FLUORESCENCE STUDIES ON THE STRUCTURE OF BACTERIAL ENVELOPES

A thesis submitted by

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for the degree of

DOCTOR OF PHILOSOPHY

of the University of Aston in Birmingham

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TO MY WIFE

AND

MY PARENTS

The University of Aston in Birmingham

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The quantitative nutritional requirements for the growth of Pseudomonas cepacia NCTC 10661 were determined. The ability of P. cepacia to store iron was indicated.

The electrophoretic mobilities of logarithmic and nutrient-depleted cells of P. cepacia decreased in the following order: logarithmic, carbon, oxygen, sulphate, phosphate, nitrogen, iron and magnesium-depleted cells. The surface hydrophobicity of the same cell types was determined using contact angles with cell layers and the angles decreased as followed: phosphate, oxygen, nitrogen, carbon, magnesium, sulphate, logarithmic and iron-depleted cells.

The killing of logarithmic-phase cells (P. cepacia) by whole blood was increased when the temperature was increased. Increase in resistance to killing by whole blood was in the following order: carbon, iron, sulphate, nitrogen, phosphate and magnesium-depleted cells.

The permeability barrier of the outer membrane (OM) was disrupted by Tris-buffer as studied by fluorescent probes. The change of the envelope of P. cepacia at different stages of the growth curve was studied with 3,3'-dipentyl-oxacarbocyanine iodide. Fluorescence intensity was highest at the turn-off point and then decreased depending on the nutrient being depleted. The OM was three times as viscous as the cytoplasmic membrane (CM) in magnesium and iron-depleted cells. Sarkosyl increased the microviscosity of the OM as studied with several fluorescent probes.

Separation of the OM from the CM was performed by sucrose density gradient centrifugation. The OM protein profile of magnesium-depleted cells was much simpler than that of iron and oxygen-depleted cells. The synthesis of the protein with molecular weight of 66,000 daltons were induced by iron depletion and two OM proteins (17,000 and 18,500 daltons) were induced by oxygen depletion. The OM consisted of 50% protein and 20% phospholipid (PL) and the rest was probably LPS while the CM consisted of 80% PL and 20% protein. The OM was enriched in phosphatidylethanolamine while the CM was enriched in phosphatidylglycerol.

Key words: Pseudomonas cepacia, nutrient depletion, electrophoretic mobility, contact angle, fluorescence.

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ABBREVIATIONS

ANS	6-anilino-1-naphthalene sulphonic acid
CDM	chemically-defined medium
c	circa
^{o}C	degrees centrigrade
CC_5	3.3'-dipentyloxacarbocyanine
CM	cytoplasmic membrane
DPG	diphosphatidyl glycerol
DPH	1,6-diphenyl-1,3,5-hexatriene
EDTA	ethylenediaminetetraacetic acid
g/l	grammes per litre
X g	relative centrifugal force
hr	hour(s)
KDO	2-keto-3-deoxyoctonic acid
L	litre(s)
LPS	lipopolysaccharide
ln	logarithms to base e
M	molar concentration
µg	microgrammes
µM	micromolar concentration
µl	microlitre(s)
min	minute(s)
N	normal concentration
NA	nutrient agar
NAD	nicotinamide adenine dinucleotide
NB	nutrient broth
NPN	N-phenyl-1-naphthylamine
nm	nanometres
nmol	nanomoles
OD_{470}	optical density at 470 nanometres
OD_{600}	optical density at 600 nanometres
OM	outer membrane
PC	phosphatidyl choline
PE	phosphatidyl ethanolamine
PG	phosphatidyl glycerol
PL	phospholipid

PMN	polymorphonuclear leukocytes
SDS	sodium dodecyl sulphate
TEMED	N,N,N',N'-tetramethylethylene diamine
TNS	2p-toluidinyl-naphthalene-6-sulphonate
V/V	volume per volume
W/V	weight per volume

ORIGIN AND SCOPE OF THE WORK

The fluorescent probing technique is known to be applicable in the study of the structure and dynamic functions of biological membranes. Unfortunately, after being first introduced by Newton (1954), there was a gap of approximately fifteen years before being used again by Stryer (1968) as a tool for the investigation of the biological systems. Together with other techniques such as electron spin resonance (ESR), electron microscopy (EM) and nuclear magnetic resonance (NMR), much information concerning the structure and dynamic functions of biological membranes have been obtained. Cheng et al. (1974) employed the fluorescent probing technique to investigate the physical properties of the outer membrane (OM) and the cytoplasmic membrane (CM) of Escherichia coli. They determined the microviscosity of the OM and CM of E. coli and found that the OM was twice as viscous as the CM. The mechanism of action of colicin was also studied by using the fluorescent probing technique. Colicin was found either to induce the increase of the microviscosity of the cell envelope of E. coli or cause the redistribution of N-phenyl-1-naphthylamine (NPN) to regions of the envelope with higher microviscosity (Helgerson et al., 1974). Other applications of the fluorescent probing technique include monitoring the trans-membrane potential and the effects of hormones on the functions of the

membranes etc. It is extremely difficult, if not impossible, to measure the membrane potential of single cells such as lymphocytes. Bramhall et al. (1976) successfully applied the fluorescent probing technique in the study of the change of the trans-membrane potential in lymphocytes following various treatments.

The potential of this technique has inspired us to employ it in the study of the physical properties of the membranes of Gram-negative bacteria. Most of the studies have been done on Pseudomonas cepacia NCTC 10661 concentrating on the effects of growth environment on the phenotypic variation of the bacterial envelope. Brown and Melling (1969 a;b) first observed that when P. aeuginosa was grown under magnesium depletion, it became resistant to polymyxin and EDTA. The effects of nutrient depletion on drug resistance have also been studied in P. mirabilis (Al-Dujaili, 1979), E. coli (Ismail, 1977) and P. cepacia (R.M. Cozens, H. Anwar and M.R.W. Brown, In preparation).

The nutritional requirements of P. cepacia have been studied in batch culture. Surface charge and hydrophobicity and phagocytosis of this organism grown under different nutrient depletions have been investigated. The effects of Tris-buffer and sarkosyl on the cell envelope have been studied with the fluorescent probe technique. Fluidity of the OM and CM has been studied by using fluorescence polarization. Separation of the OM

away from the CM has been successfully performed by sucrose density gradient centrifugation. The protein patterns of the OM and CM have been verified by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and the protein and phospholipid contents in these membranes have been assayed.

1. INTRODUCTION

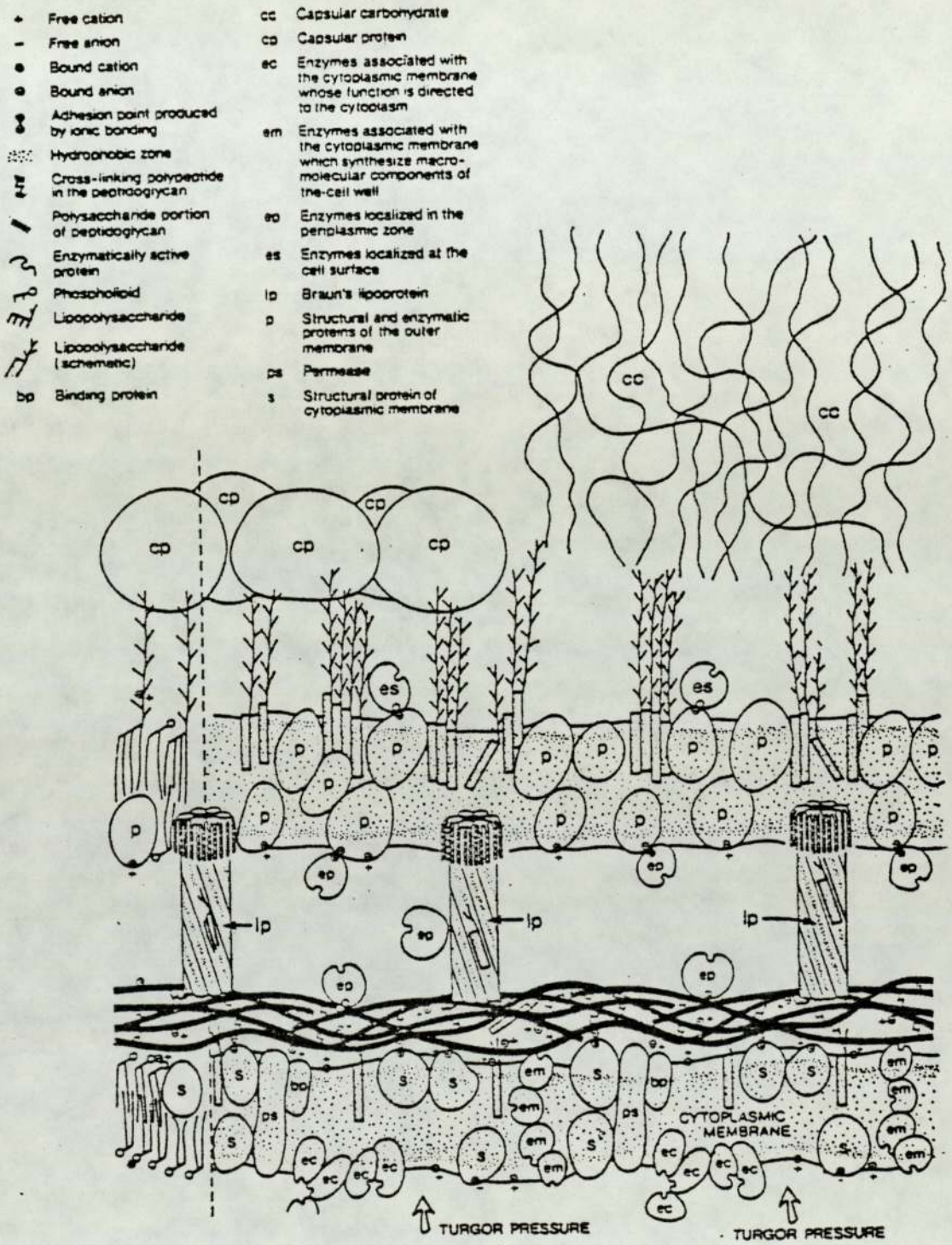
1.1 The Gram-Negative Cell Envelope

1.1.1 Introduction

The cell envelope of a Gram-negative bacterium is known to be a complex structure. It includes the cytoplasmic membrane, the rigid murein-lipoprotein complex, the periplasmic space, the outer membrane and those structures external to the outer membrane such as the carbohydrate slime layer. The molecular arrangement of this multilayer complex forming the cell envelope of Gram-negative bacteria will be discussed in the following sections. There has been increasing interest in the study of its structure and functions, because this multilayer complex is known to play an important role in regulating the internal environment of the cell and in the interactions of the cell with its external environment. It is well known that the cell envelope of Gram-negative bacteria may also act as a permeability barrier which hinders the passage of certain antibiotics across the outer membrane (Brown, 1975 ; Sawai et al., 1979).

Costerton and Cheng (1975) proposed a model for an idealised Gram-negative bacterial cell envelope (Fig. 1). Although recent research in this field has inspired Nikaido and Nakae (1979) to make modifications in some aspects of ideas of the outer membrane, it is still a useful basis for discussion. Reviews of the chemistry (Salton,1964; Martin, 1966; Rogers and Perkins,1968; Braun and Hantke,1974) and of the structure and function (Glauert and Thornley, 1969;

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



Reaveley and Burge, 1972; Braun, 1973; 1978 ; Leive, 1974; Costerton et al., 1974) of the Gram-negative bacterial cell envelope have been published. An excellent book on the bacterial outer membrane in terms of biogenesis and functions has been edited by Inouye (1979). DiRienzo et al. (1978) published an excellent review on the outer membrane proteins of Gram-negative bacteria.

The cell envelope of Gram-positive bacteria has long been shown to be different from that of Gram-negative bacteria. Costerton and Cheng (1975) reviewed the cell envelope of these organisms concentrating on their roles in antibiotic resistance.

1.1.2 The cytoplasmic membrane (CM)

The cytoplasmic membrane (CM), also known as the inner membrane, is located between the cell wall and the cytoplasm. The structure of the CM of Gram-negative bacteria may well be described by the liquid-crystalline model (see Fig. 2 a) proposed by Singer (1972 ; 1974). The phospholipids of the membrane are arranged in the form of a liquid hydrophobic bimolecular layer, in which the hydrocarbon chains are arranged approximately at right angles to the plane of the membrane, with the globular proteins partially embedded in the phospholipid matrix and partially extended from it (see Fig. 2b). These membrane proteins have been classified in two general categories, viz ' extrinsic ' and ' intrinsic ' proteins, based on their ease of dissociation from the membrane (Harrison and Lunt, 1975). Extrinsic protein (Fig.3)

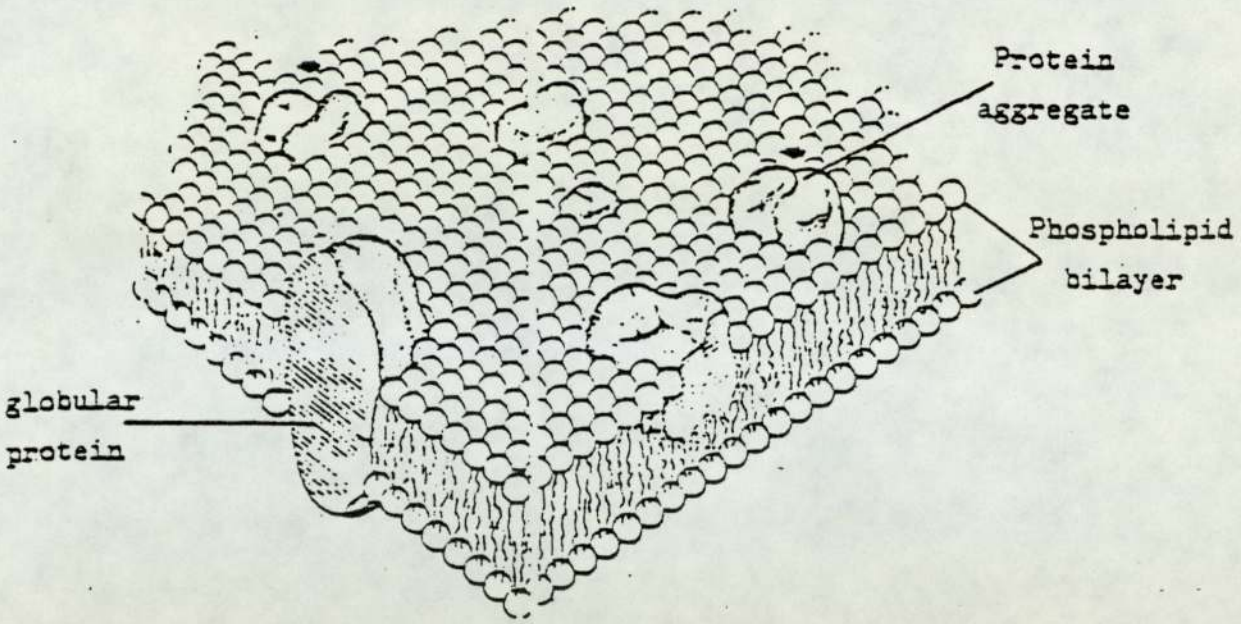


Fig 2a. A schematic representation of the three-dimensional organization of the cytoplasmic membrane (Singer, 1972).

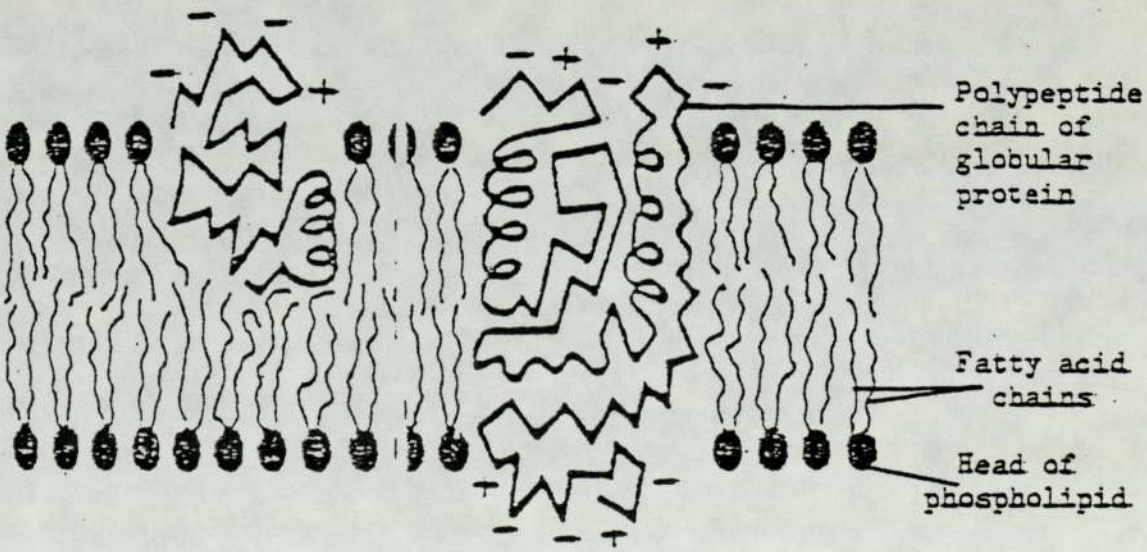


Fig 2b. A diagramatic representation of a cross section of the cytoplasmic membrane (Singer, 1972).

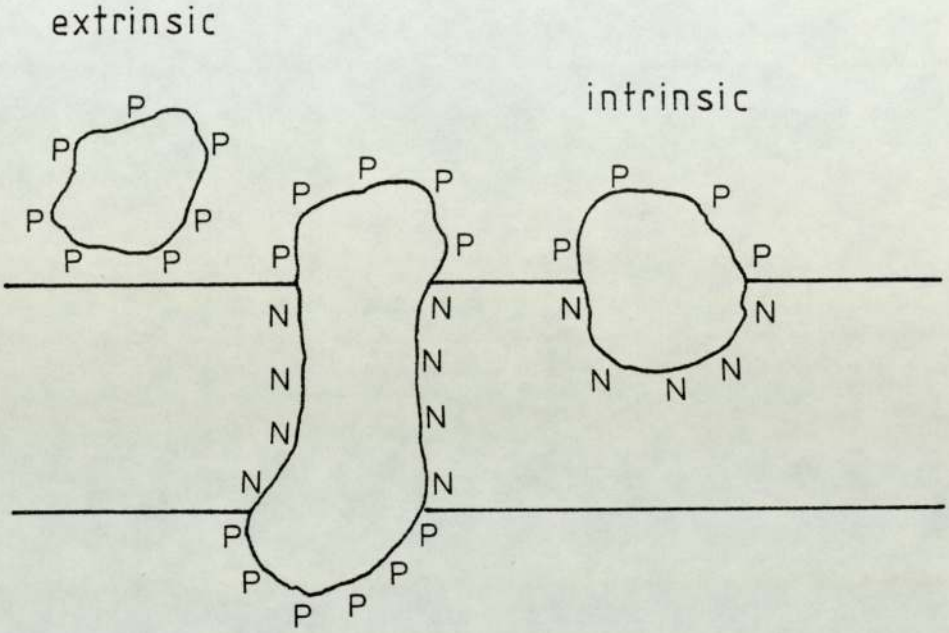


Figure 3 : Location of extrinsic and intrinsic proteins relative to the lipid bilayer of the membrane.
The distribution of polar (P) and non-polar (N) amino acid residues is shown schematically.

also referred to as peripheral or membrane-associated proteins, are relatively easily dissociated by mild treatment, such as exposure of the membrane to media of very low or very high ionic strength or extremes of pH. These proteins are then released in a soluble,non-aggregated form and free from contaminating lipid. Intrinsic (or integral) proteins generally need more dramatic treatment with reagents such as detergents or chaotropic agents before they can be dissociated from the membrane. The proteins of the membrane have an amphipathic structure, in which their ionic and highly polar groups are located on the membrane outer surface in contact with the aqueous medium, whereas their non-polar residues are located in the membrane interior. The chemistry, structure, as well as the function of the CM of Gram-positive bacteria have been shown to be similar to that of Gram-negative bacteria (Reaveley and Burge,1972). Because of the elastic properties of the CM, osmotic forces acting on the CM cause it to form vesicles against the rigid murein-lipoprotein complex (Costerton and Thompson,1972). However, in plasmolysed cells of Escherichia coli, turgor pressure of the cytoplasm, acting at various points on the CM causes the cell wall and the CM to remain in close contact (Bayer,1968).

The relationship between the CM and the outer membrane (OM) as well as the other components of the cell envelope is well documented. The CM harbours the enzyme systems of the respiratory chain, of oxidative phosphorylation, of active transport (Kaback and Hong,1973; Kundig and Roseman,1971a;b),

and of biosynthetic pathways which lead to the synthesis of the major structural components of the cell envelope such as phospholipids (Bell et al., 1971; White et al., 1971), murein-lipoprotein complex (Rogers,1970; Braun, 1975), OM proteins (Randall and Hardy,1977; Randall et al.,1978) and lipopolysaccharide (Osborn et al.,1972). Quite recently Raetz (1978) reviewed the enzymes involved in phospholipid biosynthesis and breakdown, possible ways of getting genetic mutations that lead to the change in phospholipid synthesis and the regulation of its synthesis. It is believed that the molecules of the cell wall components are synthesized and may be partially assembled within the CM and then translocated to the appropriate sites of the cell wall. For example, LPS is synthesized in the CM and then translocated to the OM (Osborn et al., 1972). Exportation of proteins and LPS into the OM appears to occur specifically at regions of contact between the inner and outer membranes, which is known as ' zones of adhesion' (Muhlradt et al.,1973; Muhlradt et al., 1974; Bayer, 1979). The enzymic composition of the CM is adapted to the growth conditions, which can cause phenotypic changes. For example, it differs greatly when the terminal electron acceptor is either oxygen or nitrate (Spencer and Guest,1974). The composition of the membrane is also not fixed in a stoichiometric way; whole enzyme systems can be suppressed and new ones can be inserted either in addition to existing ones or by replacing them. It is known that the enzyme systems seem to vary in accordance to physiological

needs. Based on this fact, it is logical that one can also predict that environmental conditions which can affect the enzymic systems at the CM, may possibly affect the cell wall in terms of its composition, structure and function, in order to adapt to the new environment, so that survival can be maintained. Gilleland et al.(1974) have shown that when Pseudomonas aeruginosa was grown in a culture under magnesium depletion, freeze-etch studies indicated a change in both the OM and CM. The appearance of the CM changed from an ordered net-like structure to one of disorder and large smooth areas were observed. The appearance of the large plaque areas in the CM of P. aeruginosa was possibly due to the loss of the CM proteins. A similar phenomenon has in fact been observed by Fiil and Branton (1969) in E.coli, when this organism was grown under conditions of magnesium depletion.

1.1.3 The murein-lipoprotein complex

The murein-lipoprotein complex is located in the innermost layer of the cell wall. In E. coli , it has been calculated to have approximately 250,000 lipoprotein molecules evenly distributed over a one-layered murein - net (Braun, 1973). This lipoprotein molecule, also known as ' Braun lipoprotein', has been found to have fifty eight amino acid residues in E. coli. It has been extensively reviewed by Braun (1975). The structural gene for this lipoprotein has been studied by Inouye et al.(1977) and

they found that it was located at 36.5 min on the *E. coli* chromosome. Inouye *et al.*(1972) have proposed that the lipoprotein is first synthesized in the free form and then covalently attached to the murein. It appears that the reaction between the two is reversible and there is a dynamic equilibrium between the two forms of protein in the envelope. It is known that about two thirds of the lipoprotein exists in free form and about one third of it is covalently linked to the peptidoglycan. There is strong evidence that lipoprotein is synthesized as a precursor containing 20 extra amino acids (Halegoua and Inouye, 1979) by the polysomes attached on the inner surface of the CM. Several models show the possible mechanisms of translocation of this protein across the cytoplasmic membrane to be assembled in the cell wall (Halegoua and Inouye,1979).

