THE EFFECT OF ENVIRONMENT ON VIRULENCE FACTORS AND RESISTANCE OF MUCOID STRAINS OF *PSEUDOMONAS AERUGINOSA*

A thesis submitted by

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TO MY HUSBAND

AND

MY PARENTS

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M7, a mucoid variant of *Pseudomonas aeruginosa* NCTC 6750 isolated *in vitro*, was compared to its non-mucoid parent strain (WT). Their nutritional requirements showed slight differences. The non-mucoid had a growth rate advantage over the mucoid variant.

M7 retained its mucoid character both in batch and in continuous culture. Using continuous culture, the mucoid organism was shown to produce several phenotypes which showed differences in their susceptibility to carbenicillin, polymyxin and deoxycholate with and without added magnesium. WT produced two major phenotypes which differed in colony size and appearance on nutrient agar.

Both showed differences in the production of the virulence factors, protease, elastase, lipase, phospholipase C and pyocyanine and fluorescent pigments under different nutrient limitations. Growth conditions also affected exopolysaccharide production by M7. In general, phosphate and iron limitation resulted in enhanced production of the extracellular products, while magnesium limited cells produced the least amounts. M7 showed lower amounts of extracellular enzymes in culture supernatants but higher levels of cell bound enzymes.

The sensitivity to polymyxin was markedly affected by nutrient limitation. The resistance of magnesium > sulphate > carbon > phosphate > iron = nitrogen limited cells. The order was the same for both organisms. Sulphate and magnesium limited cells of M7 were more resistant than similarly limited WT. In contrast, iron and nitrogen limited M7 were more sensitive than WT cells. The results suggest differences in cell envelope structures between M7 and WT.

Batch grown M7 was more resistant than WT to the killing effect of blood, but this difference was reduced when cells were grown in nutrient depleted medium. Using chemostat cultures, magnesium limited cells showed highest sensitivity while iron and nitrogen limited cells were least sensitive. Iron limited cells showed an optimum dilution rate at which both M7 and WT cells were least sensitive to the killing effect of blood.

The results demonstrate differences between mucoid and non-mucoid *P. aeruginosa*, and the need for defined growth conditions on work on virulence determinants and pathogenic mechanisms of *P. aeruginosa* and probably other bacteria.

Key words: *Pseudomonas aeruginosa*, mucoid, nutrient depletion, extracellular products, resistance.

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ABBREVIATIONS

ADP	adenosine diphosphate
c	circa
°C	degrees centigrade
C-lim	carbon (glucose) limited growth
DCA	deoxycholate citrate agar
DCAM	deoxycholate citrate agar with magnesium
DPG	diphosphatidylglycerol
EDTA	ethylenediaminetetraacetic acid
F'	fluorescent pigment
Fe-lim	iron limited growth
g/l	grammes per litre
g	relative centrifugal force
hr	hour(s)
KDO	2-keto-3-deoxyoctonic acid
LPS	lipopolysaccharide
ln	logarithms to base e
M	molar concentration
mM	millimolar concentration
µM	micromolar concentration
MA	minimal agar
MLA	milk agar
Mg-lim	magnesium limited growth
min	minute(s)
N	normal solution
NA	nutrient agar
NAD	nicotinamide adenine dinucleotide
NB	nutrient broth
N-lim	nitrogen limited growth
nm	nanometres
nmol	nonomoles
OD470	optical density at 470 nanometres
PC	pyocyanine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PL	phospholipid
P-lim	phosphate limited growth
PMN	polymorphonuclear leucocytes
S-lim	sulphate limited growth

ORIGIN AND SCOPE OF THE WORK

Cystic fibrosis (CF) is the most common fatal inherited sease in the Caucasian population, with an estimated frequency 1 in 2000 live births (Pennington et al., 1979). No single ochemical or structural factor has been proved to provide the derlying defect but many clinical abnormalities have been described cluding exocrine gland dysfunction, thick, sticky secretions, ectrolyte abnormalities, increased tracheo-bronchial mucous ycoproteins and impaired mucociliary transport (di Sant'Agnese al., 1953; Wood, 1979).

CF in the respiratory system manifests itself with initial lmonary lesions and mucous plugging of peripheral airways which on lead to infection. Further obstruction, chronic infection d tissue damage result in loss of pulmonary function eventually ading to death. Although patients with CF presumably have rphologically normal lungs at birth, it has been shown that vivo airway clearance is markedly impaired (Reynolds et al., 75) and this renders the lung susceptible to infection. Chronic lmonary disease is thus the major factor contributing to morbidity d mortality (Wood et al., 1976).

The infecting organisms are greatly influenced by therapy. the 1940s - 1950s, the predominant pathogen was Staphylococcus reus (Iacocca et al., 1963). There was also a high incidence of emophilus influenzae with an increase in Pseudomonas aeruginosa olation as the disease progressed. In the last 10 - 15 years, ere has been a significant decrease in the frequency of isolation Staph. aureus and an increase in P. aeruginosa in the most verely affected patients (Mearns et al., 1972). At present aeruginosa appears to be the most common pathogen isolated from

CF patients. This usually appears during therapy, and once established, is very difficult to eradicate. Other species that have been found in CF include Klebsiella, Escherichia coli, Proteus and Streptococci.

A peculiar feature of P. aeruginosa infection in CF is the isolation in very high frequency of mucoid strains that are rarely isolated from other human sources (Doggett et al., 1966; May et al., 1972). In the cystic fibrotic patient, mucoid P. aeruginosa may be over 60% (di Sant Agnese & Davis, 1976), this is in contrast to approximately 2% in other patients (Iacocca et al., 1963; Doggett et al., 1964; Burns & May, 1968; Doggett, 1969; McCrae & Raeburn, 1974). Mucoid strains are characteristically unstable on subculture. Nutrient depletion, especially iron (Weinberg, 1974; Sussman, 1974; Weinberg, 1978), has been implicated as a body defence mechanism and may well influence mutant selection as well as the production of virulence factors, drug resistance and phagocytosis. It was the object of this study, therefore, to investigate the properties of the mucoid form of P. aeruginosa in comparison with the non-mucoid wild type. The nutritional requirements have been studied in batch culture. Using continuous culture, the effect of different nutrient depletions on the stability and production of extracellular products was examined. The effect of nutrient limitation and growth rate on the sensitivity to antimicrobial agents and to killing by blood has also been investigated.

1. INTRODUCTION

1.1 Mucoid Pseudomonas aeruginosa

.1.1 Introduction

Pseudomonas aeruginosa, the organism of blue pus, is an ctively motile, Gram negative non spore forming oxidase positive acillus. It is widely found in water and soil and often as a ormal inhabitant of the skin and intestinal tract in man and nimals. The organism possesses an exotoxin and other extracellular roducts that are thought to be related to its pathogenicity.

Pseudomonas aeruginosa is an opportunist pathogen and infection usually occurs when several host defences are impaired, for example umoral and cellular immunity, or when anatomical barriers have een breached. Patients with cystic fibrosis (CF), acute leukemia, evere thermal injury and patients with renal transplants are articularly susceptible to P. aeruginosa infection (Aduan & Reynolds, 979).

Colonization and chronic infection of respiratory tract by . aeruginosa is very common in patients with CF (Reynolds et al., 975a; Zierdt & Williams, 1975; Wood et al., 1976). This is ccompanied by high levels of a mucoid variant rarely found in non F patients (Zierdt & Williams, 1975; Doggett, 1966). There is lso a high incidence of mucoid strains present at death of CF atients (Wood et al., 1976; May et al., 1972; Reynolds et al., 975a). A relatively low incidence in older patients has suggested hat patients with mucoid P. aeruginosa die earlier (Wood et al., 976). Although no difference in virulence of mucoid and non mucoid trains was demonstrated (Doggett, 1966), it appears that the mucoid trains may cause destructive changes of pulmonary tissues.

.1.2 Selection of mucoid P. aeruginosa in CF

Almost always, the initial colonization of the CF patient is y the non-mucoid strain of P. aeruginosa, the mucoid is generally ssociated with a more chronic colonization (Doggett et al., 1966; oggett & Harrison, 1969). The reason why the unstable (in vitro) ucoid variants are so frequently in the respiratory tract of CF atients is not known, but is perhaps multifactorial. Several ossible explanations have been proposed (Hoiby, 1977), including:

i) Cross infection between CF patients at the out-patient's linic or during hospitalization through inhalation equipment. here is however, a very low frequency of mucoid variants in other atients. Moreover, parents or relatives of CF patients generally o not harbour mucoid variants and do not produce significant ntibodies against P. aeruginosa (Laraya-Cuasay et al., 1976). hese observations suggest that the CF patients have factors which electively favour mucoid variants (Doggett & Harrison, 1969; artin, 1973; Costerton et al., 1979).

ii) Selective environmental factors: Govan (1975) demonstrated n increased stability of the mucoid strain of P. aeruginosa in ulture media containing various surfactants including dipalmitoyl ecithin, which is present in the lungs. Since mucoid P. aeruginosa s so rarely isolated in other chronic diseases, for the mucoid train to prevail in the CF patients the surfactant in such lungs as to be different from that of normal lungs. Whether this is the ase, it is not yet known.

iii) Selection by antibacterial therapy: although differences n drug sensitivity between mucoid and non-mucoid P. aeruginosa re not always observed (May & Ingold, 1973; Anderson, 1974; arkowitz et al., 1980), it has been demonstrated that some mucoid

isolates have a small but significant increase in resistance to carbenicillin, gentamicin and tobramycin (Govan, 1976; Govan & Fyfe, 1978). By employing this difference, Govan (1976) was able to select mucoid variants from non-mucoid strains of P. aeruginosa in vitro using carbenicillin. The significance of this slightly increased resistance is in the advantage it would give to the mucoid variant in presence of low drug levels.

There is often a difficulty in eradicating P. aeruginosa from the respiratory tract of CF patients by antibiotics to which conventional clinical methods indicate that the culture should be sensitive. Unresponsiveness to the appropriate antibiotic has been ascribed to low antibiotic concentrations caused by low diffusion rates or intrinsic properties of CF sputa. Mark et al. (1971) in a pharmacological study of carbenicillin in CF patients, observed that whilst satisfactory levels of antibiotic could be achieved in serum and urine, maximum levels achieved in the lung, even with six times the recommended dose, never reached the minimum inhibitory concentrations of carbenicillin for most strains of P. aeruginosa. In a similar study on the penetration of antimicrobial agents into bronchial secretions, Wong et al. (1975) found the levels of ampicillin, cephalothin and gentamicin in the tracheo-bronchopulmonary secretions and exudates differed from those in the serum. The amounts, compared to corresponding serums, were 10%, 25% and >40% with ampicillin, cephalothin and gentamicin respectively. An effect of sputum from patients with CF on the activity of some antimicrobial drugs on P. aeruginosa was demonstrated by Davis & Theodore Bruns (1978). Using twelve strains of P. aeruginosa, they showed that CF sputum significantly increased the minimum bactericidal concentration of polymyxin B and neomycin, and had a smaller effect on gentamicin

d tobramycin. The organisms multiplied rapidly in broth alone
in the presence of 50% sputum. Polymyxin B (5 µg/ml) was rapidly
ctericidal to the organism in broth but addition of 50% sputum
mpletely blocked the bactericidal activity. Pooled saliva from
althy adults did not show this activity. Presumably, the
tagonistic effect of CF sputum was by binding the antimicrobial
ug, thus preventing its uptake by the organism. Therefore
rains that were classified as sensitive *in vitro* to polymyxin
d neomycin would all be resistant in CF sputum. Similar binding
antimicrobial agents to purulent material (Bryant & Hammond, 1974)
cus (Saggers & Lawson, 1966; Kellaway & Marriot, 1975) and even
ltration material (Wagman *et al.*, 1975) has been reported.

Cations have been shown to affect the activity of polymyxin
ewton, 1954; Klemperer *et al.*, 1979). The decreased polymyxin
tivity may have been due to the high cation content in CF sputum
Wood *et al.*, 1976).

There is therefore evidence, at least for some drugs, that
latively low drug levels are achieved in the respiratory tract
d sputa of CF patients. These low levels, in combination with
her factors, would favour the more resistant mucoid variant. In
her body fluids, for example serum and urine, the drug levels
uld greatly exceed their minimum inhibitory concentrations and
e small advantage would be of no consequence. This would explain
e relative absence of mucoid strains in other chronic diseases.
coid variants had been reported before the use of antibiotics
onnenschein, 1927) and in patients reported to have had no
tibiotic treatment (Doggett & Harrison, 1969). The results of
van & Fyfe (1978) and Markowitz *et al.* (1980) suggest antimicrobial
lection may be a factor in the emergence of some mucoid strains

f P. aeruginosa, but, as suggested by Markowitz et al. (1980), ultiple factors are probably more important.

iv) Specific selective factors: small particles (0.5 - 3 μ) n air (including bacteria), are deposited in the terminal airway here mucociliary removal and other means of mechanical clearance re thought not to operate. Clearance of microorganisms is initiated y alveolar macrophages which are thought to be the main defence echanisms in the respiratory secretions (Reynolds & Thompson, 1973). ell and complement mediated lysis seem to be less important (Young Armstrong, 1972; Forsgren & Quie, 1974). A number of changes nvolving the defence mechanism in CF patients have been described. hese possibly act together in enhancing the selection of the mucoid ariant.

A pronounced antibody response in most CF patients harbouring he mucoid strain but not in those harbouring the non-mucoid forms ave been described by several workers. Doggett & Harrison (1972) easured the circulating and secretory antibodies in several patients. hey found CF patients had increased levels of circulating and ecretory antibodies of the IgA and IgG classes. Serum precipitins o P. aeruginosa were more common in those patients with the mucoid orms. McCrae & Raeburn (1974) also reported raised levels of mmunoglobulins and presence of precipitins in severe respiratory nfections. Other groups including Hoiby & Axelsen (1973), Burn May (1968) and May et al., (1972) have demonstrated significant ncreases in precipitins against P. aeruginosa in patients with ucoid strains.

The bactericidal effect of serum from normal individuals and rom patients with CF or other disease was studied by Hoiby & Olling 1977). Sera from some CF patients chronically infected with

P. aeruginosa and which had many precipitins against these bacteria, showed low or no bactericidal activity against the patients own Pseudomonas isolate. There was no significant difference between mucoid and non-mucoid variants in their sensitivity to the bactericidal activity of normal serum. Furthermore, a decreased or inhibited opsonic capacity of CF serum to support phagocytosis of P. aeruginosa was reported by both Biggar et al. (1971) and Boxerbaum et al. (1973). The former group found that six out of nine sera of CF patients failed to support normal phagocytosis of P. aeruginosa by rabbit alveolar macrophages. Normal phagocytosis of Pseudomonas by blood polymorphonuclear leucocytes was unaffected. They postulated that cystic fibrosis patients may have a quantitative or functional defect of IgG antibodies, specific for Pseudomonas, and possibly of importance in the pathogenesis of their pulmonary disease. A selective inhibition of phagocytic activity of rabbit alveolar macrophages against P. aeruginosa by fresh CF serum was also observed by Boxerbaum et al. (1973). Not only were fewer bacteria engulfed, but also of the engulfed bacteria, few were destroyed. This effect appeared to be specific for P. aeruginosa. In addition, the exopolysaccharide formed by this organism has been shown to be antiphagocytic (Schwartzmann & Boring, 1977; Costerton et al., 1978) as described in section 1.2.1.3.

The pronounced humoral immune response, opsonic defect or inhibition of antibodies of the CF respiratory secretions and the antiphagocytic properties of the mucoid substance, led Hoiby & Axelsen (1973) to propose that these play a major role in favouring mucoid strains. It is generally known that microbial immunogens have to pass through the mucous epithelium into the lamina propia to evoke considerable immune response. The number of different

ntibody responses produced by mucoid strains suggest that these rganisms cause active tissue damage in the lungs. It appears that he non-mucoid strain of P. aeruginosa represents surface colonization n this disease, whereas the mucoid strain possesses properties nabling it to invade the respiratory lamina propia. The non-mucoid train may enter the lamina propia after tissue damage, but these re readily phagocytized by macrophages. Although the patients era may not promote immune elimination of the bacteria, the humoral esponse possibly keeps the infection localized in the respiratory ract (Hoiby, 1974; Schiotz & Hoiby, 1975).

Postmortem examination of lung tissue from CF children with . aeruginosa have shown that the bacteria occur in microcolonies, hich sometimes appear to adhere to the alveolar wall (Costerton t al., 1979). In addition, the exopolysaccharide of mucoid cells an cause increased viscosity of the bronchial secretions. This ay lead to worsening of both the small airway obstruction and the bnormal mucociliary clearance mechanism. Furthermore, the mucoid aterial probably protects the bacteria from the opsonizing effect f the antibodies and from complement lysis. Under these conditions, he mucoid strain would appear to be more pathogenic than the non-ucoid type (Kilbourn, 1970).

.1.3 Types of P. aeruginosa isolated from patients with cystic fibrosis

Various serotypes, pyocin types and phage types of P. aeruginosa ave been isolated from cystic fibrotic patients, although some ypes or groups may predominate in any single material (Doggett et l., 1966; William and Govan, 1973). Diaz et al. (1970) isolated erogroups II, III and IV while Zierdt and Williams (1975) found ostly Homma serotype 8 as the most common in this disorder. The

mucoid and non-mucoid variants of P. aeruginosa isolated from the same specimen are usually of the same type and have the same biochemical properties (Zierdt & Schmidt, 1964; Bergan & Hoiby, 1975).

Due, in part, to the fact that most serotyping, pyocine typing and phage typing revealed a single type for both mucoid and non-mucoid isolates, it has been generally assumed that cystic fibrotic patients are colonized by only one strain of P. aeruginosa. Recently there have been some reports that suggest that cocolonization of cystic patients by two or more strains of P. aeruginosa may be more frequent than initially thought (Diaz et al., 1970; Bergan & Hoiby, 1975; Seale et al., 1979). Seale et al. (1979) showed a high frequency of heterogeneity in their isolates, on the basis of differences in antibiotic resistance, colony morphology, pigmentation and serotype. Two or more isolates with different but stable susceptibilities to carbenicillin, gentamicin, streptomycin, tetracycline, chloramphenicol and sulfamethoxazole plus trimethoprin were detected in 38% of the sputa. Two or more serologically distinct strains were present in about 50% of the sputa. In agreement with other workers (Bergan & Hoiby, 1975) they found that the non-mucoid derivatives of mucoid isolates are also of the same type as the parent mucoid variant.

P. aeruginosa is known to have a diversity of colonial types including rough, smooth, mucoid, gelatenous, enterobacter (rough-smooth) and dwarf colonies (Zierdt & Schmidt, 1964; Wahba & Darrell, 1965; Maybury et al., 1979; Thomassen et al., 1979). Zierdt and Schmidt (1964) observed that 66% of cultures showed more than one colony type. These were capable, at varying rates, of giving rise to large numbers of phenotypes, this occurring more

frequently in broth (submerged) than surface culture. The phenotypes differed in their pigmentation and some had increased antibiotic sensitivities. Bacteriophage typing confirmed the identity of the strain of P. aeruginosa and also revealed differences in phage patterns among phenotypes from the same parent while retaining the major patterns of the parent. They suggested that P. aeruginosa is capable of undergoing dissociation in vivo and in vitro and this may give false impressions of the presence of different bacterial species. The isolation in CF patients of various morphological varieties of non-mucoid P. aeruginosa has also been suggested as arising from one dissociating strain (Thomassen et al., 1979).

Usually two non-mucoid colonial variants are commonly observed from a culture of P. aeruginosa. These have been described as a large, greenish, flat, moist colony producing a green pigment and a small round khaki-green, rough colony with a purple-green pigment (Shionaya & Homma, 1968; Lui, 1961; Brown, 1977). These were shown to have no differences in serological specificity but had a difference in bacteriophage and pyocin sensitivities (Shionaya & Homma, 1968). Brown (1977) observed that the small colony type was the most stable form in normal culture medium and also the most resistant to benzalkonium chloride. The colonial difference was discernible after long incubation.

1.1.4 Stability of mucoid P. aeruginosa

Mucoid isolates either from patients with cystic fibrosis or isolated in vitro are very unstable, quickly reverting to non-mucoid forms on repeated subculturing (Govan, 1975; Martin, 1973; Zierdt & Schmidt, 1964). Reversion to the non-mucoid form was curtailed by incooperation of surfactants in the maintenance media (Govan 1975).

The concentration of dipalmitoyl-lecithin which gave maximum stabilization was approximately the same as that found in normal lungs. This suggested a possible role of surfactants in stability of mucoid forms *in vivo*. Increased stability was also described in nutrient broth cultures when they were suitably agitated (Govan *et al.*, 1979). In addition to the increased stability in shaken cultures, they were also able to show that the non-mucoid revertants had a growth rate advantage over the mucoid parent strain. This advantage was more pronounced in unshaken than in shaken cultures. They suggested that the instability of mucoid *P. aeruginosa* also results from spontaneous mutation back to the non-mucoid form, which then has a selective growth advantage in unshaken cultures. In a shaken culture, physical dispersion of cell bound polysaccharide resulted in improved oxygen and nutrient uptake. The polysaccharide surrounding the cell probably acts as a diffusion barrier to oxygen transfer. In unshaken cultures, this barrier results in a decreased growth rate for the mucoid variant. A higher growth rate is achieved in shaken cultures due to the increased dissolved oxygen concentration.

Little is known about the influence of nutrient depletion or of growth rate either on stability or on production of exopolysaccharide of mucoid *P. aeruginosa*. Using continuous culture, the mucoid form was found to be relatively stable when grown under sulphate, magnesium and iron limitation (Jones *et al.*, 1977). Phosphate, nitrogen and carbon limitation resulted in reversion to non-mucoid forms. Similar instability under nitrogen, carbon and potassium limitation, and increased stability under sulphate limitation has also been reported for mucoid strains of *Xanthomonas juglandis* and *Xanthomonas campestris* (Evans *et al.*, 1979). Magnesium and phosphate limitation

nowed intermediate stabilities, with variants occurring after
nree week's growth at D = 0.03 hr^{-1}. Mian *et al.* (1978), also
eported instability under nitrogen limitation of mucoid *P. aeruginosa*.
ney found that the rate of increase of non-mucoid organisms in
n initially totally mucoid, ammonium limited continuous culture,
o be consistent with a mutation to a subsequently advantaged
on-mucoid form.

1.2 Extracellular Products of P. aeruginosa

Pseudomonas aeruginosa produces a large number of extracellular products which include exotoxin A, protease, elastase, phospholipase C, phytotoxic factors, pigments, hydrocyanic acid and exopolysaccharide. Some of these products, for example the proteolytic enzymes and exotoxin A have been shown or implicated to contribute to the virulence of the organism. An enterotoxin (Kubota & Liu, 1971), a luecocidin (Scharmann, 1976a; 1976b; Lutz, 1979) and the LPS have also been shown to have some toxicity, but their role in the pathogenicity of P. aeruginosa is difficult to assess or too low to be of much significance.

1.2.1 Exopolysaccharide

Microbial extracellular polysaccharides (exopolysaccharides) are surface structures of the procaryotic cell that surrounds the bacteria as a capsule or slime. The capsule is usually a discrete covering on the cell surface that is morphologically well defined and often stains negatively with capsule stains. The slime does not form a distinct layer on the cell surface and can disperse readily from the bacteria. The exopolysaccharides range in complexity of their chemical structures from homopolysaccharides with one or more types of linkage to heteropolysaccharides containing several different monosaccharides. In some cases non-sugar residues may be present (Sutherland, 1977). The exact nature of the exopolysaccharide produced by an organism depends both on the species and on the physiological state of the microorganisms. These carbohydrate polymers are thought to serve as receptors for bacteriophages and bacteriocins (Smith, 1977), to determine the adhesion of bacterial cell to surfaces (Gibbons & Van Houte, 1975; Costerton et al., 1978; 1979), to establish symbiotic or competitive relations with other

cells and to determine the susceptibility of the organism to host defence mechanisms (Smith, 1977; Costerton et al., 1978).

1.2.1.1 Composition of P. aeruginosa exopolysaccharide

The majority of non-mucoid isolates of P. aeruginosa are not copious producers of exopolysaccharide except under specific cultural conditions. The reports on composition of exopolysaccharide from P. aeruginosa have shown some variations. The exopolysaccharide produced by P. aeruginosa was composed entirely of mannose with small amounts of protein and nucleic acid (Eagon, 1956; Eagon, 1962). Using P. aeruginosa isolated from cystic fibrotic patients, Doggett et al. (1964) obtained exopolysaccharide material that was an insoluble elastic gel in ethanol or ethanol/benzene mixtures. The capsular mucus of P. aeruginosa from non cystic fibrotic patients did not produce the insoluble material in ethanol and produced only a small amount of non-fibrillar precipitate in ethanol/benzene mixtures. The ethanol insoluble material had fucose, mannose, galactose, glucose and certain other hexosamine together with two unidentified compounds. The unidentified compounds were not present in exopolysaccharide from P. aeruginosa of non cystic fibrotic patients.

Bartell et al. (1970) described a slime polysaccharide from P. aeruginosa containing rhamnose, glucosamine, galactosamine, glucuronic acid and glucose. Brown et al. (1969) isolated from eight different strains of P. aeruginosa. a polysaccharide which contained mainly glucose and mannose. Small amounts of hyaluronic acid, protein and contaminating intracellular deoxyribonucleic acid were also present.

The disparities in exopolysaccharide composition of non-mucoid strains of P. aeruginosa suggest a possibility that different

strains produce different exopolysaccharides. The various methods of isolation and purification and growth conditions (see also section 1.2.4.1) may also significantly affect the results.

In contrast to the disparities shown for non-mucoid exopolysaccharides, there is agreement on the composition of exopolysaccharide from mucoid variants. The exopolysaccharide produced by both mucoid P. aeruginosa isolated from natural habitat and induced in vitro has been shown to be a partially acetylated alginate-like polymer, first reported by Linker and Jones (1964). The studies on its composition, infra-red spectra and alginase digestion (Linker & Jones, 1966) confirmed the resemblance to alginic acid. The polysaccharide of Pseudomonas contains O-acetyl groups which are not present in alginates, and the acetylated polymers did not precipitate in acid medium. Similar polysaccharides containing O-acetylated groups have been isolated from Azotobacter vinelandii (Gorin & Spencer, 1966; Jarman, 1979).

The production of alginate-like polysaccharide by P. aeruginosa has been substantiated by several other workers (Carlson & Matthews, 1966; Evans & Linker, 1973; Murakawa, 1973; Martin, 1973; Mian et al., 1978). Using a non-mucoid type P. aeruginosa IFO 3445, and a mucoid type from a cystic fibrotic patient, Murakawa (1973) reported that the exopolysaccharide from the non-mucoid consisted of DNA and a small amount of protein while the mucoid exopolysaccharide was composed of polyuronic acid. Martin (1973) reported very similar results. Mucoid strains produced two very distinct precipitates when the precipitating mixture was added slowly. The first precipitate (A) was stringy and could be removed by a rotating glass rod. This contained considerable amounts of uronic acids (17.5 - 27%). The second precipitate (B) which was formed after further addition of

the precipitating mixture, was flocculent and could be removed by centrifugation. This had little uronic acid (<2.5%) and showed the same O-specificity as the whole cells.

Evans and Linker (1973) using mucoid strains obtained from chronic infections including cystic fibrosis, were able to show similarities and differences between polyuronides from Pseudomonas of various sources. They also showed the differences between them and Azotobacter vinelandii exopolysaccharide and the alginates. They all had the same major linkages in having β-configuration as well as the 1→4 position of uronidic linkages. The differences are in the acetyl content (none in alginates) and in the mannuronic to guluronic acid ratios. The ratio can vary with strain (Linker & Jones, 1966) or with the amount of anions and cations present in the medium (Larsen & Haug, 1971). The degree of acetylation is also variable, for example the O-acetyl content of P. aeruginosa varied with bacterial strain between zero and 13.4 $^{w}/_{w}$ (Evans & Linker, 1973).

.2.1.2 Control of exopolysaccharide production

The mechanism of control of exopolysaccharide synthesis under conditions of optimal yield are not clear. The control of production probably involves various aspects which may include substrate uptake and polysaccharide synthesis and release from the cell (Sutherland, 1979). Substrate is committed to either anabolic or catabolic processes. The extent to which substrate is converted into polymer to give high yields suggests that little is catabolized or converted into cell material other than exopolysaccharide. As sugar nucleotides are the immediate precursors of the oligosaccharides, they represent a possible site of control for example through

nthesizing enzymes. Another key intermediate in exopolysaccharide nthesis where control can be applied is the lipid-linked oligo- ccharide. In Gram negative bacteria, the isoprenoid lipids are ed to form intermediates in the formation of peptidoglycan, lipo- lysaccharide and exopolysaccharide. All these polymers are mposed, at least partially, of repeating glycan units and are cated outide the cytoplasmic membrane. Sutherland (1977, 1979) s suggested that, in those species where exopolysaccharide is served late in the logarithmic or in the early stationary phase, nthesis might be controlled by limitation of the available oprenoid lipid. In the growing cell, synthesis of peptidoglycan d lipopolysaccharide would have priority over the exopolysaccharide, d therefore, no isoprenoid lipid would be available until the rst two are satisfied. It was argued that at lower temperatures, ss isoprenoid lipid is needed for peptidoglycan and lipopolysaccharide nthesis, both of which occur at a reduced rate, and therefore it available for exopolysaccharide synthesis. This would probably ply to those organisms showing increased exopolysaccharide synthesis sub-optimal growth temperatures.

2.1.3 Role of exopolysaccharide in *P. aeruginosa*

The expression of alginate production in *P. aeruginosa* occurs der specific conditions, for example in cystic fibrotic patients under specific growth conditions (Brown *et al.*, 1969). The fact at most strains of *P. aeruginosa* contain the genetic information cessary for alginate synthesis, would indicate that it has some portance in the survival of the organism. There have been several ggested roles of bacterial exopolysaccharides. Costerton *et al.* 978) and Lam *et al.* (1980) suggested that the exopolysaccharide

is involved in the bacterial-host interactions. Fibres of polysaccharide or branching sugar molecules, extend from the bacterial surface to form a felt-like glycocalyx surrounding an individual cell or colony of cells. The glycocalyx attaches the bacteria onto the host thus preventing its removal. The pronounced specificity of some bacteria and viruses to attack a particular host tissue may well be explained by the specificity of the glycocalyx of the host cells.

Apart from positioning the bacteria, the fibres may also conserve and concentrate the digestive enzymes released by the bacteria and direct them against the host cell (Costerton et al., 1978). The polysaccharide, being negatively charged, acts like an ion-exchange resin and can bind nutrient ions and molecules that are in the environment or are produced by the bacterial degradation. This helps the availability of nutrients for the bacteria. In a similar way, the polysaccharide may be involved in the trapping and detoxification of harmful ions and molecules in the environment (Den Dooren de Jong, 1971).

The polysaccharide may also have a protective role against body defences (Schwartzmann & Boring, 1971; Roe & Jones, 1974; Smith, 1977; Costerton et al., 1978). When single uncoated P. aeruginosa cells were introduced into the lung of a rat, the bacterial were quickly phagocytized by the rat's white blood cells (Costerton et al., 1978). The white blood cells were unable to eradicate the same bacteria when they were enclosed in a glycocalyx. Similarly, an antiphagocytic effect of exopolysaccharide was demonstrated by Schwartzmann and Boring (1971). Using cells of P. aeruginosa, E. coli and Staph. aureus, they observed a rapid phagocytosis by rabbit polymorphonuclear leucocytes in the absence

f slime. Addition of relatively small amounts of slime from ucoid P. aeruginosa to the bacteria leucocyte mixture, resulted n inhibited phagocytosis as measured by phagocytic killing of he organisms. They suggested that the antiphagocytic effect of xopolysaccharide was probably due to an alteration or masking of urface charges necessary for phagocytosis in vitro and thus slime ay be considered a virulence factor in the respiratory tract of ystic fibrotic patients. It has been speculated that the mucoid ell is probably protected against phagocytosis by the thick alginic cid coat which might resist rapid enzymatic degradation as human issue is not known to possess alginases (Doggett & Harrison, 1972).

The exopolysaccharide probably functions as a general protective arrier employing all or some of the above properties.

The exact role of the exopolysaccharide in the virulence of he organism is however unclear, since large amounts of exopoly-accharide do not appear sufficient to make the organism more irulent. For example, a large number of mucoid strains of . aeruginosa have been isolated from the respiratory tracts of ystic fibrotic patients, but they do not show higher virulence han the non-mucoid type when injected intraperitonially into mice Liu, 1979). The exopolysaccharide has none-the-less been emonstrated to have its own toxicity (Liu et al.,1961; Sensakovic & artell, 1974; Lynn et al., 1977). Intraperitonial injection of urified slime glycolipoprotein (GLP), devoid of any accompanying rotein and different from lipopolysaccharide, induced leucopenia nd death in mice, showing an effect similar to that obtained by nfection with viable organisms (Lynn et al., 1977). There was lso a decrease in polymorphonuclear leucocytes (PMN) and immunization ith the glycolipoprotein enhanced survival of the animals. The

neutropenia was attributed to removal of PMN from the circulation by formation of a PMN-GLP complex. Earlier, Sensakovic and Bartell (1975) had obtained results suggesting that the carbohydrate moiety of GLP was responsible for the antigenic and phagocytosis inhibition property while the lipid component was probably associated with the leucopenia and lethal effects.

In a similar study, Dimitracopoulos et al. (1974) observed that exopolysaccharide inhibited haemagglutination and this activity was unaffected by heating at 100°C for 15 min. Furthermore, exopolysaccharide was rapidly disseminated into peripheral circulation and a rapid coating of cells by the polysaccharide occurred both in vivo and in vitro. Production of exopolysaccharide by P. aeruginosa in vivo was also observed (Dimitracopoulos et al., 1974; Marrie et al., 1979).

The toxic effects described, the inhibition of phagocytosis and intracellular killing observed in experimental infections (Schwarzmann & Boring, 1971; Roe & Jones, 1974; Sensakovic & Bartell, 1974) and the immunogenic properties of exopolysaccharide from both mucoid and non-mucoid strains of P. aeruginosa (Alms & Bass, 1967a, 1967b; Maresz-Babczyszyn & Sokalska, 1979) tend to suggest that the exopolysaccharide does play a role in the toxicity and pathogenesis of P. aeruginosa. The major role of the exopolysaccharide in the pathogenicity of this organism is probably in the protection of the organism from destruction and thus indirectly enhancing the activity of the other virulence factors. Liu et al (1961) suggested that the toxicity of crude slime was probably a reflection of the presence of exotoxins. This is probably partly true. The presence of large amounts of exopolysaccharide would concentrate such factors as proteases around the immediate environment of the

organism where they would be most beneficial to the organism. Furthermore, exopolysaccharide has been implicated in the release of exolipase from both Serratia marcescens and P. aeruginosa (Winkler & Stuckmann, 1979; Winkler et al., 1979; Bohne & Winkler, 1979). The later group incubated P. aeruginosa cells in the presence and absence of glycogen, hyaluronate or the organism's own exopolysaccharide. They observed enhanced yield of exolipase activity by a factor of 5 to 10. The exopolysaccharide derived from the growth medium of P. aeruginosa was more effective than glycogen. It has been suggested that the polysaccharide stimulates formation of exolipase by detaching exolipase molecules from temporary storage sites on the surface of the cells (Winkler & Stuckmann, 1979). This is achieved either by competition for the site or by changing the conformation of the exolipase. Alternatively, the polysaccharide may protect nascent chains of exolipase from proteolysis during secretion (Braatz & Heath, 1974).

1.2.2 Extracellular proteins

1.2.2.1 Exotoxin A

Exotoxin A is the most toxic and lethal product produced by P. aeruginosa and whose role in virulence of P. aeruginosa is well documented (Liu, 1979; Pavlovskis et al., 1978; Pavlovskis et al., 1977; Saelinger et al., 1977; Snell et al., 1978). It is produced by a majority of P. aeruginosa strains in vitro (Pollack et al., 1977) and serological evidence suggests that it is also released in vivo in most clinical infections (Pollack et al., 1976; Pollack & Taylor, 1977). The toxin inhibits protein synthesis (Pavlovskis et al., 1978) and has been demonstrated to interfere with bacterial clearance. The response of animals to injection with exotoxin A

occurs rapidly. In the dog, it causes a rapid rise in portal blood pressure followed by a decline in peripheral blood pressure. The animals eventually die of shock. Killed animals show necrosis of liver and in some, lung haemorrhage and necrosis of kidney tubules may also be present.

The mode of action of exotoxin A has been elucidated (Inglewski & Kabat, 1975; Inglewski et al., 1977; Pavlovskis et al., 1978). The toxin catalyses the transfer of the ADP-ribosyl moiety of NAD to form a covalent linkage with elongation factor 2 (EF-2). The active EF-2 levels are reduced and thereby synthesis of protein at ribosomal level is inhibited. The impairment of phagocytosis by macrophages after exposure to low doses of toxin (Pollack & Anderson, 1978) probably arises from the impaired protein synthesis. Macrophages play a protective role in pulmonary infections (Reynolds & Thompson, 1973) and polymorphonuclear leucocytes are thought to represent the main cellular defence against pseudomonas infections (Young & Armstrong, 1972). The cytopathic effects of exotoxin A on these cells might significantly enhance pathogenesity of P. aeruginosa.

1.2.2.2 Proteases

One of the best known characteristics of P. aeruginosa is its proteolytic activity. This was first reported by Morihara (1956) who used liquefaction of gelatin as a marker of proteolytic activity. It has since been shown that there are at least three distinct types of proteases produced by P. aeruginosa, designated fractions I, II and III (Morihara, 1964; Kreger & Griffin, 1974; Wretlind & Wadstrom, 1977). Protease I is produced in very small quantities and is considered to be of no major significance (Morihara,

1964). Fraction III protease is a protein with molecular weight f 48,400, an isoelectric point below 4.08 and an optimum temperature or activity of 60°C (Liu, 1979).

The fraction II which shows elastase activity is protein of olecular weight of 39,500, an isoelectric point of 5.9 and an ptimum pH of activity at about 8 (Morihara et al., 1965). The enzyme hows a broad activity against various substrates including elastin, asein, haemoglobin, egg albumin and fibrin. P. aeruginosa strains hich are elastase positive produce all three enzymes. Elastase egative strains produce only fractions I and III (Morihara, 1964).

The production of proteolytic enzymes is of obvious importance n the ecological role of these organisms. The enzymes may play role as agents of saprophytic protein solubilization in the nvironment and the production of such toxic exocellular proteins ay explain the success of P. aeruginosa as an opportunist pathogen. here are a large number of reports on the biological activities f P. aeruginosa proteases (Fisher & Allen, 1958; Liu, 1974, 1966a; edberg et al., 1969; Muszynski & Kedzia, 1974; Kreger & Griffin, 974; Kawaharajo et al., 1974, 1975; Meinke et al., 1970; Kreger & ray, 1978). Initially the role of proteolytic enzymes as virulence actors was in some dispute. Hedberg et al. (1969) observed no orrelation of virulence with the enzymes while Liu and Hsieh (1969) ound a negative correlation, where high protease producers were ess virulent than low producers. They suggested that high titres f protease resulted in destruction of exotoxin A. In contrast oth Muszynski (1973) and Al-Dujaili (1976) found a correlation etween protease production and virulence. The reasons for the onflicting reports may be several. It has been shown that the mount of protease production is influenced by experimental conditions

Morihara, 1964; Wretlind & Wadstrom, 1977, Jensen et al., 1980a). Also differences in susceptibility to pseudomonal infections exist within the same species of animal (Kawaharajo & Homma, 1977) and normal uncompromised animals are highly resistant to pseudomonal infection. Most experimental models used to study psuedomonas pathogenesis are inadequate substitutes for clinical infections since unrealistic large doses of organisms or virulence enhancing factors for example mucin are required. In addition, use of severely traumatized animals makes interpretation of results difficult since the injury may contribute to death.

The use of mouse burn infection has alleviated most of these problems. This model, while compromising the host without trauma, closely resembles human burn wound sepsis. Using such a model, Pavlovskis and Wretlind (1979) showed that in experimental P. aeruginosa infection in mice, protease enhances the virulence of the organism. The average LD_{50} for mice infected with a protease (elastase) producing organism was at least one log lower than LD_{50} of protease deficient mutant. Addition of purified protease to an infecting inoculum of a protease negative strain reduced the LD_{50}. There were also fewer viable bacteria in the blood of mice infected by a protease negative strain than the positive strains. Inoculation of both protease positive and negative organisms, caused an increase in the number of protease negative organisms while protease positive remained static thus suggesting that protease contributed to the invasiveness of the organism. Pavlovskis and Wretlind (1979) also showed that surivival of mice infected by protease producing Pseudomonas was enhanced by use of antiprotease serum. Such serum had no effect of mice infected with protease negative bacteria. Similar results were obtained by Holder and Haidaris (1979) who

showed that the mortality of burned mice infected with non-lethal inocula of toxin producing but protease negative *P. aeruginosa* was increased significantly when a variety of solutions containing protease and elastase were added. Injection of enzyme solutions alone were non-lethal. Injection of α-macroglobulin, which was shown to inhibit proteolytic activity, with a strain of *P. aeruginosa* producing both enzyme and toxin, caused a significant delay in mortality when compared to a control group. Enhanced activity of exotoxin A by protease was also shown by Snell *et al.* (1978).

Destruction of corneal tissue has been demonstrated as one of the pathological effects of proteases (Fisher & Allen, 1958; Gray & Kreger, 1975; Kreger & Gray, 1978). Kreger and Gray (1978) showed that *Pseudomonas* protease was responsible in part for the rapid and extensive liquifaction necrosis observed in pseudomonas-induced keratitis. The proteases elicited the severe corneal damage by causing loss of corneal proteoglycan ground substance. This resulted in dispersal of undamaged collagen fibres, weakening of corneal stroma and subsequently led to corneal perforation by the anterior chamber pressure. Later the same workers (Gray & Kreger, 1979) presented results that supported the idea that *in vivo* production and activity of *P. aeruginosa* proteases is important, at least in part, in eliciting lung damage characteristic of pseudomonal pneumonia. They showed that purified protease caused intra-alveolar haemorrhage, injury and necrosis of alveolar septal cells and infiltration of mononuclear cells in the lung. Furthermore, intranasal administration of *P. aeruginosa* proteases into mice was observed to produce focal and confluent haemorrhagic lung lesions identical to those seen in human with *P. aeruginosa* pneumonias (Fetzer *et al.*, 1967; Meinke *et al.*, 1970; Liu, 1966a; Shimizu *et al.*,

1974). Haemorrhagic lesions into skin have also been attributed to these enzymes (Liu, 1966a).

Reports on collagenolytic activity of P. aeruginosa proteases have been contradictory (Morihara, 1956; Carrick & Berk, 1975; Wretlind & Wadstrom, 1977). The controversies have probably been due to different definitions of collagenase. Proteases of P. aeruginosa appear not to act as classical collagenase which liberates proline or hydroxyproline from collagen. These enzymes attack native collagen only on the non-helical region which makes up about 10% of the molecule (Jensen et al., 1980a). The destruction of arteriola elastin reported by both Mull & Callahan (1965) and Lawaharajo et al. (1975) but not observed by Meinke et al. (1970) or Oakley and Banerjee (1963) have led to the suggestion that pseudomonal elastases do not readily digest native elastin in vivo. Route of administration may also be important. Normal rabbit serum was observed to inhibit proteases in vitro (Fisher & Allen, 1958). It is possible that intravenous administration of elastase may result in its inhibition or destruction and this barrier is by-passed in intranasal administration.

The precise mechanism by which protease exerts its toxic action is not known. Pavlovskis et al. (1978), working with non-toxigenic protease producing strains, showed that protein synthesis inhibition by protease during an infection was minimal and that very large doses were required to kill mice as compared to toxigenic strains. Further studies (Pavlovskis & Wretlind, 1979) suggested that protease may be important in overcoming the hosts initial defence mechanisms. This may be accomplished either by proteolytic activity which provides additional nutrients or by destruction of anatomical barriers. One possible target could be at the level of

acid mucopolysaccharide that constitutes the cell coat, as supported by the results of Leake et al. (1978). This group observed that purified protease from P. aeruginosa caused agglutination and vacuolization of rabbit alveolar macrophages after incubation. Macrophages that had adhered to glass coverslips when incubated in the presence of active enzymes showed thread-like material extending in all directions and joining adjacent and distant cells. These changes did not occur in the presence of enzymatically inactive proteases. They suggested that the purified proteases induced changes of the cell membrane resulting in clumping, vacuolization and increased stickiness of the alveolar macrophages.

The destruction of an anatomical barrier was further indirectly supported by Wretlind and Kronevi (1978) who found no difference in LD_{50} between protease producing P. aeruginosa and protease deficient mutant when the bacteria were injected in with mucin or in cyclophosphamide treated mice. In both cases, the host's initial defenses had been overcome either by lowering with cyclophosphamide or by increasing host sensitivity using mucin. In such cases any advantage a protease-producing strain might have had was eliminated.

Inactivation of vital plasma components may also be involved in the action of proteases. Human plasma α_1 proteinase inactivator (α_1PI) is genetically controlled and its deficiency has been associated with pulmonary emphysema. Morihara et al. (1979) showed that elastase of P. aeruginosa is a very potent inactivator of α_1PI and they suggested that elastase may disrupt the delicately regulated balance between serine proteinases and α_1PI in body tissues. Assuming therefore that the pseudomonal proteases are produced and can inactivate α_1PI in vivo during pseudomonal disease,

the loss of α_1PI activity may permit the endogenous serine proteases to cause tissue destruction. Schultz and Miller (1974), observed that purified elastase inactivated over 90% of all nine complement components. The enzyme inactivated fluid phase complement components, generated and destroyed the chemotactic fragment from C5 and reduced the ability of polymorphonuclear leucocytes to phagocytize sensitive pseudomonas because of its action on cell bound C3.

The evidence so far has indicated that both the proteolytic enzymes and exotoxin A are required for full expression of virulence of P. aeruginosa infections. The report of elevated antibody titres to pseudomonal proteases and exotoxin A in cystic fibrosis pulmonary infections (Klinger et al., 1978; Pollack et al., 1976) indicate that these factors serve as significant virulence factors in vivo in these chronic infectious states. The activity of proteases probably involves a combination of several factors. Although the proteases have a low toxicity compared to exotoxin A (Liu, 1979) the demonecrotic and cornea damaging effects suggests a role in localized infections where serum protease inhibitors may not be present in sufficient amounts. Vasil et al. (1977) reported the production of exotoxin A as an inactive exoenzyme which was activated by Pseudomona proteases. It seems likely therefore that proteases may serve to activate more toxic factors. The proteases probably act more as virulence enhancing factors rather than major virulence factors on their own.

1.2.2.3 Phospholipase

Pseudomonas aeruginosa produces two types of haemolysins, one heat resistant and one which is heat labile (Liu, 1957). The heat labile haemolysin was found to be phospholipase C (Esselmann

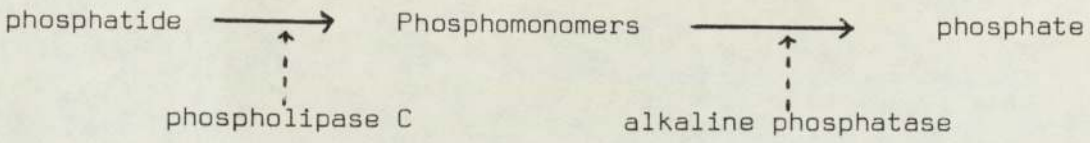
& Liu, 1961) and the two types are usually produced together in an environment of very low phosphate and high carbohydrate (Liu, 1964). The heat stable haemolysin, a glycolipid, enhances the phospholipase C activity due to its detergent-like activity in solubilizing phospholipids (Kurioka & Liu, 1967). When a culture of P. aeruginosa produces phospholipase C and the glycolipid, it also produces a phosphatase that splits inorganic phosphate from phosphorylcholine, and the three substances appear to co-operate to yield phosphate from lecithin.

When phospholipase C was injected into the skin of rabbits and guinea pigs, it induced necrosis of the area and a central zone of erythema, these effects taking longer to reach maximum effect than those produced by proteases. When injected intraperitonially, it produced hepatic necrosis and adematous lungs (Liu, 1966a). Phospholipase activity was, however, not demonstrated in heart blood of animals that had died after multiple injections of P. aeruginosa (Liu, 1966b). Since many strains of P. aeruginosa are sensitive to serum (Muschel et al., 1969) and are therefore unlikely to achieve a high amount of phospholipase in vivo, this enzyme is probably not a lethal factor in infection by P. aeruginosa in skin or bloodstream of normal men and animals.

Phospholipase most probably plays a significant role in the pathology of pneumonia due to P. aeruginosa. The alveolar membrane is usually covered with a surfactant, a substance that functions to reduce tension of alveoli and prevent atelectasis (collapse). The main component is lecithin, and therefore, the production of phospholipase may result in destruction of pulmonary surfaces with subsequent atelectasis. The production of phospholipase by P. aeruginosa has, in fact, been stimulated by the use of pulmonary

surfactant as substrate. Concomitant production of the glycolipid would further enhance the destruction of the surfactant. Since phospholipase can also exert a necrotizing effect on lung tissue itself, the combined effect of destruction of surfactant and lung tissue can lead to considerable damage.

Phospholipase C is probably produced by P. aeruginosa as a phosphate scavenging mechanism. Under phosphate depletion, the phospholipase C genes may be derepressed and synthesis and excretion initiated. Phosphate is therefore possibly supplied by the reaction:



1.2.3 Pigments

1.2.3.1 Pyocyanine

Pyocyanine (1-hydroxy-5-methylphenazine) is the major phenazine pigment produced by P. aeruginosa. Some strains produce other types of phenazine pigments such as α-carboxylic acid, chlororaphine and pyomelanin. Although several different organisms can produce the same compound, under appropriate cultural conditions, a particular organism can produce a variety of phenazines (Kanner et al., 1978; Byng et al., 1979; Propst & Lubin, 1979). Interest in pyocyanine derives from its intense colour, on some claims of its toxicity and on its well established antibiotic properties.

There have been a few claims on the toxicity of pyocyanine. This pigment was reported to be toxic to skin cells and leucocytes cultured in vitro (Cruickshank & Lowbury, 1953) and to inhibit mouse liver mitochondria (Armstrong et al., 1971). The toxicity of pyocyanine is however so low that large amounts can accumulate

in live animals without any harm (Liu, 1979). Although 50% of clinical isolates of P. aeruginosa were shown to produce this compound (Knight et al., 1979) there is no conclusive evidence of toxicity or of large production of pyocyanine in vivo.

The antimicrobial properties of phenazines have long been known (Waksman & Woodruff, 1942; Young, 1947; Schoental, 1941; Knight et al., 1979; Hassan & Fridovich, 1980). A complex extract of P. aeruginosa called pyocyanase, whose active fraction was found not to be an enzyme, but α-oxyphenazine (Schoental, 1941) had been in use in treating bacterial infections long before penicillin. Pyocyanine inhibits a wide array of bacterial species including E. coli, Staph. aureus, Mycobacter smegmatis and Proteus species (Young, 1947; Knight et al., 1979).

The antibiotic action of pyocyanine has been proposed to be due to the toxicity of O_2^- and H_2O_2 produced in increased amounts in its presence (Hassan & Fridovich, 1980). Pyocyanine was shown to increase the cyanide resistant respiration of E. coli and increased biosynthesis of superoxide dismutase and catalase. It was inferred that pyocyanine diverted electrons from the normal cytochrome pathway to an O_2^- and H_2O_2 producing pathway. The direct reduction of pyocyanine by NADH produced the means for this diversion of the electron flow. Extracellular O_2^- and H_2O_2 formed by diffused pyocyanine is thought to make a large contribution to the lethal effect of pyocyanine or other bacteria. P. aeruginosa was found to produce 62% higher catalase when grown under phosphate limitation, a condition conducive to pyocyanine production. In addition, compared to E. coli, the organism was less permeable to pyocyanine. These are probably means by which P. aeruginosa protects itself from the activity of the pigment. When growing in

the presence of other cells, P. aeruginosa could gain nutrients by eliminating competition and even gain access to nutrients released by the dead cells. The significance of the phenazine pigments in pathogenesis may well be in the suppression of other bacterial flora and their replacement by P. aeruginosa.

1.2.3.2 Fluorescent compounds

When P. aeruginosa is grown under conditions of low iron, it produces a water soluble fluorescent pigment which has been called fluorescein. This term however has led to confusion with the chemically synthesized product and the term pyoverdine has been proposed (Meyer & Abdullah, 1978). Use of a suffix indicating the producing species will differentiate products of different organisms, for example, pyoverdine$_{pf}$ for the compound produced by P. fluorescens.

Using P. aeruginosa, Cox and Graham (1979) showed that purified pyoverdine$_{pa}$ had a molecular weight between 400 - 600 and had an emmission maximum at 442 nm when excited at 352 nm. The compound became non fluorescent when iron was added and the iron-pyoverdine complex was more soluble than the free compound. Synthesis of pyoverdine occurred early in culture but reached a maximum when cells entered stationary phase. Addition of pyoverdine to cells resulted in decreased lag phase and an increased growth rate. Meyer and Abdullah (1978) using pyoverdine$_{pf}$, showed that the free compound had absorption peaks at 230 and 385 nm. The longer wavelength peak was however sensitive to pH, shifting to a longer wavelength (402 - 410 nm) at pH >7. The iron-pyoverdine complex had absorption spectra maxima at 235 and 403 and a shoulder at 450 nm. Under alkaline conditions, the pyoverdine was transformed into a number of degradation products.

The exact chemical nature of pyoverdines is not fully established, but studies indicate that they have molecular weights in the range of 1000, and consist of a peptide associated with a fluorescent chromophore (Leisinger & Margraff, 1979). Partial determination of the structure of pyoverdine$_{Pf}$ (Meyer, 1977) showed presence of the unusual amino acid 5-N-hydroxyornithine, which is a constituent of several hydroxamate iron binding compounds including ferribactin (Maurer et al., 1968) and ferrichrome A (Zalkin et al., 1966). The pyoverdine of P. aeruginosa purified by Cox and Graham (1979) (referred to as pyochelin) had M. wt. of 400 - 600, possessed phenolic characteristics with little or no similarity to dihydroxybenzoate and no indication of a hydroxamate group.

The pyoverdines have been shown to be powerful chelators of iron, forming stable complexes with Fe^{3+} (Meyer & Abdallah, 1978; Leisinger & Margraff, 1979; Cox & Graham, 1979). Pyoverdine$_{Pf}$ was found to form a ferric complex with a stability constant of the order of 10^{32}, which was comparable to those of siderophores ferroxamine B and ferrichrome (Meyer & Abdallah, 1978). In contrast, the fluorescent pigment of P. aeruginosa (pyochelin, Cox & Graham, 1979) had an iron binding coefficient in ethanol of 2.4×10^{5}. The compound was, however, able to reverse the growth inhibition of ethylenediamine-di-(o-hydroxyphenylacetic acid (EDDA), an iron chelator with binding coefficient of $10^{33.9}$. The authors suggested that either the binding coefficient was higher in aqueous solution, or that the reversal of EDDA inhibition indicates some specificity in the interaction of P. aeruginosa with ferripyochelin.

