections 2 and 3. Samples of the inoculum and harvested cells were used to prepare viable counts on nutrient agar and nutrient agar containing 200 U/ml carbenicillin. The number of colonies surviving on the carbenicillin plates were expressed as a percentage of the colonies on the nutrient agar plates to determine the percentage of the population that contained the R-factor.

The extent and initial rate of lysis were determined from Fig. 52, and are presented in Table 31.

Ethylenediaminetetra-acetic acid mediated lysis.

It can be seen from Fig. 52 that *P.aeruginosa* $RP1^+$ grown in Mg^{2+}-adequate chemically defined media was markedly resistant to lysis by this chelating agent when compared with *P.aeruginosa* $RP1^-$ grown and lysed under similar conditions.

Figure 52. Lysis of P. aeruginosa RP1^{+} and P. aeruginosa RP1^{-} mediated by EDTA, EGTA or Polymyxin.

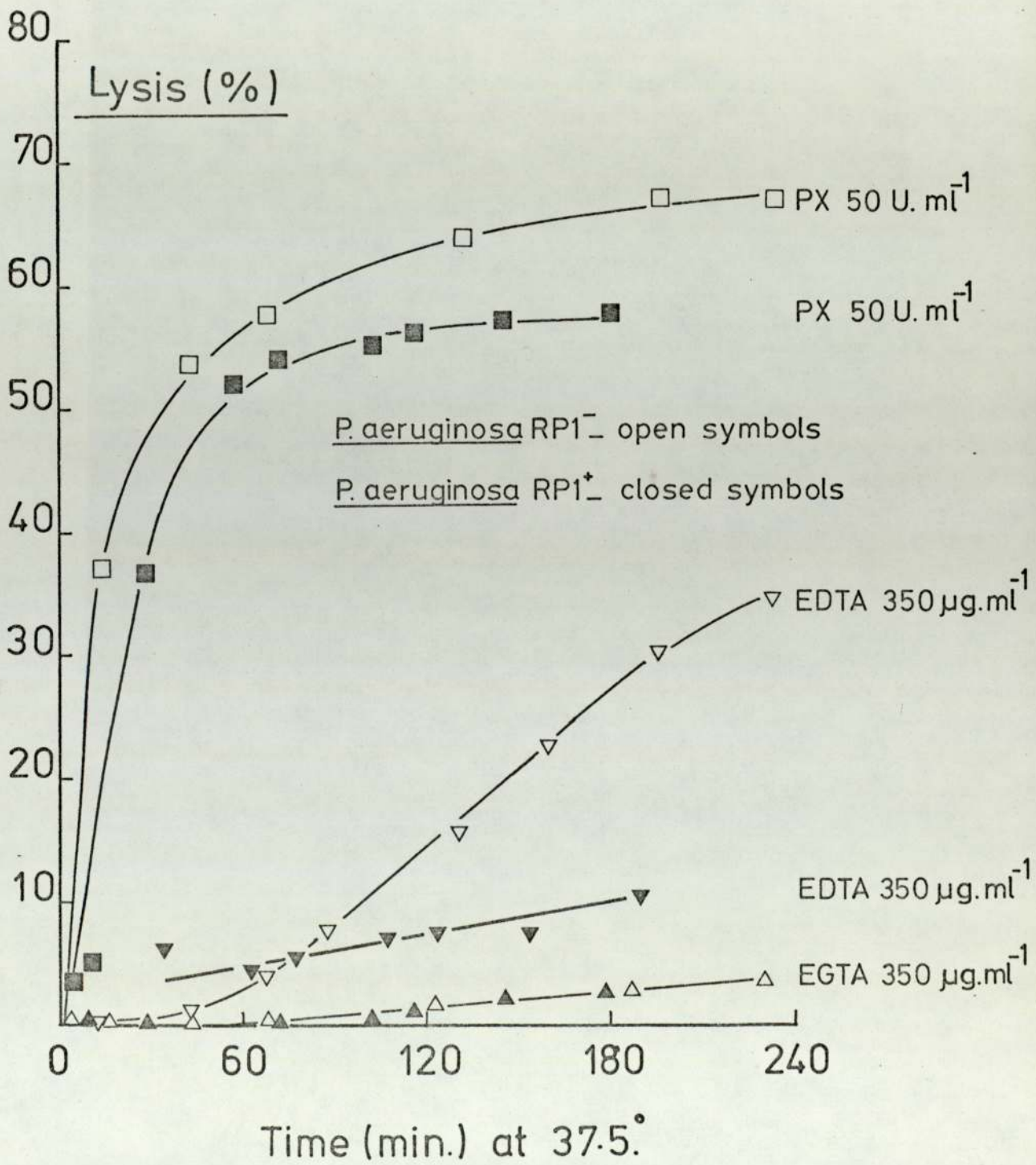


TABLE 31

Initial lysis and extent of lysis of E.D.T.A., E.G.T.A. and P.X. treated cultures of P.aeruginosa $RP1^+$ and P.aeruginosa $RP1^-$ grown in magnesium-adequate chemically defined media.

Culture / Lytic agent	Without RP1		With RP1	
	a	b	a	b
E.D.T.A.	7.8	27.5	63.0	10.0
E.G.T.A.	> 220	2.1	>220	2.1
P.X.	1.0	66.5	6.0	57.5

The data in this Table was determined from Fig. 52.

a Time (min) for first 5% lysis to occur.

b Percent lysis after 180-min.

Ethyleneglycol-Bis-(β-aminoethylether)-N:N'-Tetra-acetic acid mediated lysis.

The culture of P.aeruginosa $RP1^+$ was as resistant to lysis by this drug as P.aeruginosa $RP1^-$. The kinetics of lysis were similar in both strains.

Polymyxin mediated lysis.

Fig. 52 shows that P.aeruginosa $RP1^+$ was less sensitive to lysis by P.X. than P.aeruginosa $RP1^-$. The initial rate of lysis, and the extent of lysis were lower than for the parent strain. It appeared that the possession of the R-factor RP1 by P.aeruginosa decreased the sensitivity of the organism to polymyxin by a small extent

(approximately 15%). Viable counts, using nutrient agar and carbenicillin impregnated agar, showed that 97% of the cells in the P.aeruginosa $RP1^{+}$ culture possessed the R-factor.

10. RESISTANCE STUDIES ON COLD SHOCKED CULTURES OF *P.aeruginosa* POSSESSING THE R-FACTOR RP1.

The cold shocked *P.aeruginosa* $RP1^+$ culture showed the same extensive autolysis upon rewarming (to 37.5°) as *P.aeruginosa* $RP1^-$. The two lysis curves are not significantly different (Fig. 53). The viabilites however, showed a marked difference; *P.aeruginosa* $RP1^+$ had a survivor percentage of 0.03% compared with 0.35% for the parent strain *P.aeruginosa* $RP1^-$. Only 21% of the cells of *P.aeruginosa* $RP1^+$ that survived cold shock were able to grow on carbenicillin agar.

Ethylenediaminetetra-acetic acid mediated lysis.

P.aeruginosa $RP1^+$ lysed in much the same manner as *P.aeruginosa* $RP1^-$ cultures (Fig. 53). The extent of lysis was greater for *P.aeruginosa* $RP1^+$ than for *P.aeruginosa* $RP1^-$ grown in a similar medium (no supplement).

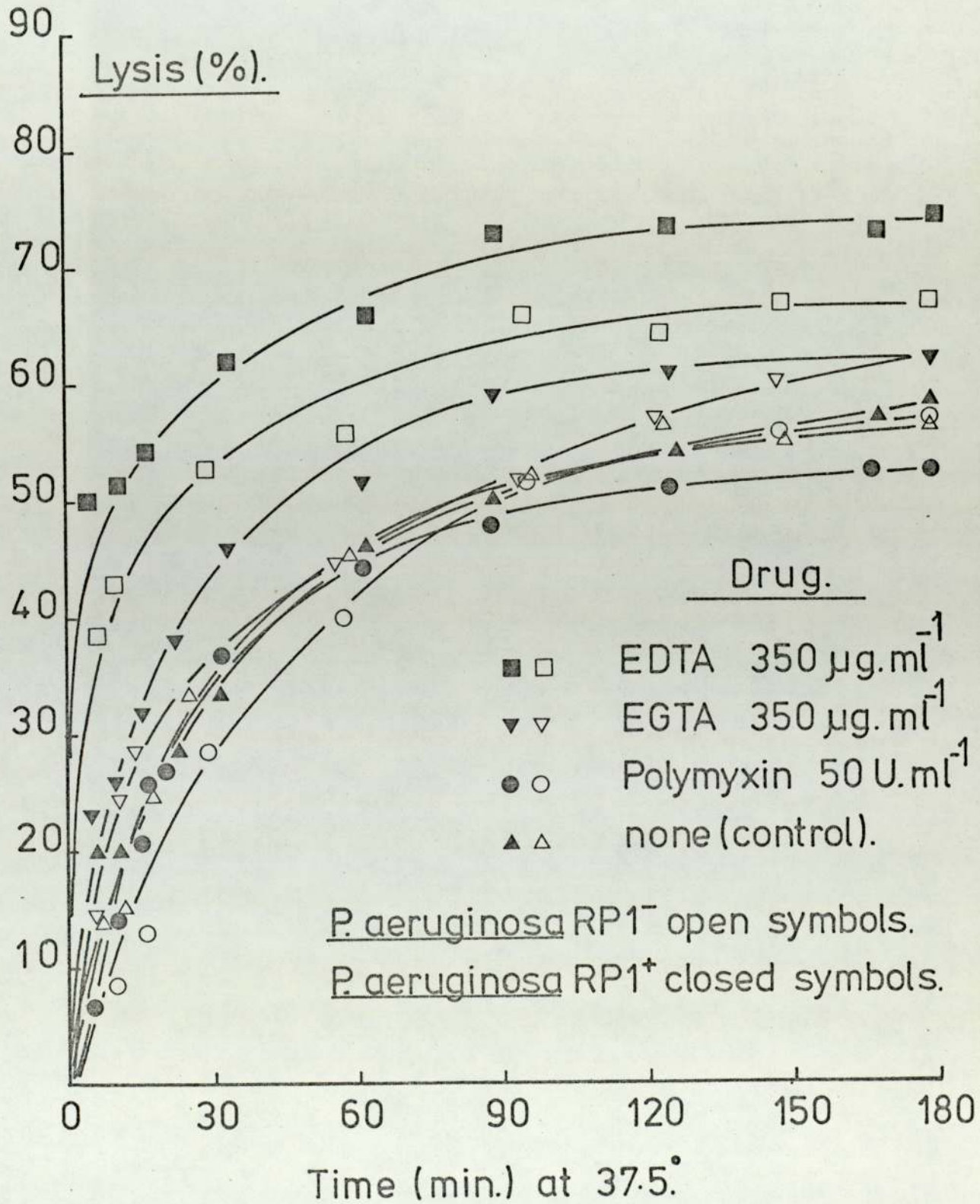
Ethyleneglycol-Bis-(β-aminoethylether)-N:N'-Tetra-acetic acid mediated lysis.

The percent lysis after 180-min was the same for both the cultures (Fig. 53). The initial rate of lysis of *P.aeruginosa* $RP1^+$ was greater than for *P.aeruginosa* $RP1^-$, but this rate decreased almost to zero after 180-min, whereas the rate for *P.aeruginosa* $RP1^-$, although decreasing had not approached zero at this time.

Polymyxin mediated lysis.

There was little difference between the initial lysis rates and the percent lysis at 180-min for the cultures *P.aeruginosa* $RP1^+$ and *P.aeruginosa* $RP1^-$ (Fig. 53), although the latter was lysed to the greatest extent.

Figure 53. Lysis of cold shocked P. aeruginosa RP1⁻ and P. aeruginosa RP1⁺ by EDTA, EGTA or Polymyxin.



11. CHEMISTRY OF CULTURES OF *P.aeruginosa* POSSESSING THE R-FACTOR RP1.

The preparation of whole cells, cell walls, lipid fractions and the chemical assay methods have been described previously. The results of the assays (Tables 32 - 35) are expressed in the same units as those used for *P.aeruginosa* $RP1^-$ cultures described in section 8. The data representing *P.aeruginosa* $RP1^-$ in Tables 32 - 35 were taken from Tables 23, 25, 27 & 28 (Culture number 1).

TABLE 32

Chemistry of whole cells from cultures of P.aeruginosa with and without the R-factor RP1 grown in Mg^{2+}-adequate chemically defined media.

Assay \ Culture	$RP1^-$	$RP1^+$
cell wall	18.3	18.0
carbohydrate	7.8	7.4
P	2.7	2.3
† Mg^{2+}	10.3	10.3
† Ca^{2+}	3.0	2.0
† Mn^{2+}	ND	ND
R.E.L.	11.6	13.8
P.L.	-	3.8
* P.L.	-	27.8
* F.A.N.	-	41.1
* E.I.F.	-	33.5
** P.S.	ND	3.2
** P.C.	ND	ND
** lys.P.G.	ND	7.4
** P.G.	ND	ND
** P.E.	25.4	36.9
** D.P.G.	52.4	52.6

† nmoles cation/100 µg cell weight

* as percent of R.E.L.

** as percent of P.L.

TABLE 33

Chemistry of cell walls prepared from cultures of P.aeruginosa, with and without the R-factor RP1, grown in Mg^{2+}-adequate chemically defined media.

Assay \ Culture	$RP1^-$	$RP1^+$
P	2.7	2.5
Mg^{2+}	16.0	13.2
Ca^{2+}	9.2	8.0
Mn^{2+}	0.2	ND
K.D.O.	2.1	1.1
Hexosamine	5.3	5.8
Carbohydrate	10.5	13.0
Protein	46.5	45.7
D.A.P.	1.8	2.9
R.E.L.	21.0	18.0
P.L.	3.8	4.3

TABLE 34

Phosphorus and cation content of defatted* cell walls of P.aeruginosa, with and without the R-factor RP1, grown in Mg^{2+}-adequate chemically defined media.

Assay \ Culture	$RP1^-$	$RP1^+$
P	2.0	2.4
Mg^{2+}	2.5	14.7
Ca^{2+}	4.5	10.5
Mn^{2+}	ND	ND

* wall after removal of R.E.L. by solvent extraction

TABLE 35

Lipids extracted from cell walls of P.aeruginosa cultures, with and without the R-factor RP1, grown in Mg^{2+}-adequate chemically defined media.

Assay \ Culture	$RP1^-$	$RP1^+$
*P.L.	17.9	28.6
*F.A.N.	82.0	41.2
*E.I.F.	ND	33.8
**P.S.	9.0	7.1
**P.C.	1.6	2.1
**lys.P.G.	1.6	6.7
**P.G.	ND	ND
**P.E.	18.3	45.2
**D.P.G.	61.3	35.9

* as percent of R.E.L.

** as percent of P.L.

DISCUSSION

1. NUTRIENT DEPLETION STUDIES ON P.aeruginosa IN DEFINED MEDIA.

Glucose depletion.

The relation between glucose concentration of the growth medium and the maximum growth ($O.D._{470}$ max) of P.aeruginosa was linear when all other nutrients were present in excess (Fig. 21). Extrapolation of the line back to the origin of the 'x'-axis indicates that there were insufficient amounts of a suitable carbon source, present as a contaminant of the other nutrients, to support visible growth. The observed linear relation is compatible with the results of similar experiments with the same organism described by Melling (1968), Watkins (1970) and Boggis (1971). The two former authors however obtained a lower maximum growth for a given glucose concentration. For instance, Fig. 21 shows that an initial glucose concentration of 10^{-3} M resulted in an $O.D._{470}$ 0.26, whereas Melling (1968) and Watkins (1970) found the same concentration resulted in values of 0.19 and 0.21 respectively. This discrepancy may be the result of the low growth rate (doubling times between 95 - 100-min) observed by Melling (1968). Although the growth rate observed by Watkins (1970) appears compatible with the observed rate in these experiments (doubling time approximately 63-min), this author did not state the shaking rate of the incubator. Both Boggis (1971) and the present author used rates of 120 throws per minute, and the maximum growth obtained were similar (0.27 and 0.26 respectively). It is conceivable that if Watkins (1970) was using a slower shaking rate the decreased aeration may have sufficiently altered the efficiency with which glucose was

converted to cell mass without significantly altering the growth rate. The relation illustrated in Fig. 21 was drawn using the data obtained from small scale (25 ml) experiments. Fig. 28 shows that a similar relation was observed when the depletion experiments were performed using 6 L batches of media. The line does not extrapolate through the orgin, but at a value of $O.D._{470}$ that would be expected from 9×10^{-5} M-glucose. This 'contamination' of the media is presumably an artifact due to the large inoculum size. The $O.D._{470}$ of the medium upon inoculation was approximately 0.02 which corresponds to the intercept on the 'y'-axis of Fig. 28. Both Fig. 21 and 28 show that the relation between initial glucose concentration and maximum growth is linear to $O.D._{470}$ 1.0. This is not compatible with the results of Melling (1968) who observed that linearity was lost at $O.D._{470}$ 0.23; this was presumably due to the significant drop in pH observed above this value, which would tend to inhibit growth. The compatibility between the effects of glucose depletion on growth observed using 25 ml and 6 L systems indicates that the alteration of the growth conditions, i.e. aeration by stirring not shaking and the increase in volume do not appear to affect the utilization of the glucose by cultures of P.aeruginosa. The relation between maximum growth and glucose was linear up to $O.D._{470}$ 1.0 but no data was obtained for values greater than 1.0. It is probable however, that the linearity of the relation would be lost at $O.D._{470}$ values greater than 1.3, since it is at this value that the growth rate of cultures of P.aeruginosa, grown in chemically defined media (C.D.M.) containing sufficient glucose to

reach O.D.$_{470}$ 4.0 and sufficient other nutrients to reach O.D.$_{470}$ 6.5, departed from the maximum exponential rate (see Fig. 29), and growth was limited by some other factor, probably lack of aeration or low pH. Since the reason for this decrease in growth rate is unclear, it was decided to stop the growth of magnesium (Mg^{2+}) manipulated cultures at O.D.$_{470}$ 1.0. Growth was stopped by glucose depletion at this point. Cultures harvested at O.D.$_{470}$ 1.0 therefore were assumed to be depleted of only glucose or Mg^{2+} and glucose. However, it is conceivable that the drop in growth rate observed at O.D.$_{470}$ 1.3 may be due to lack of aeration initially occuring at an O.D.$_{470}$ below 1.0, and cells at 1.0 may be exposed to some degree of oxygen depletion. The culture was thought not to be affected by low pH since the pH of Mg^{2+}-adequate cultures at O.D.$_{470}$ 1.0 was never found to be lower than 7.4.

Magnesium-depletion.