The murein layer is composed of alternating molecules of N-acetylglucosamine and N-acetylmuramic acid, about 12 - 13 Å apart and linked by 1,4 glycosidic bonds. These rigid chains of polysaccharides are cross-linked by a peptide bond between the meso-diaminopimelic and the D-alanine of the neighbouring peptide side chains. Murein is known to be the target of the action of penicillin and other antibiotics (Ghuysen and Shockman, 1973; Blumberg and Strominger, 1974) and was first visualised by Weidel *et al.*(1960). A three dimensional atomic model of the murein layer has been deduced from X-ray diffraction and infra-red data by Formanek *et al.* (1974). Fig. 4

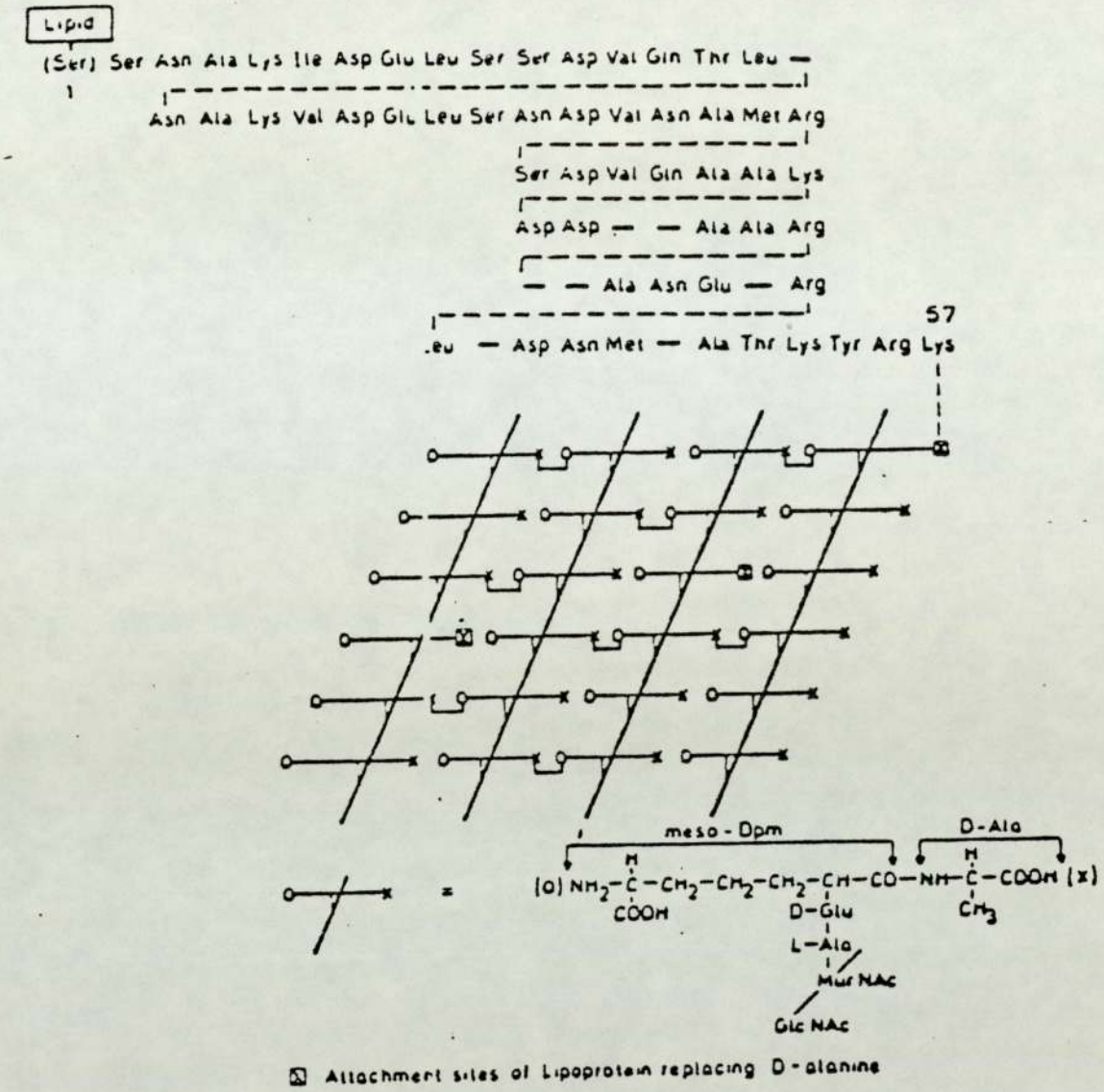


Fig 4 The lipoprotein-murein complex of E. coli (Braun, 1973).

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

shows the model of the murein-net proposed by Braun (1973) together with the chemical structure of one attached lipoprotein molecule which is a major protein in E. coli, located in the periplasmic space. One end of it is covalently linked to the murein-net (Braun and Hantke, 1975). Schnaitman (1971) has proposed that the covalently linked lipid component of this molecule serves to anchor the OM by hydrophobic interactions with the phospholipids of this layer. The observation that proteolytic digestion of the lipoprotein causes the separation of the OM from the murein layer supported this theory (Braun and Sieglin, 1970).

The main function of the murein-lipoprotein complex is believed to maintain cell rigidity. Forsberg et al. (1970) found that lysozyme acted by cleaving acetylglucosamine from acetylmuramic acid and muramic acid and diaminopimelic acid were lost. Lysozyme treated cells were converted to spheres. Isolated 'murein sacculi' retain the shape of the cells from which they were derived (Forsberg et al, 1972). Besides its function in the maintenance of cell rigidity, it may also act as a barrier to the passage of certain antibiotics (Burman et al.,1972; Tseng and Bryan, 1974) possibly by adsorptive effects of their structural polymers.

Martin et al. (1972) reported that Proteus mirabilis and E. coli were rich in lipoprotein covalently linked to the murein-net whereas this was less in P. aeruginosa. Stinnett et al. (1973) proposed that this might be a

possible reason why P. aeruginosa showed particular sensitivity to lysis by EDTA. It was shown by Braun and Sieglin (1970) that in the case of the peptidoglycan of E. coli , approximately one out of the ten diaminopimelate residues bears one of these proteins attached via lysine. This was the same phenomenon observed by Heilmann (1972) in P. aeruginosa. Besides that Heilmann (1972) was also able to show that divalent metal cations played no structural role in this complex, as it could be shown that adding chelating agent such as EDTA did not induce changes in shape or size of the peptidoglycan. Schwarz and Leutgeb (1971) found that when E. coli was grown in poor or rich media, analysis of the murein composition at the different stages of growth showed significant variations, whereas the general shape of the cells remained unchanged.

1.1.4 The periplasmic space

Mitchell (1961) described what we know to be the periplasmic space of Gram-negative bacteria as an enzyme-containing compartment bounded on the outside by a 'molecular sieve' and on the inside by the cytoplasmic membrane. The molecular sieve is equivalent to the outer membrane of Gram-negative bacteria described by Forge and Costerton (1973). The periplasmic space is now believed to be the area between the cytoplasmic membrane and the outer membrane, so that it is the area in which the murein-

lipoprotein complex is located. Brockman and Heppel (1968) found that guanosine-5- triphosphatase is located in this space. Besides that 5-nucleotidase, 3-nucleotidase, acid phosphatase, alkaline phosphatase and ribonuclease-1 have also been found in the periplasmic space of E. coli (Cerny and Teuber, 1972), and several other Gram-negative bacteria such as P.aeruginosa (Cheng et al., 1970 ; Cerny and Teuber, 1972). Periplasmic enzymes also play an important role in antibiotic resistance. Specific enzymes denature streptomycin (Lundback and Nordstrom, 1974), chloramphenicol (Benveniste and Davies, 1973), gentamicin, kanamycin and neomycin (Doi et al., 1968) by either acetylation or phosphorylation. Various β-lactamases degrade penicillins and cephalosporins (Nordstrom and Sykes, 1974) are known also to be located within this zone. This mode of bacterial resistance to antibiotics is very effective if the limited penetrability of the OM restricts the number of antibiotic molecules that can reach the periplasmic space and specific periplasmic enzymes then inactivate these few molecules to provide double protection for the resistant cells (see Brown, 1975 ; Richmond, 1975).

Quite recently, Stock et al. (1977) worked out the volume of the periplasmic space in E. coli and S.typhimurium. They found that it comprised from 20 to 40% of the total cell volume, depending on the growth conditions and strains used. However, Nikaido's group (Nikaido and Nakae, 1979) found that the space never exceeds 5% of the total cell

volume, except in starved cells or stationary-phase cells in which up to 13% of the total cell volume became the periplasmic space. The cause of this discrepancy is not clear at present. Stock et al. (1977) also showed that there is a Donnan equilibrium between the periplasm and the extracellular fluid, and that the periplasm and cytoplasm are isoosmotic. A corollary of their findings was that an electrical potential exists across the OM , as measured by determining the distributions of Na^+ and Cl^- between the periplasm and the cell exterior. The potential varied with the ionic strength of the medium; for cells in minimal salts medium it was approximately 30mV, negative inside.

1.1.5 The outer membrane (OM)

The OM of Gram-negative bacteria has received much attention in the last decade. It may be due to its involvement in resistance to antimicrobial agents and body defence mechanisms. The OM is unique to Gram-negative bacteria and has not been observed in the cell envelope of Gram-positive bacteria, hence it acts as a major distinguishing feature of the cell wall of Gram-negative bacteria. Fig. 5 shows a suggested structure of the OM of Gram-negative bacteria (Brown et al., 1979).

The structure and composition of the OM of Gram-negative bacteria are well understood, but credit must be given to the pioneering work of Miura and Mizushima (1968;

Fig. 5 Outer membrane of Gram-negative bacteria
(Brown et al., 1979)

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

TPP, Trimer of porin protein.

STP, Specific transport protein.

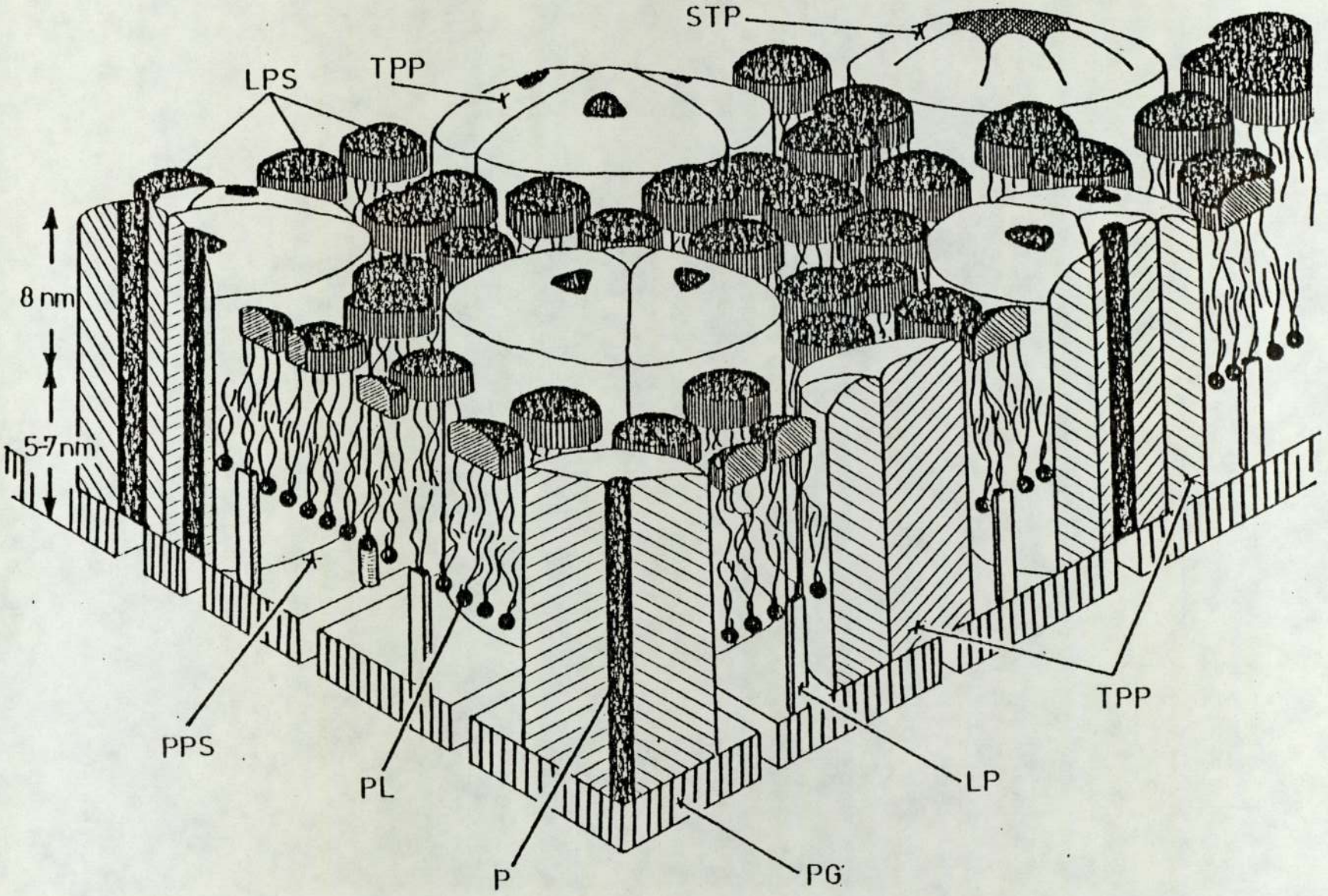
PPS, Periplasmic space.

PL, Phospholipid.

P, Hydrophilic pore.

PG, Peptidoglycan.

LP, Braun's lipoprotein.


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

1969), who first attempted to separate it from the CM of *E. coli* . Today, there are several methods that can be applied to obtain pure outer membranes of Gram-negative bacteria. These are :

I. Methods based on the buoyant density differences (Miura and Mizushima, 1968 ; Osborn *et al.*, 1972).
The outer membrane is known to have a higher density than the CM , presumably because it contains much more carbohydrate in the form of lipopolysaccharide (LPS) and possibly more proteins than does the CM, so that the membranes can be separated by an equilibrium sucrose density gradient centrifugation.

II. Methods based on the differences in electric charges (White *et al.*, 1972; Osborn and Munson, 1974).
The presence of LPS in the OM does not only create the differences in the density of the OM and CM , but it also results in the differences in electric charge. The OM has a large number of negatively charged groups and can be separated from the less negatively charged cytoplasmic membrane by use of a preparative particle electrophoresis apparatus.

III. Methods based on specific solubilisation with detergents (Filip *et al.*, 1973).
The stabilization of the structure of the OM presumably relies on the ionic interactions of its constituents, which often resists attacks by non-ionic detergents. However, several ionic detergents such as Triton X-100

in the presence of Mg^{2+} (Schnaitman, 1971) and sarkosyl (Filip et al., 1973) have been found to solubilise the CM completely, while leaving the OM to retain its morphological integrity.

The major constituents of the OM are proteins, phospholipids and LPS. Interestingly, LPS has only been observed in the OM and not in the CM. The structure of the LPS has been the subject of a number of recent reviews (Galanos et al., 1977; Orskov et al., 1977; Wilkinson, 1977 ; Osborn, 1979). The number of molecules of the major components in 1 μm^2 section of the OM of E. coli and S.typhimurium is of the order of 10^5 for LPS (Muhlradt et al., 1974 ; Smit et al., 1975), 10^5 for proteins (Braun and Rehn, 1969 ; Rosenbusch, 1974 ; Inouye et al., 1972) and 10^6 for phospholipids (Smit et al., 1975).

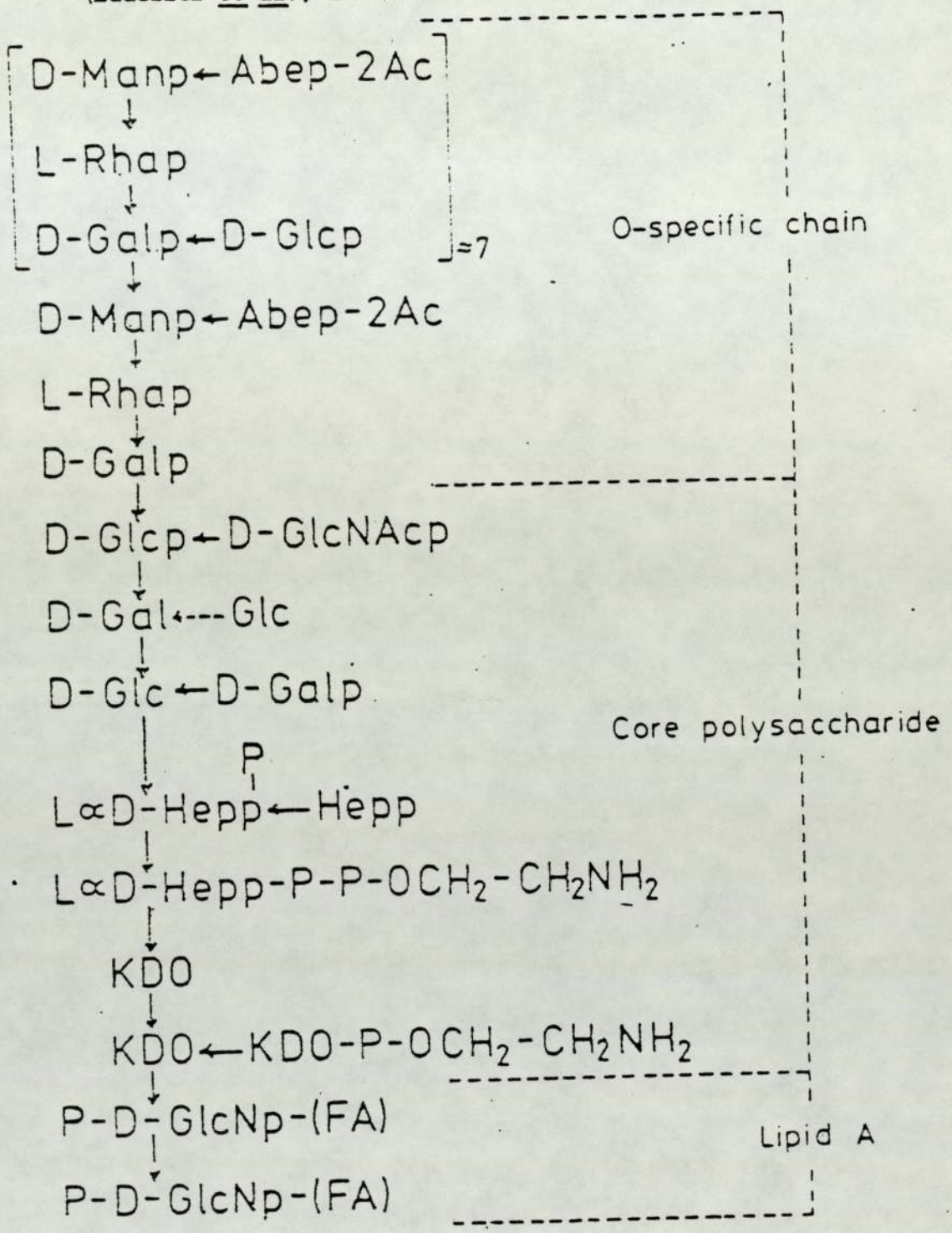
Fig. 6 shows the chemical structure of the LPS of S. typhimurium (Luderitz et al., 1974). Three regions are recognisable, these are :

a) The surface O- antigen-specific side chain, which is the hydrophilic part of the molecule, usually used in serological typing of the strains, especially in Enterobacteriaceae (Wilkinson, 1977).

b) The core polysaccharide, which also corresponds to the hydrophilic part of the molecule, protrudes into the surrounding environment (Glauert and Thornley, 1969).

c) The lipid A endotoxic region, which is the hydrophobic core of the OM (Glauert and Thornley, 1969).

Fig. 6 Structure of S. typhimurium lipopolysaccharide (Luderitz et al., 1974).



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

These three regions of LPS molecule are known to occur in the LPS of all bacteria being studied, except slight variation in chemical composition has been found within species (Meadow, 1974). This may be due to the difference in cultural conditions as well as methods of extractions.

The lipid bilayer of the OM appears to be asymmetric in Enterobacteriaceae (Nikaido and Nakae, 1979), in which the outer half of the membrane is composed predominantly of LPS and proteins whereas phospholipid is located only in the inner half in wild type S.typhimurium, as shown by the cyanogen bromide labelling technique (Kamio and Nikaido, 1976). However, mutations of the LPS or OM proteins result in the redistribution of the phospholipids to the outer leaflet of the membrane, therefore reducing the extent of asymmetry (see review by Nikaido and Nakae, 1979). The electrostatic repulsion caused by charged centres in the outer leaflet of the membrane is stabilized by divalent cations, probably through neutralization of and bridging between anionic groups (Gray and Wilkinson, 1965). Treatment of the cells with EDTA, which removes these cations from the outer leaflet, will result in the release of predominantly LPS (Leive, 1965) in E. coli and LPS-protein complexes in P. aeruginosa (Rogers et al., 1969), presumably because the electrostatic repulsion becomes stronger than the cohesive forces of hydrophobic interaction.

There were conflicting data on the lipid compositions of the outer and inner membranes. Osborn et al. (1972) reported that the OM had a higher proportion (80 versus 60%) of phosphatidylethanolamine (PE) than did the CM and less phosphatidylglycerol (PG) and cardiolipin (CL) than the CM. White et al. (1972) reported that the OM was deficient in PE when compared to the CM. Recent data (Joseleau - Petit and Kepes, 1975 ; Goodell et al., 1974 ; Lugtenberg and Peters, 1976) strongly supported the findings of Osborn's group. Furthermore, Lugtenberg and Peters (1976) found that the PE of the CM had a somewhat higher proportion of unsaturated fatty acids than did the PE of the OM. The discrepancy in the data reported by White et al. (1972) was probably skewed by the large amounts of lyso PE and ' unidentified phospholipids' found in their lipid analyses.

Current reviews on the OM proteins of Gram-negative bacteria have been published (DiRienzo et al., 1978 ; Halegoua and Inouye, 1979 ; Osborn and Wu, 1980). Rapid progress has been made in this area, mainly due to the technique of sodium dodecyl sulphate (SDS)- polyacrylamide gel electrophoresis (Schnaitman, 1970 ; Lugtenberg et al., 1975 ; Pugsley and Schnaitman, 1979).

Braun lipoprotein which is one of the major OM proteins in Gram-negative bacteria has been discussed in section 1.1.3.

The migration of several OM proteins is known to be

affected by the temperature of denaturation in the presence of SDS , They are usually known as ' heat-modifiable' proteins, with molecular weights in the range of 32 - 37,000 daltons. One of these proteins, Omp A in E. coli , on SDS - polyacrylamide gel electrophoresis moved like an approximately 25,000 dalton protein when the samples were solubilized in SDS at room temperature, but migrated more slowly when the samples were heated in SDS prior to electrophoresis. Factors that can affect the migration of OM proteins in SDS - polyacrylamide gel electrophoresis in E. coli (Pugsley and Schnaitman, 1979) and in P. aeruginosa (Hancock and Carey, 1979) have been published. Another group of proteins that has received much attention is the pore-forming proteins or porins. In SDS-polyacrylamide gel electrophoresis, they move with apparent molecular weights in the range of 33 - 40,000 daltons. They are named ' porins', because they have the unusual property of producing transmembrane diffusion pores when added to a phospholipid - lipopolysaccharide mixture (Nakae, 1976 a ; b). The functional unit of the porin is believed to be a trimer (Benz et al., 1980 ; Ishii and Nakae, 1980). The pore size probably varies with organisms, as P. aeruginosa was shown to allow the passage of molecules below 9000 daltons (Hancock et al., 1979) while E. coli and S. typhimurium allow only molecules of less than 600 daltons (Nakae, 1975). Their data were obtained by using plasmolysed cells or incorporating pore-forming OM proteins

into the phospholipid- lipopolysaccharide vesicles. Finally, there is a group of proteins that facilitates specific diffusion of certain substrates. When E. coli was grown in the presence of maltose, it induced the production of lam B or λ-receptor protein (Braun and Krieger-Brauer, 1977). Several OM proteins in the range of 70 - 85,000 daltons were produced when E. coli was deprived of available sources of iron (Rosenberg and Young, 1974).