Totter and Moseley (1953) suggested that the fluorescent pigment probably appeared as partial substitute for iron containing

cytochrome and related systems when some precursor of iron was limiting. In their study, Meyer and Hornsperger (1978) showed that purified pyoverdine in external media facilitated iron transport into P. fluorescens and the rate of uptake of iron increased when pyoverdine was added to washed cells. Similarly, addition of pyoverdine$_{Pa}$ (pyochelin) to iron poor cultures of P. aeruginosa resulted in a promoted growth (Cox & Graham, 1979). Pyoverdines therefore act as siderophores, a term describing microbial metabolites produced for the purpose of iron chelation and transport. These include enterobactin (enterochelin) of several enteric bacteria, schizokinen produced by species of Bacillus and mycobactins from Mycobacterium species (Weinberg, 1978).

Since host iron is usually bound to transferrin and other iron binding proteins, most microbes are unable to obtain sufficient iron for growth in tissue fluids (Weinberg, 1974). Liu and Shokrani (1978) observed that strains of P. aeruginosa able to grow in serum produced siderophores in large quantities, enabling them to extract iron from transferrins. Also these serum resistant strains tended to be more virulent. Serum sensitive P. aeruginosa and other Gram-negative cells could grow in serum when pyoverdine was added. The role of pyoverdine in the pathogenicty of P. aeruginosa seems to be in enabling the organism to grow under conditions of low iron. Such a role has also been suggested for the other organisms producing siderophores (Weinberg, 1970).

1.2.4 Production of extracellular products under various environmental conditions

The synthesis and secretion of exocellular products by

bacteria is of interest, not only because of its importance in manufacturing industry and in bacterial identification and classification, but also because the product(s) may represent causative toxin(s) in some diseases. Many exocellular enzymes are hydrolytic, acting on large insoluble substrates, and production of these enzymes when inorganic or small organic nutrients are in short supply suggests an obvious role for the enzymes in the life of the cell (Tempest, 1970). The exoenzymes may enable the organism to utilize macromolecules in the environment (Engelking & Seidler, 1974; Mandelstam, 1969) or the enzyme or other product may act to kill neighbouring cells, thus reducing competition or gaining nutrients from the lysed cells (Hassan & Fridovich, 1980). Exoenzymes are also involved in spore germination (Tempest, 1970).

Although there are differences among organisms, it has been found that the synthesis of many exocellular products is influenced, at least in part, by the level of individual nutrients in the extracellular environment.

1.2.4.1 Exopolysaccharide

The effect of environmental conditions on the production of exopolysaccharide has been studied for a number of microbial species. Strains differ in their responses to effects of changes in the environment and their carbon source utilization, mineral requirements and temperature optima for both growth and polysaccharide synthesis (Sutherland, 1972, 1979). Cultural conditions can affect both the properties of the exopolysaccharide and the degree of production.

There are three possible types of structures that can be derived from the two monomers of alginates: polymannuronic acid

(-M-M-M-M-), polyguluronic acid (-G-G-G-G-) or alternating portions (-M-G-M-G-). The viscosity of the exopolysaccharide depends both on the ratio of D-mannuronic acid to L-guluronic acid, the proportion of the three possible structures and on its molecular weight. By appropriate variations of culture conditions, the arrangement of the two uronic acids in the block structures can be varied, and a range of alginates can be produced (Larsen & Haug, 1971; Deavin et al., 1977). A high proportion of polyguluronic acid blocks results in stronger and more rigid gels formed in the presence of calcium ions (Smidsrod, 1974). Such changes can be affected by the medium used. For example, increasing the concentration of calcium in the growth medium of Azotobacter vinelandii caused an increase in guluronate content in the uronic acid (Larsen & Haug, 1971). Molecular weight can be altered by growth rate as exemplified by the shorter O-antigenic side chains in the LPS of Salmonella enteritidis cell walls obtained when growth rate was increased (Collins, 1964). It is possible that shorter chain extracellular polysaccharides may be produced with increased growth rate.

Different nutrient limitations may also result in the production of different polysaccharides having different properties (Markovitz & Sylvan, 1962). Evans et al., (1979) noted that the ratio of two polysaccharides which were formed by Xanthomonas campestris in chemostats varied with the cultural conditions. The ratio was highest when the cells were carbon limited and lowest when they were sulphate limited. Under potassium limitation, the material precipitated by acetone was poorly soluble in water while the exopolysaccharide formed under carbon limitation differed from that obtained under excess carbon. Under carbon limitation, the material produced had very low viscosity.

The yield of exopolysaccharide is markedly affected by the mineral and carbon content in the medium, and by the temperature, pH, aeration and growth phase of the culture.

The effect of mineral limitation on exopolysaccharide production differs between strains. There is however, a general agreement that a high carbon : nitrogen ratio results in increased polysaccharide production by some facultative anaerobic bacteria and species of Pseudomonas (Evans & Linker, 1973; Duguid & Wilkinson, 1953; Goto et al., 1973; Neijssel & Tempest, 1975; Williams & Wimpenny, 1977, 1978; Mian et al., 1978). Potassium, magnesium and calcium were shown to be essential for maximum synthesis of polysaccharide by a pseudomonad and by Xanthomonas juglandis (William & Wimpenny, 1977; Evans et al., 1979). Sulphate limitation has been shown either to stimulate or to inhibit, depending on strain and polymer formed (Duguid & Wilkinson, 1953; Markovitz & Sylvan, 1962; Evans et al., 1979). Ferrous iron was reported to stimulate production of exopolysaccharide by P. aeruginosa (Palumbo, 1972, 1973; Goto et al., 1971, 1973) while its omission led to increased polysaccharide synthesis by Chromobacterium violaceum (Corpe, 1964). The effects of phosphate limitation have varied from increased production (Duguid & Wilkinson, 1953; Duguid, 1948) to having no effect (Williams & Wimpenny, 1978). Goto et al. (1973) found they had to add phosphate for their non-mucoid strain to produce exopolysaccharide, but whether the phosphate was limiting was not known. Addition of phosphate was not necessary for the production of exopolysaccharide by mucoid cultures.

Both the type of carbon source and its availability affect exopolysaccharide production. Using eight strains of non-mucoid P. aeruginosa grown in chemically defined medium, Brown et al. (1969)

obtained exopolysaccharide when the carbon source was gluconate, acetate, citrate, glycerol or other low molecular weight organic compounds. No polysaccharide was formed when glucose or other sugar was the carbon source. Similarly, Hayne (1951) had found all strains of P. aeruginosa he tested produced exopolysaccharide in a gluconate-tryptone broth but not in glucose media. In contrast, Williams and Wimpenny (1977, 1978) found that their non-mucoid strain produced exopolysaccharide with glucose. Goto et al. (1973) found that when organic acids, such as quinic acid or gluconate were used as the carbon source, their non-mucoid strain of P. aeruginosa produced considerable amounts of exopolysaccharide. The organism non-the-less produced the exopolysaccharide when either glucose, xylose or glycerol were used but of much less quantity.

The effect of carbon limitation on exopolysaccharide also varies with strain. Some exopolysaccharides are not formed under carbon limitation (Williams & Wimpenny, 1978; Neijssel & Tempest, 1975). When a species of non-mucoid Pseudomonas was continuously cultured under ammonia and phosphate limitation, it produced a galactoglucan. This was not produced under carbon limitation (Williams & Wimpenny, 1978). In contrast, both Azobacter vinelandii and mucoid P. aeruginosa produced exopolysaccharide in carbon limited continuous culture studies (Jarman et al., 1978; Mian et al., 1978).

Temperature has also been shown to affect exopolysaccharide production by some strains. Williams and Wimpenny (1978) and Goto et al. (1971) reported a higher yield of exopolysaccharide at 37^{o}C than at 25^{o}C by Pseudomonas PB1 and P. aeruginosa IFO3445. The strain of P. aeruginosa used by Evans and Linker (1973) produced

more exopolysaccharide with decrease in temperature. Other bacteria strains producing higher polysaccharide at lower temperatures include *E. coli* and *Salmonella* species (Wilkinson *et al.*, 1954).

Other factors that may affect exopolysaccharide production include oxygen (Duguid & Wilkinson, 1953; Dudman, 1964; Jarman *et al.*, 1978) and pH (Williams & Wimpenny, 1978; Sutherland, 1979). Growth rate (Williams & Wimpenny, 1978, 1980) and growth phase (Duguid & Wilkinson, 1953; Bukantz *et al.*, 1941; Foster, 1968) were also shown to affect polysaccharide production. In most cases, the polymer formation was maximal in the stationary phase. Exopolysaccharide production was concomitant with growth for *Xanthomonas campestris* (Moraine & Rogovin, 1973) and with a mucoid strain of *P. aeruginosa* (Mian *et al.*, 1978).

1.2.4.2 Extracellular enzymes and pigments

Some enzyme synthesis has been associated with stationary phase cells (Hagihara *et al.*, 1958; Lane & Pirt, 1973; Bjorn *et al.*, 1979; Stinson & Hayden, 1979; Jensen *et al.*, 1980a). In these cases, little or no exoenzyme is observed during the lag or logarithmic growth phase. As the mean generation time increases, synthesis is initiated and becomes maximal during early stationary phase. There are however, several reports on exoprotein synthesis following growth cycle (Welker & Campbell, 1963; McDonald, 1965; Dean, 1972; Morgan & Priest, 1979). In some cases reports have differed in whether exoenzyme was formed in logarithmic or stationary phase, for example, amylase synthesis by *Bacillus licheniformis* (Meers, 1972; Morgan & Priest, 1979). Confusion has probably arisen because of use of media of differing complexities. Metabolic activity and stable RNA content of cells grown in complex and in

minimal media differ (Herbert, 1961) and will therefore affect enzyme synthesis.

Many exoenzymes are subject to end-product repression, for example the repression of exoprotease of several bacterial strains by amino acids (May & Elliot, 1968; Hofsten & Tjeder, 1965; Glenn, 1976) and of alkaline phosphatase of P. aeruginosa, E. coli and Micrococcus sodonesis by inorganic phosphate (Cheng et al., 1970; Torriani, 1960; Glew and Heath, 1971). Other enzymes are subject to catabolite repression, for example the α-amylase of several species of Bacillus (Priest, 1977) and Vibrio parahemolyticus (Tanaka & Uichi, 1971) is repressed by glucose. In addition to repression by amino acids, protease synthesis in many organisms is also glucose repressible (Heineken & O'Connor, 1972; Daatsellar & Harder, 1974; Hofsten & Tjeder, 1965). Induction, by end products, of synthesis of some enzymes has also been observed. Amino acids stimulated the synthesis of protease by a Gram negative marine bacteria (Daatselaar & Harder, 1974), and the hydrolysis products of α-amylase have been shown to induce the synthesis of this enzyme (Glenn, 1976).

It appears therefore that there are variations in control mechanisms and under appropriate conditions, an organism may secrete exoprotein throughout the growth cycle, even though the rate of synthesis may be higher either in logarithmic or stationary phase, or may secrete no exoenzyme at all.

The effect of cultural condition on exoprotein and pigment production by P. aeruginosa in continuous culture has received little attention. A few studies have been carried out using batch cultures.

.2.4.2 i Growth in complex and simple salts media

The complexity of culture media has been found to affect the roduction of extracellular products of P. aeruginosa. The amount nd variety of proteases produced varied depending on whether a omplex or simple salts media was used. While no protease was roduced in a simple glucose-ammonium-salts medium (Liu, 1964; ensen et al., 1980a), the production of protease fraction III was nhibited by a complex medium and was synthesized in a synthetic edium containing Ca^{++} (Morihara, 1964). This effect of medium robably accounts for the varying reports on the number of proteases roduced by P. aeruginosa (Morihara, 1964; Wretlind et al., 1977; ensen et al., 1980a). The production of leucocidin was inhibited n synthetic medium (Scharmann, 1976a) and complex medium inhibited yocyanine production (Young, 1947). Liu (1966b) found that the roduction of exotoxin A required nutrients that were present in xtracts of natural protein. In a defined medium, this product as absent (Liu, 1973) or of much lower quantity than that produced n trypticase soy broth dialysate (DeBell, 1979). The addition f the amino acids alanine, aspartic acid and glutamic acid into synthetic medium containing glycerol resulted in a high yield f exotoxin A (Liu, 1964; DeBell, 1979), but nucleic acids, which nhanced growth, inhibited exotoxin production (Liu, 1979). An nhanced pyocyanine production was also observed when the amino cid DL alanine was added to simple salts medium.

.2.4.2 ii Influence of the carbon and energy source

The kind and amount of carbon source affects both the roduction of exoproteins and pigments. Glucose was found to be ore important than just an energy source, and its presence was

necessary for the production of haemolysin and lecithinase (Liu, 1964; Stinson & Hayden, 1979). In contrast, high levels of glucose inhibited formation of pyocyanine and fluorescent pigment (Young, 1947) and growth of P. aeruginosa in low levels of glucose resulted in increased protease and alkaline phosphatase production (Liu, 1964, 1966a). When the carbon source was changed from glucose to glycerol, a higher level of exotoxin A production was achieved and this effect could not be replaced by hexoses or pentoses (Liu, 1973). Similarly, the replacement of glucose by sodium succinate caused a higher production of phospholipase C.

The effect of carbon source on the quality of the product formed is illustrated by pigment formation by P. aeruginosa. When the organism was grown at 37°C with shaking in a medium containing glucose as the carbon source, phenazine pigment was formed. Under the same growth conditions using glycerol, the pigment was not formed. When the growth temperature was changed to 28°C, oxychlororaphin precursor was formed with glucose and fluorescein with glycerol (Kanner et al., 1978).

1.2.4.2 iii Growth under iron and phosphate limitations

The concentration of iron and phosphate have been shown to be very critical in the production of exoproteins and pigments by P. aeruginosa (Leisinger & Margraff, 1979). The effect of iron on production of exotoxin A, total protease, elastase, haemagglutinin and total protein have recently been studied (Bjorn et al., 1978; Bjorn et al., 1979). In both studies, a marked reduction of exotoxin A was observed when iron concentration in the medium was increased from 0.05 µg/ml to >0.15 µg/ml. This effect was confirmed by the study of DeBell (1979). In addition, Bjorn et al. (1978)

showed that the intracellular toxin levels were also reduced by high iron concentration in the culture medium, although iron did not alter the enzyme activity. They suggested that the rate of production or rate of intracellular degradation of exotoxin A was regulated by the concentration of iron in the culture medium. In the later study (Bjorn et al., 1979) they examined production of exoproteins by seven strains of P. aeruginosa. All seven strains showed a reduction in production of exotoxin A and total extracellular proteins in high iron, and four strains showed a decreased yield of haemagglutinin. In all but one out of the seven, there was a similar decrease in protease production in the presence of relatively large amounts of iron and a study on two of them showed a similar decrease in elastase activity. The one strain that differed showed an increase of both protease and elastase with increase in iron concentration. The production of exotoxins by other bacterial strains have also been observed to be affected by iron. An increased production of exotoxin by Corynebacteria diptheriae and Clostridium tetani (Locke & Main, 1931; Pappenheimer & Johnson, 1936), shiga toxin by Shigella dysenteriae (Dubos & Geiger, 1946) and of protease by a strain of Vibrio (Wiersma & Harder, 1978) was observed under low iron concentrations.

Although other factors have been noted to affect pigment production, the main nutritional condition controlling the biosynthesis and excretion of fluorescent compounds by Pseudomonas species has been found to be the availability of iron (Totter & Moseley, 1953; King et al., 1948; Garibaldi, 1967; Weinberg, 1970; Palumbo, 1972; Cox & Graham, 1979). In all cases, an inverse relationship existed between available iron and fluorescent pigment production. The production of pyocyanine was however, shown to require an optimum

supply of iron of > 0.5 mg/l (Korth, 1971). Its production decreased when iron was the growth limiting factor in the medium (Korth, 1971). Similarly, the production of pyocyanine was observed to increase linearly as the ferric ion was increased from 1×10^{-6} M to 1×10^{-5} M (Kurachi, 1958, quoted by Weinberg, 1970). The addition of iron to washed cells previously grown in synthetic medium without added iron, was found to stimulate pyocyanine production (Frank & DeMoss, 1959).

Most extracellular products are also markedly affected by the concentration of phosphate in the medium. Liu (1966a, 1964) observed that production of phospholipase C by P. aeruginosa was inhibited by various phosphates, both organic and inorganic. He suggested that presence of phosphate repressed secretion of this enzyme. In support of this idea, Stinson and Hayden (1979) observed repression of phospholipase C during early logarithmic growth of P. aeruginosa when inorganic phosphate concentration was 0.15 mM. After its depletion, repression was relieved and synthesis and secretion initiated. Addition of phosphate before onset of secretion delayed the appearance of phospholipase C and addition during secretion resulted in synthesis stopping within an hour. The concentration of phosphate was also critical in the production of haemolysin (Liu, 1964; DeBell, 1979), protease (Liu, 1964), exotoxin A (DeBell, 1979) and alkaline phosphatase (Cheng et al., 1970). In all cases, decreasing the amount of phosphate resulted in increased yield of the exoproduct.

A similar effect by phosphate on production of exoenzymes by other organisms has been reported. The rate of synthesis by Bacillus subtilis of ribonuclease was reduced in the presence of inorganic phosphate and increased under phosphate starvation

(May et al., 1968). Alkaline phosphatase synthesis was repressed by inorganic phosphate in E. coli, Bacillus subtilis and P. fluorescens, but the alkaline phosphatase formed during sporulation of B. subtilis was unaffected (Glenn & Mandelstam, 1971).

The effect of phosphate limitation on production of phenazine pigments is well known. When P. aeruginosa is grown aerobically in a phosphate poor medium, it secretes copious amounts of phenazine pigments (Frank & DeMoss, 1959; Ingram & Blackwood, 1962; Chang & Blackwood, 1969; Ingledew & Campbell, 1969). High phosphate concentrations inhibit pyocyanine production and these observations have led to the suggestion that with P. aeruginosa, phosphate depletion is the initiator of phenazine biosynthesis (Ingram & Blackwood, 1970).

1.2.4.2 iv Requirement for cations and anions

Several other cations and anions are required for the production of some of the extracellular products of P. aeruginosa. Using washed cell suspensions, Stinson and Hayden (1979) reported that a high rate of secretion of phospholipase C was observed when K^+, NH_4^+, Mg^{2+} and Ca^{++} were present in the resuspending medium. Deletion of any of these compounds from the medium resulted in the reduction in the rate of phospholipase C secretion. Of the divalent cations tested, calcium was most effective in supporting enzyme secretion, followed by magnesium and strontium. The presence of calcium was also essential for the production of protease III (Wilson, 1930; Morihara, 1956, 1962). The requirement for Mg^{2+} in addition to Ca^{2+} in the synthesis and secretion of alkaline phosphatase and cellular nuclease by Micrococcus sodonesis led to the suggestion that synthesis, permeation and release of extracellular

enzymes of this organism were magnesium dependent (Glew & Heath, 1971). Magnesium was also required for the production of fluorescent pigment (Meyer & Abdallah,1978) and was also important in determining the final state of the pigment (Chodat & Gonda, 1961). Frank and DeMoss (1959) observed that the omission of magnesium greatly reduced or completely inhibited the production of pyocyanine.

Both nitrogen, which is required for the synthesis of nitrogenous compounds of the cell and sulphur, which is present in many essential components of protoplasm (for example cystine, methionine, thiamine) are essential for the growth of the organism. Ammonium ion was found to cause an increased rate of secretion of phospholipase C but a decreased protease production (Liu, 1973 ; Liu & Hsieh, 1969). Sulphate was found to be necessary in the elaboration of pyocyanine, with production increasing with increasing sulphate concentration (Frank & DeMoss, 1959). In a similar study, Palumbo (1972) found the production of pyocyanine and fluorescent compound by a stain of P. aeruginosa depended both on iron and the source of sulphur used. When sulphite was used without added iron in the medium, a blue fluorescent pigment with excitation maxima at 365 nm and emission at 440 nm was formed. In the presence of iron a yellow-green fluorescent pigment with excitation wavelength of 410 and an emission wavelength of 525 nm was obtained. No pyocyanine was formed when sulphite was used. When sulphate was used, the fluorescent pigment was not produced but pyocyanine production was enhanced.

1.2.4.2 v Other factors

A number of other environmental factors affect extracellular product production including aeration, light, temperature, pH and Eh. Aeration and light markedly affect production of both pyocyanine

(Meyer & Abdullah, 1978) and phenazine pigments (Kanner et al., 1978; Propst & Lubin, 1979). The production of pyocyanine, chlororaphin and oxychlororaphin by P. aeruginosa varied dependent on whether the culture was shaken or stationary and on the temperature (Kanner et al., 1978). At 37°C, a shaken culture produced pyocyanine and when stationary oxchlororaphin was produced. At 28°C the shaken culture produced oxychlororaphin precursor while a stationary culture produced first oxychlororaphin followed after some time by chlororaphin. Oxygen tension and temperature also affected exotoxin A production by P. aeruginosa (Liu, 1966b, 1973) and protease production by Vibrio (Wiersma et al., 1978). The production of phospholipase C (Stinson & Hayden, 1979), alkaline phosphatase (Cheng et al., 1970) and fluorescent pigment (Meyer & Abdullah, 1978; Palumbo, 1972) were all affected by medium pH.

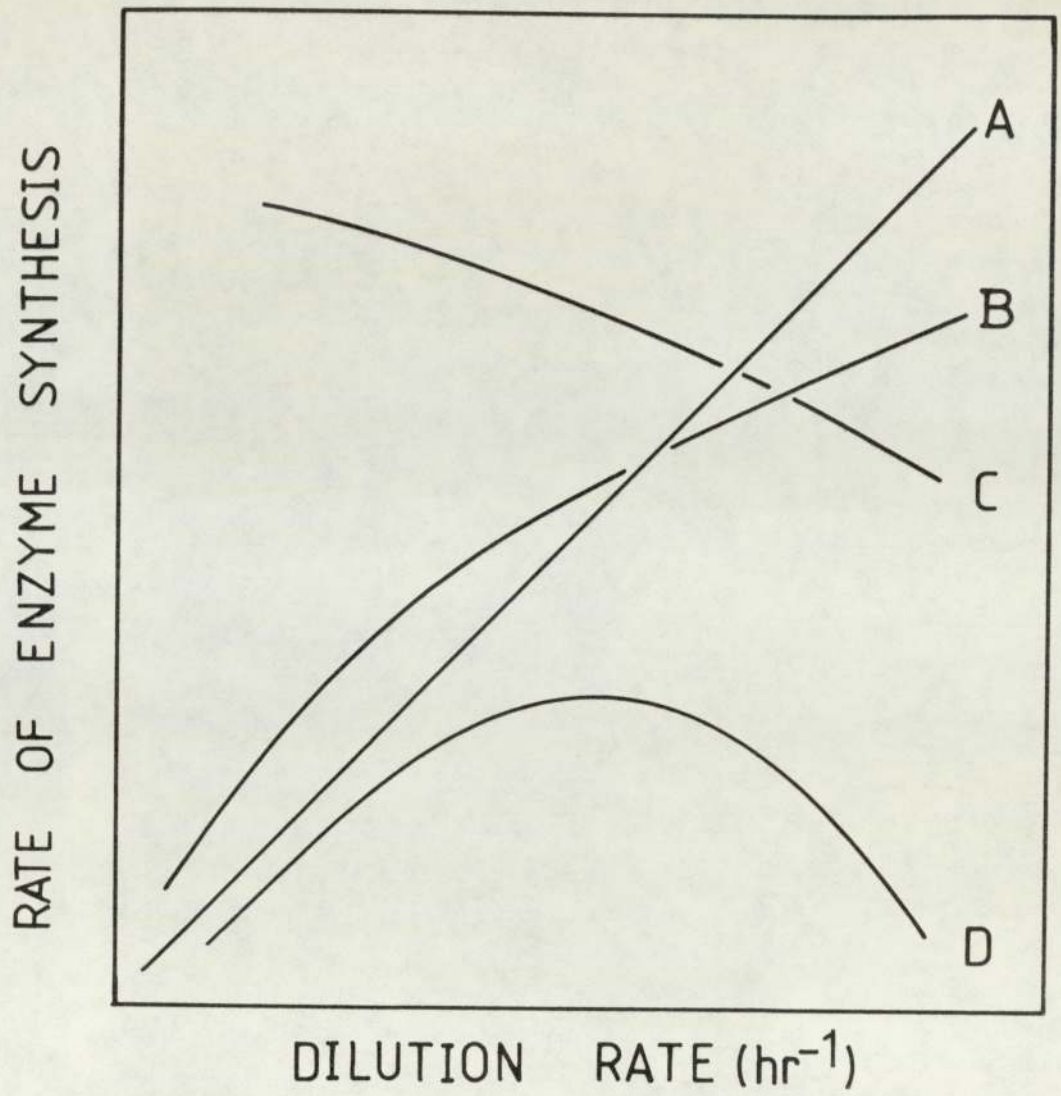
1.2.4.2 vi Growth rate and production of enzymes

The production of microbial extracellular enzymes under nutrient depletion can follow one of four general patterns (Figure 1) and the effect of growth rate (dilution rate) depends on the characteristics of the regulatory mechanisms involved (Wiersma & Harder, 1978).

Pattern A applies to constitutive enzymes, the rate of enzyme production is a function of the product of the cell concentration and growth rate. The rate of enzyme production therefore increases linearly with increase in dilution rate. This has been observed for penicillinase production by Bacillus licheniformis (Wouters & Buysman, 1976, 1977) and by several other enzymes (Dean, 1972).

In the case of an inducible enzyme where the inducer is the growth limiting substrate, a non-linear relation between the rate of enzyme production and dilution rate has been found (curve B) (Dean, 1972).

Figure 1. Possible patterns of extracellular enzyme production under nutrient limitation.



As the dilution rate increases, the steady state concentration of limiting nutrient increases (Tempest, 1970). The synthesis of an enzyme controlled by catabolite repression by the growth limiting substrate may therefore be expected to decrease as dilution rate increases (Curve C). Such a pattern was found for the production of extracellular agarase of Cytophaga flevensis (van der Meullen & Harder, 1975) and for some intracellular enzymes (Dean, 1972).

An optimum dilution rate leading to maximum enzyme production has been reported for the protease production by Vibrio SAI (Wiersma & Harder, 1978), extracellular pullulanase by Klebsiella aerogenes (Dean, 1972) and the extracellular protease, phosphatase and ribonuclease excreted by Bacillus licheniformis (Wouters & Buysman, 1976, 1977). This can occur when the enzyme is less sensitive to catabolite repression by the growth substrate and the pattern D is observed. This is, in fact, a combination of A and C for constitutive enzymes, or of B and C for an inducible enzyme.

The production of amidase by P. aeruginosa in continuous culture has also been shown to follow pattern D. Clarke et al. (1968) used three types of P. aeruginosa cells to demonstrate the effect of dilution rate and the balance between induction and catabolic repression. The wild type cells showed increased amidase production as dilution rate increased to an optimum at $D = 0.3 - 0.35\ hr^{-1}$. Higher dilution rates resulted in a decreased synthesis. A full constitutive mutant that was susceptible to catabolic repression showed the highest specific amidase activity at a lower dilution rate ($D = 0.06\ hr^{-1}$). The third type of cell was an altered catabolic mutant. This showed a maximum at $D = 0.2\ hr^{-1}$

but amidase activity decreased much less rapidly than the wild type as the dilution rate was increased. These results supported those of Boddy *et al.* (1967) who reported that the synthesis of *P. aeruginosa* amidase was affected by dilution rate of the culture. They concluded that the extent of catabolite repression was related to dilution rate and suggested that at high dilution rate, high concentration of intermediary metabolites prevented enzyme induction by inducer. In addition, the results of Clarke *et al.* (1968) show that the balance between induction and catabolic repression of an enzyme is also important in the regulation of the synthesis of an inducible enzyme in a culture growing at a steady state.

1.3 The Cell Envelope of Gram-negative Bacteria: Role in Resistance to Antimicrobial Agents and Phagocytosis

1.3.1 The envelope: an introduction

The cell envelope of Gram-negative bacteria is a complex and multilayered structure, differing from the Gram-positive bacteria by having an outer membrane in addition to the peptidoglycan layer (Freer & Salton, 1970; Costerton, 1977). This envelope can be divided into a number of layers, namely, the cytoplasmic membrane, the periplasmic space within which the peptidoglycan is found, the outer membrane and other structures external to the outer membrane. These layers differ in their chemical composition and physical nature (Costerton et al., 1974; Costerton, 1977; Nikaido & Nakae, 1979) but they are intimately associated in their protective role to the organism. Various aspects of Gram-negative cell envelope have been reviewed including the chemistry (Martin, 1966), structure and function (Glauert & Thornley, 1969; Braun, 1973; Costerton et al., 1974; Nikaido & Nakae, 1979), biogenesis (DiRienzo et al., 1978) and genetics (Stocker & Makela, 1978). The discussion will therefore be confined to a summary of the role of the cell envelope, particularly as an antibiotic barrier and phagocytosis surface.

1.3.1.1 Cytoplasmic membrane

The cytoplasmic membrane is the innermost structure of the cell envelope and separates the cytoplasm from the cell wall. The structure is best represented by the Singer model (Singer, 1972, 1974) in which phospholipids form a liquid hydrophobic bimolecular layer which is modified by proteins floating in it. The enzymes of the respiratory chain and active transport (Fox, 1972; Osborn

et al., 1972) and the synthesis and assembly of structural components of the cell envelope are located in this layer (Rogers, 1970; Bell et al., 1971; Hinckley et al., 1972).

Since the cytoplasmic membrane is hydrophobic, it is readily penetrable by hydrophobic antibiotics (Teuber & Miller, 1977) but acts as an effective barrier to polar molecules. Hydrophilic antibiotics must therefore be able to mimic a natural substrate in order to pass through the cytoplasmic membrane via the transport system (Costerton & Cheng, 1975; Shipley & Olsen, 1974). Changes in the cytoplasmic membrane which confer resistance may include deletion (Chopra et al., 1974) or the addition of a new protein (Levy & McMurray, 1974). Penetrability is also affected by alteration in chain length and degree of saturation of its component fatty acids (McElhaney et al., 1973).

1.3.1.2 Periplasmic space

The periplasmic space is that area lying between the outer membrane and the cytoplasmic membrane. It includes the peptidoglycan layer and many molecules and enzymes released into it by the cytoplasmic membrane (Costerton et al., 1974; MacAlister et al., 1977). Among the enzymes enclosed in this area, are those that can alter antibiotic molecules including β-lactamases (Smith & Wyatt, 1974; Bryan, 1979), acetylases (Brzezinska et al., 1972; Ingram & Hassan, 1975); phosphorylases (Doi et al., 1968; Bryan et al., 1972) and adenylases (Lundback & Nordstrom, 1974). Most of these are plasmid controlled and are especially effective in backing up a partial barrier to antibiotics at the level of outer membrane (Lundback & Nordstrom, 1974; Richmond, 1975).

In addition to conferring shape and mechanical strength, the peptidoglycan can also play a role in controlling the permeability of molecules. The predominantly negative charge can act as a barrier by absorbing molecules onto the structural polymers and impede movement by the Donnan effect (Rogers, 1970; Scherrer & Gerhardt, 1971; Sasaki, 1972; Tseng & Bryan, 1974). By holding together the outer layers, the peptidoglycan acts indirectly as a barrier since damage to the peptidoglycan of several Gram-negative bacteria was observed to cause changes in penetrability of the outer membrane (Burman et al., 1972).

1.3.1.3 The outer membrane

The major components of the outer membrane are proteins, lipopolysaccharide (LPS) and phospholipids (PL). In members of Enterobacteriaceae, the phospholipid composition of the outer membrane is very similar to that of inner membrane. Using Salmonella typhimurium, grown in complex medium, Osborn et al. (1972), found that the PL are mostly phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and very small amounts of diphosphatidylglycerol (DPG). The LPS is an amphipathic molecule with a hydrophilic polysaccharide (O-specific chain and R-core) and a hydrophobic portion (lipid A). In P. aeruginosa, the LPS is heavily substituted with phosphate (Eagon et al., 1975). Among the major outer membrane proteins are the peptidoglycan-associated proteins (porins) which form diffusion channels (Nikaido, 1976; Nikaido & Nakae, 1979). Other proteins include the Braun lipoprotein, heat modifiable proteins, minor proteins and a few enzymes (Schnaitmann, 1973a, 1973b; Braun, 1975; Palva, 1978; Nikaido & Nakae, 1979).

The outer membrane differs from other biological membranes in that its lipid bilayer appears to be asymmetric, as shown by studies on Enterobacteriaceae (Nikaido & Nakae, 1979). Studies on S. typhimurium and E. coli show that the outer half is composed of polyanionic molecules of the LPS and the strongly acidic porin proteins. The protruding polysaccharide fibres make this face hydrophilic. The electrostatic repulsion between the charged centres is stabilized by divalent cations, probably through neutralization of, and bridging between, anionic groups (Gray & Wilson, 1965). Braun lipoprotein probably pulls down the outer membrane layer onto the peptidoglycan layer. Evidence based on total phospholipid and freeze fracture studies suggest that in wild type S. typhimurium, the phospholipids are located in the inner half of the membrane. The asymmetry can be reduced by appearance of phospholipids in the outer leaflet as in deep rough mutants or in mutants with reduced outer membrane proteins (Ames et al., 1974; Koplow & Goldfine, 1974; Smit et al., 1975). Some nonenteric bacteria for example, P. aeruginosa and Neisseria gonorrhoeae show evidence of containing some phospholipid in the outer leaflet (Gilleland et al., 1973; Nikaido & Nakae, 1979).

Quantitatively, the outer membrane, in particular the proteins and the LPS, is the most important penetration barrier to antibiotics. The porin proteins aggregate to form water-filled pores through which hydrophilic molecules could pass through. The pore size probably varies with organisms, as P. aeruginosa was shown to allow passage of molecules below 9000 daltons (Hancock & Nikaido, 1978) while E. coli and S. typhimurium allow only molecules of less than 600 daltons. Due to the hydrophilic outer face, the outer membrane excludes hydrophobic molecules even though their size may be small.

Thus compounds like puromycin (M. wt. 491), Crystal violet (M. wt. 408), erthromycin (M. wt. 1411) are excluded while carbenicillin (M. wt. 378), tetracycline (M. wt. 444) and sucrose (M. wt. 342) are allowed to pass (Payne & Gilvarg, 1968; Costerton et al., 1974; Nikaido & Nakae, 1979). The outer membrane acts as a simple sieve with respect to hydrophilic antibiotics and a barrier to ~~hydrophilic~~ hydrophobic antibiotics. It is also as effective against outward movement and serves to retain enzymes and structural components within the periplasmic space. Genetic defects or environmentally induced damage to this barrier often results in a release of periplasmic enzymes into the medium (Cheng et al., 1972; Ames et al., 1974).

Changed antibiotic sensitivity or antigenic properties have been correlated with changes in protein composition and architecture of the outer membrane (Hancock et al., 1976; Tsang et al., 1976; Gilleland, 1977; Gilleland & Lyle, 1979; Darveau et al., 1980; Nicas & Hancock, 1980). In their study, Gilleland and Lyle (1979) observed a decreased sensitivity of P. aeruginosa to polymyxin with a changed protein composition. They suggested that loss of major proteins may be correlated with altered polymyxin resistance through loss of pores in the outer membrane which either one or more of these proteins would normally produce. In this way, passage of drugs normally using these pores (hydrophilic pathway) would therefore be reduced. An involvement of outer membrane proteins in pathogenicity was demonstrated for N. gonorrheae where the presence of opacity protein (Walstad et al., 1977; Swanson, 1978) was found to make the bacteria surface very adhesive to surface epithelial cells of the host (Nikaido & Nakae, 1979). Resistance was also affected since cells showing altered protein

content had altered serum and antimicrobial sensitivities (James & Swanson, 1978; Lambden et al., 1979; Guynon et al., 1978).

The entry of hydrophobic molecules into the bacteria ~~are~~ is markedly affected by the LPS. This has been demonstrated by changes in penetrability of the outer membrane observed in LPS mutants of both E. coli (Tamaki & Matsuhashi, 1973; Koplow & Goldfine, 1974) and S. typhimurium (Tamaki et al., 1971; Sanderson et al., 1974; Nikaido, 1976; Roantree et al., 1977; Stan-Lotter et al., 1979). The susceptibility to most antibiotics was increased in LPS defective or deep rough mutants. Similar results, using either deep rough mutants or cells whose LPS has been removed by ethylenediaminetetraacetic acid (EDTA), and with a variety of hydrophobic compounds have been reported (MacGregor & Elliker, 1958; Rapaske, 1958; Sheu & Freese, 1973; Leive, 1974).

The effect of LPS on phagocytosis has also been demonstrated using LPS-mutants of S. typhimurium (Friedberg & Shilo, 1970; Stendahl & Edibo, 1972; Cunningham et al., 1975). The results showed that the least virulent mutant with the shortest LPS side chain was most rapidly engulfed, even in the absence of normal serum, compared to the virulent smooth strains. Serum enhanced the phagocytosis of smooth strains (Stendahl & Edebo, 1972; Cunningham et al., 1975). The polysaccharide of the LPS, as with capsular polysaccharide, is thought to increase the hydrophilicity of the cell surface (see section 1.3.2) and thus enable the cell to escape phagocytosis (Cunningham et al., 1975; van Oss et al., 1975).

In addition, the O-specific chain shows great diversity and this may be a selective factor in pathogenicity. Attachment of specific antibodies to bacterial surfaces enhances phagocytosis

by promoting attachment on the phagocyte surface (Silverstein et al., 1977) and by decreasing the hydrophilicity of bacterial surface (van Oss & Gillman, 1972b). Introduction of a pathogenic bacteria with an O-chain to which the host animal has not been exposed and to which it has no antibodies, would therefore favour the organism's survival. Furthermore, the polysaccharide fibres may prevent both complement and antibody from reaching the antigenic determinant on the cell. This was demonstrated by Reynolds et al. (1975b) who observed that the protruding distal polysaccharide elements of LPS of smooth strains of S. typhimurium reacted with serum complement so that the complement could not penetrate to its targets on the envelope.

Changes in other components of outer membrane, particularly cations and phospholipids, have also caused changed antibiotic penetrations (Brown & Watkins, 1970; Burman et al., 1972; Gilleland et al., 1974; Kenward et al., 1978; Gilbert & Brown, 1978a).

1.3.1.4 External layers

A number of species have cell wall layers (Watson & Remsen, 1969; Fletcher & Floodgate, 1973; Cheng & Costerton, 1973) or proteinaceous coats outside the LPS (Murray, 1963; Buckmire & Murray, 1973). Other organisms have a thick carbohydrate layer, as a capsule or slime (Shands, 1966; Cheng & Costerton, 1973; Fletcher & Floodgate, 1973; Sutherland, 1977). The elaboration of layers of protein or carbohydrate may serve to protect the organism against large molecules or predators (Starr & Huang, 1972). Furthermore, the radial protrusion of the polysaccharide fibres means that this layer is the structure which actually makes contact with other cell surfaces, and it has been shown to be involved in adhesion (Fletcher & Floodgate, 1973; Breznak & Pankratz, 1977;

Gibbons, 1977; Smith, 1977; McCowan et al., 1978; Costerton et al., 1979).

The polysaccharide layer is particularly important in phagocytosis of bacteria. The susceptibility of the cells to phagocytosis and the virulence of some organisms has been shown to be markedly affected by the presence of this layer or of the LPS polysaccharide (Wood & Smith, 1949; Reynold & Pruul, 1971; Davies et al., 1973; Melly et al., 1974; Reynolds et al., 1975b; Onderdonk et al., 1977; Costerton et al., 1978). Most virulent bacteria are endowed with capsules (Davies et al., 1973) and the exopolysaccharide has been shown to prevent phagocytosis (Schwartzmann & Boring, 1971; Smith, 1977; Costerton et al., 1978). One mechanism of phagocytosis inhibition has been proposed as the increased hydrophilicity of the bacterial cell surface due to the exposed hydrophilic polysaccharide fibres, with subsequent reduction in phagocytosis (see section 1.3.2) (Stinson & van Oss, 1971; van Oss & Gillman, 1972b). In these studies, the presence of capsule was found to increase surface hydrophilicity as exemplified by the low contact angle and low phagocytosis of Staph. aereus strain Smith. Removal of the capsule greatly increased the contact angle (indicating hydrophobicity) and the bacteria were easily phagocytized. Furthermore, masking of active sites for antibodies and complement by polysaccharide layer has been observed in a number of organisms (van Oss & Stinson, 1970; Stinson & van Oss, 1971; Peterson et al., 1978; Wilkinson et al., 1979).

The open structure of the capsular material does not represent a simple penetration barrier to antibiotics, but due to its predominantly anionic sugar molecules, extensive adsorption of antibiotic molecules can occur in a manner similar to gentamicin

which is firmly bound to the polysaccharide fibres of cellular filters (Wagman et al., 1975). The number of antibiotic molecules penetrating into the cell can therefore be reduced and this may be a significant factor in determining minimum inhibitory concentrations (Costerton et al., 1979). Increased resistance to polymyxin, gentamicin, kanamycin and carbenicillin of encapsulated (mucoid) P. aeruginosa or of cells grown in a polysaccharide enhancing medium have been reported (Feary, 1975; Govan & Fyfe, 1978; Costerton et al., 1979).

1.3.2 Phagocytosis

Phagocytosis is the ingestion of particles by single cells and is the oldest and most fundamental defence mechanism of the host against an invader. The polymorphonuclear leucocyte (PMN) constitute about 60 to 70% of the total circulating phagocytic cells and may be subdivided into three types: øesinophils (stain bright orange-red with Wright/Giemsa stains), basophils (stain blue-black) and neutrophils, which possess granules that do not stain intensely. The vast majority (>90%) consist of neutrophils, which contribute most to the host defence mechanism against bacteria.

The PMNs are generally considered the first line of defence against bacteria invasion, and the phagocytic event initially involves contact of the neutrophil with the invading particle. The phagocytic cell extends pseudopods around the particle which meet and fuse to enclose the particle in a vacuole. Fusion of granules present in the cell and the phagocytic vacuole occur, bringing the contents of granules into contact with the particle. The changes are accompanied by biochemical changes that lead to

the ingestion, killing and digestion of the particle (Gadebusch, 1979).

A wide variety of particles including polystyrene spheres, carbon particles, bacteria, yeast and immune complexes can be phagocytized (Rabinovich, 1968; Lehrer, 1973; Turner, 1973) but they are not all taken up with equal avidity. Two mechanisms for attachment of particles to phagocytic cells have been proposed: i) specific recognition by the phagocyte membrane for the Fc part of IgG and for activated factor C3 of complement system (Rowley & Turner, 1966; Lay & Nussenzweig, 1968; Messner & Jelinek, 1970) and ii) aspecific adhesion based on physicochemical characteristics of the particle and phagocyte surface, such as shape, charge or hydrophobicity (Mudd et al., 1934; Stendahl et al., 1974; van Oss et al., 1975).

Early investigation had shown that a relationship existed between physical and surface characteristics and phagocytosis of a cell (Mudd & Mudd, 1933; Rabinovich, 1968). In an attempt to explain how the decision for phagocytosis is made and to quantitate the results of the earlier investigators, van Oss and his associates (van Oss & Stinson, 1970; Stinson & van Oss, 1971; van Oss & Gillman, 1972a, 1972b; van Oss et al., 1972a, 1972b; Cunningham et al., 1975; van Oss et al., 1975) have proposed that phagocytosis occurs primarily because of differences in surface free energy of the particle and of the phagocyte. This could be quantified easily by use of contact angles which is defined as the angle between a drop of physiological saline and a slide surface covered with a dry suspension of the particles/phagocytes under test. If the substance is hydrophobic, the liquid drop tends to round up and the contact angle is large. A hydrophilic substance yields a flat

drop and a small contact angle. Using this method, the group determined the contact angles of a large number of bacteria and found a striking correlation between hydrophilicity and phagocytosis. Those bacteria with angles greater than 18^{o} (more hydrophobic than the neutrophil) were readily phagocytized while those with angles less than 18^{o} were phagocytized with difficulty. Thus, the lower the contact angle of the bacteria, the less susceptible it was to phagocytosis. Encapsulated organisms had lower contact angles than decapsulated cells of same strain and were markedly more resistant to phagocytosis (Stinson & van Oss, 1971). Furthermore, both antibodies (IgG and IgM) and complement were found to increase the contact angle of bacteria. Bacteria that are poorly ingested in the absence of serum had contact angles less than that of neutrophils. The addition of serum markedly increased the contact angle and simultaneously enhanced phagocytosis (van Oss & Gillman, 1972a, 1972b; Cunningham et al., 1975). Thus it appears that one mechanism of opsonization involves increasing the hydrophobicity of the particle thereby increasing its susceptibility to phagocytosis. The size of electrical surface charge and the cell shape play a role in determining whether contact between the two cells is possible before engulfment or adhesion can proceed. The results appear to link both mechanisms of attachment proposed and therefore opsonization, adherence and ingestion cannot readily be separated from each other.

Since the efficiency of phagocytosis is dependent on the surface properties of the particle to be ingested, changes in the surface of bacteria would be expected to alter their interaction with phagocytes. Surface changes were observed by Lacey (1953, 1961) who found reversible changes in antigen composition of

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Haemophilus species induced by variations in the proportion of Na^{+}, Mg^{++}, Cl^{-}, SO_4^{2-} and temperature of growth medium. Pirt et al. (1961) using Pasturella pestis found that the antigenic production in organisms grown in continuous culture varied with temperature, pH and oxygen. Cultural conditions have been shown to affect cell envelope composition (Holme, 1972, Ellwood & Tempest, 1972; Brown, 1977) and the effect of these changes on antibiotic sensitivity and on phagocytosis are discussed in section 1.3.3.

1.3.3 Influence of environment on the cell envelope composition and properties

The cell envelope structures are complex and interdependent and the whole protective bacterial envelope can be rendered ineffective by the alteration of one vital component such as the peptidoglycan (Burman et al., 1972; Nordstrom & Sykes, 1974) or the outer membrane (Costerton et al., 1974). Such changes can be brought about by both genetic and nutritional factors.

Genetic changes are usually effected by R-factors, causing increasing drug destructive enzymes (Bryan & van Den Elzen, 1977; Bryan, 1979) or envelope variants (Richmond & Clarke, 1975; Kenward et al., 1978; Gilleland & Lyle, 1979).

When faced with lack of an essential nutrient, a growing bacterium changes, not only its general biochemistry, but also manufactures an envelope characteristic of that particular depletion (Brown, 1975, 1977). Significant changes in envelope composition have been observed to occur in response to depletion of cations, anions, carbon source and specific growth factors as well as to changes in temperature, pH and aeration (Costerton, 1970;

Holme, 1972; Ellwood & Tempest, 1972; Schnaitmann, 1974; Melling & Brown, 1975; Lugtenberg et al., 1976; Van Alphen & Lugtenberg, 1977). The effect of inorganic ions and the growth rate on the cell envelope serve to show some of the changes that have been observed.

1.3.2.1 Effect of nutrient limitation

The divalent cations, particularly magnesium, calcium, manganese, iron and zinc are present in significant amounts in Gram-negative bacteria (Rouf, 1964; Brown & Wood, 1972). In P. aeruginosa, Eagon (1969) reported that magnesium and calcium are major elements, while iron was a minor element. Other metals were present as trace elements. Both calcium and magnesium have been shown to be important in the stability of the structural arrangement of larger molecules (Michaels & Eagon, 1966; Costerton et al., 1974). The removal of these cations and associated macromolecules is the suggested mechanism of action of EDTA (Asbell & Eagon, 1966; Eagon et al., 1975; Wilkinson, 1975).

Brown and Melling (1969a, 1969b) first demonstrated that growth of sensitive P. aeruginosa in magnesium depleted medium resulted in acquired resistance to polymyxin and EDTA. These effects are observed for both batch and chemostat cultures (Gilleland et al., 1974; Finch & Brown, 1975). Calcium was shown to be able to replace magnesium in the cell in certain conditions and this had an effect on the cell's subsequent sensitivity to EDTA (Finch & Brown, 1975; Boggis et al., 1979). Furthermore, polymyxin resistant mutants of P. aeruginosa (Brown & Watkins, 1970) and other Gram negative bacteria (Brown & Wood, 1972) possess low levels of magnesium in the cell walls. These observations

were due to changed cell envelope structure and composition as has been demonstrated by several groups (Gilleland et al., 1974; Gilleland, 1977; Gilbert & Brown, 1978b; Kenward et al., 1979; Nicas & Hancock, 1980). In their study, Gilleland et al. (1974) found that the outer membrane of magnesium depleted cells contained more carbohydrate and KDO but less phosphorus and a qualitative difference in proteins. The changes were associated with the presence of an increased number of highly compact spherical units in the middle layer of outer membrane. The particles were insensitive to EDTA. Similar changes have been observed in E. coli (Tsang et al., 1976; Verkleij et al., 1977) and the particles are thought to comprise of protein - LPS complex (Rogers et al., 1969; Verkleij et al., 1977). Recently, Nicas and Hancock (1980) found that decreased magnesium concentration in the envelope varied in a linear reciprocal relationship with protein HI. They suggested that this protein replaces magnesium in its LPS sites and render the cells EDTA resistant and protect against polymyxin B, colistin and gentamicin. Polymyxin and EDTA resistant mutants show a decreased number of particles and altered protein content (Gilleland, 1977; Gilleland & Lyle, 1979). It has been suggested that the loss of LPS - protein particles alters the primary site of EDTA and also makes the cell less permeable to polymyxin.

Phosphorus is present in the cell envelope of Gram-negative bacteria as a constituent of PL and LPS. The high phosphate content in LPS of several species of Pseudomonas has been correlated with their high sensitivity to EDTA (Key et al., 1970; Wilkinson, 1975). Divalent cations are thought to be associated with the phosphate groups therefore forming inter and intramolecular cross-linking of the cell wall components.

Phosphate limitation affects drug sensitivity and cell wall composition. Boggis (1971) observed that phosphate-depleted P. aeruginosa was insensitive to lysis by EDTA and polymyxin. The sensitivity increased linearly with increase in phosphate concentration in the medium. Dorrer and Teuber (1977) demonstrated that phosphate depleted cultures of Pseudomonas fluorescens produces less PE, PG and DPG but synthesized a positively charged ornithine amide lipid. The chemistry of the cell wall of Bacillus subtilis was also changed by phosphate concentration. Under conditions of phosphate limitation, teichoic acid was replaced by teichuronic acid, a non-phosphate containing anionic polymer (Ellwood & Tempest, 1972).

Iron limitation has been shown to affect enzyme activities and growth efficiency of a number of bacteria and yeasts (Light & Clegg, 1974). Morphological changes were also observed for Mycobacterium smegmatis (Winder & O'Hara, 1961, 1962), Clostridium perfringens (Pappenheimer & Shaskan, 1944) and Torulopsis utilis (Light) & Clegg, 1974). There is evidence that iron is growth limiting in serum in vivo (Bullen et al., 1974) and that infection may result in a drop in the concentration of iron available to the invading organisms (Sussman, 1974). A similar in vivo growth limitation by phosphate and even zinc has also been suggested (Weinberg, 1974). The effect of limitation by these ions of cells growing at slow growth rates would therefore provide useful and relevant information in vitro, which may be correlated with in vivo situations.

1.3.2.2 Effect of growth rate

Growth rate has also been shown to affect the sensitivity

of bacteria grown in continuous culture, and this has depended both on the limiting nutrient and the drug. Both Melling et al. (1974) and Finch and Brown (1975) observed that carbon limited cells of P. aeruginosa became progressively more resistant to lysis by EDTA as the growth rate was decreased. In the same organism, the sensitivity of carbon limited cells to polymyxin showed a 30-fold difference between the dilution rates of 0.05 hr^{-1} and 0.7 hr^{-1} (Melling et al., 1974).

Gilbert and Brown (1978b) found that fast-growing cultures of P. aeruginosa were more sensitive to 3-chlorophenol and 4-chlorophenol than slower growing ones. They also noted that 3-chlorophenol had greater activity than 4-chlorophenol at slow growth rates, but at faster rates of growth, their activity was similar. Since these drugs uncouple oxidative phosphorylation (Weinbach & Garbus, 1965) the variations in sensitivity reflected exclusion of the drugs from their site of action by the outer envelope. Gilbert and Brown (1978b) found the total phospholipid content decreased and fatty acid content increased with increasing growth rate. The LPS content decreased with increasing growth rate and correlated significantly with drug uptake and sensitivity and it appeared to determine the degree of penetration of the cell envelope to the chlorinated phenols.

Similar decreases in total lipid content with increasing growth rate have been observed for Bacillus megaterium (Sud & Schaecter, 1964), E. coli (Ballesta & Schaecter, 1972) and Streptococcus faecum (Carson et al., 1979). Changes in protein content and certain sugar components of cell walls associated with dilution rates were observed by Collins (1964). He also observed that faster growing

cells had shorter LPS and underwent partial smooth to rough antigenic variations which were reversed when the dilution rate was decreased. Serological studies on chemostat grown cultures of Lactobacillus fermentum also revealed differences between fast and slow growing cells (Knox et al., 1979).

1.4 Bacterial Growth in Continuous Culture

1.4.1 Introduction

In batch culture during logarithmic growth, nutrients are continuously taken up and end products of metabolism excreted therefore causing a continuous change of the environment. The organisms therefore pass through a number of phases in which the physiological and biochemical conditions of the cell are continuously changing. The growth rate is only related to a limiting nutrient at low concentrations. The chemostat, first described by Novick and Szilard (1950) and Monod (1950) allows growth of bacteria to be maintained at submaximal rates by controlling the concentration of the one limiting nutrient below that which is necessary for maximal growth rate. Unlike batch culture, factors such as pH, oxygen and population densities are kept constant and can easily be controlled. Microbial growth in continuous culture therefore occurs at a constant rate in a constant environment.

1.4.2 Theory

Exponential growth of a bacterial culture is given by the equation:

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

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

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

In equation (2), μ and t_d are constant only when all substrates necessary for growth rate are present in excess (Herbert et al., 1956).

Monod (1942, 1950) first demonstrated the relationship between specific growth rate and the concentration of an essential nutrient when all other nutrients are in excess. Here the growth rate is dependent on one particular nutrient, called the growth limiting nutrient, and is represented by the equation:

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

where μ_m is the maximum growth rate, S is the concentration of the growth-limiting substrate and K_s is the saturation constant, numerically equal to the substrate concentration giving half the maximum rate. The specific growth rate therefore varies with the concentration of the growth-limiting nutrient and is proportional to the substrate at low concentrations but approaches a maximun value μ_m as the substrate concentration increases. Monod (1942) also found that in batch culture there is a simple relationship between growth and utilization of substrate. The growth rate is a constant fraction, Y, of the substrate utilization rate:

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

where Y is the yield constant =

$$\frac{\text{weight of bacteria formed}}{\text{weight of substrate used}}$$

In a chemostat vessel, assuming perfect mixing and constant

volume, V, medium flows into and out of the vessel at a steady rate, f. The growth rate of bacteria is governed by the dilution rate, D, defined as f/v. D is the number of complete volume changes per unit time and is usually expressed as h^{-1}. Thus the rate at which particles are lost in the effluent is proportional both to the number remaining, x, and to D.