The shape of the growth curves for Mg^{2+}-depleted cultures (Fig. 22 & 24) were markedly different from glucose-depleted cultures. The gradual decrease in the growth rate observed after maximum exponential growth had ceased, may be due in part to a corresponding decrease in the synthesis of ribonucleic acid (RNA). Tempest, Hunter & Sykes (1965) observed that the RNA content of Aerobacter aerogenes cells limited by Mg^{2+} in a chemostat decreased as the degree of limitation increased. A similar result was reported for Pseudomonas putida (Sykes & Tempest, 1965). These results indicated that RNA synthesis, including ribosomal RNA, was controlled by the Mg^{2+} content. McCarthy (1962) and Morgan, Rosenkranz, Chan & Rose (1966)

have shown that protein synthesis in Mg^{2+}-depleted Escherichia coli was proportional to the ribosome content of the cells. The observed decrease in growth rate after the onset of Mg^{2+}-limitation (Fig. 24) may be explained by a decrease in ribosome content and hence protein synthesis. At each successive division of the Mg^{2+}-depleted cells the cellular content of Mg^{2+} and ribosomes will progressively decrease, resulting in a corresponding decrease in the growth rate. This is seen in the growth curves as a continuous hyperbolic function since the cultures are dividing asynchronously.

The relation between intial Mg^{2+} concentration and the maximum growth ($O.D._{470}$ 3-hr after the onset of Mg^{2+}-depletion) is shown to be linear up to a concentration of 1.645×10^{-5} M (Fig. 23 & 25). The $O.D._{470}$ max at which the relation becomes non-linear was 1.3, the same value at which maximum exponential growth begins to decrease in a fully adequate medium (see Fig. 29). The relation between onset of Mg^{2+}-depletion ($O.D._{470}$ onset) and initial Mg^{2+} concentration is also linear to 1.645×10^{-5} M, corresponding to an $O.D._{470}$ onset 0.8. This suggests that the growth limiting factor that causes an observed decrease in growth rate at $O.D._{470}$ 1.3 affects the onset of growth depletion due to lack of Mg^{2+} at populations greater than $O.D._{470}$ 0.8. The total growth however is unaffected presumably while glucose is present in sufficient excess. The relation between onset of Mg^{2+}-depletion and initial Mg^{2+} concentration in 6 L batches is also linear (Fig. 31) and compatible with the 25 ml batch results. The linearity of the relation in 6 L batches also ceases at $O.D._{470}$ 0.8.

Batches of Mg^{2+}-adequate and Mg^{2+}-depleted cells, harvested at $O.D._{470}$ 1.0, may have been exposed to different conditions prior to the cessation of growth due to glucose depletion. Magnesium-depleted cultures grow at a progressively decreasing rate, and require much more glucose to reach $O.D._{470}$ 1.0 (1.2×10^{-2} M-glucose as opposed to 3.8×10^{-3} M for Mg^{2+}-adequate cultures). The extra glucose is presumably required to maintain respiration of the cells dividing at a slower rate. This would indicate that the rate of respiration has remained unchanged, the amount of glucose merely reflecting the time taken for Mg^{2+}-depleted cultures to reach an $O.D._{470}$ 1.0. The other possibility is that, faced with Mg^{2+}-depletion, the cells rely upon a less efficient form of energy production (e.g. fermentation), and thus require more glucose for the same amount of energy. The latter case may also be brought about if the increased requirement for oxygen, to oxidize the increased glucose, cannot be met by the aeration system. In either case the larger amount of glucose consumed may be expected to result in a decrease in the pH, and it was found that Mg^{2+}-depleted cultures at $O.D._{470}$ 1.0 were at pH 7.1 - 7.2. Thus, it must be borne in mind that Mg^{2+}-depleted cultures may also have been physiologically different in that the method of glucose utilization was altered and may also have been oxygen limited to a certain extent and exposed to a drop in pH. Recently Minnikin & Abdolrahimzadeh (1974) have found that whole cells of Bacillus subtilis exhibited altered phospholipid content when the pH was altered, irrespective of the type of nutrient limitation imposed.

The alteration of phospholipids will have an effect upon the action of polymyxin, since it has been shown to bind to membrane phospholipids (Hsu-Chen & Feingold, 1972; Bader & Teuber, 1973).

The effect upon growth of P.aeruginosa of calcium and manganese supplementation of Mg^{2+}-depleted C.D.M..

The growth curves illustrated in Fig. 32 show that supplementation by calcium (Ca^{2+}) and manganese (Mn^{2+}), singly or in combination, had no significant effect upon the onset of Mg^{2+}-depletion, the maximum growth, or the growth rate before and after the onset of Mg^{2+}-depletion. In the cultures supplemented with Ca^{2+} alone this was compatible with previous results (P.aeruginosa: Brown & Melling, 1969b; E.coli: Lusk, Williams & Kennedy, 1968), and may be explained either by the fact that Ca^{2+} does not wholly replace Mg^{2+} in ribosomes, either structurally or functionally (Weiss, Kimes & Morris, 1973) or the lack of enzymes in which Mg^{2+} can be totally replaced by Ca^{2+} (Dixon & Webb, 1964). Boggis (1971) however, noted that increasing the concentration of Ca^{2+} increased the maximum growth of the Mg^{2+}-depleted cultures; this effect may have been due to contaminating Mg^{2+} in the batch of Ca^{2+} salt used and/or the distilled water used. As discussed in the previous paragraph, Mg^{2+} depletion resulted in a decrease in functional ribosomes. Weiss et al. (1973) have shown that Ca^{2+} could not wholly replace Mg^{2+} in E.coli ribosomes (30 S and 50 S) in vitro. Williams & Wacker (1967) suggested that Ca^{2+} was maintained at sites exterior to the cytoplasm of living cells, and so it would be expected

on this basis to find that Ca^{2+} would be taken up by the cell wall. As can be seen from Table 26 this was shown to be the case in the present study. The cultures supplemented with Mn^{2+} alone would be expected to show some differences. Manganese can replace Mg^{2+} quite effectively in many enzyme systems, and has been found to totally replace Mg^{2+} in E.coli ribosomes in vitro (Weiss et al., 1973). Boggis (1971) has found that supplementation of the growth medium with Mn^{2+} (1.4×10^{-5} M) partially restored the sensitivity of Mg^{2+}-depleted P.aeruginosa to polymyxin (P.X.), ethylenediaminetetra-acetic acid (E.D.T.A.) and ethyleneglycol-bis-(β-amino-ethylether)-N:N'-tetra-acetic acid (E.G.T.A.), but did not report any affect upon growth rate although he found no significant increase in the maximum growth of Mg^{2+}-depleted cultures. Fig. 32 shows that Mn^{2+} had no significant effect upon growth rate, explained by the low level used (2×10^{-5} M). Higher concentrations of Mn^{2+} in an otherwise adequate C.D.M. resulted in precipitation of the phosphate. The cell walls from cells of Mn^{2+} supplemented Mg^{2+}-depleted cultures were found to contain significant amounts of Mn^{2+} (Table 26). As may be expected from the results of supplementation by Ca^{2+} and Mn^{2+} individually, the result of supplementation by both cations also produced no effect upon the growth rate and on set of growth limitation. Both cations were present in significant amounts in the cell walls, and as will be discussed in a later section they had marked effects upon the resistance of Mg^{2+}-depleted P.aeruginosa to P.X., E.D.T.A. and E.G.T.A..

The effect upon growth of P.aeruginosa of Ca^{2+} and Mn^{2+} supplementation of Mg^{2+}-adequate C.D.M..

The growth curves in Fig. 33 indicates that C.D.M. supplemented with Ca^{2+} and Mn^{2+}, singly or together, had no significant effect upon the growth rate or cut off of Mg^{2+}-adequate cultures of P.aeruginosa. Although these cations do not affect the growth of the organism, reference to Table 25 shows that they are nevertheless taken up by the cell walls. Fig. 34 shows that the sensitivity to E.D.T.A. is enhanced, indicating a requirement for these cations, even in the presence of excess Mg^{2+}.

2. RESISTANCE STUDIES OF P.aeruginosa GROWN IN MAGNESIUM-ADEQUATE AND MAGNESIUM-DEPLETED CHEMICALLY DEFINED MEDIA SUPPLEMENTED WITH CALCIUM OR MANGANESE.

General observations.

The lytic action of ethylenediaminetetra-acetic acid (E.D.T.A.), ethyleneglycol-bis-(β-amino-ethylether)-N:N'-tetra-acetic acid (E.G.T.A.) and polymyxin (P.X.) on the cultures was followed by measuring the decrease in optical density at 470 nm ($O.D._{470}$) of cultures treated with the drugs. It is possible that these changes in $O.D._{470}$ were drug induced changes in the light scattering properties of the cells and not lysis of the cells *per se*, or a combination of both of these effects. Direct microscopical examination of treated cultures revealed that significant lysis had occured, but this was not quantified by total or viable counts. Brown & Melling (1969b) however have shown that the decrease in $O.D._{470}$, leakage of 260 nm-absorbing material and drop in colony count were compatible with one another for E.D.T.A. and P.X. treated cultures of *P.aeruginosa* grown in Mg^{2+}-adequate and Mg^{2+}-depleted chemically defined media (C.D.M.). This has been reproduced using total counts by Boggis (1971). Another disadvantage of using $O.D._{470}$ measurements to follow lysis is that both Mg^{2+}- adequate and Mg^{2+}-depleted cultures were harvested at $O.D._{470}$ 1.0, and identical $O.D._{470}$ readings do not necessarily mean similar cell concentrations. Tempest, Hunter & Sykes (1965) have found that the cell size of *Aerobacter aerogenes* cultures decreased with increasing Mg^{2+}-limitation in a chemostat. If the cell size decreases, then there

would be a corresponding increase in the surface area, and hence the light scattering properties of the culture. Thus, it may be expected that if the Mg^{2+}-depleted cultures used in this study contained significantly smaller cells than the Mg^{2+}-adequate cultures, then there would be less Mg^{2+}-depleted cells per ml at any given $O.D._{470}$ value. However, upon direct microscopical (phase contrast) examination the Mg^{2+}-depleted cells did not appear to be markedly smaller than Mg^{2+}-adequate cells at the same $O.D._{470}$, and Boggis (1971) found little difference between the total counts of Mg^{2+}-adequate and Mg^{2+}-depleted P.aeruginosa suspensions of similar $O.D._{470}$.

Cells were not washed prior to lysis experiments to avoid any change in sensitivity due to the washing procedure (Brown, 1968). The cultures were diluted five-fold prior to lysis experiments in the growth C.D.M. lacking glucose (Mg^{2+}-adequate cultures) or lacking both glucose and Mg^{2+} (Mg^{2+}-depleted cultures). The dilution with the C.D.M. lacking the growth rate limiting nutrient was done in order that the cultures were diluted into a system closely resembling the growth medium at the time of harvesting. This may not have been the case for Mg^{2+}-depleted cultures, since there would have been a sudden, slight pH change (from 7.1 to 7.8) which may have had some effect upon the sensitivity of Mg^{2+}-depleted cultures. The resistance of the cultures was determined in the presence of different concentrations of various cations according to which growth medium was used. It is therefore likely that some of the differences in sensitivity observed

between cultures may be due, in part, to antagonism of the drugs by cations.

The extent of lysis observed in samples taken from 6 L batch cultures in this work were not compatible with the results reported by Boggis (1971) for samples from 8 L batch cultures grown in similar media. The present results were compatible with the results obtained by Boggis (1971) from resistance experiments performed on small scale (100 ml) cultures grown up to an $O.D._{470}$ 0.2 and treated directly with the lytic agent. Boggis (1971) suggested that the discrepancies he observed between the lysis samples from 8 L and 100 ml cultures may have been due to the following reasons: 1) the 8 L cultures were harvested at $O.D._{470}$ 2.0 and Mg^{2+}-adequate cultures were not observed to grow at maximum exponential rate between $O.D._{470}$ 1.3 and 2.0 and were presumably oxygen depleted, 2) the pH of 8 L batch cultures at harvesting was low (6.8), 3) the samples were diluted ten-fold in 0.0075 M ammonium acid phosphate buffer, pH 7.8, thus exposing the cells simultaneously to a sudden increase in pH (6.8 to 7.6) and a sudden decrease in the molarity of the medium (from approximately 0.04 M to 0.011 M). These points may also explain the discrepancies between the results in this thesis and those reported by Boggis (1971) since only the former effect (a sudden change in pH) is likely to have occured in the present study.

There were certain features of the lysis curves of E.D.T.A. and E.G.T.A. treated cultures that were common to all the cultures. All the Mg^{2+}-adequate cultures exhibited a slow initial rate of lysis that gradually

increased until a faster, constant, secondary rate occured. This would indicate that the cations to be removed are not immediately accessible to the chelating agent, but once the cation removal had been initiated then the remaining cations were removed with greater ease. With Mg^{2+}-depleted cultures the initial rate of lysis is very rapid, falling progressively to a slower, constant rate of lysis. These results suggest that a few of the cations are quickly removed from superficial sites and that most of the cations are more firmly bound at a less accessible site. An alternative explanation is that the sudden change in pH from 7.1 - 7.2 to 7.8 may have rendered a small percentage of the population more sensitive to the chelating agents. The action of P.X. upon Mg^{2+}-adequate cultures was very rapid, the initial rate of lysis being responsible for most of the lysis. No 'lag' was observed suggesting that P.X. binding sites were readily available. It was not possible to note if there was an increase in the initial rate of lysis of Mg^{2+}-depleted cultures since they were either extremely resistant (no supplement) or as sensitive (Ca^{2+} supplemented) as the Mg^{2+}-adequate cultures. From these observations the major changes in sensitivity to the drugs appeared to involve the initial rate of lysis, and the extent of lysis. Consequently, the sensitivity of the cultures was quantified by two means: 1) the time taken (in min) for the first 5% lysis to occur, and 2) the percent lysis observed 180-min after the addition of the drug. The first parameter indicates the change in initial rate and the second parameter, change in the extent of lysis. These parameters are presented in Tables 36 and 37.

TABLE 36

Initial lysis and extent of lysis for E.D.T.A., E.G.T.A. and P.X. treated cultures of P.aeruginosa grown in Mg^{2+}-adequate C.D.M. supplemented with Ca^{2+} and/or Mn^{2+}.

Culture / Lytic agent	1*		3*		5*		7*	
	a	b	a	b	a	b	a	b
E.D.T.A.	78.0	27.5	60.0	35.5	60.0	31.0	53.0	39.5
E.G.T.A.	>220	2.1	>220	2.1	>220	3.2	190	4.5
P.X.	1.0	66.5	10.0	65.0	3.0	66.5	10.0	62.5

The data in this table was determined from Fig. 34, 36 and 38.

* These numbers signify the type of cation supplementation and are as described for Tables 23 - 30 inclusive.

a Time (min) for first 5% lysis to occur.

b Percent lysis after 180-min.

TABLE 37

Initial lysis and extent of lysis for E.D.T.A., E.G.T.A. and P.X. treated cultures of P.aeruginosa grown in Mg^{2+}-depleted C.D.M. supplemented with Ca^{2+} and/or Mn^{2+}.

Culture / Lytic agent	2*		4*		6*		8*	
	a	b	a	b	a	b	a	b
E.D.T.A.	>220	4.5	10.0	16.7	134	6.5	10.0	26.0
E.G.T.A.	>220	4.2	15.0	13.0	85.0	10.6	26.0	17.0
P.X.	54.0	6.0	11.0	59.5	54.0	17.5	11.0	59.0

The data in this table was determined from Fig. 35, 37 and 39.

* These numbers signify the type of cation supplementation and are as described for Tables 23 - 30 inclusive.

a Time (min) for first 5% lysis to occur.

b Percent lysis after 180-min.

E.D.T.A. mediated lysis of Mg^{2+}-adequate and Mg^{2+}-depleted cultures of P.aeruginosa supplemented with Ca^{2+} or Mn^{2+}.

A prominent effect of cation supplementation of Mg^{2+}-adequate cultures was upon the sensitivity to E.D.T.A. (Fig. 34). The cultures supplemented with Ca^{2+} were significantly more sensitive to E.D.T.A. than the control culture. Reference to Fig. 33 and Table 25 shows that the supplement does not affect the growth rate, but does

alter the cell wall content of Ca^{2+}. These results suggest that although Mg^{2+} may be the major structural cation in P.aeruginosa cell walls, other cations (e.g. Ca^{2+} and Mn^{2+}) may also be required. From the data in Table 25 it can be determined that the increased uptake of Ca^{2+} is at the expense of a small amount of Mg^{2+}, and that Mn^{2+} is also taken up at the expense of Mg^{2+}. The total cation content of Ca^{2+} supplemented cell walls is 30% greater than the controls. This suggests that there were sites within, or on the wall that could only be filled by Ca^{2+} and others that were occupied by Ca^{2+} in preference to Mg^{2+}. The sensitivity of the cultures reflect to some extent the affinity of E.D.T.A. to the cations (Mn > Ca > Mg). The cultures with Ca^{2+} were more sensitive than the control. The culture with Mn^{2+} was also more sensitive than the control, but less sensitive than Ca^{2+}. This may indicate that Mn^{2+} was either not as important in the structure of the cell wall as Ca^{2+}, or that the amount used was insufficient for it to completely fulfil a structural role in the wall. When Ca^{2+} and Mn^{2+} were used to replace Mg^{2+} in Mg^{2+}-depleted cultures (Fig. 35) the same pattern (Ca-Mn > Ca > Mn > Mg) of sensitivity was found as for Mg^{2+}-adequate cultures, although the extent and rate of lysis was lower in all cultures. These results also suggest that Mg^{2+} is not the sole cation required by cell walls, and that the most sensitive cell walls require the presence of all these cations. Apart from the unsupplemented control cultures the amount of Ca^{2+} in the cell walls of Mg^{2+}-adequate and Mg^{2+}-depleted cultures was similar, suggesting that the deficiency

caused by Mg^{2+} depletion was not completely met by Ca^{2+} and/or Mn^{2+}, again supporting the view that there are Mg^{2+} and Ca^{2+} (and possibly Mn^{2+}) specific sites in the cell wall. The lack of an initial lag period in the lysis curves seen in Fig. 35 suggests that either a small percentage of the cells were at a stage of cell division that rendered them more sensitive to E.D.T.A. (this may or may not be enhanced by the sudden increase in pH imposed by the experimental conditions) or, since the effect was more noticeable with Ca^{2+}-supplemented cultures, that in the absence of Mg^{2+} some Ca^{2+} superficially occupied sites with a preference for Mg^{2+}, and thus were more easily removed. It is noticeable that the Mg^{2+} content of Mg^{2+}-depleted cell walls was dependant upon the Ca^{2+} concentration, the Mg^{2+} content being raised in the presence of Ca^{2+} (the Mg^{2+} may have been a contaminant in the Ca^{2+} salt used). This suggests a further explanation for the increased initial rate of lysis. The sudden increase in pH prior to the lysis experiment may have weakened the binding of the Mg^{2+} ions present in the wall, thus making them more susceptible to the action of E.D.T.A. which would then act on the Ca^{2+} and Mn^{2+} bound more tightly, or at different sites. The lower rate of lysis observed in Mg^{2+}-depleted cultures may reflect interference of the E.D.T.A. action by organic cations replacing the Mg^{2+} in the wall (Wilkinson & Galbraith, 1975). The view that Ca^{2+} is required for structural roles in P.aeruginosa cell walls is supported by the findings of Asbell & Eagon (1966) and Eagon (1969) who found that Ca^{2+} was the major cation present in the cell wall. The present results

also support the structural role of Ca^{2+} in P.aeruginosa cell walls since it can be seen that the addition of this cation did not significantly affect the growth rate or cut off points of the cultures (Fig. 32 & 33) but did render both Mg^{2+}-adequate and Mg^{2+}-depleted cultures more sensitive to E.D.T.A. (Fig. 34 & 35). This lack of effect upon the growth rate or cut off is not surprising in view of the fact that Ca^{2+} was assimilated in the cell wall at the expense of only a small amount of Mg^{2+}. The significant contamination of non-supplemented Mg^{2+}-adequate cell walls by Ca^{2+} (Table 25) makes it difficult to determine the role of Mg^{2+} alone in E.D.T.A. sensitivity. This contamination may be present in the Mg^{2+} solution since a decrease in the Mg^{2+} supplied to the cultures not only resulted in a decrease in the wall content of Mg^{2+}, but also of Ca^{2+} (Table 26). Alternatively, the assimilation of Ca^{2+} and Mg^{2+} may be linked (Williams & Wacker, 1967).