The OM , due to its unique structure, has been known to be a barrier to the penetration of many antibiotics. LPS was found to interact with gentamicin in P. aeruginosa (Day,1980). The interaction has been proposed to be partially ionic and probably involved the core region of the LPS molecule. The amount of organic phosphate associated with the LPS appeared to play a role in the interaction and it could be disrupted by lysozyme and magnesium. The availability of single-step mutants with defined biochemical defects in LPS synthesis made it possible experimentally to alter the structure of LPS of the OM and to study the effect of such alterations on the permeability of the membrane. Mostly the entry of molecules affected by the structure of LPS are hydrophobic in their nature. Wild type strains of S. typhimurium and E. coli are much more resistant than most Gram-positive bacteria to such antibiotics as actinomycin D, erythromycin, novobiocin and rifamycin SV, to such dyes as crystal violet and to such detergents as bile salts and sodium dodecyl sulphate (SDS). The sensitivity of these agents, however, increased markedly

in ' deep rough' mutants of S.typhimurium (Roantree et al., 1977) that produced extremely defective LPS of Rd_1 , Rd_2, and Re types and also in E. coli treated with EDTA, which resulted in the removal of large part of the LPS, would render the cells sensitive to actinomycin D, novobiocin, rifampicin, long-chain fatty acids and detergents. Nikaido and Nakae (1979) proposed a model for the structure of the OM of wild type S. typhimurium or E. coli, in which the outer leaflet was mainly covered by LPS and proteins. Mutations involving the LPS or treatment with EDTA would result in the appearance of phospholipid in the outer leaflet of the OM and this increased the penetration of hydrophobic molecules. From the observations, one can draw the mechanism of the entry of hydrophobic molecules across the membrane, i.e, by first dissolving in the hydrophobic interior of the membrane, then crossing the thickness of this hydrocarbon layer by simple diffusion which is probably helped by dynamic movement of phospholipid, and finally redissolving in the aqueous phase bathing the other side of the membrane. The process is markedly affected by close stacking of LPS at the outer leaflet of the OM.

We have discussed the mechanism of the entry of hydrophobic molecules across the OM. The remaining questions focus on the entry of hydrophilic molecules. To our knowledge, it can be divided into two pathways.

(I) Specific transport pathway.

The specific transport system has a very high affinity

towards the substrate encountered. It was first discovered by Bradbeer's group (White et al., 1973) that protein, located in the OM , could function in the transport of vitamin B_{12} (1357 daltons) across the OM. The iron transport system has been studied in great detail, it involves several OM proteins in the 70 - 85,000 dalton range (for review see Rosenberg and Young, 1974). Lam B or λ-receptor protein has been known to be involved in the transport of maltose and maltodextrins (Braun and Krieger-Brauer, 1977). Another OM protein, T_6-receptor protein or tsx protein, was found to facilitate the diffusion of nucleosides across the OM (Hantke, 1976). Albomycin, which has the chemical structure quite similar to that of ferrichrome, is believed to be transported across the OM of E. coli by the same uptake system as the iron-suppling ferrichrome complex (Hartmann et al., 1979).

(II) Non-specific diffusion pathway for hydrophilic molecules

The pioneering studies of Nikaido and Nakae and their co-workers demonstrated the existance of hydrophilic pores across the OM (Nakae and Nikaido, 1975 ; Decad and Nikaido, 1976) and established the function of the omp C and F proteins in pore formation in vivo (Nikaido et al., 1977) and in reconstituted membrane systems (Nakae, 1976a). This diffusion pathway is not only required for a number of hydrophilic antibiotics, which must penetrate through the outer membrane in order to reach their targets of action,

but it is also essential for nutrients including sugars, amino acids and inorganic salts as well as for waste products of metabolism. Nikaido et al. (1980) proposed that porin channels are essentially cylindrical channels with no specific combining sites.

To study the penetration of hydrophilic molecules through the OM, it was necessary to use oligosaccharides that neither penetrated through nor were actively transported across the CM. Nikaido's group (see Nikaido and Nakae,1979) found that oligosaccharides of the sucrose-raffinose series fulfilled these conditions in S. typhimurium and were used in their experiments. Raffinose (galactosyl-sucrose), stachyose (galactosyl-galactosyl-sucrose), and verbascose (galactosyl-galactosyl-galactosyl-sucrose) have an added advantage that they can be easily labeled with tritium by oxidation of C-6 of the terminal galactose residue with galactose oxidase, followed by its reduction by NaB^3H_4 . Since the space between the OM and the CM is small (in contradiction to Stock et al. (1977), see section 1.1.4), the diffusion into this space was difficult to measure. They therefore expanded the periplasmic space by plasmolysing cells in 0.3-0.5 M NaCl or in 0.5 M sucrose (Decad and Nikaido, 1976). Under these conditions, 40-50% of the cell volume was occupied by the periplasmic space, and the extent of penetration of oligosaccharides into this space could easily be measured by centrifuging down plasmolysed cells after incubation with the radioactive

oligosaccharides and by determining the concentration of the radioactive substance both in the supernatant and in the pellet. Corrections for intercellular space in the pellet were made by adding to the incubation mixture, large, uncharged polymers (^{3}H dextran), which were assumed to be impermeable through the OM.

Their studies showed the existence of a clear size limit in the penetration process through the hydrophilic pores. E. coli and S. typhimurium allow only molecules of less than about 600 daltons to pass through the pores, while P. aeruginosa allows the passage of molecules below 9000 daltons (Hancock et al., 1979). The diffusion through hydrophilic pores is not affected by the presence of LPS.

1.1.6 External layers

Several Gram-negative bacteria have a protein layer attached externally to the LPS layer (Thornley et al., 1974). Other organisms have a loosely associated capsule or slime layer composed of carbohydrate. The production and composition of slime in P. aeruginosa may be affected by cultural conditions (Brown and Scott-Foster, 1971 ; Goto et al., 1973). The slime of P. aeruginosa may have an important role in toxic properties (Sensakovic and Bartell, 1974). It seems likely that the attachment of carbohydrate at the outermost surface of the cell envelope will result in an increase of hydrophilicity of the surface, which will become much less readily engulfed

by phagocytes (Cunningham et al.,1975). Furthermore, the radial protrusion of the polysaccharide fibres means that this layer is the structure which first makes contact with other cell surfaces, and it has been shown to be involved in adhesion (Costerton et al., 1979).

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

1.1.7.1 Introduction

It has long been known that divalent metal cations such as magnesium are essential for the growth of bacteria in simple salts media (Webb, 1949). Metal cations although constituting only about 4% of the dry mass of the cells of E. coli (Luria, 1966), play an important role in enzymatic function and general cellular physiology. Rouf (1964) found that divalent cations such as magnesium, calcium, manganese, iron and zinc were present in significant quantities in the whole cells of E. coli and Sphaerotilus natans. Eagon (1969) found that in the cell walls of P. aeruginosa , magnesium and calcium were major elements, iron a minor element, whereas zinc, lead, copper, manganese and strontium were present as trace elements. The metal cation content in the cells of E. coli grown exponentially and synchronously was analysed by Kung et al. (1976), using X-ray fluorescent spectrophotometry and atomic absorption spectrophotometry. They found that cellular potassium, calcium and magnesium content increased smoothly

during the cell cycle, but cellular zinc showed an increase about 10 to 15 minutes after cell division in a culture having a doubling time of 47 minutes.

The absolute requirement for magnesium in bacteria is probably mainly due to the specific requirement for the maintenance of the intact structure and activity of the ribosome (Tissieres et al., 1959), the activity of many enzyme systems (Dixon and Webb, 1964), stability and permeability of the membrane (Lederberg, 1956 ; Brock, 1962 ; Hassan, 1976) and nucleic acid synthesis (Cohn and Ennis, 1967). Interestingly, magnesium is known to be involved in the activity of those enzymes responsible for the synthesis of the cell wall components such as fatty acids (Knivett and Cullen, 1967), phospholipids (White et al., 1971), murein (Garratt,1967), and LPS (Edstrom and Heath, 1967). Magnesium has also been suggested to be a structural component of the OM of Gram-negative bacteria (Costerton et al., 1974) , especially in P. aeruginosa (Brown and Melling, 1969a;b ; Boggis et al., 1979). Quite recently, Nicas and Hancock (1980) found that OM protein H_1 production was induced when P. aeruginosa was grown in magnesium deficient medium and they proposed that protein H_1 acted by replacing magnesium ion at a site on the LPS which can otherwise be attacked by the cationic antibiotics or EDTA. Zinc has been found to be associated with the adsorption of T_2 and T_4 bacteriophages to E. coli (Kozloff and Lute, 1957), and

also found in various metalloenzymes in E. coli . It is important for the assembly and activity of alkaline phosphatase (Torriani, 1968) and the regulating activity of aspartate transcarbamylase (Nelback et al., 1972). Calcium has been suggested to be involved in the maintenance of the structural organisation of the wall of P. aeruginosa (Asbell and Eagon, 1966) and E.coli (Leive, 1968).

1.1.7.2 Divalent cations and the cell envelope

Divalent cations are known to play several important roles in the bacterial cell envelope. They are essential in maintaining the stability of the CM (Lederberg, 1956), regulating the activity of several enzyme systems, especially in those enzymes which are involved in the synthesis of the cell wall (see section 1.1.7.1), and as structural components, providing cross-linkages between LPS components (Wilkinson and Galbraith, 1975) and the rest of the cell wall (Asbell and Eagon, 1966 ; Costerton et al., 1974).

The role of magnesium in the maintenance of the stability as well as permeability of the membranes, especially the outer membrane, has been observed by treating the cells with EDTA or polymyxin, which acts initially at a common site of action, namely the Mg^{2+} and Ca^{2+} cross bridges that stabilize components of the OM (Brown and Melling, 1969a ; b ; Boggis et al., 1979). This may then cause damage to the OM and therefore induces the change of

the permeability of the OM.

Leive (1965) found that EDTA could remove the LPS from E.coli and Leive (1968) concluded that EDTA removed magnesium from the OM which then underwent a steric or chemical change which required energy metabolism for its reversal. Repaske (1958) found that treatment of E.coli or P. aeruginosa with anionic exchange resin (Dowex 50) as well as EDTA caused the removal of cations from their cell walls, which would then follow by the increase of the sensitivity to lysozyme. Since Lysozyme is known to act on the murein layer of the bacteria, she concluded that metal cations prevented the action of lysozyme by steric hindrance. Neu and Heppel (1964;1965) found that EDTA caused the release of alkaline phosphatase which is known to be located in the periplasmic space.

1.1.8 Phagocytosis

Phagocytosis is the process whereby certain cells convey solid objects from external milieu to their interior, and then subject those objects, which are surrounded by an envelope of plasma membrane (the phagosome), to chemical and enzymatic attack . Cells that can perform this function are termed 'phagocytes'. Phagocytosis, the cellular events that occur just before, during and after the actual ingestion of particles, has long been recognized as a key process in combating infections in humans and animals. The polymorphonuclear leukocytes (PMNs), which are generally considered

the first line of defense against bacterial invasion, constitute about 60-70% of the circulating phagocytic cells and may be subdivided into: eosinophils (stain bright orange-red with Giemsa stain), basophils (stain blue-black) and neutrophils, which possess granules that do not stain intensely. Neutrophils constribute most to the host defense mechanism against bacteria.

An excellent review on phagocytosis has been published (De Chatelet, 1979). Two mechanisms for adhesion of bacteria to the phagocytes have been suggested: 1. specific recognition for the F_c part of IgG and for activated complement system on the surface of bacteria by phagocytes (Rowley and Turner,1966; Messner and Jelinek, 1970) and 2. Aspecific adhesion based on physicochemical characteristics of the bacteria and phagocyte surface, such as shape, charge or hydrophobicity (Mudd et al.,1934; Stendahl et al.,1974; Van Oss, 1978).

The first mechanism is based on the receptor theory. The surface of bacterial cells has the receptors for the F_{ab} part of the antibody and for the complement system. Bacterial cells coated with antibodies and complement system provide the binding sites (F_c part of the antibody) which fit the receptors on the surface of the phagocytes, as a key fits a lock. This process is known as opsonization. The hypothesis can well explain most observations, in which enhancement of phagocytosis is observed when the cells are opsonized. However, it becomes very complicated by the

findings that phagocytes can also engulf various synthetic plastic beads such as polystyrene latex particles and some microorganisms are well phagocytized without being opsonized (Van Oss, 1978).

Early investigators emphasized that physical and surface characteristics of cells involved in the phagocytic process (Mudd and Mudd,1933). In the past decade, Van Oss and his associates have done the most work with surface phenomena and their findings have been published in an excellent monograph (Van Oss et al.,1975). Their basic hypothesis is that phagocytosis occurs primarily because of difference in surface free energies of the particle and of the phagocyte. This could be quantified by layering the bacteria concerned on a membrane filter and measuring the angle formed between a drop of saline and the dried bacterial surface with an ophthalmic instrument known as goniometer. If the surface of the bacteria is hydrophobic, the saline drop tends to round up and the contact angle is relatively large. On the other hand, if it is relatively hydrophilic, the droplet tends to flatten out and the contact angle is small. Based on this principle, they determined the contact angles of a large number of bacteria and found a striking correlation between hydrophobicity and phagocytosis. Those bacteria with contact angles greater than that of neutrophils which have the contact angle of 18^{o} were readily phagocytized, while those with angles less than 18^{o} were phagocytized with difficulty. There was a good degree of correlation

between the difference in contact angle and the relative ease of ingestion; the lower the contact angle of the bacteria, the less susceptible it was to phagocytosis. A striking example is Staphylococcus aureus (Smith strain), which has a pronounced capsule, is thus quite hydrophilic (contact angle of 16.5°), and strongly resists phagocytosis, whereas after decapsulation, it acquires a high contact angle (26°) and becomes easily phagocytized. Thus, the hydrophobicity of bacteria appears to determine the degree to which they are susceptible to phagocytosis. Furthermore, at least one action of both antibodies and complement involved increasing the contact angle of the bacteria. Bacteria that are poorly ingested in the absence of serum have contact angles less than that of neutrophils. The addition of serum markedly increased the contact angle and simultaneously enhanced the engulfment (Van Oss et al., 1975). Thus it appears that one mechanism of opsonization involves increasing the hydrophobicity of the bacteria, so that it will be susceptible to engulfment by neutrophils.

Stendahl and Edebo (1972) studied the phagocytosis of a well documented range of cell wall mutants of S. typhimurium by PMN leukocytes. These workers found that the least virulent mutant with the shortest LPS side chain was most rapidly engulfed by PMN leukocytes compared with the virulent smooth (MS 395) strain with a complete LPS structure. Phenotypic changes caused by variation in growth environment can alter the antigen composition of Haemophilus

species (Lacey, 1954;1961). Finch and Brown (1978) found that slow growing (D=0.05 h^{-1}),magnesium-limited cells of P. aeruginosa were significantly more resistant to the lethal effect of the phagocytes than were fast-growing magnesium-limited cells.

No goal is too high if we climb with care and confidence.

1.2 The Fluorescent Probing Technique

1.2.1 Introduction

Luminescence attracted the attention of scientists a long time ago. In the early years , not much in its application was observed. This might be due to the lack of knowledge involved in light emission effects and the lack of suitable apparatus such as light sources, light filters and photometers. The developments over the last twenty years of photomultipliers and of commercial fluorometers and spectral dispersing instruments has attracted a great interest in the study of luminescent processes.

Photoluminescence can be defined as the radiation emitted by a molecule or an atom, after it has absorbed radiant energy and been raised to an excited state. It consists of fluorescence and phosphorescence. Fluorescent technique is applicable to the study of biological membranes (see reviews by Radda, 1971 ; 1975).

Fluorescent probing techniques were first introduced by Newton (1954) to investigate the action of antibiotics on bacteria. He found that fluorescent intensity of N-tolyl-α-naphthylamine-8-sulphonic acid (TNS) was increased when polymyxin was added to the suspensions of P. aeruginosa. The increase of fluorescence intensity of TNS upon addition of ovalbumin or bovine serum albumin was first observed by Weber (1952). Unfortunately, there was

a gap of approximately fifteen years, before it was reintroduced as a tool for the investigation of biological systems (Stryer, 1968). Today much information concerning biological membranes such as polarity, microviscosity, lipid-phase transition have been obtained through this technique (Trauble and Overath, 1973 ; Cheng et al., 1974).

In order to understand the application of the fluorescent technique in the study of biological membranes, it is necessary to become familiar with the nature of the electronic and excited state processes.

1.2.2 Electronic states

Electronic states are concerned with properties of all of the electrons in a molecule in all of the electronic orbitals. As an electron is moved from one orbital to another, the state of the molecule is changed. Electronic states of most organic molecules can be grouped into two broad categories of singlet and triplet states. In a singlet state, all of the electrons in the molecule have their spins paired, the resulting spin is zero, whereas in a triplet state one set of electron spin become unpaired, that is all electrons in the molecule except two have paired spins. In other words, the difference between singlet and triplet states is their multiplicity (orbital angular momentum) of the state. If the multiplicity of the state is one, then it is called singlet state, if the

multiplicity of the state is three, then it is called a triplet state. The singlet and triplet states are different in their properties and in their energies. Triplet states always lie lower in energy than their corresponding singlet states. The emission lifetime of a triplet state is longer than its corresponding singlet state.

1.2.3 Fluorescence and phosphorescence processes

The processes of fluorescence and phosphorescence may be represented in a schematic diagram (see Fig. 7).

The absorption of appropriate radiant energy by a molecule raises the molecule from the zero vibrational level of the ground state to one of many vibrational levels in one of the excited states, usually the first excited state, S_1 . The absorption step occurs within 10^{-15} second. A number of vibrational levels of the excited state are populated immediately following absorption. However, molecules in higher vibrational levels will soon return to the lowest vibrational level of the excited state by transferring their excess energy to other molecules through collisions, as well as by partitioning the excess energy to other possible modes of vibration or rotation within the excited molecule.

Fluorescence results from spontaneous radiative transition that occurs when molecules return to the ground electronic level. This radiative process (from $S_1 \rightarrow S_o$)

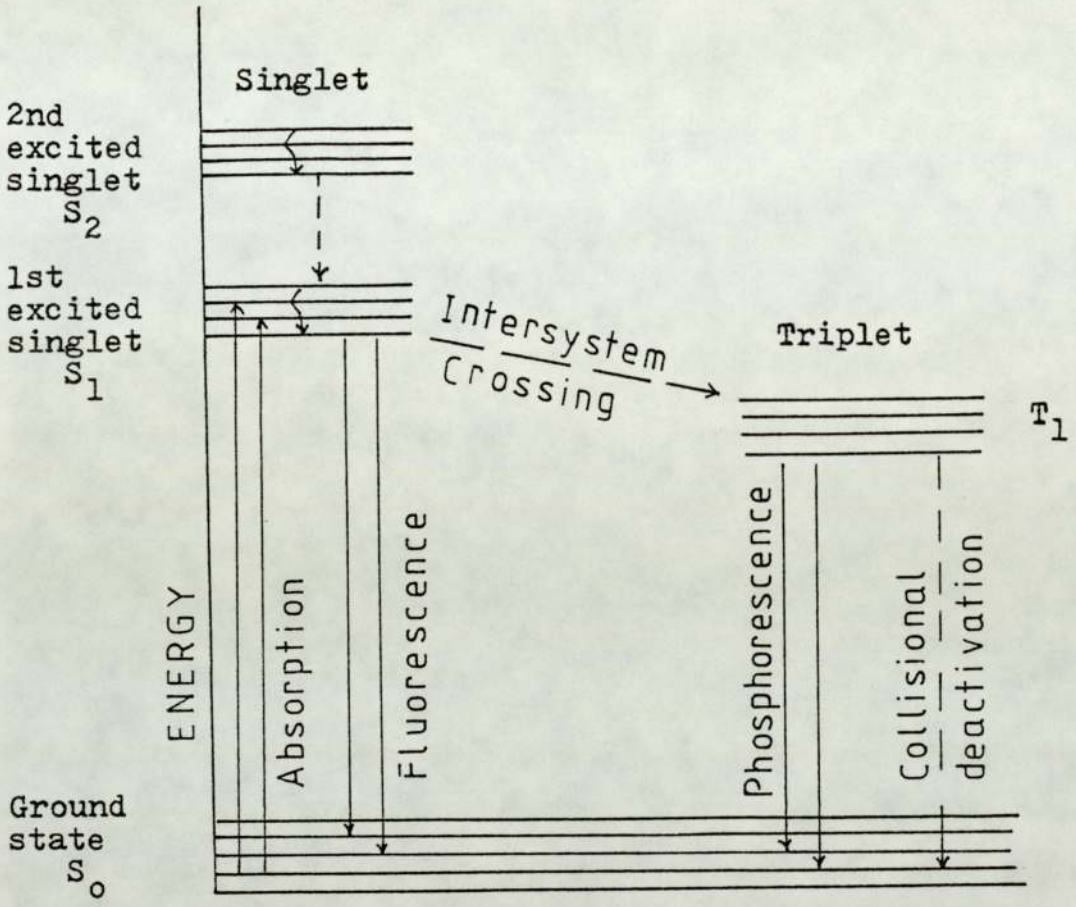


Fig. 7 : Potential energy diagram
Detailed description of the processes is given in section 1.2.3.

has a lifetime of about 10^{-8} second, so that in many molecules it can compete effectively with other processes capable of removing the excitation energy such as internal conversion or intersystem crossing. Of course, if the absorption process leads to an electronic state in which the energy exceeds the bond strength of one of the solute's linkages, then excitation energy is lost before

fluorescence can take place.

If the potential energy curve of the singlet state crosses that of the triplet state, some molecules in the singlet excited state may pass over to the lowest energy of the triplet state via an intersystem crossing which is concerned with vibrational coupling between the excited singlet state, S_1, and the triplet state, T_1. Singlet-triplet transitions are known to be forbidden processes, but there is some probability that an internal conversion from the excited singlet to the excited triplet will occur, because the energy of the lowest vibrational level of the triplet state is lower than that of the singlet excited state. The probability of the intersystem crossing is greater when the potential energy curves cross at the lowest point on the excited singlet curves. Once indirect occupation of the triplet state has been achieved, further vibrational energy will be lost by radiationless transitions to the vicinity of the zero point vibrational level of the triplet state, phosphorescence occurs. Again it is a transition that will occurs with low probability since spin reversal must once more occur. Consequently, the triplet state persists for a relatively long lifetime and the rate of phosphorescence emission is very low (10^{-2}-100 seconds). Spin-orbit coupling, which is a magnetic perturbation capable of flipping spins, is believed to be the main source of phosphorescence transitions back to the ground state , while some

deactivation of excited-state molecules may occur as a result of collision with solvent molecules before the energy is re-emitted; this effect becomes very important in phosphorescence due to the long lifetimes of $S_1 \longrightarrow T_1$ and $T_1 \longrightarrow S_o$ transitions.

Phosphorescence is virtually unknown for dissolved molecules and can only be observed on any significant scale when phosphor is frozen into glass at low temperatures so that collisional deactivation is prevented or at least severely restricted.

In both fluorescence and phosphorescence, the lower energy photon is emitted in an arbitrary direction and at wavelength longer than the excitation wavelength. Since raising a molecule to its excited state is a matter of total energy required, the emitted photoluminescence is at the same wavelength of the absorbed energy. Fluorescence and phosphorescence each provide two kinds of spectra for identification, these are excitation and emission. Fluorescence measurements can be made under a wider range of conditions than phosphorescence measurements. This may be why fluorescence spectroscopy is more extensively used.

1.2.4 Parameters of fluorescence

The fluorescence parameters which can be measured are the excitation spectrum, emission spectrum, quantum yield, lifetime and polarization. The excitation (or absorption) and emission spectra describe the dependence of fluorescence

intensity on the wavelength of exciting and emitted light respectively. The intensities of the various vibrational bands in the two spectra depend on the shape of the potential energy diagram for the ground and excited states (see section 1.2.2.).

The quantum yield of fluorescence is defined as:

q = quanta emitted / quanta absorbed

and it depends on the relative rates of the emission, internal and external conversion processes, which can be expressed as :

$$q = \frac{K_e}{K_e + \Sigma K_{internal} + \Sigma K_{external}}$$

where K_e is the rate of emission, $\Sigma K_{internal}$ may contain several rate processes: intersystem crossing into the triplet state or radiationless inactivation through the vibrational levels of the two electronic states. But radiationless inactivation has been argued to be relatively slow (10^{-5} second) (Brocklehurst, 1970), so that the main contribution to $\Sigma K_{internal}$ is intersystem crossing to the triplet state. External competition for energy takes place by collision with solvent or chemical reaction.

The lifetime of the excited state is the time taken for fluorescence to drop to 1/e of its initial value after the excitation light is switched off. It depends on the rates of transitions which depopulate the lowest excited singlet

state. If the only process leading to energy loss is fluorescence (i.e., when $K_{internal}$ and $K_{external}$ are both zero), the ' natural lifetime ' is obtained (τ_o).

The fluorescence lifetime is related to the quantum yield through the equation:

$$q = \tau / \tau_o \quad ,$$

where q = quantum yield, τ is the fluorescence lifetime and τ_o is the natural fluorescence lifetime, $\tau_o = 1 / K_e$, K_e is the rate constant for the emission processes. The measurement of τ gives information concerning the processes of depopulation of the lowest excited singlet state rather than the fluorescence process, and is valuable to decide whether a change in quantum yield is apparent (ground state formation of a complex with a quencher, changes in the occupancy of the sites in which the probe has high fluorescence yield, trivial effect such as changes in absorption of exciting and emitted light) from those in which a quantum yield change is real (energy transfer, dynamic quenching, solvent interactions with excited state molecules,etc).