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

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

In a chemostat, the organisms are growing according to equation (2) and are simultaneously washed out at a rate determined by equation (5). The increase in concentration of bacteria is therefore shown by the balance equation:

$$\text{increase} = \text{growth rate} - \text{rate of output}$$

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

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

When $\mu = D$, $\frac{dx}{dt} = 0$ and since x is constant, the culture will be in a steady state. The dilution rates at which steady state is possible can be determined from the effect of dilution rate on the concentration of substrate in the culture vessel, since μ_m depends on S (equation 3). In a steady state

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

$$\text{and} \quad \mu = D = \frac{\ln 2}{t_d}$$

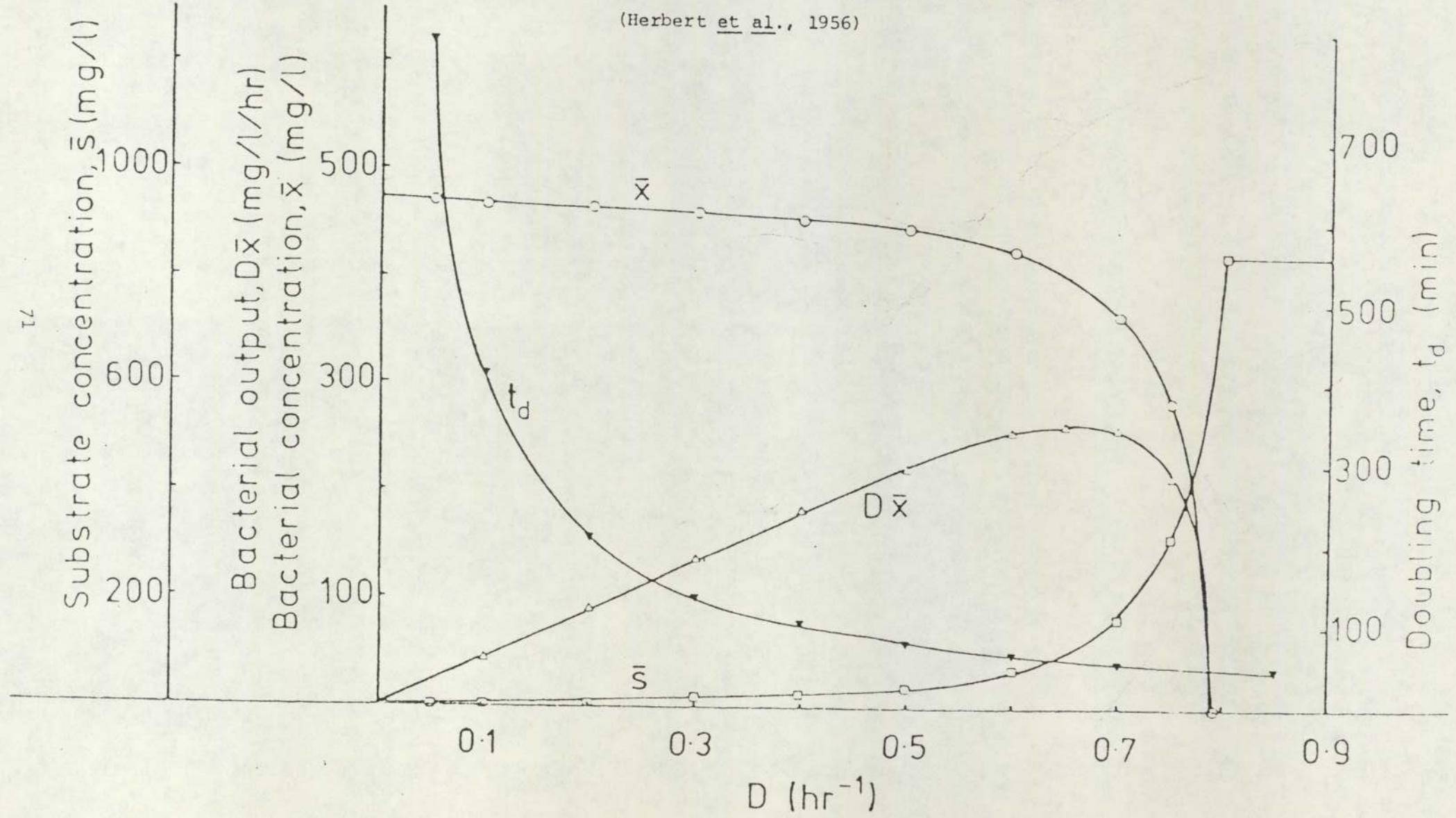
$$\text{therefore,} \quad t_d = \frac{0.693}{D}$$

and from equation 3

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

Fig. 2. Theoretical steady-state relationships in a continuous culture

(Herbert et al., 1956)



culture vessel can be predicted for any value of D by using equations (11) and (12) provided that the growth constants μ_m, K_s and Y and the concentration of inflowing substrate (Sr) are known. The steady state substrate concentration, $\bar{S}$, is independent of both $\bar{x}$, Y and Sr (equation 11).

Using values of K_s, Y and μ_m determined from batch culture, curves can be plotted for $\bar{x}$ and $\bar{S}$ at a chosen value of Sr over a range of values of D (fig. 2). It can be seen that there is a critical value of D at which complete washout of bacteria occurs; it is equal to the highest value of μ which is attained when $\bar{S}$ has its highest value, Sr. This critical value, designated D_c was shown by Herbert et al. (1956) to be given by:

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

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

The quantity of bacteria produced in unit time by the chemostat is obviously equal to the product of the flow rate and the concentration of the organisms within the vessel. The value of D at which the maximum amount of bacteria per unit time is produced, designated D_m, was found by Herbert et al. (1956) to be given by:

$$D_m = \mu_m - \left(1 - \sqrt{\frac{K_s}{K_s + S_r}}\right) \qquad (14)$$

It is possible to determine the value of S_r for any desired concentration of bacteria at the maximum output value, i.e. when $D = D_m$ by substituting $\mu_m \left(1 - \sqrt{\frac{K_s}{K_s + S_r}}\right)$ for D in equation (11)

and solving for S_r which gives:

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

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

The general growth formulae derived by Monod (1942, 1950) and as described above, does not take into account diffusion effects, endogenous metabolism or the accumulation of storage products, all of which are observed in practice with the chemostat. They none-the-less form an adequate basis for continuous culture studies. More detailed equations designed to take account of these effects have been described by several groups (Neijssel & Tempest, 1976; Powell, 1967; Van Uden, 1969).

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Organisms

The two organisms used throughout this study were Pseudomonas aeruginosa NCTC 6750, originally obtained from the National Collection of Type Culture, Central Public Health Laboratory, Colindale Avenue, London NW9 5HT, and M7, a mucoid variant of P. aeruginosa NCTC 6750, isolated in the laboratory by the method described by Govan (1976). The organisms were maintained in the following manner: colonies of M7 or P. aeruginosa NCTC 6750 (hereafter referred to as the wild type and abbreviated WT) from agar plates grown overnight at 37°C, were inoculated into 3 ml of 3% sterile skimmed milk contained in Bijoux bottles. After freezing in dry ice, the bottles were immediately transferred to a deep freezer (-20°C). For routine use, a bottle of each organism was kept at 4°C and replaced after a maximum of 12 weeks.

2.1.2 Media

Nutrient broth (NB): Oxoid nutrient broth, CM 1.

Nutrient agar (NA): Oxoid nutrient agar, CM 3.

Deoxycholate citrate agar (Haynes modification) (DCA) Code CM 227.

Diagnostic sensitivity agar Code CM 261. All above were obtained from Oxoid Ltd., London SE1 9HF, and were prepared according to the manufacturer's instructions.

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

Milk agar (MLA) 10% $^{w}/_{v}$ was prepared by mixing steam sterilized 20% $^{w}/_{v}$ skimmed milk with an equal volume of sterile half strength NB containing Lab M agar. Both preparations were allowed to cool

to 60°C before mixing.

Minimal agar (MA) was based on that described by Vogel and Bonner (1956) and contained:

$MgSO_4.7H_2O$	0.2 g
Citric acid (anhydrous)	2 g
$NaNH_4HPO_4$	3.5 g
K_2HPO_4	6.0 g

These were autoclaved together. Glucose 2% and Lab M agar, 15 g were autoclaved separately and the three solutions mixed and made up with sterile water to a final volume of 920 ml.

Lecithin quenching medium contained 0.5% lecithin and 4.0% glycerin made up in 0.8% NB. The lecithin was first dissolved in a small amount of absolute alcohol, then glycerin and a small amount of NB added. The alcohol was removed by heating over a steam bath for 15 min with constant stirring. The preparation was sterilized by autoclaving.

Bacto Pseudomonas Isolation agar (PIA) from Difco Laboratories, Detroit, Michigan, USA.

Calbiochem Aquaside III from Calbiochem Behring, Corp., La Jolla, Ca 92037.

Chemically defined medium (CDM): Table 1 describes the basic CDM used routinely for culturing the organisms. This was modified for resistance and chemostat studies as described in sections 3.2.2 and 3.5.1.1.

MOPS (3-(N-morpholino)propanesulphonic acid), a zwitterionic buffer (Sigma Chemicals Company, P.O. Box 14508, St. Louis, Mo. USA) was employed to avoid buffer changes for different limitations. It has a useful buffering range of 6.5 - 7.9 (pka 7.2) and pH was adjusted with NaOH.

Table 1

Basic chemically defined medium

Ingredient	Final Concentration (M)
KCl	3.0×10^{-3}
$(NH_4)_2SO_4$	1.2×10^{-2}
$MgSO_4.7H_2O$	3.2×10^{-3}
$FeSO_4.7H_2O$	2.0×10^{-5}
Glucose	2.0×10^{-2}
K_2HPO_4	1.2×10^{-3}
NaCl	3.0×10^{-3}
MOPS	5.0×10^{-2}

All the medium constituents except MOPS and $FeSO_4.7H_2O$ were sterilized by autoclaving at 121°C for 15 min. MOPS and $FeSO_4.7H_2O$ (to which concentrated H_2SO_4 acid (1 ml/L) was added to prevent ferric salt precipitation) were sterilized by filtration through a 0.2 µm membrane filter.

2.1.3 Chemicals

All chemicals used in the preparation of CDM were Analar Grade supplied by British Drug Houses Ltd., Poole, Dorset or Fison's Scientific Apparatus Ltd., Loughborough, Leicestershire.

Other chemicals were obtained as follows:

Polymyxin B sulphate was kindly given by Dr. Jill Gurney, Burroughs Wellcome & Co., London, in vials containing 500,000 international units.

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

Glycerol BP, Macarthys, Romford, Essex.

Elastin congo red, crystalline bovine serum albumin, casein and E. coli alkaline phosphatase were purchased from Sigma Chemicals Company.

2.1.4 Apparatus

Optical density and absorbance measurements were carried out in 1 cm matched glass cuvettes on a Unicam SP 600 spectrophotometer supplied by Pye-Unicam Instruments Ltd., Cambridge.

Batch cultures were grown in a Mickle reciprocating shaker bath obtained through Camlab Ltd., Nuffield Road, Cambridge.

Millipore membrane filtration apparatus was obtained from Millipore U.K. Ltd., Wembley, Middlesex. Membrane filters were obtained from Sartorius, Gottingen, West Germany. Before use, the membrane filters were boiled in three changes of distilled water before sterilization to remove wetting agents and other chemicals (Brown, Farwell & Rosenbluth, 1969).

The chemostats used were constructed by the university glass blower according to the design described by Gilbert and Stuart (1977) with slight modifications.

Other apparatus used included:

Phase contrast microscope, 'Wild' model M 20 binocular: Micro Instruments (Oxford) Ltd., Oxford, OX1 2PH.

pH meter: Corning - EEL model 5: Evans Electroselenium Ltd., Halstead, Essex, England.

IEC centrifuge: International Equipment Company, 300 Second Avenue, Needham Heights, Massachusetts, USA.

Aminco-Bowman Spectrophotofluorometer from American Instruments Company Inc., Silver Springs, Maryland, USA.

Soniprobe Type 7530A, Dowe Instruments Ltd., England.

Unicam SP 8000 spectrophotometer from Pye-Unicam Instruments Ltd., Cambridge.

Blood sample tubes, with and without lithium heparin were obtained from Searle and Company Ltd., P.O. Box 53, Lane End Road, High Wycombe, Bucks.

2.1.5 Treatment of Glassware

Glassware and tubing used were totally immersed in 5% v/v Decond 90 (Decon Laboratories Ltd., Brighton BN4 1EQ) overnight at room temperature or for 30 min at 100°C. They were then rinsed once in distilled water, totally immersed in 1% v/v HCl, rinsed six times in distilled water and finally twice in deionized distilled water. They were then dried at 60°C, sealed with aluminium foil and sterilized in hot air oven at 160°C for 3 hr. Heat sensitive material was autoclaved for 20 min at 121°C.

2.2 Basic Experimental Methods

2.2.1 Optical measurements of growth: an introduction

All batch culture growth experiments were performed using 25 ml medium in 100 ml conical flasks. Except in the study of iron requirement, the inoculum was grown in CDM, centrifuged at 25° at 10,000 g for 20 min and the pellet washed twice in medium lacking the nutrient under investigation. Media, at 37°C, containing graded concentrations of the nutrient under investigation, were inoculated with the prepared inoculum to give an initial OD_{470} of 0.03 to 0.05. These were incubated at 37°C in a shaking water bath with a shaking rate of 110 x 5.0 cm throws per minute. During growth, samples were removed at timed intervals with a Pasteur pipette, placed in a glass cuvette and the OD_{470} determined. Undiluted samples were returned to their respective flasks. The cuvette was rinsed thoroughly with deionized distilled water and allowed to drain onto an absorbant paper. Diluted samples were discarded.

In the study of iron requirement, Pseudomonas aeruginosa wild type was grown in 500 ml CDM without added iron for 18 hr, the culture centrifuged and the pellet discarded. The supernatant was sterilized by centrifugation through a 0.2 µm membrane filter and steamed for 15 min. This was then used for both WT and M7 as the growth medium to which graded amounts of iron were added. The inoculum was prepared by growing M7 or WT in CDM without added iron and after centrifugation, washing the pellet with the previously prepared iron depleted medium. Growth and optical density were measured as previously described. Iron depleted medium could not be prepared from M7 due to large amounts of exopolysaccharide produced (see also section 3.4.1).

2.2.2 Measurement of growth

Bacterial growth in liquid medium was determined by measuring the optical density of the cell suspension.

When a monochromatic beam of light passes through a bacterial suspension, part of it will be scattered and diverted from the light path because of the refractive index difference between the cell surface and the medium (Monod, 1949). This scattering can be quantified by measuring the undeviated light emerging from the suspension. At relatively low cell concentrations, a relationship exists between the incident light (I_o) and the emerging light (I), and is described by the Beer-Lambert law:

$$\log_{10} \frac{I_o}{I} = e.l.c.$$

where e is the extinction coefficient, l is the distance the light travels through the culture and c the concentration of organisms in the culture. The term $\log_{10} (I_o / I)$ is referred to as the optical density (OD).

Spectrophotometers that give readings in terms of $\log(I_o/I)$ are considered the most suitable for measurements of turbidity (Monod, 1949) and the Unicam SP 600, which measures optical density over the wavelength range of 350 to 1000 nm was used for all turbidity measurements during this study.

2.2.2.1 Selection of wavelength

Maximum sensitivity to changes in optical density, and little or no absorption from medium constituents and metabolic products, are important factors which need to be considered when selecting a suitable wavelength for measuring turbidity of a bacterial culture. The total amount of light scattered is directly proportional to the

ratio of cell size to the wavelength of the incident light (Koch, 1961). Therefore, the shorter the wavelength employed for a given bacteria, the more sensitive the instrument will be to optical density changes. The use of wavelengths below 380 nm is not practical because proteins and nucleic acids absorb in this region. When using non-pigmented organisms, other workers have found that 420 nm is the lowest practical wavelength (Hodges, 1973; Handley, Quesnel & Sturgiss, 1974). Pseudomonas aeruginosa produces a pigment, pyocyanine, which was shown to exhibit an absorption maximum at 390 nm (Finch, 1976). The lowest practical wavelength at which no absorption due to the pigment occurred was found to be 470 nm, and this was the wavelength used to measure bacterial growth in the present study.

2.2.2.2 Relation between optical density (OD) and cell concentration

Kenward (1975) showed that the relationship between OD and cell concentration obeyed the Beer-Lamberts' law up to an OD_{470} of 0.3. Above this value, the relationship is non-linear, with increase in OD less than predicted in proportion to increase in cell concentration. Linearity was restored when the suspension was diluted with water to give readings in the range OD_{470} 0.03 to 0.28. To minimise the amounts of dilutions required when several cultures were being studied, the method described by Lawrence and Maier (1977) was used. This involved reading % Transmission on the spectrophotometer and then converting the reading to OD on a calibration curve. The even spacing on the transmission scale offered an added advantage. Measurements of transmission below 20% were none-the-less diluted.

2.2.3 Viable counts

Viable counts of cultures were obtained by the spread plate method (Crone, 1948) and the Miles and Misra method (Miles & Misra, 1938).

The spread plate method consisted of spreading known volumes of the diluted culture over the surface of overdried nutrient agar plates. Serial dilutions of ten or one hundred fold were made in nutrient broth or lecithin glycerol so that 0.1 or 0.25 ml samples gave 30 - 300 colonies per plate. At each dilution level three or five replicate plates were used and the plates incubated at 37°C for 24 hr. The viable count was calculated from the mean number of colonies per plate, assuming that one colony was formed by the growth of one organism.

In the Miles and Misra method, drops of 0.02 ml of diluted culture were deposited on the dry agar plates. One plate had up to nine drops, consisting of three drops of each of three dilutions. After incubation for 15 hr at 37°C, counts were made in the drop areas showing the largest number of colonies without confluence (15 to 30 colonies) and the mean count per drop gave the viable count per 0.02 ml of that dilution. All counts were performed in triplicates.

The reproducibility of the counting procedures was tested by performing five replicate counts and the results subjected to an analysis of variance (Tables 2 and 3).

Table 2

Variation of replicate viable counts of 24 hr cultures of WT and M7 (spread plate method)

COUNT / REPLICATE PLATES	WT					M7				
	1	2	3	4	5	1	2	3	4	5
1	100	102	90	97	100	95	101	103	111	101
2	94	103	101	103	108	99	105	100	106	106
3	106	106	101	100	101	105	101	101	104	95
4	104	100	97	93	100	104	108	109	110	100
5	96	105	90	94	103	100	109	100	99	107
MEANS (x)	100	103	96	97	102	101	105	102	106	102
STANDARD DEVIATION (Sx)	5.1	2.4	5.5	4.2	3.4	4.0	3.8	3.8	4.8	4.9
COEFF. OF VARIATION	5.1%	2.3%	5.6%	4.3%	3.3%	4.0%	3.6%	3.7%	4.6%	4.9%
MEAN OF POPULATION (μ)	99.6					103.2				
STANDARD DEVIATION OF POPULATION (δ)	3.0					2.2				
COEFF. OF VARIATION OF MEANS	3.1%					2.1%				

Table 3

Variation of replicate viable counts of 24 hr cultures of WT and M7 (Miles and Misra method)

REPLICATE DROPS \ COUNT	WT					M7				
	1	2	3	4	5	1	2	3	4	5
1	16	18	17	19	16	21	15	16	18	17
2	17	15	15	18	23	15	19	20	22	20
3	18	18	18	15	15	20	20	16	17	18
4	22	19	22	18	21	16	22	20	17	17
5	16	15	20	18	22	19	18	21	18	17
6	18	21	22	16	17	17	19	20	19	18
7	21	15	16	22	17	15	19	19	21	18
8	18	15	15	20	19	15	20	20	17	16
9	17	17	18	17	19	17	18	21	20	17
MEANS (x)	18	17	18	18	19	17	19	19	19	18
STANDARD DEVIATION (Sx)	2.1	2.2	2.7	2.1	2.8	2.3	1.9	1.9	1.9	1.1
COEFFICIENT OF VARIATION	11.7%	12.9%	15.0%	11.7%	14.7%	13.2%	10.0%	10.0%	10.0%	6.4%
MEAN OF POPULATION (μ)	17.96					18.4				
STANDARD DEVIATION OF POPULATION (δ)	0.64					0.89				
COEFFICIENT OF VARIATION	3.6%					4.9%				

2.3 Assay Methods for Extracellular Products

2.3.1 Preparation of assay samples

M7 and WT were allowed to grow under nutrient limitation in chemostats or in batch cultures. The chemostats were emptied as soon as they had reached steady state (5 - 7 volume changes). Batch cultures were grown for 48 hr at 37^{o}C on a shaking water bath. Cells were removed by centrifugation at 5,000 g for 20 min and the clear supernatants obtained were retained. From each sample, 10 ml were retained without further treatment for use in pigment assays. The supernatants of the mucoid variant were treated with calcium chloride to a final concentration of 0.1 M to gel any polysaccharide formed. Non-mucoid cultures given this treatment produced no polysaccharide and neither did they show any significant difference in enzyme activity from those not subjected to this step. The gel was removed by centrifugation for 20 min at 5,000 g. All the supernatants were then concentrated by dialysing at 4^{o}C for 24 hr against polyethylene glycol, and finally made up to half the original volume with distilled water. The samples were finally divided into 2 ml portions and immediately stored frozen (-20^{o}C). Assay samples were allowed to thaw at 4^{o}C and any unused portion was discarded.

The amount of cell bound enzymes was studied using batch grown cells. After centrifugation, the supernatants were retained and treated as described above. The cells were washed once in 10 ml water, recentrifuged at 5,000 g for 2 min and the pellet finally resuspended in fresh 10 ml water. After thorough mixing, 2 x 1 ml samples were placed in weighed containers. Excess water was allowed to evaporate at 50^{o}C and the cells dried to a constant weight in vacuo over P_2O_5 and the weight of cells per ml of suspension determined. The cells in the remaining 8 ml were broken in an ultrasonic disintegrator set at maximum amplitude for 10 - 20 x 30 seconds, the suspension being cooled in ice. Disintegration (>90%) was confirmed by observing the cells under

a microscope. Cell debris was removed by centrifugation at 38,000 g for 1 hr at 4°C. The clear supernatant obtained was divided into 1 ml portions and immediately stored at -20°C. Samples for assay were thawed at 4°C and any part left after thawing was discarded.

2.3.2 Determination of exopolysaccharide yields

The method used for extraction of exopolysaccharide was based on that of Evans and Linker (1973). Cultures were obtained either from the washout of each chemostat which had been collected over ice for 12 hr or from 48 hr batch cultures. The media used in nutrient limited batch cultures was identical to that used in chemostats. The cultures were centrifuged at 5,000g for 2 hr and both supernatants and pellets retained. Very viscous cultures were diluted 5 times to enable cell separation.

The pellet was washed once with distilled water, recentrifuged in weighed tubes at 5,000 g for 2 min and dried to a constant weight *in vacuo* over P_2O_5. To the supernatant, 3 volumes of 95% alcohol were added slowly with stirring and the precipitate allowed to settle for 2 hr at room temperature. The precipitate was collected by centrifugation and similarly dried to constant weight.

2.3.3 Total protein assay

The total protein content of the samples was estimated by the method of Lowry *et al.* (1951). A standard curve was obtained by using a solution of bovine serum albumin containing 0 - 500 µg/ml protein and reading the OD at 750 nm. A linear relationship was obtained for concentrations of 0 to 100 µg/ml. Sample determinations were performed in duplicates and a fresh standard curve was prepared each time the assay was performed.

2.3.4 Enzyme activity assays

2.3.4.1 Total proteolytic activity (protease)

Protease activity was assayed according to the method of Kunitz (1946 /1947) and as modified by Wretlind and Wadstrom (1977). The extent of proteolysis was determined by reading the absorbance of perchloric acid-soluble degradation products at 280 nm. To minimise variations, a single batch of casein was used throughout the study.

Casein (10 g/l) was dissolved, with stirring in 0.05 M sodium phosphate buffer, pH 7.4 supplemented with 1 mM $CaCl_2$. To 4 ml of casein solution preincubated at 37°C, 0.2 ml sample was added. The reaction was terminated after 30 min by addition of 3 ml 1 M perchloric acid, the precipitate removed by centrifugation and the absorbance of the supernatant read at 280 nm. A blank was prepared for each sample by using phosphate buffer. One unit of protease activity is defined as increase in $A^{10\ mm}_{280\ nm}$ = 1.0 in 30 min at 37°C. The reaction is linear to $A^{10\ mm}_{280\ nm}$ = 0.5.

2.3.4.2 Elastase activity

This was determined by the method given by Bjorn et al. (1979) using elastin congo red as a substrate. 10 mg elastin congo red was weighed into individual stoppered 15 ml centrifuge tubes. To this, 2 ml of tris-maleate buffer (0.1 M, pH 7.0 and supplemented with 1 mM $CaCl_2$) was added. The sample being assayed (1 ml) was added to the buffer-substrate preparation, and the mixture incubated for 2 hr at 37°C in a waterbath with rapid shaking. The reaction was terminated by addition of 2 ml of sodium phosphate buffer (0.7 M, pH 6.0) and the precipitate removed by centrifugation. Blanks were prepared by using 3 ml buffer containing 10 mg elastin

congo red. The absorbances of the supernatants were read at 495 nm. One unit elastase activity is defined as the amount that released soluble dye from 1 mg elastin congo red at 37°C in 2 hr corresponding to $A^{10\ mm}_{495\ nm} = 0.25$.

2.3.4.3 Lipase activity

Lipase activity was determined by a modification of the method described by Staeudinger et al. (1973) and as given by Wretlind et al, (1977).

0.1 ml of a solution of p-nitrophenyl caprylate, 7 mM, in dimethyl sulphoxide was mixed with 1.0 ml of 0.1 M phosphate buffer, pH 7.4. To this, 0.1 ml of sample was added and the mixture transfered to a cuvette in a recording spectrophotometer. The linear increase in $A^{10\ mm}_{410\ nm}$ was measured. A standard curve was prepared by reading the absorbance at 410 nm of 0 to 0.7 µmole/ml p-nitrophenol. Using this curve, the amount of released p-nitrophenyl and hence amount of hydrolysed substrate was determined. One unit of enzyme activity was defined as the amount of enzyme causing the hydrolysis of 1 µ mole of substrate per min at 25°C.

2.3.4.4 Phospholipase C activity

The method given by Stinson and Hayden (1979) was used to determine phospholipase C activity. A solution of phosphatidyl-choline was prepared by dissolving 100 mg phosphatidylcholine (egg yolk) in 1 ml absolute alcohol. To this 5 ml of 0.25 M Tris-hydrochloride buffer, pH 7.2 were added and a homogenous suspension obtained by ultrasonication for 10 x 30 sec at 4°C. Sodium deoxycholate was added to a concentration of 0.25% in final volume of 5.5 ml. This solution was prepared fresh each day. The enzyme mixture contained in 100 ml of 0.25 M trishydrochloride buffer

pH 7.2, 0.183 g $CaCl_2.H_2O$, 0.4 g bovine serum albumin and 4 units of E. coli alkaline phosphatase.

To 0.2 ml phosphatidylcholine solution, 0.5 ml enzyme solution and 0.3 ml of sample were added and the reaction mixture incubated for 30 min at 37°C. Blanks were prepared with 0.3 ml of tris-HCl buffer replacing the sample. The reaction was terminated by the addition of 1 ml trichloroacetic acid and the precipitate removed by centrifugation.

The amount of phosphate concentration was determined as described by Bartlett (1959). To the supernatants obtained above, 0.5 ml of 10 N H_2SO_4 was added and the mixture placed in 12 ml marble covered conical centrifuge tube. This was heated in a 150 - 160°C oven for at least 3 hr. Two drops of 30% H_2O_2 was added and the solution returned to the hot oven for at least 1.5 hr to ensure complete combustion and decomposition of all the peroxide. To the oven treated samples, 4.6 ml of 0.22% ammonium molybdate and 0.2 ml of Fiske Subbarow reagent were added and mixed thoroughly. The mixture was heated for 7 min at 100°C on a water bath and the OD at 830 nm was recorded. A standard curve was prepared by using inorganic orthophosphate solution of 0 to 1.5 µ mole. Solutions were made up to 4.1 ml with water and 0.5 ml 10 N H_2SO_4 added. 0.2 ml of 5% ammonium molybdate and 0.2 ml Fiske Subbarow reagent were added in succession with mixing. The solutions were heated for 7 min at 100°C and the colour read at 830 nm. The amount of phosphate released was thus determined. One unit of phospholipase C is defined as the amount of enzyme that hydrolyzes 1 nmol of substrate per min at 37°C.

2.3.5 Pigment assays

2.3.5.1 Pyocyanine

Pyocyanine is soluble in chloroform under neutral or alkaline conditions and insoluble under acidic conditions. This property was employed in the extraction of this pigment from the culture supernatants as described by Burton et al. (1947).

To 5 ml of culture supernatant, 5 x 2 ml volumes of chloroform were added and shaken in a 25 ml separating funnel. After separating, the bulked chloroform extracts were added to 5 ml of 0.1 N HCl in a separate funnel and shaken. The pyocyanine went into the aqueous layer and showed a change of colour from blue-green to red. The chloroform layer was discarded and 5 ml of 0.1 N NaOH added to the aqueous layer to neutralize it and the colour reverted to blue-green. A UV spectrum was obtained and the peak arising at 310 nm was used quantitatively to estimate pyocyanine content of the cultures.

2.3.5.2 Fluorescing compounds

A fluorescent, water soluble, chloroform insoluble pigment was produced by the organisms and was estimated in an aqueous solution in an Aminco-Bowman spectrophotofluorometer. After the chloroform extraction of pyocyanine, the aqueous layer was recentrifuged to remove debris and traces of chloroform. The clear solution was used to estimate the amount of fluorescent compound. Maximum response was found when excitation wavelength was 400 nm and the emmission was measured at 465 nm.

3. EXPERIMENTAL AND RESULTS

3.1 Nutrient Depletion Studies of *P. aeruginosa* NCTC 6750 WT and M7 in batch culture

The final cell density reached by a culture of *P. aeruginosa* has been found to be dependent, below certain levels, on the initial concentration of a growth limiting nutrient in the medium (Brown and Melling (1969a), Boggis (1971) and Finch (1976). The object of this study was therefore to investigate and compare the nutritional requirement of the mucoid and non-mucoid types of *P. aeruginosa* grown under identical conditions.

3.1.1 Experimental

The medium used for the study of glucose, magnesium, nitrogen and phosphate is that described in Table 1. Preparation of medium for iron growth curves has been discussed in section 2.2.2. Table 4 shows the medium used for the sulphate study. Experimental details have been described in section 2.2.1.

Table 4

Chemically Defined Medium used for Sulphate Study

Ingredient	Final Concentration (M)
$(NH_4)_2SO_4$	1.2×10^{-2}
$MgCl_2.6H_2O$	1.6×10^{-4}
$FeCl_2.4H_2O$	2.7×10^{-4}
Glucose	2.0×10^{-2}
KCl	3.0×10^{-3}
NaCl	3.0×10^{-3}
$(NH_4)_2HPO_4$	1.852×10^{-2}
$NH_4H_2PO_4$	4.8×10^{-4}
	pH 7.8

3.1.2 Carbon depletion studies

The growth curves for carbon requirement are shown in Figures 3 and 4 where either glucose (Figure 3) or gluconate (Figure 4) was the carbon source. A rapid cessation of growth was demonstrated by cultures with low levels of carbon in their media. Cultures with a high concentration of carbon behaved differently. After logarithmic growth, there was a decline in growth rate but growth did not stop, indicating that carbon was no longer the growth limiting nutrient. The behaviour was similar for both organisms. The relationship between initial glucose or gluconate concentration and the onset of non-linear growth is shown in Figure 5. There was little or no difference between the two carbon sources and the onset of non-linear growth for any of the two organisms. The relationship was linear to an OD_{470} of 1.5 for WT and of 1.0 for M7 when glucose was the carbon source. A slightly higher OD was reached when gluconate was used.

3.1.3 Magnesium depletion studies

Figures 6 and 7 show the growth curves for magnesium study, with glucose or gluconate (36.6 mM) as the carbon source. Unlike glucose, the growth did not cease after a period of exponential growth, but continued at a gradually decreasing rate. The onset of magnesium limitation was therefore taken to occur when growth ceased to be exponential. This value varied with initial magnesium concentration. The relationship between onset of non-linear growth and initial magnesium concentration is shown in Figure 8. As with carbon requirement, a higher growth was achieved when gluconate was the carbon source. Also, irrespective of the carbon source, M7 grew to a higher OD_{470} than WT for the same magnesium concentration.

3.1.4 Iron depletion studies

The growth curves for iron limitation (glucose carbon source) are shown in Figure 9. Figure 10 shows the relationship between onset of non-linear growth and added iron concentration. There was no difference between M7 and WT to an OD_{470} of 1.0. Further addition of iron resulted in a slightly higher OD for WT.

3.1.5 Sulphate depletion studies

The growth curves for sulphate limitation are shown in Figure 11and the relationship between OD onset of non-linear growth and sulphate concentration is shown in Figure 12. M7 showed a higher OD_{470} onset than WT for the same amount of sulphate. WT showed only a slightly higher OD than M7 at high sulphate concentration.

3.1.6 Phosphate depletion studies

Growth curves describing phosphate limitation (Figure 13) were similar to those of magnesium and iron limitations, exhibiting a progressive decrease in growth rate after onset of limitation. Figure 14 shows the relationship between onset of non-linear growth and added phosphate in the medium. As with sulphate, M7 showed a higher OD_{470} onset than WT for the same amount of phosphate at low ODs. Above OD_{470} of 0.65,further addition of phosphate did not cause an increase in OD onset of M7. WT grew to an OD_{470} of 1.0 before OD onset ceased to be affected by further addition of phosphate.

3.1.7 Nitrogen depletion studies

The growth curves and the relationship between OD at onset and $(NH_4)_2SO_4$ concentration in the medium are illustrated in

Figures 15 and 16. The growth curves are similar to those of glucose and sulphate depleted studies, with growth ceasing when nitrogen became limiting. There was very little difference between WT and M7 at low ODs, with WT showing a slightly higher OD. Above an OD_{470} 0.7, further addition of nitrogen did not affect M7. WT grew to an OD_{470} 0.85 before OD onset ceased to be affected by further addition of ammonium sulphate.

3.1.8 Optical density and viable count relationship

When the two organisms were grown to the same optical density or were resuspended to identical optical density, they showed similar viable counts. Differences however existed between the limitations. C-lim cells showed a higher and P-lim a lower viable count when compared to the other limitations of same optical density.

3.1.9 Saturation constants and maximum growth rate

The saturation constants, K_s, and the maximum growth rate, μmax, for glucose, iron, magnesium, sulphate, nitrogen and phosphate were calculated using Monod's equation (Monod 1942; 1950).

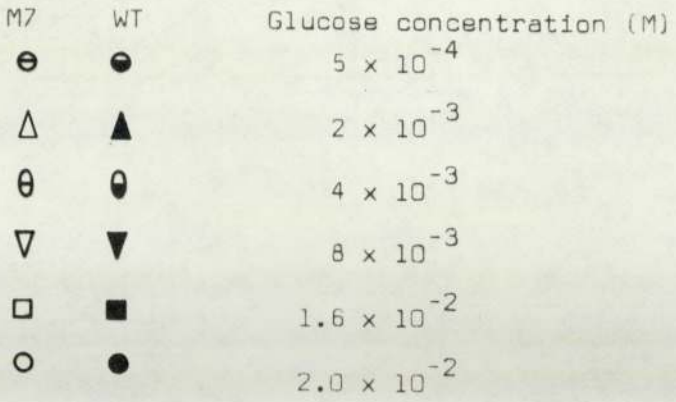
$$\mu = \mu m \left(\frac{S}{K_s + S}\right)$$

and its rearrangement as suggested by Lineweaver and Burke:

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

A plot of $\frac{1}{\mu}$ versus $\frac{1}{S}$ will give a graph with line of slope $\frac{K_s}{\mu m}$ and intercept on the Y axis of $\frac{1}{\mu m}$. Using figure 17 (A - F), the two constants were calculated for both M7 and WT and are summarized in Table 5. Under all conditions, the specific growth rate was higher for WT than M7.

Figure 3. Growth of *P. aeruginosa* in CDM with graded concentrations of glucose.



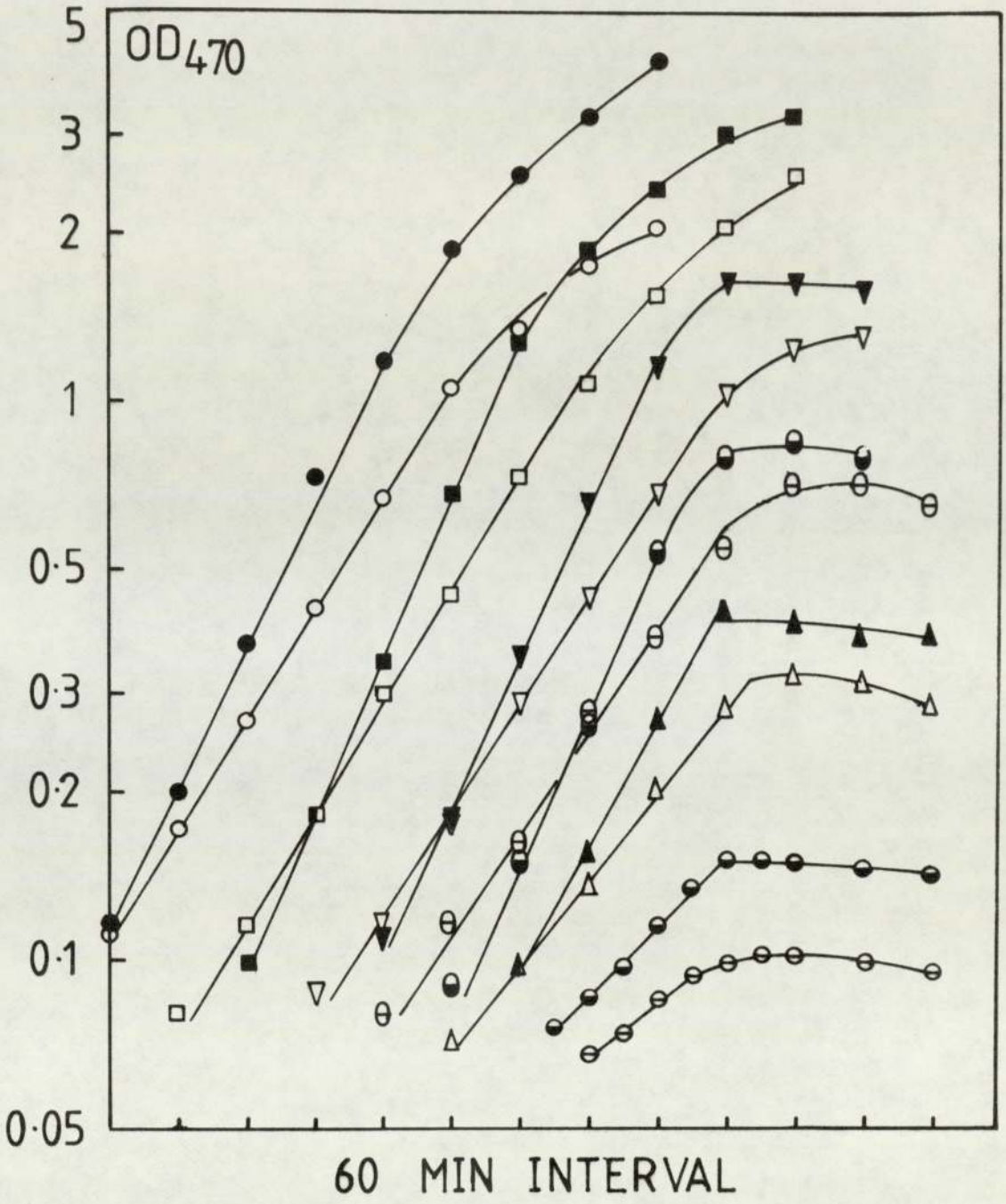
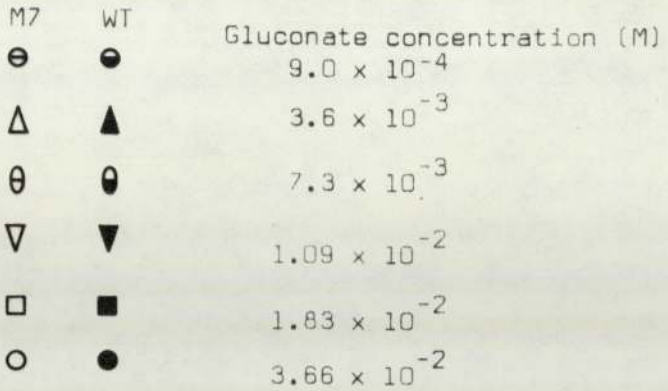

5
3
2
1
0·5
0·3
0·2
0·1
0·05
OD470
60 MIN INTERVAL

Figure 4. Growth of P. aeruginosa in CDM with graded concentrations of gluconate.



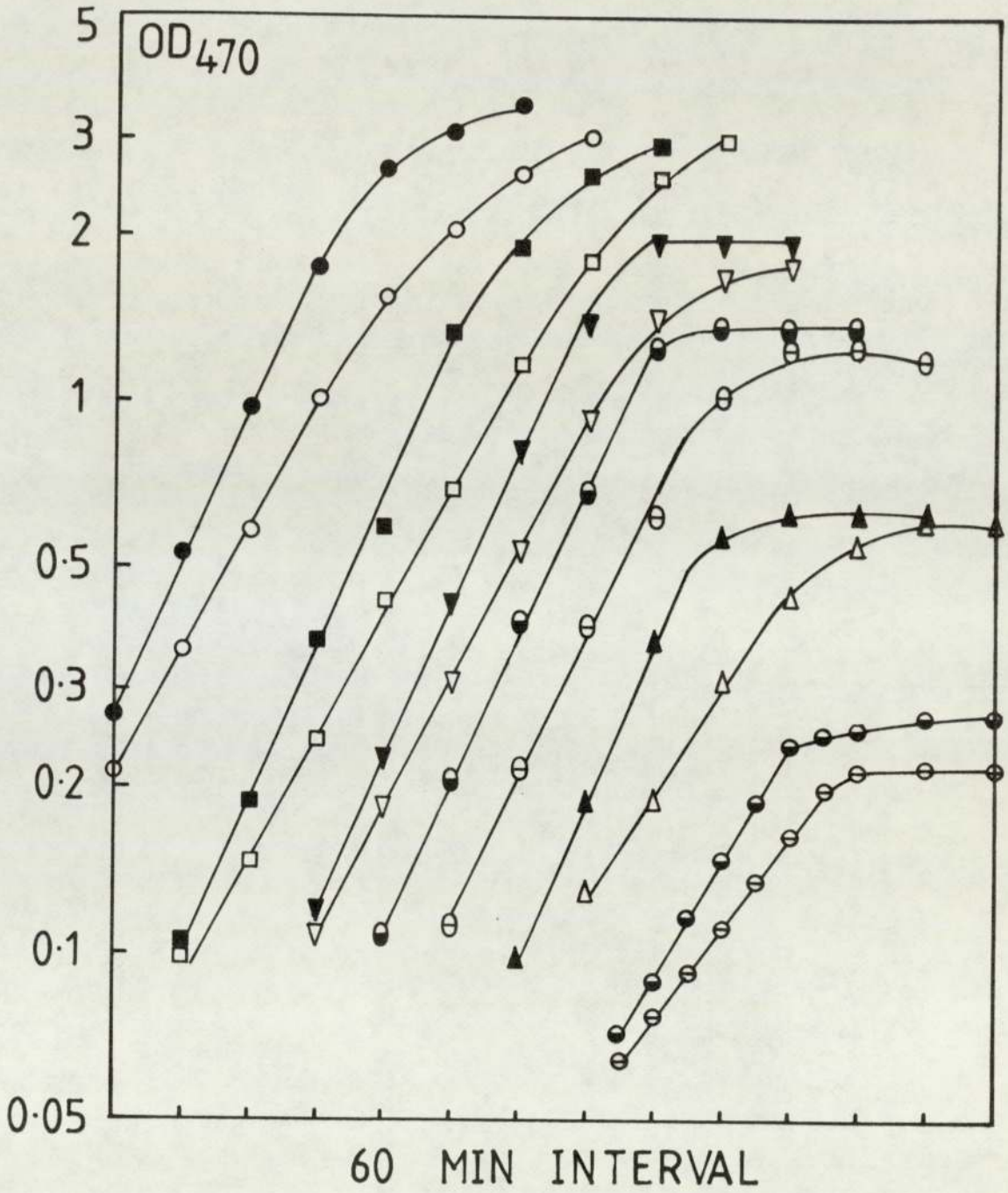
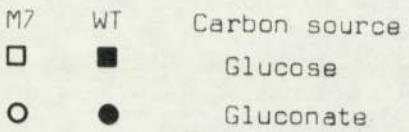

5
3
2
1
0·5
0·3
0·2
0·1
0·05
OD470
60 MIN INTERVAL

Figure 5. Relation between onset of non-linear growth of *P. aeruginosa* and initial carbon concentration.



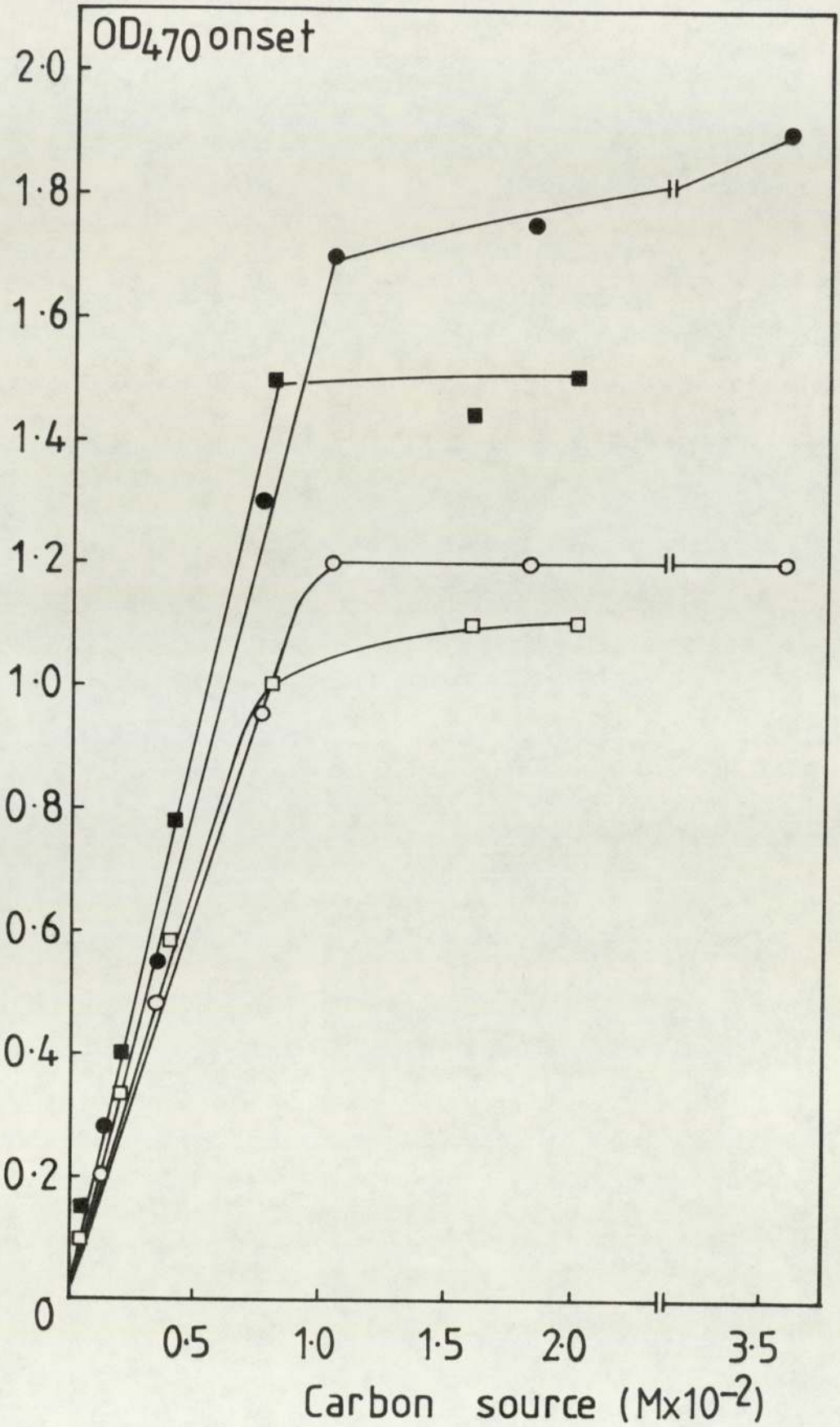
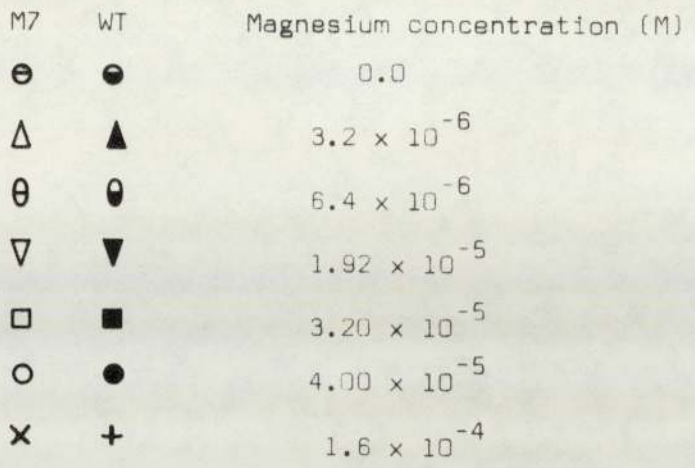

OD470 onset
2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
0·5
1·0
1·5
2·0
3·5
Carbon source (Mx10⁻²)

Figure 6. Growth of P. aeruginosa in CDM with graded concentrations of magnesium (glucose carbon source).



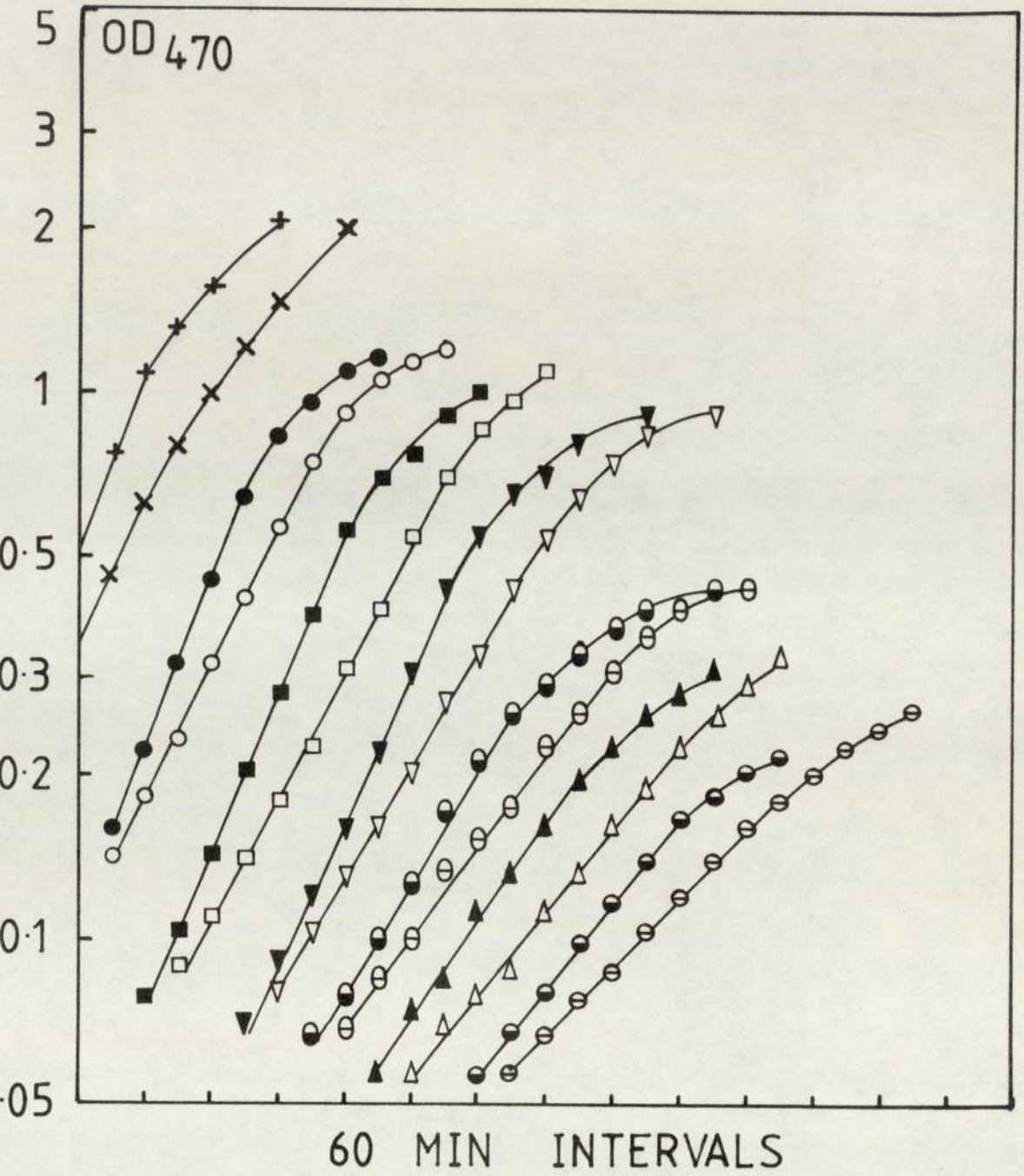
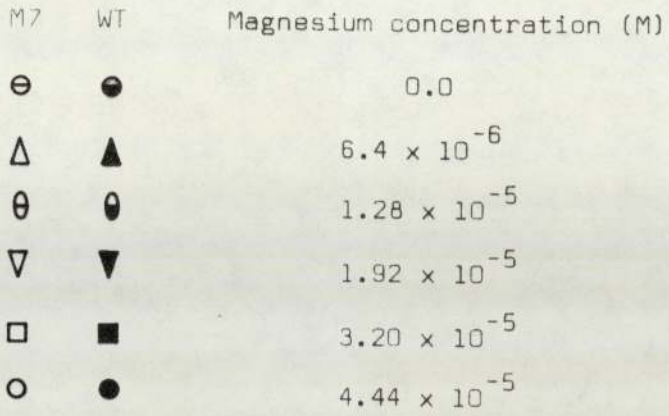

OD 470
5
3
2
1
0·5
0·3
0·2
0·1
·05
60 MIN INTERVALS

Figure 7. Growth of P. aeruginosa in CDM with graded concentrations of magnesium (gluconate carbon source).