E.G.T.A. mediated lysis of Mg^{2+}-adequate and Mg^{2+}-depleted cultures of P.aeruginosa supplemented with Ca^{2+} or Mn^{2+}.

The most striking aspect of the resistance of the Mg^{2+}-adequate cultures is the difference for the percent lysis of the Ca^{2+}-supplemented culture studied by the present author and that studied by Boggis (1971). The latter found that Ca^{2+}-supplemented, Mg^{2+}-adequate cultures were significantly sensitive to E.G.T.A. (approximately 30% lysis in 180-min) whereas in the present work all the Mg^{2+}-adequate cultures were resistant to E.G.T.A. (Fig. 36). There may be several explanations for this discrepancy. First, the experimental conditions are markedly different. These differences have already been

described (see general observations, this section). In describing the results of E.D.T.A. lysis it has been noted that the difference in initial shape of the curves may have been due to the sudden change in pH for Mg^{2+}-depleted cultures. This change in pH occurred for both Mg^{2+}-adequate and Mg^{2+}-depleted cultures studied by Boggis (1971), and it may be significant that the lysis curves for both cultures were similar, and resembled those in Fig. 37. All the 8 L batch cultures studied by Boggis (1971) were subjected to a four-fold drop in molarity, so that the osmotic pressure was lower than that observed in the present study, and would be expected to support lysis to a greater extent. This difference in molarity may also explain the different results obtained by Boggis (1971) in 8 L and 100 ml batch culture resistance studies. Thus, in conclusion in may be the sudden change in pH and molarity experienced by the system used by Boggis (1971) which rendered the Mg^{2+}-adequate cells more sensitive to E.G.T.A. by weaking the bonds of either Mg^{2+} and/or Ca^{2+} in the walls. The lag period observed in Fig. 36 may be explained on the basis that Mg^{2+} and Ca^{2+} are bound at sites relative to one another such that the Mg^{2+} must be removed before access is gained to the Ca^{2+}. This prior removal of Mg^{2+} by E.G.T.A. would be a slow process due to the low affinity for Mg^{2+} compared to the affinity for Ca^{2+}. It is conceivable that the increase in the rate and extent of lysis observed towards the end of the lysis curves (Fig. 36) reflects the penetration of E.G.T.A. to the Ca^{2+}. This theory would also explain the similar, but shorter lag period observed for the action of E.D.T.A.

in Fig. 34. In both Fig. 34 & 36 the 'lag' is shorter for the Ca^{2+}, Mn^{2+}-supplemented cultures, possibly indicating that Ca^{2+} has been occupying sites normally occupied by Mg^{2+} when no Ca^{2+} is available.

The affinity of E.G.T.A. for Mn^{2+} is the same as for Ca^{2+} (log k = 11.0), which is significantly higher than that for Mg^{2+} (log k = 5.2). It would be expected therefore, that the lysis of Mn^{2+}-supplemented cultures would be similar to Ca^{2+}-supplemented cultures. This pattern was not observed (see Fig. 37), although the rates of lysis were similar. These results may indicate that Mn^{2+} plays a minor role in the structure of cell walls, or that the concentration used was too low for the Mn^{2+} to completely fulfil its structural role. As with the results for E.D.T.A. the lysis of cells by E.G.T.A. reflects the amount of Ca^{2+} in the walls. For both E.D.T.A. and E.G.T.A. the differences in the shapes of the lysis curves may be due to cation antagonism of the chelating agents, since the menstruum of the Mg^{2+}-adequate cultures contained a greater concentration of cations. This is unlikely however since in Fig. 34 & 36 it can be seen that the cultures with the most cations present in the menstruum were the most sensitive. It can be seen from Fig. 36 & 37 that Mg^{2+}-depleted cultures of P.aeruginosa, whether supplemented with Ca^{2+} or Mn^{2+}, were significantly more sensitive to E.G.T.A. than the corresponding Mg^{2+}-adequate cultures. This is compatible with the findings that the cell walls of Mg^{2+}-depleted cultures contained less Mg^{2+}, but similar molar concentrations of Ca^{2+}. Presumably the lack of Mg^{2+} ensured adequate access of the E.G.T.A.

to the Ca^{2+} ions. Even the non-supplemented Mg^{2+}-depleted control lysed more than the Mg^{2+}-adequate culture, suggesting that the lack of Mg^{2+} allowed the E.G.T.A. access to the small amount of Ca^{2+} in the wall of Mg^{2+}-depleted cells (presumably there due to contamination from other medium components). These data are compatible with the theory that Mg^{2+} must be removed from cell walls before Ca^{2+} can be removed, and that given free access to a source of Ca^{2+}, this cation contributes significantly to the structure of the cell wall.

Polymyxin mediated lysis of Mg^{2+}-adequate and Mg^{2+}-depleted cultures of P.aeruginosa supplemented with Ca^{2+} or Mn^{2+}.

The only observed effect of supplementation upon the sensitivity of Mg^{2+}-adequate cultures to P.X. was the decrease in the rate of lysis of Ca^{2+} and Ca^{2+}, Mn^{2+}-supplemented cultures (Fig. 38). This effect was probably not due to a change in the sensitivity of the cells, but due to antagonism of the P.X. by the cations present in the menstruum. Cations have been found to antagonise the action of a variety of antibacterial agents (E.D.T.A.: Brown & Melling, 1969a,b; Davis, Iannetta & Wedgwood, 1971; P.X.: Newton, 1954). Newton (1954) concluded that it was unlikely that the antagonism of P.X. by cations (Mg^{2+} and Ca^{2+}) was due to complex formation between P.X. and the cations, but was more probably due to competition for anionic sites on, or within the cell wall. Mg^{2+}-depleted cultures without supplementary cations were found to be very resistant to P.X. (Fig. 39). This is compatible with the findings of previous workers that the sensitivity in P.aeruginosa is dependent in part upon the level of Mg^{2+}

in the growth medium (Newton, 1954; Melling, 1968; Brown & Melling, 1969b; Boggis, 1971). When the Mg^{2+}-depleted C.D.M. was supplemented with Mn^{2+} the sensitivity was restored to some extent. The lysis rate was significantly lower than that observed in the corresponding Mg^{2+}-adequate cultures, and was maintained at a constant rate throughout the duration of the experiment. The Mn^{2+} apparently allowed some of the wall to be stabilized. An equimolar concentration of Mn^{2+} (1.645×10^{-4} M) may have resulted in a greater degree of lysis and/or an increased rate. Both the cultures supplemented with Ca^{2+} showed almost complete restoration of sensitivity as measured by the rate and extent of lysis. One other notable effect of Ca^{2+} replacement was that upon addition of P.X. there was an immediate increase in the $O.D._{470}$ of the cell suspension, which was probably due to an optical effect caused by the P.X. binding with the cell surface. This is compatible with the theory that increased uptake of P.X. alters the optical properties of the cell surface, an effect that has been observed by Hugo & Longworth (chlorhexidine: 1964) and Brown & Melling (P.X.: 1969b). Boggis (1971) observed that increasing the concentration of P.X. caused this effect, but with Mg^{2+}-depleted cultures the phenomenon occurred at lower concentrations of P.X. (i.e. 30 U/ml). In the present study however, the concentration of P.X. used was invariably 50 U/ml which suggests that the Ca^{2+} replaced cells possessed a greater capicity for binding the P.X. This theory is compatible with the increased amounts of lysyl-phosphatidyl-glycerol (ly.P.G.) found in the cell walls of Mg^{2+}-depleted

cultures. This phospholipid (P.L.) was found in the highest amounts in Ca^{2+}-supplemented cultures (Table 24), but not in significant amounts in Mg^{2+}-adequate cultures (Table 25). It is conceivable that lys.P.G. plays an important role in the assimilation of cations. The net charge exerted by the molecule would be negative, hence attracting cations. Minnikin & Abdolrahimzadeh (1974) and Minnikin, Abdolrahimzadeh & Baddily (1972) have observed that Bacillus subtilis var. and Aerobacter aerogenes will increase the content of negatively charged P.L. under conditions of Mg^{2+}- depletion in batch culture and Mg^{2+}-limitation in continuous culture. These authors suggested that this was to increase the efficiency of the cells to accumulate cations in limiting circumstances. Presumably, if a P.L. can attract Mg^{2+} and other cations there is no reason to suppose that it will not also attract P.X. which is basically charged. It is evident from Tables 25 & 26 that P.aeruginosa increased its content of wall lys.P.G. upon Mg^{2+}-depletion, and that when Ca^{2+} is added to Mg^{2+}-depleted C.D.M. the level of lys.P.G. is increased even more despite the fact that there are now excess cations present. This may be explained by the theory that most cells maintain a Mg^{2+}:Ca^{2+} balance (Williams & Wacker, 1967). Addition of Ca^{2+} to a Mg^{2+} depleted environment will upset this balance even further and the cells will attempt to attract Mg^{2+} to restore the balance. It is not known if lys.P.G. binds Mg^{2+} with greater affinity than Ca^{2+}, but Gordon & MacLeod (1966) have found that Mg^{2+} was associated with a P.L. which they suggested may have been derived from diphosphatidyl-

glycerol (D.P.G.). The level of D.P.G. in Mg^{2+}-depleted cells was found to be less than in Mg^{2+}-adequate cells, and it is possible that the cells possess the necessary enzymes to breakdown D.P.G. when the environment makes this advantageous, or that the environment with high Ca^{2+} and low Mg^{2+} inhibits the dimerase. At first glance D.P.G. may be thought to be more effective at accumulating Mg^{2+} than P.G. or lys.P.G. since it carries two negative charges. However, any Mg^{2+} bound by D.P.G. may not be available for cross linking or transfer into the cell, since the configuration of D.P.G. may be such that the Mg^{2+} is firmly bound by both negative charges.

Although Mg^{2+}-depleted cultures with no added cations possessed increased amounts of lys.P.G. in the wall, thus presumably attracting and binding P.X. more effectively than the corresponding Mg^{2+}-adequate culture, little lysis took place. This indicates that it may not be the binding of P.X. to P.L. in the wall *per se* that initiates the disruption of the outer membrane. Feingold, Hsu-Chen & Sud (1974) suggested that it was the phosphatidyl-ethanolamine (P.E.) in *Escherichia coli* membranes that was attacked by P.X.. It was found that all the Mg^{2+}-adequate (P.X. sensitive) cultures in this study possessed significant amounts of P.E.. It thus appears that the action of P.X. upon *P.aeruginosa* cannot be correlated to any one parameter of P.L. composition of the walls since Ca^{2+}-supplemented, Mg^{2+}-depleted cells were as sensitive to P.X. as Mg^{2+}-adequate cells but possessed very little P.E. It appears that Mg^{2+}-depleted cells, in the absence of an alternative source of divalent

metal cations, adapt the structure of the cell wall (but not necessarily the gross chemistry) in two ways. One is to increase the lipid components capable of attracting specific metal cations, the other is to stabilize the outer membrane by means other than divalent metal ion cross linking. Wilkinson & Galbraith (1975) have suggested polyamines and other similar organic cations may take the role of Mg^{2+} to some extent. These could render Mg^{2+}-depleted cultures resistant to P.X. by either hindering access of P.X. to the initial binding site, or binding more tightly than P.X. to the same site. If alternative cations are supplied (e.g. Mn^{2+}) then the sensitivity is increased. Boggis (1971) found that the sensitivity of Mg^{2+}-depleted cultures to P.X. increased as the amount of Ca^{2+} added to the C.D.M. was increased. This may indicate that the low level of Mn^{2+} added was insufficient to restore full sensitivity. Boggis (1971) found that comparatively low levels of Mn^{2+} (1.3×10^{-5} M) restored the lysis of Mg^{2+}-depleted P.aeruginosa by P.X. to approximately 55%. The reasons for the discrepancy between these results and those presented in this thesis are unclear. The reason may be found in the experimental methods (see discussion for E.D.T.A. and E.G.T.A. results). Boggis (1971) obtained his data from small scale experiments which involved limiting the growth of P.aeruginosa cultures at $O.D._{470}$ 0.1, and limiting total growth at $O.D._{470}$ 0.2 by glucose-depletion. Thus, the cultures studied by Boggis (1971) were grown in conditions of Mg^{2+}-depletion for much less time than those in this study ($O.D._{470}$ 0.4 to 1.0 took approximately 10-hr).

Brown & Melling (1969b) have found that the degree of resistance depends upon the time the cultures have been growing in Mg^{2+}-depleted C.D.M.. The short duration of Mg^{2+}-depleted growth may be another explanation of the comparative sensitivity of the Mg^{2+}-depleted, Mn^{2+}-supplemented cultures studied by Boggis (1971).

3. RESISTANCE STUDIES ON COLD SHOCKED CULTURES OF *P.aeruginosa* GROWN IN MAGNESIUM-ADEQUATE AND MAGNESIUM-DEPLETED CHEMICALLY DEFINED MEDIA SUPPLEMENTED WITH CALCIUM OR MANGANESE.

General observations.

All the Mg^{2+}-adequate cultures were markedly sensitive to the lethal effects of cold shock. Upon rewarming, the cold shocked cultures underwent extensive autolysis (Fig. 41). This lysis may have been either enzymatic or osmotic, or a combination of both effects. The extensive lysis observed upon rewarming indicates an enzymatic mode of lysis. At the end of the cold shock treatment the cultures appeared less dense than control cultures, although no optical density readings were taken. This visual effect may have been due to lysis during the cold shock procedure, or alternatively, a change in the surface properties of the cold shocked cells may have altered the light scattering properties of the cells. This lysis, and the drop in viable counts, indicates that *P.aeruginosa* in this study was extremely sensitive to cold shock. This is compatible with the results of Gorrill & McNeil (1960) and Farrell & Rose (1968) who found marked decreases in the viability of cold shocked *P.aeruginosa*. These authors however, found little or no evidence of lysis of the cells from exponential phase cultures, and noted that stationary phase cells were resistant to cold shock. The cells used in this study were glucose-depleted or glucose and Mg^{2+}-depleted, and were in the stationary phase. MacKelvie, Gronlund & Campbell (1967) found that carbon-limited *P.aeruginosa*

underwent significant lysis when cold shocked. Strange & Dark (1962) noted that stationary phase cultures of Aerobacter aerogenes became sensitive to cold shock when maintained at 35°in aerated, buffered saline for 18-hr prior to cold shock. It is possible that the marked sensitivity of Mg^{2+}-adequate, glucose-depleted cultures in this study was due, in part, to the effects of glucose-depletion as well as the effects of cold shock upon the structural arrangement of the cell wall (see section on cold shock). In view of the probable depend nce of P.aeruginosa upon the outer membrane for structural integrity of the cell (see section on cell wall), the observed lysis may be expected from the disorganisation of this membrane.

The reduced rate and extent of post cold shock lysis of all the Mg^{2+}-depleted cultures (Fig. 42) suggests that these cells possessed envelopes with a composition or structure better able to withstand the effects of cold shock. It may be significant that all the Mg^{2+}-depleted cell walls contained a greater proportion of lysyl-phosphatidyl-glycerol (lys.P.G.) than cell walls of Mg^{2+}-adequate cells. This phospholipid (P.L.) may well be able to adapt to cold shock conditions without too much disorganisation or loss of 'fluidity' of the fatty acid side chains. The reduction of post cold shock lysis was accompanied by an increase in the percentage survivors from cold shock. Farrell & Rose (1968) found that growth of P.aeruginosa at low temperature (15°) resulted in a decreased sensitivity to the lethal effects of cold shock. They attributed this to the increased

degree of unsaturated fatty acids in the P.L. of the envelopes.

The lysis curves in Fig. 41 show that the post cold shock autolysis of Mg^{2+}-adequate cultures was not the same pattern for all the cultures. Those cultures supplemented with calcium (Ca^{2+}) or manganese (Mn^{2+}) exhibited a significantly greater extent and rate of lysis compared with the control (no supplement) and Ca^{2+} with Mn^{2+} supplemented cultures. Tempest & Strange (1966) found that Mg^{2+} adsorbed on the surface of A.aerogenes cells protected them against cold shock, and there may be a similar effect with the cold shocked cells. The cells were washed with lysis medium prior to cold shock and the high ammonium ion content of this medium may have removed surface adsorbed cations. Even if it is supposed that the surface adsorbed cations were not removed by washing, it is difficult to see why Ca^{2+} in combination with Mn^{2+} was less effective in protecting against cold shock than when these two cations are present individually. A more likely explanation is that the P.L. content of the cell walls may determine the extent to which the cells are damaged by cold shock. Leder (1972) suggested that dehydration of Escherichia coli cells caused the cell membranes to contract, thus effectively increasing the membrane lipid concentration. These dehydrated cells were less sensitive to cold shock than control cells, but the effect may have been due to some other effect of membrane contraction. From Table 25 it can be seen that the mean value for P.L. of Mg^{2+}-adequate cell walls (as percentage dry weight) were 6.1, 3.8, 2.3 and 6.6

for control, Ca^{2+}-supplemented, Mn^{2+}-supplemented and Ca^{2+} with Mn^{2+}-supplemented cultures respectively. Thus the cultures with the two lowest cell wall P.L. content were the most sensitive to post cold shock autolysis. Similarly, the two cultures with the greatest proportion of cell wall P.L. lysed the least. This is compatible with the observations of Leder (1972), but the variation in the individual P.L. values (Table 25) may make the correlation less significant. When the average values for the wall P.L. of Mg^{2+}-depleted cultures were determined (from Table 26) the percentages were 5.1, 6.3, 6.2 and 3.8% for control, Ca^{2+}-supplemented, Mn^{2+}-supplemented and Ca^{2+} with Mn^{2+}-supplemented cultures respectively. Reference to Fig. 42 shows that these values correlated with the observed lysis pattern of the Mg^{2+}-depleted cultures. It seems reasonable to hypothesise therefore that the wall P.L. of these cultures play a part in the effect of cold shock upon the cells. Why the P.L. pattern should vary in the way it did is not clear, and it may not be related to the cation supplement of the media. The pattern of lysis observed in Fig. 41 was repeated when cold shocked, Mg^{2+}-adequate cultures were treated with ethylenediaminetetra-acetic acid (E.D.T.A.), ethyleneglycol-bis-(β-amino-ethylether)-N:N'-tetra-acetic acid (E.G.T.A.) or polymyxin (P.X.). This is to be expected since it can be seen from Fig. 40 that the contribution of the E.D.T.A. to the lysis of cold shocked Mg^{2+}-adequate cultures of P.aeruginosa was not very extensive, and thus the autolysis pattern would tend to dominate the lysis of the cultures.