Fluorescence polarization measurements can give information about the mobility of the probe, its orientation as well as the microviscosity of its environment. In the common fluorescence of aromatic compounds, the processes of absorption and emission of electromagnetic radiation are associated with transition dipoles of a well-defined orientation on the molecular frame. To each electronic

absorption band, an 'excitation dipole' is assigned, whereas the fluorescence is generally assigned to a single 'emission dipole'. Both the excitation and emission dipoles lay on the plane of the molecule and are displaced by an angle α. The common fluorescence polarization can be measured by excitation performed with monochromatic light which is vertically polarized and the emission measured through an analyser oriented parallel or perpendicular to the direction of the excitation light. In general, the emission is detected at the right angle to the excitation, and the direction of polarization $I_{\parallel}$ and $I_{\perp}$ are Z and Y (see figure 8). Absolute values for fluorescence polarization are generally presented as $I_{\parallel} / I_{\perp}$, P or r.

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} = \frac{I_{\parallel}/I_{\perp} - 1}{I_{\parallel}/I_{\perp} + 1} \equiv \text{degree of fluorescence polarization}$$

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} = \frac{I_{\parallel}/I_{\perp} - 1}{I_{\parallel}/I_{\perp} + 2} \equiv \text{fluorescence anisotropy}$$

The total fluorescence intensity, F , is given by $F = I_{\parallel} + 2I_{\perp}$.

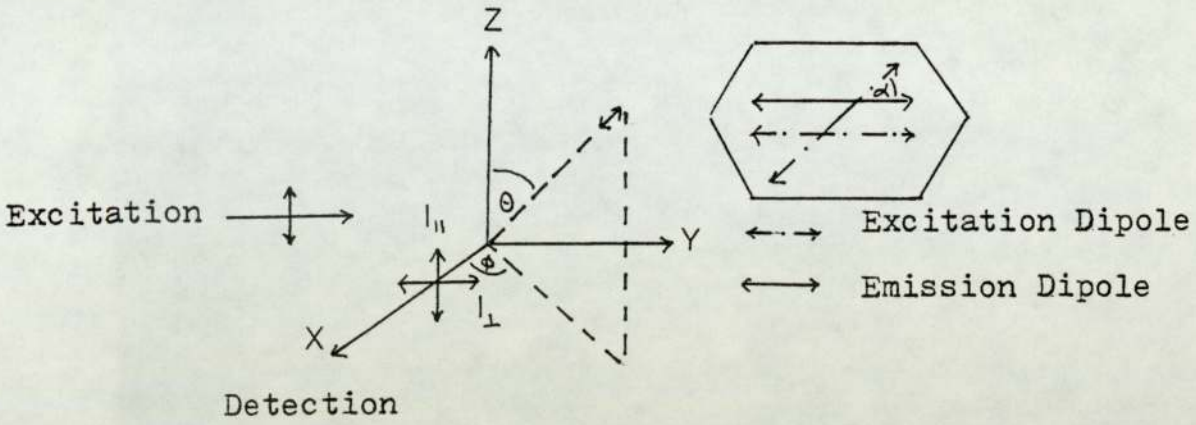


Figure 8. Transition dipoles in polar coordinates.

1.2.5 The application of fluorescent probes in the study of membrane

Fluorescent probing techniques have been widely accepted as a tool for the investigation of membrane structure as well as membrane function.

A probe of a biological system can be defined as a small molecule which when introduced into a biological system containing an organised aggregate of macromolecules like a membrane, will give us information, mainly through its spectroscopic properties, concerning its environment or even changes in it. The information that one can obtain through fluorescent probing techniques concerns membrane polarity , membrane fluidity, accessibility of the membrane by certain molecules, distances between macromolecules and electric potential.

(1) Membrane polarity:

If the dipole moment of the ground and excited states of a fluorophore differ, the interaction between polar solvent molecules and the fluorophore in these two electronic states will be different as well. It is known that the position of the emission maximum will reflect environmental polarity (Radda , 1975). For example, with 1-anilino naphthalene-8-sulphonate (ANS), the emission maximum and quantum yield of this probe are blue shifted and increased respectively when the probe is transferred from a polar to a non-polar environment (Stryer, 1965). Similar effects are observed when the probe is added to suspensions of lipids or membranes such as erythrocytes (Brocklehurst et al., 1970), or bacterial membranes (Cramer and Phillips, 1970). These two parameters have been used as a basic statements concerning the nature of 'hydrophobic binding sites' in membranes. However, there is a lot of evidence that other factors may be involved, so that interpretation of the membrane polarity based on these two parameters may be mistaken (see reviews by Radda, 1971 ; 1975 ; Azzi, 1975). For example, a polar solvent relaxing slowly may simulate a non-polar environment, or a high quantum yield may not be due to the absence of polar molecules only, but also to shielding from quencher or to particular geometries imposed by constraint upon the probe (Penzer, 1972). Hence it is necessary that before any definite conclusion can be drawn as to the physical nature of the change, other parameters

such as the affinity or the number of binding sites that probably have any influence on quantum yield and emission maximum should be taken into account. The dependence of the emission maximum of N-phenyl-1-naphthylamine (NPN) in various solvent systems is shown in Table 1.

(2) Membrane fluidity:

Membrane fluidity is determined by the components of the membrane and the way they interact with each other. For example, the length and the degree of unsaturation of fatty acid chains in membrane lipids have a profound effect on membrane fluidity (Stryer, 1981). Membrane fluidity is believed to regulate the membrane function. There are some factors that can influence membrane fluidity. These are:

(a) Effect of temperature

A temperature dependence crystalline-liquid-crystalline phase transition produces remarkable changes in the physical state and in the organisation of membrane lipid molecules.

Changes in both the hydrocarbon core and the polar interphase of the membrane at the phase transition might affect binding of ligands and the interactions among membrane components. These effects are expected to influence the permeability properties of the membrane below and above phase transition. Fluorescent probes are useful to study these properties, since lipids per se do not have optical or fluorescent properties.

Trauble and Overath (1973) used several probes

Table 1. Emission maxima of N-phenyl-1-naphthylamine (NPN) in various solvents*

Solvent	Emission (nm)	Maximum (CM^{-1})
water	460	21,700
20% ethanol	458	21,800
40% ethanol	449	22,300
60% ethanol	439	22,800
80% ethanol	431	23,200
Methanol	426	23,500
Ethanol	419	23,900
Propan-1-ol	421	23,800
Propan-2-ol	418	23,900
2-methylpropan-2-ol	416	24,600
Acetone	416	24,600
Ethandiol	430	23,260
1,2-propandiol	426	23,470
Glycerol	432	23,100

* Data taken from Radda (1975).

including ANS and NPN to investigate the lipid-phase transition of the fatty acid auxotroph of E. coli strain K 1062. From lipid-phase transition data, they predicted the structure of the membrane of E. coli. Besides that, they also predicted that (1) A fraction p= 80 $\pm$ 6% of the membrane lipids takes part in the lipid-phase transition. (2) A fraction μ= 53 $\pm$ 6% of these lipids is directly accessible to ANS molecules. NPN was employed to detect bacterial thermosensitivity by Mantsala and Lang (1973). Transition temperatures were different for different organisms tested, ranging from 30°C for Pseudomonas fluorescens and 44°C for Streptococcus thermophylus.

(b) Microviscosity

Microviscosity of the membrane, which can indicate the mobility or orientation of the fluorophores in the membrane, is an interesting phenomenon to be studied by the fluorescent probing techniques. Several probes that may be used in the investigation of microviscosity are retinol, perylene, ANS, 2-methyl anthracene, 1,6-phenyl 1,3,5 hexatriene (DPH) (Shinitzky and Barenholz, 1978). The microviscosity can be calculated from fluorescence polarization data by the relationship given in the Perrin equation:

$$\frac{\frac{1}{P} - \frac{1}{3}}{\frac{1}{P_0} - \frac{1}{3}} = 1 + \frac{KT\tau}{\eta V_0}$$

Where P_o is the degree of polarization measured in an extremely viscous medium, τ is the average lifetime of the probe molecules in the excited state, V_o is its effective volume, and η is the viscosity.

Cheng *et al* (1974) used 2-methyl anthracene to investigate the microviscosity of outer and inner membranes of *E. coli* . They found that the microviscosities were 37 and 73.5 cP for inner and outer membrane vesicles, respectively, illustrating the less rigid structure of the inner membrane. They also found that inner membrane vesicles are more permeable than the outer membrane vesicles to the probes used from quenching experiments. Helgerson *et al*. (1974) found that when colicin E_1 was added to *E. coli*, it caused an increase in the polarization of fluorescence of the cell-bound probe, NPN. They observed that colicin caused the rotational relaxation time to increase from 3.6 to 6.5 nsec and from 6.6 to 10.5 nsec when added at 21° and 12.5°C respectively. They suggested that it was due to (1) a colicin-induced increase in microviscosity in the cell envelope or (2) a redistribution of NPN to regions of the envelope with higher microviscosity. Fluorescence polarization is the technique most often used in the study of the viscosity of the biological membranes.

(c) Effect of ions

The fluorescence intensity of ANS in the presence of phospholipid vesicles or natural membrane is strongly increased by cations (Vanderkooi and Martonosi,1969).

This has been attributed to an increase of ANS binding facilitated by the suppression of electrostatic repulsion between the anionic ANS and the negatively charged phosphate or carboxylate of the lipid.

Rudy and Gitler (1972) observed small changes in fluorescence polarization of ANS upon addition of KCl to erythrocyte membranes. They also found that the viscosity of the membrane was reduced from 132 to 120 cP.

Trauble and Eibl (1974) investigated the effect of ions and pH on the ordered-fluid transitions of phospholipid in membranes. They found that when the pH was increased from 7.0 to 9.0 in phosphatidic acid, its transition temperature dropped by about 20°C. In this pH region, a small change in pH was enough to induce such a significant phase transition at constant temperature. On the other hand, monovalent cations such as lithium, sodium and potassium lowered the transition temperature and could be effective in inducing an ordered to fluid transition at constant temperature. Hence it can be inferred that small changes in the ionic environment can induce dramatic alteration in the membrane structure, monovalent and divalent cations being antagonists in this respect.

(d) Effect of cholesterol

The insertion of cholesterol into phospholipid vesicles has been shown to immobilise a spin labelled fatty acid (Waggoner et al, 1969). It stimulated the investigation of the molecular events following the insertion of cholestrol

in lipid membranes. This phenomenon has also been observed by fluorescence polarization studies(Badley at al., 1972). They employed ANS as a probe and demonstrated an increase in polarization, a decrease in lifetime and a decrease in the estimated actual motion of the probe of about 10^{o} as a consequence of the presence of cholesterol in lecithin membranes. In bacteria, cholesterol has not been detected as a component of the membrane. Those who are interested in the effect of cholesterol on the membrane fluidity, should consult the reviews by Radda (1971; 1975) and Azzi (1975).

The physical parameters mostly utilised for obtaining information about membrane fluidity are fluorescence polarization, time resolved emission anisotropy, excimer emission (Galla and Sackman, 1974) and fluorescence quenching (Radda and Vanderkooi, 1972).

(3) Accessibility of the membrane by certain molecules

Fluorescent probes bound to membrane can be used to investigate the accessibility of their binding sites by a number of molecules, which can alter the fluorescent behaviour of the probe. An example of this is the solvent isotope effects on the fluorescence of ANS. When the probe is bound to membranes it becomes less accessible to the isotope, as indicated by the decreased value of the fluorescence when the membranes are in $^{2}H_{2}O$ with respect to the value of the unbound probe in water (Radda and Vanderkooi, 1972). Fluorescent quenchers have also been

used in the study of accessibility of the membrane, and the data have been used to predict the location of the probes in the membrane.

(4) Distance between macromolecules

The distance between macromolecules and the probe can be predicted by energy transfer occurring between the probe and some components of the macromolecules, such as tryptophan residues of the protein, as the probe relaxes from the excited state to the ground state.

A structural difference between the membranes of E. coli and Bacillus megaterium was inferred from the presence of tryptophan-ANS energy transfer in the former but not in the latter (Ballard et al., 1972).

From the use of energy transfer in membranes, some proximity relationships can be calculated in a qualitative way. Therefore by selecting an appropriate fluorophore, it may be possible to measure the distances between proteins and lipids and their changes associated with function.

To believe is difficult. Not to believe is impossible.
Victor Hugo.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Organisms

The organism used throughout the study was Pseudomonas cepacia NCTC 10661

It was maintained on nutrient agar slopes. Fresh nutrient agar slope cultures were prepared from single colony isolates at approximately monthly intervals. It was kept at 4° in the refrigerator.

Prior to an experiment, a sample from an agar slope was streaked onto a nutrient agar plate, incubated overnight at 37° and a single colony inoculated into chemically-defined medium (CDM) and cultivated in an orbital shaker at 37°. A further subculture in CDM was used as inocula for growth experiments for a period of one week, after that time it was discarded and a fresh inoculum was prepared by the same method.

Pseudomonas aeruginosa PAO 1 and E. coli NCIB 8277 were kindly provided by Dr. P.A. Lambert (Department of Pharmacy, University of Aston in Birmingham, England).

2.1.2 Media

Nutrient broth (NB) : Oxoid nutrient broth, code CM_1.

Nutrient agar (NA) : Oxoid nutrient agar, code CM_3.

They were purchased from Oxoid Ltd, London SE1 9HF and were prepared according to the manufacturer's instructions.

Chemically-defined medium : The basic CDM used for the routine subculturing of P. cepacia is described in Table 2. Modifications of this medium were used for growth and membrane studies and will be discussed in the relevant sections.

Table 2 : Basic Chemically-defined medium

Ingredient	Final concentration (M)
Glucose	0.02
KCl	0.003
NaCl	0.003
$MgSO_4.7H_2O$	32×10^{-4}
$(NH_4)_2SO_4$	0.012
$FeSO_4.7H_2O$	2×10^{-5}
K_2HPO_4	12×10^{-4}
MOPS	5.0×10^{-2}

Medium pH 7.4

MOPS (3-(N-morpholino)propane sulphonic acid : It was obtained from Sigma Chemicals Company, Poole, Dorset, England. It is an organic buffering agent with a pKa of 7.2 (range 6.5 - 7.9). Its pH was adjusted with NaOH and it was used at a concentration of 5.0×10^{-2}M to buffer media containing low concentrations of phosphate.

All the medium constituents except MOPS were separately sterilised by autoclaving at 121° for 20 minutes. MOPS and phosphate buffer which was used in the study of sulphate limitation were sterilised by filtration through a 0.2 um pore size membrane filter.

2.1.3 Chemicals

Water : Water used throughout the study was first deionised and then distilled in a glass still.

All chemicals used in the preparation of CDM were of Analar grade, except for the study of iron limitation, in which Aristar grade were used (when available). Both grades were obtained from British Drug Houses Chemicals Ltd, Poole, Dorset, England.

Organic solvents used were obtained from British Drug Houses, Chemicals Ltd, Poole, Dorset, England.

Tris(hydroxymethyl)aminomethane (Tris) : obtained from Sigma Chemicals Company, Poole, Dorset, England.

Acrylamide : British Drug Houses, Chemicals Ltd, Poole, Dorset, England.

N,N'-methylene-bisacrylamide (Bis): obtained from Sigma Chemicals Company, Poole, Dorset, England.

Ammonium persulphate: British Drug Houses, Chemicals Ltd, Poole, Dorset, England.

2-mercaptoethanol : Sigma Chemicals Ltd, Poole, Dorset.

N,N,N',N'-Tetramethylethylene diamine (TEMED) : British Drug Houses, Chemicals Ltd, Poole, Dorset, England.

Coomassie brilliant blue R-250 : obtained from Sigma Chemicals Company, Poole, Dorset, England.

Folin-Ciocalteu phenol reagent : British Drug Houses, Chemicals Company, Poole, Dorset, England.

Cupric sulphate : British Drug Houses, Chemicals Ltd.

N-phenyl-1-naphthylamine : obtained from Koch-light Laboratories Ltd, Coinbrook Busks, England. It was further purified by recrystallizing it twice from methanol-distilled water, dried in vacuum dessicator with phosphorus pentoxide and then kept at $4^{o}C$ in the refrigerator.

3,3'-dipentyloxacarbocyanine iodide (CC_5) : was generous gift of Dr. A.Z. Britten, Department of Pharmacy, University of Aston in Birmingham, England.

2p-toluidinyl-naphthalene-6-sulphonate (potassium salt) : Sigma Chemicals Company, Poole, Dorset, England.

6-anilino-1-naphthalene-sulphonic acid : Sigma Chemicals Ltd, Poole, Dorset, England.

Bovine serum albumin : Sigma Chemicals Company.

SDS-molecular weight markers: Sigma Chemicals Company.

Sucrose : Analar grade, obtained from British Drug Houses, Chemicals Ltd, Poole, Dorset, England.

Pancreatic deoxyribonuclease : Sigma Chemicals Company.

Pancreatic ribonuclease : Sigma Chemicals Company.

N-Lauroyl Sarcosine (sodium salt): Sigma Chemicals Company.

Sodium gluconate : British Drug Houses, Chemicals Ltd.

Sodium succinate : British Drug Houses, Chemicals Ltd.

Solution of sodium lactate : about 70% (W/V), obtained from British Drug Houses, Chemicals Company, Poole, Dorset.

Lysozyme : Crystalline, from egg white, obtained from British Drug Houses, Chemicals Company, Poole, Dorset.

Phospholipid standards: Sigma Chemicals Company, Poole, Dorset, England.

2-methyl anthracene : obtained from Aldrich Chemical Company Ltd, The Old Brickyard New Road, Gillingham, Dorset, SP8 4BR, England.

1,6-Diphenyl-1,3,5-hexatriene (DPH) : obtained from Aldrich Chemical Company Ltd.

2.1.4 Apparatus

Spectrophotometer : Unicam SP 600 supplied by Pye-Unicam Instruments Ltd., York St, Cambridge, England, or Cecil, model CE 373, Cecil Instruments Ltd, Milton Industrial Estate, Cambridge Road, Milton, Cambridge.

Spectrophotofluorometer : Aminco-Bowman Spectrofluorometer and a Hewlett Packard 7035B X-Y recorder were used to obtain the fluorescence spectra.

UV-spectrophotometer : Unicam 8000 Pye-Unicam Instruments Ltd., was used to obtain the UV spectra.

pH meter : Pye model 290 pH meter, obtained from Pye-Unicam Instruments Ltd.

Blood sample tubes : with or without lithium heparin, obtained from Searle & Co Ltd, P.O.Box 53, Lane End Road, High Wycombe, Bucks.

Electrophoresis power supply : model SAE 2761, Shandon Southern Products Ltd, 95 Chadwick Road, Astmoor Industrial Estate, Runcorn, Cheshire, WA7 1PR or model 500, Bio-Rad Laboratories Ltd, 27 Homesdale Road, Bromley, Kent, BR2 9LY.

Water bath : Mickle reciprocating shaker water bath, obtained from Camlab Ltd, Cambridge, England.

Centrifuge : model B20, International Equipment Company, 300 Second Avenue, Needham Heights, Massachusetts, U.S.A. or MSE, model 18, MSE, Manor Royal, Crawley, Sussex,RH10 2QQ, England.

Cuvettes : For measurement of the growth of P. cepacia, glass cuvettes obtained from Pye-Unicam Instruments Ltd., were used. For the study of fluorescence, fluorescence cuvettes were obtained from Hellma (UK) Ltd.

Automatic MLA pipettes : 0.1 ml and 0.25 ml sizes and tips (can be sterilised by autoclaving), obtained from Frost Instruments Ltd., Wokingham, Berkshire, RG11 1BZ, England.

Whirlimixer : obtained from Fison Scientific Apparatus Ltd.

Glassware : Corning Glass Ltd., (Pyrex) Laboratory Division, Wearglass Works, Sunderland, England.

Chart recorder : model 28000, Bryans Southern Instruments Ltd., Willow Lane, Mitcham, Surrey, CR4 4UL.

Syringes : 5 ml, 10 ml and 20 ml syringes, obtained from Gillette Surgical, Gt West Road, Isleworth, Middx.

Millipore membrane filtration apparatus : Millipore

Ltd., Wembley, Middlesex.

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

French press : model J4-3398A, Aminco, American Instruments Company., Inc., Silver Spring, Maryland, USA.

Protractor eyepiece : for the goniometer, obtained from Ealing Beck Ltd, 15 Greycaine Road, Watford, WD2 4PW.

2.1.5 Treatment of Glassware

Glassware was rinsed with tap water and totally immersed in 5% V/V Decon 90 (Decon Laboratories Ltd., Ellen Street, Portslande, Brighton BN4 1EQ), at room temperature overnight. It was then rinsed sequentially with distilled water, 1%(V/V) hydrochloric acid, six times in distilled water and twice in deionised distilled water. After that, it was dried at 60°, covered with aluminium foil and sterilised by dry heat (at 160° for three hours).

2.2 Basic Experimental Methods

2.2.1 Viable counts

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

The spread plate method consisted of spreading known volumes of the diluted culture on overdried nutrient agar plates. Serial dilutions of ten or one hundred fold were made in nutrient broth so that 0.1 or 0.25 ml samples gave 50 - 200 colonies per plate. At each dilution three replicate plates were made and the plates incubated at 37^{o} for 24 h. 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 20 µl of diluted culture were deposited on overdried nutrient agar plates. One plate had up to nine drops consisting of three drops of each of three dilutions. After incubation for 18 h at 37^{o}, counts were made in the drop areas showing the largest number of colonies without confluence and the mean count per drop gave the viable count per 20 µl of that dilution. All counts were performed in triplicate.

The reproducibility of the counting procedures was tested by performing five replicate counts and the results subjected to an analysis of variance (Tables 3 and 4).

Table 3

Variation of replicate viable counts of culture of P.cepacia (spread plate method)

REPLICATE PLATES \ COUNT	A	B	C	D	E
1	104	99	99	95	102
2	100	109	106	99	98
3	104	91	82	89	109
4	89	112	91	111	98
5	97	101	109	94	102
MEANS (x)	98.7	102.4	97.4	97.6	101.8
STANDARD DEVIATION (Sx)	6.2	8.3	11.1	8.3	4.5
COEFF. OF VARIATION	6.3%	8.2%	11.3%	8.5%	4.4%
MEANS OF POPULATION (μ)			99.6		
STANDARD DEVIATION OF POPULATION			2.4		
COEFF. OF VARIATION OF MEANS			2.4%		

Table 4

Variation of replicate viable counts of culture of P.cepacia (Miles & Misra method)

REPLICATE PLATES \ COUNT	A	B	C	D	E
1	22	19	18	25	22
2	19	21	20	21	18
3	24	23	19	21	21
4	23	18	23	18	18
5	18	18	21	23	22
6	21	22	20	20	19
MEANS (x)	21.2	20.2	20.2	21.3	20
STANDARD DEVIATION (Sx)	2.3	2.1	1.7	2.4	1.9
COEFF. OF VARIATION	11%	10.6%	8.5%	11.4%	9.5%
MEAN OF POPULATION (μ)			20.6		
STANDARD DEVIATION OF MEANS			0.6		
COEFF. OF VARIATION OF MEANS			3%		

2.2.2 Measurement of bacterial cell concentration

The concentration of a bacterial suspension is conveniently determined by optical density measurement with a spectrophotometer or by total count with a microscope. Electronic counting (Coulter) was not done in this study.

In optical density measurements, when a monochromatic beam of light passes through a suspension of bacterial cells, part of the light will be scattered due to the difference in the refractive index between the cell surface and the medium. Hence the photometric determination of bacterial concentrations depends primarily on light scattering rather than light absorption. At relatively low concentrations, the light scattered by the bacterial cells is directly proportional to the concentration of the cells in the suspension, in which a useful relationship similar to the Beer-Lambert law of absorption exists. It may be expressed as following:

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

Where

I = Intensity of emerging light.

I_o = Intensity of incident light.

c = Concentration of bacteria in the suspension.

l = Path length (distance travelled by the light through the cell suspension).

e = The extinction coefficient.

This relationship holds at low bacterial concentrations. At high concentrations, the Beer-Lambert law is no longer

obeyed. This has been suggested to be due to secondary scattering of the light by the cells (Meynell and Meynell, 1970).

2.2.2.1 Selection of wavelength

The amount of light scattered by a bacterial suspension is directly proportional to the ratio of the cell size to the wavelength of the incident light (Koch , 1961).