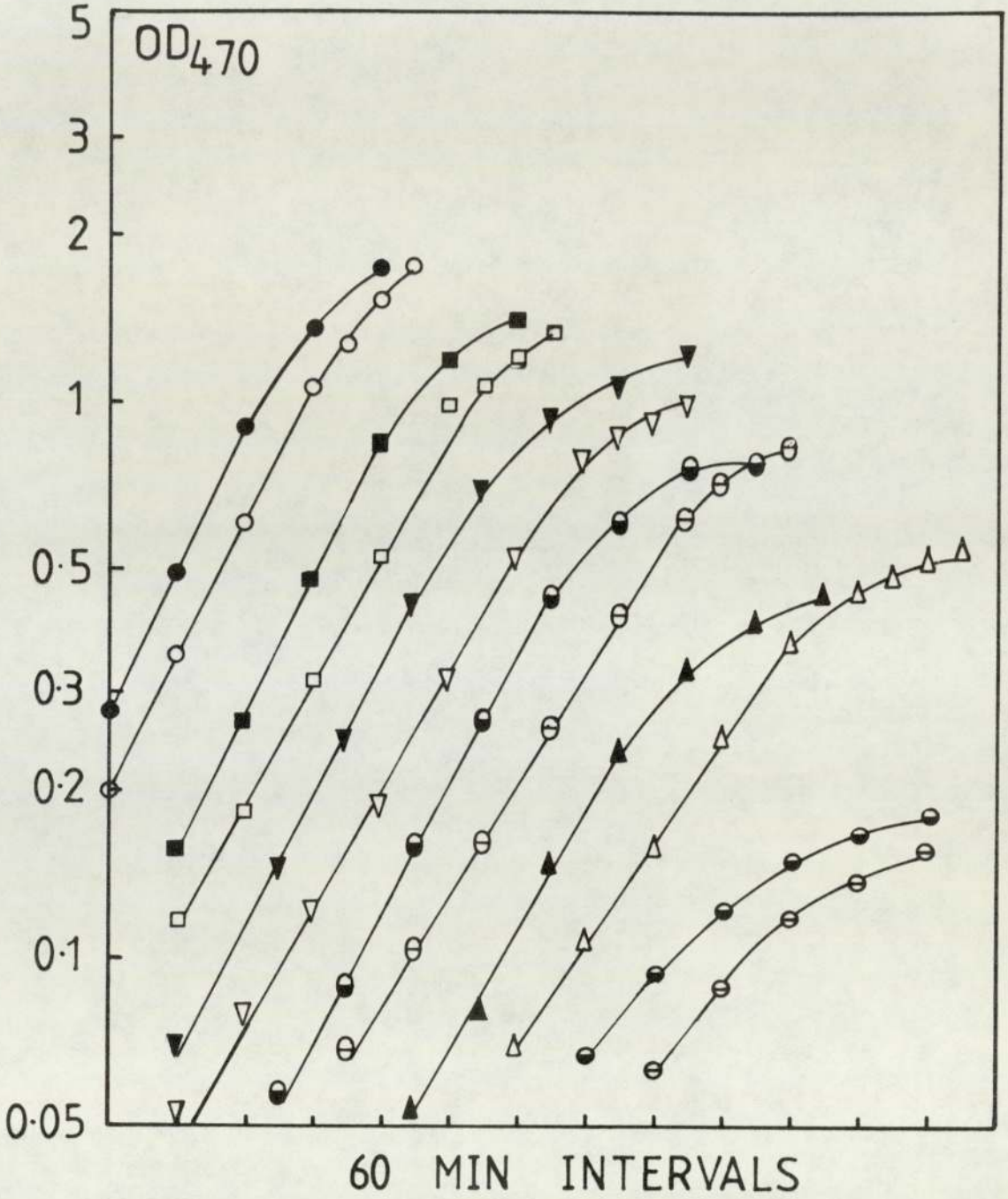
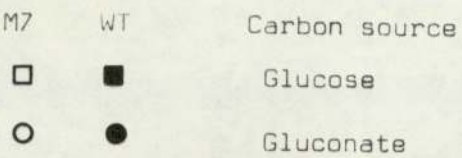

5
3
2
1
0·5
0·3
0·2
0·1
0·05
OD470
60 MIN INTERVALS

Figure 8. Relation between onset of non-linear growth of *P. aeruginosa* and initial magnesium concentration.



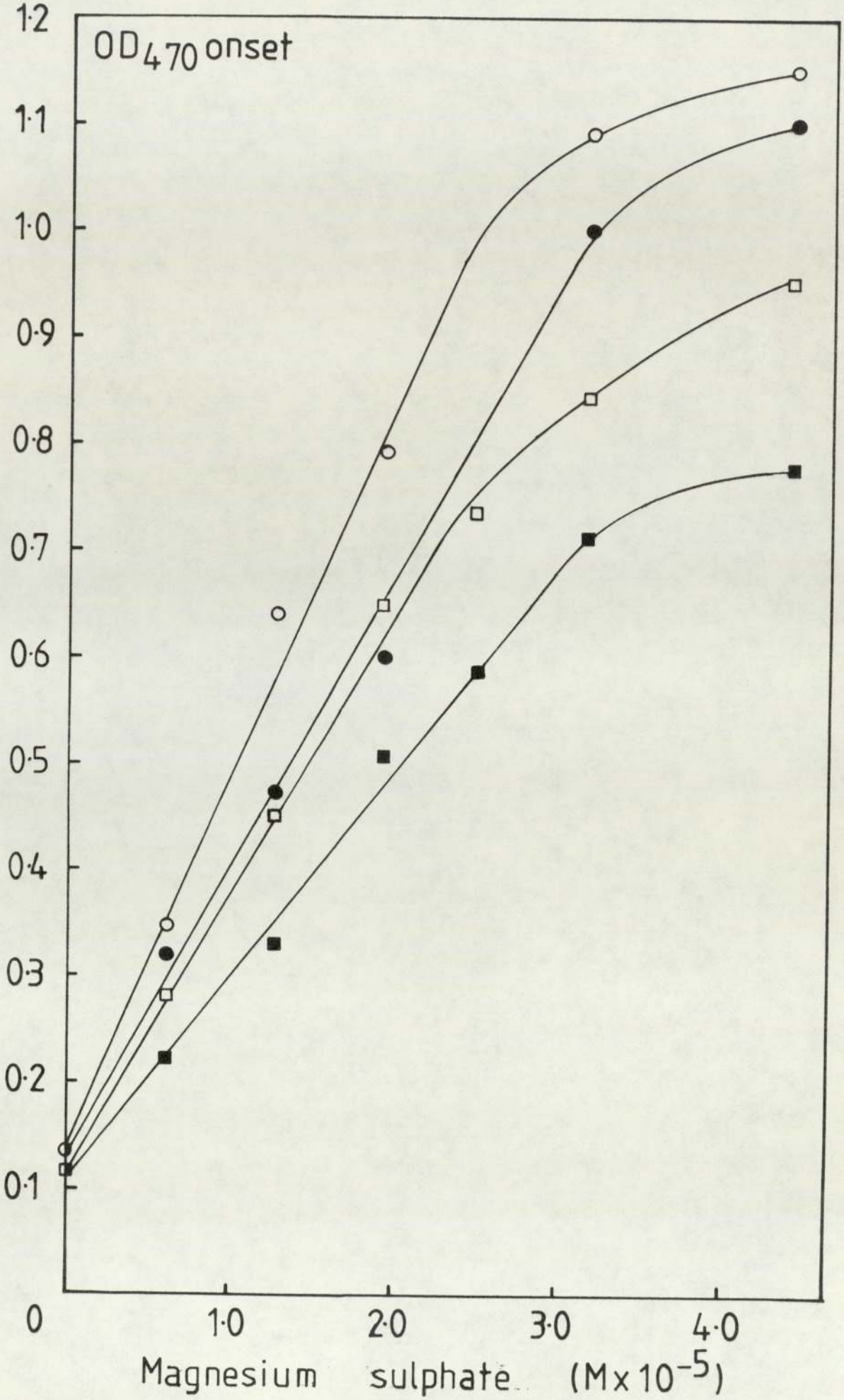
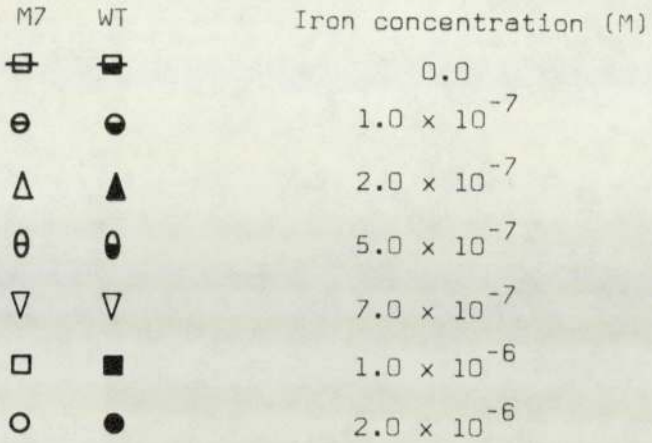

OD470 onset
1·2
1·1
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
1·0
2·0
3·0
4·0
Magnesium sulphate (M x 10⁻⁵)

Figure 9. Growth of P. aeruginosa in CDM with graded concentrations of iron (glucose carbon source).



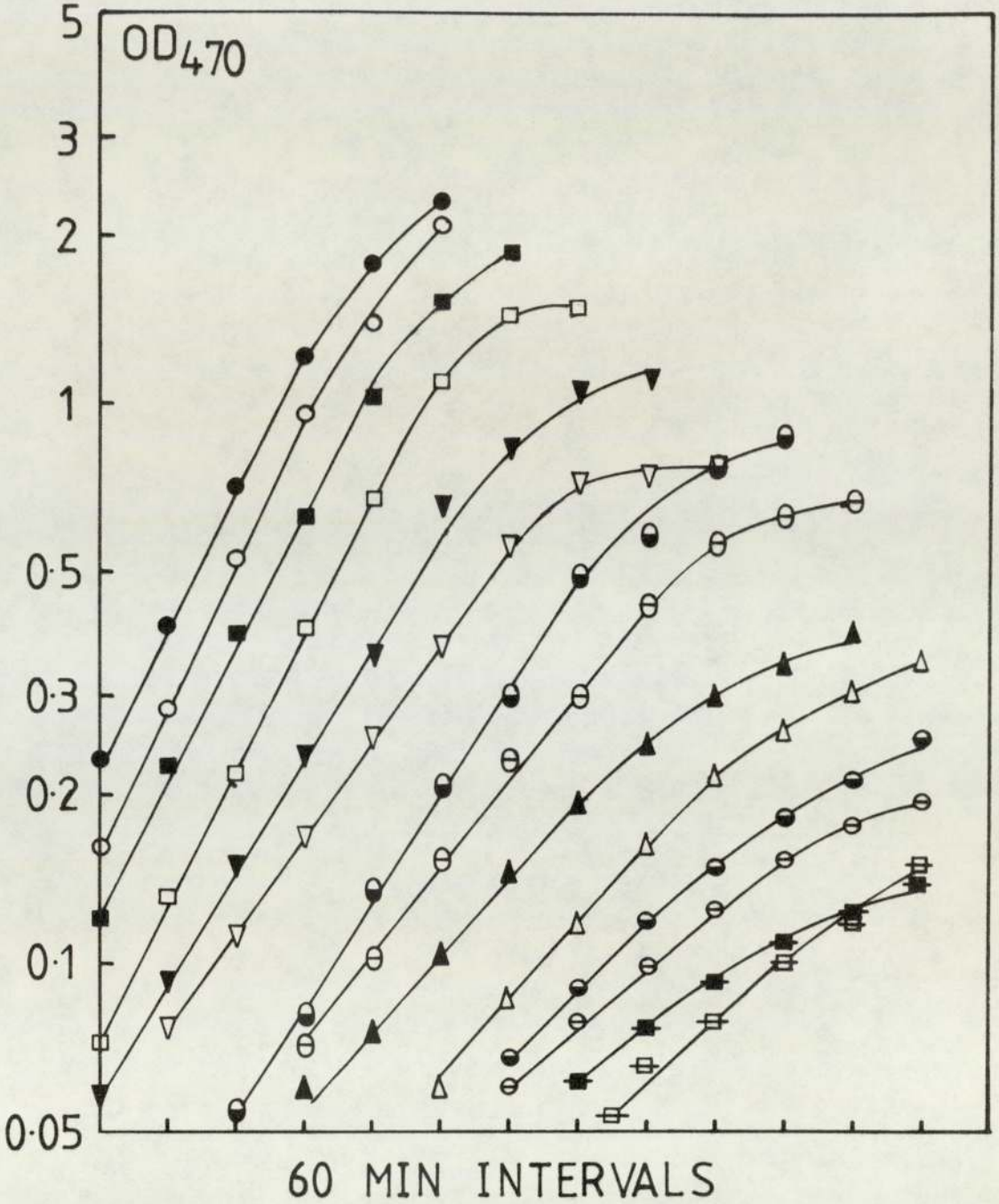
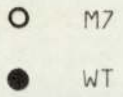

5
OD470
3
2
1
0·5
0·3
0·2
0·1
0·05
60 MIN INTERVALS

Figure 10. Relation between onset of non-linear growth of *P. aeruginosa* and initial iron concentration.



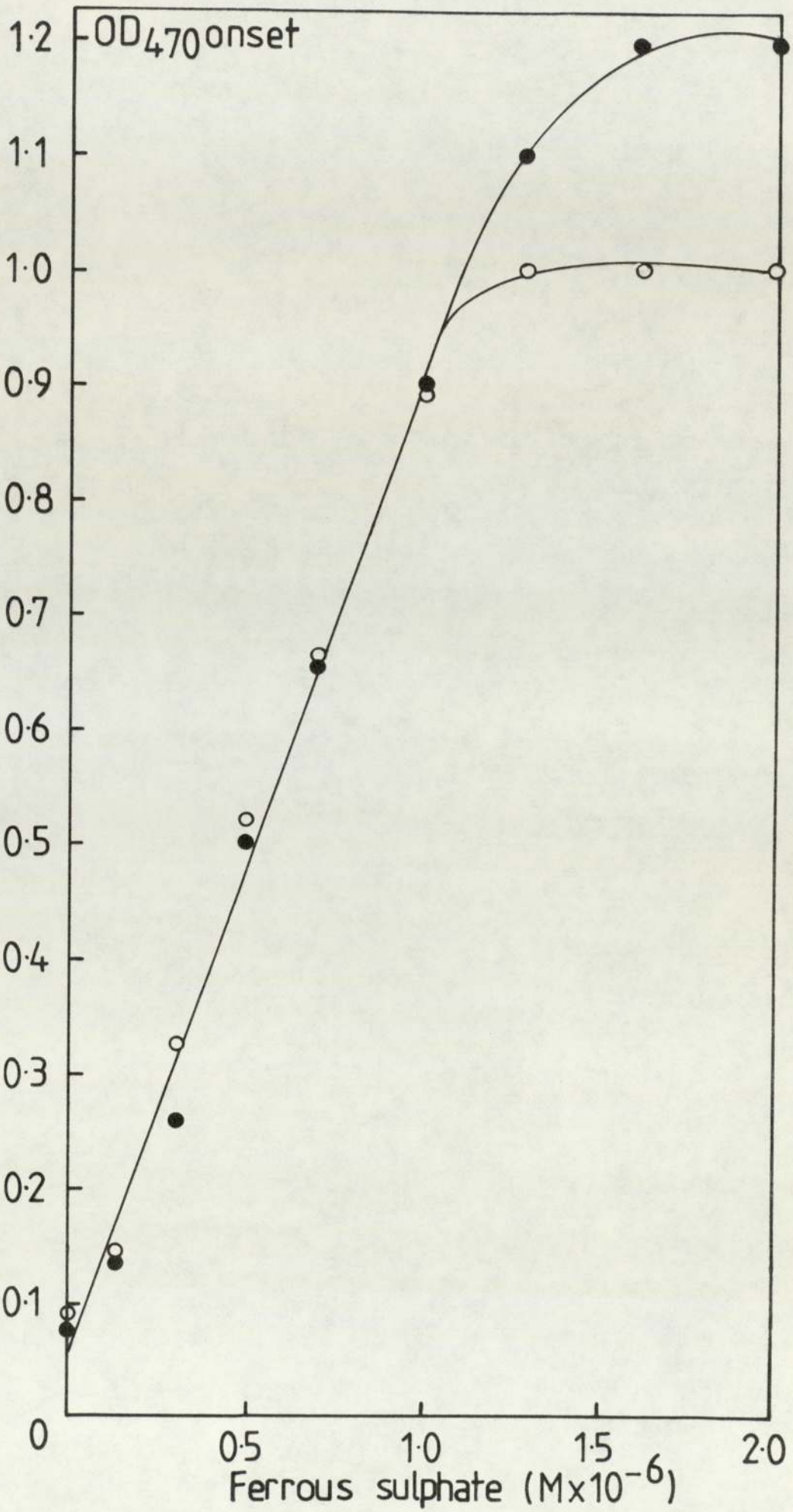
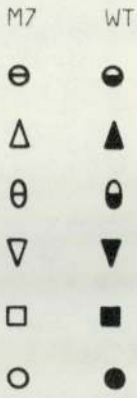

OD470onset
1·2
1·1
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
0·5
1·0
1·5
2·0
Ferrous sulphate (M x 10⁻⁶)

Figure 11. Growth of P. aeruginosa in CDM with graded concentrations of sulphate (glucose carbon source).



M7	WT	Sulphate concentration (M)
⊖	⊖	6.0×10^{-6}
△	▲	1.2×10^{-5}
θ	θ	2.4×10^{-5}
▽	▼	3.6×10^{-5}
□	■	6.0×10^{-5}
○	●	9.6×10^{-5}

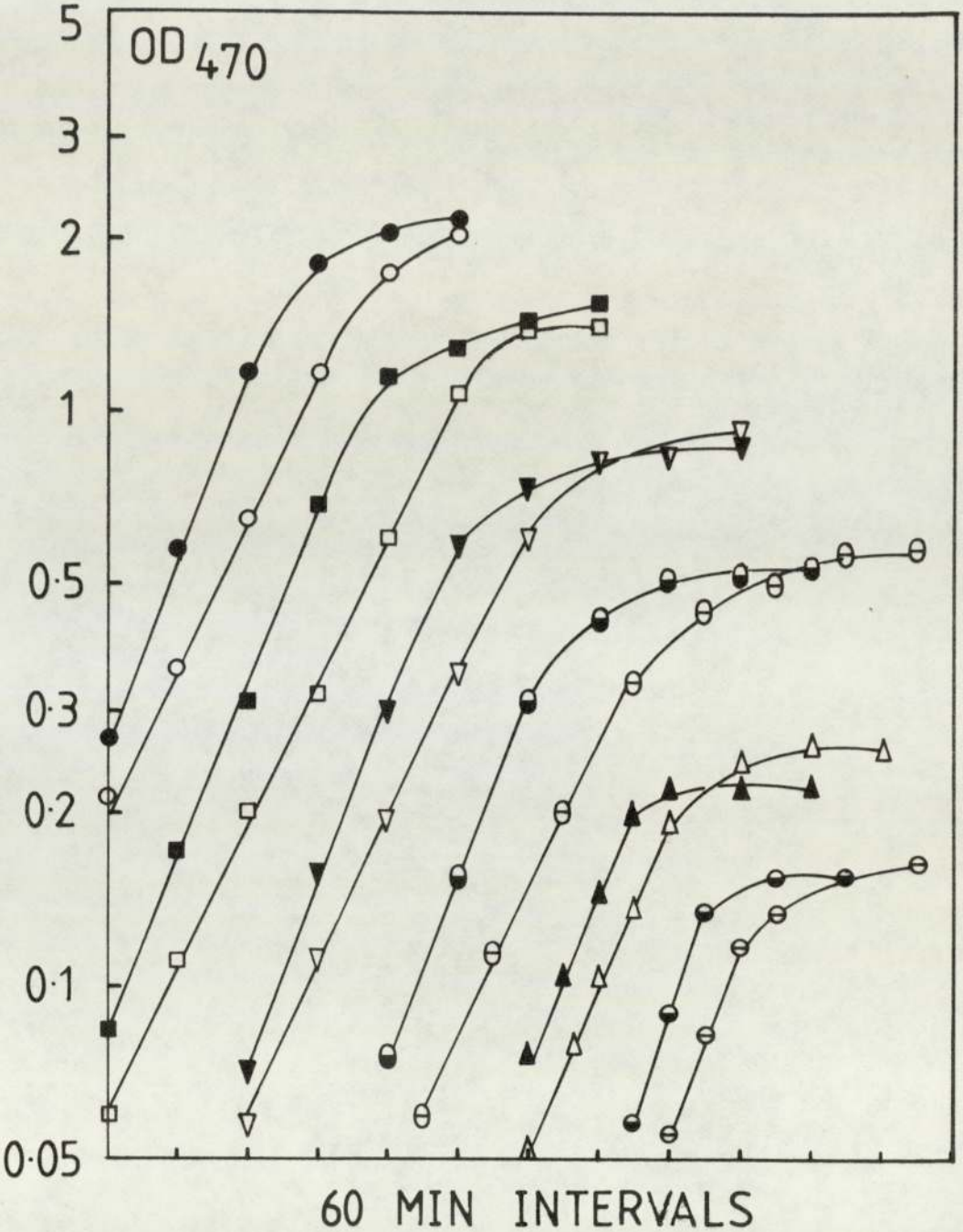
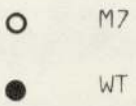

OD 470
5
3
2
1
0·5
0·3
0·2
0·1
0·05
60 MIN INTERVALS

Figure 12. Relation between onset of non-linear growth of P. aeruginosa and initial sulphate concentration.



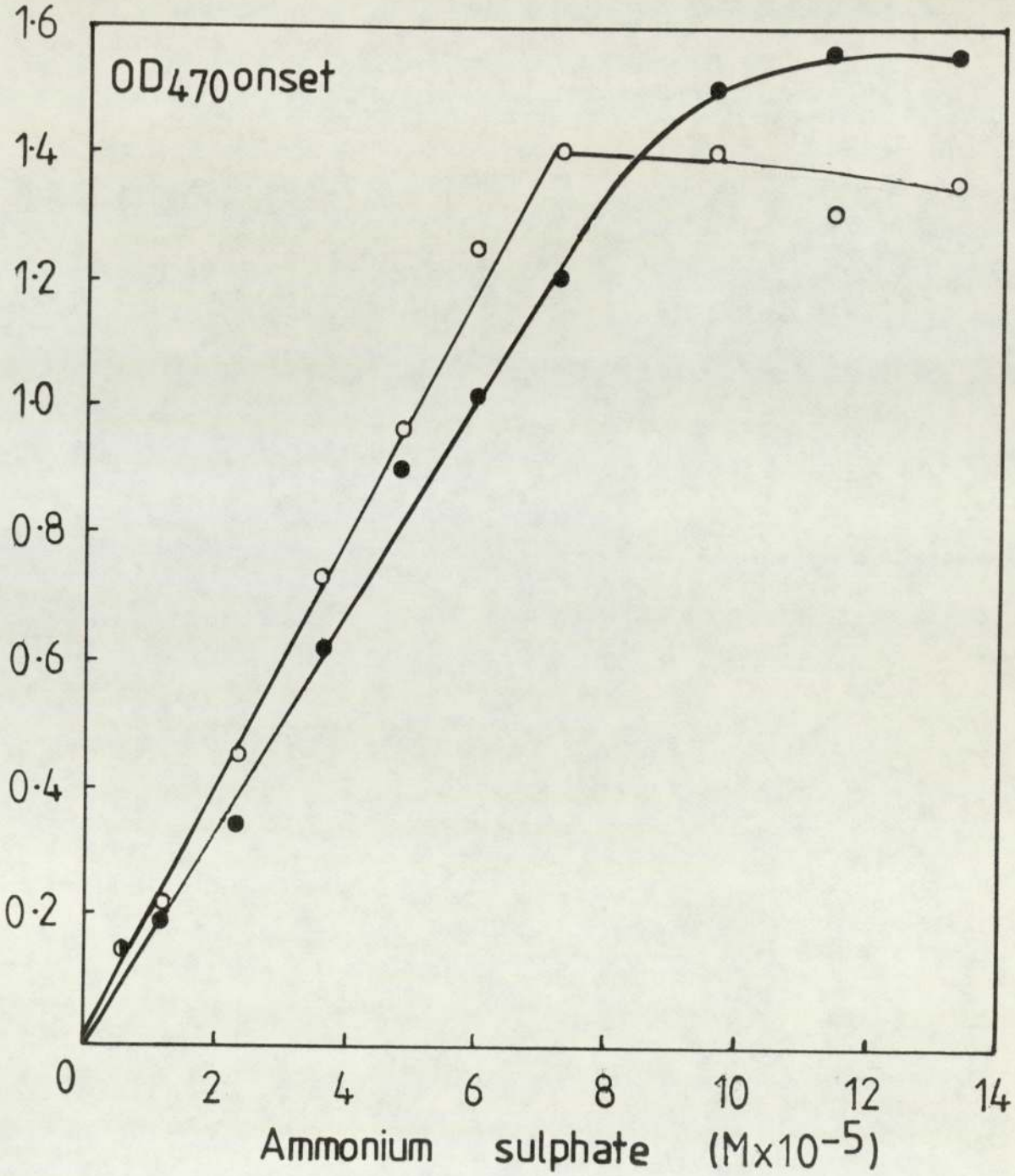
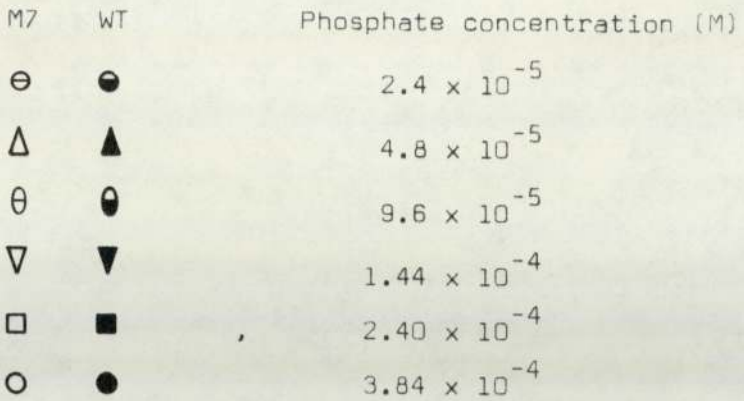

1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
OD470onset
2
4
6
8
10
12
14
Ammonium sulphate (M x 10-5)

Figure 13. Growth of P. aeruginosa in CDM with graded concentrations of phosphate (glucose carbon source).



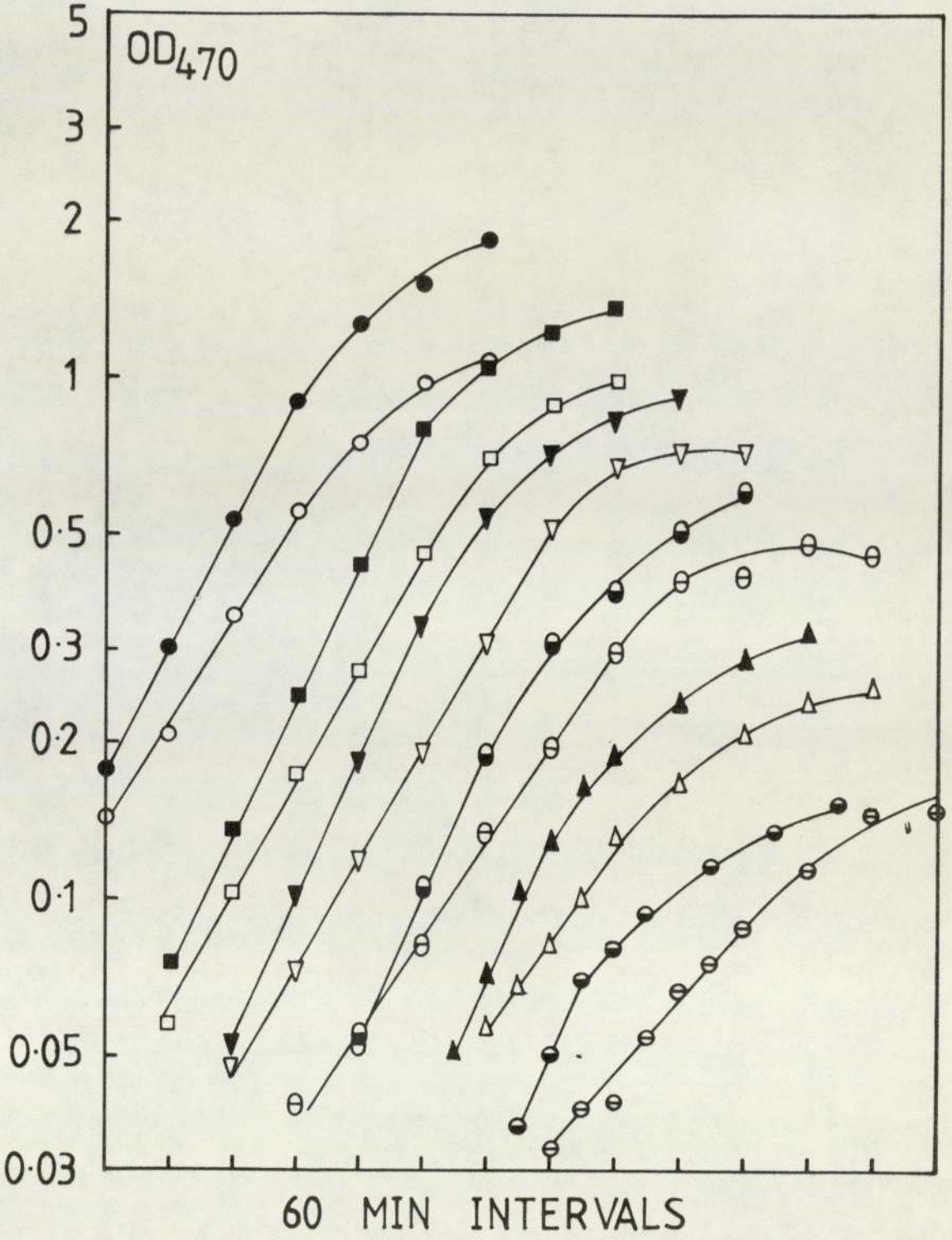

5
3
2
1
0·5
0·3
0·2
0·1
0·05
0·03
OD470
60 MIN INTERVALS

Figure 14. Relation between onset of non-linear growth of *P. aeruginosa* and initial phosphate concentration.

○ M7

● WT

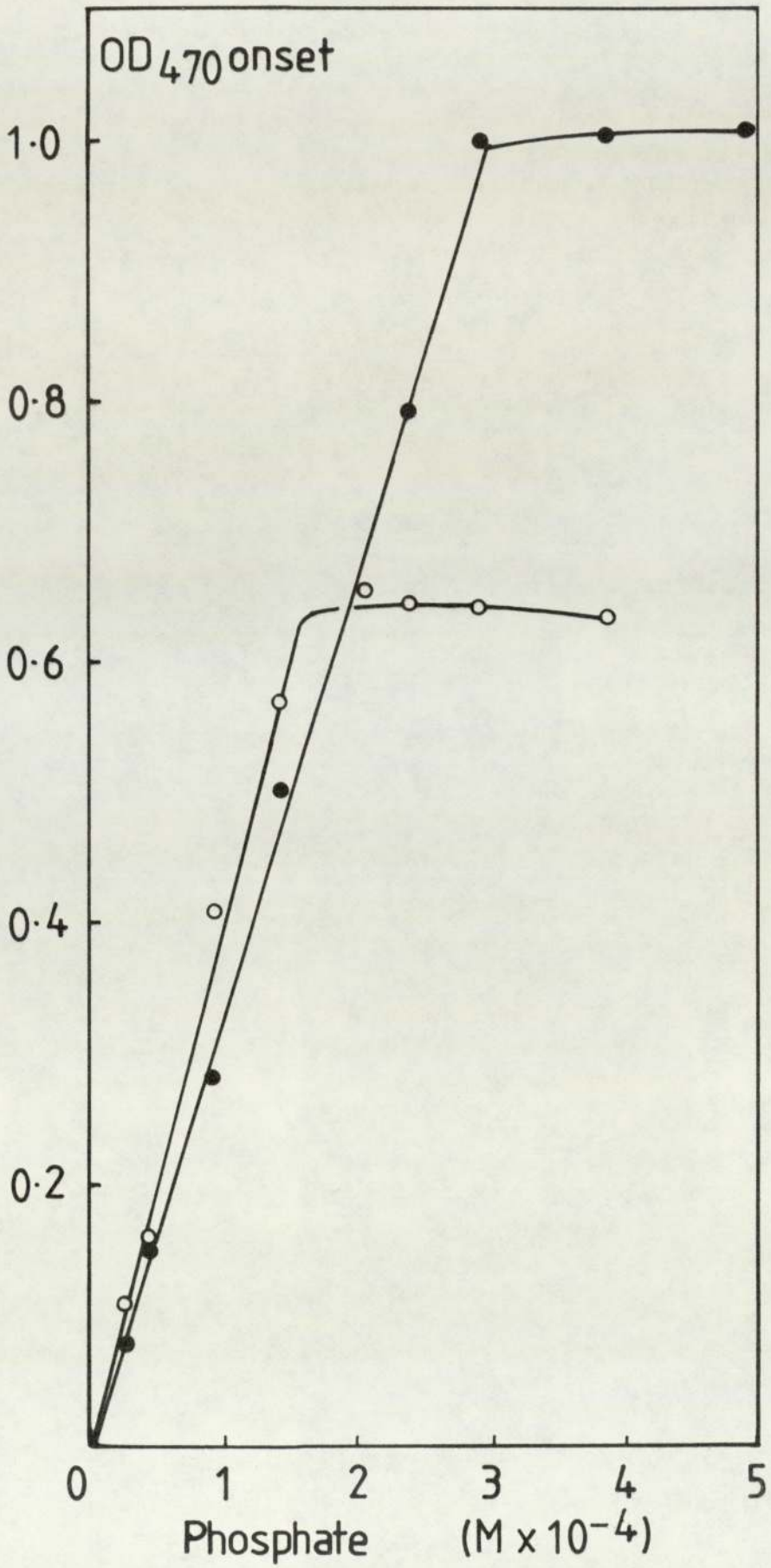
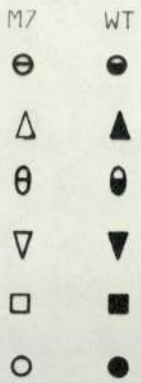

OD 470 onset
1·0
0·8
0·6
0·4
0·2
0
1
2
3
4
5
Phosphate (M x 10⁻⁴)

Figure 15. Growth of P. aeruginosa in CDM with graded concentrations of ammonium ion (glucose carbon source).



M7	WT	Ammonium sulphate concentration (M)
⊖	◒	1.2×10^{-4}
△	▲	2.4×10^{-4}
θ	◒	7.2×10^{-4}
▽	▼	1.44×10^{-3}
□	■	2.88×10^{-3}
○	●	4.80×10^{-3}

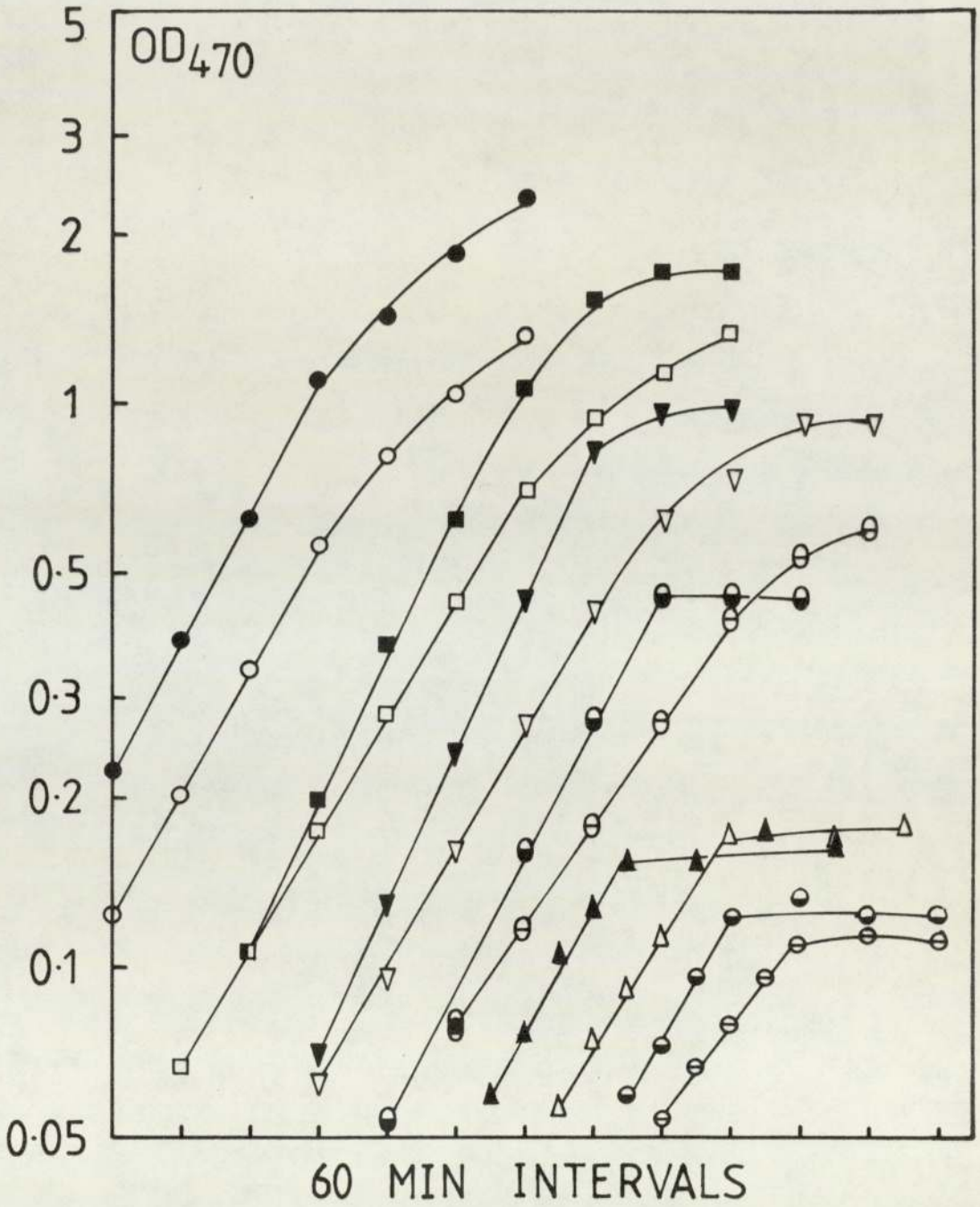
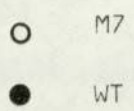

5
3
2
1
0·5
0·3
0·2
0·1
0·05
OD470
60 MIN INTERVALS

Figure 16. Relation between onset of non-linear growth of *P. aeruginosa* and initial ammonium concentration.



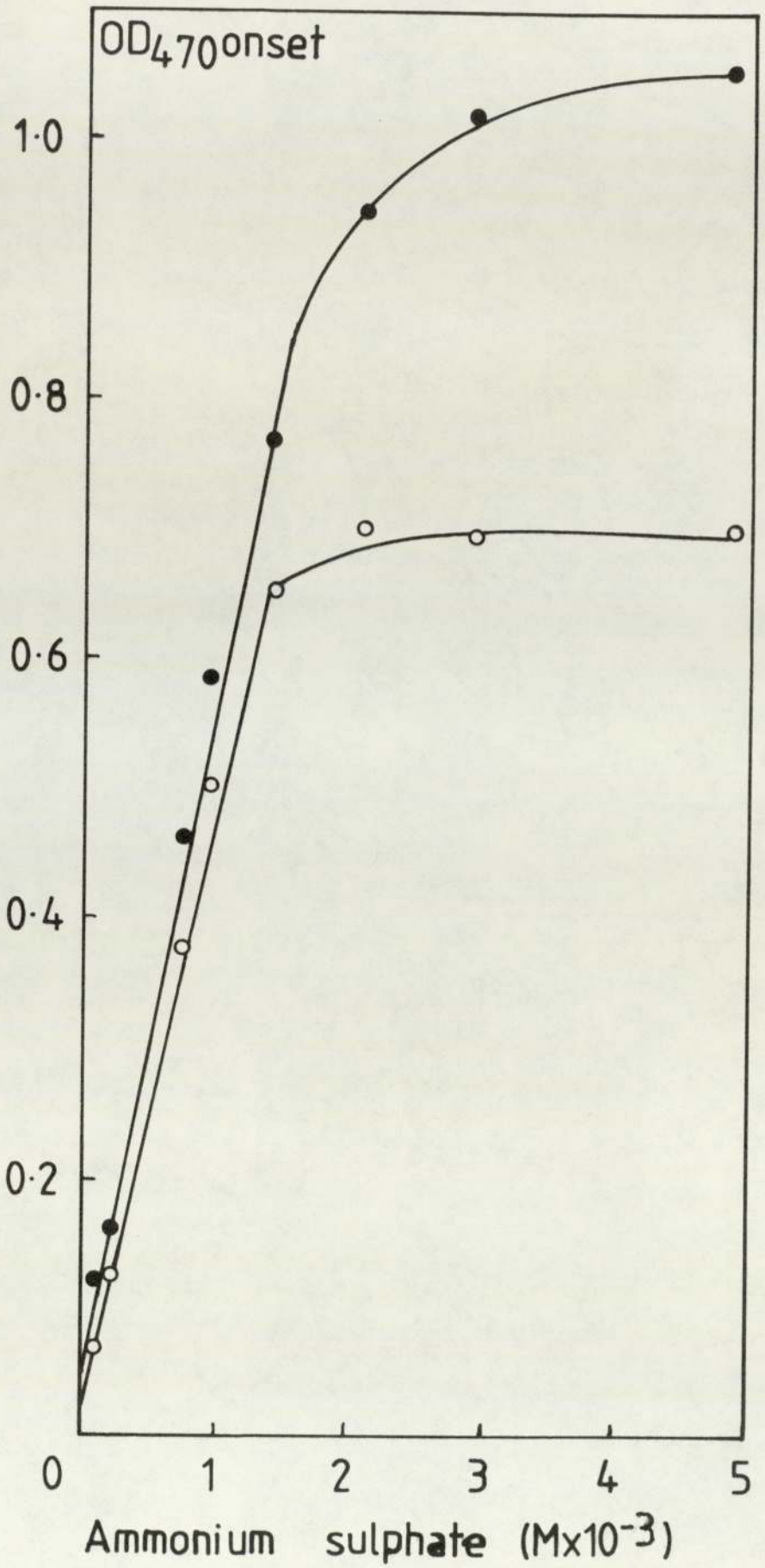

OD470onset
1·0
0·8
0·6
0·4
0·2
0
1
2
3
4
5
Ammonium sulphate (Mx10-3)

Figure 17. Double reciprocal plot of P. aeruginosa growth rate versus medium substrate concentration.

M7 ○ WT ●

Substrate

A Carbon

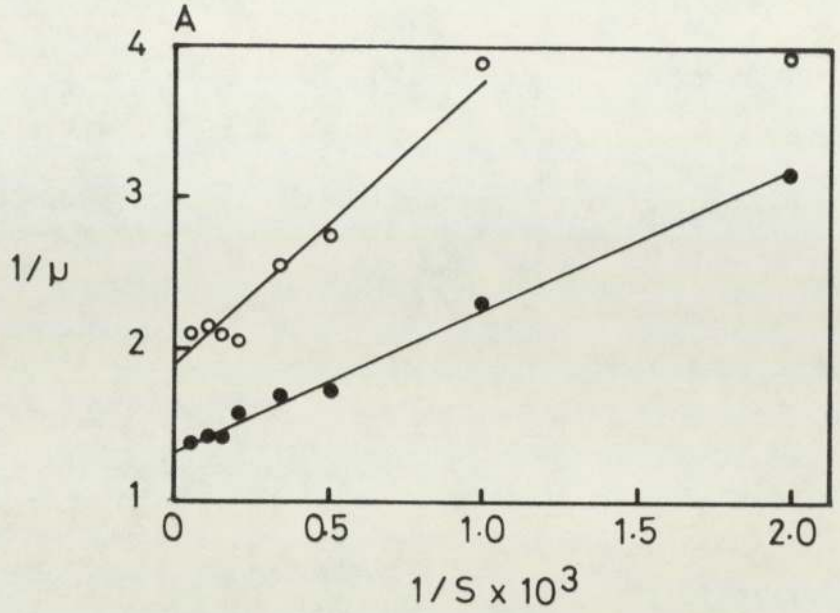
B Iron

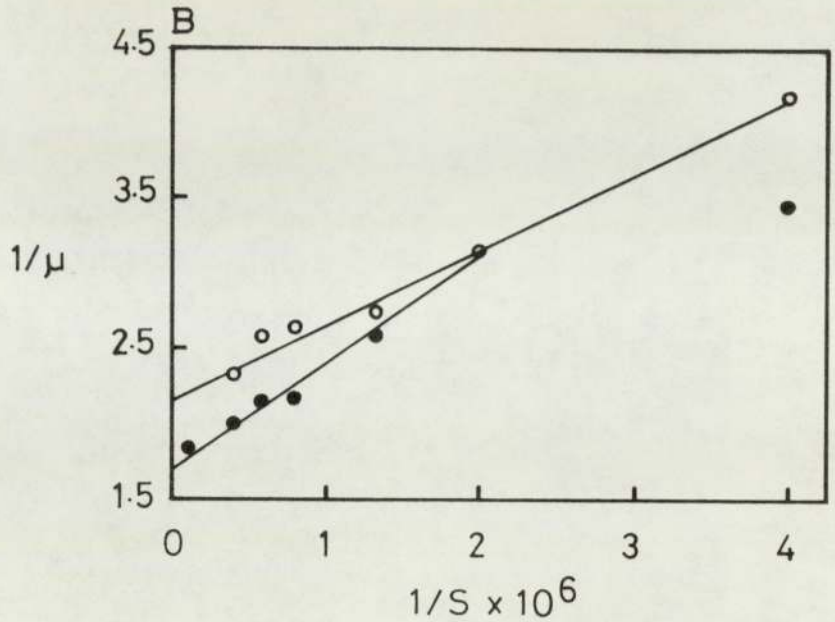
C Magnesium

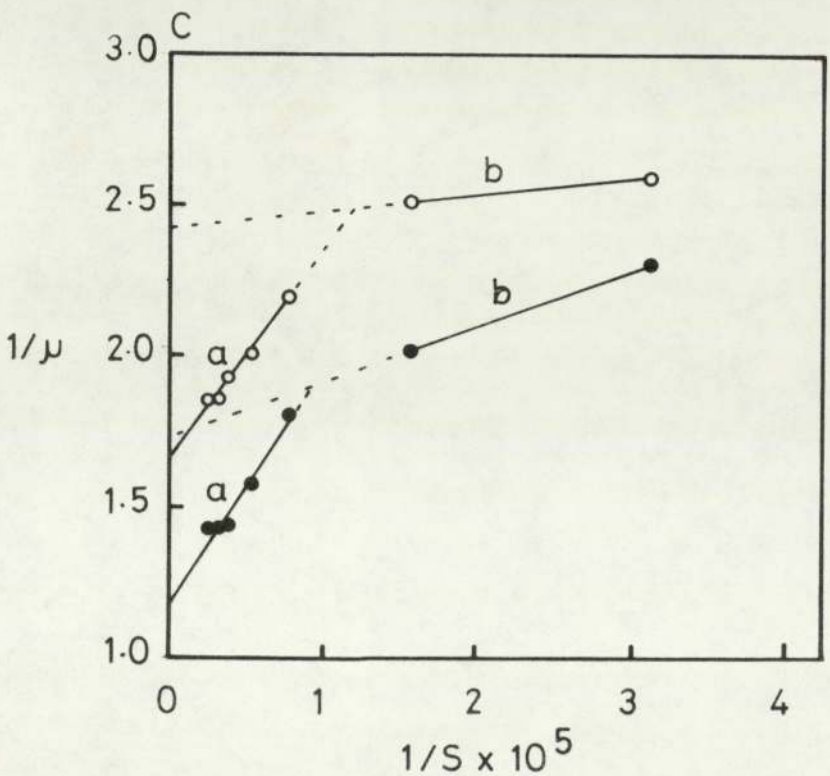
D Sulphate

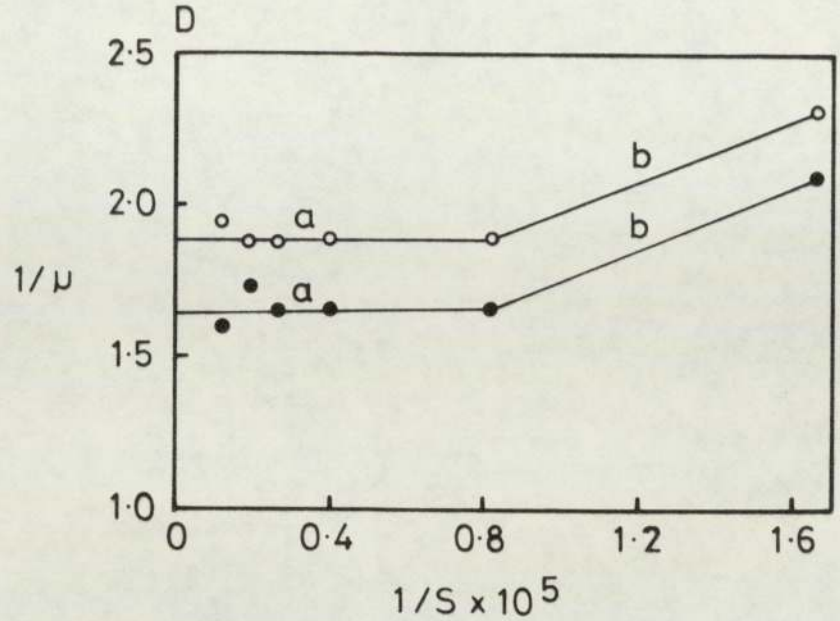
E Ammonium ion

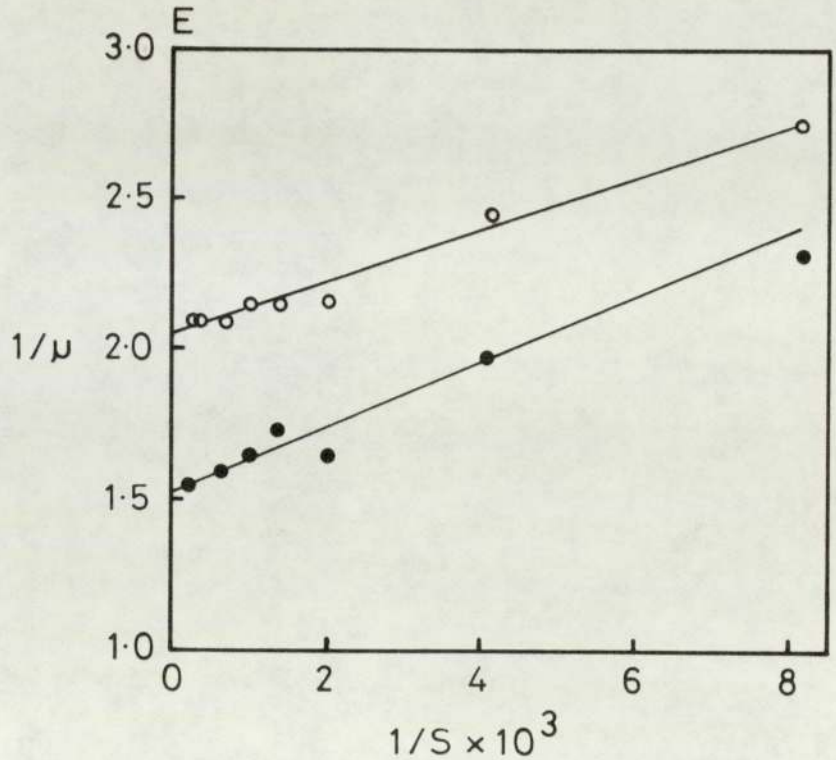
F Phosphate


A
4
3
2
1
1/μ
0
0.5
1.0
1.5
2.0
1/S x 10^3


B
4.5
3.5
2.5
1.5
1/μ
0
1
2
3
4
1/S x 10^6


C
3.0
2.5
2.0
1.5
1.0
1/μ
a
a
b
b
0
1
2
3
4
1/S x 10^5


D
2·5
2·0
1·5
1·0
1/μ
a
a
b
b
0
0·4
0·8
1·2
1·6
1/S x 10^5


E
3·0
2·5
2·0
1·5
1·0
1/μ
0
2
4
6
8
1/S x 10^3

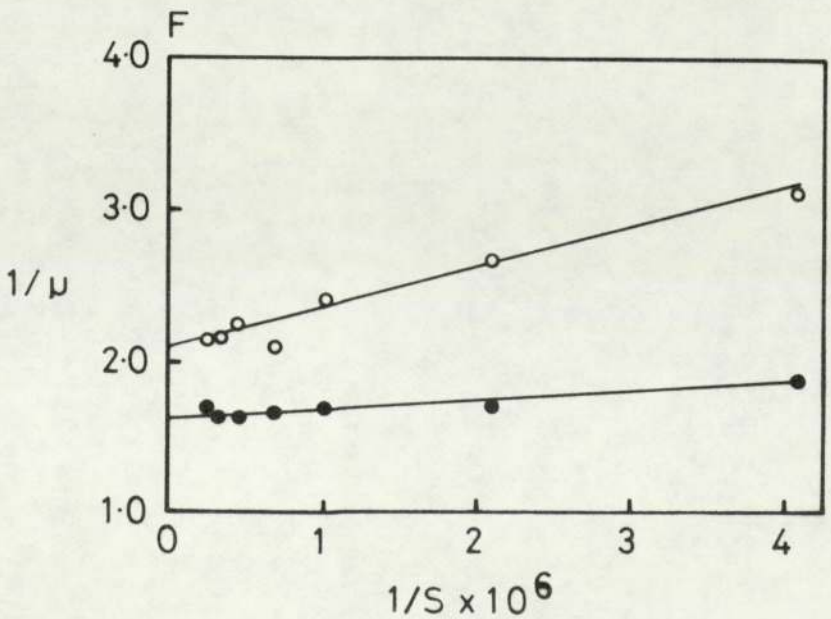

F
4·0
3·0
2·0
1·0
1/μ
0
1
2
3
4
1/S x 10^6

Table 5

Growth rate constant, μm, and saturation constant, K_s, of M7 and WT grown under identical conditions

Limiting Nutrient	Organism	Part of curve*	μm	K_s (M)
C	M7		0.52	9.52×10^{-4}
	WT		0.75	6.89×10^{-4}
Fe	M7		0.45	3.13×10^{-7}
	WT		0.58	3.98×10^{-7}
Mg	M7	a	0.57	3.95×10^{-6}
	WT	a	0.83	6.85×10^{-6}
	M7	b	0.41	2.29×10^{-7}
	WT	b	0.56	9.32×10^{-7}
S	M7	a	0.53	-
	WT	a	0.61	-
	M7	b	0.68	3.64×10^{-7}
	WT	b	0.82	3.49×10^{-7}
N (as $(NH_4)_2SO_4$)	M7		0.48	4.04×10^{-7}
	WT		0.64	6.90×10^{-7}
P	M7		0.48	1.28×10^{-6}
	WT		0.62	4.14×10^{-7}
O_2	M7		0.58	-
	WT		0.73	-

* a and b refer to the different parts of the biphasic curves as shown in Figure 17.