The sensitivity of the cultures to cold shock was also assessed by the percent survivors, using the data from colony counts of cold shocked cultures. Cultures grown in Mg^{2+}-adequate media were more susceptible to cold shock than Mg^{2+}-depleted cultures (see Tables 21 & 22) with the exception of the Ca^{2+} with Mn^{2+}-supplemented culture. The patterns of lysis in Fig. 41 & 42 did not correlate with the percent survivors. In view of the low survivor ratio it is not surprising that there was no correlation. A study of the cold shock resistance against time may well give more information as to the effect of the Mg^{2+}-depletion and supplementation by Ca^{2+} and Mn^{2+}. The results for the percent survivors were compatible with results obtained for P.aeruginosa by previous workers (Gorrill & McNeil, 1960; Meynell, 1958). These workers however, studied cultures in the exponential phase of growth and reported that when stationary phase cells were studied the effects of cold shock were very much decreased. Strange & Dark (1962) have noted that stationary phase A.aerogenes maintained at 35.0° for several hours in aerated, buffered saline, became as sensitive to cold shock as exponential cells. The cultures used in this study have been maintained in depleted chemically defined media (C.D.M.) for one hour prior to cold shock, and this in combination with the fragile nature of the cell wall may explain the sensitivity of stationary phase cultures of this organism to cold as observed in this study and previously by MacKelvie et al., (1967).

The lysis curves presented in Fig. 34 - 39 were

all corrected for the lysis (rarely more than 10%) of the control (no drug) cultures. The degree of lysis of the cold shocked, Mg^{2+}-adequate control cultures however, was very extensive (60 - 70%, Fig. 41). The lysis curves in Fig. 40 represent the lysis of Mg^{2+}-adequate cultures in the absence of E.D.T.A. and its presence, and the result of correcting the E.D.T.A. curve for control lysis. In the interpretation of the corrected curve it must be borne in mind that this curve merely represents the contribution of E.D.T.A. to the lysis of the culture at any given time. It may be reasonable to assume that the cold shocked culture contained a high proportion of cells that had a weakened or damaged outer membrane, which would facilitate the removal of wall cations by E.D.T.A., hence the very rapid initial rate of lysis. At the same time the cells were lysed at a significant rate due to autolysis, and the contribution of E.D.T.A. declined, perhaps due to antagonism from intracellular cations released from the lysed cells. This apparent negative lysis was not an artifact due to the use of percent lysis data in making the correction, since the same effect was observed when actual optical density readings were used.

Action of E.D.T.A. upon cold shocked cultures of *P.aeruginosa* grown in Mg^{2+}-adequate and Mg^{2+}-depleted C.D.M. supplemented with Ca^{2+} or Mn^{2+}.

The lysis curves obtained for Mg^{2+}-adequate cultures (Fig. 43) closely resemble the lysis observed in the untreated cultures (Fig. 41). This may be expected since the E.D.T.A. had very little effect upon the initial rate. The major influence appeared to be autolysis of the cells.

The pattern of lysis for the Mg^{2+}-depleted cultures (Fig. 44) was markedly different from that for Mg^{2+}-adequate and untreated, Mg^{2+}-depleted cultures (Fig. 42). This suggests that Mg^{2+} depletion results in cells possessing a cell envelope better able to withstand the effects of cold shock. The corrected lysis curves (Fig. 45), although similar with respect to supplementation by Ca^{2+} and Mn^{2+} are dissimilar in shape to non-cold shocked cultures (Fig. 35). As may be expected, the cold shocked cultures were more sensitive, indicating that the walls had been weakened by cold shock. This action of cold shock in sensitising resistant cultures (Mg^{2+}-depleted) would indicate that the resistance induced by Mg^{2+}-depletion was due, in part, to a structural alteration of the cell wall. It has been shown that cold shock damages or disorganises the outer membrane, thus leading to a greater penetration of the E.D.T.A. to sites that were previously less accessible. The role of post cold shock autolysis must also be considered since it was still significant in the Mg^{2+}-depleted cultures (Fig. 42). The contribution by E.D.T.A. (Fig. 45) was still greater in cold shocked cultures than in non-cold shocked cultures. In considering the differences between the lysis curves in Fig. 45 & 35 the differences in the lysis media must be considered. Cold shocked cultures were washed in C.D.M. lacking divalent cations (Mg^{2+}, Ca^{2+} and Mn^{2+}) and glucose, and resuspended in this medium. The same medium was used in the lysis experiments of cold shocked cells. The non-cold shocked cultures however were lysed in growth medium lacking Mg^{2+} and glucose only. The

increased lysis of the cold shocked cultures may therefore be due to the lack of antagonism by free cations in the medium. Such antagonism seem unlikely however, since reference to the non-supplemented, Mg^{2+}-depleted curves (Fig. 35), measured in essentially the same medium, shows that these cultures did not lyse as extensively as cold shocked cultures, indicating that the increased lysis is probably due to the effects of cold shock upon the cell walls and cell viability.

Action of E.G.T.A. upon cold shocked cultures of P.aeruginosa grown in Mg^{2+}-adequate and Mg^{2+}-depleted C.D.M. supplemented with Ca^{2+} or Mn^{2+}.

The Mg^{2+}-adequate cultures lysed in the presence of E.G.T.A. (Fig. 46) in a manner that still appeared to be dictated largely by the autolysis observed when no drugs were present (Fig. 41). There is an important difference however that appears to be mediated by E.G.T.A.. The lysis of the control (no supplement) culture is the same with, or without E.G.T.A., suggesting that E.G.T.A. is unable to exert a lytic effect before the cells lyse of their own accord. When the cultures were supplemented with Ca^{2+} and Mn^{2+} the extent of lysis was increased in the presence of E.G.T.A.. This increase appears to be the result of an increase in the initial rate of lysis. It is conceivable that the E.G.T.A. could readily gain access to the Ca^{2+} and Mn^{2+} in the weakened wall of these cells, and thus was able initially to lyse the cells at a rate greater than the autolysis that was occurring. Cultures supplemented with Mn^{2+} showed a slight increase (3.5%) in the extent of lysis in the presence of E.G.T.A..

This may be experimental error, or may reflect the slight role of Mn^{2+} in the structure of the wall. When the cultures were supplemented with Ca^{2+} however, there was an increase in the extent and initial rate of lysis in the presence of E.G.T.A., presumably reflecting the ready accessibility of Ca^{2+} in the damaged cell walls.

When the lysis of cold shocked Mg^{2+}-depleted cultures was observed (Fig. 47) it was noted that the control culture (no supplement) and the Mn^{2+}-supplemented culture were markedly more resistant to E.G.T.A. than the Ca^{2+} and Ca^{2+} with Mn^{2+}-supplemented cultures, which were in turn only slightly less sensitive than the corresponding Mg^{2+}-adequate cultures. As with the action of E.D.T.A., the E.G.T.A. appeared to exert an influence far in excess of that exerted by autolysis. Calcium supplementation appeared to have produced cells with walls that, though they were not greatly sensitive to post cold shock autolysis, nevertheless were damaged sufficiently to allow the extraction of Ca^{2+} with subsequent lysis. When the corrected curves in Fig. 48 are compared with those for similar, non-cold shocked cultures (Fig. 37), it can be seen that cold shock resulted in a greater sensitivity to E.G.T.A.. The control (no supplement) culture and Mn^{2+}-supplemented cultures showed similar patterns of lysis whether they were cold shocked or not, but the extent was significantly greater for cold shocked cells. The lysis of Ca^{2+} and Ca^{2+} with Mn^{2+}-supplemented cold shocked cells as well as being far greater in extent, was also a different pattern, showing a very rapid initial rate that decreased to zero progressively. This very

rapid initial rate suggests that the damage done by cold shock gave the E.G.T.A. ready access to the Ca^{2+}. The smaller extent of lysis of Mn^{2+}-supplemented cultures again suggests that either the role of Mn^{2+} was less significant, or that the amount of Mn^{2+} used was insufficient for it to exert its full influence. Although the initial rates of lysis for the Ca^{2+} and Ca^{2+} with Mn^{2+}-supplemented cultures were similar, the extent of lysis was less for the Ca^{2+} with Mn^{2+}-supplemented culture. This may reflect the lower concentration of Ca^{2+} in the walls of this culture (17.1 nmoles/100 µg as opposed to 19.2 nmoles/100 µg).

Action of P.X. on cold shocked cultures of P.aeruginosa grown in Mg^{2+}-adequate and Mg^{2+}-depleted C.D.M. supplemented with Ca^{2+} or Mn^{2+}.

The lysis curves in Fig. 49 show only slight differences from the control (no drug) curves (Fig. 41). The culture containing no supplement exhibited lysis with P.X. that was not distinguishable from the lysis of the same cultures without P.X.. This suggests that P.X. had no action on Mg^{2+}-adequate cold shocked cells, but this is unlikely since it would be expected that damage to the outer membrane would render the cells more sensitive to P.X.. One explanation may be that the drug lysed the cells at a similar rate to the lysis taking place in the absence of the drug. Comparison of Fig. 49 with Fig. 38 shows that the cold shocked cells appeared to lyse less with P.X. than non-cold shocked cells which is unexpected. The damage caused by cold shock to the cell envelope may have disorganised the

outer membrane, such that P.X. is unable to bind to the lipid A of the lipopolysaccharide or P.L. of the outer membrane. Such disorganisation however, would be expected to facilitate P.X. access to the cytoplasmic membrane. The rapid autolysis of the cold shocked cells would result in the release of intracellular components some of which (ribosomal and nucleic phosphates and membrane fragments) may have antagonised the action of P.X. Reference to Fig. 5 shows that the menstruum from the cold shocked cell suspension exerted little or no inhibitory effect upon the lysis of P.aeruginosa by P.X.. The cold shock cell suspension however was spun down and removed as soon as the cells had rewarmed to 37.5°, and will have contained less cell contents than a similar suspension harvested after 3-hr at 37.5°. The supplemented cultures in Fig. 49 showed significantly less lysis in the presence of P.X. than in its absence. This apparent protective effect of P.X. against post cold shock lysis may only be due to an optical effect caused by the binding of the P.X. to the cell wall fragments. It may also be an artifact due to the antagonism of P.X. just described, or a combination of both effects.

The lysis of the Mg^{2+}-depleted, cold shocked cultures by P.X. (Fig. 50) was very similar for all the supplemented cultures and was significantly less than for the Mg^{2+}-adequate cultures. The cell walls of the Mn^{2+}-supplemented culture were as sensitive to P.X. as the Ca^{2+} and Ca^{2+} with Mn^{2+}-supplemented cultures. This effect was not apparent in the action of E.D.T.A. or E.G.T.A. treated cultures, and would tend to indicate that the

cation content of the cell walls of cold shocked cultures did not influence the sensitivity of these walls to P.X.. This is compatible with the reports of membrane and envelope P.L. involvement in P.X. action (Newton, 1956; Hsu-Chen & Feingold, 1972; Bader & Teuber, 1973). Comparision of the corrected lysis curves of cold shocked, P.X. treated, Mg^{2+}-depleted cultures of P.aeruginosa (Fig. 51) with similar non-cold shocked cultures (Fig. 39) reveals a number of significant differences produced by cold shock. The most noticeable effect is that all the cold shocked cultures showed a similar pattern of lysis, and the maximum lysis reached was quite comparable (allowing for the effects of correction discussed earlier). From this result another point arises, i.e. that the Mn^{2+}-supplemented and the non-supplemented cultures were apparently more sensitive to P.X. after cold shock, whereas the Ca^{2+} and the Ca^{2+} with Mn^{2+}-supplemented cultures appeared less sensitive after cold shock. These rather contradictory effects may find an explanation if the optical effects of P.X. binding to Ca^{2+} and Ca^{2+} with Mn^{2+} supplemented, Mg^{2+}-depleted cultures is considered. The cold shocked cells may well have been more sensitive to P.X., as would be expected from the damaged cell walls, but the cell walls containing Ca^{2+} may have bound a higher proportion of P.X., thus altering the optical properties of the cells and the cell wall fragments. The optical effects would be expected to lead to an apparent resistance, since it gave rise to an increase in the optical density at 470 nm.

4. CHEMISTRY OF *P.aeruginosa* CULTURES GROWN IN MAGNESIUM-ADEQUATE AND MAGNESIUM-DEPLETED CHEMICALLY DEFINED MEDIA SUPPLEMENTED WITH CALCIUM OR MANGANESE.

Many of the assays used in this study were of a non-specific nature; in particular the assays for protein and carbohydrate. The carbohydrate assay was based on the charring effect of concentrated sulphuric acid upon carbohydrates. It may reasonably be expected that lipids, proteins and nucleic acids would also contribute to the charring. The assays for hexosamine, meso,2,6-diaminopimelic acid (D.A.P.) and 2-keto,3-deoxyoctonic acid (K.D.O.) were also non-specific, and employed the use of internal standards to try and overcome interference due to other chemical species that interact with the reagents. The standards and internal standards used were one of a range of the possible amino sugars, diamino-acids and deoxyoctonic sugars present in bacterial cells, and as a result may not reflect the overall picture. These deficiencies in the assays used will naturally tend to cause a loss of definition, and a slight, though significant change in one or more of these components may not have been detected.

The results for the lipid content of the cells and cell walls were gravimetric, and as such subject to the errors involved in the measurement of small weights (i.e. of the order of 1 - 10 mg). The estimation of the percentage composition of the individual phospho-lipids (P.L.) in the total P.L. fraction however was not gravimetric but densitometric. In this study the separation of the P.L. closely followed the method of

Minnikin & Abdolrahimzadeh (1971). These authors found that the error in a quantitative densitometric assay was ± 5%, and a similar densitometer was used in this study. The variation observed between the batch results for the readily extractable lipids (R.E.L.) and P.L. fractions may well be methodological, i.e. differences between extractions and weighings.

As described in section 2 the whole cells were chilled slowly, washed and then stored deep frozen until being used for cell wall preparation and chemical assays. This procedure was used to prevent physiological changes of the culture. This treatment however, may have caused a loss of cell wall and surface components in addition to any losses incurred during cell wall preparation. The slow drop in temperature from 37.5° to 15° would be expected to retard cell metabolism, but it may have had the additional effect of damaging the cell envelope in a manner similar to, but less drastic than, cold shock. The subsequent washing with water at 4° may have resulted in the loss of some envelope components concerned with permeability control of the cell. Leder (1972) observed that the temperature and composition of the washing medium significantly affected the transport and permeability function of Escherichia coli. Several workers have noted that Pseudomonad sp. lose periplasmic enzymes of the cell wall upon washing in various media (Cheng, Ingram & Costerton, 1970; Fitzgerald & Laslie, 1975). It is therefore possible that subunits of the outer membrane (e.g. lipopolysaccharide) were partially lost during the harvesting. Crude cell walls were washed with water at

4° and then incubated with trypsin and ribonuclease before final washes in saline and water. If the cell surface had been weakened by the harvesting procedure, it is possible that significant losses of components occurred during this stage of the cell wall preparation.

Reference to Tables 23 to 30 show that cultures of P.aeruginosa grown in Mg^{2+}-depleted chemically defined media (C.D.M.) without supplementary cations differed significantly from cultures grown in a similar Mg^{2+}-adequate C.D.M.. The most outstanding differences, in addition to the resistance to ethylenediaminetetra-acetic acid (E.D.T.A.) and polymyxin (P.X.) (Tables 36 & 37), were in the cation content of the cell walls and in the composition of the P.L. fraction of the cell wall lipids. It is tempting to suggest that the resistance of the Mg^{2+}-depleted cultures is reflected by the P.L. content of the cell walls. Several workers have shown a correlation between the lipid content and the resistance of various strains of bacteria (Brown & Watkins, 1970; Pechey, Yau & James, 1974). The P.L. content of whole cells of E.coli, Aerobacter aerogenes and Bacillus subtilis have been shown to be significantly altered in conditions of Mg^{2+}-depletion (Günther, Richter & Schmalbeck, 1975; Minnikin, Abdolrahimzadeh & Baddiley, 1972). In the present study however, the cell wall P.L. content was found to be related to the presence of Mg^{2+} in the growth medium. The changes in resistance observed upon Mg^{2+}-depletion were not related to the P.L. content of the cell walls. Reference to Table 26 shows that all the Mg^{2+}-depleted cell walls had a similar pattern of P.L.

although the amounts of individual lipids present were subject to variation, apparently related to the type of cation present as a supplement. Althouth all the Mg^{2+}-depleted cultures possessed cell walls with similar P.L. contents, only the control cultures were resistant to E.D.T.A. and P.X. (Table 37). The Mg^{2+}-depleted, Ca^{2+}-supplemented (and to a limited extent Mn^{2+}-supplemented) cultures, although possessing a 'resistant' (i.e. Mg^{2+}-depleted) cell wall P.L. pattern were sensitive to E.D.T.A., ethyleneglycol-Bis-(β-aminoethylether)-N:N'-Tetra-acetic acid (E.G.T.A.) and P.X.. It appears from these results that although cell wall P.L., in part, plays a role in the exclusion of certain drugs from P.aeruginosa, it cannot be correlated per se with resistance. A similar conclusion was reached by Winshell & Neu (1974) regarding the relation between cell wall lipids of Serratia marcescens strains resistant or sensitive to several antibiotics. The constancy of the P.L. pattern of cell walls from all the Mg^{2+}-depleted cultures indicates that the synthesis of the P.L. was largely controlled by the amount of Mg^{2+} available, even in the presence of excess Ca^{2+} and Mn^{2+}. This is compatible with the observations that E.coli P.L. are altered by Mg^{2+}-depletion (Günther et al., 1975) and that the cell free synthesis of E.coli P.L. was also affected by Mg^{2+}-depletion (Benns & Proulx, 1974). The observation that Mg^{2+}-depleted, Ca^{2+}-supplemented cultures of P.aeruginosa were sensitive to E.D.T.A. and P.X., whilst retaining a cell wall P.L. composition similar to Mg^{2+}-depleted control cells (resistant), suggests that the sensitivity of P.aeruginosa depended to a large extent

upon the availability of cations, presumably required for cross-linking and stabilization of the wall rather than for synthetic enzymes. The results also suggest that the type of P.L. that was predominant in the walls was not necessarily related to the sensitivity of the cells, although this may be the case in genotypically altered cells. Brown & Watkins (1970) noted that strains of P.aeruginosa resistant to P.X. contained less cell wall P.L. than sensitive strains. The resistant strains also possessed significantly less cell wall Mg^{2+}. The composition of the P.L. fractions from the two strains was however similar, and the resistance may have been due to differences in other cell wall components (e.g. lipo-polysaccharides) or to the low wall Mg^{2+} content.