In selection of a suitable wavelength for the measurement of the turbidity of a cell suspension, several factors should be taken into account. These are medium constituents, pigments produced by the bacteria or bacterial metabolic products. They may absorb light of certain wavelengths, so that the wavelength should be selected that absorption by these materials is no longer measured. The use of wavelength below 380 nm is not practical, since proteins and nucleic acids absorb within this range. P. aeruginosa under certain growth conditions produces a pigment called pyocyanin which has absorption maxima around 390 nm and can still be detected at 460 nm (Watkin, 1970), but is undetectable at 470 nm. This is why 470 nm is selected wavelength for the measurement of growth or lysis in P. aeruginosa (Kenward, 1975). This wavelength was also used throughout this study in the measurement of growth or bacterial concentrations.

2.2.2.2 Relationship between optical density (OD) and cell concentration

Kenward (1975) found that the relationship between OD and cell concentration obeyed the Beer-Lambert law up to an OD of 0.3 with P.aeruginosa, above which the OD increased less than was predicted in proportion to the increase in cell concentration. However, when these cell suspensions were diluted with CDM without glucose to give readings in the range of optical densities 0.03 to 0.28, then linearity was restored. To test whether the same relationship occurred in P.cepacia, it was grown in basic CDM (Table 2) overnight. The cells were harvested by centrifugation at 8000 x g for 10 min, washed twice with normal saline and resuspended in this solution. From it a series of dilutions of the bacterial suspension were prepared using normal saline as diluent and the OD at 470 nm measured (Fig. 9). P.cepacia was found to behave similarly to P.aeruginosa. Therefore in the measurements of cultures above an OD of 0.3, they should be diluted to give readings in the range of optical densities 0.03 to 0.28.

2.2.2.3 Relationship between OD and dry weight at different stages of the growth curve

This was done with 1250 ml of the medium (Table 5) in a 5 litre flask. It was done in duplicate.

The medium was designed to support P.cepacia 10661 to grow linearly to an OD of about 1.0 before Mg^{2+} became

Fig. 9 Relationship of optical density and cell concentration (*P.cepacia* NCTC 10661).

●——● , Diluted suspension

□——□ , Undiluted suspension

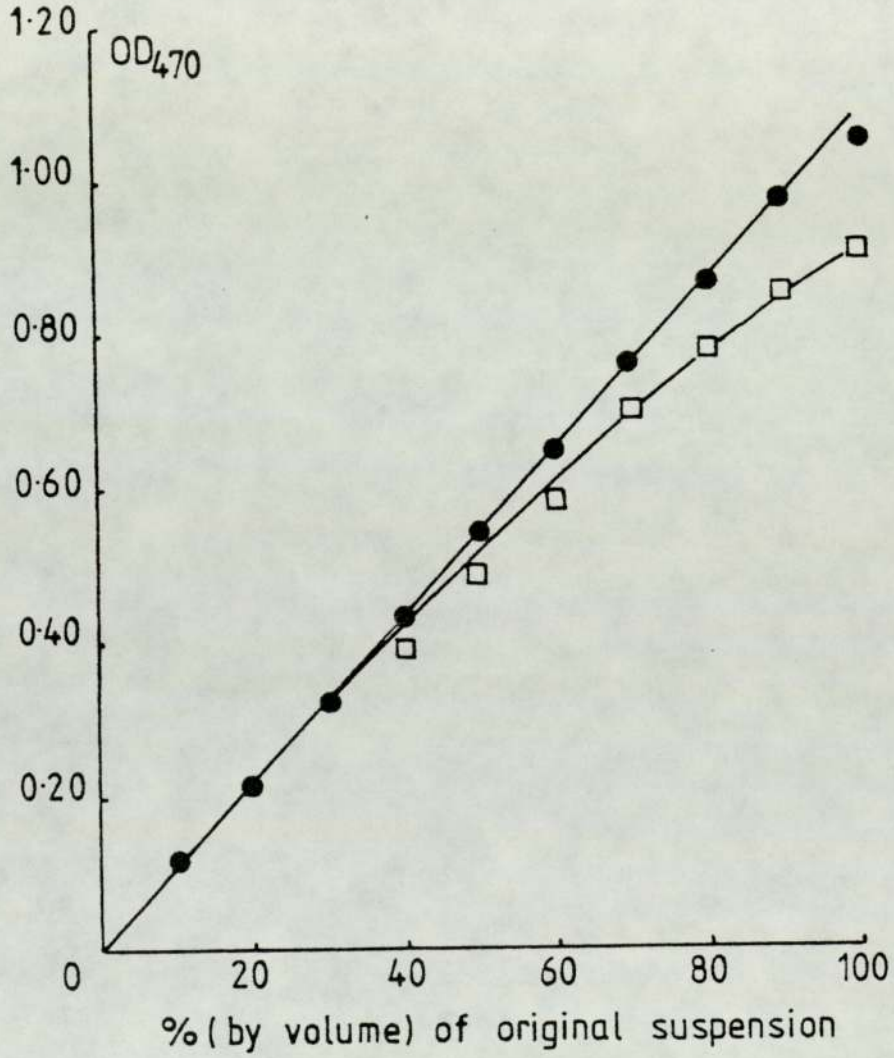

1·20
OD470
1·00
0·80
0·60
0·40
0·20
0
20
40
60
80
100
% (by volume) of original suspension

Table 5

CDM used for the study of the relationship between OD_{470} and dry weight

Ingredient	Final concentration (M)
Glucose	0.02
KCl	0.003
NaCl	0.003
$MgSO_4.7H_2O$	1.9×10^{-5}
$(NH_4)2SO_4$	0.012
$FeSO_4.7H_2O$	2×10^{-5}
K_2HPO_4	12×10^{-4}
MOPS	0.05

medium pH=7.4

limited in the culture. The medium was stirred vigorously with a magnetic stirrer at 37° in the incubator room. Accurate volumes of the culture were removed at different stages of the growth curve to a pre-weighed centrifuge tube. As the cells were removed from the flask, 40% formaldehyde solution (V/V) was added to the cell suspension to final concentration of 5% (V/V). The cells were centrifuged at 10,000 x g for 10 min, washed once with distilled water and then kept in a vacuum dessicator containing phosphorus pentoxide until constant weight was obtained. The results are presented in figures 10 and 11. A linear relationship between OD and dry weight was obtained along the logarithmic

Fig. 10 Growth of P. cepacia in CDM containing 19.2 μM magnesium sulphate in 1.25 L culture.

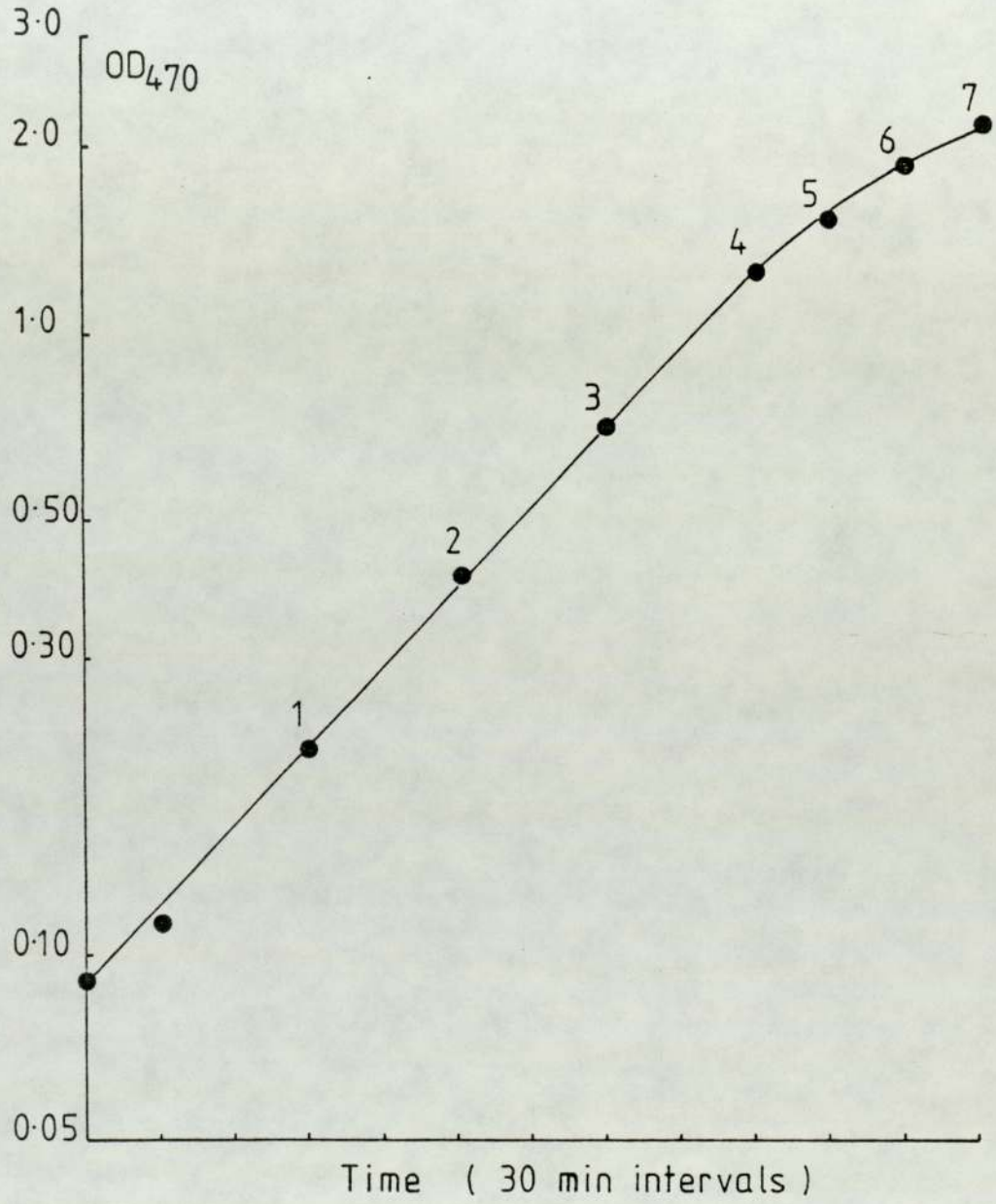
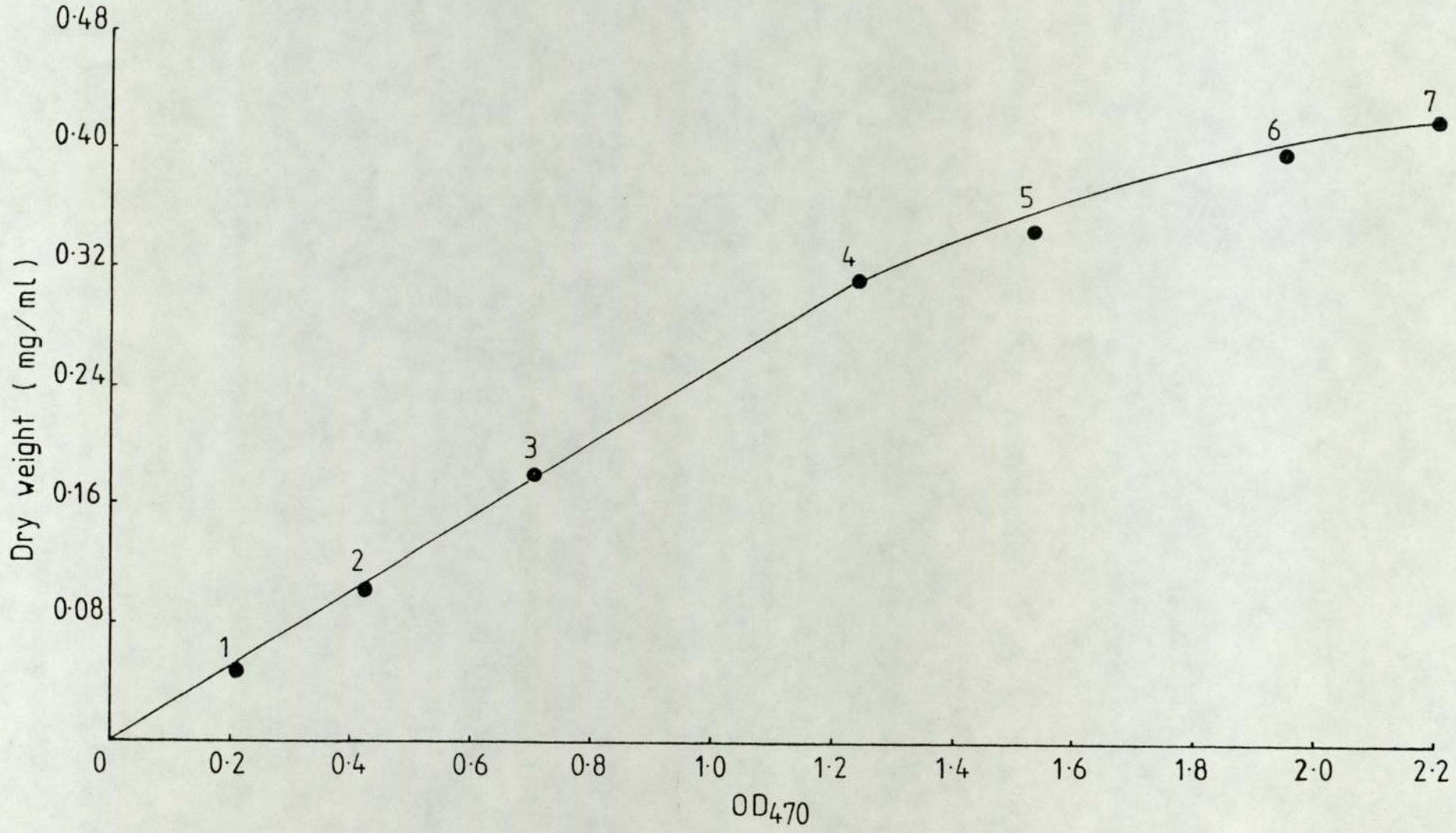

3·0
OD470
2·0
1·0
0·50
0·30
0·10
0·05
1
2
3
4
5
6
7
Time (30 min intervals)

Fig. 11 Relationship between OD and dry weight at different stages in the growth curve of batch grown, magnesium-depleted cultures of *P. cepacia* .

Note: Numbers correspond to points on the growth curve (see Fig. 10).


0·48
0·40
0·32
0·24
0·16
0·08
Dry weight (mg/ mL)
1
2
3
4
5
6
7
0
0·2
0·4
0·6
0·8
1·0
1·2
1·4
1·6
1·8
2·0
2·2
OD470

phase of the growth curve. However, when the cells entered the stationary phase due to the lack of magnesium in the culture, the OD increased less than that was predicted in proportion to the increase in dry weight. This probably is due to the difference in cell mass or surface/volume ratio between the cells in logarithmic phase and those in the stationary phase, also possibly surface changes. In another experiment, the dry wet of cells growing exponentially to OD_{470} of 1.0 was 0.28 $\pm$ 0.006 mg/ ml.

2.2.2.4 Relationship between optical density and viable counts at different stages of the growth curve

The relationship between optical density and the viable count at different stages of the growth curve was found to be linear in P.aeruginosa by Kenward (1975). However, he performed the experiments within the logarithmic phase. In this experiment, it was attempted to study the relationship up to the stationary phase. This was done by cultivating P.cepacia 10661 in 100 ml volumes in 500 ml conical flasks. The formula of the medium is shown in Table 5, except that the magnesium sulphate concentration was reduced by half (8 μM). It was cultivated in a shaking water bath at 37^{o} with shaking rate of 120 throws per minute. Every 30 minutes, in addition to taking the optical density measurements, suitable dilutions of 1 ml samples of the growing culture were made to prepare five replicate plates for a viable count determination. Growth curves were constructed by plotting

log viable count and log OD_{470} against time (Fig. 12). Both curves exhibited similar characteristics and the exponential growth rate constants were the same (3.19×10^{-3}/min ≡ to a doubling time of 90 min). The relationship between viable count and optical density was linear (Fig. 13). The data used to construct Fig. 13 was used to calculate the correlation coefficient 'r' using the formula :

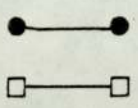
$$'r' = \frac{C}{S_x^2 \cdot S_y^2} \quad ,$$

Where 'C' is the covariance, $(X.Y - \bar{X}.\bar{Y})/n-1$, '$S_x^2$' the variance of the optical density readings, $\Sigma(X-\bar{X})^2/n-1$, and 'S_y^2' the variance of the viable count determinations, $\Sigma(Y - \bar{Y})^2/n-1$. From the data, C=1.30, S_x^2 =0.137 and S_y^2 = 12.715. The calculated value of 'r' for n-2 degree of freedom, at 1% and 5% significant levels is 0.736 and 0.523 respectively. Thus, the correlation between optical density and viable counts was significant within these levels. In another experiment, it was found that there were 8.13×10^8 cells/ml, for cells growing exponentially to OD_{470} of 1.0.

2.2.3 Batch cultures for the study of growth

Growth experiments were done by using as an inoculum a culture of P.cepacia NCTC 10661 grown overnight in complete CDM (Table 2), which had been washed twice in a medium lacking the nutrient under study and resuspended in this medium to give an OD of about 2.5. 0.25 ml of this cell

Fig. 12 Growth curves of P.cepacia constructed from optical density and viable count data.



Optical density data

Viable count data

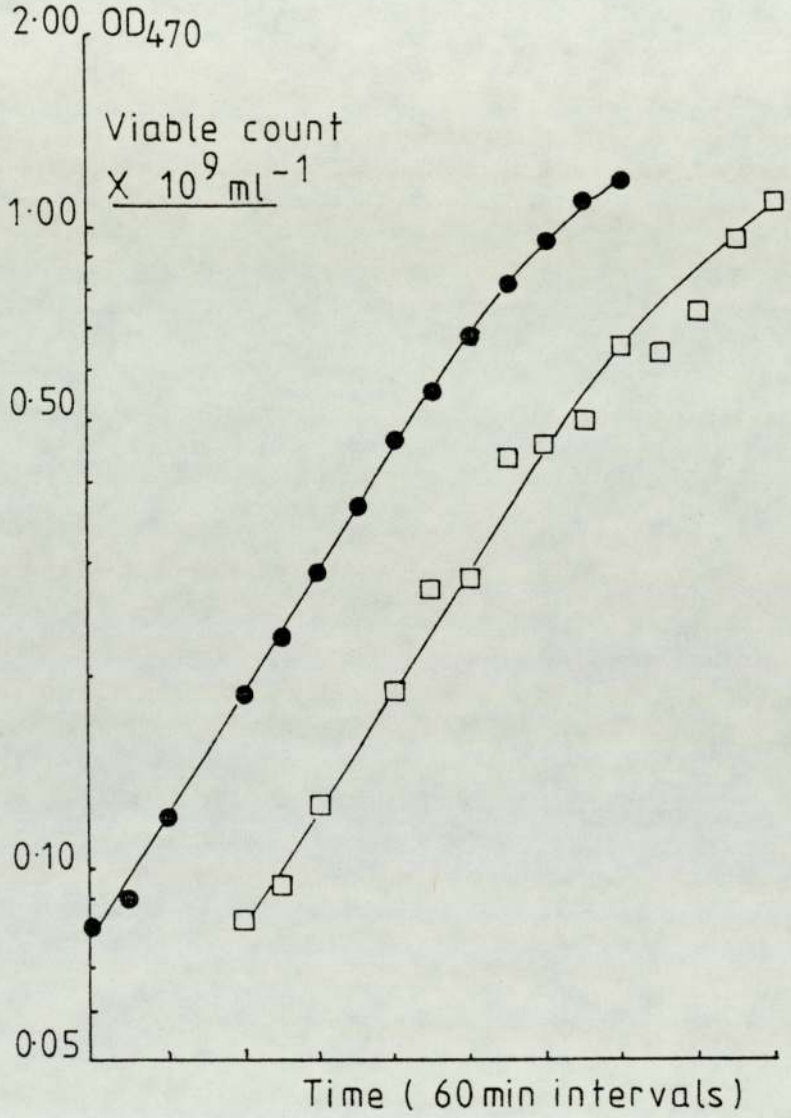
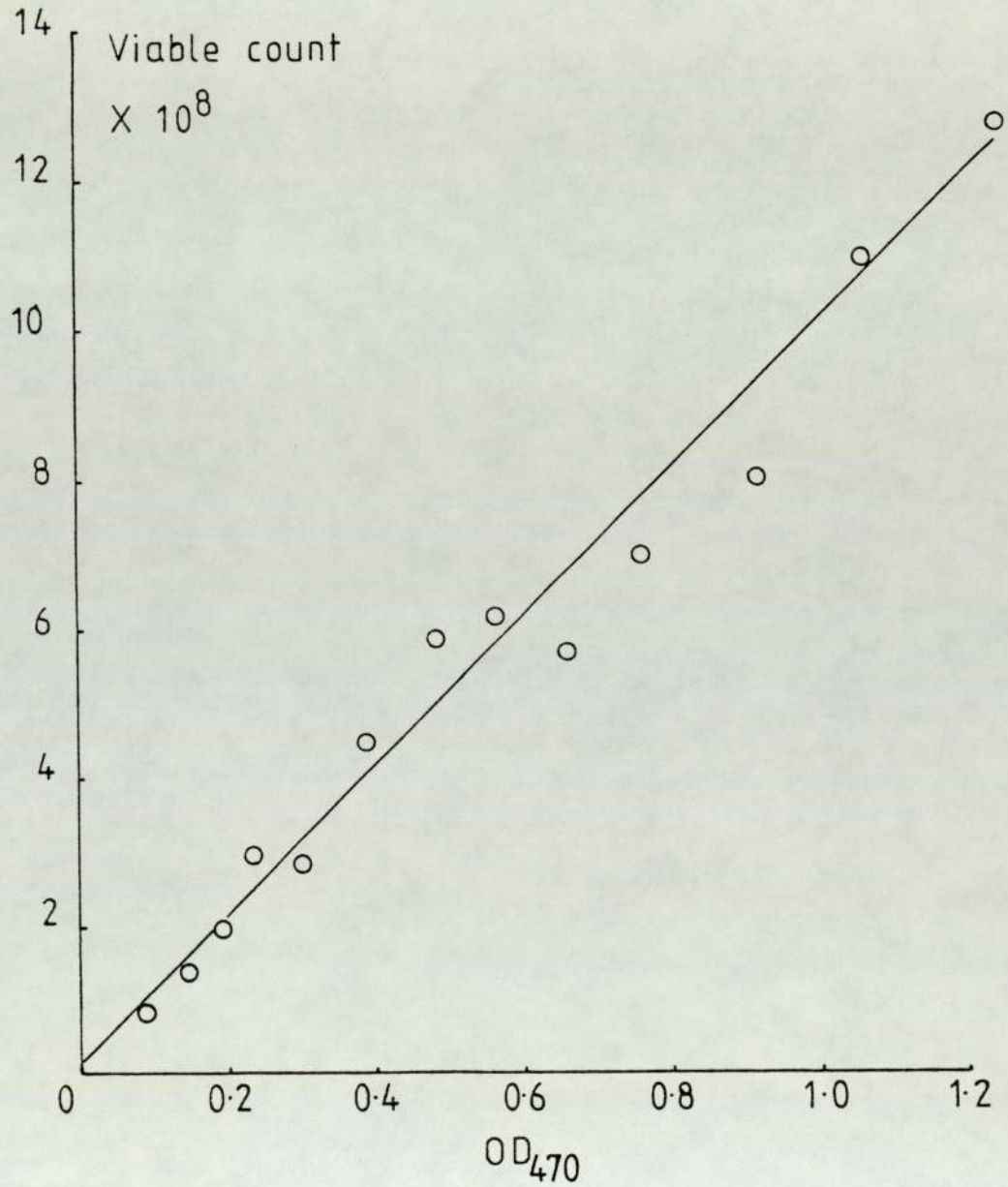

2·00
OD470
Viable count
X 10^9 ml^-1
1·00
0·50
0·10
0·05
Time (60 min intervals)

Fig. 13 Relationship between OD_{470} and viable count in P. cepacia at different stages of growth curve.


Viable count
X 10^8
14
12
10
8
6
4
2
0
0·2
0·4
0·6
0·8
1·0
1·2
OD_{470}

suspension was used to inoculate 24.75 ml of the appropriate medium (see Table 6) in 100 ml flasks.

Flasks were incubated at 37° in a water bath with a shaking rate of 120 throws per minute. Growth was then followed by taking the optical densities of these cultures with a pasteur pipette at timed intervals. When optical densities of these cultures reached above 0.3, samples taken for optical density measurements were diluted appropriately. Undiluted samples were returned to the flasks to prevent the excessive reduction of culture volumes, but samples which had been diluted with distilled water were subsequently discarded.