3.2 The Growth of M7 and WT in Chemostats

The theory of bacterial growth in continuous culture has been discussed in section 1.4.

3.2.1 Growth in chemostats

Twelve chemostats (50 ml) were designed using the model described in Gilbert and Stuart (1977) with slight modifications. Sintered glass was used on the air inlet to ensure adequate and even aeration and mixing. A water cooled unit was added on the air and spent medium outlet to reduce evaporation. Where possible, a water jacket surrounded the culture vessel through which water at 37°C could be circulated. The chemostats were assembled and autoclaved at 121°C for 20 min. Those chemostats without water jackets were immersed in a water bath at 37°C. Water from the same water bath was circulated in jacketed chemostats. Air could only enter the chemostats through sterile bacteriological filters.

The chemostats were filled with fresh media by a calibrated peristaltic pump and the air pump switched on. The vessels were inoculated aseptically with several ml of Pseudomonas aeruginosa WT or M7 from a batch culture grown in CDM. The bacteria were allowed to grow as a batch culture overnight. When visible growth was observed in the vessels, the pump was set to a dilution rate of 0.05 hr^{-1} using a calibration curve. When a faster dilution rate was required, the pump was turned from slow to fast gradually. For each dilution rate, the chemostats were allowed to equilibrate for at least five complete media changes through the vessel. This was a minimum of 100 hr when D = 0.05 hr^{-1} and of 20 hr when D = 0.25 hr^{-1}. Further checks on dilution rates were performed by measuring washout volumes.

Table 6

Media used in chemostat studies of *P. aeruginosa*

INGREDIENT*	Carbon limited growth	Phosphate limited growth	Nitrogen limited growth	Iron limited growth	Sulphate limited growth	Magnesium limited growth
KCl	3×10^{-3}	3×10^{-3}	3×10^{-3}	3×10^{-3}	3×10^{-3}	3×10^{-3}
$(NH_4)_2SO_4$	1.2×10^{-2}	1.2×10^{-2}	9×10^{-4}	1.2×10^{-2}	2.25×10^{-5}	1.2×10^{-2}
$MgSO_4.7H_2O$	3.2×10^{-3}	3.2×10^{-3}	3.2×10^{-3}	3.2×10^{-3}	-	1.6×10^{-5}
$FeSO_4.7H_2O$	2.0×10^{-5}	2.0×10^{-5}	2.0×10^{-5}	None added	-	2×10^{-5}
Glucose	6×10^{-3}	2.0×10^{-2}	2.0×10^{-2}	2.0×10^{-2}	2×10^{-2}	2.0×10^{-2}
K_2HPO_4	1.2×10^{-3}	1.05×10^{-4}	1.2×10^{-3}	1.2×10^{-3}	-	1.2×10^{-3}
NaCl	3.0×10^{-3}	3.0×10^{-3}	3.0×10^{-3}	3.0×10^{-3}	3×10^{-3}	3.0×10^{-3}
MOPS	5.0×10^{-2}	5.0×10^{-2}	5.0×10^{-2}	5.0×10^{-2}	-	5.0×10^{-2}
$MgCl_2.6H_2O$	-	-	-		1.6×10^{-4}	-
$FeCl_2.4H_2O$	-	-	-		2.7×10^{-4}	-
$(NH_4)_2HPO_4$	-	-	-		1.852×10^{-2}	-
$NH_4H_2PO_4$	-	-	-		4.8×10^{-4}	-

*All ingredients expressed in Molar concentrations

Periodically, samples were removed from the chemostats and purity checked by plating on nutrient and Pseudomonas isolation agar plates. Further characterisation of phenotypes is discussed in section 3.3.3.1. Pseudomonas aeruginosa WT and M7 grown in chemostats using the CDM described (section 3.2.2), did not produce significant amounts of foam.

3.2.2 Media for chemostat cultures

Six types of media were used. These were media limited by glucose (C-lim), phosphate (P-lim), nitrogen (N-lim), iron (Fe-lim), sulphate (S-lim) or magnesium (Mg-lim). The concentration of the growth limiting substrates (Table 6) were a modification of those previously used in our laboratory (Finch, 1976; Idziorek-Kirby, unpublished). The formation of exopolysaccharide by M7 and the problems of aeration which may be associated with very dense cultures, made it convenient to use cultures of low density. The media used gave a culture density of approximately 0.5. Growth was monitored by optical density and viable count measurements. The low OD was itself an assurance of nutrient limitation. Nevertheless, to check that a particular component was growth limiting during preliminary experiments, a few drops of a concentrated solution of that component were injected into the culture. The component was growth limiting if the cell concentration increased.

3.2.3 Preparation of media

Four litres each of the six media were prepared by dissolving the components in fresh deionized glass distilled water. 12 x 2 l bottles were fitted with a cotton wool glass vent and millipore filter apparatus and autoclaved at 121^{o}C for 20 min. After autoclaving,

a sterile 0.2 µm filter was aseptically placed on the filter holder and, by applying a vacuum at the vent, media was sterilized and filtered into the bottle. The filter holder was then aseptically replaced with a sterile plug. Two identical bottles were prepared for each type of media.

3.3 Stability and Phenotype Selection of Nutrient Depleted M7 and WT Grown in Batch and Continuous Culture

3.3.1 Stability of M7 in batch culture

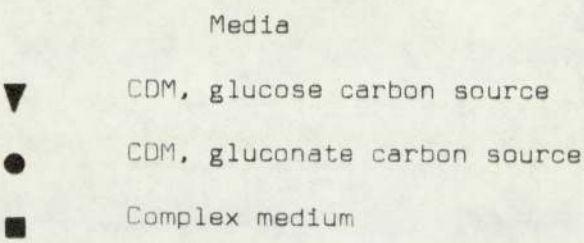
Stability of M7 in batch culture was carried out in both a chemically defined medium (CDM, Table 1) with glucose or gluconate, 36.6 mM, as the carbon and energy source, and in a complex medium (nutrient broth). Media (25 ml) was inoculated in triplicate from a mucoid colony of M7 and incubated for 18 hr at 37^{o}C in a shaking water bath. Samples of 0.1 ml were serially diluted in nutrient broth and 0.1 ml spread on NA plates and incubated for 18 hr at 37^{o}C. The percentage of mucoid colony forming units was calculated from the average count of five plates at a dilution yielding 100 - 200 colonies per plate. All mucoid colonies were counted together irrespective of their phenotypic classification (see section 3.3.3.1). A loopful of the liquid culture was inoculated into fresh medium and re-incubated for subsequent subcultures.

Typical results are shown in Figure 18. In both types of CDM, the percentage of mucoid colonies showed an initial fall to a minimum after three to four subcultures. This initial fall was followed by a stable increase in the percentage of mucoid colonies to over 90%. Bacteria subcultured in nutrient broth showed no stability of mucoid form and total reversion was evident within five subcultures.

3.3.2 Stability of M7 in continuous culture

M7 was grown in chemostats under the six nutrient limitations described, at a dilution rate of 0.12 hr^{-1}. A sample (0.1 ml) was

Figure 18. Stability of mucoid *P. aeruginosa* (M7) subcultured, in batch, in a chemically defined or complex media.



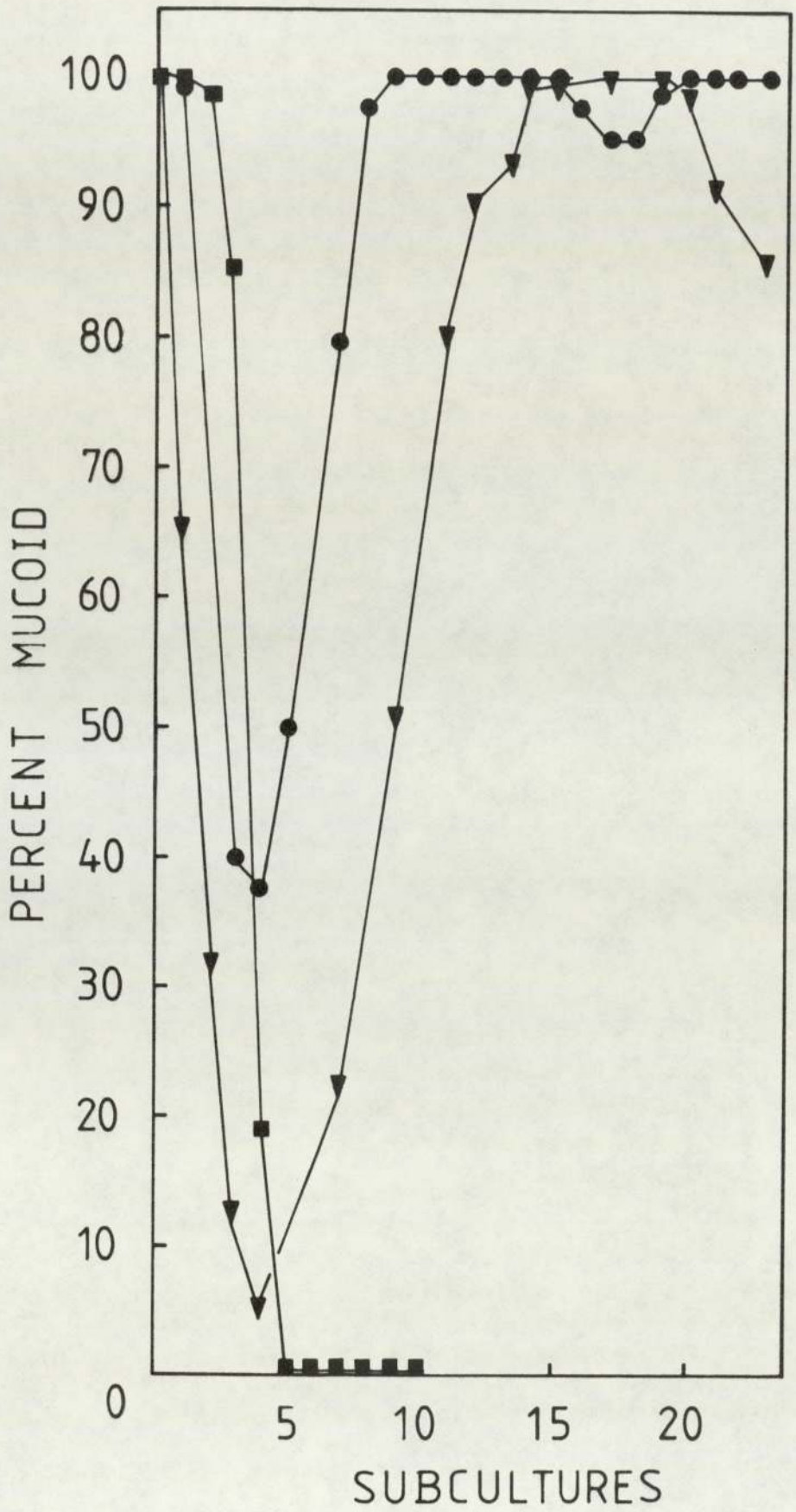
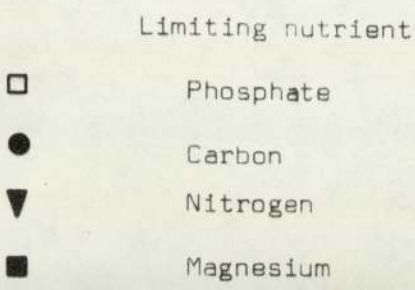

100
90
80
70
60
50
40
30
20
10
0
PERCENT MUCOID
5
10
15
20
SUBCULTURES

Figure 19. Stability of mucoid P. aeruginosa (M7) in chemostat (D = 0.12 hr^{-1}).



(arrow: addition of 1% or 10% non-mucoid cells)

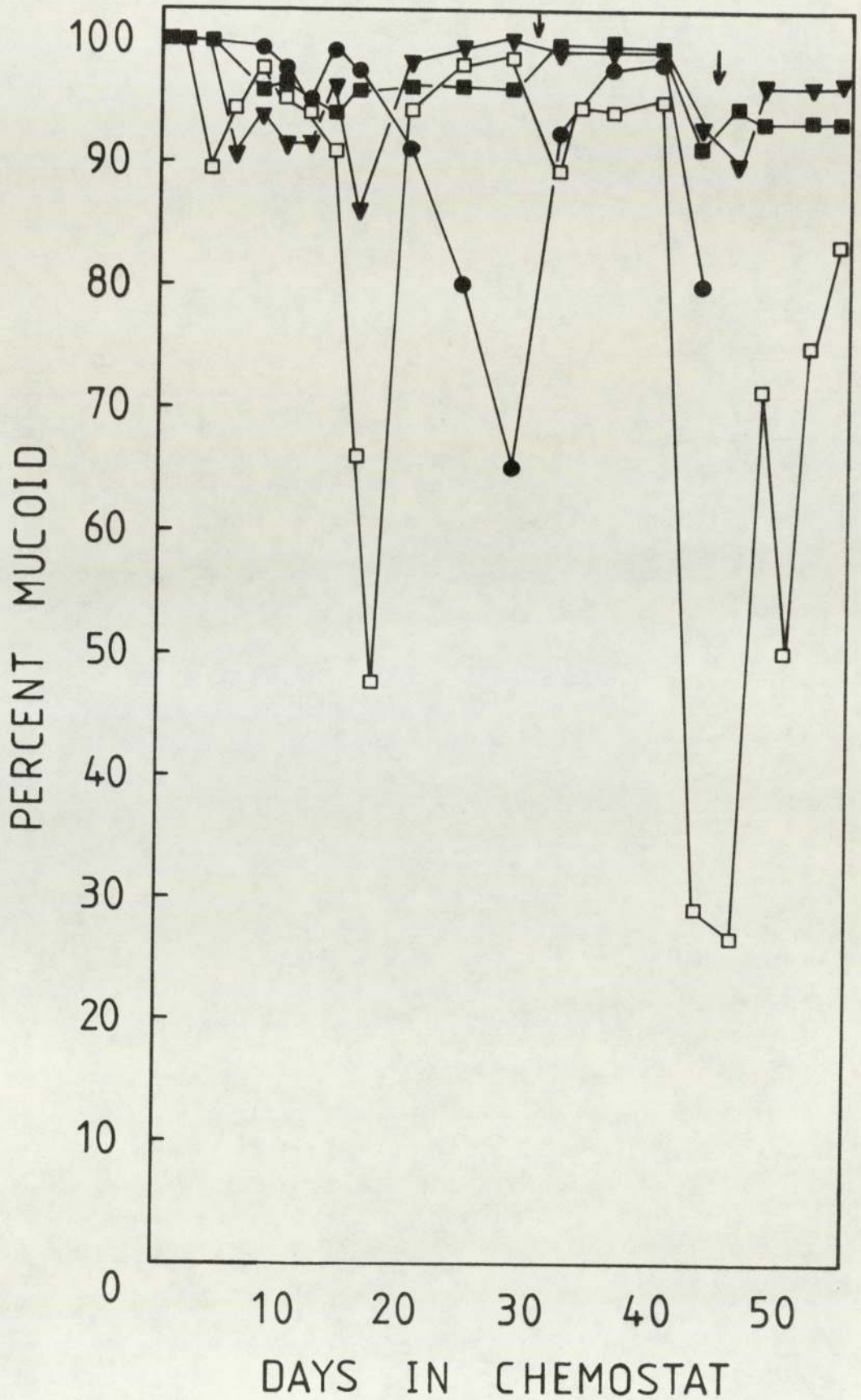
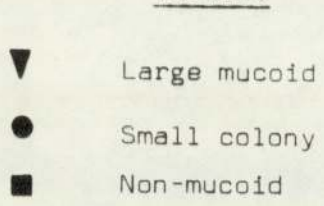

PERCENT MUCOID
100
90
80
70
60
50
40
30
20
10
0
10
20
30
40
50
DAYS IN CHEMOSTAT

Figure 20. Stability of mucoid *P. aeruginosa* (M7) variant in chemostat (nitrogen limited, D = 0.12 hr^{-1})



(arrow: addition of 10% non-mucoid cells)

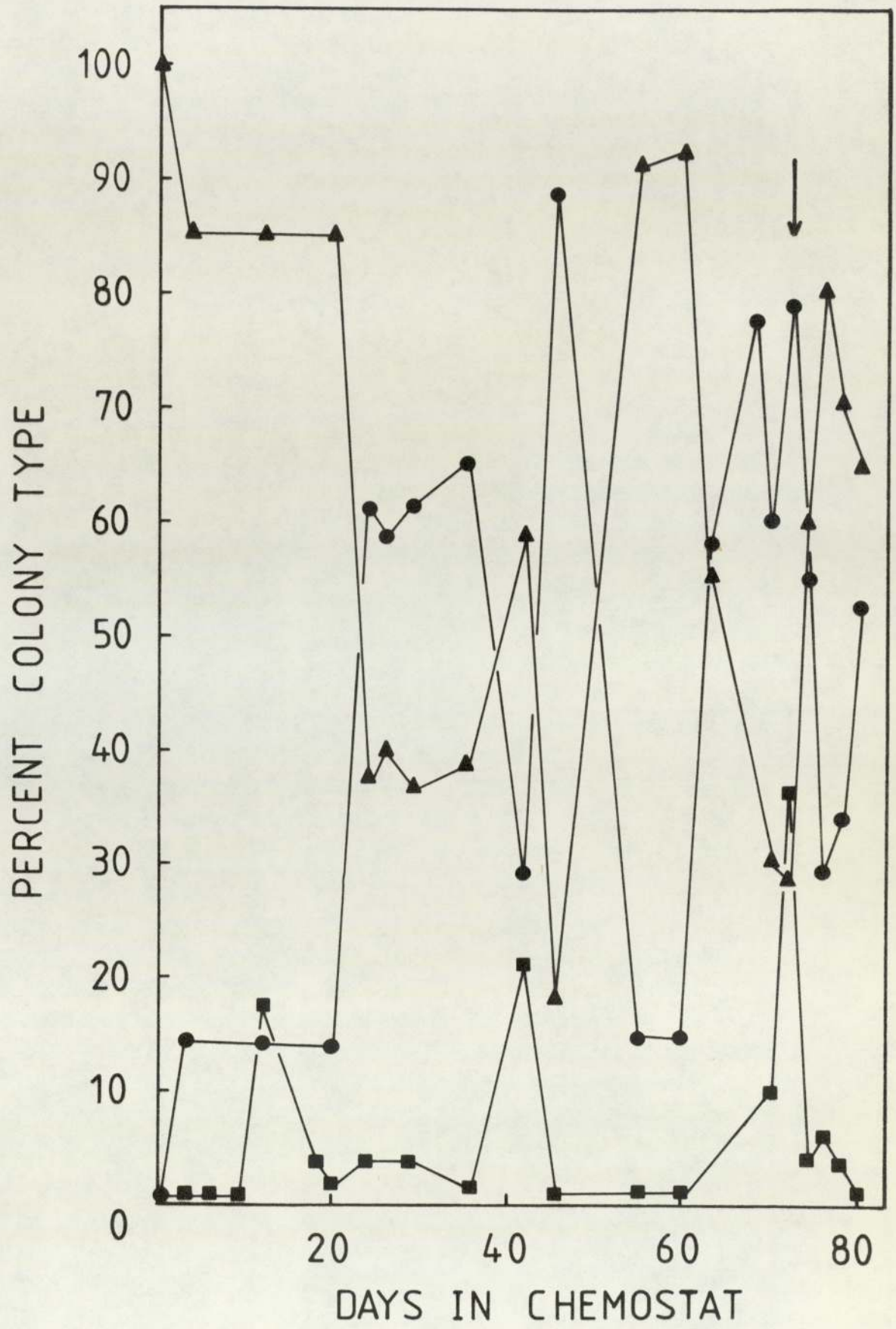

PERCENT COLONY TYPE
100
90
80
70
60
50
40
30
20
10
0
20
40
60
80
DAYS IN CHEMOSTAT

Table 7

Percentage of non-mucoid variant isolated in culture vessel (CV) and in culture washout (CW) of nutrient limited M7

LIMITING NUTRIENT	SAMPLE SOURCE	DAYS IN CHEMOSTAT				
		9	11	13	15	32
C	CV	2	4	4	3	7
	CW	22	10	15	21	40
P	CV	5	6	6	9	5
	CW	19	31	63	76	86
N	CV	9	9	4	14	1
	CW	13	22	10	30	2
Fe	CV	3	3	3	4	4
	CW	3	9	6	17	7
S	CV	1	1	1	2	1
	CW	1	1	1	2	1
Mg	CV	3	6	5	5	1
	CW	21	28	15	19	8

aseptically transferred from the culture vessel, diluted and the percent mucoid cells calculated as described for batch culture. The experiment could be run for up to ten weeks and was terminated when wall growth was significant, contamination occurred or when a small colony variant (see section 3.3.3.1) took over. Figure 19 shows the pattern of one of four similar experiments. Only carbon, phosphate, nitrogen and magnesium limited cultures are shown. Iron and sulphate cultures showed the same pattern as magnesium limited cultures and are excluded for visual clarity.

Further checks on stability were performed by addition of 1% and 10% (arrows) WT cultures grown under the same nutrient limitation. This addition appeared to make no significant difference to the percentage of mucoid colonies eventually recovered. Neither did high levels of non-mucoid variants in the culture vessel lead to eventual loss of the mucoid strain.

When the culture washout was subjected to the same test, a higher percentage of non-mucoid variant was isolated under all limitations except for sulphate limited cells which showed no difference (Table 7).

3.3.3 Colony type (phenotype) selection

Although the mucoid character was retained when M7 was grown in chemostats, two distinctly different colony types were frequently displayed. Both M7 and WT were therefore grown in continuous culture under the six limitations at $D = 0.12\ hr^{-1}$. At weekly intervals, colonies were isolated and subjected to various tests designed to distinguish phenotypes (J. Govan, personal communication).

Samples (0.1 ml) were removed from the culture vessel, diluted and plated on nutrient agar (NA) and allowed to grow for 24 hr.

Three plates of each, containing 70 - 150 colonies per plate were replica plated on the following solid media: Pseudomonas isolation agar (PIA); Deoxycholate citrate agar (DCA); DCA supplemented with 0.1% $MgSO_4.7H_2O$ (DCAM); Minimal agar (MA) (Vogel and Bonner, 1956) and milk agar (MLA). They were also replica plated on Diagnostic sensitivity agar containing doubling concentrations of carbenicillin ranging from 16 to 512 µg/ml or polymyxin ranging from 1.25 to 40 µg/ml. After incubation for 18 hr at 37°C, the plates were examined for growth of each colony. The phenotypes thus isolated were also subjected to conventional biochemical tests (Cowan, 1974) and phage and serotyping.

3.3.3.1 M7 colony types

Six colony types (phenotypes) were isolated from M7 and were designated by the letters A to F.

Colony type A: Type A was the typical mucoid (M7) colony. It grew to a large, opaque mucoid colony on NA, showed little or no lysis on MLA after 18 hr and a small zone of lysis after 48 hr. The colony grew well on MA, DCA and DCAM but appeared non-mucoid. Growth on carbenicillin plates ceased between 64 and 128 µg/ml and on polymyxin between 2.5 and 5 µg/ml.

Colony type B: On NA, type B appeared as a small transparent colony after 18 hr incubation at 37°C and as a large, transparent mucoid colony after a further incubation at room temperature. It showed very slight or no growth on MA, DCA or DCAM. It showed increased sensitivity to carbenicillin and polymyxin with growth ceasing at <16 - 64 µg/ml and 0 - 2.5 µg/ml respectively.

Colony type C: The colonial appearance of type C on NA was indistinguishable from that of type A. Type C however did not grow on DCA unless supplemented with magnesium (DCAM). Its sensitivity

to carbenicillin was increased (16 - 64 µg/ml) but had the same sensitivity to polymyxin as type A (2.5 - 5 µg/ml).

Types B and C showed similar behaviour on MLA as type A. All 3 types showed mucoid growth on PIA with formation of blue/green pigment.

Colony types D and E: Both D and E appeared as small white non-mucoid colonies on NA but mucoid on MA. They did not grow on DCA or DCAM. They both had very high resistance to polymyxin with 40 µg/ml causing no visible inhibition of growth. Type D was, however, sensitive to carbenicillin (32 - 64 µg/ml) while type E was very resistant (>256 µg/ml).

Colony type F: This gave a small opaque mucoid colony on NA and MA but did not grow on DCA or DCAM. It had increased resistance to polymyxin (>40 µg/ml) and its sensitivity to carbenicillin was similar to that of type A (64 - 128 µg/ml).

Colony types D, E and F did not grow on PIA and formed no zones of lysis on MLA even after 7 days incubation.

3.3.3.2 WT colony types

The wild type showed good stability in continuous culture and only occasionally produced a variant irrespective of the limiting nutrient. This variant (designated type P) differed from the rough colonial type of WT (designated Q) by being a smoother and larger colony. They both showed good growth on NA, DCA, DCAM, MA and large lysis zones on MLA after 18 hr incubation. Both grew well on PIA with formation of blue/green pigment. Growth on carbenicillin plates ceased between 64 and 128 µg/ml and on polymyxin between 2.5 and 5 µg/ml. On a few occasions, under sulphate and nitrogen limitations, a small colony appeared which behaved as if it were a mixture of types D and E described above.

3.3.3.3 Biochemical characteristics

Biochemical tests were performed on the phenotypes described and on a non-mucoid revertant of M7. Several metabolic differences were observed as shown on Table 8. All M7 phenotypes did not produce pigments P' and F', and the formation of acid from mannitol was absent in all but one. Of the large mucoid colony types, type C showed the least changes when compared to Pseudomonas aeruginosa NCTC 6750. Small colonies showed the highest number of changes and all shared the inability to form acid from glucose and to reduce both nitrate and nitrite. Few changes were observed for type P and the M7 revertant (R). Type Q had some biochemical characteristics as those expected from P. aeruginosa NCTC 6750.

3.3.3.4 Phage and serotyping

Table 9 shows the phagetypes and serotype of the different colony types. Some differences existed even between colonies which belonged to the same colony type and are described as subgroups. Cultures from the different colonies grew poorly in broth and formed a thin granular lawn on phage typing plates. They also gave granular unstable suspensions in saline and varied in strength of agglutination reaction from each other. The types however were found to belong to the same strain.

3.3.3.5 Effect of nutrient limitation on phenotype selection of M7

The colony types appeared and disappeared in an irregular manner. There was however a tendency for some of the phenotypes to appear in particular limitations as illustrated in Table 10.

Small colonies appeared very frequently under sulphate and nitrogen limitations, their pattern of appearance varying with that of the large mucoid type (Figure 20). Small colonies were observed

Table 8

Biochemical characteristics of M7 and WT phenotypes isolated in chemostats

TEST	*A	B	C	D	E	F	P	Q	R	NCTC 6750
Pigment P'	-	-	-	-	-	-	+	+	+	+
F'	-	-	-	-	-	-	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+
pHB	-	-	-	-	-	-	-	-	-	-
Growth at 5°C	-	-	-	-	-	-	-	-	-	-
42°C	+	-	+	-	+	+	+	+	+	+
on MCC	+	+	+	+	-	-	+	+	+	+
in KCN	+	-	+	-	+	-	+	+	+	+
Citrate utilization	-	+	+	+	-	+	+	+	+	+
Acid from: Glucose	-	+	+	-	-	-	+	+	+	+
Lactose	-	-	-	-	-	-	-	-	-	-
Maltose	+	-	-	-	-	+	-	-	-	-
Mannitol	-	-	-	-	-	+	-	+	-	+
Salicin	-	-	-	-	-	-	-	-	-	-
Sucrose	-	+	-	-	-	-	-	-	-	-
Xylose	+	-	+	+	-	+	-	+	+	+
Starch hydrolysis	-	-	-	-	-	-	-	-	±	-
Nitrate reduction	+	+	+	-	-	-	+	+	+	+
Nitrite reduction	+	+	+	-	-	-	+	+	+	+
Gelatine hydrolysis	+	+	+	-	+	-	+	+	+	+
Casein hydrolysis	+	+	+	-	+	-	+	+	+	+
Urease	+	+	+	+	-	+	+	+	+	+
Arginine hydrolase	+	+	+	±	-	±	+	+	+	+
Lysine decarboxylase	-	-	-	-	+	-	-	-	-	-
Orn "	-	-	-	-	-	-	-	-	-	-
Egg-yolk reaction	-	-	-	-	+	-	-	-	-	-
Tween 80 hydrolysis	+	+	+	-	+	-	+	+	+	+

*A - F: colony types described in section 3.3.3.1

P - Q: colony types described in section 3.3.3.2

R: non-mucoid revertant of M7

NCTC 6750: expected results of P. aeruginosa NCTC 6750

Table 9

Serological and phage types of main phenotypes of M7 and WT

Colony type	Sub-group	R.T.D.									
		7	24	31	44	F.8	109	352	1214	M4	188/1
M7 (grown on NA)					±	+	++	++			++*
WT (grown on NA)							++	±			+
A	1				++	++	++	+	++		++
	2		+				++	++			++
	3			++	±		++	±			++
	4				±	±	++	±			+
	5		++		++	++	++	++	+	±	++
	6					++	++	++			++
B	1				±	++	++	++			++
	2				++	++	++	++	++		++
C	1				±	±	++	±			±
D	1					+	++	++			++
	2				±	+	++	++			++
E	1	++			+	±	++				++
F	1			++	±	++	++	++			++
P	1	++					++			++	
	2	++			±		++			+	
	3	++	+	++	±	++			++	++	
Q	1					+	++	++			++
	2					±	++	±			++
	3			++	±	++	++	++			++

Serological type (of all) O : 6 : H : NT

* ++ = Strong reaction; +, ± = weak reaction;

RTD = Routine Test and Dilution

Table 10

Appearance of main phenotypes of M7 in chemostats (D = 0.12 hr^{-1})

COLONY TYPE	WEEK No	PERCENT COLONY TYPE PRESENT					
		C	P	N	Fe	S	Mg
A	1	100	98	85	100	91	100
	2	97	79	82	98	100	65
	3	71	63	87	98	71	99
	4	84	67	30	98	58	98
	5	89	100	36	97	75	98
B	3	26	7				
	4	14					
	5	6					
C	2		19				
	3		29				
	4		5				
D	1			15		6	
	2			15		0	31
	3			13		22	
	4			58		38	
	5			64		20	
E	4		25			4	
	5					5	
F	4			7			

less frequently and in low percentages in all the other limitations, usually showing drug sensitivity pattern of type E.

Iron and magnesium limitations showed the most stable retention of the original colony type (type A) while phosphate and carbon limitations often showed type B. Usually phosphate limitation exhibited the largest variety of phenotypes.

3.4 Production of Extracellular Products (Virulence Factors) by Nutrient Limited M7 and WT

3.4.1 Exopolysaccharide

The non-mucoid organism (WT) produced negligible amounts of exopolysaccharide under all nutrient limitations when glucose was used as the carbon source (Brown et al., 1969).

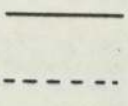
Two distinct precipitates were formed when alcohol was added to the culture supernatants of M7. These consisted of a stringy gelatenous precipitate which could easily be removed by a glass rod. This was formed under all growth conditions. The second, a finer, flocculent precipitate was formed in small quantities occasionally and randomly among the limitations. It was however much more consistently observed under carbon limitation. In the present study, all alcohol precipitable material was collected together as exopolysaccharide.

Table 11a shows the amounts of exopolysaccharide formed by M7 in batch and in continuous culture ($D = 0.12\ hr^{-1}$). In batch, P-lim, N-lim and Fe-lim resulted in high production of the exopolysaccharide with P-lim showing the most. Mg-lim and S-lim resulted in less exopolysaccharide levels. C-lim cells produced similar amounts as unlimited (or O_2-limited) cultures.

The same pattern was observed in all the chemostats during the first generations. As time progressed, the amount of exopolysaccharide produced was markedly reduced in all limitations.

Table 11b shows the effect of dilution rate on slime production of iron limited cultures. The exopolysaccharide was produced at all dilution rates. At high dilution rates, the amount of exopolysaccharide formed was equal to or greater than at lower dilution rates.

Figure 21. Infra red spectra of calcium alginate and calcium gelled slime from iron limited M7.



Calcium alginate

M7-slime

Table 11a

Exopolysaccharide production in nutrient limited M7 in batch and chemostat (D = 0.12 h^{-1}) cultures. Values are mg exopolysaccharide per mg dry weight bacteria

	Nutrient limiting growth						
	C	P	N	Fe	S	Mg	O_2
BATCH	1.28 ± .50	6.05 ± 1.74	3.94 ± 1.24	3.55 ± 0.94	0.44 ± 0.27	0.47 ± 0.4	1.72 ± 0.9
2*	11.35	29.55	15.78	10.24	1.10	0.50	-
4	2.60	30.50	16.00	16.00	-	0.37	-
6	0.06	11.20	12.80	7.40	0.81	0.23	-
8	1.60	5.60	8.70	1.30	0.52	0.72	-
10	1.30	1.80	3.00	1.10	0.10	0.09	-
12	1.10	1.40	3.00	2.20	0.32	0.08	-

*DAYS IN CHEMOSTAT

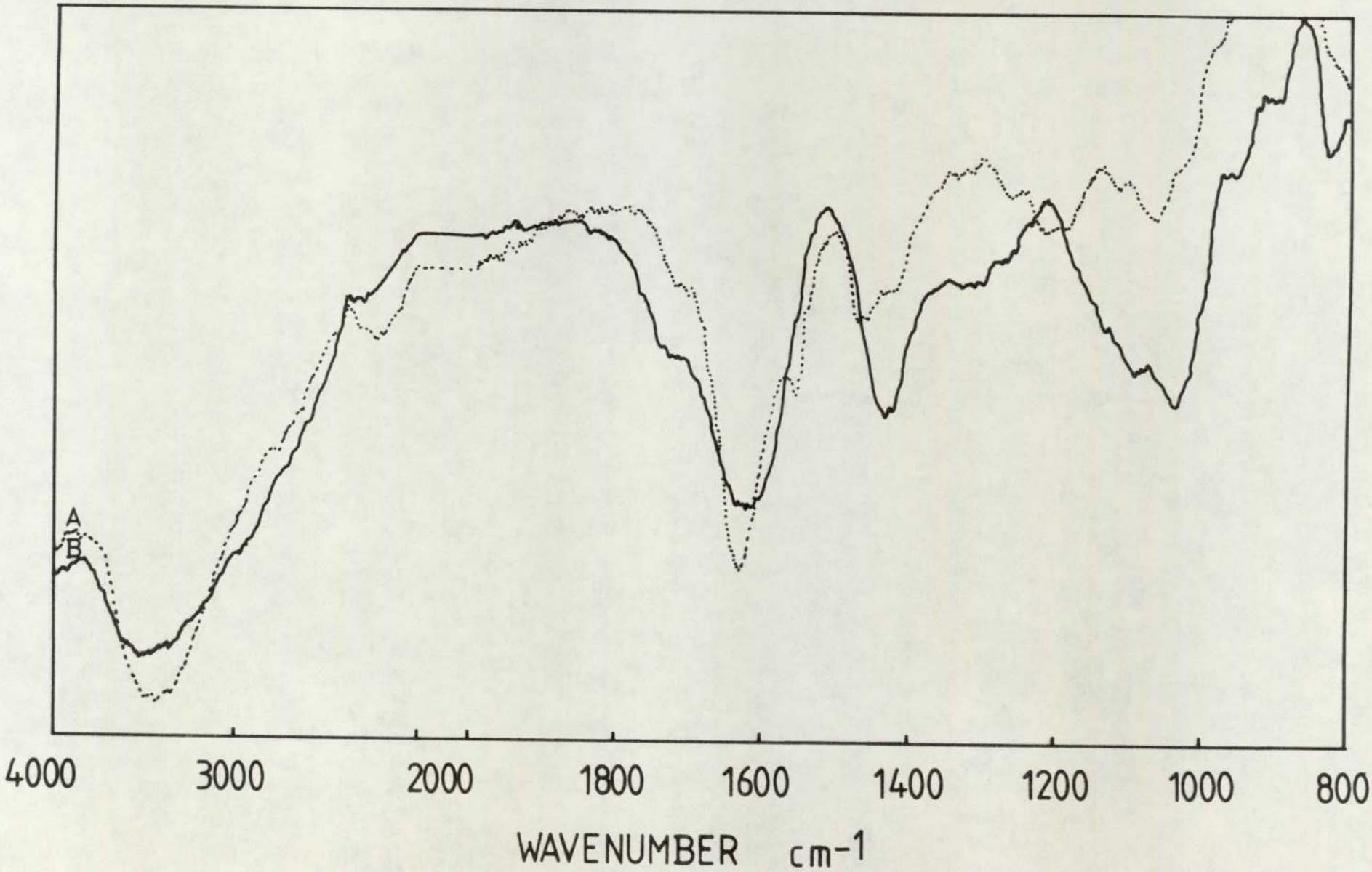
Table 11b

Effect of dilution rate on production of exopolysaccharide by iron limited M7

D hr^{-1}*			
0.02 - 0.05	0.08 - 0.12	0.15 - 0.20	0.25 - 0.35
5.48 ± 0.07	4.86 ± 1.46	4.37 ± 1.93	9.4 ± 4.35

(Exopolysaccharide determined after 5 volume changes.)

*Due to slight variations in flow rate between experiments, ranges of dilution rates are quoted.


A
B
4000
3000
2000
1800
1600
1400
1200
1000
800
WAVENUMBER cm-1

The ability of the exopolysaccharide to be precipitated by alcohol and to be gelled by Ca^{++}, and the IR pattern observed for the exopolysaccharide of Fe-lim cells (Figure 21) demonstrate an alginate-like nature.

3.4.2 Extracellular enzymes

The extracellular activities of protease, elastase, lipase and phospholipase C of cultures grown in chemostats and in batch are shown in Tables 12 - 15. The second value was that obtained when the chemostats were dismantled and the entire procedure repeated or of repeated batch cultures.

3.4.2.1 Protease

Protease production is shown in Table 12. At the lower dilution rate, WT showed more protease activity than M7 at all limitations with two exceptions. C-lim M7 showed higher activity than WT and the protease activity of N-lim cells of the two organisms was similar. At the higher dilution rate, protease activity was reduced in all cases except Fe-lim M7 cells, where activity was increased. At this growth rate (0.25 hr^{-1}), unlike the lower growth rate, C-lim M7 showed lower activity than WT. Maximum protease activity was observed from P-lim WT grown at low dilution rate. Magnesium limitation showed least protease activity.

The protease activity of supernatants of batch grown cells was relatively low and therefore difficult to compare. Broken cells showed a pattern of activity different from that of supernatants of chemostat cultures. Under all limitations, broken cells of M7 showed higher protease activity than the wild type. Nitrogen, iron and phosphate limited M7 showed high activities, while phosphate and sulphate limited WT showed least activity.

Table 12

Extracellular protease activity of M7 (mucoid) and WT (non-mucoid) P. aeruginosa grown in chemostats and in batch

METHOD		Nutrient limiting growth											
		C		P		N		Fe		S		Mg	
		M7	WT	M7	WT	M7	WT	M7	WT	M7	WT	M7	WT
CHEMOSTATS* Dilution rate (hr^{-1})	0.05	2.17	0.633	0.247	3.190	1.800	2.83	0.049	2.57	0.172	0.56	0.028	0.08
		1.195	0.380	0.325	3.875	1.325	1.30	0.039	0.895	0.062	1.035	ND	ND
	0.25	0.016	0.060	0.051	0.262	0.02	0.490	0.309	0.080	0.007	0.026	0.010	0.043
		0.010	0.070	ND	0.155	0.085	0.290	0.415	0.065	0.005	ND	ND	ND
BATCH:* Culture Supernatant		ND	ND	ND	0.0012	ND	ND	0.0005	0.0051	0.0052	ND	0.0028	ND
		0.0108	ND	0.0212	0.0006	0.0299	0.0006	0.0004	0.0286	ND	ND	ND	ND
BATCH:** Broken Cells		0.1524	0.1299	0.3714	0.0880	0.4197	0.1341	0.5341	0.1013	0.2571	0.0566	0.1628	0.0807
		0.1957	0.1060	0.1894	0.0518	0.4389	0.0949	0.2167	0.1554	0.1153	0.0243	0.1390	0.0929

*Values are units per mg protein per 10^9 cells

**Values are units per mg dry weight

ND = not detectable

3.4.2.2 Elastase

Elastase activity (Table 13) was very low in both batch and chemostat cultures and comparison was therefore difficult. The enzyme activity of broken cells showed that M7 produced more activity than WT under all other limitations except carbon and nitrogen, where activities were similar. Iron and phosphate limited M7 showed highest activities, while magnesium limited WT showed the least.

3.4.2.3 Lipase

With two exceptions, lipase activity (Table 14) was higher for WT than M7 at both dilution rates. Mg-lim M7 however, showed a higher activity than WT, while N-lim cells of both organisms showed similar activities. The lipase activity of carbon limited cells at the lower dilution rate could not be determined due to formation of a precipitate during assay procedure. At the low dilution rate, highest activity was shown by P-lim WT.

When dilution rate was increased, lipase activity was reduced under all limitations except P-lim M7 which showed an increased activity. At the higher dilution rate, P-lim M7, and both M7 and WT C-lim cells, showed high lipase activity.

Culture supernatants of batch grown cells showed little activity. The enzyme activity of S-lim and Mg-lim broken cells of both M7 and WT were similar. M7 however, showed higher activity than WT under phosphate, nitrogen and iron limitations. Only under carbon limitation did WT show higher activity than M7. Of the broken cells, P-lim M7 and C-lim WT showed highest activity while both M7 and WT S-lim cells, together with Fe-lim and N-lim of WT showed least activity.

Table 13

Extracellular elastase activity of M7 (mucoid) and WT (non-mucoid) *P. aeruginosa* grown in chemostats and in batch

METHOD		Nutrient limiting growth											
		C		P		N		Fe		S		Mg	
		M7	WT	M7	WT	M7	WT	M7	WT	M7	WT	M7	WT
CHEMOSTATS* Dilution rate (hr^{-1})	0.05	0.025	0.822	0.017	0.017	<0.018	0.058	0.014	0.012	<0.007	<0.006	0.033	<0.008
		0.005	0.010	0.006	0.090	<0.005	0.023	<0.005	0.005	<0.005	<0.005	<0.005	<0.005
	0.25	0.019	0.010	<0.012	0.112	<0.047	<0.050	0.012	<0.029	<0.007	0.001	<0.006	0.001
		<0.005	<0.005	<0.005	<0.005	<0.005	<0.006	<0.006	<0.006	<0.006	<0.005	<0.005	<0.005
BATCH:* Culture Supernatant		0.0008	<0.0005	0.0008	0.0005	<0.0005	<0.0005	<0.0005	0.0005	0.0028	<0.0005	<0.0005	<0.0005
		0.0007	<0.0005	0.0010	0.0008	0.0010	<0.005	<0.0005	0.0023	0.0023	0.0005	0.0006	<0.0005
BATCH:** Broken cells		0.0068	0.0062	0.0147	0.0014	0.0072	0.0033	0.0191	0.0034	0.0117	0.0025	0.0030	0.0006
		0.0143	0.0067	0.0138	0.0006	0.0040	0.0049	0.0155	0.0050	0.0046	0.0014	0.0005	0.0001

*Values are units per mg protein per 10^9 cells.

**Values are units per mg dry weight

Table 14

Extracellular lipase activity of M7 (mucoid) and WT (non-mucoid) *P. aeruginosa* grown in chemostats and in batch

METHOD		Nutrient limiting growth											
		C		P		N		Fe		S		Mg	
		M7	WT	M7	WT	M7	WT	M7	WT	M7	WT	M7	WT
CHEMOSTATS* Dilution rate (hr^{-1})	0.05	-	-	0.020	1.574	0.315	0.273	0.032	0.514	0.032	0.356	0.274	<0.008
		-	-	0.008	1.107	0.222	0.222	0.026	0.412	0.025	0.296	0.360	ND
	0.25	0.053	0.083	0.061	<0.008	<0.007	<0.004	<0.021	0.045	0.037	0.039	<0.024	<0.017
		0.030	0.061	0.040	ND	ND	ND	<0.020	<0.030	<0.030	<0.040	<0.020	<0.010
BATCH:* Culture Supernatant		0.0010	<0.0005	0.0129	0.0051	0.0025	0.0050	<0.0005	0.0020	0.0063	ND	0.0009	0.0031
		ND	0.0009	ND	0.0025	0.0026	0.0015	<0.0005	0.0052	0.0042	0.0078	0.0007	0.0011
BATCH:** Broken Cells		0.0089	0.0739	0.1410	0.0294	0.0579	0.0086	0.0791	0.0059	0.0089	0.0068	0.0615	0.0458
		0.0549	0.1190	0.0858	0.0430	0.0306	0.0087	0.0450	0.0077	0.0052	0.0053	0.0314	0.0330

*Values are units per mg protein per 10^9 cells

**Values are units per mg dry weight

ND = not detectable

Table 15

Extracellular phospholipase C activity of M7 (mucoid) and WT (non-mucoid) *P. aeruginosa* grown in chemostats and in batch

METHOD		Nutrient limiting growth											
		C		P		N		Fe		S		Mg	
		M7	WT	M7	WT	M7	WT	M7	WT	M7	WT	M7	WT
CHEMOSTATS*		ND	ND	0.078	0.010	0.052	0.237	0.009	0.007	0.007	0.025	0.007	ND
Dilution rate (hr^{-1})	0.05	ND	ND	0.043	ND	0.027	0.100	ND	0.008	0.005	0.022	0.007	ND
	0.25	0.093	0.143	0.084	0.347	0.062	0.013	0.089	0.082	0.100	0.140	0.082	0.007
		0.082	0.097	0.124	0.248	0.071	0.012	0.087	0.094	0.104	0.140	0.076	0.002
BATCH:* Culture Supernatant		2.75	1.54	6.71	2.90	2.40	2.38	0.025	0.625	9.01	5.70	1.10	0.875
		3.19	0.625	5.80	2.95	2.35	1.48	0.025	0.850	11.45	9.33	6.73	3.65
BATCH:** Broken Cells		14.90	10.20	14.60	9.60	16.00	15.10	19.00	13.90	23.30	29.80	9.70	9.10
		18.50	27.00	19.00	13.80	15.70	31.20	17.70	29.60	34.50	41.20	12.70	18.90

*Values are units per mg protein per 10^9 cells

**Values are units per mg dry weight

ND = Not detectable

3.4.2.4 Phospholipase C

With the exception of nitrogen limitation, phospholipase C activity was low at the slower dilution rate and increased at the faster dilution rate (Table 15). N-lim M7 showed a slight increase in activity with increase in dilution rate. The opposite was true for N-lim WT. At the lower dilution rate, this showed the highest activity. But activity decreased at the faster dilution rate.

At the faster dilution rate, where more phospholipase C activity was observed, the two organisms showed similar activities under carbon, sulphate and iron limitations. Only with phosphate limitation, which showed the highest activity, did WT show greater activity than M7. Mg-lim WT showed the least activity.

Phospholipase C activity of batch culture supernatants was of much higher magnitude than for chemostat grown cells. At all limitations except Fe-lim, M7 showed higher activity than the wild type. Sulphate limitation showed the highest while iron showed the lowest activity in both organisms.

High activity was also shown by broken cells. As with culture supernatant, sulphate limitation resulted in highest activity. No big differences were observed between M7 and WT.

3.4.3 Pigment production

3.4.3.1 Pyocyanine

Table 16 shows the amount of pyocyanine produced in chemostats and in batch culture. At both dilution rates phosphate limitation led to the largest production of this pigment, wild type cultures showing the maximum amount. Magnesium limitation was the only other condition that resulted in formation of relatively large amounts of the pigment.

Table 16

Pyocyanine production by chemostat and batch grown cells of M7 (mucoid) and WT (non-mucoid) *P. aeruginosa*. Values are A_{310} nm units per 10^9 viable cells per ml

METHOD	Nutrient limiting growth													
	C		P		N		Fe		S		Mg		O_2	
	M7	WT	M7	WT	M7	WT	M7	WT	M7	WT	M7	WT	M7	WT
CHEMOSTAT D hr^{-1}														
0.05	0	0	3.87	6.31	0	0	0	0	0	0	0.28	0.15	-	-
	0	0	2.13	5.11	0	0	0	0	0	0	0.45	0.32	-	-
0.25	0	0.08	1.04	6.29	0	0	0	0	0	0	0	0.53	-	-
BATCH CULTURE	0	0.16	0.37	2.09	0	0	0	0.05	0	0.42	0	0.04	0	0.15
		0.08	0.30	3.14	0	0	0	0.04	0	0.16	0	0.035	0	0.08

In batch cultures, the pigment was formed by WT under all limitations except under nitrogen limitation. As with chemostat cultures, most pyocyanine was produced under phosphate limitation. Mucoid type produced the pigment only under phosphate limitation.

3.4.3.2 Fluorescent pigment

The fluorescent compound was found to differ from fluorescein in its emission as shown by Figure 22. Maximum response was found when excitation wavelength was 400 nm and the emission was measured at 465 nm.

The compound was produced to varying degrees by both organisms at all limitations (Table 17). Only iron limited cells showed consistently large amounts in both chemostats and in batch cultures. Other limitations showed larger amounts at specific dilution rates. Except for nitrogen limitation, WT produced larger quantities than M7 at both dilution rates.

The UV spectrum of the fluorescent compound was found to differ with nutrient limitation. Mucoid cells limited by phosphate or magnesium at the slower dilution rate showed a shift in absorption maximum from 400 nm to approximately 360 nm (Figure 23). Non-mucoid cells limited by magnesium showed a shift to approximately 360 nm while those of phosphate shifted to approximately 310 nm (Figure 24). Non-mucoid cells showed the same shift at the higher growth rate.

Batch cultures had the fluorescent pigment under all limitation except oxygen and was produced in much greater quantities by iron limited cells. Iron limited M7 cultures produced about double the compound than did WT.

Table 17

Production of water soluble fluorescent pigment by chemostat and batch grown cells of M7 (mucoid) and WT (non-mucoid) *P. aeruginosa*. Values are emission 465 nm per 10^9 viable cells per ml

METHOD	Nutrient limiting growth													
	C		P		N		Fe		S		Mg		O_2	
	M7	WT	M7	WT	M7	WT	M7	WT	M7	WT	M7	WT	M7	WT
CHEMOSTAT $D\ hr^{-1}$ 0.05	822	1933	31	226.5	1693	68	40	346	8.5	40	1	4	-	-
	403	1240	40.2	187	1283	179	56	138	5.8	3	3	3	-	-
0.25	41.5	16	11.5	112	2710	347	145	380	17	62	2	10	-	-
BATCH CULTURE	13.0	11.3	4.78	19.25	3.51	0.55	59.5	25.9	0.41	0.62	0.69	3.19	0	0
	8.73	9.90	2.94	11.61	2.22	0.34	48.0	22.6	1.58	0.54	0.78	0.96	0.1	0.3

Figure 22. Emission spectra of fluorescein and the water soluble fluorescent pigment produced by P. aeruginosa.

A Water soluble pigment

B Fluorescein

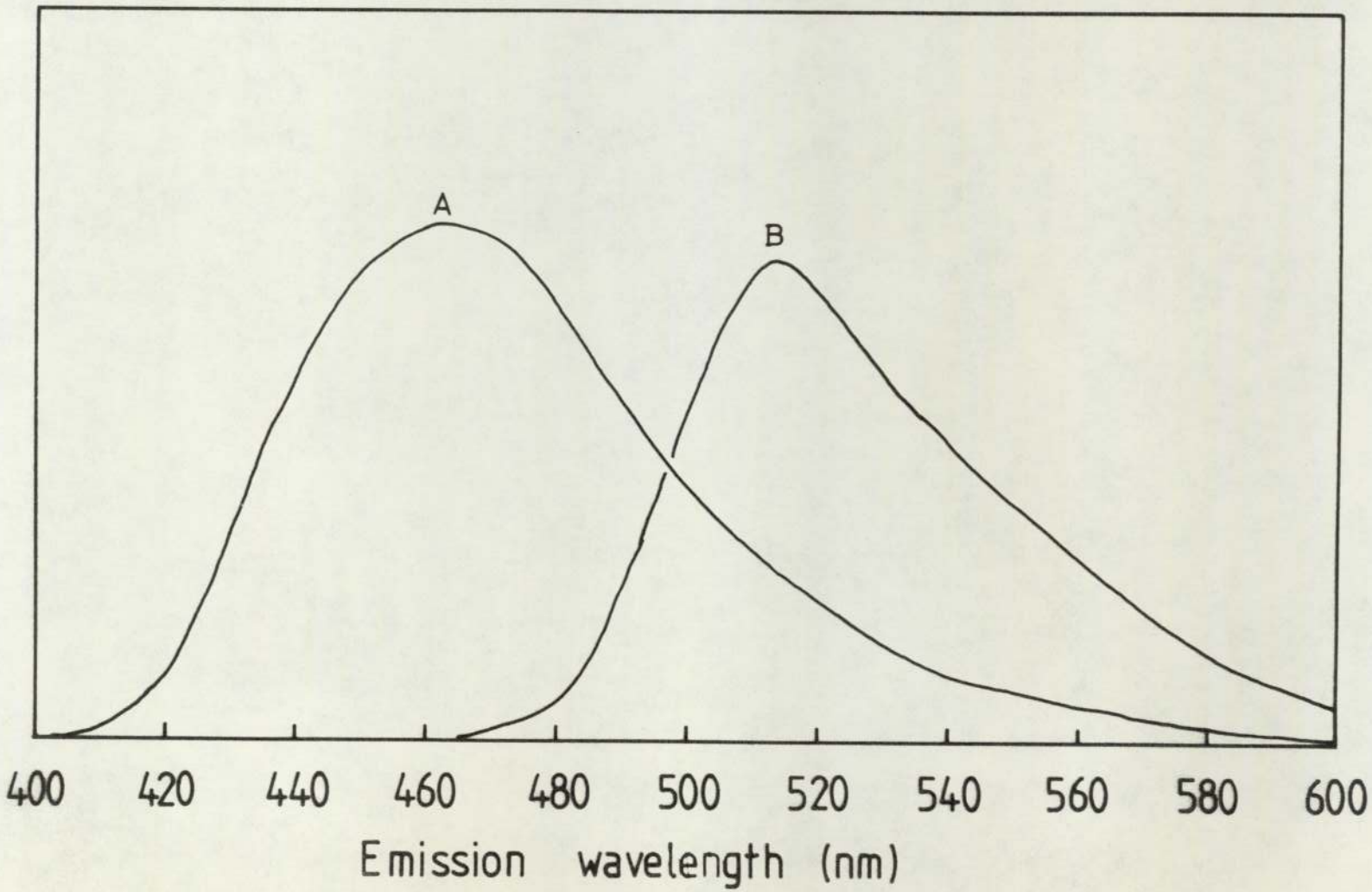

A
B
400
420
440
460
480
500
520
540
560
580
600
Emission wavelength (nm)

Figure 23. Ultraviolet spectra of the water soluble fluorescent pigment produced by nutrient limited M7.