Although the P.L. may be a factor in the permeability of the cell envelope, the O antigen specific polysaccharide chain has also been shown to affect the passage of drugs into Salmonella species and P.aeruginosa (McQuillen, 1956; Sanderson, MacAlister, Costerton & Cheng, 1974). Since the polysaccharide chains extend out from the cell wall for a considerable distance, it would be expected that they might interfere with the passage of a drug to a target within the cell wall, cell membrane or cytoplasm. Bader & Teuber (1973) has shown that the length of the O antigen chain of Salmonella typhimurium species was related to the sensitivity to, and binding of, P.X.. If the resistance of the Mg^{2+}-depleted cultures grown without cation supplement may be due in part to an increase in the O antigenic side chain length or some other change in the lipopolysaccharide (L.P.S.). The assay of K.D.O.

gives an indication of the amount of core polysaccharide present in the L.P.S., and a change in the side chain may possibly be reflected in a change in the carbohydrate. Reference to Tables 25 & 26 show that there were only slight increases in the content of wall carbohydrate in Mg^{2+}-depleted cultures. The Mg^{2+}-depleted control (no supplement) culture contained the highest amount of cell wall carbohydrate. This may be significant since the culture was the most resistant to lysis by E.D.T.A. and P.X.. The increased amounts of carbohydrate in the cell walls of Mg^{2+}-depleted cells may have reflected (due to the non specific nature of the assay the increase in cell wall R.E.L.. However, the solvent extracted cell walls of Mg^{2+}-depleted cultures contained significantly more carbohydrate than Mg^{2+}-adequate cell walls. Again it was found that the Mg^{2+}-depleted control culture possessed extracted cell walls with the most carbohydrate. The relation between the results and the resistance of Mg^{2+}-depleted cultures to E.D.T.A. and P.X. suggest that the presence of more firmly bound carbohydrate may have contributed in part to the resistance of Mg^{2+}-depleted cultures. It is not clear which component of the cell wall may be responsible for the increase in the carbohydrate content as measured in this study. It may be more firmly bound lipid, or an increase in the O antigen specific side chain of the L.P.S. (note that the K.D.O. values were similar for both Mg^{2+}-adequate and Mg^{2+}-depleted cultures, suggesting that the L.P.S. content <u>per se</u> did not increase). The increase in carbohydrate may also reflect the presence of an organic cation to replace Mg^{2+}

in the cell wall (as suggested by Wilkinson & Galbraith, 1975). It is unlikely that all the carbohydrate was present as such an organic cation, since Mg^{2+}-depleted cultures supplemented with Ca^{2+}, although retaining most of the sensitivity to E.D.T.A. and P.X., still had a significantly higher cell wall content of firmly bound carbohydrate. The content of carbohydrate for these cell walls however, was significantly lower than for Mg^{2+}-depleted control cell walls. This would suggest that only part of the carbohydrate was present as an organic molecule capable of replacing Mg^{2+} or Ca^{2+} in the cell wall.

The murein region of the bacterial cell wall has been found to be intimately involved in the maintenance of cell shape and cell wall rigidity (Rogers & Perkins, 1968; Costerton, Ingram & Cheng, 1974; Preusser & Katz, 1972). It has been suggested that the low amounts of murein and lipoprotein complex in P.aeruginosa cell walls may in part result in the murein of this species playing a smaller role in the structure of the cell wall than in other Gram-negative organisms (Meadow, 1974). In the present study it was found that the control culture had less D.A.P. (a marker for murein: Rogers & Perkins, 1968) than the Mg^{2+}-adequate cultures supplemented with Ca^{2+} or Mn^{2+} (Table 25). The Mg^{2+}-adequate control culture was also the least sensitive to E.D.T.A.. It appears that the presence of Ca^{2+} or Mn^{2+} in some way increased the amount of D.A.P. in the cell walls. The diamino acid, D.A.P., is located in the pentapeptide side chains of the murein macromolecule. The 'backbone' of this

macromolecule is a series of polysaccharide chains composed of alternating molecules of N-acetyl glucosamine and N-acetyl muramic acid. The assay for total amino sugars showed that there was a small degree of variation between the Mg^{2+}-adequate cultures, but this did not correlate with either the cation supplement or the resistance to E.D.T.A.. The D.A.P. content of the Mg^{2+}-depleted cell walls showed little variation (Table 26). The assay for hexosamines however, revealed that the Mg^{2+}-depleted control culture contained less hexosamine than the supplemented, Mg^{2+}-depleted cultures. The variation in the cell wall hexosamine content of Mg^{2+}-depleted cultures appeared to be related to the variation in sensitivity to E.D.T.A. and E.G.T.A.. The reason for this relation is not clear. An increase in hexosamine value of the cell wall might indicate that the murein content had been increased. This might be expected to lead to a decrease in sensitivity. However, an increase in the murein content need not necessarily lead to a more stable structure. The degree of intramolecular cross-linking and the degree of cross-linking via lipoprotein to the outer membrane would ultimately determine the stability of the murein. The apparent increase of the hexosamine content may possibly be due also to interference from other cell wall components, e.g. the polysaccharide chains of the L.P.S. may have been hydrolysed to component sugars which, in combination with amino acid residues lysed from cell wall proteins, would interfere with the assay.

The D.A.P. content of solvent extracted walls was found to be significantly greater for Mg^{2+}-adequate

cultures than for Mg^{2+}-depleted cultures. Extracted cell walls from Mg^{2+}-depleted and Mg^{2+}-adequate controls (no supplement) were found to have less D.A.P. than the supplemented cultures (Tables 27 & 28). The value for the D.A.P. content of the extracted cell walls correlates with the sensitivity of the cultures to E.D.T.A.; those cultures with the least amount of D.A.P. being the least sensitive to the action of the chelating agents. The results also indicate that, although Ca^{2+} rendered the cells more sensitive to E.D.T.A. it also made the D.A.P. and murein more resistant to solvent extraction. It might be expected that cells with cell walls that lose D.A.P. upon solvent extraction would have a weaker murein region, and would consequently be less resistant to lytic agents. Reference to Tables 27 & 28 show that the low D.A.P. levels in Mg^{2+}-depleted cultures corresponded with a high level of carbohydrate and vice versa for the Mg^{2+}-adequate cultures. It is reasonable to assume from this that part of the structural role played by the murein may have been replaced by another organic molecule, as suggested by Wilkinson (1975) for the stabilization of L.P.S.. This substituting organic molecule may be less sensitive to E.D.T.A. and, in the absence of Ca^{2+}, to P.X.. Alternatively, the synthesis of D.A.P. and murein may be reduced, or the incorporation into the wall inhibited (Garret, 1969; Rogers, 1970) by lack of Mg^{2+} which coincidently, produced cell walls resistant to P.X. and E.D.T.A..

The cell walls of Mg^{2+}-depleted and Mg^{2+}-adequate cultures of P.aeruginosa were found to show increased

amounts of Ca^{2+} and Mn^{2+} when the media was supplemented with these cations (Tables 25 & 26). It appears from Tables 29 & 30 that some of the extra cations were bound to lipids in the R.E.L. fraction. It can be seen from these Tables that the amounts of Mg^{2+}, Ca^{2+} and Mn^{2+} associated with wall R.E.L. was lower for Mg^{2+}-depleted cultures than for Mg^{2+}-adequate cultures. These results are compatible with those reported by Brown & Watkins (1970) that a P.X. resistant strain of P.aeruginosa was found to have less Mg^{2+} associated with wall R.E.L. than was a sensitive strain of P.aeruginosa. The wall phosphorus (P) was also found to be decreased in Mg^{2+}-depleted cultures (Tables 25 & 26). It can be seen that this decrease in wall P corresponded to the decrease in the P content of the wall R.E.L. (Table 30), and it would appear that this decrease was due to a decrease in the P content of the P.L. fraction. The low P content of R.E.L. and P.L. from the cell walls of Mg^{2+}-depleted cultures may be due to the low level of diphosphatidyl-glycerol (D.P.G.) in these walls. The two cultures with the lowest levels of P.L. and R.E.L. P also had walls with the two lowest levels of D.P.G. and the two highest levels of lysyl-phosphatidyl-glycerol (lys.P.G.). Supplementation of Mg^{2+}-depleted cultures with Ca^{2+} resulted in the uptake of the Ca^{2+} into the cell wall, a partial restoration of sensitivity to E.D.T.A. and a complete restoration of sensitivity to P.X.. It can be seen from Tables 25 & 26 that the amounts of Ca^{2+} in the walls of Mg^{2+}-depleted cultures supplemented with Ca^{2+} was not significantly lower than in similarly supplemented Mg^{2+}-adequate

cultures. However, less of the Ca^{2+} was associated with the cell wall R.E.L. and P.L. of Mg^{2+}-depleted cultures compared with Mg^{2+}-adequate cultures (Tables 29 & 30). This is compatible with the hypothesis that Ca^{2+} (and probably Mg^{2+}) is associated with other less readily extracted components of the cell wall such as L.P.S. (Brown & Watkins, 1970; Wilkinson & Galbraith, 1975).

5. RESISTANCE STUDIES ON CULTURES OF P.aeruginosa POSSESSING THE R-FACTOR RP1.

General observations.

The colony counts on carbenicillin agar (200 U/ml) and nutrient agar revealed that the R-factor was present in about 97% of the organisms in the culture designated P.aeruginosa $RP1^+$. The parent strain, designated P.aeruginosa $RP1^-$, did not grow at all on the carbenicillin agar. These results make it highly probable that the differences observed between the two strains were, in part, R-factor mediated. The resistance to carbenicillin was shown to be associated with the action of a cell wall located (presumably periplasmic) penicillinase.

Ethylenediaminetetra-acetic acid mediated lysis of P.aeruginosa $RP1^+$.

P.aeruginosa $RP1^+$ was significantly less sensitive to ethylenediaminetetra-acetic acid (E.D.T.A.) than P.aeruginosa $RP1^-$. The lysis curves of P.aeruginosa $RP1^+$ (Fig. 52) resemble the E.D.T.A. mediated lysis of Mg^{2+}-depleted $RP1^-$ (Fig. 35), but the chemistry of the walls (Table 33) did not indicate that a lack of cell wall Mg^{2+} (or other cations) was responsible for the loss of sensitivity. The cation content of P.aeruginosa $RP1^+$ cell walls may be implicated in the resistance to E.D.T.A. however, since it can be seen from Table 34 that little or no cation was lost during the solvent extraction. This was in contrast to P.aeruginosa $RP1^-$, in which 80% and 60% of Mg^{2+} and Ca^{2+} respectively was lost from the cell wall during lipid removal. These results indicate that Mg^{2+} and Ca^{2+} probably contributed little to the

cross-linking of phospholipid (P.L.) molecules in the cell wall, or were firmly bound to a less readily extractable lipid or component. This is compatible with the results of Brown & Watkins (1970) who reported that P.aeruginosa, trained to a polymyxin (P.X.) resistance of 6000 U/ml, lost significantly less of the wall Mg^{2+} upon solvent extraction than did the sensitive strain. These authors suggested that the Mg^{2+} in both strains, and particularly the resistant strain, was not associated with the R.E.L. of the cell wall, but may have been associated with the lipopolysaccharide (L.P.S.). Alternatively, the cations in P.aeruginosa $RP1^+$ may have been bound at sites similar to those in P.aeruginosa $RP1^-$, but the sites may have been obscured by increased amounts of outer membrane components. However, the decreased quantities of R.E.L. and 2-keto,3-deoxyoctonic acid (markers for L.P.S.) make this unlikely, although the P.L. did increase slightly and may have had some effect.

Ethyleneglycol-Bis-(β-aminoethylether)-N:N'-Tetra-acetic acid mediated lysis of P.aeruginosa $RP1^+$.

There was no significant difference between the lysis of P.aeruginosa $RP1^+$ and P.aeruginosa $RP1^-$ cultures when treated with ethyleneglycol-Bis-(β-aminoethylether)-N:N'-Tetra-acetic acid (E.G.T.A.) (Fig. 52). This is compatible with the suggestions above that P.aeruginosa $RP1^+$ bound Mg^{2+} and Ca^{2+} more firmly, or that Mg^{2+} and Ca^{2+} were inaccessible to E.G.T.A. or played little part in the structural integrity of the cell wall.

P.X. mediated lysis of P.aeruginosa $RP1^+$.

P.aeruginosa $RP1^+$ culture lysed in a similar

manner to P.aeruginosa $RP1^-$, but the initial rate and extent of lysis was lower (Fig. 52). The lysis of P.aeruginosa $RP1^+$ resembled that of P.aeruginosa $RP1^-$ grown in Mg^{2+}-adequate, chemically defined media supplemented with Ca^{2+}. The observed effects were unlikely to be the result of Mg^{2+} antagonism, unless there were fewer binding sites per cell, leading to a lower level of cations being required to compete successfully with P.X. for the sites. The slight loss of sensitivity to P.X. may have been due to the different lipid patterns observed. The proportion of wall R.E.L. that was found to be P.L. was greater in P.aeruginosa $RP1^+$ than in P.aeruginosa $RP1^-$, and may indicate that there was a greater P.L. barrier in the cell wall of P.aeruginosa $RP1^+$ impeding the passage of P.X. through the wall to the cell membrane. This is not compatible with the findings of Pechey, Yau & James (1974) who found a decreased P.L. content in whole cells of a gentamicin-resistant P.aeruginosa. Brown & Watkins (1970) found that the cell walls and whole cells of a P.X.-resistant strain of P.aeruginosa contained more R.E.L., but less P.L., than the sensitive strain. This mutant was isolated by serial subculture in a chemically defined media containing progressively higher concentrations of the drug. It is possible that growth in the presence of P.X. may have resulted in a different type of exclusion mechanism than that mediated by the $RP1^+$ R-factor. It is unlikely that the differences in cell wall lipids between P.aeruginosa $RP1^+$ and P.aeruginosa $RP1^-$ were the sole explanation for the loss of sensitivity of the former to P.X.. The marked resistance to E.D.T.A. and retention

of wall cations during lipid removal, suggest that the P.L. and other components of the outer membrane and cell wall of P.aeruginosa $RP1^+$ may have been stabilized by a mechanism not involving Ca^{2+} and Mg^{2+}, and that these cations were involved elsewhere in the cell wall. The cell wall may therefore have been of a more stable nature and not so sensitive to the action of P.X.. It is conceivable that such a cell wall may lyse into larger fragments, and the reduced lysis may have been an optical effect due to these fragments. Such optical effects have been reported by Eagon & Carson (1965) who observed less of a decrease in optical density in P.aeruginosa cultures treated with E.D.T.A. compared with similar cultures treated with E.D.T.A. plus lysozyme. When the action of E.D.T.A. and E.D.T.A. plus lysozyme was followed by viable counts these authors observed identical death rates. They concluded that the discrepancy between the optical density results was due to the large cell wall fragments resulting from the E.D.T.A. treatment. The slight drop in sensitivity of P.aeruginosa $RP1^+$ may be due to an optical effect caused by binding of the P.X. to the cell wall. The content of lysyl-phosphatidyl-glycerol was slightly greater in P.aeruginosa $RP1^+$ and the cells and cell wall fragments may have absorbed most of the drug. Such increased binding would be compatible with the results of Watkins (1970) that P.aeruginosa 6750 trained to P.X. resistance bound P.X. more effectively than sensitive cells.

6. RESISTANCE STUDIES ON COLD SHOCKED CULTURES OF *P.aeruginosa* POSSESSING THE R-FACTOR RP1.

General observations.

Extensive autolysis was observed when cold shocked cultures of *P.aeruginosa* $RP1^+$ were rewarmed to 37.5°. There was no significant difference between the autolysis exhibited by *P.aeruginosa* $RP1^+$ and *P.aeruginosa* $RP1^-$ (Fig. 53). Although the post cold shock autolysis of these two strains was similar, *P.aeruginosa* $RP1^+$ was significantly more sensitive to the lethal effects of cold shock as determined by viable counts. The percent survivors for *P.aeruginosa* $RP1^+$ and *P.aeruginosa* $RP1^-$ were 0.03 and 0.35% respectively. This sensitivity is surprising since the high phospholipid (P.L.) content of the cell walls of *P.aeruginosa* $RP1^+$ would be expected to protect against the effects of cold shock (Leder, 1972). This protective effect may prevent killed cells from lysing, but need not necessarily be concerned with the viability of the cells. Indeed, it would seem that possession of the R-factor rendered *P.aeruginosa* more susceptible to the lethal effects of cold shock. Of the survivors of the *P.aeruginosa* $RP1^+$ culture only 21% exhibited resistance to carbenicillin (200 U/ml). The inability of the other 79% of the survivors to grow on carbenicillin agar does not necessarily indicate the elimination of RP1 from the cells. It is probable that these carbenicillin sensitive survivors, although they retained viability, nevertheless had cell walls that were sufficiently damaged by cold shock to allow the carbenicillin increased access to the murein of the wall.

This increased concentration of carbenicillin may have been sufficiently high to be unaffected by the periplasmic β-lactamase. The β-lactamase itself may have been rendered inactive, either by conformational changes induced by distortion of the membrane during cold shock, or by dilution due to loss of periplasmic enzymes after cold shock (cold osmotic shock is a well proved method for the isolation of periplasmic enzymes: Heppel, 1967). Some of the carbenicillin sensitive survivors of cold shocked P.aeruginosa $RP1^+$ may have been authentic P.aeruginosa $RP1^-$ since approximately 3% of the initial population of P.aeruginosa $RP1^+$ were carbenicillin sensitive, and therefore presumably P.aeruginosa $RP1^-$. Assuming a survivor rate of 0.35%, the contribution of P.aeruginosa $RP1^-$ to the surviving fraction of P.aeruginosa $RP1^+$ would be approximately 35%. This would result in a corrected percent survivor value of 0.02% for P.aeruginosa $RP1^+$. Since 79% of the P.aeruginosa $RP1^+$ cold shock survivors were sensitive to carbenicillin, and 35% may have been authentic P.aeruginosa $RP1^-$, it is probable that the remaining 45% were P.aeruginosa $RP1^+$ that possessed slightly damaged cell walls. However, it is also possible that the 3% carbenicillin sensitive cells in the initial P.aeruginosa $RP1^+$ were not authentic P.aeruginosa $RP1^-$, but were cells that at the time of testing were at a stage of cell growth or division that rendered them susceptible to the carbenicillin.

Ethylenediaminetetra-acetic acid mediated lysis of cold shocked cultures of P.aeruginosa $RP1^+$.

Cold shocked cultures of P.aeruginosa $RP1^+$ were

significantly more sensitive to the lytic effects of ethylenediaminetetra-acetic acid (E.D.T.A.) than were cold shocked cultures of P.aeruginosa $RP1^-$. This is in contrast to the non-cold shocked culture which was significantly more resistant to E.D.T.A. than was P.aeruginosa $RP1^-$. It has been suggested that this resistance of the non-cold shocked P.aeruginosa $RP1^+$ may have been due, in part, to other components of the outer membrane restricting the access of the E.D.T.A. to the cations (see Discussion, section 5). On the basis of this hypothesis, it may be expected that cold shock, in damaging the outer membrane would indeed render P.aeruginosa $RP1^+$ more sensitive to E.D.T.A. than the non-cold shocked culture. Reference to Fig. 53 shows that both the initial rate and extent of E.D.T.A. mediated lysis was greater for cold shocked P.aeruginosa $RP1^+$ than it was for cold shocked P.aeruginosa $RP1^-$. This indicates either, that cations are relatively more important in maintaining the structure of P.aeruginosa $RP1^+$ cells, or that the cells lysed into smaller fragments.