The above procedures are suitable in the study of glucose, sulphate, magnesium, nitrogen and phosphate limitations in P.cepacia. However, it was not a success in the study of iron limitation, so that a slight modification of the above procedure was done. P. cepacia grown overnight in iron-depleted medium was used as an inoculum. The reason for this modification will be discussed in section 2.2.4.

2.2.4 Growth of P. cepacia in the supernatant of iron-depleted medium

P. cepacia NCTC 10661 was grown in an iron-depleted medium and also in complete CDM (Table 2). Next morning the OD of the iron-depleted culture was measured at 30 min intervals, until no further increase of the OD between two readings was observed. For the complete CDM culture, the OD

Table 6

Chemically-defined media used to quantify nutrient requirements of P.cepacia

Ingredient (Molar)	Medium component under investigation					
	Glucose	NH_4^+	PO_4^{3-}	SO_4^{2-}	Mg^{2+}	Fe^{2+}
KCl	0.003	0.003	0.003	0.003	0.003	0.003
NaCl	0.003	0.003	0.003	0.003	0.003	0.003
$MgSO_4 . 7H_2O$	0.0032	0.0032	0.0032	-	*	0.0032
$(NH_4)_2SO_4$	0.012	*	0.012	*	0.012	0.012
$FeSO_4.7H_2O$	2×10^{-5}	2×10^{-5}	2×10^{-5}	-	2×10^{-5}	*
Glucose	*	0.02	0.02	0.02	0.02	0.02
K_2HPO_4	12×10^{-4}	12×10^{-4}	*	-	12×10^{-4}	12×10^{-4}
MOPS	0.05	0.05	0.05	-	0.05	0.05
$MgCl_2.6H_2O$	-	-	-	16×10^{-5}	-	-
$FeCl_2.4H_2O$	-	-	-	27×10^{-5}	-	-
$(NH_4)_2HPO_4$ **	-	-	-	1.8×10^{-2}	-	-
$(NH_4)H_2PO_4$ **	-	-	-	4.8×10^{-4}	-	-

* Component added at various concentrations., see Figs. 18 - 32.

** It was used in the study of sulphate limitation. Also used as a buffer system.

was only taken once. Both cultures were harvested by centrifugation, washed twice with the supernatant of the iron-depleted medium and resuspended in this washing medium to give an OD_{470} of 2.0.

The supernatant of the iron-depleted medium was divided into two in 4 x 100 ml conical flasks (two flasks in each group). The first group had 24.75 ml of the supernatant and the second group had 20 ml of the supernatant in which 4.75 ml sterilised ferrous sulphate solution was added to give a final concentration of 2×10^{-5}M. 0.25 ml of the cells grown in the iron-depleted culture was then inoculated to one of each group. A similar procedure was applied to the cells grown in complete CDM. The growth of these cells was then followed by measuring the optical densities at 30 min intervals. The result is presented in Fig. 14.

2.2.5 Growth of 8 litre batch cultures for membrane separation (Sucrose gradient centrifugation method)

The composition of the media are shown in Table 7. The components of the medium, except glucose, were mixed in 4 x 5 litre conical flasks with magnetic followers inside, and autoclaved at 121° for 30 min. At the time of inoculation, sterile glucose solution was added and the final volume of each culture was 2 litres. These flasks were then put in the incubator room at 37° over the magnetic stirrer for cultivation.

An overnight culture in complete CDM was used as an

Fig. 14 Growth of P. cepacia in the supernatant of iron-depleted culture.

●——● , Cells grown in iron-depleted medium, no iron added.

■——■ , Cells grown in iron-depleted medium, $2x10^{-5}$M $Fe^{2+}$was added.

○——○ , Cells grown in CDM, no iron added.

□——□ , Cells grown in CDM, $2x10^{-5}$M $Fe^{2+}$was added.

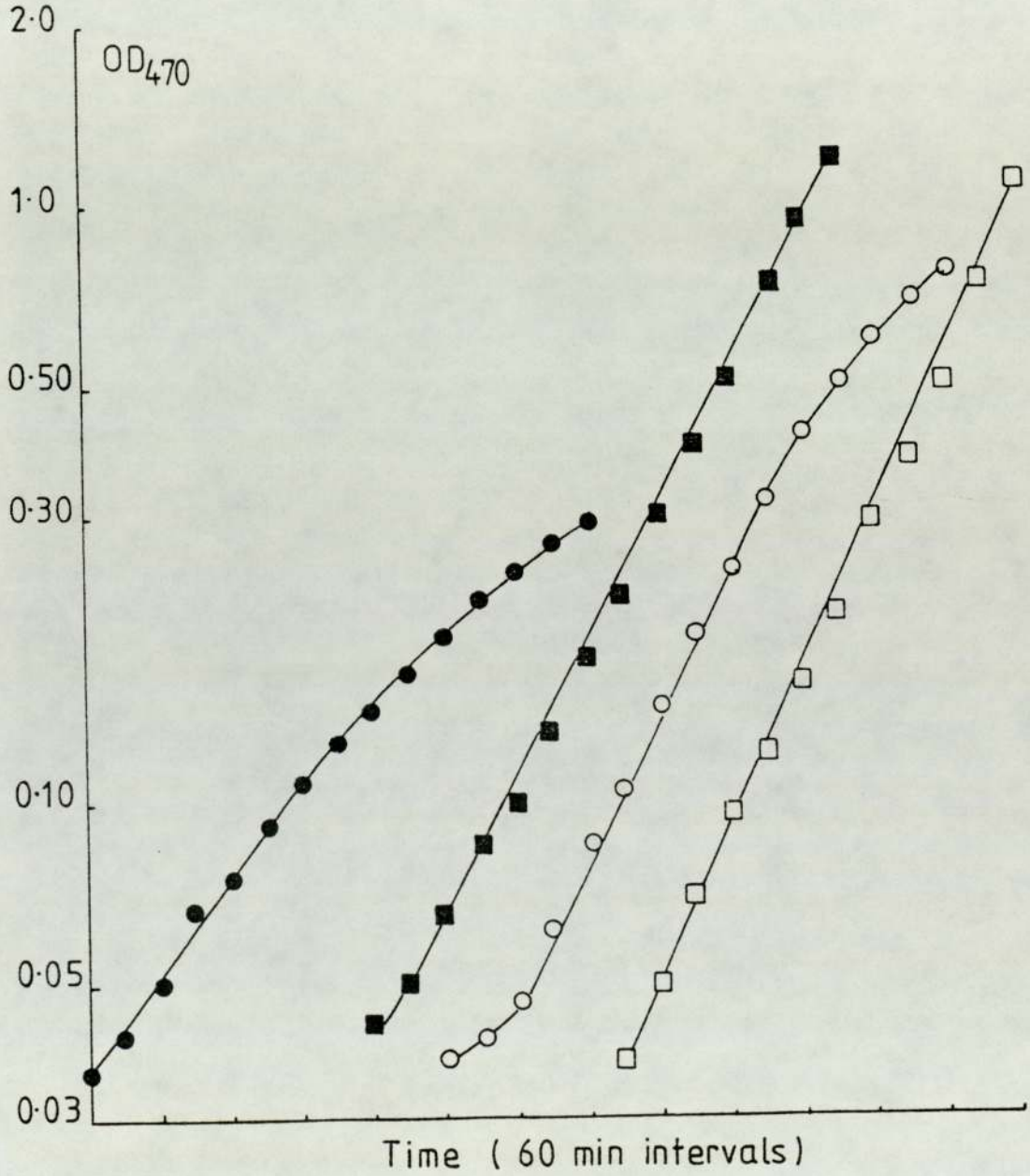

2·0
OD470
1·0
0·50
0·30
0·10
0·05
0·03
Time (60 min intervals)

Table 7

Chemically-defined medium used for 8 L culture of Pseudomonas cepacia

Ingredient (Molar)	Limiting Nutrient	
	Mg^{2+}	Fe^{2+}
Glucose	0.02	0.02
KCl	0.003	0.003
NaCl	0.003	0.003
$MgSO_4.7H_2O$	1.9×10^{-5}	32×10^{-4}
$(NH_4)_2SO_4$	0.012	0.012
K_2HPO_4	12×10^{-4}	12×10^{-4}
$FeSO_4.7H_2O$	2×10^{-5}	4×10^{-7}
MOPS	0.05	0.05

Medium pH 7.4

inoculum in the case of magnesium-depleted cultures. For iron-depleted cultures, overnight iron-depleted culture was used as an inoculum. The cells were harvested at 8,000 x g for 10 min, washed once with sterile normal saline and resuspended in this solution to give OD of approx. 2. 3 ml of this bacterial suspension was inoculated into each of 2 l medium described above. The speed of the magnetic follower was adjusted so that the vortex extended down to the follower. This provided a large surface area for gaseous exchange between the atmosphere and the culture, due to the numerous air bubbles resulting from the vortex touching the follower. The cultivation of the cells was performed in the

incubator room at 37 ± 1° for 24 hr. The maximum OD reached by Mg^{2+}-depleted culture was approx. 1.2 and was 0.8 for iron-depleted cultures (OD reached by oxygen-depleted complete CDM and nutrient broth cultures was approx. 3). These cultures were harvested and used for the preparation of outer and cytoplasmic membranes as will be discussed in section 2.2.6.

2.2.6 Preparation of the cell envelopes for the separation of the outer and cytoplasmic membranes (Sucrose density gradient centrifugation)

The method was described by Hancock and Nikaido (1978) with some modifications.

Eight litres of cells grown as described in section 2.2.5 were harvested by centrifugation at 8,000 x g for 10 min. This and all subsequent operations were done at 4°. The reagents and French pressure press were pre-cooled by keeping in the refrigerator for overnight. The cells were washed once with 30 mM Tris-buffer, pH 8.0 and resuspended in 20 ml of 20% (W/V) sucrose in 30 mM Tris-buffer, pH 8.0 containing 2 mg deoxyribonuclease and 2 mg ribonuclease. The cells were then passed three times through a French pressure press at 15,000 lb/in^2 (1055 kg/cm^2), after which 4 mg of egg white lysozyme was added and incubated in the ice-bath for 20 min. The preparation was then diluted with 40 ml of 30 mM Tris-buffer, pH 8.0. Cell debris was re moved by centrifugation at 1,500 x g for 10 min, usually it was necessary to repeat this procedure once. The supernatant was then centrifuged

at 38,000 x g for 60 min. The pellet was then resuspended in 25 ml 20%(W/V) sucrose in 30 mM Tris-buffer,pH 8.0. Two millilitres were layered onto a sucrose step gradient containing 9 ml each of 70% (W/V) sucrose and 60% sucrose in 30 mM Tris-buffer,pH 8.0. The gradient was centrifuged at 100,000 x g in a MSE 20° angle rotor for 14 to 17 hrs. Three bands were observed and removed by suction with a pasteur pipette from above and the individual samples were diluted with cold distilled water, centrifuged at 38,000 x g for 60 min and washed twice with distilled water to remove traces of sucrose. The resultant pellets were resuspended in a small amount of distilled water and frozen at -20°C. The success of separation was checked by analysing several enzymes as described in section 2.2.9.

2.2.7 Specific solubilization of the cytoplasmic membrane of Gram-negative bacteria by Sarkosyl

Organisms used in this study were P.cepacia NCTC 10661, P. aeruginosa PAO 1 and E. coli NCIB 8277.

P.aeruginosa PAO 1 and E.coli NCIB 8277 were grown overnight in double strength nutrient broth at 37°, whereas P.cepacia NCTC 10661 was grown in 100 ml medium in 500 ml conical flasks (see Table 8) or described in section 2.2.5.

The cells were harvested by centrifugation at 8,000 x g for 10 min. This and all subsequent procedures were done at 4°C. The pellet was washed once with cold distilled water and resuspended in 10 ml cold distilled water. The cells

Table 8

Quantities of nutrients to achieve growth limitation of P.cepacia at OD 1.0

Ingredient (Molar)	Limiting nutrient					
	Mg^{2+}	Fe^{2+}	PO_4^{3-}	SO_4^{2-}	NH_4^{+}	C
KCl	0.003	0.003	0.003	0.003	0.003	0.003
NaCl	0.003	0.003	0.003	0.003	0.003	0.003
$MgSO_4.7H_2O$	1.9×10^{-5}	0.0032	0.0032	0.0032	0.0032	0.0032
$(NH_4)_2SO_4$	0.012	0.012	0.012	0.012	0.0022	0.012
$FeSO_4.7H_2O$	2×10^{-5}	4×10^{-7}	2×10^{-5}	2×10^{-5}	2×10^{-5}	2×10^{-5}
Glucose	0.02	0.02	0.02	0.02	0.02	0.004
K_2HPO_4	12×10^{-4}	12×10^{-4}	6×10^{-5}	-	12×10^{-4}	12×10^{-4}
MOPS	0.05	0.05	0.05	-	0.05	0.05
$MgCl_2.6H_2O$	-	-	-	16×10^{-5}	-	-
$FeCl_2.4H_2O$	-	-	-	27×10^{-5}	-	-
$(NH_4)_2HPO_4$	-	-	-	1.8×10^{-2}	-	-
$(NH_4)H_2PO_4$	-	-	-	4.8×10^{-4}	-	-

Medium pH 7.4

were either broken by sonication for 3 min or as described in section 2.2.6. Cell debris was removed by centrifugation at 1,500 x g for 10 min. 1 ml of 20% sarkosyl solution was added to the supernatant (final concentration 2%) and incubated at room temperature for 30 min. It was then centrifuged at 38,000 x g for 60 min. The supernatant was collected in a universal vial and stored at -20°. The pellet was washed once with cold distilled water and resuspended in small amount of cold distilled water and kept at - 20°C. The proteins in the sarkosyl insoluble materials were studied by SDS-polyacrylamide gel electrophoresis as will be described in section 2.2.10.

2.2.8 Total protein assay

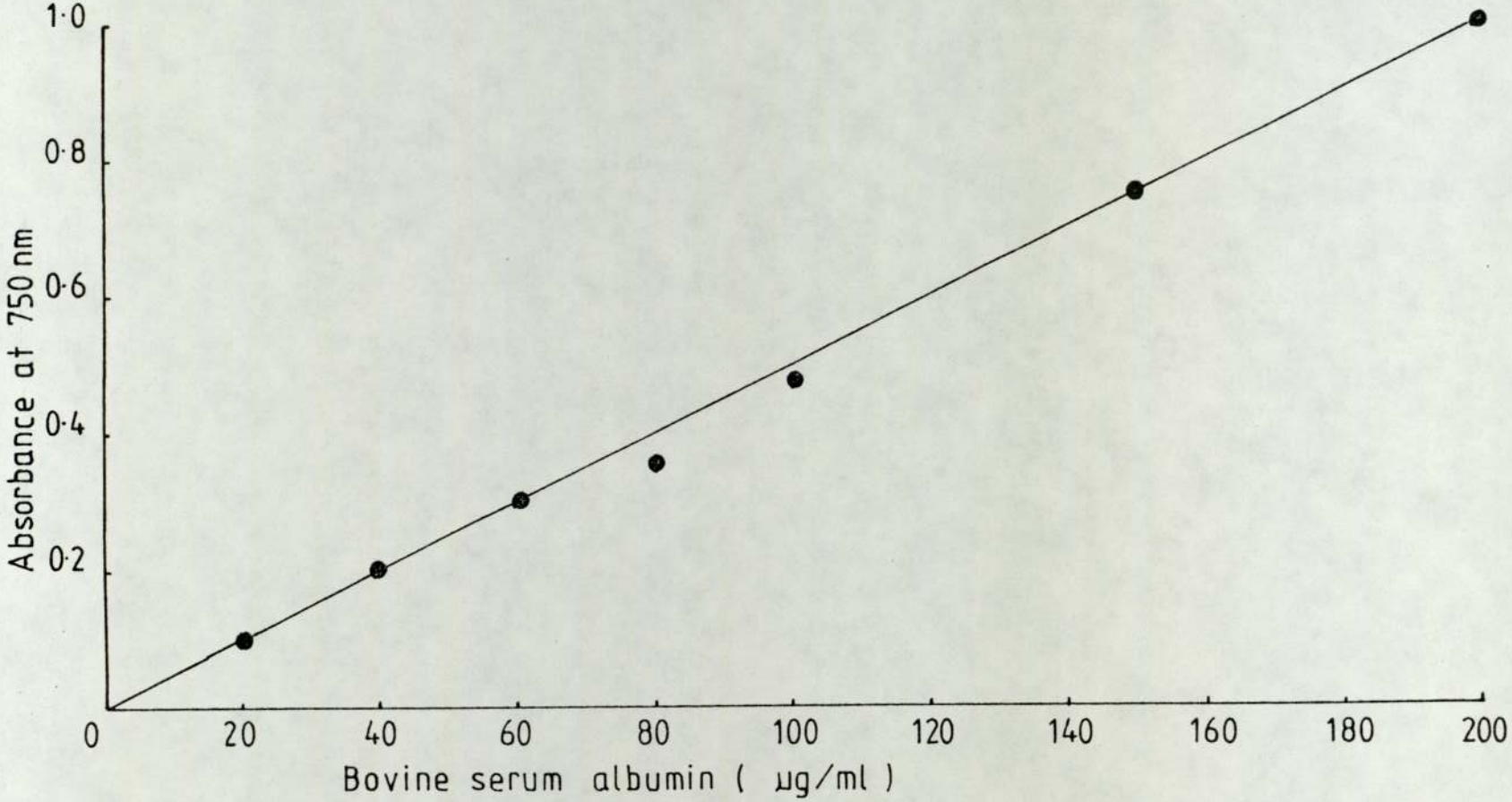
The total protein content of the outer and cytoplasmic membranes was estimated by the method of Lowry et al.(1951). A standard curve was obtained by using a solution of bovine serum albumin containing 0 - 200 µg/ml protein and reading the OD at 750 nm (Fig. 15). A linear relationship was obtained for concentrations of 0 to 200 µg/ml. Sample determinations were performed in triplicates and a fresh standard curve was prepared each time the assay was done.

2.2.9 Enzyme assays of outer and cytoplasmic membranes

The enzymes were assayed according to the procedures described by Mizuno and Kageyama (1978).

Succinate dehydrogenase, D-lactate dehydrogenase,

Fig. 15 Protein assay calibration curve.


1·0
0·8
0·6
0·4
0·2
0
Absorbance at 750 nm
20
40
60
80
100
120
140
160
180
200
Bovine serum albumin (µg/ml)

gluconate dehydrogenase and glucose dehydrogenase were determined by incubating mixtures containing 60 mM phosphate buffer (pH 7.2), 10 mM KCN, 10 µg phenazine methosulphate, 20 µg dichlorophenol indophenol, 25 mM succinate (2.5 mM D-lactate, 20 mM gluconate or 30 mM glucose) and the membrane fraction (50 to 100 µg protein) in a volume of 1 ml. The rate of decrease in absorbance at 600 nm was recorded for more than 10 min at room temperature.

Nicotinamide adenine dinucleotide oxidase was determined by incubating mixtures containing 50 mM Tris-HCl (pH 8.0). 0.12 mM NADH, 0.2 mM dithiothreitol and membrane fraction (50 to 100 µg protein) in a volume of 1 ml. The rate of decrease in absorbance at 340 nm was recorded at room temperature for more than 10 min.

2.2.10 SDS-polyacrylamide gel electrophoresis (PAGE)

The method was described by Lugtenberg et al. (1975) with slight modifications.

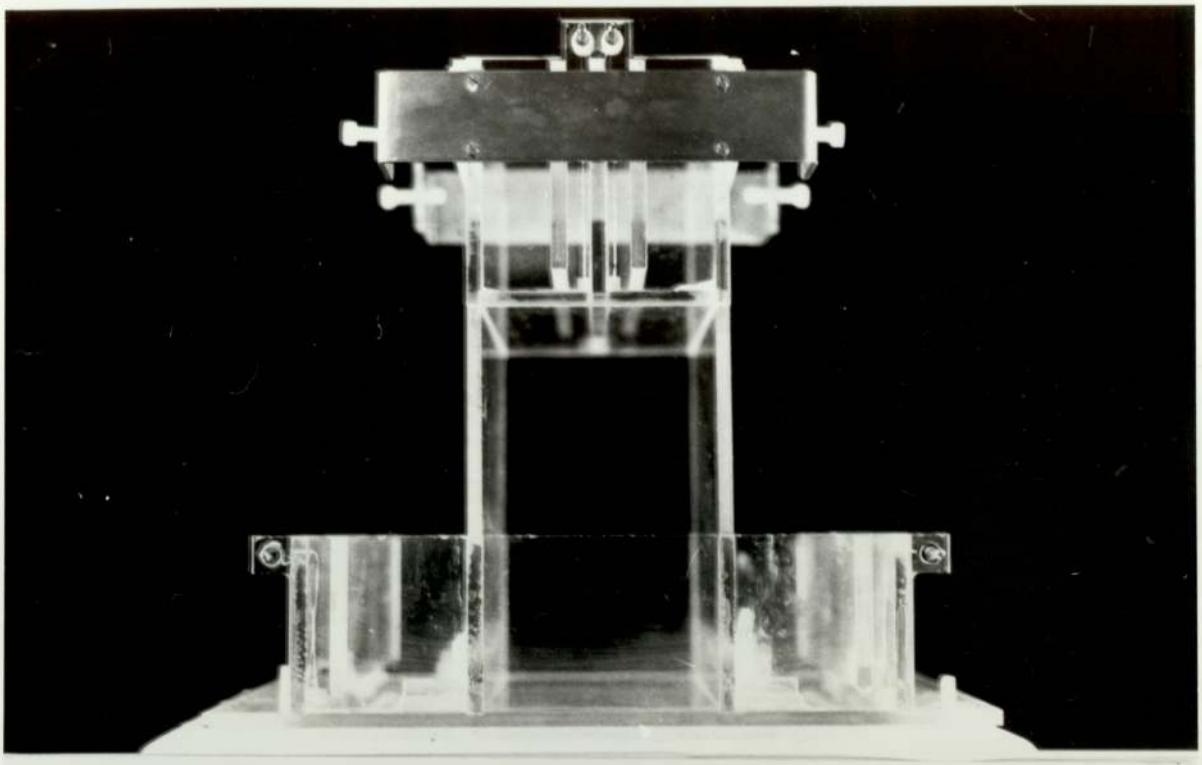
The slab gel electrophoresis apparatus used throughout the study is shown in Fig. 16. It was constructed similarly to the commercially available apparatus, except that it can hold two plates in a single run. For the preparation of running and stacking gels, the following solutions were used: stock solution I contained 44 g acrylamide and 0.8 g methylene-bisacrylamide while stock solution II contained 30 g acrylamide plus 0.8 g methylene bisacrylamide. The volume of both solutions were adjusted to 100 ml with distilled water.