	Limiting nutrient
Mg	Magnesium
P	Phosphate
Fe	Iron
C	Carbon
N	Nitrogen
S	Sulphate

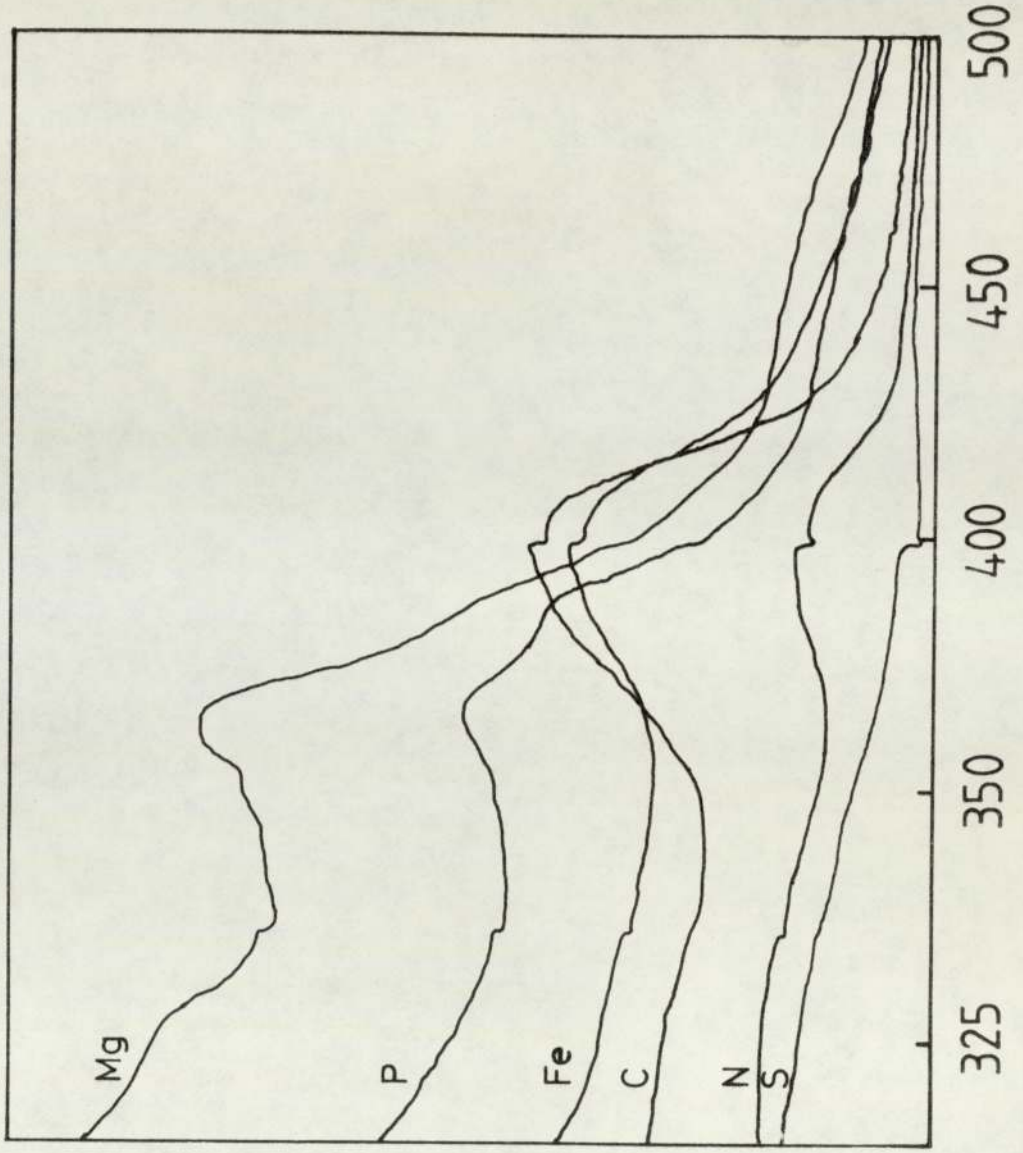
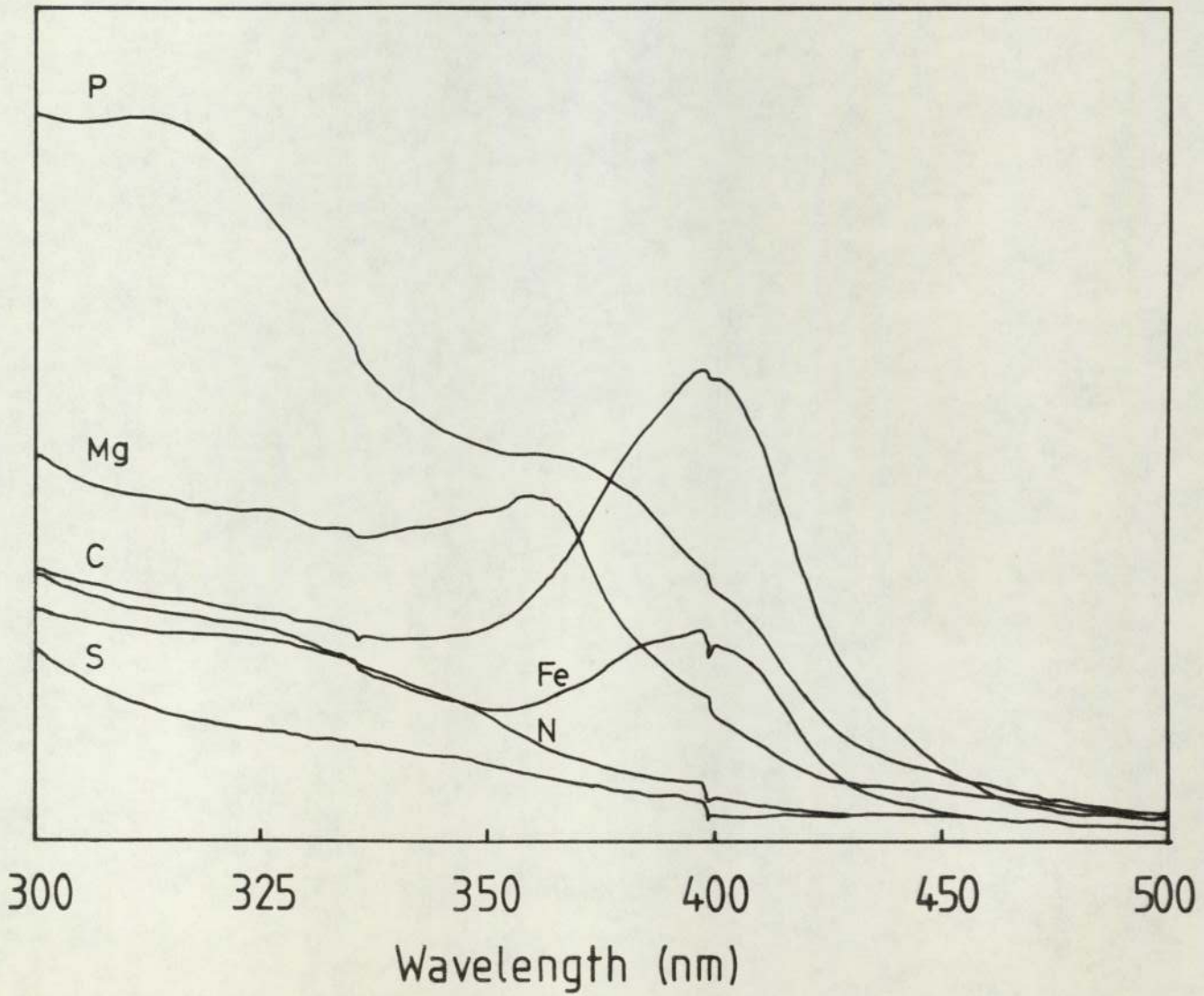

Mg
P
Fe
C
N
S
325
350
400
450
500
Wavelength (nm)

Figure 24. Ultraviolet spectra of the water soluble fluorescent pigment produced by nutrient limited WT.

	Limiting nutrient
P	Phosphate
Mg	Magnesium
C	Carbon
Fe	Iron
N	Nitrogen
S	Sulphate


P
Mg
C
S
Fe
N
300
325
350
400
450
500
Wavelength (nm)

3.5 Resistance of Nutrient Limited M7 and WT to Antimicrobial Agents and to Killing by Blood

3.5.1 Antimicrobial agents

3.5.1.1 Collection and preparation of cells

During growth in chemostats, the concentration of the non-limiting nutrients vary in an unpredictable manner. Some of these components, for example magnesium, phosphate and potassium may affect the sensitivity of cells to antimicrobial agents (Klemperer et al., 1979), therefore making comparisons difficult. To overcome this media effect, cells from the chemostat were washed and resuspended in medium of known composition prior to testing with the antimicrobial agents.

Nutrient depleted cultures from the chemostats growing at D = 0.05, 0.1, 0.15, 0.25 or 0.31 hr^{-1} were centrifuged at 5,000 g for 20 min at 37°C, washed and resuspended in a prewarmed glucose-free and divalent cation-free medium (resuspending medium Table 18). Microscopical examination of cells indicated no lysis of cells during the washing and resuspension.

Table 18

Resuspending medium

Ingredient	Final concentration (mM)
Ammonium acid phosphate buffer	7.20
NaCl	1.00
KCl	1.00
$(NH_4)_2SO_4$	0.10
	pH 7.7

An inoculum (0.1 ml) calculated to give approximately 2×10^7 cells/ml was added to 1.4 ml CDM containing 70 µg/ml carbenicillin or 25 µg/ml tetracycline. A similar inoculum was added to CDM without any drug. In the study of the effect of EDTA and polymyxin, the inoculum was added to 1.4 ml resuspending medium containing 350 µg/ml EDTA or 5 µg/ml polymyxin. The controls contained cells in a drug free resuspending medium. The cells were then incubated in a shaking water bath at $37^{o}C$ and samples removed at timed intervals.

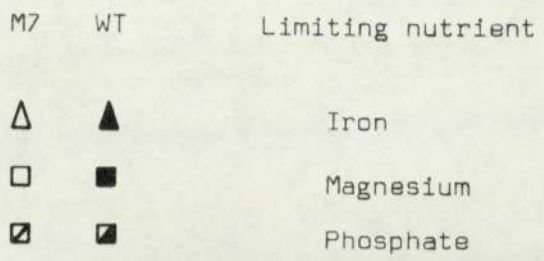
3.5.1.2 Carbenicillin and Tetracycline

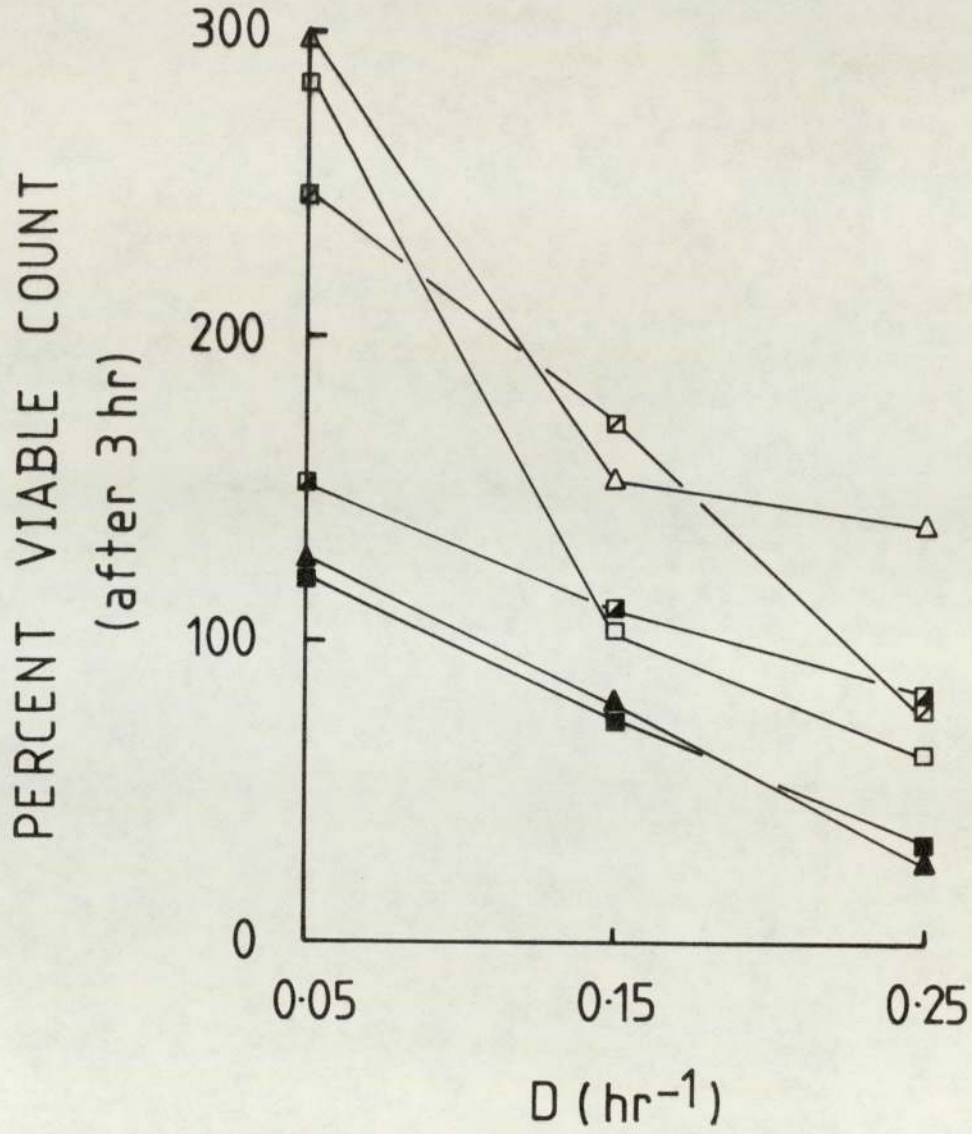
At time 0 min and at 1.5 hr intervals, 0.1 ml samples were removed and diluted in nutrient broth containing penicillinase (a 1 : 250 dilution) or Mg^{2+} (0.1 mM) to inactivate carbencillin and tetracycline respectively. Viable counts were obtained and the percentage of surviving bacteria calculated.

The effect of carbenicillin was not evident until after 3 to 4.5 hr. The different limitations did not show large differences in their sensitivities to carbenicillin (appendix 1 - 4). There was however, an increase in sensitivity as dilution rate was increased as shown for iron, magnesium and phosphate limited cells in Figure 25. At all limitations, the mucoid was more resistant than the wild type. The difference was minimised or abolished at higher dilutions.

The effect of tetracycline was evident between 1.5 to 3 hr. As with carbenicillin, no large differences were observed between the limitations. There was no difference between mucoid and non-mucoid cells and the effect of dilution rate was not clearly defined (appendix 1 - 4).

Figure 25. Effect of dilution rate on resistance to carbenicillin.




PERCENT VIABLE COUNT
(after 3 hr)
300
200
100
0
0·05
0·15
0·25
D (hr⁻¹)

3.5.1.3 EDTA

At time 0 and at 40 min intervals, 0.1 ml samples from EDTA treated cells were diluted in nutrient broth containing 0.1 mM Mg^{2+} to inactivate EDTA, plated out and viable counts obtained. Figure 26 illustrates the results obtained at different dilution rates after 80 min. There were no differences between the mucoid and non-mucoid cells and no big differences between limitations were observed. Except for iron limitation, there was little or no effect of dilution rate on the percent survivors (appendix 5 - 7).

Iron limited cells showed an increase in sensitivity at 0.15 hr^{-1}. This increase was lost when cells were growing at a faster dilution rate. Further investigation using cells grown at different dilution rates at the same time showed the pattern of results shown in Table 19. For WT, there was an increase in sensitivity between cells grown at 0.02 hr^{-1} to 0.1 hr^{-1}. There was however little change between the dilution rates of 0.1 and 0.37 hr^{-1}. Similarily, M7 showed an increased sensitivity at $D \simeq 0.1\ hr^{-1}$. This was however, lost at higher dilution rates.

3.5.1.4 Polymyxin

Samples (0.1 ml) of polymyxin treated cells were diluted in freshly made lecithin - glycerol inactivating medium and viable counts obtained for every 20 min. Figures 27 to 30 show typical curves obtained. Large variations were observed in different experiments although relative sensitivity of cells remained the same.

Differences between mucoid and non-mucoid strains existed for sulphate and iron limited cells. S-lim M7 was more resistant than S-lim WT at all dilution rates although at the highest dilution rate studied (0.31 hr^{-1}) this difference was reduced and was only

evident after 60 min. The reverse was true when the cells were iron limited. Fe-lim WT was more resistant than Fe-lim M7 at dilution rates 0.15, 0.25 and 0.31 hr^{-1}. At D = 0.05 hr^{-1}, the order was reversed, with M7 showing higher resistance.

Nitrogen limited cells showed a similar behaviour as iron limited cells at the higher dilution rates. At D = 0.05 hr^{-1}, little or no difference was observed between the two organisms. At D = 0.15 and 0.31 hr^{-1}, WT was more resistant than M7.

There were also differences between the limitations. At all the dilution rates and for both organisms, the resistance was in the order Mg-lim > S-lim > C-lim > P-lim > Fe-lim = N-lim. The differences for all but Mg-lim WT and for C-lim, P-lim and N-lim M7 were reduced at 0.05 hr^{-1}.

The difference between the most resistant cells (Mg-lim) and the least resistant (Fe-lim) is further illustrated in Figure 31.

After 160 min, the counts in the controls were always above 70% of initial count, except for P-lim cells which showed some cell death (≈50%). The sensitivity of P-lim cells should therefore be viewed with this in mind. For the purposes of comparison, however, the same resuspending medium was used for all cells.

3.5.2 Phagocytosis and killing by blood

3.5.2.1 Method

The method used in the study of phagocytosis and killing by blood was a modification of that described by Jones *et al.* (1979).

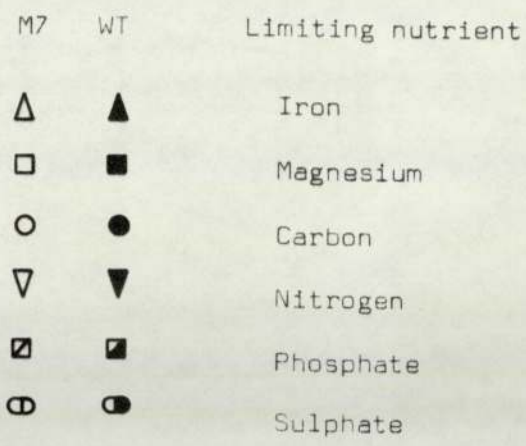
Cells were harvested by centrifuation at 5,000 g for 20 min, washed once and resuspended in normal saline to an OD_{470} of 0.2. Whole blood was collected into a heparinized sterile plastic tube and equal volumes of blood (0.25 ml) and bacteria suspension were

Table 19

The effect of dilution rate on the sensitivity of iron limited M7 and WT to EDTA

D hr^{-1}	% Viable Count (120 min)	
	M7	WT
0.02	56	120
0.04	60	79
0.09	26	60
0.17	38	-
0.21	-	61
0.25	39	-
0.33	-	88
0.35	55	-
0.37	-	61

Figure 26. Effect of nutrient limitation and dilution rate on resistance to EDTA.



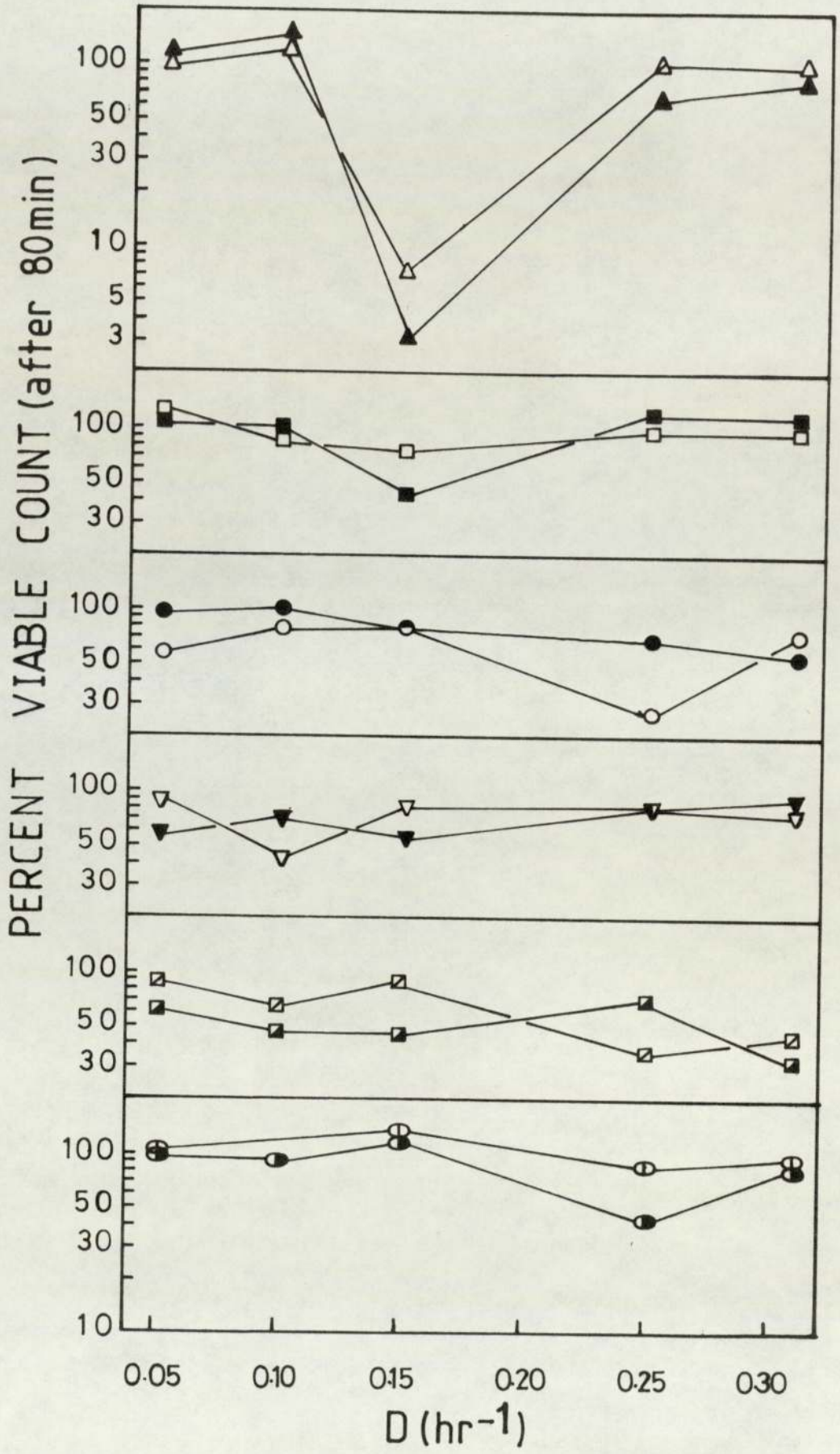
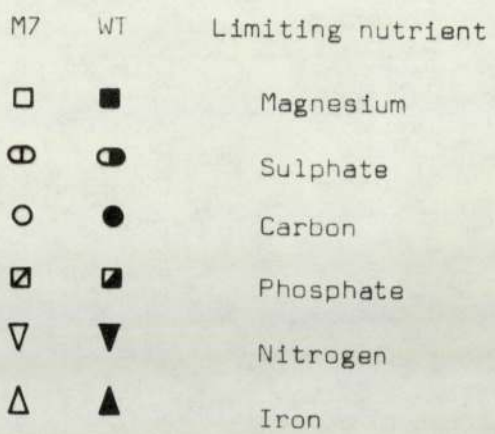

PERCENT VIABLE COUNT (after 80min)
100
50
30
10
5
3
100
50
30
100
50
30
100
50
30
100
50
30
100
50
30
10
0·05
0·10
0·15
0·20
0·25
0·30
D (hr⁻¹)

Figure 27. Effect of polymyxin (5 µg/ml) on M7 and WT grown in chemostats at D = 0.05 hr^{-1}.



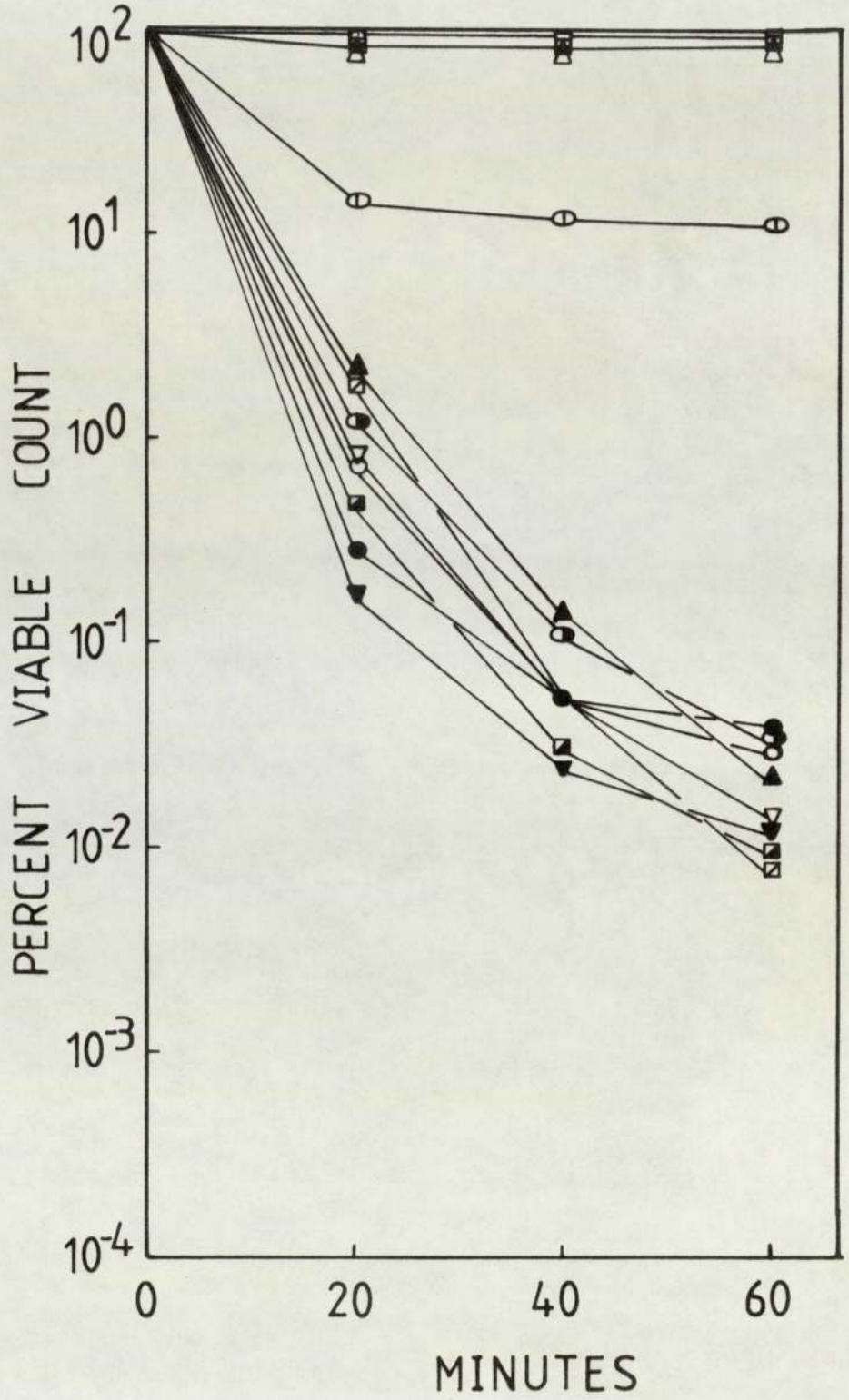
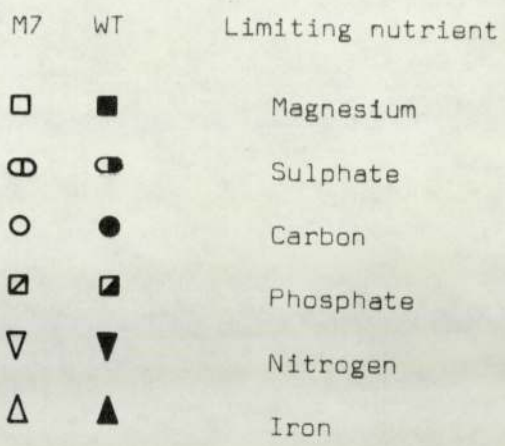

10^2
10^1
10^0
10^{-1}
10^{-2}
10^{-3}
10^{-4}
PERCENT VIABLE COUNT
0
20
40
60
MINUTES

Figure 28. Effect of polymyxin (5 µg/ml) on M7 and WT grown in chemostats at D = 0.15 hr^{-1}.



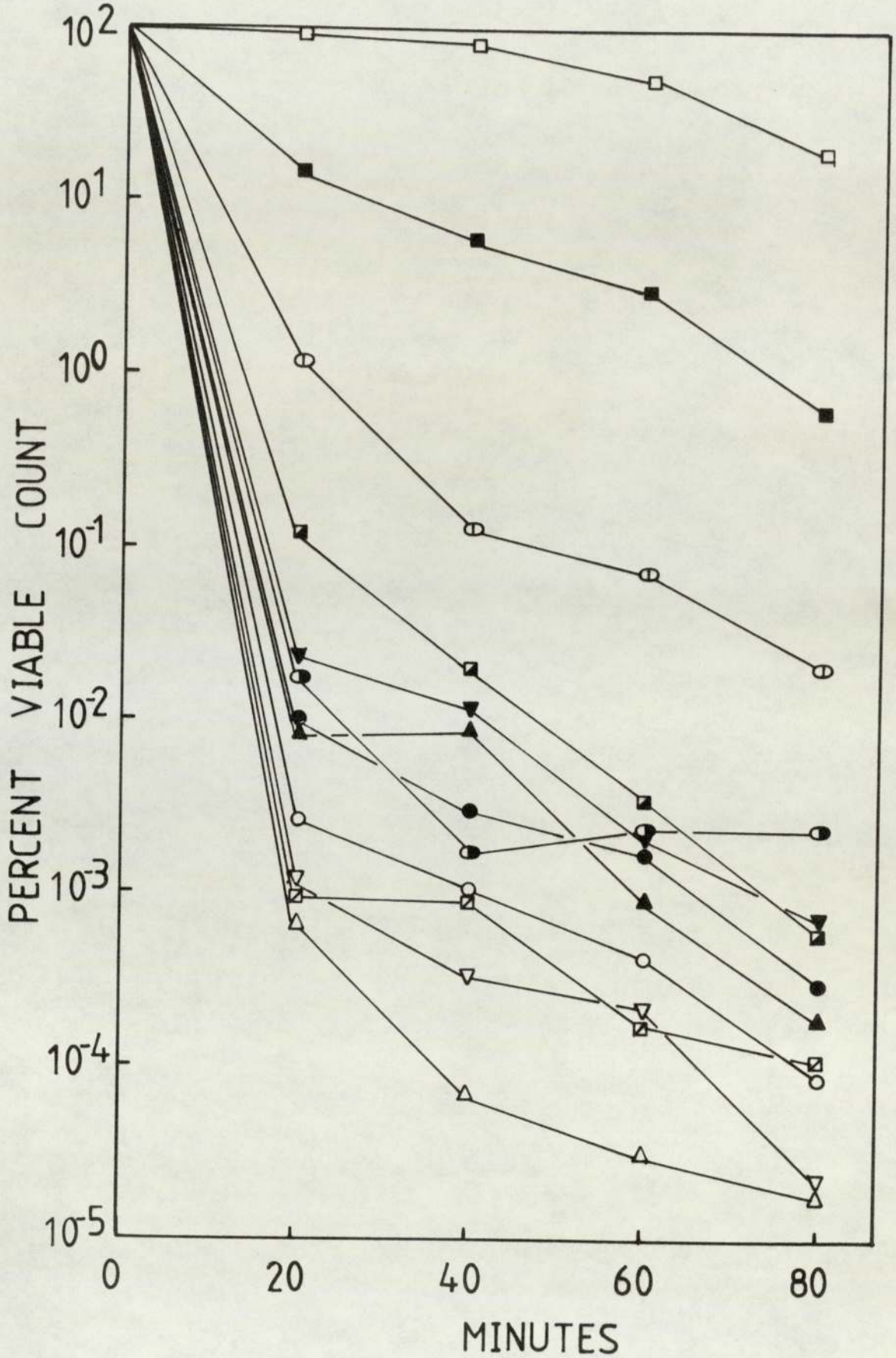
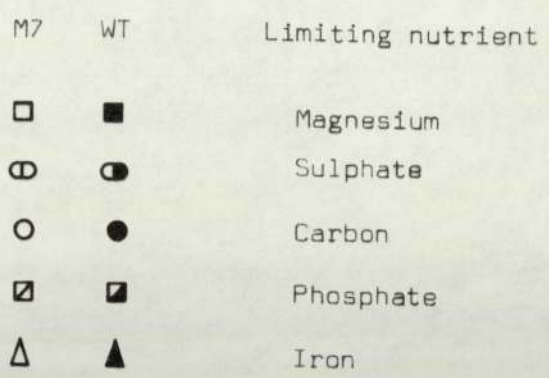

PERCENT VIABLE COUNT
10^2
10^1
10^0
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
0
20
40
60
80
MINUTES

Figure 29. Effect of polymyxin (5 µg/ml) on M7 and WT grown in chemostats at D = 0.25 hr^{-1}.



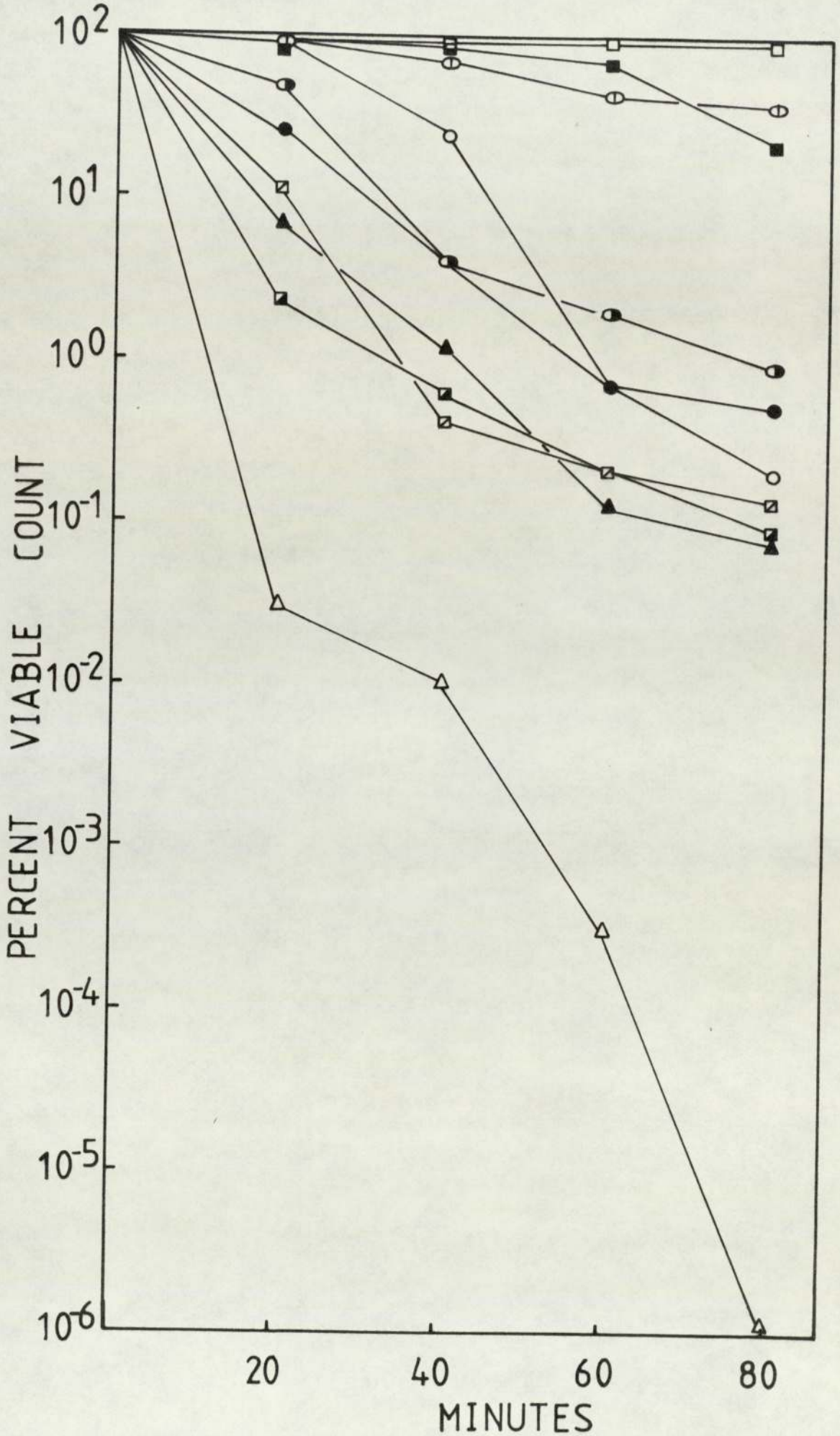
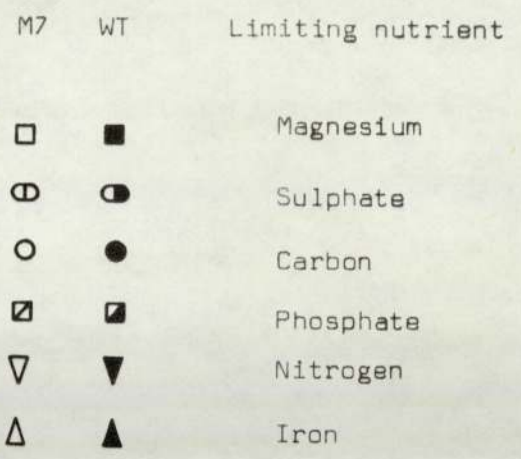

PERCENT VIABLE COUNT
10^2
10^1
10^0
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
20
40
60
80
MINUTES

Figure 30. Effect of polymyxin (5 µg/ml) on M7 and WT grown in chemostats at D = 0.31 hr^{-1}.



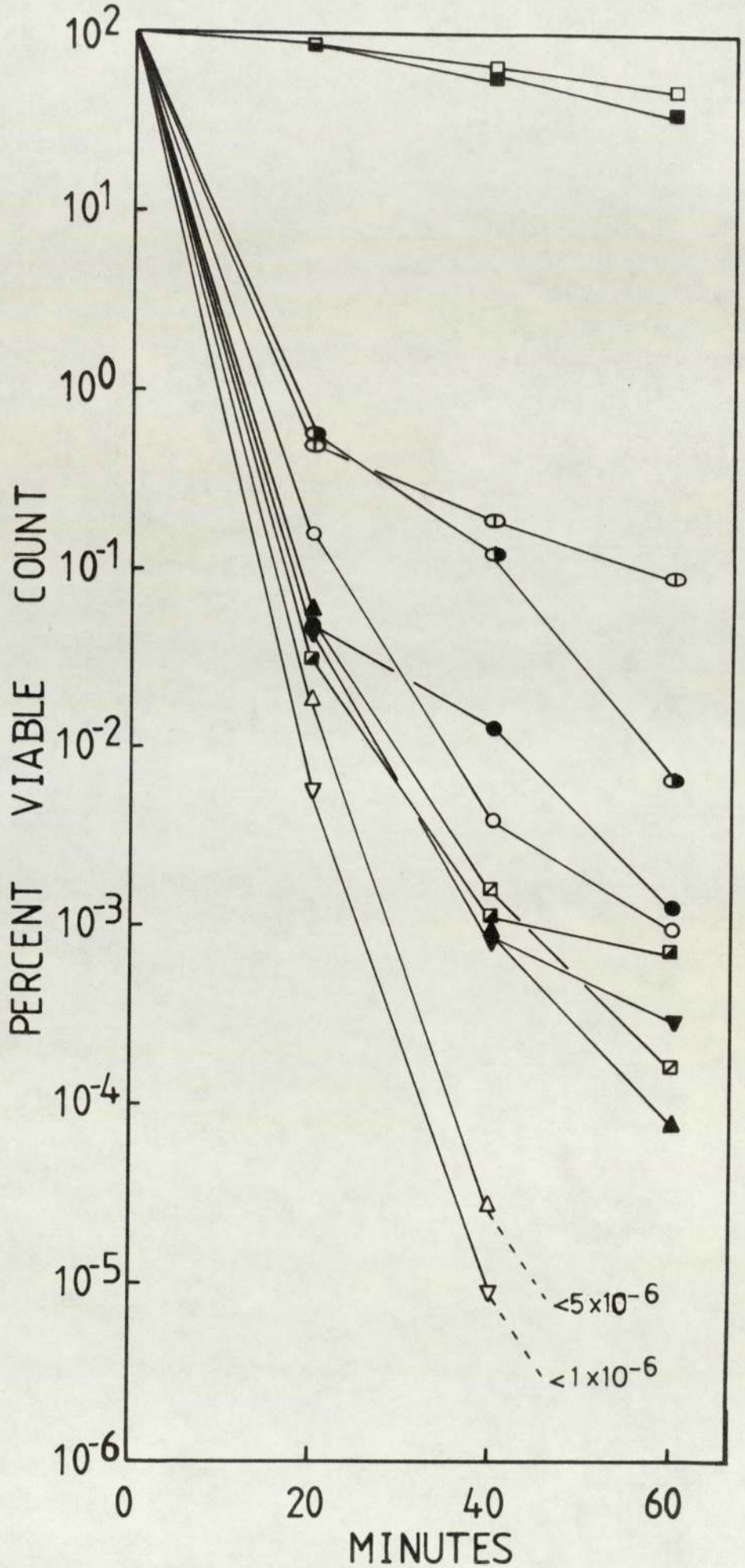
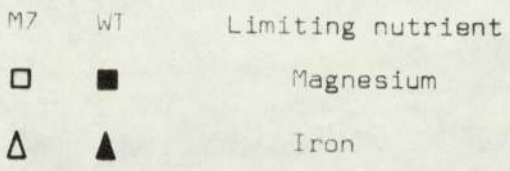
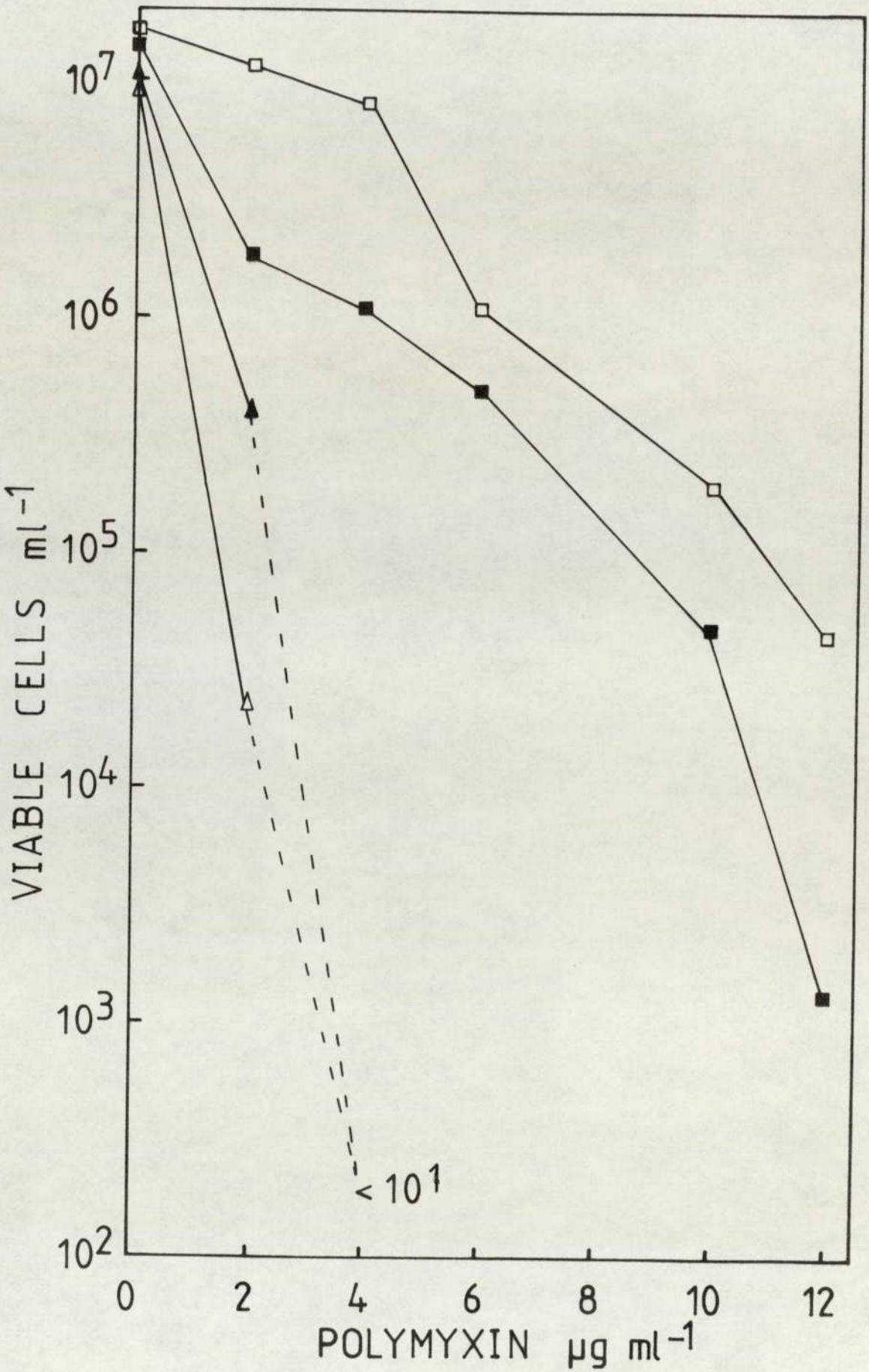

PERCENT VIABLE COUNT
10^2
10^1
10^0
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
$<5 \times 10^{-6}$
$<1 \times 10^{-6}$
0
20
40
60
MINUTES

Figure 31. Sensitivity of magnesium and iron limited M7 and WT grown in chemostats at D = 0.25 hr^{-1} to different concentrations of polymyxin. (Exposure time: 30 min).




VIABLE CELLS ml^{-1}
10^7
10^6
10^5
10^4
10^3
10^2
$< 10^1$
0
2
4
6
8
10
12
POLYMYXIN µg ml^{-1}

mixed together in a plastic tube. The blood/bacteria mixtures (in triplicates) were incubated in a shaking water bath at 37°C. At the start of incubation (0 min) 0.1 ml sample was removed and transferred into 0.9 ml of sterile water, mixed well and allowed to stand for 10 min at room temperature. The time interval was to allow lysis of blood cells without killing of bacteria. At timed intervals, 0.1 ml samples were removed from the phagocytic mixture. These were first transferred into 0.9 ml of sterile water at 37°C to avoid cold shock and then left for 10 min at room temperature. Samples were further diluted to 10^5 in half strength nutrient broth. Using the Miles and Misra method described in section 2.2.3, viable counts were made. Plates were incubated for 15 hr only to avoid confluent growth of the mucoid colonies. From the counts obtained for time 0 min and at other time intervals, the percentage of surviving bacteria was calculated.

A small sample was removed after 30 min incubation, dried on a slide and stained with Giemsa stain. An estimate of the phagocytic index was made by counting the number of bacteria in randomly dispersed polymorphonuclear leucocytes (PMN) and also the number of PMNs without bacteria. This was subsequently abandoned as results varied considerably. This was probably due to the difficulty in distinguishing intracellular and extracellular bacteria on the PMN.

3.5.2.2 Reproducibility of the method

Initial experiments on the same kind of cells were found to give absolute survival figures that differed more than would be expected from day to day experimental errors. It was subsequently found that the diluting medium, temperature and the age of blood sample could significantly affect the results. Shaken and unshaken

samples also gave different results. Using water for all dilutions was found to cause some cell death after approximately 25 min, hence half strength nutrient broth was used after the first dilution. Temperature was also found to have a large effect especially for the wild type (see section 3.5.2.4). Strict temperature controls were therefore essential. The day to day variation due to blood samples were minimized by using a single blood donor and collecting the blood at the same time each day. Although the killing activity of blood was found to be unaffected after 4 hr, it was none-the-less used within one hour of collection.

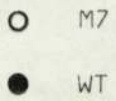
3.5.2.3 Killing of overnight culture of M7 and WT

To 25 ml of CDM, an inoculum of M7 or WT was added to give an initial OD_{470} of approximately 0.05. This was incubated overnight (16 hr) at 37°C in an orbital incubator at 140 rpm, the cells harvested and the blood killing determined. Samples were removed at 7.5 min intervals for 60 min. Figure 32 shows the results of one of three experiments. The same pattern was observed with the other two experiments, with WT showing a higher sensitivity to killing by blood than those of M7.

3.5.2.4 Effect of temperature

The effect of temperature on killing by blood was carried out using overnight cultures of M7 and WT. The blood/bacteria mixtures were incubated at 33°C, 37°C and 41°C and samples removed at 15 and 30 min. At 41°C, the survival was reduced in both organisms when compared to survival at 37°C as shown in Figure 33. At 33°C, the survival of WT was enhanced but M7 showed little difference between 33°C, 35°C and 37°C. At 39°C, a slight decrease in survival was observed. The wild type showed a slight increase

Figure 32. Killing of batch grown *P. aeruginosa* by whole blood.



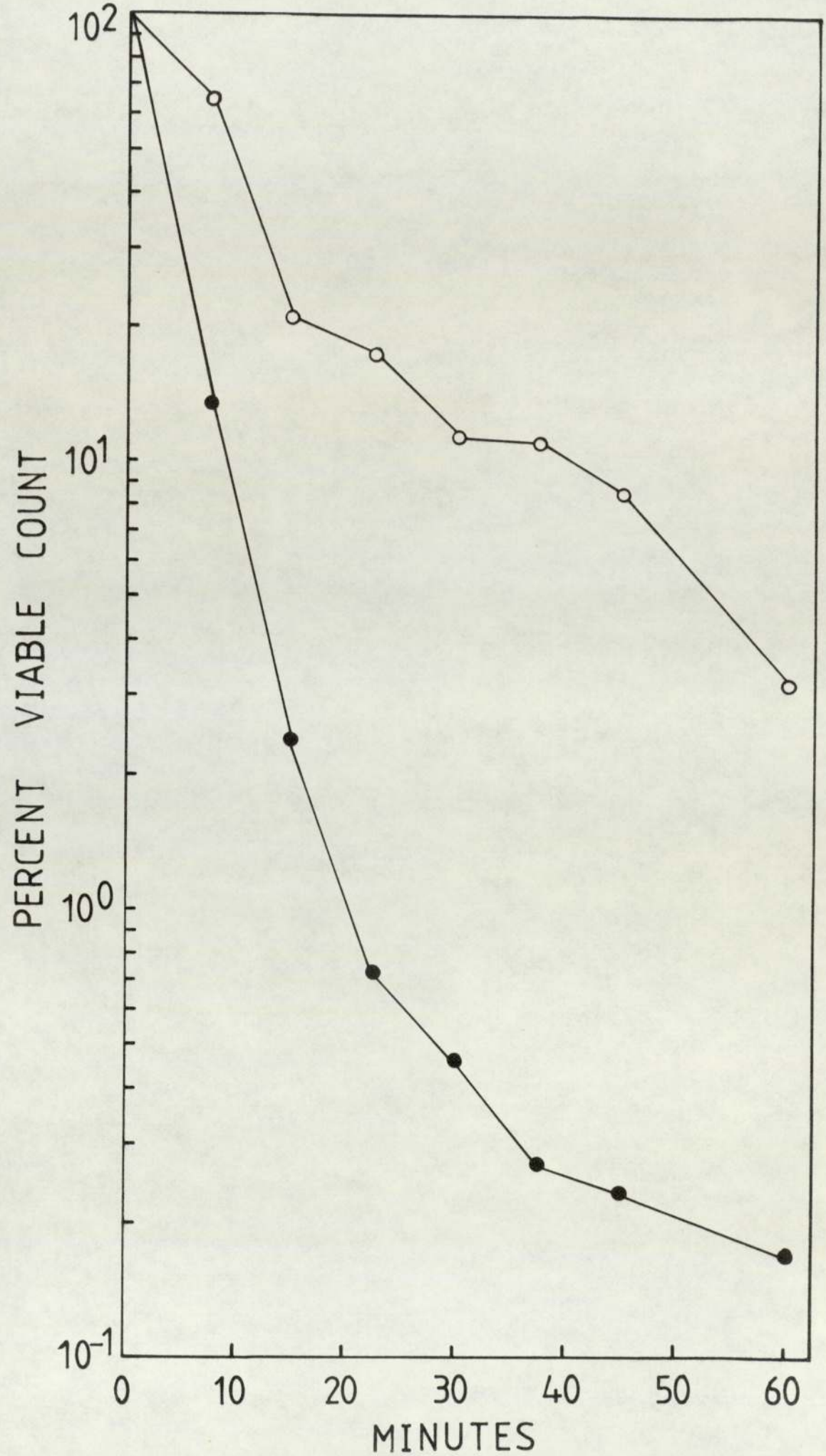
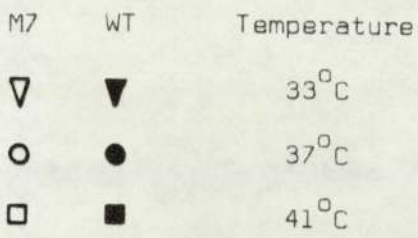
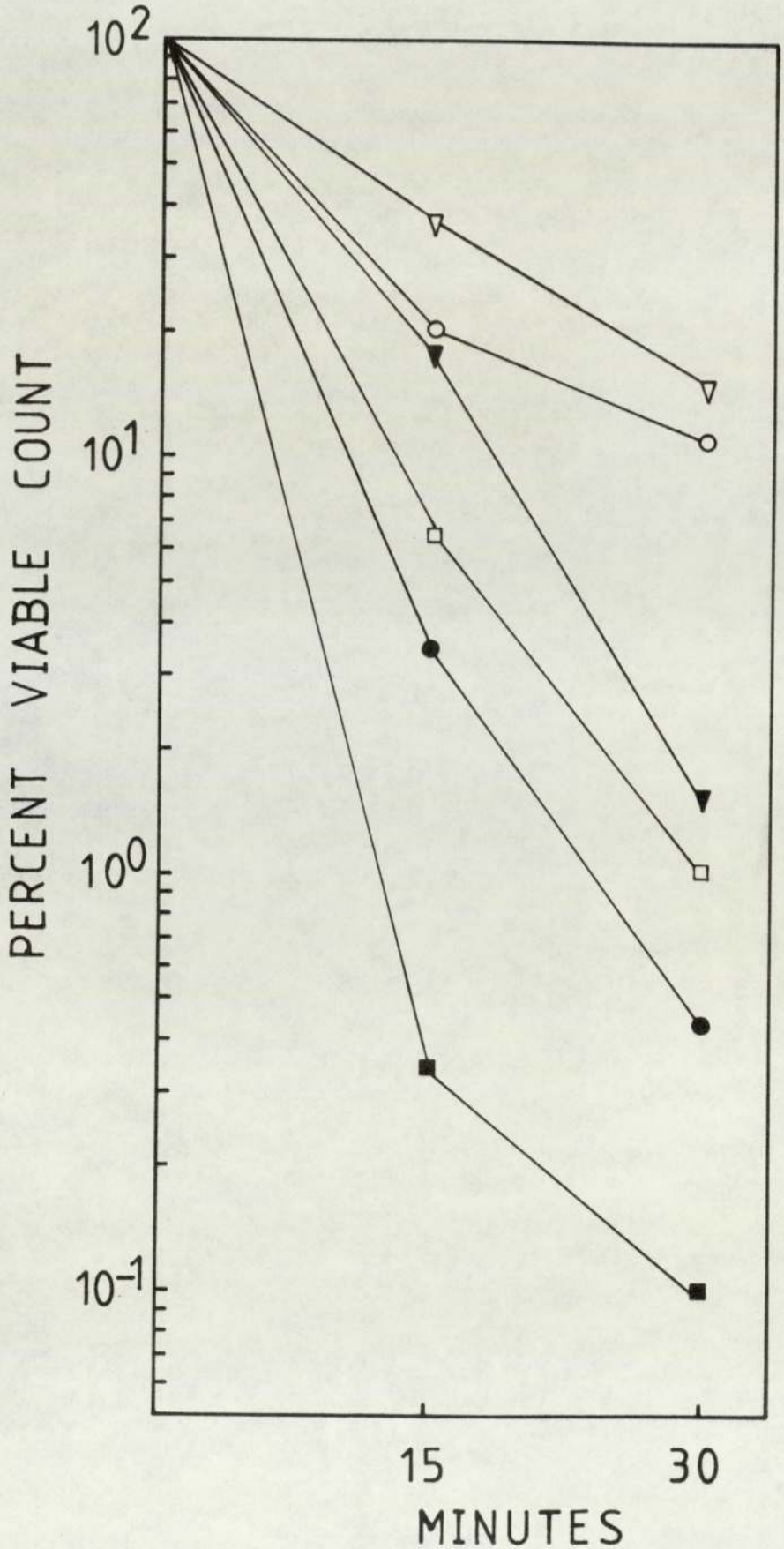

10^2
10^1
10^0
10^{-1}
PERCENT VIABLE COUNT
0
10
20
30
40
50
60
MINUTES

Figure 33. Effect of temperature on killing of batch grown *P. aeruginosa* by whole blood.