Ethyleneglycol-Bis-(β-aminoethylether)-N:N'-Tetra-acetic acid mediated lysis of cold shocked cultures of P.aeruginosa $RP1^+$.

The lytic action of ethyleneglycol-Bis-(β-amino-ethylether)-N:N'-Tetra-acetic acid (E.G.T.A.) upon cold shocked P.aeruginosa $RP1^+$ was similar to that of E.D.T.A. upon this culture. The culture exhibited a faster initial rate of lysis, but the extent after 180-min was similar to that of P.aeruginosa $RP1^-$. This seems to indicate that the membrane disruptive effect of cold

shock exposed, or weakened, the calcium cations to the action of the chelating agents. Unfortunately the cultures were autolysing so rapidly that little information could be gained about the effect of both E.D.T.A. and E.G.T.A. other than they seemed initially to enhance the lysis.

Polymyxin mediated lysis of cold shocked cultures of P.aeruginosa $RP1^{+}$.

The initial rate of polymyxin (P.X.) mediated lysis of cold shocked P.aeruginosa $RP1^{+}$ was faster than for cultures of P.aeruginosa $RP1^{-}$. The extent of lysis after 180-min was less than it was for P.aeruginosa $RP1^{+}$, but this may have been the result of a change in optical properties due to P.X. binding to cell wall fragments of P.aeruginosa $RP1^{+}$ to a greater extent than to fragments from P.aeruginosa $RP1^{-}$.

7. CHEMISTRY OF CULTURES OF *P.aeruginosa* POSSESSING THE R-FACTOR RP1.

There were few chemical differences between the cell walls of *P.aeruginosa* $RP1^+$ and the cell walls of *P.aeruginosa* $RP1^-$. The readily extractable lipids (R.E.L.) and the 2-keto,3-deoxyoctonic acid (K.D.O.) content were both less for *P.aeruginosa* $RP1^+$ cell walls, but the phospholipid (P.L.), meso,2,6-diaminopimelic acid (D.A.P.) and carbohydrate content were all increased compared with *P.aeruginosa* $RP1^-$. The slight difference in the lipids may explain the slight loss of sensitivity to polymyxin (P.X.) shown by *P.aeruginosa* $RP1^+$, but the P.L. cannot be considered in isolation (as explained in section 1 of the discussion). The low K.D.O. and high carbohydrate values may indicate that less lipopolysaccharide (L.P.S.), with longer 'O'antigen specific side chains, may have been present in the walls of *P.aeruginosa* $RP1^+$. It has been shown for *Salmonella typhimurium* strains that the sensitivity to P.X. is inversely proportional to the length of the 'O' antigen side chain of the L.P.S., even though the site of P.X. binding was in the lipid A region of the L.P.S. molecule (Bader & Teuber, 1973). It is reasonable to hypothesise that if the increased carbohydrate of the cell wall of *P.aeruginosa* $RP1^+$ was due to the presence of longer 'O'antigen side chains, these would interfere with the access of P.X. and ethylenediaminetetra-acetic acid (E.D.T.A.) to the target sites. This interference, together with any permeability effects of the increased P.L., may explain the resistance of *P.aeruginosa* $RP1^+$ to P.X. and E.D.T.A..

Extraction of the cell walls of P.aeruginosa $RP1^+$ with chloroform:methanol (2:1 by volume) to remove the R.E.L. was found to release little, or none, of the wall magnesium (Mg^{2+}) and calcium (Ca^{2+}) when compared with similarly treated walls of P.aeruginosa $RP1^-$ which lost 80% Mg^{2+} and 60% Ca^{2+} (Table 34). These results tend to suggest that the association between the cations and P.L. was minimal in the cell walls of P.aeruginosa $RP1^+$. This is compatible with the observation that P.X. resistant strains of P.aeruginosa had significantly less Mg^{2+} associated with the cell wall lipid when compared with P.X. sensitive strains (Brown & Watkins, 1970). This observation that cell wall cations of P.aeruginosa $RP1^+$ are apparently more firmly bound to a less readily extractable component of the wall may, in part, explain the resistance of this strain to E.D.T.A.. Possible reasons for the resistance to E.D.T.A. may be 1) that the cations are more firmly bound at a similar site occupied in the cell walls of P.aeruginosa $RP1^-$, 2) access of E.D.T.A. is restricted by another cell wall component, 3) the cations are bound at a different site deeper in the cell wall, or 4) the cations have a much reduced role in the maintenance of cell shape and structure. The last reason is improbable since cold shocked cultures of P.aeruginosa $RP1^+$ lysed significantly faster, and to a greater extent in the presence of E.D.T.A. and ethyleneglycol-Bis-(β-aminoethylether)-N:N'-Tetra-acetic acid (E.G.T.A.) compared with similarly treated P.aeruginosa $RP1^-$ cultures, indicating that the cations play a significant role in the structure of the cells.

The first and third reasons seem valid since the cations were not removed by solvent treatment of the walls. The first reason may not be as relevant as the third however, since to assume the cations are bound at the same site is to assume some are bound to P.L. and would be extracted with the R.E.L.. The second reason may also be likely since there is little evidence to suggest that a component that can effectively shield cations from E.D.T.A. should also shield P.L. or lipid A regions of L.P.S., and lipid membranes from P.X.. Nevertheless Sanderson, MacAlister, Costerton & Cheng (1974) have found that the 'O'antigen specific side chain of S.typhimurium L.P.S. restricted the access of a variety of drugs to their targets. Therefore the significant sensitivity of P.aeruginosa $RP1^+$ to P.X. compared with E.D.T.A. may indicitate that exclusion by the cell wall components played a less important role in the resistance of P.aeruginosa $RP1^+$ to E.D.T.A. than the relocation or firmer binding of the cations in the wall. The high D.A.P. content of cell walls from P.aeruginosa $RP1^+$ may also offer an explanation of the resistance to E.D.T.A.. It is conceivable that the structure of the cell wall and the maintenance of cell shape may, in common with other Gram-negative species, depend to a large extent upon the murein of the cell wall. There is reason to believe that in P.aeruginosa $RP1^-$ the outer membrane plays a major role in cell rigidity (Carson & Eagon, 1966; Wiklinson & Galbraith, 1975). If the greater D.A.P. content of P.aeruginosa $RP1^+$ reflects an increase in the murein, then this layer may have assumed greater

importance in the rigidity of the cell, and it may be at this site that the cations are involved. The lack of a significant difference in the hexosamine content between P.aeruginosa $RP1^+$ and P.aeruginosa $RP1^-$ however, indicates that the murein content may be similar, and that the increased D.A.P. content reflects a greater degree of intra- and intercellular cross-linking of the murein. This too is compatible with the murein playing an essentially important part in maintaining the structure of the cell in P.aeruginosa $RP1^+$.

8. CONCLUDING DISCUSSION.

Growth of Pseudomonas aeruginosa in chemically defined media (C.D.M.) ultimately depleted in magnesium (Mg^{2+}) and glucose has resulted in cells, the walls of which were characterised by a few marked changes in their chemistry. Most notable of these changes were the cell wall cation and lipid content, and the composition of the phospholipids (P.L.). The corresponding changes in the sensitivity of the Mg^{2+}-depleted cultures to lysis by ethylenediaminetetra-acetic acid (E.D.T.A.) and polymyxin (P.X.) did not correlate with any one particular chemical change. This is quite acceptable, since it is conceivable that the lytic action of a chelating agent or P.X. upon P.aeruginosa will be dependant upon the presence (or absence) of several different cell wall components. This point is illustrated by the cell wall P.L. composition of P.aeruginosa grown in Mg^{2+}-depleted C.D.M. without added calcium (Ca^{2+}) or manganese (Mn^{2+}). The content of lysyl-phosphatidyl-glycerol (lys.P.G.) was significantly higher in the Mg^{2+}-depleted culture compared with Mg^{2+}-adequate culture. Since the Mg^{2+}-depleted culture was very resistant to P.X. and E.D.T.A. it would seem likely that the P.L. composition, or content, affects the resistance. This correlation has been noted in several resistant strains of P.aeruginosa (Brown & Watkins, 1970; Pechey, Yau & James, 1974). When Ca^{2+} was added to the Mg^{2+}-depleted growth medium the growth rate and cut off of the culture were unaffected. The lys.P.G. content of the cell walls was greater than that of unsupplemented cells, and resistance to E.D.T.A. and P.X. was lost.

Thus it was not the P.L. per se, but the presence of divalent metal cations that influenced the sensitivity, while the presence of Mg^{2+} seemed obligatory to alter the P.L. content and composition. This latter point has been observed in several species of bacteria (Escherichia coli: Günther, Richter & Schmalbeck, 1975; Benns & Proulx, 1974; Bacillus subtilis: Minnikin & Abdolrahimzadeh, 1974). It is interesting to note that a P.X. resistant strain of P.aeruginosa possessed cell walls with much less Mg^{2+} and a different P.L. content, but not P.L. composition, compared with walls from a sensitive strain (Brown & Watkins, 1970). It is conceivable that this resistant strain was deficient in accumulating or utilizing Mg^{2+} in the cell wall and that this in turn may have altered the P.L. content of the walls. The cations Mg^{2+} and Ca^{2+} also appear to have some relevance to the resistance of P.aeruginosa $RP1^{+}$ to E.D.T.A.. The wall content of these cations differed little from those of the parent strain, P.aeruginosa $RP1^{-}$, but none of the cations were lost during the extraction of the readily extractable lipids (R.E.L.) from the walls. This indicated that the cations were bound at a deeper site within the wall, and may have been associated with the murein region, since the diaminopimelic acid (D.A.P.) content suggested a greater degree of cross-linking in this region of P.aeruginosa $RP1^{+}$.

Another significant effect of Mg^{2+}-depletion was to render P.aeruginosa less susceptible to both the lethal effects of cold shock and post cold shock lysis. In this respect it was found that there was a correlation between

P.L. content of the wall and the effects of cold shock. The composition of the cell wall P.L. fraction however did not appear to correlate with the sensitivity to cold shock of the Mg^{2+}-depleted cultures, although it is probable that an analysis of the fatty acid side chains might have shown a lesser degree of saturation in the more resistant cultures. The resistance of Mg^{2+}-depleted cultures to E.D.T.A. and P.X. may probably be explained by a mechanism involving exclusion of the drug and a more stable wall structure. If the resistance was due solely to exclusion, it would not be expected that Mg^{2+}-depleted cells would be any less sensitive to post cold shock autolysis than Mg^{2+}-adequate cells. Since the Mg^{2+}-depleted cells did show less post cold shock autolysis it would seem that the components of the cell walls were more firmly bound to one another. The component responsible for this firm binding may well have been an organic molecule (Wilkinson & Galbraith, 1975) which could conceivably exert some type of exclusion mechanism too.

The increased sensitivity to E.D.T.A. of even Mg^{2+}-adequate cultures when grown in the presence of Ca^{2+} or Mn^{2+} correlated with an increase in the wall content of these cations, apparently with a slight loss of Mg^{2+}. The loss of Mg^{2+} did not match the increase in added cation, suggesting that there were two sorts of cation binding sites in the cell wall of P.aeruginosa. First, there must be sites that can be occupied by any similar divalent cation, and secondly, there may be sites that are cation specific. The evidence for the second sort of site is in the observation that the wall content of

Ca^{2+} in cells from Ca^{2+}-supplemented, Mg^{2+}-adequate culture differs little from the content of cells from Ca^{2+}-supplemented, Mg^{2+}-depleted cultures.

From the action of E.D.T.A. upon P.aeruginosa $RP1^{+}$ and P.aeruginosa $RP1^{-}$ it would appear that the hypothesis that E.D.T.A. acts by removal of structurally important cations was valid. It was apparent from the chemistry of defatted cell walls of P.aeruginosa $RP1^{+}$ that Mg^{2+} and Ca^{2+} were firmly bound, possibly at a deeper site within the wall. This in combination with the resistance to E.D.T.A. seems to confirm the action of E.D.T.A. upon sensitive strains in which it is thought that the cell rigidity is dependant upon the outer membrane to a great extent (Carson & Eagon, 1966), which itself may be stabilized to a greater extent by cation mediated cross-linkages in in the lipopolysaccharide (L.P.S.) region (Wilkinson & Galbraith, 1975). The relation between the sensitivity of P.aeruginosa to P.X. and the cation content of the growth medium is not clear. It has been suggested that P.X. competes with extracellular Mg^{2+} and Ca^{2+} for sites on, or in, the cell wall (Newton, 1956; Brown & Melling, 1969b). It has been shown that in Salmonella typhimurium P.X. binds with the lipid A region of L.P.S. (Bader & Teuber, 1973) and phosphatidyl-glycerol (P.G.) in the cell wall (Teuber, 1973). Wilkinson (1975) has suggested that L.P.S. in the outer membrane of P.aeruginosa is stabilized by cationic cross-links (see also Costerton, Ingram & Cheng, 1974). Polymyxin may well displace those cations bound to the lipid A region or to P.G., and so disrupt the outer membrane. If these sites are

occupied by a different (organic) cation, or are obscured, the cell would be resistant to P.X.. The very firm nature of the alternative binding in the cell wall of Mg^{2+}-depleted P.aeruginosa may have been demonstrated by the relative resistance to post cold shock autolysis of these cultures compared to Mg^{2+}-adequate cultures. It is conceivable therefore, that P.X. bound to its primary site on the outer membrane, but that the 'stronger' Mg^{2+}-depleted cell wall did not disrupt.

It seems that in the presence of available cations the cell walls of P.aeruginosa may be stabilized by weak cationic cross-links that are easily disrupted by physical effects (cold shock: Farrell & Rose, 1968; pH, temperature and tonicity: Brown & Winsley, 1969). The cations themselves may be removed without too much difficulty by E.D.T.A., or displaced by P.X.. When no divalent metal cations are available the cell wall changed, and may have been stabilized by much stronger bonds which were less sensitive to the effect of cold shock, and presumably not mediated by metal cations since E.D.T.A. had little, or no effect. Polymyxin also had no effect on P.aeruginosa grown in Mg^{2+}-depleted C.D.M., but there was little evidence to suggest that sites were not available, although the possibility of exclusion by organic cations cannot be ruled out.

Cultures of P.aeruginosa $RP1^{+}$, although significantly resistant to E.D.T.A. were only slightly less sensitive to P.X., which might suggest that the firmly bound nature of Mg^{2+} and Ca^{2+}, in conjunction with the high D.A.P. content of the walls, reflected a greater dependance upon the

murein layer for cell rigidity. If this situation was so, then it would not be expected to alter significantly the sensitivity of P.aeruginosa $RP1^+$ to P.X..

In conclusion, it seems that although no one component of the cell walls from P.aeruginosa correlated with the resistance to E.D.T.A. or P.X., the divalent cations were found to have two important roles. First, the P.L. content and composition of the walls was dependant upon the amount of Mg^{2+} available in the growth medium. The P.L. per se did not correlate with resistance. Second, other cations (Ca^{2+} and Mn^{2+}), although not replacing Mg^{2+} in a metabolic or synthetic function, were incorporated into the cell walls of Mg^{2+}-depleted and Mg^{2+}-adequate cultures, and restored sensitivity (Mg^{2+}-depleted) or enhanced sensitivity (Mg^{2+}-adequate) to E.D.T.A. and P.X.. Thus the availability of additional cations was a major factor in determining the sensitivity of P.aeruginosa to chelators and P.X.. When cold shock was studied however, it appeared that the P.L. rather than cations contributed to resistance to cold shock. An important range of cell wall components (the individual sugars of L.P.S., fatty acid side chains in P.L. and cell envelope proteins) were not studied, and may have been found to contribute to the sensitivity, particularly in regard to the role that L.P.S. plays in the exclusion of drugs.

SUGGESTIONS FOR FURTHER WORK.

1) A detailed study of the component sugars of the O antigen specific side chain and core polysaccharide from Mg^{2+}-adequate and Mg^{2+}-depleted cultures of P.aeruginosa in conjuction with antisera and fluorescent antibody studies of the cell surface may yield useful information regarding the nature of the changes mediated by Mg^{2+}-depletion.

2) Further useful studies that may be informative about the cell surface in Mg^{2+}-depleted cells are electrophoretic mobility and the binding or adsorption of lipopolysaccharide and lipoprotein-specific bacteriophages.

3) A study of the sensitivity of Mg^{2+}-adequate and Mg^{2+}-depleted cultures to drugs that act on a variety of targets, both within and at the cell wall, may be useful in determining the role played by exclusion in resistance due to Mg^{2+}-depletion.

4) A kinetic study of cold shock upon Mg^{2+}-adequate and Mg^{2+}-depleted P.aeruginosa, and the effect of various cold shock procedures upon drug sensitivity, especially in conjunction with a chemical study of the cellular material released, may yield useful information about the cell wall of P.aeruginosa.

5) A detailed study of the fatty acid content of the wall phospholipids would be beneficial in the interpretation of the effect that Mg^{2+}-depletion has upon the sensitivity of P.aeruginosa to cold shock or antimicrobial agents.

REFERENCES.

ASBELL, M.A. & EAGON, R.G. (1966). Role of multivalent cations in the organisation, structure and assembly of the cell wall of Pseudomonas aeruginosa. J. Bacteriol. 92, 380-387.

BADER, J. & TEUBER, M. (1973). Action of Polymyxin B on bacterial membranes. I. Binding to the 'O' antigenic lipopolysaccharide of Salmonella typhimurium. Z. Naturforsh. 28c, 422-430.

BAGINSKI, E.S., FOÀ, P.P. & ZAK, B. (1967). Micro-determination of inorganic phosphate, phospholipids and total phosphate in biological materials. Clin. Chem. 13(4), 326-332.

BELL, R.M., MAVIS, R.D., OSBORN, M.J. & VAGELOS, P.R. (1971). Enzymes of phospholipid metabolism: Localisation in the cytoplasmic and outer membrane of the cell envelope of Escherichia coli & Salmonella typhimurium. Biochim. Biophys. Acta. 249, 628-635.

BENNS, G. & PROULX, P. (1974). The effect of ATP and Mg^{2+} on the synthesis of phosphatidyl-glycerol in Escherichia coli preparations. Biochim. Biophys. Acta. 337, 318-324.

BEUMER, J., DIRKX, J. & BEUMER-JOCHMANS, M.P. (1957). Role of cations in phage absorption to sensitive bacteria. Nature. 180, 83-85.

BOGGIS, W. (1971). The effect of metal cations and phosphate upon EDTA and polymyxin mediated lysis of Pseudomonas aeruginosa. Ph.D. Thesis. Bath University of Technology.

BRAUN, V. (1973). Molecular organisation of the rigid layer and the cell wall of Escherichia coli. J. Infect. Dis. 128, Suppl. S9-S16.