Fig. 16 Slab gel electrophoresis apparatus

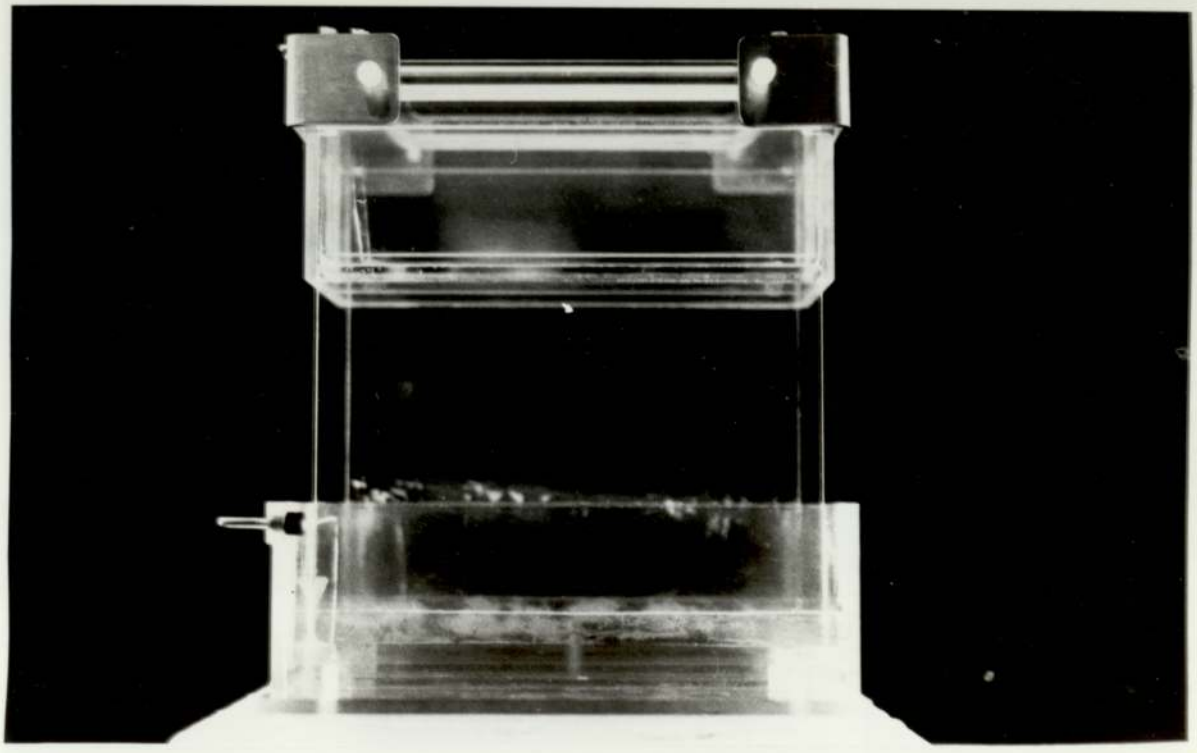
A Side view

B Front view

A



B



The solutions were kept at 4° and could be used for 2 months. A fresh solution of ammonium persulphate (100 mg/ml) was prepared each time the gel was performed. Running gel solution contained: Stock solution I, 12.5 ml; ammonium persulphate, 0.12 ml; 10% (W/V) SDS, 1 ml; 1.5 M Tris-HCl, pH 8.8, 12.5 ml and distilled water, 22 ml. Stacking gel solution contained: Stock solution II, 5 ml; ammonium persulphate, 0.1 ml; 10% SDS, 0.3 ml; 0.5 M Tris-HCl, pH 6.8, 7.5 ml and distilled water, 16 ml. Both solutions were well mixed by using a magnetic stirrer. Polymerization was started by the addition of N,N,N',N'-tetramethylene diamine (TEMED). 0.1 ml and 80 µl of TEMED was added to the running and stacking gel solutions respectively. Both electrode buffers contained 0.025 M Tris; 0.19 M glycine and 0.1% SDS. The pH of this buffer was 8.3. The sample buffer contained: 0.5 M Tris-HCl, pH 6.8, 5 ml; 10%(W/V) SDS, 10 ml; 2-mercaptoethanol 0.5 ml or without; glycerol, 5 ml and distilled water, 10 ml. The sample was diluted in the ratio of 1:1 with the sample buffer, 20 µl of bromophenol blue solution was added to each sample. The samples were boiled for 5 min at 100° or 30 min at 60°. Approximately 75 µg of protein was applied to each slot. Sample containing a mixture of standard proteins of known molecular weights were prepared as described above.

Electrophoresis was carried out at room temperature using a constant current of 40 mA per gel. The electrophoresis was stopped when the tracking dye had moved approx. 12 cm from the top of the running gel, which usually happened

after 3.5 hr. Gels were stained for proteins overnight in a solution of 0.1% Brillient Commassie Blue R-250 in 50% methanol-10% acetic acid, then destained in 5% methanol-10% acetic acid. Gels were also stained for carbohydrate by the method described by Steck et al. (1971). The photographs of the gels were taken by using diffuse light from the bottom.

2.2.11 Lipid extraction from the OM and CM

The method was described by Ames (1968). It was first reported by Bligh and Dyer (1959) for fish.

The method consisted of an extraction of the lipids in a monophasic system in which methanol, chloroform and water (membrane suspension) were in the proportion 2:1:0.8 (V/V) in a separating funnel. If a very dense suspension of membrane was used, a correction should be made (1 g of wet weight of sample contained 0.8 ml of water). The lipids were separated from the water soluble materials by diluting the mixture with 1 volume of chloroform followed by 1 volume of water. The chloroform layer (bottom layer) was separated and evaporated to dryness in a rotary evaporator at 10° or less. The lipids were dried over P_2O_5 in vacuo to a constant weight (readily extractable lipids, REL). It was then dissolved in 1 ml of solvent and 20 ml of acetone at -10° was added to precipitate phospholipids (PL) for overnight. PL was removed by centrifugation in a tared centrifuge tube and dried over P_2O_5 in vacuo to a constant

weight.

2.2.12 Assay of phospholipids

The PL fractions were dissolved in 1 ml of chloroform and methanol (1:2) and stored at -20° until required. Ascending thin-layer chromatography was used to fractionate them into their component PL. Glass plate, 20 x 20 cm, were spread with a 0.25 mm layer of silica gel PF_{254} (E. Merck, Darmstadt, West Germany). The slurry was prepared by mixing 60 g of silica gel with 120 ml of distilled water; the plates were air-dried for overnight and then activated by heating at 70° for 90 min. The samples and standards were separately loaded on the plate. They were developed in a chromatography tank using solvent 1 (chloroform-methanol-water (65:25:4) described by Ames (1968). After development, the plates were air-dried, sprayed with phosphate spray reagent (Fig. 17). The ratio of individual PL, identified by co-chromatography with standards, was determined by densitometry with a chromatoscan 200 integrating microdensitometer (Joyce Loebl & Co. Ltd., Gateshead, Northumberland, England). The area under each peak was cut off and weighed. The ratio of PE/PG+PC was calculated. After that the plate was heated at 160° for 2 h, no extra spots were observed.

Fig. 17 Phospholipid Assay: developed chromatogram of sample phospholipids.

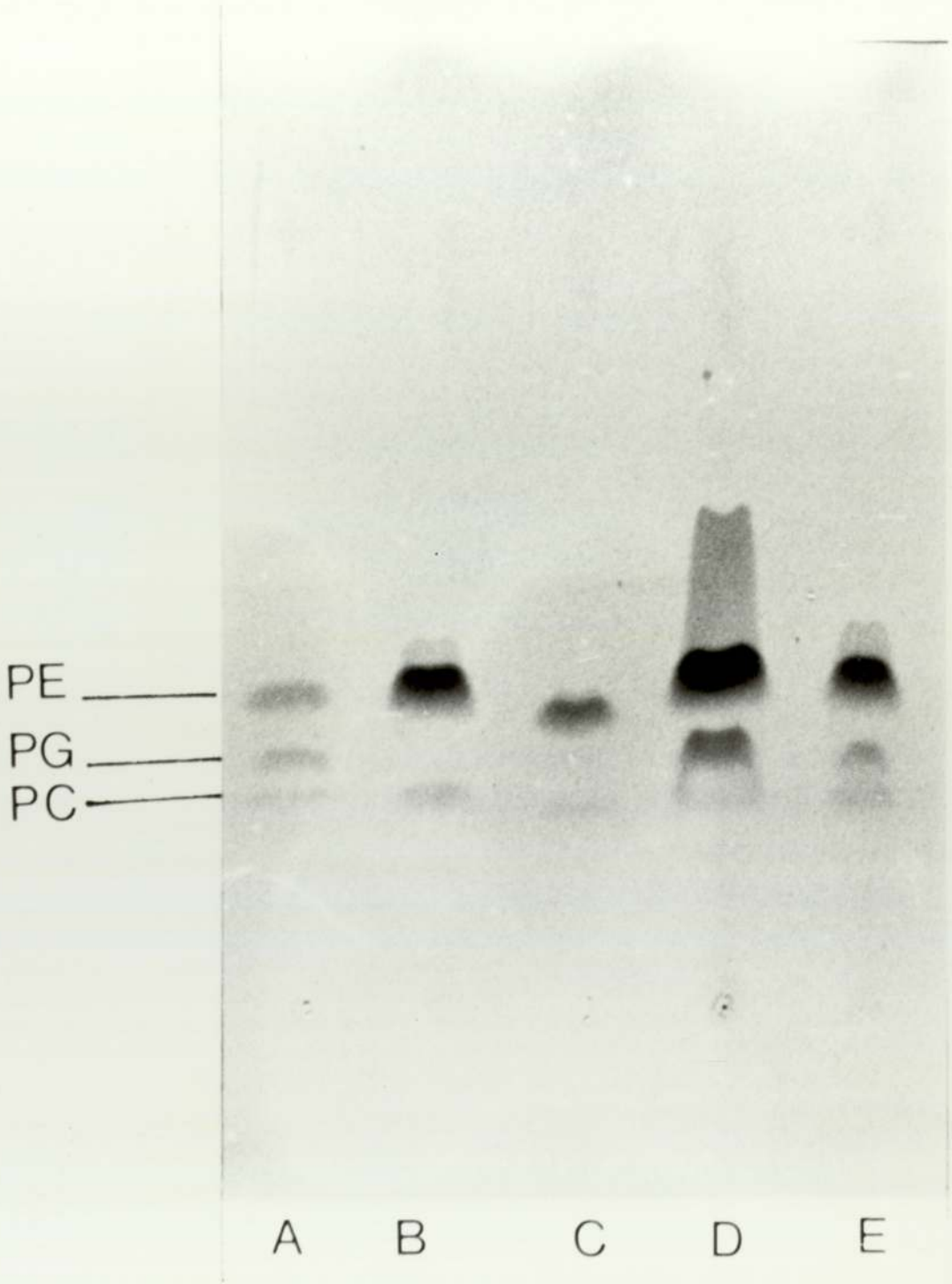
Slot A : The cytoplasmic membrane of magnesium-depleted cells.

Slot B : The outer membrane of iron-depleted cells.

Slot C : The cytoplasmic membrane of iron-depleted cells.

Slot D : The outer membrane of nutrient broth grown cells (oxygen-depleted).

Slot E : The cytoplasmic membrane of nutrient broth grown cells.


PE
PG
PC
A
B
C
D
E

3. EXPERIMENTAL AND RESULTS

3.1 Nutrient Depletion Studies of P. cepacia NCTC 10661 in Batch Culture

The requirement of certain nutrients in P. aeruginosa has been studied by several workers (Brown and Melling, 1969a; Boggis, 1971; Finch, 1976). They concluded that the final cell density reached by a culture of P.aeruginosa was dependent, below certain levels, on the initial concentration of a growth limiting nutrient in the medium. The object of this study was to investigate the nutritional requirements in P. cepacia, to achieve a suitable medium for its growth.

3.1.1 Experimental

The medium used for the study of glucose, magnesium, nitrogen, phosphate, sulphate and iron is that described in Table 6. Experimental details have been described in section 2.2.3.

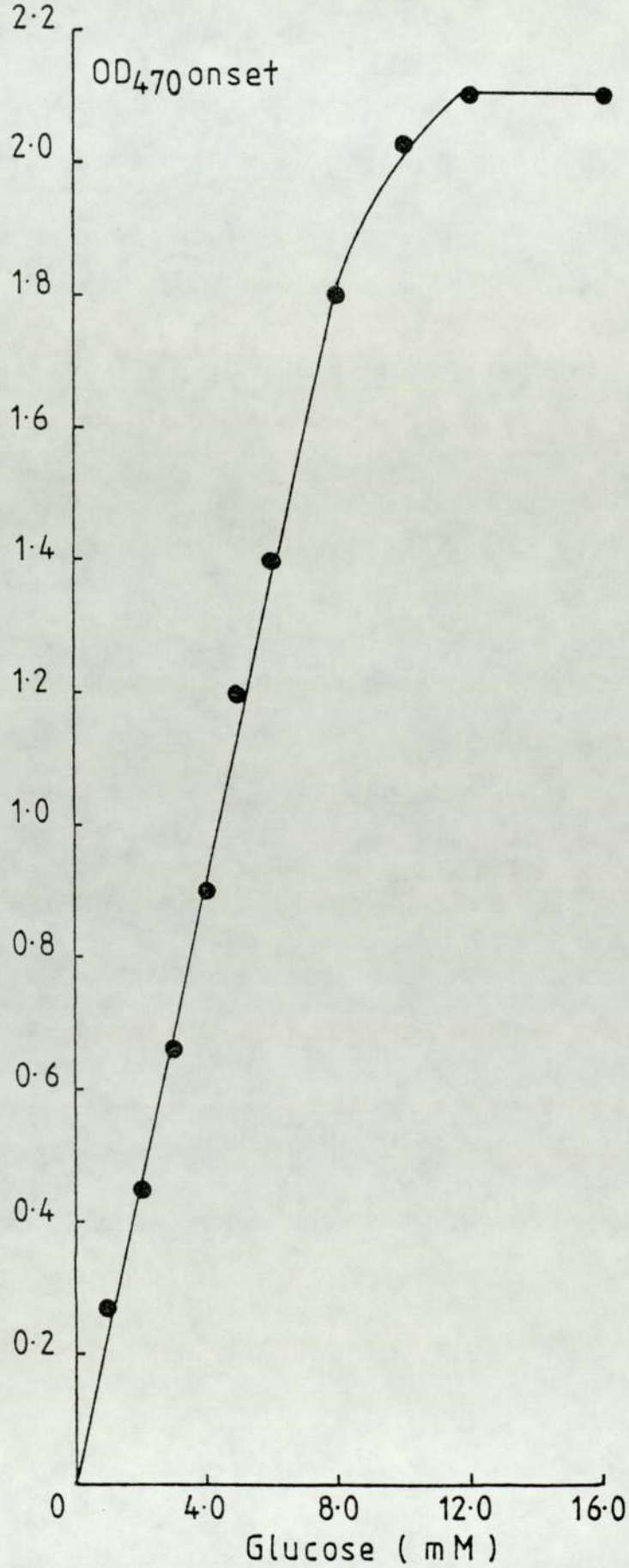
3.1.2 Carbon depletion studies

The growth curves for carbon requirement are shown in Fig. 18. Glucose was the only carbon source in this study and all other nutrient limitation studies. A rapid cessation of growth was observed by cultures with low levels of carbon in their media. At higher glucose levels, there was a decline in growth rate but growth did not stop, indicating that glucose was no longer the growth limiting factor. The relationship between initial glucose concentration and the onset of non-linear growth is shown in Fig. 19. The

Fig. 18 Growth of P.cepacia in CDM with graded concentrations of glucose.

	Glucose concentration (mM)
▌	1.0
◇	2.0
▲	3.0
□	4.0
○	5.0
◪	6.0
◒	8.0
◆	9.0
▮	10.0
■	12.0
●	16.0

Fig. 19 Relation between onset of non-linear growth of *P.cepacia* and initial glucose concentration.


2·2
OD470 onset
2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
4·0
8·0
12·0
16·0
Glucose (mM)

relationship was linear to an OD_{470} of 2.1 under the conditions used in the study.

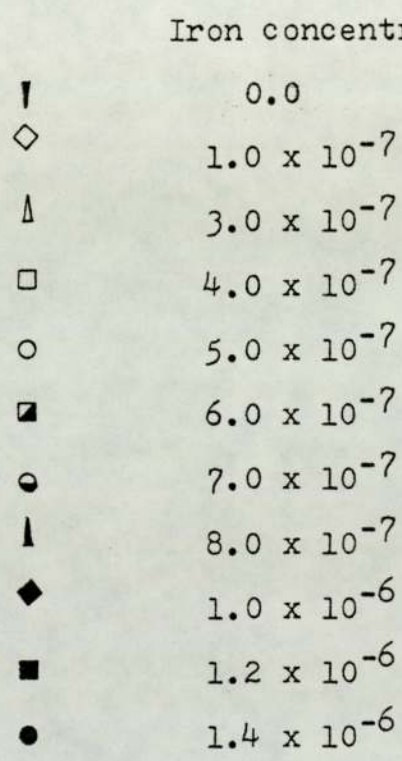
3.1.3 Iron depletion studies

There was a problem in the study of growth under iron limitation due to media contamination by iron. However, it was solved (see section 2.2.4) by using iron-depleted cultures as inocula. The growth curves for iron limitation are shown in Fig.20. Fig.21 shows the relationship between onset of non-linear growth and added iron concentration. Double reciprocal plots of the growth rate versus added iron concentration are shown in Fig.22. Fig.14 shows the comparison of the growth of P.cepacia, grown in iron-depleted culture or complete CDM, in the supernatant of iron-depleted medium. The procedure for this study has been described in section 2.2.4. The doubling time in iron-depleted culture was 110 min and was 60 min for complete CDM. Addition of 2×10^{-5}M of ferrous sulphate to the media of both types of cells resulted in the decrease of doubling time for the cells from iron-depleted cultures. It dropped from 110 min to 60 min, while it remained unchanged for the cells from complete CDM.

3.1.4 Magnesium depletion studies

Fig. 23 shows the growth curves for magnesium study. Unlike glucose, the growth did not cease abruptly after a period of exponential growth, but proceeded at a gradually decreasing growth rate. The onset of magnesium limitation

Fig. 20 Growth of P. cepacia in CDM with graded concentrations of iron (ferrous sulphate).



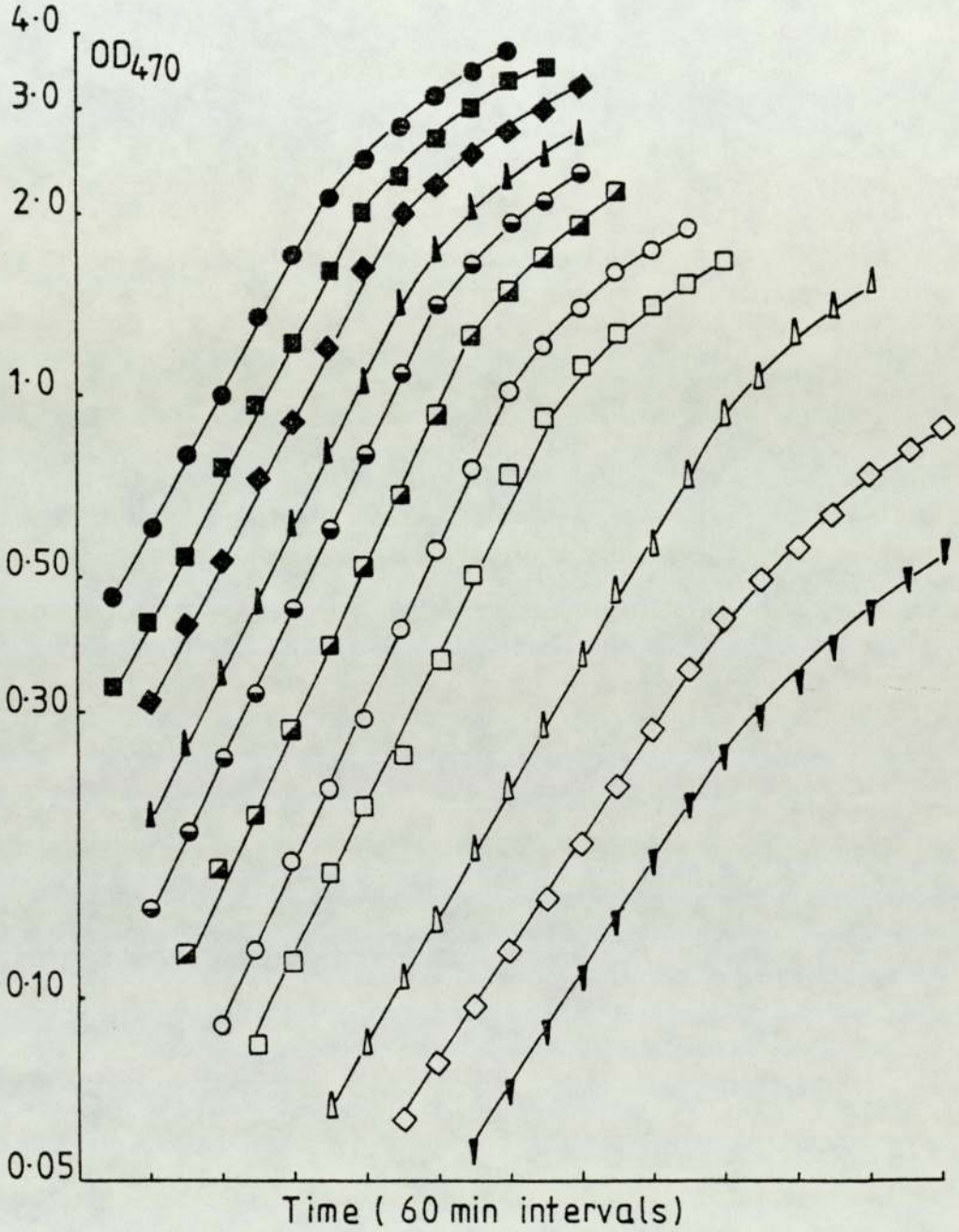

4·0
3·0
2·0
1·0
0·50
0·30
0·10
0·05
OD470
Time (60 min intervals)

Fig. 21 Relation between onset of non-linear growth of *P. cepacia* and initial iron concentration.

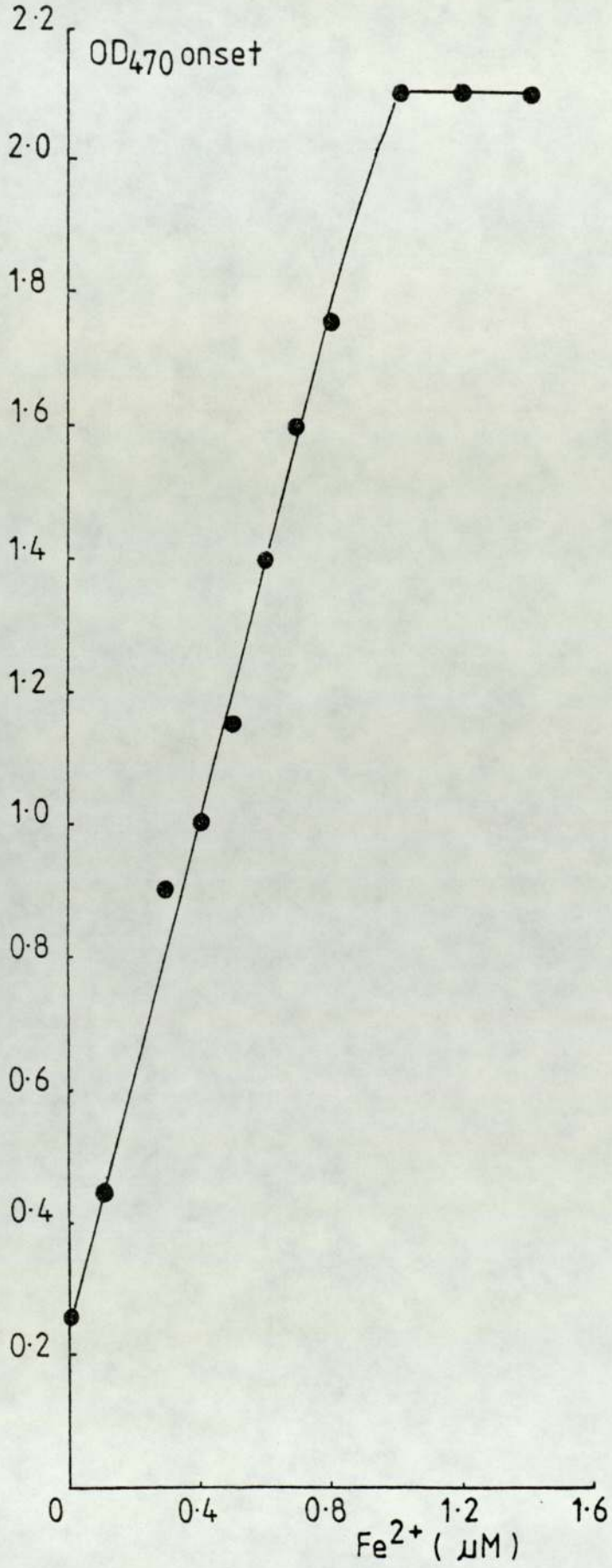
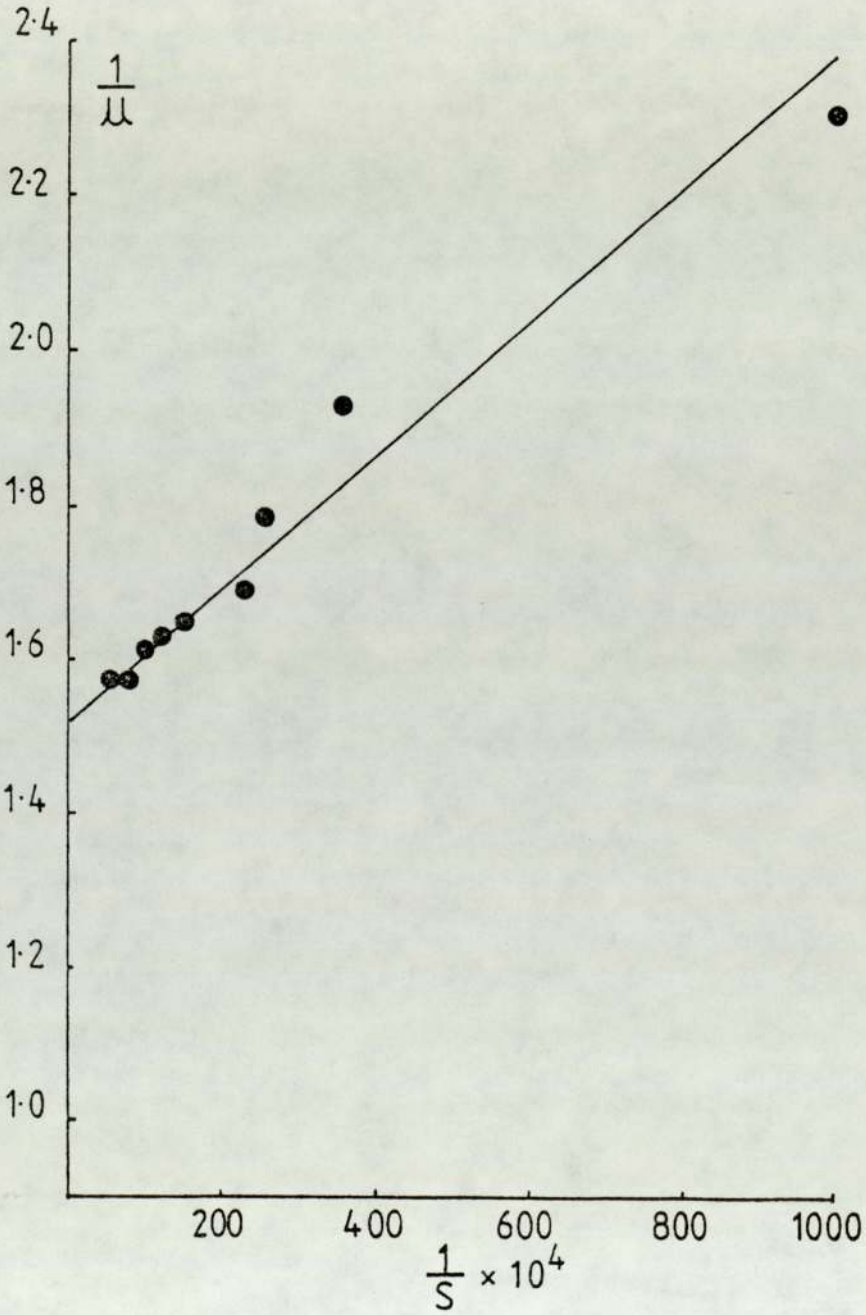

2·2
OD470 onset
2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
0·4
0·8
1·2
1·6
Fe2+ (µM)

Fig. 22 Double reciprocal plot of *P. cepacia* growth rate versus medium iron concentration.