10^2
10^1
10^0
10^{-1}
PERCENT VIABLE COUNT
15
30
MINUTES

in survival at 35°C and a large decrease in survival at 39°C. At all temperatures studied, M7 was more resistant than WT.

3.5.2.5 Effect of nutrient limitation on killing by blood: batch cultures

M7 and WT were grown in nutrient limited media in an orbital incubator (140 rpm) at 37°C for 36 hr. The killing by blood of these cells is shown in Figure 34A. Mucoid cells showed a higher resistance than the non-mucoid but the difference was reduced as compared to the complete medium (oxygen limited cells). Nitrogen limited cells of WT were consistently more sensitive. The killing of mucoid cells was low under all limitations. To determine whether the low killing shown in Figure 34A was due to the fact that the cells were in stationary phase, early stationary phase cells were used. The state of the cells was determined by observing the growth by optical density measurements. The cells were said to be in early stationary phase when growth ceased to be linear. Growth was then followed for a further 1.5 hr, the cells harvested and the experiment carried out as previously described. Figure 34B shows the survival of these cells. In this case, N-lim cells of both M7 and WT had the lowest survivors. Although there was a slight increase in sensitivity compared to stationary phase cells, no clear separation of the different limitations was evident. The limitations however reduced or eliminated the difference in killing that was observed between M7 and WT.

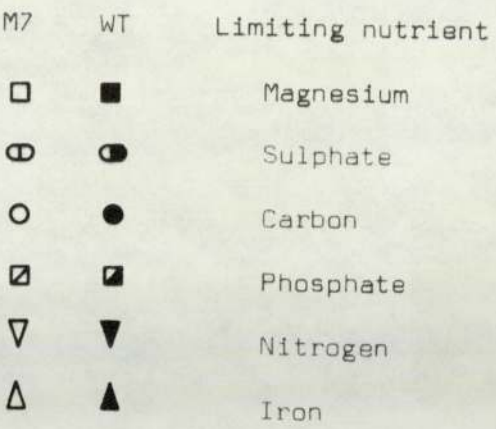
3.5.2.6 Effect of nutrient limitation on killing by blood: chemostat cultures

Studies on nutrient limited cells grown in chemostats were performed mostly using cells grown at the slow dilution rate of

Figure 34. Effect of nutrient limitation on killing of batch grown *P. aeruginosa* by whole blood.

A Stationary phase cells

B Early stationary phase cells



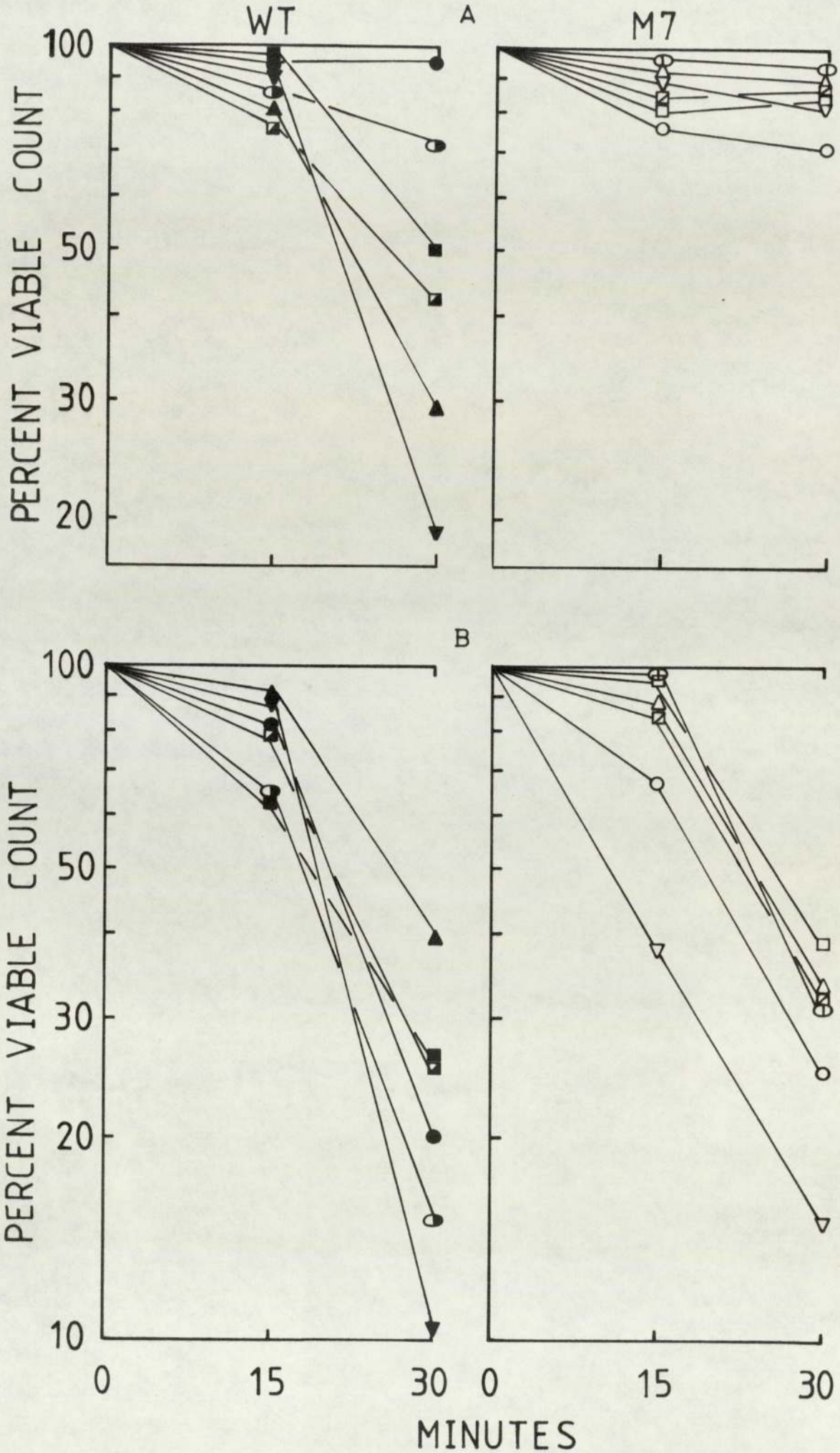
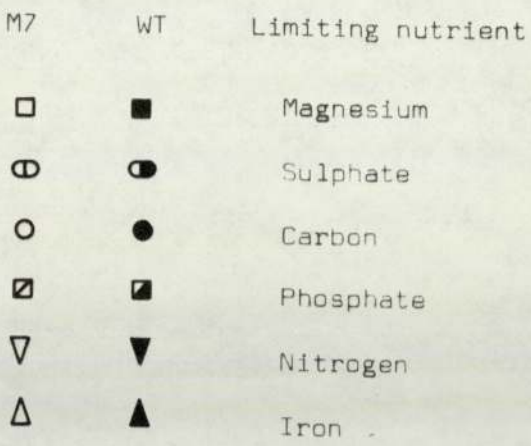

WT
M7
A
B
100
50
30
20
10
PERCENT VIABLE COUNT
0
15
30
MINUTES

Figure 35. Sensitivity of nutrient limited M7 and WT grown in chemostats at D = 0.05 hr^{-1} to whole blood.



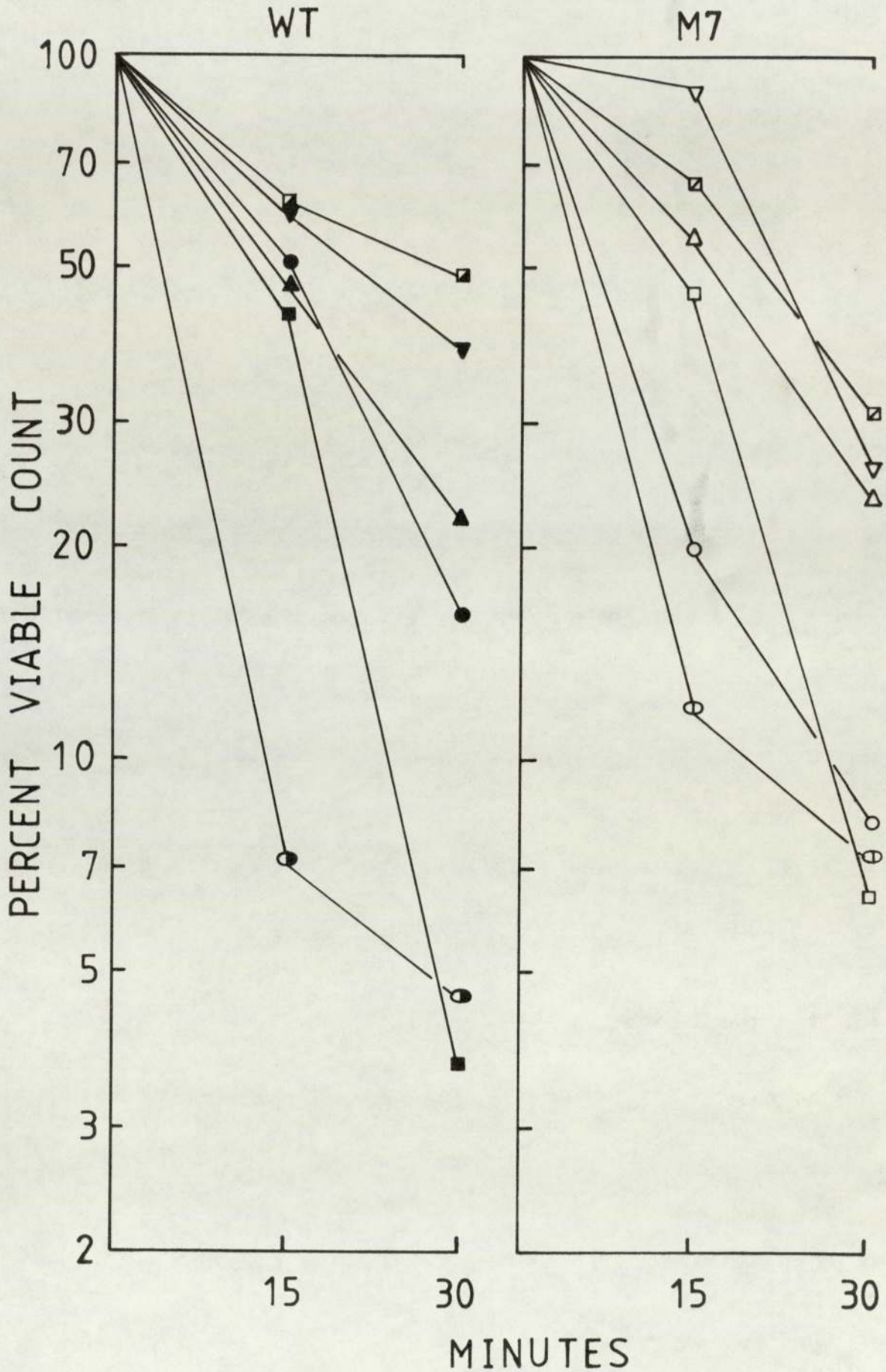
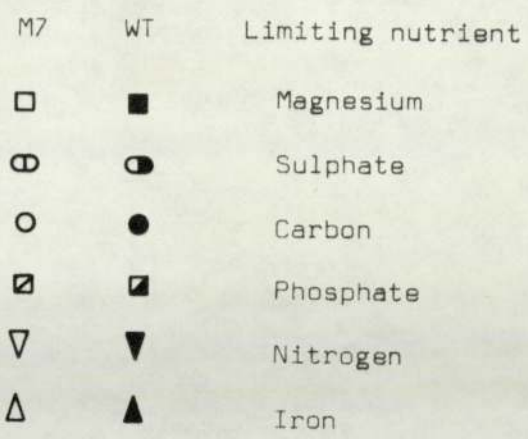

WT
M7
100
70
50
30
20
10
7
5
3
2
PERCENT VIABLE COUNT
15
30
15
30
MINUTES

Figure 36. Sensitivity of nutrient limited M7 and WT grown in chemostats at D = 0.15 hr^{-1} to whole blood.



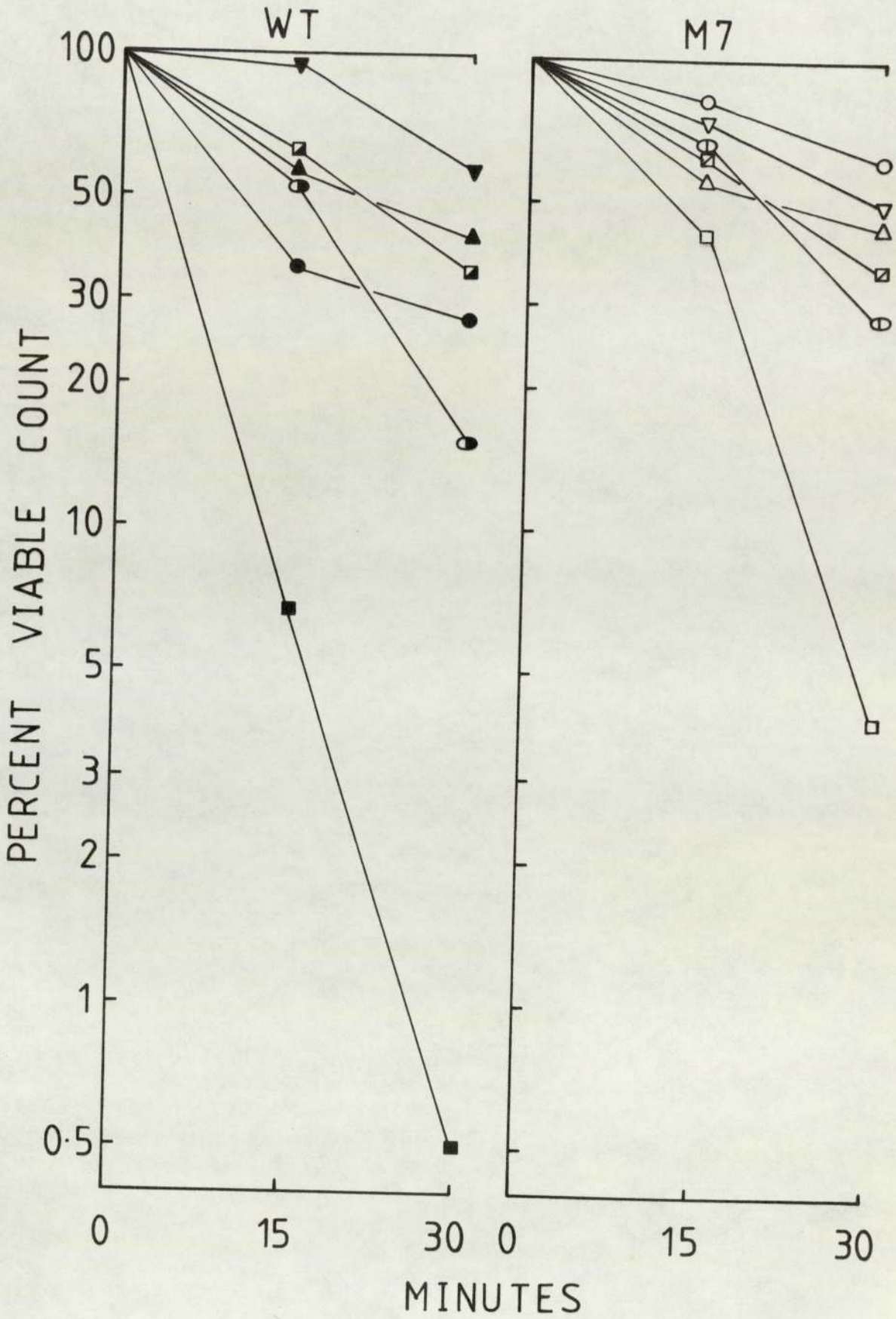
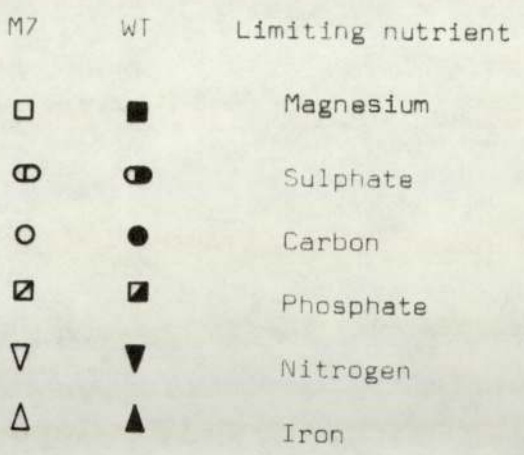
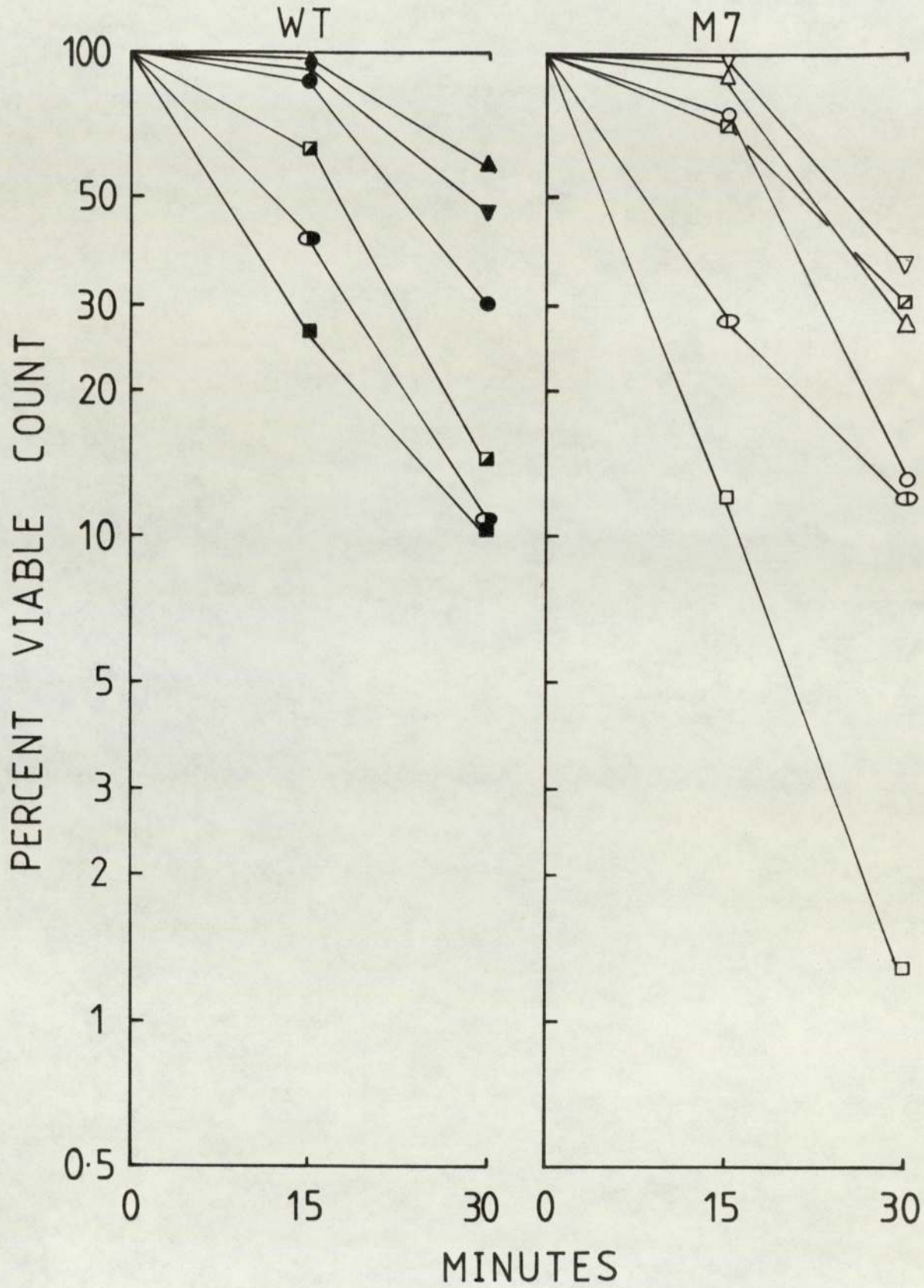

WT
M7
100
50
30
20
10
5
3
2
1
0·5
PERCENT VIABLE COUNT
0
15
30
0
15
30
MINUTES

Figure 37. Sensitivity of nutrient limited M7 and WT grown in chemostats at D = 0.25 hr^{-1} to whole blood.




WT
M7
100
50
30
20
10
5
3
2
1
0·5
PERCENT VIABLE COUNT
0
15
30
0
15
30
MINUTES

0.05 hr^{-1}. This was in attempt to mimic conditions *in vivo* where microorganisms have a slow growth rate. Samples were collected over ice after the cultures had reached steady state. Figure 35 shows the pattern observed in four replicate experiments. The order of sensitivity could be placed into two groups. Nitrogen and phosphate and possibly iron limited cells consisted of resistant cells whilst magnesium, sulphate and possibly carbon limitation resulted in sensitive cells. It was observed, for both organisms, that most of the killing of sulphate limited cells occurred within the first 15 min. For these cells, there was little change in the percentage survivor between 15 min and 30 min.

At the faster dilution rates of 0.15 hr^{-1} (one experiment) and 0.25 hr^{-1} (two replicate experiments) the two orders of sensitivities remained the same as shown in Figures 36 and 37. Magnesium limited cells were the most sensitive. There was no significant difference between M7 and WT cells at any of the dilution rates.

3.5.2.7 Effect of dilution rate on killing by blood of iron limited cells

The effect of day to day variations made it impossible to compare the effect of growth rate on experiments done on different days. To overcome this, iron limited cells grown at different dilution rates were studied at the same time.

Iron limited M7 or WT were allowed to grow to steady state in six chemostats at different rates. Growth was monitored by viable and optical density measurements. Both organisms showed the same behaviour in chemostats under the dilution rates studied as shown in Figure 38. To achieve steady state conditions at the same time, the chemostats were inoculated on different days starting

with the slowest dilution rate. Cells for tests were obtained not from the overflow but by using culture from the chemostat chamber.

The consistent pattern of killing of Fe-lim M7 (three replicate experiments) and Fe-lim WT (two replicate experiments) is shown in figures 39 and 40. The highest and lowest dilutions showed no difference in percentage survivors. A difference was however observed with intermediate dilution rates. As the dilution rate increased from $\simeq$ 0.02 to 0.1 hr^{-1}, the percent survivors increased two to three fold. Figure 41 shows the effect of dilution rate, using data from figures 39 and 40, as shown by both organisms. Dilution rates between 0.1 hr^{-1} and 0.15 hr^{-1} showed an optimum state where the cells were more resistant to the killing effect of blood.

Figure 38. Observed behaviour of chemostat grown, iron limited *P. aeruginosa*.

M7	WT	Measurement
□	■	Viable count
○	●	Optical density

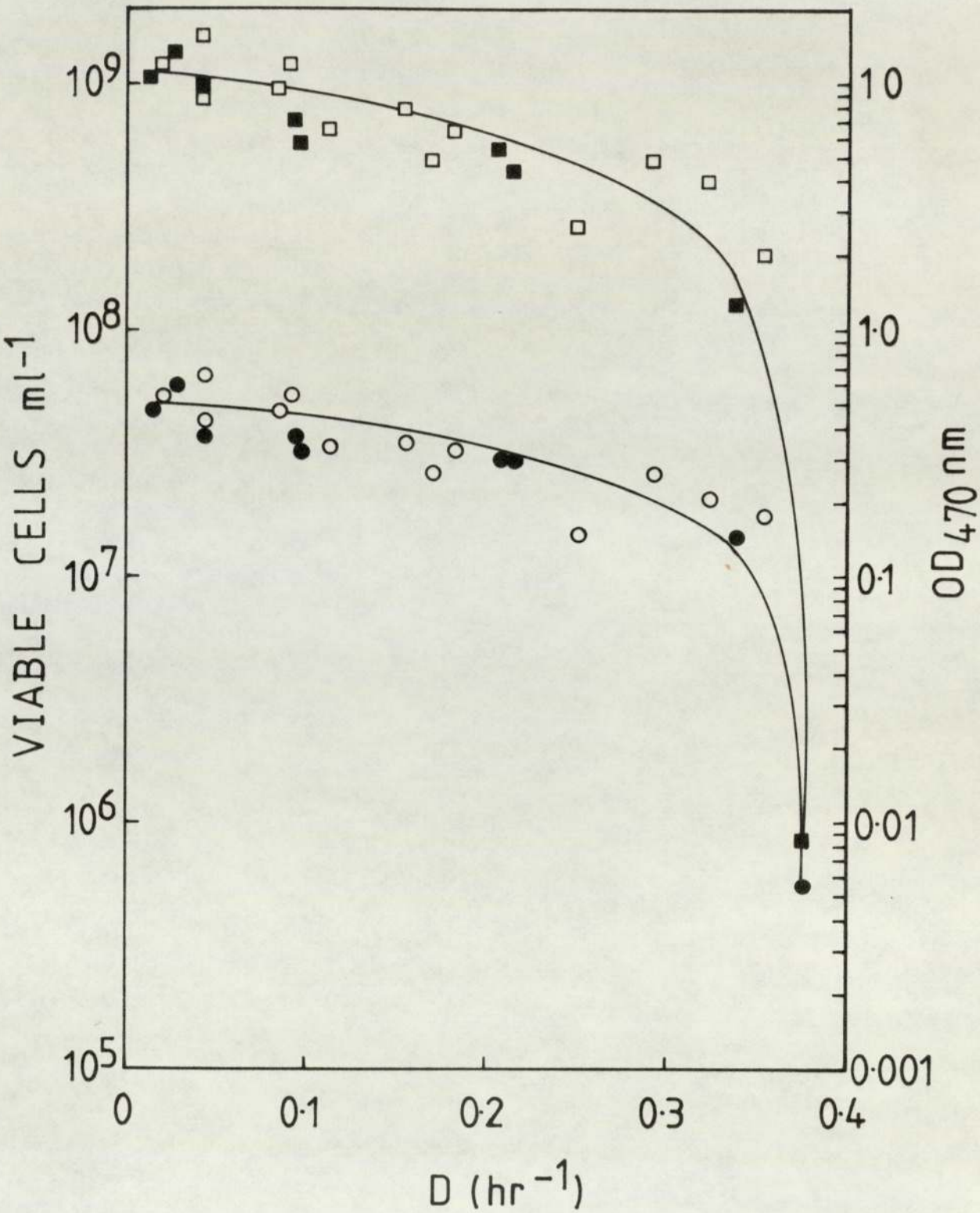
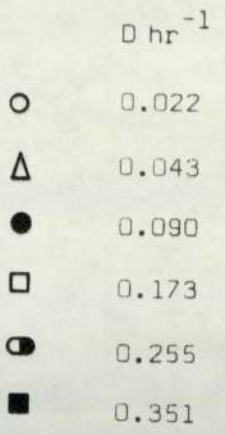

VIABLE CELLS ml-1
10^9
10^8
10^7
10^6
10^5
1·0
0·1
0·01
0·001
OD470 nm
0
0·1
0·2
0·3
0·4
D (hr^-1)

Figure 39. Sensitivity of iron limited M7 grown in chemostats at different dilution rates to whole blood.



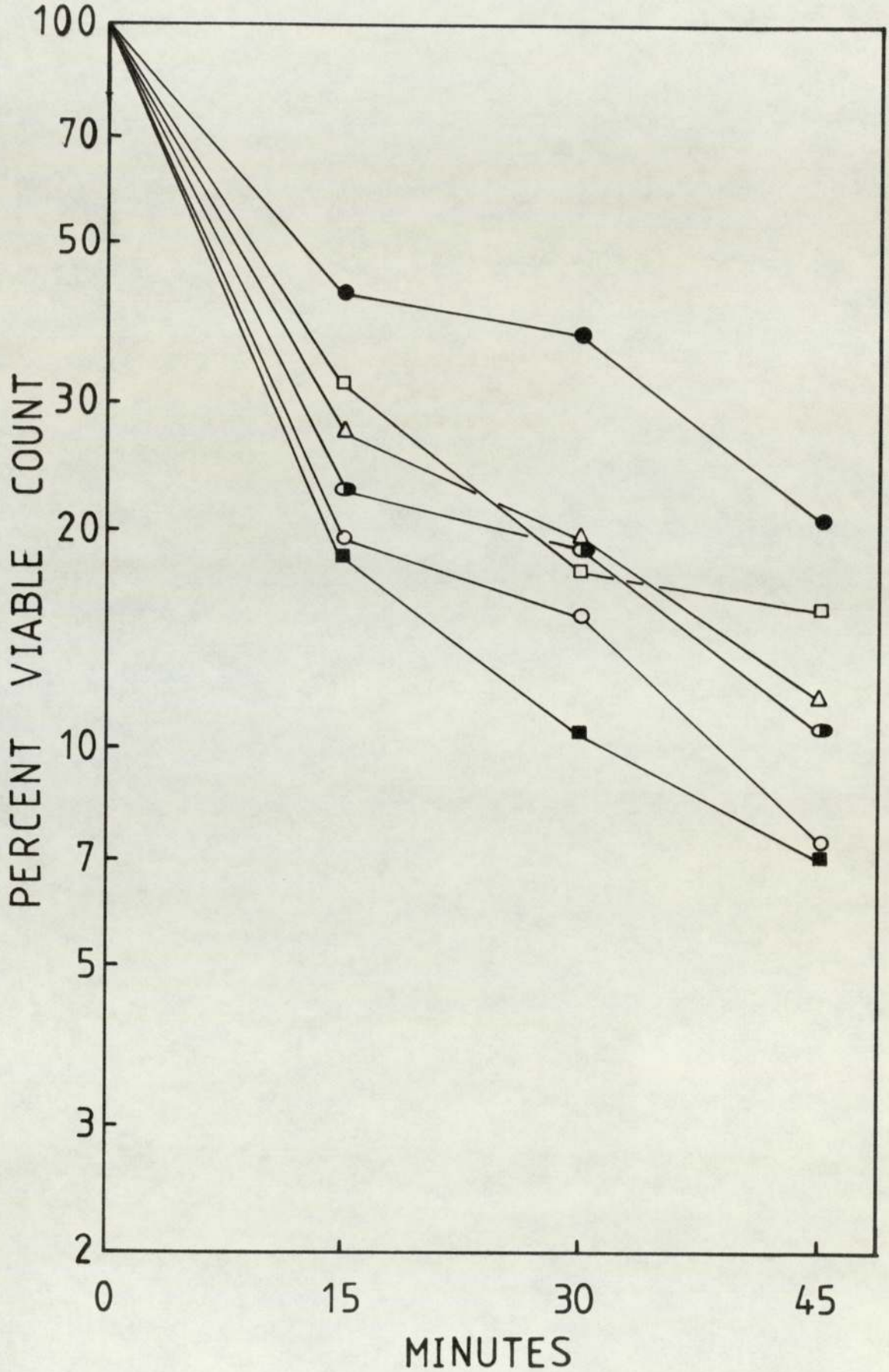
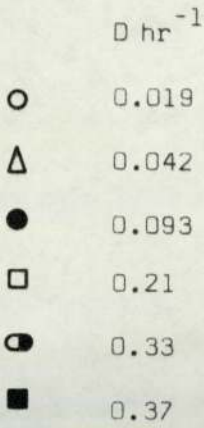

100
70
50
30
20
10
7
5
3
2
PERCENT VIABLE COUNT
0
15
30
45
MINUTES

Figure 40. Sensitivity of iron limited WT grown in chemostats at different dilution rates to whole blood.



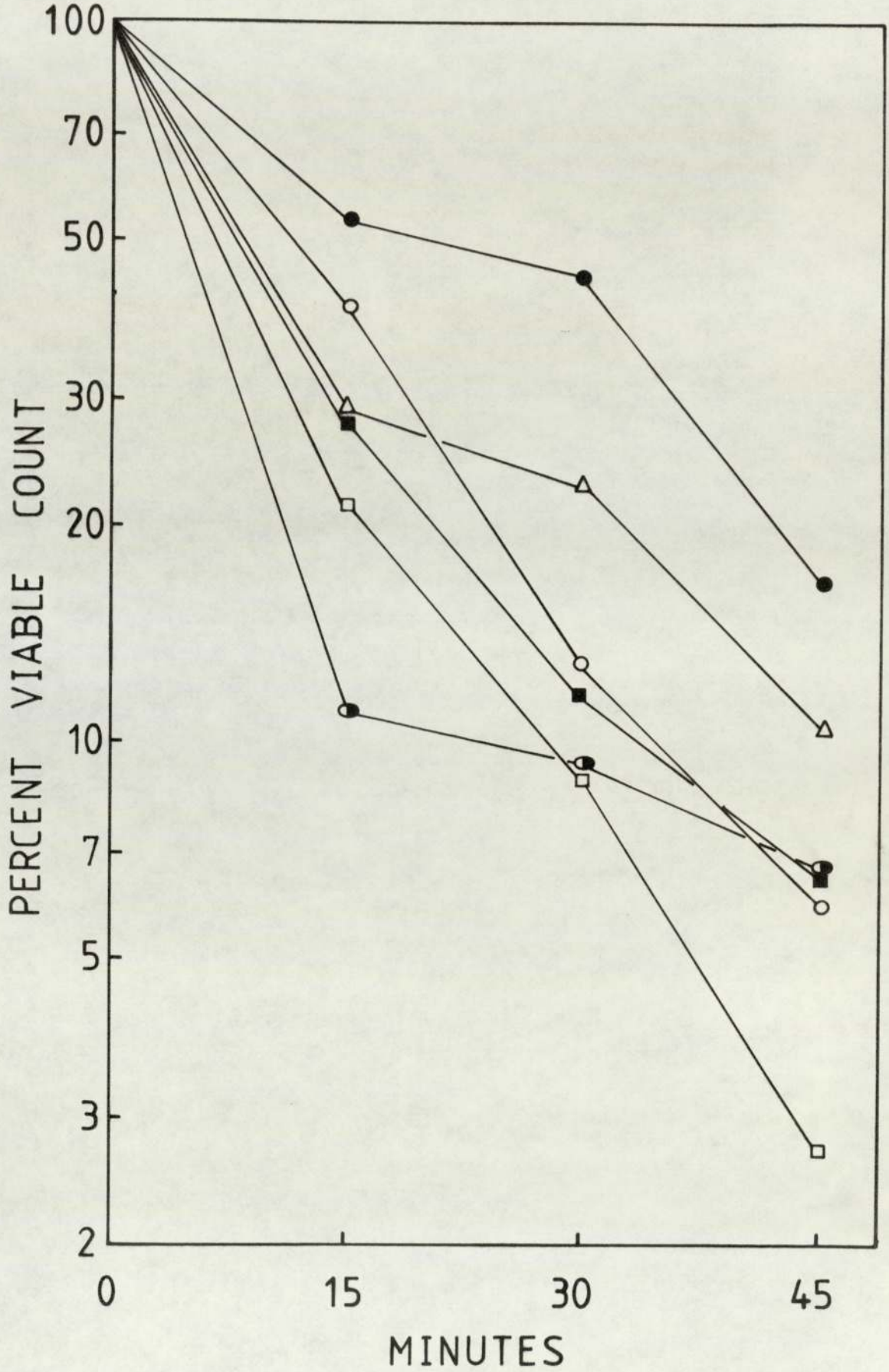
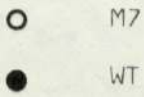
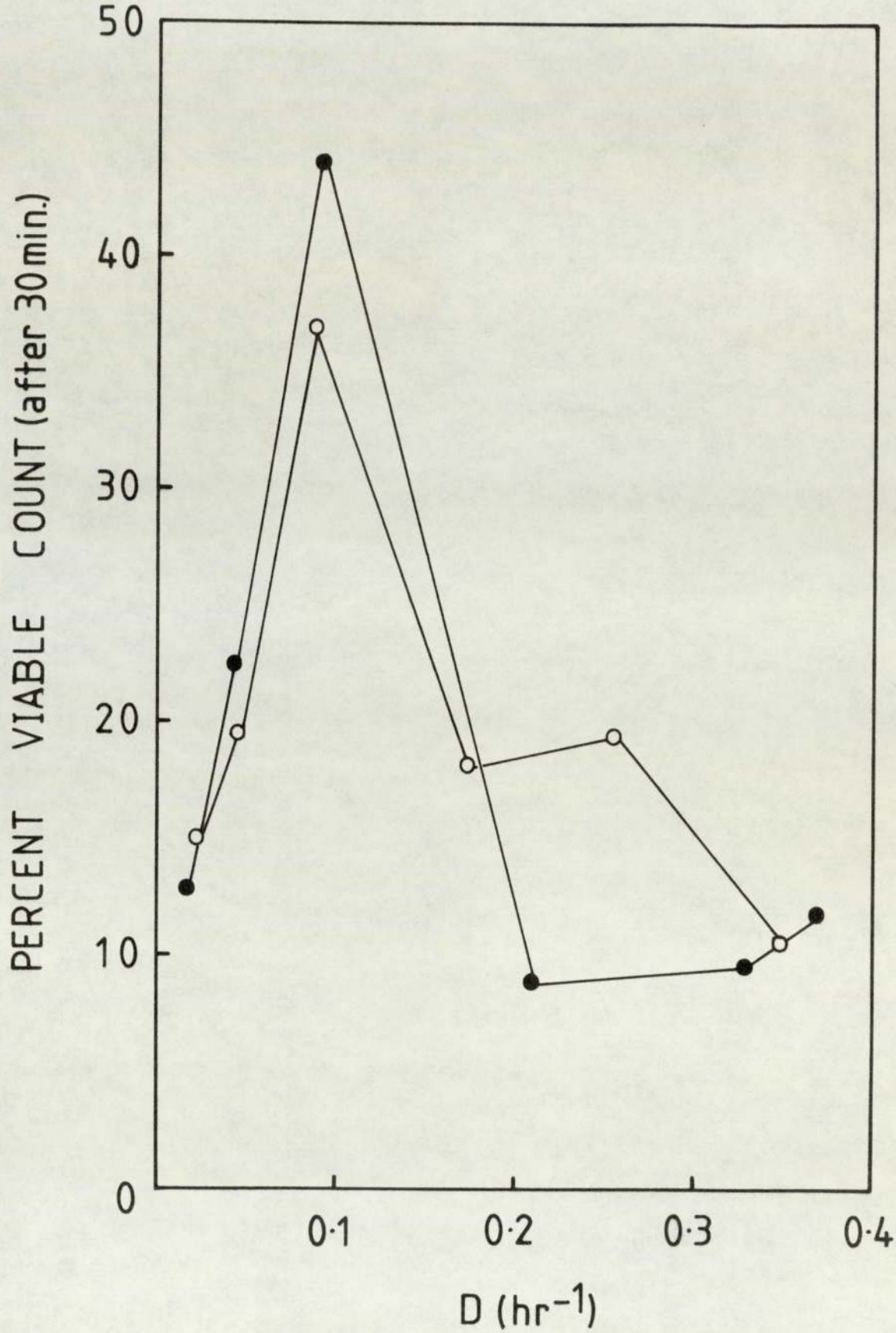

100
70
50
30
20
10
7
5
3
2
PERCENT VIABLE COUNT
0
15
30
45
MINUTES

Figure 41. Effect of dilution rate on killing by whole blood of iron limited *P. aeruginosa*.




PERCENT VIABLE COUNT (after 30 min.)
50
40
30
20
10
0
0·1
0·2
0·3
0·4
D (hr⁻¹)

4. DISCUSSION

4.1 Nutrient Requirements of *P. aeruginosa* WT and M7

4.1.1 General

Natural environments rarely contain all those nutrient substances that are necessary for cell synthesis in concentrations sufficient to permit microbial growth to proceed to its maximum rate. The effectiveness with which organisms can compete with one another for the limited supply of a nutrient present in the medium, will therefore depend critically on the relative affinities of their uptake systems. Thus, where two organisms are competing, their nutrient requirement may affect their chances of survival. The nutrient depletion studies carried out on M7 and WT showed that there were some differences in their nutritional requirements. Two general statements can be made regarding their oxygen requirements and the maximum growth rate constants.

4.1.1.1 Oxygen requirement

With the exception of magnesium limitation (Figure 8), M7 showed a lower optical density than the WT at relatively high optical densities, when the specific nutrient under investigation was no longer limiting (Figures 5, 10, 12, 14, 16). The two organisms however, showed similar viable counts for the same optical density (section 3.18). This indicated that some other factor, to which M7 was more sensitive than WT has become growth limiting. To determine whether this was due to change in pH, the organisms were grown in medium buffered at pH 6.5 and 7.8 and the onset of non-linear growth observed. This occurred at OD_{470} 1.5 for WT for both pH values. The doubling times were also unaffected and the growth rate constant was 0.73. M7 showed a change in linear growth at OD_{470} 1.0 for both pH values, but the doubling

time was higher at the acidic pH and consequently the growth rate constant was 0.50 compared to 0.58 at pH 7.8. Since the low pH did not change the optical density at which the change in growth rate occurred, it appeared likely that oxygen had become the growth limiting factor. This was confirmed by growing cultures in identical media in a shaking water bath, as used in the nutrient requirement study, and also in an orbital incubator where the shaking rate was higher (140 rpm) and therefore aeration was more efficient. The optical density was higher in the orbital incubator. The lower optical densities obtained with M7 were therefore probably due to oxygen limitation. This suggested that M7 required higher concentrations of oxygen in the medium than WT.

An increased requirement for oxygen could be due to two factors. One, that M7 has an altered biochemical process compared to WT. Secondly, the organisms may use the same amount of oxygen but in cultures of M7, diffusion of oxygen is hindered. The presence of exopolysaccharide could hinder not only passage of oxygen to the cell, but also of carbon dioxide and of extracellular products. The effect of exopolysaccharide on oxygen diffusion would be more marked at higher cell densities where the viscosity would be higher and the aeration efficiency effectively reduced.

The optical density at which growth ceased to be linear differed between cultures grown in full CDM and those grown under some nutrient limitation. The relation between onset of magnesium depletion (OD_{470} onset) and initial Mg^{2+} concentration was linear only to an OD_{470} of 0.7 (Figure 8) instead of 1.5 as shown by WT grown in full CDM. Likewise, linearity ended at OD_{470} 1.0 and 0.85 for phosphate and nitrogen respectively (Figures 12, 14).

Similarly for M7, the values were 0.75, 0.65 and 0.70 for magnesium, phosphate and nitrogen respectively (Figures 8, 12, 14). This suggests that growth limitation by oxygen affects the onset of growth depletion by these nutrients at population densities lower than those obtained in fully adequate medium. Kenward (1975) observed that the growth limiting factor that caused a decrease in growth at OD_{470} 1.3 of fully adequate medium, affected the onset of growth depletion due to lack of magnesium at population greater than OD_{470} 0.8. The total growth was however unaffected while glucose was present in excess. The effect is probably due to the lower molar concentrations of the limiting nutrients compared to the amount in full CDM which may have affected the medium tonicity and hence growth pattern. Changes in medium tonicity can affect both cell composition (Tempest & Meers, 1968) and the metabolism (Pirt, 1975)

4.1.1.2 Growth rate constant (μm)

Figure 17 A-F shows the relation between the reciprocal of the specific growth rate of exponential phase cells and the reciprocal of initial concentration in the medium of carbon, iron, magnesium, sulphate, nitrogen and phosphate. Using these graphs, μ_m and K_s were calculated and are described in Table 5. In all cases, the specific growth rate was always higher for WT irrespective of the limiting nutrient. Govan et al. (1979), proposed that the instability of mucoid variants was due to a spontaneous mutation which results in non-mucoid variants which have a growth rate advantage over their mucoid parent strain and which is more pronounced in unshaken than shaken cultures. Using unshaken cultures, they observed that during the period of maximum growth from 1.5 to 6 hr, the mean generation times of mucoid and non-mucoid

were 37.5 and 30.5 min respectively. After 6 hr however, as the growth rate of mucoid strain approached zero, there was clear evidence of growth rate advantage for the non-mucoid which continued to grow until 24 hr. During the period between 6 and 12 hr, Govan et al. (1979) found that the mean generation time was 97.5 min for mucoid compared to 60 min for non-mucoid. The percentage of non-mucoid variants in the unshaken cultures of mucoid culture increased from 0.2% at the beginning of the experiment to 52% after 24 hr. In contrast, in shaken culture the growth rate advantage of the non-mucoid was less evident, with generation times of 23.7 compared to 26.7 in the first 6 hr of growth. After 6 hr, the non-mucoid approached zero growth rate only slightly ahead of non-mucoid strain. The percentage of non-mucoid colonies was only 13.4% after 24 hr. The present results support the theory of growth rate advantage for non-mucoid forms. This is further confirmed by the observed higher oxygen requirements shown by M7 as discussed in section 4.1.1.1. The fact that the growth rate difference was diminished by shaking, suggests that oxygen diffusion, rather than changed biochemical processes is the major controlling factor. Oxygen may therefore be the rate limiting factor that causes a lower μ_m for M7.

The maximum growth rate in full CDM was 0.73 for WT and 0.58 for M7. Since μ_m is the maximum value of the growth rate constant, it would be expected that the values of μ_m would be the same irrespective of the limiting nutrient and to be equal to the growth rate constant of full CDM. The values of μ_m however varied between 0.58 and 0.83 for WT and 0.45 and 0.57 for M7 (Table 5). Using Proteus, Al-Dujaili (1979) found μ_m was 0.66, 0.7 (0.49) and 0.65 when medium was limited by glucose, magnesium (two values) or

phosphate. Similarly, Finch (1976) using P. aeruginosa, found μm was 0.797, 0.73 and 0.815 when medium was glucose or magnesium limited or in full CDM respectively. The value of μ_m can be affected by oxygen, temperature, pH and medium tonicity (Pirt, 1975). Day to day variations probably affect all four factors and cause small variations in μ_m. Larger changes are probably due to tonicity effects brought about by the decreased concentration of the limiting nutrient. The effect of medium tonicity on growth rate has been investigated for a number of organisms including Salmonella newport (Christian & Scott, 1953), Staph. aureus (Scott, 1953), P. fluorescens (Wodzinski & Frazier, 1960), Klebsiella aerogenes (Wodzinski & Frazier, 1961; Pirt, 1975) and Aspergillus amstelodami (Pirt, 1975). In all cases there was an optimum tonicity value which gave a maximum growth rate. The maximum value of the growth rate could also be affected by the nature of the solute used to control the tonicity of the medium. The ability of ions to associate with water and so increase the tonicity is given by the series sulphate > acetate > chloride > nitrate and for cations, magnesium > calcium > lithium > sodium > potassium (Glasstone & Lewis, 1964). Ions high in the series such as sulphate and magnesium would have a bigger effect on the tonicity of the culture medium. The different μm observed for different nutrient limitations are probably a combination of tonicity effect and day to day variation. Iron shows the lowest μ_m. This is probably due to a larger pH effect arising from the fact that the medium for the study had previously been used. The starting pH was therefore lower (c 7.6) than that used for other studies (7.8), and therefore not a really valid comparison. Similarly, μ_m from sulphate study should not be compared with the others since the growth medium used was different (Table 4).

4.1.2 Carbon

When all nutrients required for growth were available in excess except the carbon source (glucose or gluconate), growth ceased abruptly when the latter became depleted in cultures of low initial carbon source (Figures 3 & 4). The glucose/gluconate provides the cell carbon and energy for growth and maintenance and the sudden lack of energy when carbon is depleted, resulted in end of growth. A linear relationship existed between optical density at the end of exponential growth and glucose concentration in the medium. This relationship became non linear at OD_{470} 1.0 for M7 and at OD_{470} 1.5 for WT corresponding to 7.0 mM glucose. Using gluconate, the relationship was linear to OD_{470} 1.7 for WT and OD 1.15 for M7, corresponding to 10 mM gluconate (Figure 5). For the linear part of the graph, when oxygen is not limiting, the WT reaches a higher optical density than M7 for the same amount of glucose or gluconate. This is not surprising since some of the carbon provided would be used in the synthesis of exopolysaccharide by M7 and hence would not be available for synthesis of cell constituents.

The growth rate of the culture was dependent upon the initial glucose concentration (Figure 17A) and WT showed a higher specific growth rate than M7 at all concentrations.

4.1.3 Magnesium

Figures 6 and 7 are the growth curves shown by the organisms when grown under different initial magnesium concentrations. Unlike carbon, magnesium depletion showed a progressive decrease in growth rate after the end of the exponential growth. This gradual decrease in growth rate may be due to a decrease in

synthesis of ribosomal RNA. Ribosomal function is dependent on magnesium (Sykes & Tempest, 1965; Tempest et al., 1965), and protein synthesis is proportional to the ribosomal content (Tempest & Dicks, 1967). Therefore, at each successive division of magnesium depleted cells, the cellular content of magnesium and ribosome progressively decreases, causing a corresponding fall in protein synthesis and growth rate.

Figure 8 shows the relationship between magnesium concentration and the onset of non-linear growth with glucose or gluconate as the carbon source. The lines do not extrapolate back to the origin, suggesting some contaminating magnesium, probably present as a contaminant in the medium. Since there was little difference in the level of medium magnesium between the two carbon sources, the higher optical density obtained when gluconate was used was not due to a higher magnesium contamination.

Marked differences existed between M7 and WT in their magnesium requirement. Except at very high magnesium concentrations for example 160 µm (Figure 6) or in full CDM, M7 grew to a higher optical density than WT for the same amount of magnesium even when oxygen was limiting the growth (Figure 8). Magnesium is essential for the activity of many enzymes including those involved in ATP and in the synthesis of cell wall components such as fatty acids (Knivett & Cullen, 1967), lipopolysaccharide (Edstrom & Heath, 1967), phospholipids (White et al., 1971) and peptidoglycan (Garrett, 1969). It is also essential for ribosomal integrity and activity (McCarthy, 1962; Stanier et al., 1977) and in the synthesis of ribosomes and RNA (Cohn & Ennis, 1967) and in the stability and permeability control in membranes (Brock, 1962). There is also evidence that magnesium is a structural component of the outer membrane of

Gram-negative bacteria (Costerton et al., 1974). The difference in the requirement for magnesium between M7 and WT suggests that there may be major changes, probably involving cell membrane alteration that are associated with the mucoid type of P. aeruginosa. Differences between the two organisms in sensitivity to polymyxin (section 4.4.1.2) indicate that the organisms may have different outer membranes. A lowered requirement for magnesium may therefore be due to a decreased content of the cation in the outer membrane of M7.

Finch (1976) found a linear relationship between the reciprocal of the specific growth rate of exponential phase cells and the reciprocal of magnesium concentration for P. aeruginosa. Figure 17B shows a biphasic curve for both organisms, a pattern that resembles the relation observed for Proteus by Al-Dujaili (1979). Shehata and Marr (1971) reported a biphasic relation between the growth rate of E. coli and phosphate concentration and suggested the presence of a dual transport system for phosphate of high and low affinity.

4.1.4 Iron

Bacteria have an absolute requirement for iron, the element being a constituent of cytochromes and other heme and non-heme proteins and is a cofactor for a number of enzymes (Stanier et al., 1977). Figures 9 and 10 describe the growth curves and the relationship between initial iron concentration and the onset of non-linear growth. As iron became limiting, there was a gradual decrease in the growth rate and log-linear growth was maintained to an OD_{470} of 1.0 corresponding to 1 μM Fe^{2+}. Similar results were obtained by Boggis (1971) using P. aeruginosa. The line in Figure 10 did not pass through the origin indicating presence of iron even in pre-used medium. Atomic absorption determination showed iron contamination

in CDM to which iron has not been added of 0.05 ppm (0.05 µg/ml iron). The two organisms showed similar requirements for iron.

4.1.5 Sulphate

Sulphur is required for the amino acids cystein and methionine and as a constituent of some coenzymes such as coenzyme A, cocarboxylate, biotin, ferredoxin, lipoic acid and thiamine (Pirt, 1975; Stanier et al., 1977). Growth limitation by sulphur may decrease the synthesis of these key sulphur compounds and affect their functions in the cell.

Figures 11 and 12 describe the growth characteristics of M7 and WT under sulphate depletion. The relation between OD onset of non-linear growth and the amount of sulphate added was linear to a sulphate concentration of 7×10^{-5} M for both organisms. Within this sulphate concentration, M7 grew to a slightly higher OD onset than WT. Above 7×10^{-5} M sulphate, WT showed a higher OD onset than M7, again showing the higher requirement for oxygen by M7. Figure 17D shows that the growth rate was not affected by initial sulphate concentration except at very low substrate concentration. The value of K_s for sulphate shown in Table 5 was calculated from part b of the graph (Figure 17D) assuming that growth rate varied with sulphate concentration.

4.1.6 Phosphate

The shape of growth curves for phosphate limited cultures shown in Figure 13 is similar to that of magnesium, as phosphate became limiting, there was a progressive decrease in growth rate. In bacteria there is a stoichiometry between the amounts of magnesium, potassium, phosphate and RNA which is characteristic of different groups of bacteria (Tempest & Dicks, 1967; Tempest, 1969). The

gradual slowing of growth may be explained by the reduction in the rate of protein synthesis due to the effect of decreasing phosphorus content in the ribosomal RNA. Apart from its association with ribosomes, phosphorus is present in the cell walls of Gram-negative bacteria as a constituent of the phospholipid and lipopolysaccharide components.