BRAUN, V., REHN, K. & WOLFF, H. (1970). Supramolecular structure of the rigid layer of the cell wall of Salmonella, Serratia, Proteus and Pseudomonas fluorescens. Number of lipoprotein molecules in a membrane layer. Biochemistry. 9(26), 5041-5049.

BROCK, T.D. (1962). Effects of magnesium ion deficiency on Escherichia coli and possible relation to the mode of action of novobiocin. J.Bacteriol. 84, 679-682.

BROWN, M.R.W. (1968). Survival of Pseudomonas aeruginosa in fluorescein solution: preservative action of PMN and EDTA. J. Pharm. Sci. 57, 389-392.

BROWN, M.R.W. (1971). Inhibition and destruction of Pseudomonas aeruginosa. In Inhibition and destruction of the microbial cell. 1st edition, chapter 7, Ed. Hugo, W.B. Academic Press, London.

BROWN, M.R.W. (1975). The role of the cell envelope in resistance. In Resistance of Pseudomonas aeruginosa. 1st edition, chapter 3, Ed. Brown, M.R.W. John Wiley, London.

BROWN, M.R.W., FARWELL, J.A. & ROSENBLUTH, S.A. (1969). Use of membrane filters for measurement of 260 mμ-absorbing substances from bacterial cells. Anal. Biochem. 27, 484-491.

BROWN, M.R.W., FENTON, E.M. & WATKINS, W. (1972). Tetracycline-sensitive/polymyxin-resistant Pseudomonas aeruginosa. Lancet, ii, 86.

BROWN, M.R.W. & MELLING, J. (1969a). Loss of sensitivity to EDTA by Pseudomonas aeruginosa grown under conditions of Mg^{2+}-limitation. J. gen. Microbiol. 54, 439-444.

BROWN, M.R.W. & MELLING, J. (1969b). Role of divalent cations in the action of Polymyxin B and EDTA on Pseudomonas aeruginosa. J. gen. Microbiol. 59, 263-274.

BROWN, M.R.W. & RICHARDS, R.M.E. (1965). Effect of ethylenediaminetetra-acetic acid on the resistance of Pseudomonas aeruginosa to antibacterial agents. Nature. 207, 1391-1393.

BROWN, M.R.W. & SCOTT-FOSTER, J.H. (1970). A simple diagnostic milk medium for Pseudomonas aeruginosa. J. Clin. Path. 23, 172-177.

BROWN, M.R.W. & SCOTT-FOSTER, J.H. (1971). Effect of slime on the sensitivity of Pseudomonas aeruginosa to EDTA and polymyxin. J. Pharm. Pharmac. 23, Suppl. 236S.

BROWN, M.R.W. & WATKINS, W. (1970). Low magnesium and phospholipid content of cell walls of Pseudomonas aeruginosa resistant to polymyxin. Nature. 227, 1360-1361.

BROWN, M.R.W. & WINSLEY, B.E. (1969). Effect of polysorbate 80 on the resistance of Pseudomonas aeruginosa exposed to rapid changes of pH, temperature and tonicity. J. gen. Microbiol. 56, 99-107.

BROWN, M.R.W. & WOOD, S.M. (1972). Relation between cation and lipid content of cell walls of Pseudomonas aeruginosa, Proteus vulgaris and Klebsiella aerogenes and their sensitivity to polymyxin B and other antibacterial agents. J. Pharm. Pharmac. 24, 215-218.

BURMAN, L.G., NORSTROM, K. & BLOOM, G.D. (1972). Murein and the outer penetration barrier of Escherichia coli K-12, Proteus mirabilis and Pseudomonas aeruginosa. J. Bacteriol. 112, 1364-1374.

BYRNE, P. & CHAPMAN, D. (1964). Liquid crystalline nature of phospholipids. Nature. 202, 987.

CAMPBELL, P.N. & SARGENT, J.R. (1967). Techniques of protein biosynthesis. 1st edition, volume 1, Academic Press, London.

CARSON, K.J. & EAGON, R.G. (1966). Lysozyme sensitivity of Pseudomonas aeruginosa: further evidence for the role of the non-peptidoglycan components in cell wall rigidity. Can. J. Microbiol. 12, 105 - 108.

CERNY, G. & TEUBER, M. (1971). Differential release of periplamic versus cytoplasmic enzymes from Escherichia coli B by Polymyxin-B. Arch. Mikrobiol. 78, 166 - 179.

CERNY, G. & TEUBER, M. (1972). Comparative polyacrylamide electrophoresis of periplasmic proteins released from Gram-negative bacteria by Polymyxin-B. Arch. Mikrobiol. 82, 361 - 370.

CHENG, K-. J. & COSTERTON, J.W. (1973). Localisation of alkaline phosphatase in three Gram-negative rumen bacteria. J. Bact. 116, 424 - 446.

CHENG, K-, J., INGRAM, J.M. & COSTERTON, J.W. (1970). Release of alkaline phosphatase from cells of Pseudomonas aeruginosa by manipulation of cation concentration and of pH. J. Bact. 104, 748 - 753.

CHENG, K-.,J., INGRAM, J.M. & COSTERTON, J.W. (1971). Interactions of alkaline phosphatase and the cell wall of Pseudomonas aeruginosa. J. Bact. 107, 325 - 336.

CHINARD, F.P. (1952). The photometric estimation of proline and ornithine. J. Biol. Chem. 199, 91 -95.

CLARKE, K., GRAY, G.W. & REAVELEY, D.A. (1967,a). The cell walls of Pseudomonas aeruginosa. Biochem. J. 105, 749 - 754.

CLARKE, K., GRAY, G.W. & REAVELEY, D.A. (1967,b). The extraction of cell walls of Pseudomonas aeruginosa with aqueous phenol; Material from the phenol layer. Biochem. J. 105, 755 - 758.

CLARKE, K., GRAY, G.W. & REAVELEY, D.A. (1967,c). The extraction of cell walls of Pseudomonas aeruginosa with aqueous phenol: The insoluble residue and material from the aqueous layer. Biochem. J. 105, 759 - 765.

COHN, P.S. & ENNIS, H.L. (1967). Amino acid regulation of RNA synthesis during recovery of Escherichia coli from Mg^{2+} starvation. Biochim. Biophys. Acta. 145, 300 - 309.

COSTERTON, J.W., INGRAM,J.M. & CHENG, K-.J. (1974). Structure and function of the cell envelope of Gram-negative bacteria. Bact. Rev. 38, 87 - 110.

DAVIS, S.D., IANNETTA, A. & WEDGWOOD, R.J. (1971). Action of colistin against Pseudomonas aeruginosa: Inhibition by calcium. J. Infec. Dis. 124(6), 610 - 612.

DAVIS, S.D. & IANNETTA, A. (1972). Influence of serum and calcium on the bactericidal activity of gentamycin and carbenicillin on Pseudomonas aeruginosa. Appl. Microbiol. 23(4), 775 - 779.

DIXON, M. & WEBB, E.C. (1964) Enzymes, 2nd edition, Longmans, London.

DREWRY, D.T., GRAY, G.W. & WILKINSON, S.G. (1971). Release of ethanolamine pyrophohsphate during mild acid hydrolysis of the lipopolysaccharide of Pseudomonas aeruginosa. Eur. J. Biochem. 21, 400 - 403.

DREWRY, D.T., GRAY, G.W. & WILKINSON, S.G. (1972). Low-molecular weight solutes released during mild acid hydrolysis of the lipopolysaccharide of Pseudomonas aeruginosa. Identification of ethanolamine triphosphate. Biochem. J. 130, 289 - 295.

DUBOIS, M., GILLES, K.A., HAMILTON, J.K., REBERS, P.A. & SMITH, F. (1956). Colourimetric method for the determinations of sugars and related substances. Anal. Chem. 28, 350-356.

EAGON, R.G. (1969). Cell wall associated substances from Pseudomonas aeruginosa. Can. J. Microbiol. 15, 235-236.

EAGON, R.G. & CARSON, K.J. (1965). Lysis of cell walls and intact cells of Pseudomonas aeruginosa by ethylenediaminetetra-acetic acid and lysozyme. Can. J. Microbiol. 11, 193-201.

EAGON, R.G., SIMMONDS, G.R. & CARSON, K.J. (1965). Evidence for the presence of ash and divalent metals in the cell wall of Pseudomonas aeruginosa. Can. J. Microbiol. 11, 1041-1042.

EAGON, R.G., STINNETT, J.D. & GILLELAND, H.E. (1975). Ultrastructure of Pseudomonas aeruginosa as related to resistance. In Resistance of Pseudomonas aeruginosa. 1st edition, chapter 4. Ed. Brown, M.R.W. John Wiley. London.

EDELMAN, I.S., TSO, P.O.P. & VINOGRAD, J. (1960). The binding of magnesium to microsomal nucleoprotein and ribonucleic acid. Biochim. Biophys. Acta. 43, 393-403.

EDSTROM, R.D. & HEATH, E.C. (1967). Biosynthesis of cell wall lipopolysaccharide in Escherichia coli. VI. Enzymatic transfer of galactose, glucose, N-acetyl-glucosamine and colitose into the polymer. J. Biol. Chem. 242, 3581-3588.

ELLWOOD, D.C. (1970). The distribution of 2-keto-3-deoxy-octonic acid in baterial walls. J. gen. Microbiol. 60, 373-380.

ELLWOOD, D.C., TURNER, W.H., HUNTER, J.R. & MOODY, G.R.G. (1969). Changes in the cell wall composition of a strain of Bacillus subtilis grown in a chemostat. Biochem. J. 113, 14p-15p.

ENDO, A. & ROTHFIELD, L. (1969). Studies of a phospho-lipid-requiring bacterial enzyme I: Purification and properties of UDP galactose:lipopolysaccharide 3-galactosyl transferase. Biochemistry. 8, 3500-3507.

FARRELL, J. & ROSE, A.H. (1967). Temperature effects on microorganisms. Ann. Rev. Microbiol. 28, 101-120.

FARRELL, J. & ROSE, A.H. (1968). Cold shock in a mesophilic and a psychrophilic Pseudomonad. J. gen. Microbiol. 50, 429-439.

FEINGOLD, D.S., HSU-CHEN, C.C. & SUD, I.J. (1974). Basis for the selectivity of action of the polymyxin antibiotics on cell membranes. Annals. N.Y. Acad. Sci. 235, 480-492.

FEW, A.V. (1955). The interaction of polymyxin E with bacterial and other lipids. Biochim. Biophys. Acta. 16, 137-145.

FIIL, A. & BRANTON, D. (1969). Changes in the plasma membrane of Escherichia coli during magnesium starvation. J. Bacteriol. 98(3), 1320-1327.

FISKE, C.A. & SUBBAROW, Y. (1925). The colourimetric determination of phosphorus. J. Biol. Chem. 66, 375-400.

FITZGERALD, J.W. & LASLIE, W.W. (1975). Loss of primary alkylsulphatase and secondary alkylsulphatases (S-1 and S-2) from Pseudomonas $C_{12}B$: Effect of culture conditions, cell washing procedure and osmotic shock. Can. J. Microbiol. 21, 59-68.

GARRARD, W.T. (1971). Selective release of protein from Spirillium intersonii by tris(hydroxymethyl)aminomethane and ethylenediaminetetra-acetic acid. J. Bacteriol. 105, 93-100.

GARRETT, A.J. (1968). The effect of Mg^{2+} deprivation on cell wall synthesis and accumulation of amino sugars in Bacillus subtilis. Biochem. J. 106, 40p-41p.

GARRETT, A.J. (1969). The effect of Mg^{2+} deprivation on the synthesis of mucopeptide and its precursors in Bacillus subtilis. Biochem. J. 115, 419-430.

GARROD, L.P., LAMBERT, H.P. & O'GRADY, F. (1973). Antibiotic and Chemotherapy. 4th edition, chapter 11. Eds. Garrod, L.P., Lambert, H.P. & O'Grady, F. Churchill Livingstone: Edinburgh and London.

GHALAMBOR, M.A. & HEATH, E.C. (1966). Biosynthesis of cell wall lipopolysaccharide in E.coli. IV. Purification and properties of cytidine monophosphate-3-deoxy-D-mannooctulosonate synthetase. J. Biol. Chem. 241, 3216-3221.

GILLELAND, Jr. H.E., STINNETT, J.D. & EAGON, R.G. (1974). Ultrastructural and chemical alteration of the cell envelope of Pseudomonas aeruginosa associated with resistance to ethylenediaminetetraacetate resulting from growth in a Mg^{2+}-deficient medium. J. Bacteriol. 117, 302-311.

GILLELAND, Jr. H.E., STINNETT, J.D., ROTH, I.L. & EAGON, R.G. (1973). Freeze-etch study of P.aeruginosa: Localization within the cell wall of an ethylenediaminetetraacetate-extractable component. J. Bacteriol. 113, 417-432.

GILVARG, C. (1958). The enzymatic synthesis of diaminopimelic acid. J. Biol. Chem. 233, 1501-1504.

GLAUERT, A.M. & THORNLEY, M.J. (1969). The topography of the bacterial cell wall. Ann. Rev. Microbiol. 23, 159-198.

GOOD, T.A. & BESSMAN, S.P. (1964). Determination of glucosamine and galactosamine using borate buffers for modification of the Elson-Morgan & Morgan-Elson reaction. Anal. Biochem. 9, 253-262.

GORDON, R.C. & MacLEOD, R.A. (1966). Mg^{2+} phospholipids in cell envelopes of a marine and a terrestial Pseudomonad. Biochem. Biophys. Res. Comm. 24(5), 684-690.

GORRILL, R.H. & McNEIL, E.M. (1960). The effect of cold diluent on the viable count of Pseudomonas pyocyanea. J. gen. Microbiol. 22, 437-442.

GRAY, G.W. & WILKINSON, S.G. (1965). The action of ethylenediaminetetra-acetic acid on Pseudomonas aeruginosa. J. appl. Bacteriol. 28(i), 153-164.

GÜNTHER, T., RICHTER, L. & SCHMALBECK, J. (1975). Phospholipids of Escherichia coli in magnesium deficiency. J. gen. Microbiol. 86, 191-193.

HAMILTON-MILLER, J.M.T. (1965). Effect of EDTA upon bacterial permeability to benzyl penicillin. Biochem. Biophys. Res. Comm. 20, 688-691.

HAMILTON-MILLER, J.M.T. (1966). Damaging effects of ethylenediaminetetraacetate and penicillins on permeability barriers in Gram-negative bacteria. Biochem. J. 100, 675-682.

HANDLEY, P.S., QUESNEL, L.B. & STURGISS, M.M. (1974). Ultrastructural changes produced in Proteus vulgaris by a synergistic combination of colistin and sulphurdiazine. Microbios. 10, 211-223.

HARVEY, N.L., FEWSON, C.A. & HOLMES, W.H. (1968). Apparatus for batch culture of microorganisms. Lab. Pract. 17, 1134-1136.

HEGARTY, C.P. & WEEKS, O.B. (1940). Sensitivity of Escherichia coli to cold shock during the logarithmic growth phase. J. Bacteriol. 39, 475-484.

HEPPEL, L.A. (1967). Selective release of enzymes from bacteria. Science. 156, 1451-1455.

HERBERT, D. (1961). The chemical composition of micro-organisms as a function of their environment. Symp. Soc. Gen. Microbiol. 11, 391-416.

HERBERT, D., ELSWORTH, R. & TELLING, R.C. (1956). The continuous culture of bacteria; a theoretical and experimental study. J. gen. Microbiol. 14, 601-622.

HODGES, N.A. (1973). Effect of sporulation conditions on chemical composition and biological properties of Bacillus megaterium spores. Ph.D. Thesis. University of Aston in Birmingham.

HSU-CHEN, C.C. & FEINGOLD, D.S. (1972). Locus of divalent cation inhibition of the bactericidal action of polymyxin B. Antimicrob. Agents. Chemother. 2, 331-335.

HSU-CHEN, C.C. & FEINGOLD, D.S. (1973). The mechanism of polymyxin B action and selectivity towards biologic membranes. Biochemistry. 12(ii), 2105-2111.

HUGO, W.B. & LONGWORTH, A.R. (1964). Some aspects of the mode of action of chlorhexidine. J. Pharm. Pharmac. 16, 655-662.

HUGES, A.H., STOW, M., HANCOCK, I.C. & BADDILEY, J. (1971). Function of teichoic acids and effects of Novobiocin on control of Mg^{2+} at the bacterial membrane. Nature. New Biology. 229, 53-55.

HUMPHREY, B.A., MARSHALL, K.C. & VINCENT, J.M. (1968). Electrophoretic mobility of calcium-adequate and calcium-deprived Rhizobium trifolii. J. Bacteriol. 95, 721

HUMPHREY, B.A. & VINCENT, J.M. (1962). Calcium in the cell walls of Rhizobium trifolii. J. gen. Microbiol. 29, 557- 561.

ITO, E. & STROMINGER, J.L. (1962). Enzymatic synthesis of the peptide in bacterial uridine nucleotides: II. Enzymatic synthesis and addition of D-alanyl-D-alanine. J. Biol. Chem. 237, 2696-2703.

ITO, E. & STROMINGER, J.L. (1964). Enzymatic synthesis of the peptide in bacterial uridine nucleotides: III. Purification and properties of L-lysine adding enzyme. J. Biol. Chem. 239, 210-214.

KATES, M. (1964). Bacterial lipids. Advanc. lipid Res. 2, 17-90.

KIMES, B.W. & MORRIS, D.R. (1973). Cations and ribosome structure II. Effects on the 50S subunit of substituting polyamines for magnesium ion. Biochemistry. 12, 442-449.

KIOKE, M. & IIDA, K. (1971). Effects of polymyxin on the bacteriophage receptors of the cell walls of Gram-negative bacteria. J. Bacteriol. 108, 1402-1411.

KIOKE, M., IIDA, K. & MATSUO, T. (1969). Electron microscope studies on the mode of action of polymyxin. J. Bacteriol. 97, 448-452.

KNIVETT, V.A. & CULLEN, J. (1965). Some factors affecting cyclopropane fatty acid formation in Escherichia coli. Biochem. J. 96, 771-776.

KNIVETT, V.A. & CULLEN, J. (1967). Fatty acid synthesis in Escherichia coli. Biochem. J. 103, 299-306.

KOCH, A.L. (1961). Some calculations on the turbidity of mitochondria and bacteria. Biochim. Biophys. Acta. 51, 429-441.

KOZLOFF, L.M. & LUTE, M. (1957). Viral invasion II. The role of Zn^{2+} in bacteriophage invasion. J. Biol. Chem. 228, 529-536.

KUNDIG, W. & ROSEMAN, S. (1971,a). Sugar transport I. Isolation of a phosphotransferase system from Escherichia coli. J. Biol. Chem. 246, 1393-1406.

KUNDIG, W. & ROSEMAN, S. (1971,b). Sugar transport II. Characterisation of constitutive membrane bound enzyme II of the Escherichia coli phosphotransferase system. J. Biol. Chem. 246, 1407-1418.

LEDER, I.G. (1972). Interrelated effects of cold shock and osmotic pressure on the permeability of Escherichia coli membranes to permease accumulated substrates. J. Bacteriol. 111, 211-219.

LEDERBERG, J. (1956). Bacterial protoplasts induced by penicillin. Proc. Nat. Acad. Sci. Wash. 42, 574-576.

LEIVE, L. (1965,a). Actinomycin sensitivity in Escherichia coli produced by EDTA. Biochim. biophys. Res. Comm. 18, 13-17.

LEIVE, L. (1965,b). A nonspecific increase in permeability in Escherichia coli produced by EDTA. Proc. Nat. Acad. Sci. 53, 745-750.

LEIVE, L. (1965,c). Release of lipopolysaccharide by EDTA treatment of Escherichia coli. Biochim. Biophys. Res. Comm. 21, 290-296.

LEIVE, L. (1968). Studies on permeability changes produced in coliform bacteria by EDTA. J. Biol. Chem. 243, 2373-2380.

LEIVE, L. (1974). The barrier function of the Gram-negative envelope. Annals. N.Y. Acad. Sci. 235, 109- 129.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265-275.

LUSK, J.E., WILLIAMS, R.J.P. & KENNEDY, E.P. (1968). Magnesium and the growth of Escherichia coli. J. Biol. Chem. 243, 2618-2624.

MacGREGOR, D.R. & ELLICKER, P.R. (1958). A comparison of some properties of strains of Pseudomonas aeruginosa sensitive and resistant to quarternary ammonium compounds. Can. J. Microbiol. 4, 499-503.

MacKELVIE, R.M., GRONLUND, A.F. & CAMPBELL, J.J.R. (1967). Influence of cold shock on the endogenous metabolism of Pseudomonas aeruginosa. Can. J. Microbiol. 14, 633-638.

McCARTHY, B.J. (1962). The effect of magnesium starvation on the ribosome content of Escherichia coli. Biochim. Biophys. Acta. 55, 880-888.

McQUILLEN, K. (1956). Unpublished results, quoted by Newton (1956), pg. 19.

MARTIN, H.H. (1966). Biochemistry of bacterial cell walls. Ann. Rev. Biochem. 35, 457-484.

MARTIN, H.H. (1969). Die Struktur der Zellwand bei gramnegativen Bakterien. Arzneim - Forsch. 19, 266-272.

MEADOW, P. (1974). Walls and membrane structure. In The Biochemistry and Genetics of Pseudomonas. Ed. P.H. Clarke & M.H. Richmond, John Wiley : London.

MEERS, J.L. & TEMPEST, D.W. (1970). The influence of growth-limiting substrate and medium-NaCl concentration on the synthesis of magnesium-binding sites in the walls of Bacillus subtilis var. niger. J. gen. Microbiol. 63, 325-331.

MELLING, J. (1968). The effect of growth medium on the resistance of P.aeruginosa to chemical inactivation. Ph.D. Thesis, Bath University of Technology.

MENDELSOHN, J. & TISSIÈRES, A. (1959). Variations in the amount of ribonucleoprotein particles in Escherichia coli. Biochem. Biophys. Acta. 35, 248-250.

MEYNELL, G.G. (1958). The effect of sudden chilling on Escherichia coli. J. gen. Microbiol. 19, 380-389.

MINNIKIN, D.E. & ABDOLRAHIMZADEH, H. (1971). Thin layer chromatography of bacterial lipids on sodium acetate impregnated silica gel. J. Chromatogr. 63, 452-454.

MINNIKIN, D.E. & ABDOLRAHIMZADEH, H. (1974). Effect of pH on the properties of polar lipids in chemostat cultures of Bacillus subtilis. J. Bacteriol. 120, 999-1003.

MINNIKIN, D.E., ABDOLRAHIMZADEH, H. & BADDILEY, J. (1972). Variation of polar lipid composition of Bacillus subtilis (Marburg) with different growth conditions. FEBS. Letters. 27(1), 16-18.

MONOD, J. (1949). The growth of bacterial cultures. Ann. Rev. Microbiol. 3, 371-394.

MORGAN, C., ROSENKRANZ, H.S., CHAN, B. & ROSE, H.M. (1966). Electron microscopy of magnesium-depleted bacteria. J. Bacteriol. 91, 891-895.

MÜHLRADT, P.F., MENZEL, J., GOLECKI, J.R. & SPETH, V. (1973). Outer membrane of Salmonella. Sites of export of newly synthesised lipopolysaccharide on the bacterial surface. Eur. J. Biochem. 35, 471-481.

MÜLLER, E., HINCKLEY, A. & ROTHFIELD, L. (1972). Studies of phospholipid-requiring bacterial enzymes. III. Purification and properties of uridine diphosphate glucose:lipopolysaccharide glucosyl transferase I. J. Biol. Chem. 247, 2614-2622.

NAKAJIMA, K. & KAWAMATA, J. (1966). Studies on the mechanism of action of colistin. III. Precipitation of Escherichia coli ribosomes with colistin. Biken. J. 9, 45-49.

NEU, H.C. & HEPPEL, L.A. (1965). The release of enzymes from E. coli by osmotic shock during the formation spheroplasts. J. Biol. Chem. 240, 3685-3692.

NEWTON, B.A. (1953,a). The action of polymyxin on Pseudomonas pyocyanea. J. gen. Microbiol. 8, vi-vii.

NEWTON, B.A. (1953,b). The release of soluble constituents from washed cells of Pseudomonas aeruginosa by the action of polymyxin. J. gen. Microbiol. 9, 54-64.

NEWTON, B.A. (1954). Site of action of polymyxin on Pseudomonas aeruginosa: antagonism by cations. J. gen. Microbiol. 10, 491-499.

NEWTON, B.A. (1955). A fluorescent derivative of polymyxin: Its preparation and use in studying the site of action of the antibiotic. J. gen. Microbiol. 12, 226-236.

NEWTON, B.A. (1956). The properties and mode of action of the polymyxins. Bact. Rev. 20, 14-27.

OSBORN, M.J., GANDER, J.E., PARISI, E. & CARSON, K.J. (1972). Mechanism of assembly of the outer membrane of Salmonella typhimurium: Isolation and characterisation of cytoplasmic and outer membranes. J. Biol. Chem. 247, 3926-3972.

OVERATH, P. & TRAUBLE, H. (1973). Phase transitions in cells, membranes and lipids of E. coli: Detection by fluorescent probes, light scattering and dialometry. Biochemistry. 12, 2625-2634.

PACHE, W., CHAPMAN, D. & HILLABY, R. (1972). Interaction of antibiotics with membranes: Polymyxin-B and gramicidin-S. Biochim. Biophys. Acta. 255. 358-364.

PARANCHYCH, W. (1966). Stages in phage R17 infection: The role of divalent cations. Virology. 28, 90-99.

PATCHING, J.W. & ROSE, A.H. (1971). Effct of growth temperature on cold osmotic shock in E. coli ML30. J. gen. Microbiol. 69, 429-432.

PECHEY, D.T., YAU, A.O.P. & JAMES, A.M. (1974). Total and surface lipids of cells of Pseudomonas aeruginosa and their relationship to gentamycin resistance. Microbios. 11, 77-86.

PIPERNO, J.R. & OXENDER, D.L. (1966). Amino acid-binding protein released from Escherichia coli by osmotic shock. J. Biol. Chem. 241, 5732-5743.

PREUSSER, H.-J. & KATZ, W. (1972). Electronenmikroskopiche und chemiche Untersuchungen über den Zellwandbau von Proteus mirabilis und Escherichia coli. Zbl. Bakt. Hyg. I Abt. Orig. A220, 506-512.

PROULX, P. & FUNG, C.K. (1969). Metabolism of phosphoglyceride in E. coli. IV. The positional specificity and properties of phospholipase-A. Can. J. Microbiol. 47, 1125-1128.

REAVELEY, D.A. & BURGE, R.E. (1972). Walls and membranes in bacteria. Advanc. Microbial. Physiol. 7, 1-81.

REESE, J.F., DIMITRACOPOULOS, G. & BARTELL, P.F. (1974). Factors influencing the adsorption of bacteriophage 2 to cells of Pseudomonas aeruginosa. J. Virol. 13, 22-27.

REPASKE, R. (1958). Lysis of Gram-negative organisms and the role of versene. Biochim. Biophys. Acta. 30, 225-232.

RICHMOND, M.H. (1975). Antibiotic inactivation and its genetic basis. In Resistance of Pseudomonas aeruginosa. 1st edition, chapter 1, Ed. Brown, M.R.W. John Wiley : London.

RING, K. (1965,a). Effect of low temperature on the permeability of Streptomyces hydrogenans. Biochim. Biophys. Acta. 19, 576-581.

RING, K. (1965,b). Der Einfluss der Adaptationstemperatur auf die Kältestabilität der Zellmembran von Streptomyces hydrogenans. Biochim. Biophys. Acta. 94, 598-600.

ROBERTS, N.A., GRAY, G.W. & WILKINSON, S.G. (1970). The bactericidal action of ethylenediaminetetra-acetic acid on Pseudomonas aeruginosa. Microbios. 2, 189-208.

RODGERS, A. (1964). The exchange properties of Magnesium in Escherichia coli ribosomes. Biochem. J. 90, 548-555.

ROGERS, H.J. (1970). Bacterial growth and the cell envelope. Bact. Rev. 34, 194-294.

ROGERS, S.W., GILLELAND, Jr. H.E. & EAGON, R.G. (1969). Characterisation of a protein-lopopolysaccharide complex released from cell walls of Pseudomonas aeruginosa by ethylenediaminetetra-acetic acid. Can. J. Microbiol. 15, 743-748.

ROGERS, H.J. & PERKINS, H.R. (1968). Cell Walls and Membranes. Ed. Rogers, H.J. & Perkins, H.R. E. and F.N. Spon Ltd. London.

ROUF, M.A. (1964). Spectrochemical analysis of inorganic elements in bacteria. J. Bacteriol. 88, 1545-1549.

SALTON, M.R.J. (1964). The Bacterial Cell Wall. Ed. Salton, M.R.J. Elsevier:Amsterdam.

SANDERSON, K.E., MacALLISTER, T., COSTERTON, J.W. & CHENG, K-.J. (1974). Permeability of lipopolysaccharide-deficient (rough) mutants of Salmonella typhimurium to antibiotics, lysozyme and other agents. Can. J. Microbiol. 20, 1135-1145.

SATO, M., SUZUKI, M. & TAKAHASHI, H. (1968). Cold shock of germinating Bacillus subtilis spores. Agr. Biol. Chem. 32, 1270-1274.

SATO, M. & TAKAHASHI, H. (1968,a). Two critical temperature zones in the cold shock of Bacillus subtilis and Pseudomonas fluorescens. Agr. Biol. Chem. 32, 259-260.

SATO, M. & TAKAHASHI, H. (1968,b). Cold shock of bacteria. I. General features of cold shock in Escherichia coli. J. gen. Appl. Microbiol. 14, 417-428.

SATO, M. & TAKAHASHI, H. (1969). Cold shock of bacteria. II. Magnesium-mediated recovery from cold shock and the existence of two critical temperature zones in various bacteria. J. gen. Appl. Microbiol. 15, 217-229.

SATO, M. & TAKAHASHI, H. (1970). Cold shock in bacteria. IV. Involvement of DNA ligase reaction in recovery of Escherichia coli from cold shock. J. gen. Appl. Microbiol. 16, 279-290.

SCHNAITMAN, C.A. (1971). Effect of ethylenediaminetetra-acetic acid, triton X-100 and lysozyme on the morphology and chemical composition of isolated cell walls of Escherichia coli. J. Bacteriol. 108, 553-563.

SCHWARZMANN, J.W. & BORING, J.R. (1971). Antiphagocytic effect of slime from a mucoid strain of Pseudomonas aeruginosa. Inf. Immun. 3, 762-767.

SHERMAN, J.M. & ALBUS, W.R. (1923). Physiological youth in bacteria. J. Bacteriol. 8, 127-139.

SHERMAN, J.M. & CAMERON, G.M. (1934). Lethal environmetal factors within the natural range of growth. J. Bacteriol. 27, 341-348.

SMEATON, J.R. & ELLIOT, W.H. (1967). Selective release of RNase-inhibitor from Bacillus subtilis cells by cold shock treatment. Biochem. Biophys. Res. Comm. 26, 75-81.

SPAUN, J. (1962). Problems in standardization of turbidity determinations on bacterial suspensions. Bull. Wld. Hlth. Org. 26, 219-225.

STINNETT, J.D., GILLELAND, H.E. & EAGON, R.G. (1973). Proteins released from the cell envelopes of Pseudomonas aeruginosa on exposure to ethylenediamine-tetra-acetic acid: comparison with dimethylformamide-extractable proteins. J. Bacteriol. 114, 399-407.

STRANGE, R.E. (1964). Effect of magnesium on permeability control in chilled bacteria. Nature. 203, 1304-1305.

STRANGE, R.E. & DARK, F.A. (1962). Effect of chilling on Aerobacter aerogenes in aqueous suspension. J. gen. Microbiol. 29, 719-730.

STRANGE, R.E. & NESS, A.G. (1963). Effect of chilling on bacteria in aqueous suspension. Nature. 197, 819.

STRANGE, R.E. & POSTGATE, J.R. (1964). Penetration of substances into cold shocked bacteria. J. gen. Microbiol. 36, 393-403.

SUD, I.J. & FEINGOLD, D.S. (1970). Mechanism of polymyxin B resistance in Proteus mirabilis. J. Bacteriol. 104, 289-298.

SYKES, J. & TEMPEST, D.W. (1965). The effect of magnesium and of carbon limitation on the macromolecular organisation and metabolic activity of Pseudomonas sp. strain C-1B. Biochim. Biophys. Acta. 103, 93-108.

TEMPEST, D.W. & ELLWOOD, D.C. (1969). The influence of growth conditions on the composition of some cell wall components of Aerobacter aerogenes. Biotechnol. Bioeng. 11, 775-783.

TEMPEST, D.W., HUNTER, J.R. & SYKES, J. (1965). Magnesium limited growth of Aerobacter aerogenes in a chemostat. J. gen. Microbiol. 39, 355-366.

TEMPEST, D.W. & STRANGE, R.E. (1966). Variation in content and distribution of magnesium and its influence on survival, in Aerobacter aerogenes. J. gen. Microbiol. 44, 273- 279.

TEUBER, M. (1973). Action of polymyxin B on bacterial membranes. II. Formation of lipophilic complexes with phosphatidic acid and phosphatidylglycerol. Z. Naturforsch. 28c, 476-477.

TEUBER, M. (1974). Action of polymyxin on bacterial membranes. III. Differential inhibition of cellular functions in Salmonella typhimurium. Arch. Mikrobiol. 100, 131-144.

TISSIÈRES, A., WATSON, J.D., SCHLESSINGER, D. & HOLLINGWORTH, B.R. (1959). Ribonucleoprotein particles from Escherichia coli. J. Mol. Biol. 1, 221-233.

VAN SOESTBERGEN, A.A., STEINGRÜBER, V.A., WEISS, K.K & OLES, K.M. (1968). Automated measurement of methylene blue reduction by cold shocked E. coli. Tex. Rep. Biol. Med. 26, 401-411.

VAN STEVENINCK, J. & LEDERBOER, A.M. (1974). Phase tansitions in the yeast cell membrane. The influence of temperature on the reconstitution of active dried yeast. Biochim. Biophys. Acta. 352, 64-70.

VINCENT, J.M. & HUMPHREY, B.A. (1963). Partition of divalent cations in the bacterial wall and cell contents. Nature, 199, 149.

WARREN, G.H., GREY, J. & YURCHENCO, J.A. (1957). Effect of polymyxin on the lysis of Neisseria catarrhalis by lysozyme. J. Bacteriol. 74, 788.

WATKINS, W. (1970). Polymyxin resistance in P.aeruginosa. Ph.D. Thesis, Bath University of Technology.

WEBB, M. (1949). The influence of magnesium on cell division. 3. The effect of magnesium on the growth of bacteria in simple chemically defined media. J. gen. Microbiol. 3, 418- 424.

WEBB, M. (1968). The influence of certain trace metals on bacterial growth and magnesium utilisation. J. gen. Microbiol. 51, 325-335.

WEBB, M. (1970a). Effects of magnesium defficiency on ribosomal structure and function in certain Gram-negative bacteria. Biochim. Biophys. Acta. 222, 416-427.

WEBB. M, (1970b). Interrelations between utilisation of magnesium and the uptake of other bivalent cations by bacteria. Biochim. Biophys. Acta. 222, 428-439.

WEIDEL, W., FRANK, H. & MARTIN, H.H. (1960). The rigid layer of the cell wall of Escherichia coli strain B. J. gen. Microbiol. 22, 158-166

WEISER, R., ASSCHER, A.W. & WIMPENNY, V. (1968). In vitro reversal of antibiotic resistance by ethylenediamine-tetra acetic acid. Nature. 219, 1365-1366.

WEISS, R.L., KIMES, B.W. & MORRIS, D.R. (1973). Cations and ribosome structure. III. Effect on the 30S and 50S subunits of replacing bound Mg^{2+} by inorganic cations. Biochemistry. 12(3), 450-456.

WEISS, R.L. & MORRIS, D.R. (1973). Cations and ribosome structure. I. Effect on the 30S subunit of substituting polyamines for magnesium ions. Biochemistry. 12, 435-441.

WEISSBACH, A. & HURWITZ, J. (1959). The formation of 2-keto-3deoxyheptonic acid in extracts of E. coli. J. Biol. Chem. 234, 705-709.

WHITE, D.A., ALBRIGHT, F.R., LENNARZ, W.J. & SCHNAITMAN, C.A. (1971). Distribution of phospholipid-synthesising enzymes in the wall and membrane subfractions of the envelope of Escherichia coli. Biochim. Biophys. Acta. 249, 636-642.

WILKINSON, S.G. (1967). The sensitivity of Pseudomonads to EDTA. J. gen. Microbiol. 47, 67-76.

WILKINSON, S.G. (1975). Sensitivity to ethylenediaminetetra acetic acid. In Resistance of Pseudomonas aeruginosa. 1st edition. chapter 5. Ed. Brown, M.R.W., John Wiley:London.

WILKINSON, S.G. & GALBRAITH, L. (1975). Studies of lipopolysaccharides from Pseudomonas aeruginosa. Eur. J. Biochem. In Press.

WILKINSON, S.G., GALBRAITH, L. & LIGHTFOOT, G.A. (1973). Cell walls, lipids and lipopolysaccharides of Pseudomonas species. Eur. J. Biochem. 33, 158-174.

WILLIAMS, R.J.P. & WACKER, W.E.C. (1967). Cation balance in biological systems. J.A.M.A. 201(1), 96-100.

WILLIS, J.B. (1961). Determination of calcium and magnesium in urine by atomic absorption spectroscopy. Anal. Chem. 33, 556-559.

WINSHEL, E.B. & NEU, H.C. (1974). Relation of cell wall lipid content of Serratia marcescens to resistance to antimicrobial agents. Antimicrob. Ag. Chemother. 6(1), 73-75.

YU, K.K-.Y, & JORDAN, D.C. (1971). Cell envelope of Rhizobium sp. mutants, resistant to viomycin. Can. J. Microbiol. 17, 1283-1286.