Figure 14 shows the relationship between onset of non-linear growth and initial phosphate concentration. The relationship was linear to a concentration of 1.5×10^{-4} M, corresponding to an OD_{470} of 0.65 for M7. The wild type showed a linear relationship to 2.7×10^{-4} M, OD_{470} 1.0. As with sulphate and magnesium, M7 grew to a slightly higher OD onset than WT when oxygen was not limiting. Altered outer membrane, as suggested by the polymyxin sensitivities may account for this effect. Decreased LPS or phospholipid content in the outer membrane of M7 would also reduce the amount of phosphorus needed. Figure 17F shows only a slight change in growth rate with initial phosphate concentration for WT. M7 showed a growth rate increase with initial phosphate concentration.

4.1.7 Nitrogen

Nitrogen is a constituent of proteins, nucleic acids, some phospholipids and coenzymes. The rapid cessation of the growth at the end of exponential growth after exhaustion of nitrogen, indicates an essential requirement for the element during growth (Figure 15). A linear relationship was maintained between the optical density at the end of exponential growth and nitrogen concentration up to an OD_{470} of only 0.65 for M7 and 0.85 for WT (Figure 16). Although the lines did not pass through the origin, thus indicating some nitrogenous material in the medium, cells inoculated in medium without added

nitrogen did not grow. Either the nitrogenous contaminant was not usable, or growth with very low levels of nitrogen source was too low to be detected by the experimental method used. The slightly higher requirements for nitrogen shown by M7 may be for the synthesis of the extra enzymes that would be involved in exopolysaccharide synthesis.

4.1.8 Conclusion

The nutritional requirements for WT observed in this study were similar to those previously reported for P. aeruginosa (Boggis, 1971; Kenward, 1975; Finch, 1976). M7, a mucoid variant of P. aeruginosa, showed some differences in its nutritional requirements when compared to WT, its non-mucoid parent strain. The mucoid showed a lower requirement for magnesium and also a slight decrease in its requirement for sulphate and phosphate. The organism showed a higher requirement for carbon while its requirement for nitrogen was only slightly less than WT. The requirements for iron were similar for the two organisms, M7 however, showed a higher production of the fluorescent pigment under iron limitation (Table 17). Production of fluorescent siderochromes by P. aeruginosa, P. putida and P. chlororaphis under iron deficient conditions has recently been reported (Meyer & Abdullah, 1980). If the fluorescent pigment produced by M7 and WT is a siderochrome, then M7 may have an advantage over WT in acquiring iron from the medium.

The decrease in nutritional requirement by M7 was only apparent in low cell densities, that is, when oxygen was not growth limiting. When medium contained enough nutrients to support high cell densities, M7 grew to a lower optical density than WT. This effect is probably due to an effectively lowered oxygen concentration reaching the

cell as a result of hindered diffusion by the exopolysaccharide. High oxygen tension may play a part in maintaining mucoid *P. aeruginosa* in the lungs as compared to other areas in the body. The low incidence of mucoid *P. aeruginosa* in non-CF lungs however, indicates that oxygen is not the primary factor selecting for these organisms *in vivo*.

Under all conditions studied, WT had a growth rate advantage over M7.

4.2 Stability and Phenotype Selection in Batch and Chemostats

4.2.1 Stability

In batch culture, M7 was unstable in complex medium but stable in a simple salts medium. The instability of M7 in complex media confirms the findings of other workers (Martin, 1973; Zierdt & Schmidt, 1964; Govan, 1975). The fall and rise of percentage mucoid observed when M7 was subcultured in chemically defined medium, seems to indicate some degree of selection or adaptation to a more stable phenotype. Govan (1975) similarly observed a more rapid loss of mucus production after subculture in nutrient broth compared to minimal broth.

The pattern of stability of the mucoid variant in continuous culture resembled that reported by other workers (Jones et al., 1977; Evans et al., 1979). Increased stability, similar to that reported by Jones et al. (1977) was observed with sulphate, magnesium and iron limitation. These same conditions produced the least number of phenotypic variants (Table 10). In an initial set of experiments, M7 showed decreased stability under carbon, phosphate and nitrogen limitations (see also Jones et al., 1977). This instability was lost on each of several repeat experiments and a stable pattern was consistently observed. This loss of instability in chemostats as in batch in simple salts medium is interesting. Changes in stock cultures have been reported for Xanthomonas campestris (Cadmus et al., 1976). Also, since aeration increases stability (Govan et al., 1979), stability in chemostats could have been helped by the efficient aeration achievable.

As the occurrence of mutants is a matter of chance, then it might be said that during these experiments no gross loss of the

mucoid character occurred because the appropriate mutation did not take place. This possibility was tested directly when 1% and also 10% of non-mucoid cells already adapted in separate chemostats to the particular limitation and dilution rate were added to the mucoid chemostats, this large fraction of non-mucoid cells did not take over in any limitation and did not appear to effect the population significantly. Furthermore, as much as 20% or more spontaneously occurring non-mucoid revertants appeared and disappeared in the chemostats, and they did not take over either. It seems a logical conclusion that in these systems, the mucoid form has some growth advantage over the non-mucoid form.

In the simple salts medium, the growing cell had to make all its cellular components from the salts using the glucose provided as both carbon and energy source. A complex medium however, provides a rich source of amino acids and peptides which can be deaminated to supply compounds that are more readily used by the organisms. A cell can therefore grow at its maximum rate. If therefore, the non-mucoid has a higher growth rate than the mucoid cell, it would be at an advantage. The report of Govan et al. (1979) suggests that this is indeed so. This is further supported by the results presented in Figure 17A-F. In all cases, the non-mucoid showed a higher growth rate constant than the mucoid. Subcultures into fresh complex medium therefore quickly selects for the non-mucoid form. Possibly in simple salts medium this advantage is reduced. The mucoid cells therefore remain in the culture long enough to select or adopt to a more stable form. Alternatively, some compounds in complex medium may act to depress formation of polysaccharide synthesizing enzymes or completely inhibit them.

Though this may explain the instability in complex media *in vitro*, it does not explain the stability of mucoid cells *in vivo*. In the infected lung, the growth environment would approximate more to a complex rather than a simple salts medium. If however, iron is limiting in the lung in a similar way to the serum, then iron limitation might be one of the causes of selection and stability of mucoid strains *in vivo*. Iron limitation of the mucoid strain gave increased stability, and a takeover of a non-mucoid culture by mucoid cells under iron limitation has been demonstrated (Jones *et al.*, 1977).

As the culture leaves the chemostat chamber, it passes through tubes to the overflow container. Growth can occur both in the tubes and in the collection vessel, but under these conditions, efficient aeration is no longer achieved, and other conditions no longer resemble those in the chemostat chamber. The isolation of a higher proportion of non-mucoid cells from samples taken from the collection vessel (Table 7) is therefore most likely due to the takeover by the washed out non-mucoid variants which would have a greater growth advantage under these new conditions.

4.2.2 Phenotype selection

The stability described above was defined as the retention of mucoid colony appearance on nutrient agar plates. The tests performed on randomly selected colonies however, revealed that M7 was able to give several phenotypes, some indistinguishable on nutrient agar plates. But although the phenotypes showed some differences in both biochemical tests and phage types, all variants were from the same parent strain and belonged to the same serotype. Variants showing altered phage sensitivities but retaining main parent strain characters were also reported by Zierdt and Schmidt (1964).

In their course of genetic studies into the nature of bacterial mutation responsible for exopolysaccharide production, Govan (1978) reported the observation of at least five phenotype variants of *in vitro* isolated mucoid *P. aeruginosa*. The phenotypic groups were based on their cultural requirements for exopolysaccharide production and their degree of sensitivity to chemicals such as sodium deoxycholate. Intensive examination of mucoid *P. aeruginosa* from patients revealed some phenotypes and also that, whilst individual patients invariably harboured several phenotypes, the same two phenotypes predominate in all patients.

In their study Seale *et al.* (1979) were able to show a high frequency of heterogeneity among isolates from cystic fibrotic patients. They concluded that cystic fibrotic patients may be co-colonized by more than one strain of *P. aeruginosa* more frequently than initially believed. Alternatively, phenotypically distinct strains from a single patient may arise due to a continuous segregation of a single infecting strain (Zierdt & Schmidt, 1964). The ease of selection of phenotypic variants from a single phenotype observed in this study supports the latter suggestion. Different strains of *P. aeruginosa* may coincidentally be harboured by an individual at the same time, and these may give rise to several variants, thus giving rise to the multiplicity of isolates which can be obtained from cystic fibrotic patients. The variants retain the serotyping of the parent strain. Therefore, the isolation of single pyocin or serotype (Williams & Govan, 1973; Zierdt & Williams, 1975) or of two or more serotypes (Diaz *et al.*, 1970; Seale *et al.*, 1979) depends on the number of initial strain(s) infecting the patient.

There have been several contradictory reports on the sensitivity of mucoid *P. aeruginosa* to antimicrobial agents. Zierdt and Schmidt

(1964) reported mucoid strains as more sensitive to oxytetracycline, tetracycline, chloramphenicol, polymyxin and streptomycin. Cetin et al. (1965) observed no differences in susceptibility to antimicrobial agents between mucoid and non-mucoid. In contrast, several reports have suggested that mucoid strains are more resistant (Feary, 1975; Govan, 1976; Costerton, 1977; Govan & Fyfe, 1978). Using fifteen strains of mucoid P. aeruginosa and their respective spontaneous occurring isogenic non-mucoid derivatives, Markowitz et al. (1980) reported that the mucoid strain was more sensitive than the non-mucoid to most of the antibiotics they used. They none-the-less reported several mucoid strains as being more resistant than the non-mucoid variants. Whilst, as they pointed out, the increased resistance could suggest a possible role of the exopolysaccharide capsule to antibiotic resistance, it is also likely that this represented different phenotypes. Since both resistant and sensitive strains were noted as mucoid and therefore both possessed the exopolysaccharide, resistance that can be attributed to the exopolysaccharide per se is minimal. In the present study, while M7 original phenotype was more resistant to carbenicillin than the non-mucoid WT, a more sensitive, but none-the-less mucoid phenotype was frequently isolated.

The reason why mucoid strains should be more or less sensitive to antimicrobials in some instances is not clear. It is possible that, although the presence of exopolysaccharide fails to confer selective advantage to the mucoid strain in such cases, the outer and cytoplasmic membranes are more important in relation to differential antimicrobial sensitivities. This suggests therefore that several factors both environmental, genetic and structural account for the differences in antimicrobial susceptibilities

reported. It is important therefore, in testing for antimicrobial sensitivities of P. aeruginosa mucoid strains, to select multiple isolates so as to give an adequate assessment.

The variant produced from the wild type in chemostat appeared independently of limitation. Although no large difference in pigment production was observed, it seems possible that the two variants were the same as those previously reported (Shiomaya & Homma, 1968; Brown, 1977).

Small (dwarf) colony variants, usually associated with increased antibiotic resistance, have been described for a number of organisms including Pseudomonas (Voureka, 1951; Wahba & Darrell, 1965; Annear, 1976). Proteus (Al-Dujaili, 1979) and Staph. aureus (Lacey, 1968). The appearance of small colony variants in chemostat is in agreement with the report by Silman and Rogovin (1972) on increased frequency of isolation of a small colony variant in nitrogen limited Xanthomonas campestris. They observed that the viable count decreased and increased as the small colony cell population changed. The small colony eventually took over in the chemostat and their appearance resulted in decreased culture viscosity. The accumulation of small colony variant in chemostat grown Proteus strains, was minimized by addition of 0.4 M KCl in the growth medium (Al-Dujaili, 1979). The high salt concentration was found to inhibit growth of the small colony variants. The small colony present in this study may have a selective advantage over large colony type cells when grown under chemostat growth conditions. Probably the stringent conditions imposed on the cells when grown in chemostats may encourage the growth of this otherwise uncommon variant.

4.3 Production of Extracellular Products Under Nutrient Limitations

4.3.1 Exopolysaccharide

The production of exopolysaccharide by mucoid (M7) P. aeruginosa (Table 11) shows a variation dependent on limitation, with phosphate, nitrogen and iron limitations resulting in relatively high yields and sulphate and magnesium showing low yields. Perhaps the most interesting observation is the reduction in production with time. A rapid loss of exopolysaccharide production in continuous culture has been reported by other workers. Silman and Rogovin (1972) using Xanthomonas campestris reported reduction of amount of exopolysaccharide after 'Q' values of 6.5 - 8.7, where Q value = 1 was defined as a volume of feed equal to the fermenter culture volume. Similarly Evans et al. (1979) using Xanthomonas juglandis observed a high acetone precipitable material for five days at $D = 0.03\ hr^{-1}$ which thereafter drastically decreased. In both cases, there was no evidence of contamination but colonial variants were apparent. Although no clear variant that could be specifically associated with fall in exopolysaccharide production was observed in this study, it is most likely that the organism became adapted to the conditions in the chemostats. Production was therefore greatest at the end of batch growth starting the chemostat study.

Phosphorus is involved in ribonucleic acid and cell envelope structure and function and since it is negatively charged, its limitation would be expected to result in less formation of the negatively charged exopolysaccharide. Neijssel and Tempest (1975) found less exopolysaccharide was found under P-lim compared to N-lim, and Williams and Wimpenny (1978) observed no increase in polysaccharide synthesis under phosphate limitation. Phosphate limitation of M7

resulted in highest formation of the exopolysaccharide. This organism therefore behaved very much like Xanthomonas juglandis (Evans et al., 1979) and Aerobacter aerogenes (Duguid & Wilkinson, 1953) both of which showed increased yield with phosphate limitation.

The formation of large amounts of exopolysaccharide when nitrogen was limiting is in agreement with previous reports (Williams & Wimpenny, 1978; Mian et al., 1978) that a high carbon : nitrogen ratio promotes a high exopolysaccharide yield. Probably the regulation involving nitrogen in exopolysaccharide synthesis is a common one.

Exopolysaccharide yield was minimal under magnesium and sulphate limitations. Magnesium is involved in ribosome and membrane structure, and plays an important role in controlling enzyme activity (Stanier et al., 1977). The relatively lower exopolysaccharide yield may be due to reduced level of synthesizing enzymes. Magnesium, calcium and potassium have been shown to be essential for maximal synthesis of polysaccharide (Tempest et al., 1965; Williams & Wimpenny, 1977; Sutherland, 1977). Elevated levels of cations Na^{+} and Mg^{2+} were found to stimulate the production of exopolysaccharide by P. aeruginosa isolated from cystic fibrotic patients (Costerton et al., 1979). In this context, the elevated levels of potassium, sodium and calcium ions in the secretions of cystic fibrotic patients (Wood et al., 1976) probably plays a role in the maintenance of the mucoid character.

Restriction of an organism's sulphur supply may modify the protein composition of the envelope, for example by reducing the membrane -SH groups (Pardee, 1968) or changing the total protein content (Robinson & Tempest, 1973). Sulphate limitation may also

cause a reduction in the synthesis of sulphur containing essential components of the cell such as coenzyme A, thiamine pyrophosphate and lipoic acid. It has also been observed to cause a release of protein (Neijssel & Tempest, 1975). Increased exopolysaccharide with sulphur limitation might result from increased enzyme release because of increased membrane permeability. Reduced oxidation efficiency due to enzyme restriction might leave excess glucose available for exopolysaccharide. Such an increase was reported by both Evans et al. (1979) and Markovitz and Sylvan (1962). But, as Markovitz and Sylvan (1962) observed, the effect of sulphate ion differed with different bacteria. In some bacteria, synthesis of polysaccharides containing uronic acids was inhibited by increase in concentration of sulphate ion while in others it was stimulated. If, in this organism, the synthesis of enzymes necessary for polysaccharide synthesis or their activity and release is affected by sulphate limitation, the result may be a decreased production of exopolysaccharide.

The effect of iron in the production of extracellular products is very dependent on the iron concentration (Weinberg, 1970) and its effect on exopolysaccharide may be affected in a similar manner. In this study, iron limitation of M7 resulted in an enhanced exopolysaccharide production. Iron contaminates most of the chemicals used in preparation of media and the exact amount is usually difficult to determine. In addition, organisms require only small amounts of iron (2×10^{-7} M) and some have developed mechanisms of scavenging for iron (Weinberg, 1970, 1978). The controversies on the effect of iron limitation on production of polysaccharide probably arise from strain differences and different concentrations of iron in culture media used.

It has been suggested that in K. aerogenes, exopolysaccharide is produced as an overflow metabolite (Neijssel & Tempest, 1975, 1976). M7 however, yielded, under carbon limitation, exopolysaccharide amounts that were similar to those produced by unlimited (oxygen limited) conditions. Since under this condition carbon would not be in excess, production of exopolysaccharide suggests that, in this organism, the macromolecule is unlikely to be an overflow metabolite.

Since bacterial polysaccharide synthesis shares common precursors and cofactors with cell wall biosynthesis (Sutherland, 1977), its synthesis would be expected to decrease with increase in growth rate. This pattern was observed by Williams and Wimpenny (1980). They suggested that the decreased synthesis of exopolysaccharide at higher dilution rates was due to the favouring of essential cell polysaccharide synthesis. The formation of the same amount of exopolysaccharide at slow and fast growth rates shown by iron limited M7 suggests that probably in this bacteria, competition with cell wall synthesis for precursors and cofactors does not greatly influence exopolysaccharide synthesis. An increased rate of alginate synthesis with specific growth rate in P. aeruginosa has previously been reported (Mian et al., 1978).

4.3.2 Extracellular enzymes

4.3.2.1 Effect of nutrient limitation

The extracellular products studied are secondary metabolites and the relationship between iron and phosphate in secondary metabolism is well known (Leisinger & Margraff, 1979). It is not unexpected therefore to find that iron and phosphate limited cultures produce relatively large amounts of all

four enzymes (Tables 12 - 15). The production of extracellular enzymes, with the exception of phospholipase C, was very low in batch cultures. This was in agreement with the reports of previous workers (Liu, 1964; DeBell, 1979) who found they had to add amino acids to their simple salts medium to enhance production of the virulence factors. Some of the results obtained in continuous culture confirm the results obtained in batch cultures. Total protease production was high under carbon, iron, phosphate and nitrogen limitation. This was in agreement with the results of Liu (1964, carbon, nitrogen and phosphate) and Bjorn (1979, iron). The proteases however, showed little elastase activity. As with the results of Stinson and Hayden (1979), phospholipase C production was low under conditions of carbon and magnesium limitations and high under phosphate limitation. If exopolysaccharide enhanced lipase production as suggested by Bohne and Winkler (1979), this was not obvious in these results. This may, however, be due to the preparation method as described below.

Although no clear pattern emerged linking the activity or non-activity of all these extracellular products in any one culture, the results under phosphate and magnesium are interesting. Hou et al. (1966) found that when P. aeruginosa was phosphate starved, RNA content decreased and ribosomal degradation was evident. They suggested that ribosomal material was the primary endogenous reserve in P. aeruginosa. This could satisfy both maintenance energy of the cell and provide essential metabolites like phosphate. RNA degradation would occur when cell growth had ceased as the requirement of a large complement of protein synthesizing enzymes is removed. But since secondary metabolism is stimulated by phosphate limitation, it appears that this synthesis is closely

linked to the ribosome degradation and cessation of primary protein synthesis occurring under phosphate limitation. Magnesium is needed in ribosomes and has been shown to vary with RNA (Tempest, 1970). It would be expected therefore that absence of magnesium would result in less ribosomes and therefore less of the non-essential enzymes. As the extracellular enzymes are not essential for growth, less would be expected under magnesium limitation. At higher dilution rates where there is a higher need for magnesium (Finch, 1976), the amount of exoenzyme would be expected to be even less. This is the general pattern shown in this study. With very few exceptions, magnesium limitation resulted in the lowest production of extracellular products. Therefore, even though the two nutrients, magnesium and phosphate, lead to decreased ribosome content, their effect on extracellular products is very different.

Apart from results that were in agreement with previous reports, the production of extracellular enzymes shows that nutrient limitation and cell type are independent variables affecting their synthesis. It is possible that all enzymes present in growing organisms are regulated by the intracellular level of 'pool' metabolites whose concentration are in turn prescribed by their rates of synthesis and utilization as suggested by Vogel (1957). Under nutrient limitation, intracellular 'pool' of growth limiting nutrient is very low, therefore enzyme production by the organism will differ depending on the effect of low levels of 'pool' metabolites.

4.3.2.2 Mucoid versus non-mcuoid

Large differences existed between the mucoid and non-mucoid cell types, with M7 generally showing less enzyme activity in

culture supernatants although no consistent pattern was obtained. A similar complex picture was obtained when culture supernatant of both smooth and mucoid P. aeruginosa were assayed for protease activity (Klinger et al., 1979). Protease activity varied over a 100 fold range against hide powder azure and 6.5 - 9 fold against orcein-elastin. They, too, found no consistent differences between smooth and mucoid strain in any of their assays. It is attempting to try to guess at the possible role of the polysaccharide in the functioning of these enzymes. It seems possible that the polysaccharide and enzymes will bind together and not release the enzyme free into the environment. The binding by polysaccharide of the enzymes would make a larger concentration of the enzyme to be in contact with the infected tissue. This would be an advantage to the invading organism. The pronounced antibody response observed in patients with mucoid P. aeruginosa (Hoiby, 1977) has suggested active tissue damage in the lungs. This may be a result of the concentration, by the exopolysaccharide, of virulence factors around the mucoid cell.

If the observed low levels of extracellular enzymes in culture supernatants is due to binding to polysaccharide, one might expect the two organisms to show similar activities of cell bound enzymes when grown under the same condition. To test this, cells were sonicated and the activity of cell lysate measured. In this case, with very few exceptions, M7 showed a higher activity than the wild type for all extracellular proteins and under all nutrient limitations. It would appear therefore that the mucoid type produced an equal or higher exoprotein than the wild type, but this was not released into the medium.

The method of preparing supernatant used in this study involves the removal of any polysaccharide by gelling with calcium chloride. If the polysaccharide does bind significantly to some of these enzymes, then this treatment would lead to an apparently low yield from mucoid cells. In general this appears to be the case in these experiments. The exceptions could possibly be explained by suggesting that different limitations produce different polysaccharide forms which have different affinities for the exoenzymes.

Apart from the binding of released exoenzymes to exopolysaccharide, the higher levels of cell bound exoenzymes in mucoid cells might be due to changes in cell wall resulting in exoenzyme being held in the periplasm. It has been suggested that exoenzyme synthesis occurs on polysomes associated with the cytoplasmic membrane (Boethling, 1975). The exoprotein has then to be transported from its site of synthesis to an exterior location and released in active or in some cases, in an inactive form. Association of enzymes with the cell envelope was reported for Serratia marcescens (Heller, 1979) and P. aeruginosa (Jensen et al., 1980b). It is essential therefore that the structure and function of the membrane be maintained. However, changes in envelope affecting enzyme release have been observed. In their study, Wretlind et al. (1977) obtained mutants of P. aeruginosa that had essentially the same character as the wild type in respect to growth and some antibiotic sensitivities but which had altered cell envelope and structure. These had a decreased ability to release extracellular proteins and revealed significant cell bound protease and staphylolytic activities. A similar study (Fernandes & Cuddy, 1980) demonstrated a cell envelope bound exotoxin A in a mutant of P. aeruginosa.

This mutant was avirulent, highly resistant to polymyxin B and all exotoxin A was in a membrane bound state. These results have suggested that the cell envelope of P. aeruginosa may be important in the release of virulence factors. Disturbance in the physiological balance of the organism may cause changes in membrane components and thus affect the fluidity of membrane and the transport and release of membrane bound factors. It is well known that nutrient limitation causes changes in the cell membrane (Tempest & Ellwood, 1969; Finch & Brown, 1975; Gilbert & Brown, 1978b). The membrane changes brought by nutrient limitation, together with changes that may have occurred during the change of wild type non-mucoid state to mucoid state may contribute to the complexity observed in the production of the extracellular enzymes.

Furthermore, metal ions function in microbial metabolism principally as activators of various enzymes whose production may be needed by the cell for further use. The balance of such ions may therefore be crucial in the cell's build-up and in the synthesis and release of either or both intracellular and extracellular products. Environmental factors can make the requirement of microorganisms for the metal ions vary both qualitatively and quantitatively. Changes may include partial or complete functional replacement of one ion by another (Kenward et al., 1979), enhanced requirement of one ion in the presence or absence of another (Meers & Tempest, 1970) or presence of complex organic compounds that are capable of acting as chelating agents (Weinberg, 1978). The effect of mineral limitation on the production of extracellular products is therefore likely to be intricately related to other factors and the pattern shown by one organism under various conditions may differ markedly with another organism.

4.3.3 Pigments

The enhanced production of pyocyanine under phosphate limitation and under iron limitation (Table 16) confirms the previous reports on the dependence of pigment formation on these two elements (Leisinger & Margraff, 1979). A relatively low amount of pyocyanine was produced by the wild type under all other limitations, with nitrogen limitation showing no apparent pigment. The mucoid produced significant pyocyanine only under phosphate limitation. Although the exact roles are not known, minerals are known to be required for pigment formation (Georgia & Poe, 1931; King et al., 1948; Burton et al., 1948; Chakrabarty & Roy, 1964). The decreased production of pyocyanine under nutrient limitation confirms these results.

The production of pyocyanine, an antimicrobial agent, may have an advantage for the producing cell in inhibiting competing microorganisms. Thus phosphate limited cultures may be at a distinct advantage in a mixed culture.

The production of the fluorescent pigment (Table 17) showed no consistent pattern. In chemostats, at the lower dilution rate, C-lim cultures produced higher levels of the pigment than F-lim cultures. At the higher dilution rate, the amount under C-lim was markedly reduced, and was less than that produced under iron limitation. M7, N-lim cultures also produced higher levels than F-lim at both dilution rates. In addition, N-lim M7 produced about ten times more pigment than N-lim WT. The production of the pigment under iron limitation in chemostats was higher for WT. In batch, relatively lower amounts of the pigment were produced compared to chemostat cultures. The highest level was produced by iron limited cells for both organisms, but unlike chemostats, M7 produced more than WT.

The production of an iron chelator pyoverdine and related siderophores would obviously have an advantage for iron limited cultures. Why other cultures should produce such large quantities is unknown. Williams and Wimpenny (1977) observed formation of yellow green pigment at high carbon : nitrogen ratio, but whether this pigment was fluorescent was not determined. P. aeruginosa has been shown to elaborate different types of phenazine pigments under different growth conditions (Kanner et al., 1978; Ingram & Blackwood, 1970). The organism also gives different types of pyoverdines which show different absorbances and solubilities in organic solvents (Palumbo, 1972, 1973; Lui & Shokrani, 1978). It is likely therefore that different strains under different growth conditions will produce different pyoverdines in varying proportions. The fluorescent pigment produced under other nutrient limitations might therefore not be identical to that produced under iron limitation. Furthermore, the shifts in wavelengths observed for magnesium and phosphate limitations indicate the likely elaboration of different types of pyoverdine$_{Pa}$, supporting the results of Palumbo (1972, 1973). Meyer and Abdullah (1978) showed that absorption peaks of pyoverdine$_{Pf}$ were shifted down in an alkaline pH. The media used in this study under all conditions was maintained between 7.6 - 7.8 and therefore the shift for magnesium and phosphate cannot be accounted for by this factor. Degradation products were also shown to have different absorption spectra (Meyer & Abdullah, 1978). If the changes absorption is due to degradation products, then the pyoverdines produced under these limitations are less stable than those produced under other nutrient limitations. In view of these phenotypic changes in the fluorescent pigment produced by P. aeruginosa, it would seem that

the designation of the name pyoverdine$_{Pa}$ is inadequate.

4.3.4 Effect of dilution rate on production of exoproteins and pigments

Protease, elastase and lipase activities fell with increase in growth rate while the reverse was true for phospholipase C. Pyocyanine production also fell with increase in dilution rate and the production of fluorescent pigment showed varying changes depending on the limiting nutrient.

In batch, secondary metabolism begins at the end of the logarithmic phase and after some time stops though the cell remains viable. In continuous culture, since there is no 'late log', secondary metabolism would not occur if balanced growth is continuously maintained. If the dilution rate is lowered, the culture can shift and growth of most cells would resemble 'late log' growth and therefore secondary metabolism can occur (Weinberg, 1970). One would therefore expect the extracellular products to decrease with increasing growth rate, assuming no inducer-catabolite factor is involved. This seems to have been the case, in most cases, in the production of protease, elastase, lipase and pyocyanine.

Phospholipase C production seems to behave as if this was a primary metabolite or a constitutive enzyme, showing increased activity at the higher dilution rate. Enzyme levels were also high in batch cultures. In batch culture, cells grow at a fast rate while in the logarithmic phase, although the environemnt is constantly changing. If phospholipase C is produced by growing cells as suggested by the chemostat results, then one would expect relatively high levels at the end of the logarithmic phase in batch. Stinson and Hayden (1979) observed the secretion of

phospholipase C during late log and early stationary phase. They suggested that the delay in synthesis was due to repression by a component in the culture medium. The present results suggest that when unrepressed, phospholipase C synthesis occurs during logarithmic phase. At low dilution rates, cell division is very slow and consequently, phospholipase C production would be low.

The production of low levels of phospholipase C at slow growth rates may be of particular advantage to mucoid P. aeruginosa growing in the lung. Govan (1975) showed that naturally occurring mucoid forms of P. aeruginosa have increased stability on media containing a number of different surfactants. He put forward the suggestion that the surfactants found in the lung may serve to stabilize the mucoid forms. If this is the case, then over-production of phospholipase C which could destroy these potential surfactants would be a distinct disadvantage. In many infections bacteria eventually have quite slow in vivo growth rates and it follows that in this case it may be advantageous to produce phospholipase C in low levels.

Wiersma and Harder (1978) have shown a strain of Vibrio to have an optimum growth rate for protease production. The production of amidase by P. aeruginosa also had an optimum growth rate (Clarke et al., 1968). It seems possible that a similar relationship between growth rate and extracellular enzyme activity might also exist with P. aeruginosa. This however cannot be determined using only two growth rates.

4.4 Resistance to Antimicrobial Agents and Killing by Blood of M7 and WT

4.4.1 Antimicrobial agents

4.4.1.1 Mode of action of polymyxin and EDTA

The polymyxins are a group of peptide antibiotics characterized by a cyclic heptapeptide ring bearing a tripeptide side chain with a terminal fatty acid. Polymyxin B sulphate contains 6-methyloctanoic acid as the terminal fatty acid (Storm et al., 1977). Gram-negative bacteria are generally more susceptible to polymyxin than Gram-positive, although Proteus species and Serratia marcescens are naturally resistant (Russell, 1963; Sud & Feingold, 1970). The antibiotics are bacteriostatic at low concentration and bactericidal at higher concentrations (Storm et al., 1977).

Polymyxin affects a wide variety of biochemical processes in bacteria which include selective membrane permeability, respiration, nucleic acid and protein synthesis, transport phenomena, synthesis of LPS and peptidoglycan and specific enzyme activity (Teuber, 1974). The primary site of action of polymyxin is probably at the bacterial cytoplasmic membrane (Few, 1955; Newton, 1956; Feingold et al., 1974; Teuber, 1974), although initial binding to the cell appears to be the LPS and PL of outer membrane of Gram-negative bacteria (Few, 1955; Bader & Teuber, 1973). Polymyxin causes rapid permeability changes on the cytoplasmic membrane (Teuber, 1974). The inhibition of growth and respiration are most likely secondary to the permeability changes as polymyxin can cause these effects without entering the cell (LaPorte et al., 1977). A summarised sequence of events of polymyxin action consists of initial binding to LPS of the outer membrane thus disrupting its permeability barrier

and causing leakage of periplasmic enzymes (Teuber, 1970; Cerny & Tueber, 1971); reaching and binding to the cytoplasmic membrane phospholipids with subsequent permeability changes, followed by inhibition of the biochemical processes of the cell.

Ethylenediaminetetraacetic acid (EDTA) is a tetra basic organic molecule which specifically chelates metal cations. The lytic action of EDTA is thought to result from the removal of the divalent cations Mg^{2+} and Ca^{2+} from the outer membrane (Eagon et al., 1975; Wilkinson, 1975). There is evidence that Mg^{2+} plays an important role in the outer membrane by cross-linking with anionic polymers and this is particularly important in P. aeruginosa (Asbell & Eagon, 1966; Wilkinson, 1975). The high level of phosphorus in LPS of P. aeruginosa is thought to allow for considerable metal binding, and may explain the high sensitivity of these organisms to EDTA. Removal of structural Mg^{2+} by EDTA would disrupt the outer membrane due to repulsion between the negatively charged components. Resistance to EDTA due to magnesium limitation has been postulated as due to replacement of magnesium by organic cations (Wilkinson, 1975) or protein (Nicas & Hancock, 1980) which are insensitive to EDTA.

4.4.1.2 Effect of nutrient limitation on sensitivity to polymyxin

The effect of polymyxin on nutrient limited M7 and WT is shown in Figures 27 - 31. Magnesium limited cells showed the highest resistance to polymyxin at all dilution rates. Between the two organisms, M7 was more resistant than WT (Figures 28 - 31). The extreme resistance of M-lim cultures is to be expected as the development of resistance with magnesium depletion is well recorded (Brown & Melling, 1969a, 1969b; Boggis, 1971; Gilleland et al., 1974;

Finch & Brown, 1975; Kenward, 1975; Dean et al., 1975). The present study is therefore in agreement with previous reports that magnesium limited P. aeruginosa is resistant to EDTA and polymyxin while C-lim cells are sensitive to these agents (Melling et al., 1974; Finch & Brown, 1975). In contrast to the results of Boggis (1971), P-lim cells were sensitive to polymyxin. The present results are however compatible with the results of Melling et al. (1964) who observed increased sensitivity of P-lim P. aeruginosa to polymyxin. Phosphorus is a major component of PL and LPS and therefore changes in either , would follow P-depletion and be likely to affect sensitivity to polymyxin. The sensitivity of P-lim P. aeruginosa was found to increase with increase in phosphate concentration in the medium to an optimum level, above which the cells became resistant (Noy, personal communication). The contradictory results obtained are probably due to differences in phosphate content in the media used. P-lim cells were shown to be sensitive to the resuspending medium used (section 3.5.1) and Tsai et al. (1979) reported a decreased cell viability and abnormal structure in P-lim Azolobacter vinelandii. Similar changes in P. aeruginosa would enhance sensitivity to polymyxin.

Sulphate limited cells also showed a relatively high resistance to polymyxin, M7 being more resistant than WT. Klemperer (1977) observed that progressive sulphate depletion of P. aeruginosa during transition from exponential growth to stationary phase in simple salts medium resulted in progressive increase in polymyxin resistance. Glucose depletion of resistant sulphate-depleted cells was followed by restoration of sensitivity. Morphological changes under sulphate limitation have been observed for E. coli (Poole & Haddock, 1975), M. bovis (Spitznagel & Sharp, 1959) and Klebsiella aerogenes (Robinson

& Tempest, 1973). In the latter case, slow growing S-lim cells were smaller and more round than faster growing cells or C-lim cells.

Iron and nitrogen limited cells were the most sensitive, the difference being more pronounced at faster dilution rates (Figures 28 - 30). In contrast to Mg-lim and S-lim cells, M7 was more sensitive than WT to polymyxin although in batch (Cozens, personal communication) and at the lowest dilution rate (Figure 27), F-lim M7 was more resistant. Nitrogen is essential for protein synthesis and iron is required for the many cofactors of enzymes with redox function in the cell. The restriction of their supply can therefore cause an alteration in enzyme synthesis and function in the cell, resulting in altered microbial metabolism, structure and function (Light, 1972; Light & Clegg, 1974; Sussman, 1974; Pirt, 1975). Both iron and sulphate limitation have been shown to cause a reduction in the iron-sulphur proteins of the electron transport chain and impair energy conservation (Light & Garland, 1971; Clegg & Garland, 1971; Light, 1972; Poole & Haddock, 1975). Morphological changes due to iron deficiency were observed in M. smegmatis and E. coli, where the cells had filamentous growth (Winder & O'Hara, 1962; Ratledge & Winder, 1964). These changes were attributed to DNA synthesis inhibition or direct effect on cell division and separation.

The response of the cells to polymyxin in this study showed day to day variations, probably due to the inactivating procedure. Egg yolk lecithin was used as the inactivator and it is possible that different batches varied in their affinity for polymyxin. The differently limited cells were therefore always studied on the same day with the same batch of quenching medium, but this made it difficult to compare the effect of dilution rate on polymyxin sensitivity.

4.4.1.3 Effect of nutrient limitation on sensitivity to EDTA and carbenicillin

Melling et al. (1974) studied the effect of dilution rate on the sensitivity of Mg-lim and C-lim P. aeruginosa to EDTA, streptomycin, gentamicin, tetracycline, carbenicillin and actinomycin. Mg-lim cells were more resistant than C-lim cells to EDTA and gentamicin. There was no difference in their sensitivity to carbenicillin or actinomycin. Differences in sensitivity to tetracycline occurred at higher dilution rates. Both types of cells showed increased sensitivity to EDTA and gentamicin as dilution rate was increased. Finch and Brown (1975) also observed an increase in sensitivity by C-lim cells of P. aeruginosa to EDTA with increase in dilution rate, but Mg-lim showed complete resistance at dilution rates between 0.05 and 0.6 hr^{-1}. Figure 26 shows the percent viable count of EDTA treated cells after 80 min contact. There were no marked differences between the two organisms, the different nutrient limitations or the growth rates except for iron limited cultures. The sensitivity to carbenicillin increased with increase in dilution rate (Figure 25). The mucoid variant was more resistant to carbenicillin at low dilution rates. A higher resistance to carbenicillin would be expected since this is the basis by which mucoid variants can be isolated in vitro (Govan, 1976; Govan & Fyfe, 1978).

Fe-lim cells showed the highest sensitivity to EDTA at a dilution rate between 0.1 and 0.2 hr^{-1} (Figure 26 and Table 19). This was also the dilution rate at which Fe-lim cells were most resistant to killing by whole blood (Figure 41). The sensitivity to drugs has been correlated with outer membrane components such as LPS (Gilbert & Brown, 1978b) and membrane cations (Kenward et al.,

1979), and the growth rate has been shown to affect membrane composition (Gilbert & Brown, 1978b; Carson et al., 1979). Iron limitation may affect the amount of a particular outer membrane component which changes with dilution rate. At a particular dilution rate, the proportion of the component may be such that the cells show an optimum resistance or sensitivity to some antimicrobial agents. No chemical data for Fe-lim cells was available but it seems reasonable that such an analysis may reveal whether an iron-dependent component which shows an optimum value at dilution rates between 0.1 and 0.2 exists.

4.4.1.4 Conclusion

For polymyxin to exert its primary action at the cytoplasmic membrane, it must first overcome the permeability barrier of the outer membrane. It must therefore be able to interact with the LPS or protein of the external surface. LaPorte et al. (1977), using polymyxin impregnated agarase beads, has shown that polymyxin can cause cell death by interacting with the outer membrane. Therefore the uptake and absorption of polymyxin at this site plays a major role in the resistance mechanism and changes in polymyxin sensitivity are likely to be due to altered outer membrane.

M7 and WT showed differences in resistance to polymyxin both between limitations and between the two organisms. The differences presented seem to point to some differences in the cell envelope structure which may account for changed drug sensitivities and also influence the release of extracellular products. If the polysaccharide plays a part in determination of sensitivity to antibiotics, then it probably does so by binding the antibiotic and hence preventing its access to the cell. In the present study, no effect could be attributed to the exopolysaccharide per se.

Some correlations have been made between changes in envelope composition under different growth conditions and microbial sensitivities to antimicrobial agents. Magnesium depletion has been shown to affect the ultrastructure of the cell envelope of P. aeruginosa (Gilleland et al., 1974) and chemical composition (Gilleland et al., 1974; Gilbert & Brown, 1978b; Nicas & Hancock, 1980). The envelope composition of P. aeruginosa grown in chemostats under P-, C- and Mg-limitations showed variations in the amount of ethanolamine, phosphorus, KDO, hexose and magnesium (Robinson et al., 1974; Gilbert & Brown, 1978b). There was a significantly lower phosphorus level in P-lim cell envelopes and lower hexose level in C- and P-lim envelopes. Lack of phosphorus and glucose in the polysaccharide portion of LPS was correlated with increased sensitivity to novobiocin and spiromycin (Tamaki & Matsuhashi, 1973).

Changes in cell envelope proteins have been associated with changed drug sensitivities (Gilleland & Lyle, 1979; Nikaido & Nakae, 1979; Nicas & Hancock, 1980). The amount and composition of envelope proteins has been shown to vary under sulphate limitation (Robinson & Tempest, 1973). At low dilution rates, S-lim cells had lower protein than C-lim cells ~~and~~ in the envelope and soluble proteins of S-lim cells had lower sulphur than C-lim cells. Under ammonium limitation, cell protein content decreased (Light, 1972) and nitrogen assimilation enzymes are synthesized (Brown, 1976).

4.4.2 Killing by whole blood

In batch culture (Figures 32 - 33), M7 was more resistant to the bactericidal effect of blood than WT when the organisms were grown in fully adequate medium. The sensitivity was enhanced at

temperatures higher than 37°C and decreased when the temperature was below 37°C (Figure 33). The higher resistance of M7 confirms previous reports that mucoid forms of P. aeruginosa were more resistant to phagocytosis (Schwartzmann & Boring, 1971; Costerton et al., 1978), the resistance being attributed to the exopolysaccharide. Since phagocytosis of bacteria by PMN is an active energy requiring process, it would be expected to be temperature dependent. An increased bactericidal capacity of PMN at 40°C relative to 37°C has been observed with E. coli, S. typhimurium, Listeria monocytogenes but not with Staph. aureus (Ellingson & Clark, 1942; Mandell, 1975; Roberts & Steigbigel, 1977). In infection there is usually elevated temperature and host resistance may be increased by enhancement of the bactericidal action of blood against certain organisms (Roberts, 1979).

Under nutrient limitation, both in batch (Figure 34) and in chemostats (Figures 35 - 37), the difference between M7 and WT was diminished. The pattern of resistance differed between batch and chemostat grown cultures. In batch, the percentage survivor after 30 min was least under nitrogen limitation. In chemostats, at all dilution rates studied, Mg-lim cells were most sensitive while N- and Fe-lim cells were resistant. The reasons for the differences between batch and chemostat cells are unclear.

Exopolysaccharide production by M7 was reduced under Mg- and S-lim and enhanced when cells were N- and Fe-lim. This is unlikely to explain the increased sensitivity of Mg-lim and S-lim cells to whole blood since the same order was shown by WT which did not form exopolysaccharide under any limitation. The differences therefore suggest other phenotypic changes induced by the different limitations. One mechanism that may be responsible for the

variation in susceptibility may be the possession or loss of a surface antigen which can inhibit opsonization and phagocytosis. Jones *et al.* (1972) reported that *M. pneumoniae* was more readily phagocytosed as a result of trypsin treatment and suggested that this may be due to a loss of some surface component which inhibited phagocytosis. Furthermore, specific nutrient limitations may change the hydrophobicity of the cell and hence affect phagocytosis (van Oss *et al.*, 1975).

Finch and Brown (1978) observed that slow growing Mg-lim cells were significantly more resistant to the lethal effect of phagocytes than were fast growing Mg-lim or C-lim cells (D = 0.05 and 0.5 hr^{-1}). Resistance of Mg-lim cells to killing by cationic proteins (obtained from PMN's) was growth dependent, the slowest showing the highest resistance. C-lim cells were sensitive to cationic proteins at all growth rates. Antisera raised in rabbit to C-lim and Mg-lim cells grown at fast and slow dilution rates and commercial anti-Pseudomonas serum rapidly agglutinated Mg-lim cells but failed to agglutinate C-lim cells after 1 min contact.

The differences in our results may be due to our test methods. In using whole blood, one mimics *in vivo* conditions by exposing the organisms to all components it encounters in the blood. This, however, has the disadvantage in that no single factor can be attributed to the observed effect. Young and Armstrong (1972) and Young (1974) deduced that for *P. aeruginosa*, the principal role of serum antibody appears to be the opsonization prior to phagocytosis and that the antibody present in normal human serum has an obligatory requirement for complement and other heat labile factors. Phenotypic changes may affect the opsonization stage which would affect the response of the cells to human blood but not to killing by cationic proteins.

Both LPS and divalent cations have been shown to have a marked effect on phagocytosis and the action of cationic proteins within the phagocyte (Friedberg & Shibo, 1970; Rosenthal et al., 1977; Odeberg & Olsson, 1975). Furthermore, Mg-lim cells were reported to have increased LPS content (Dean et al., 1976; Gilbert & Brown, 1978b) and this would explain the high agglutination and increased resistance of Mg-lim cells as observed by Finch and Brown (1978). The present results suggest that other factors such as opsonins in human blood or an antigen on cell surface play a significant role in determining sensitivity in vivo. It is interesting to note that the order of sensitivity to blood is the exact reversal of that seen when cells were treated with polymyxin.

Figures 39 - 41 show the effect of dilution rate on the sensitivity of iron limited cells to whole blood. As the dilution rate was increased from c 0.02 to c 0.1, the cells became more resistant. Further increases in dilution rate were accompanied by increasing sensitivity. At $D \simeq 0.35$, the sensitivity was similar to cells grown at $D \simeq 0.02$ hr^{-1} and the dilution rate showing highest resistance was approximately the same as that showing highest sensitivity to EDTA (Figure 26 and Table 19). The pattern of sensitivity to blood was reproducible in repeat experiments although absolute values of survivors varied from day to day. Variations would be expected as a result of blood variations (Alexander & Wixson, 1970).

An optimum dilution rate suggests an outer membrane composition containing optimum proportion of components that probably vary with growth rate, as discussed in section 4.4.1.3. In vivo, organisms are likely to be limited by iron (Bullen et al., 1974; Weinberg, 1978) and the growth rate is very slow, the order of

one division every 20 - 24 hr (Maynell & Subbiah, 1963; Eudy & Burrows, 1973). A dilution rate of 0.1 hr^{-1} would correspond to a doubling time of c 7 hr, which may be achieved *in vivo* near the start of an infection. An increased resistance at the beginning of an infection where the microbial population is low, may be of an advantage. The withdrawal of iron from circulation shown by the body may be an attempt to reduce the growth rate and hence increase the sensitivity of the microorganism to phagocytosis.

In conclusion, the marked variations, both in the production of extracellular products and in sensitivity to antimicrobial agents, illustrate the importance of defined cultural conditions in studying the behaviour of microorganisms.

Appendix 1

Effect of tetracycline and carbenicillin on nutrient limited M7 and WT (D = 0.05 hr^{-1})

Colony counts (% of initial)

LIMITING NUTRIENT	ORGANISM	TETRACYCLINE 25 µg/ml				CARBENICILLIN 70 µg/ml				CONTROL			
		1½*	3	4½	6	1½	3	4½	6	1½	3	4½	6
C	M7	73	98	119	176	129	171	230	319	131	221	621	1466
	WT	43	-	65	78	104	98	127	55	106	-	412	1262
P	M7	63	100	147	203	130	217	273	365	115	223	375	905
	WT	109	146	260	411	96	128	156	88	112	291	528	2138
N	M7	61	57	97	135	122	170	227	250	145	299	628	1534
	WT	41	35	71	142	127	116	139	57	135	318	702	2012
Fe	M7	28	43	59	102	120	155	315	700	99	175	460	920
	WT	27	22	22	14	127	119	124	146	133	200	509	903
S	M7	25	26	35	53	93	181	234	354	79	191	409	1091
	WT	29	34	37	33	178	150	142	71	108	167	608	1431
Mg	M7	83	163	214	358	113	185	296	318	89	198	439	780
	WT	71	54	103	147	109	123	127	127	123	211	448	848

*Time of contact in hr.

Appendix 2

Effect of tetracycline and carbenicillin on nutrient limited M7 and WT ($D = 0.10\ hr^{-1}$)

Colony counts (% of initial)

LIMITING NUTRIENT	ORGANISM	TETRACYCLINE 25 µg/ml				CARBENICILLIN 70 µg/ml				CONTROL			
		1½*	3	4½	6	1½	3	4½	6	1½	3	4½	6
C	M7	12	17	25	74	51	31	63	92	164	356	1310	3413
	WT	43	70	108	256	149	ND	150	ND	152	221	1171	2624
P	M7	55	84	177	403	39	39	147	97	134	293	1089	2439
	WT	86	107	176	395	74	33	80	ND	107	137	266	922
N	M7	62	97	156	553	113	58	74	21	180	262	747	1267
	WT	50	52	72	221	112	57	12	34	128	263	1020	2595
Fe	M7	53	160	27	38	54	75	77	61	104	161	302	821
	WT	58	56	71	185	49	63	64	ND	140	281	461	970
S	M7	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	WT	44	43	99	303	64	35	136		84.6	174	588	2694
Mg	M7	54	45	55	94	35	37	31	ND	155	285	650	3081
	WT	49	126	271	858	97	ND	150	ND	76.4	297	843	2014

*Time of contact in hr. ND = Not done

Appendix 3

Effect of tetracycline and carbenicillin on nutrient limited M7 and WT (D = 0.15 hr^{-1})

Colony count (% of initial)

LIMITING NUTRIENT	ORGANISM	TETRACYCLINE 25 µg/ml				CARBENICILLIN 70 µg/ml				CONTROL			
		1½*	3	4½	6	1½	3	4½	6	1½	3	4½	6
C	M7	17	15	22	64	ND	ND	ND	ND	131	265	739	1919
	WT	14	0.4	0.3	0.1	ND	ND	ND	ND	50	46	97	198
P	M7	53	11	12	33	67	136	174	174	88	150	268	723
	WT	48	17	1.3	0.9	118	133	113	120	104	171	350	1056
N	M7	23	22	19	ND	ND	ND	ND	ND	116	183	419	935
	WT	13	0.4	0.3	0.1	111	91	167	153	74	105	224	627
Fe	M7	27	0.3	0.1	0.02	125	154	156	160	97	113	130	351
	WT	21	0.9	0.6	3.8	128	96	79	121	57	9	13.2	36.3
S	M7	84	13	13	23	160	169	204	213	166	219	426	1081
	WT	51	7.3	11	23	117	110	191	168	110	107	136	595
Mg	M7	31	0.25	0.32	0.63	102	123	106	192	30	6.8	17	17
	WT	35	5	7	27	68	77	84	84	58	58	109	398

*Time of contact in hr. ND = Not done

Appendix 4

Effect of tetracycline and carbenicillin on nutrient limited M7 and WT (D = 0.25 hr^{-1})

Colony count (% of initial)

LIMITING NUTRIENT	ORGANISM	TETRACYCLINE 25 µg/ml				CARBENICILLIN 70 µg/ml				CONTROL			
		1½*	3	4½	6	1½	3	4½	6	1½	3	4½	6
C	M7	25	46	89	182	121	126	112	86	128	283	776	1497
	WT	73	122	236	354	140	87	80	55	106	434	1128	1384
P	M7	53	75	130	281	114	94	79	17	96	188	453	859
	WT	62.5	86	141	389	84	101	81	78	144	172	401	1097
N	M7	75	73	71	ND	137	161	101	89	160	300	615	1050
	WT	49	66	142	199	126	116	88	82	192	394	752	1432
Fe	M7	61	60	124	145	158	110	144	130	116	264	379	964
	WT	3.3	2.4	2.2	8.9	113	57	30	27	110	256	525	1389
S	M7	80	70	85	ND	135	150	150	141	98	161	250	571
	WT	177	330	634	1734	110	121	81	74	149	344	776	1320
Mg	M7	35	52	76	115	76	64	53	47	111	322	700	1407
	WT	39.5	65	159	305	100	77	34	21	112	240	460	990

*Time of contact in hr.

Appendix 5

Effect of EDTA on nutrient limited M7 and WT (D = 0.05 & 0.1 hr^{-1})

Colony count (% of initial)

DILUTION RATE		D = 0.05 hr^{-1}						D = 0.10 hr^{-1}					
LIMITING NUTRIENT	ORGANISM	EDTA 350 µg/ml				CONTROL		EDTA 350 µg/ml				CONTROL	
		40*	80	120	160	80	160	40	80	120	160	80	160
C	M7	66	53	49	48	105	70	76	79	53	65	94	74
	WT	81	88	70	79	105	90	83	95	75	87	124	93
P	M7	92	88	60	45	65	58	53	63	37	45	75	61
	WT	67	59	29	39	80	60	92	46	53	45	73	84
N	M7	68	85	74	70	108	92	42	41	37	35	107	76
	WT	87	52	80	89	82	77	87	71	56	ND	107	86
Fe	M7	118	93	ND	86	105	87	112	89	95	67	118	88
	WT	119	106	78	52	95	82	113	115	107	76	136	104
S	M7	101	100	37	43	110	89	ND	ND	ND	ND	ND	ND
	WT	91	92	98	48	96	94	87	83	81	68	100	86
Mg	M7	83	118	75	68	101	86	96	81	80	68	83	82
	WT	83	98	88	70	109	99	71	96	56	68	110	119

*Time of contact in min. ND = Not done

Appendix 6

Effect of EDTA on nutrient limited M7 and WT (D = 0.15 & 0.25 hr^{-1})

Colony count (% of initial)

DILUTION RATE		D = 0.15 hr^{-1}						D = 0.25 hr^{-1}					
LIMITING NUTRIENT	ORGANISM	EDTA 350 µg/ml				CONTROL		EDTA 350 µg/ml				CONTROL	
		40*	80	120	160	80	160	40	80	120	160	80	160
C	M7	62	80	81	82	87	64	39	25	20	19	53	53
	WT	93	74	79	72	101	72	82	67	111	59	103	91
P	M7	84	92	95	93	51	47	45	34	18	16	83	53
	WT	46	45	48	18	74	61	84	66	43	56	110	81
N	M7	88	84	108	89	92	78	107	74	60	82	ND	55
	WT	65	51	65	87	78	63	74	75	113	146	ND	114
Fe	M7	12	7	9	9	62	70	88	76	62	65	117	97
	WT	4	3	2	2	105	74	65	66	52	53	106	83
S	M7	88	116	123	115	93	91	80	82	79	ND	ND	118
	WT	100	105	105	75	113	78	60	39	39	38	110	87
Mg	M7	76	74	93	84	101	ND	104	106	105	129	81	ND
	WT	50	38	35	11	58	ND	124	128	100	139	135	ND

*Time of contact in min. ND = Not done

Appendix 7

Effect of EDTA on nutrient limited M7 & WT (D = 0.31 hr^{-1})

Colony count (% of initial)

DILUTION RATE		0.31 hr^{-1}				
LIMITING NUTRIENT	ORGANISM	EDTA 350 µg/ml			CONTROL	
		40*	80	120	60	120
C	M7	61	52	60	100	91
	WT	54	58	61	99	86
P	M7	66	52	65	84	56
	WT	37	32	23	71	76
N	M7	69	60	69	111	98
	WT	96	84	95	100	100
Fe	M7	71	76	63	91	74
	WT	105	102	99	89	73
S	M7	80	82	79	102	118
	WT	75	71	71	93	87
Mg	M7	76	87	100	98	94
	WT	105	104	97	100	101

*Time of contact in min.

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