2·4
2·2
2·0
1·8
1·6
1·4
1·2
1·0
1/μ
200
400
600
800
1000
1/S × 10⁴

was therefore taken to occur when the 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 Fig. 24. The relationship was linear to an OD_{470} of 2.1. Double reciprocal plots of the growth rate versus magnesium concentration are shown in Fig. 25.

3.1.5 Nitrogen depletion studies

The growth curves for nitrogen limitation are shown in Fig.26. The relationship between onset of non-linear growth and initial ammonium sulphate concentration is shown in Fig.27. The relationship was linear to an OD_{470} of 2.1. The contamination level of utilisable nitrogenous materials from other medium ingredients was equivalent to about 1×10^{-3}M ammonium sulphate.

3.1.6 Phosphate depletion studies

The growth curves for phosphate limitation are shown in Fig.28 and the relationship between onset of non-linear growth and phosphate concentration is shown in Fig. 29. Similar to magnesium, iron, glucose and nitrogen limitations, the relationship was linear to an OD_{470} of 2.1.

3.1.7 Sulphate depletion studies

Growth curves illustrating sulphate limitation (Fig.30) are similar to those of magnesium, iron and phosphate

Fig. 23 Growth of P. cepacia in CDM with graded concentrations of magnesium.

Magnesium concentration (M)
0.0
3.2×10^{-6}
6.4×10^{-6}
9.6×10^{-6}
1.3×10^{-5}
1.9×10^{-5}
2.6×10^{-5}
3.2×10^{-5}
5.8×10^{-5}
6.4×10^{-5}

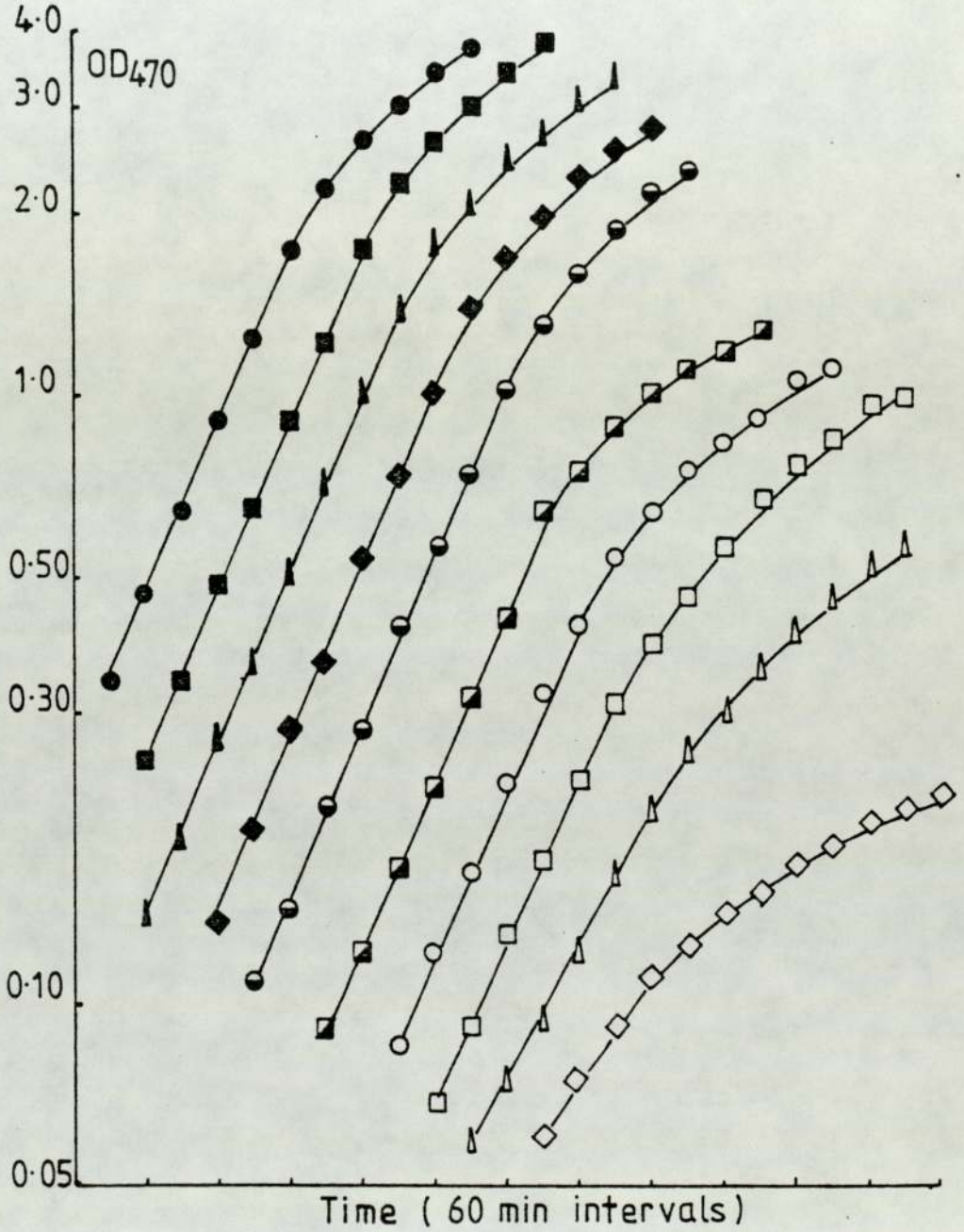

4·0
3·0
2·0
1·0
0·50
0·30
0·10
0·05
OD470
Time (60 min intervals)

Fig. 24 Relation between onset of non-linear growth of *P. cepacia* and initial magnesium concentration.

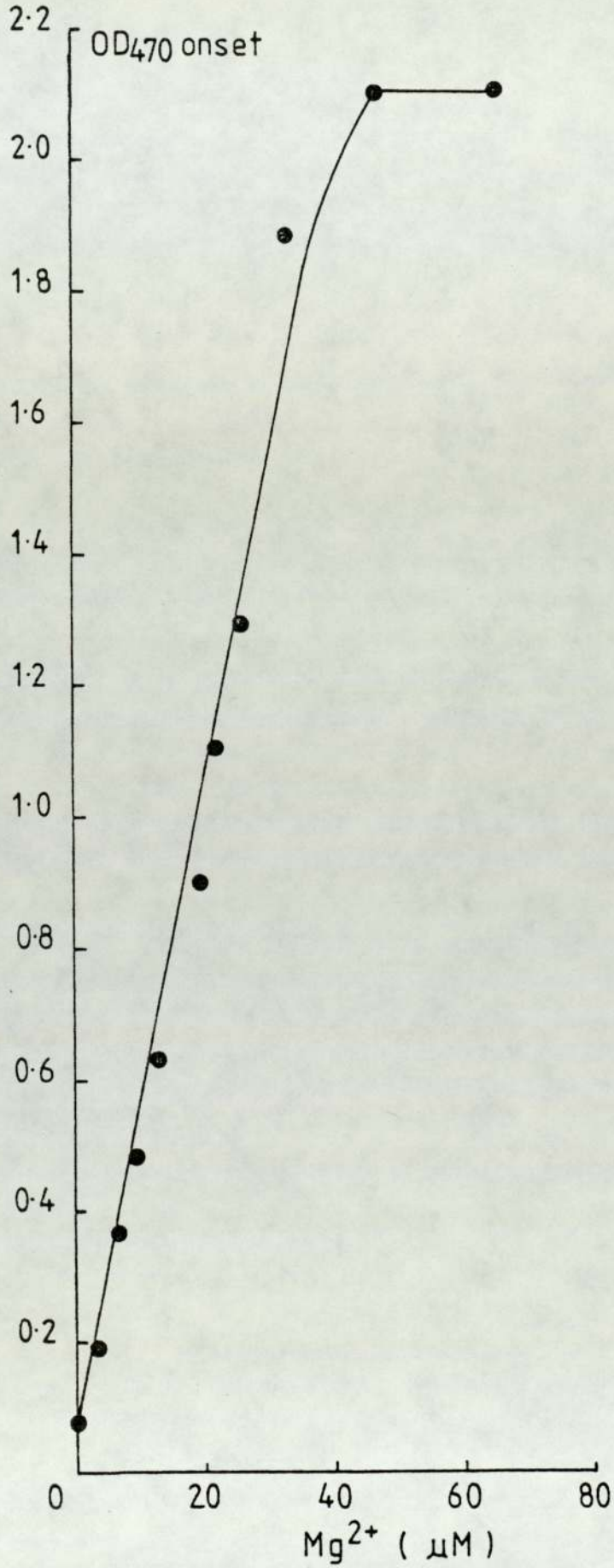

2·2
OD470 onset
2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
20
40
60
80
Mg2+ (µM)

Fig. 25 Double reciprocal plot of P. cepacia growth rate versus medium magnesium concentration.

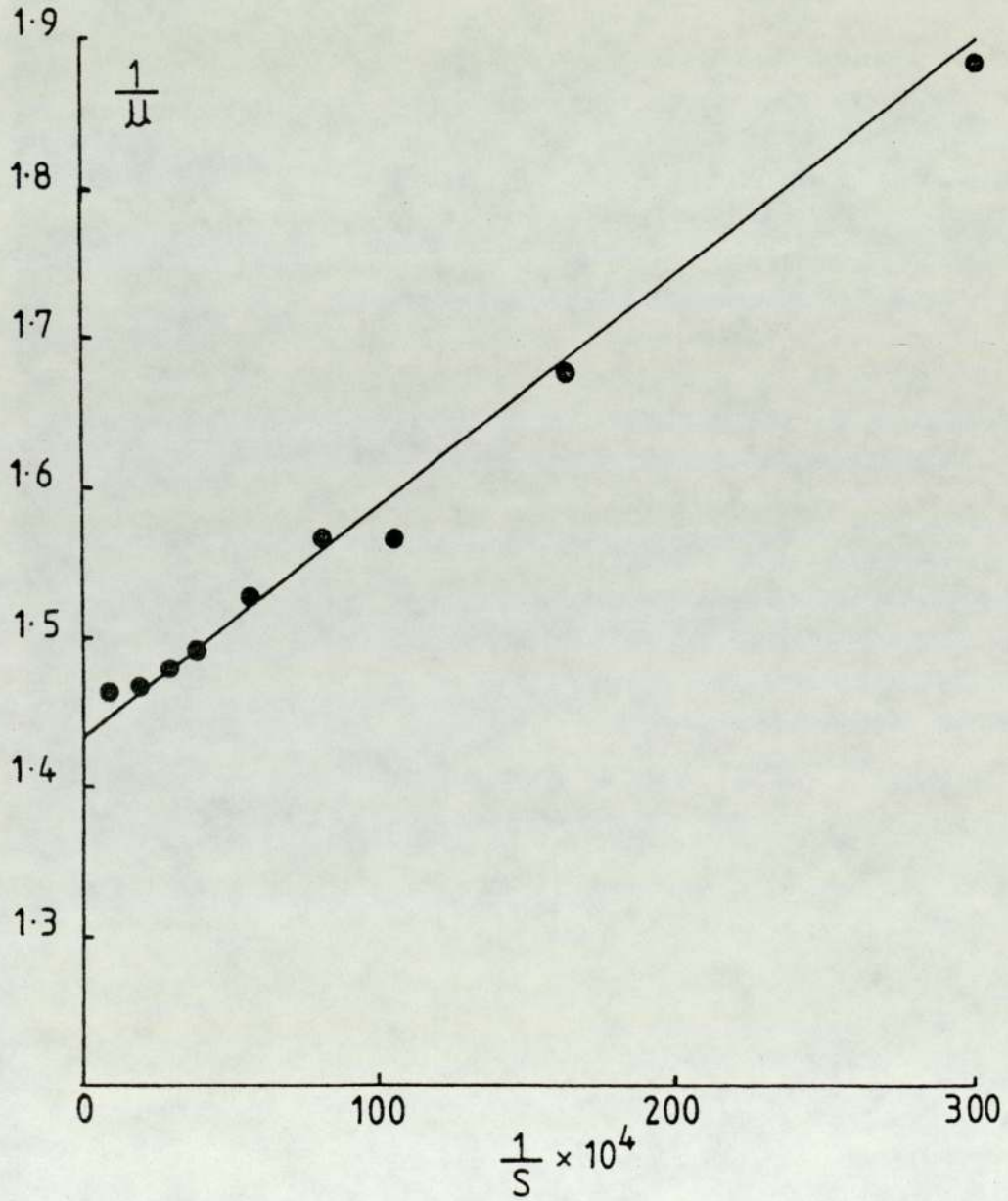
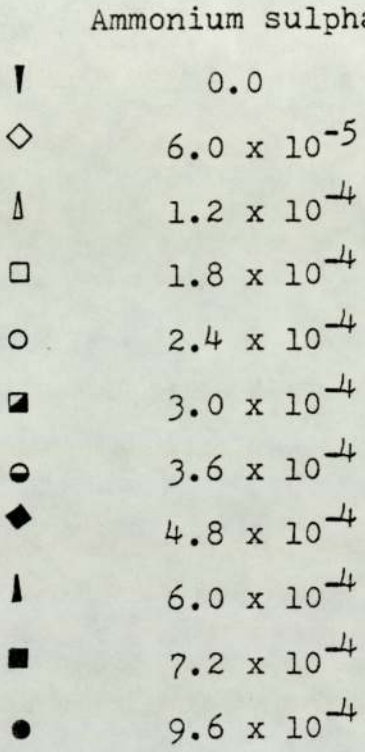

1·9
1·8
1·7
1·6
1·5
1·4
1·3
1/μ
0
100
200
300
1/S × 10⁴

Fig. 26 Growth of P. cepacia in CDM with graded concentrations of ammonium ion.



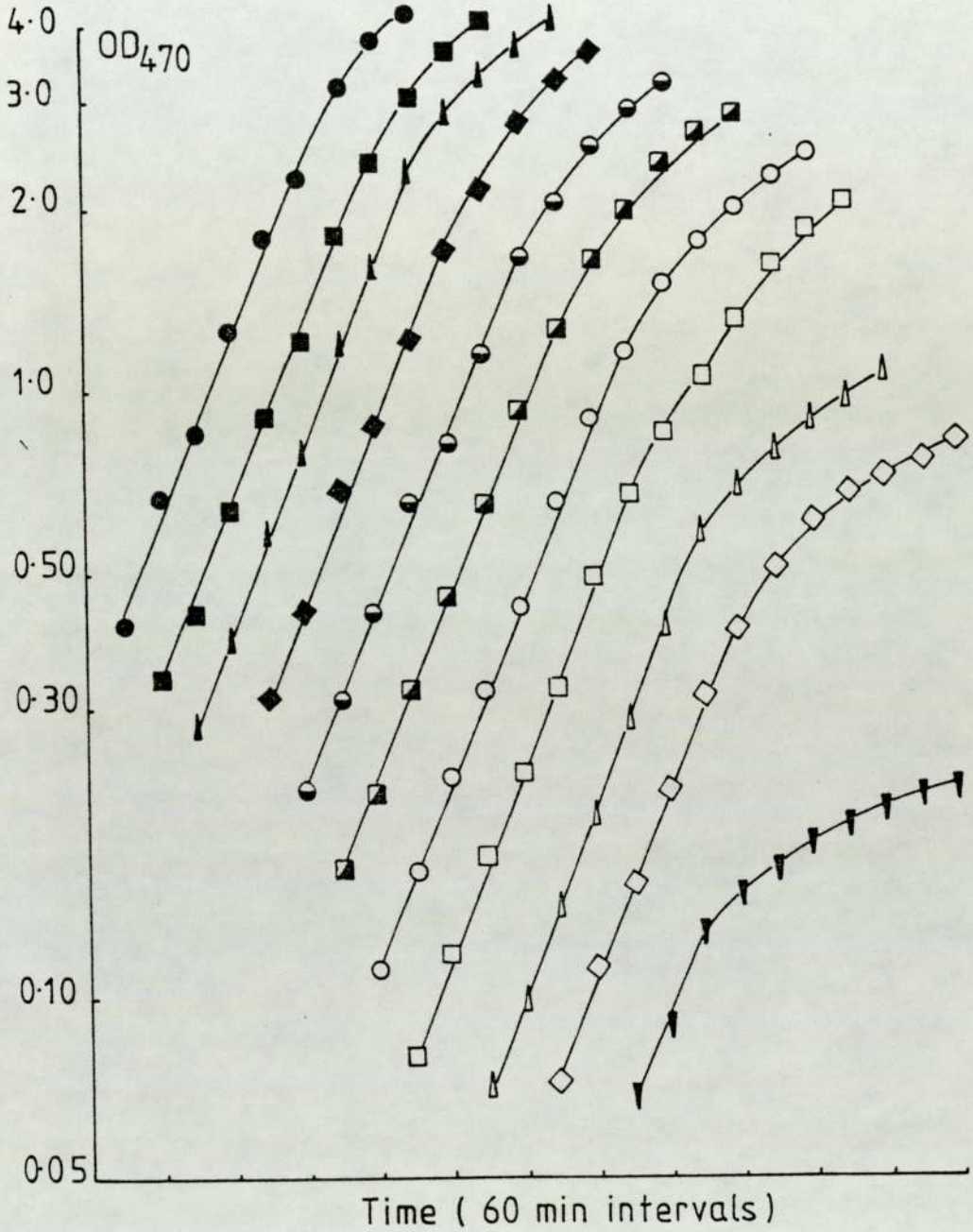

4·0
3·0
2·0
1·0
0·50
0·30
0·10
0·05
OD470
Time (60 min intervals)

Fig. 27 Relation between onset of non-linear growth of *P. cepacia* and initial ammonium concentration.

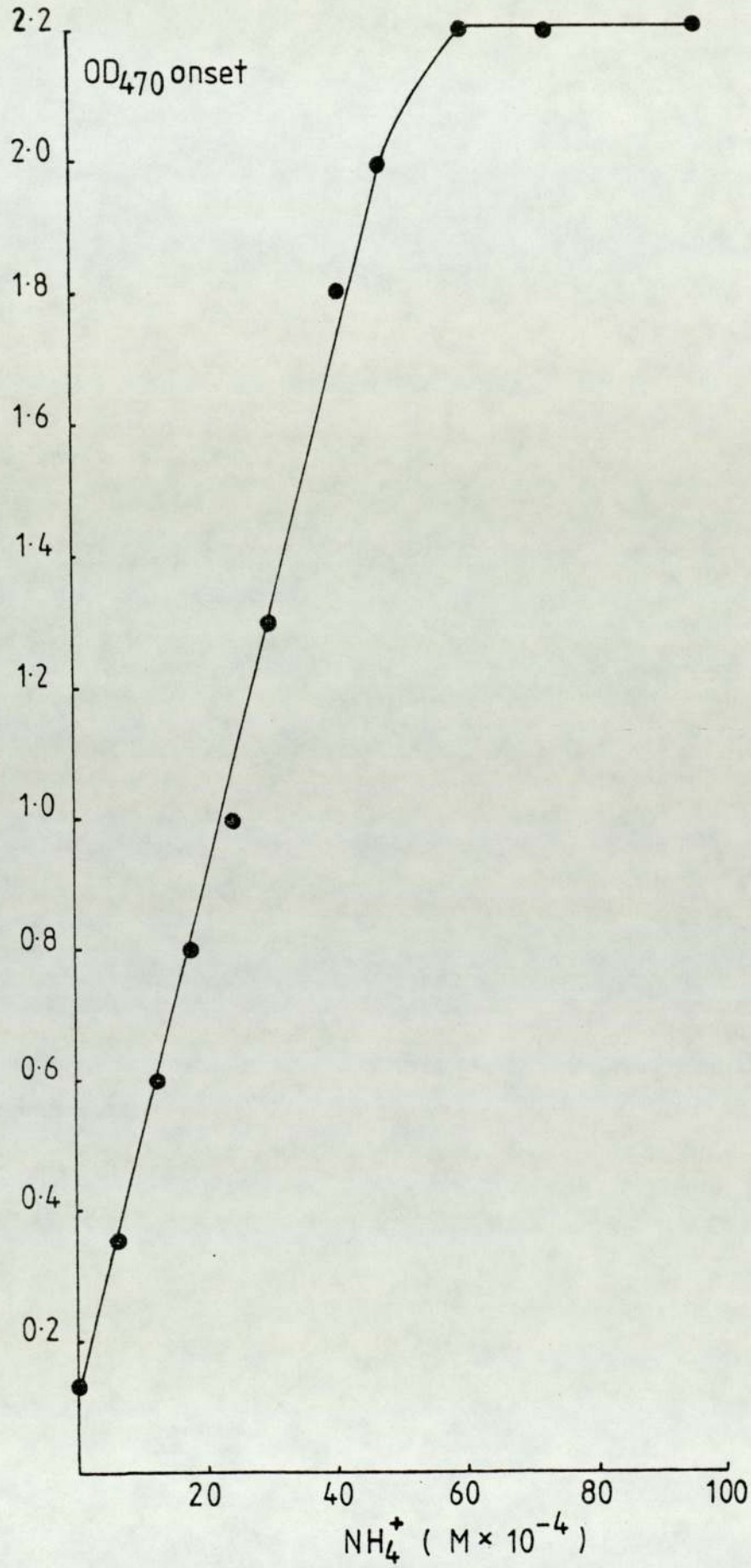

2·2
OD470 onset
2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
20
40
60
80
100
NH4+ (M × 10−4)

Fig. 28 Growth of P. cepacia in CDM with graded concentrations of phosphate.

	Phosphate concentration (M)
○	0.0
□	6.0×10^{-5}
◐	1.2×10^{-4}
◪	1.8×10^{-4}
▵	2.4×10^{-4}
◆	3.0×10^{-4}
▽	3.6×10^{-4}
◇	4.8×10^{-4}
▮	6.0×10^{-4}
■	7.2×10^{-4}
●	9.6×10^{-4}

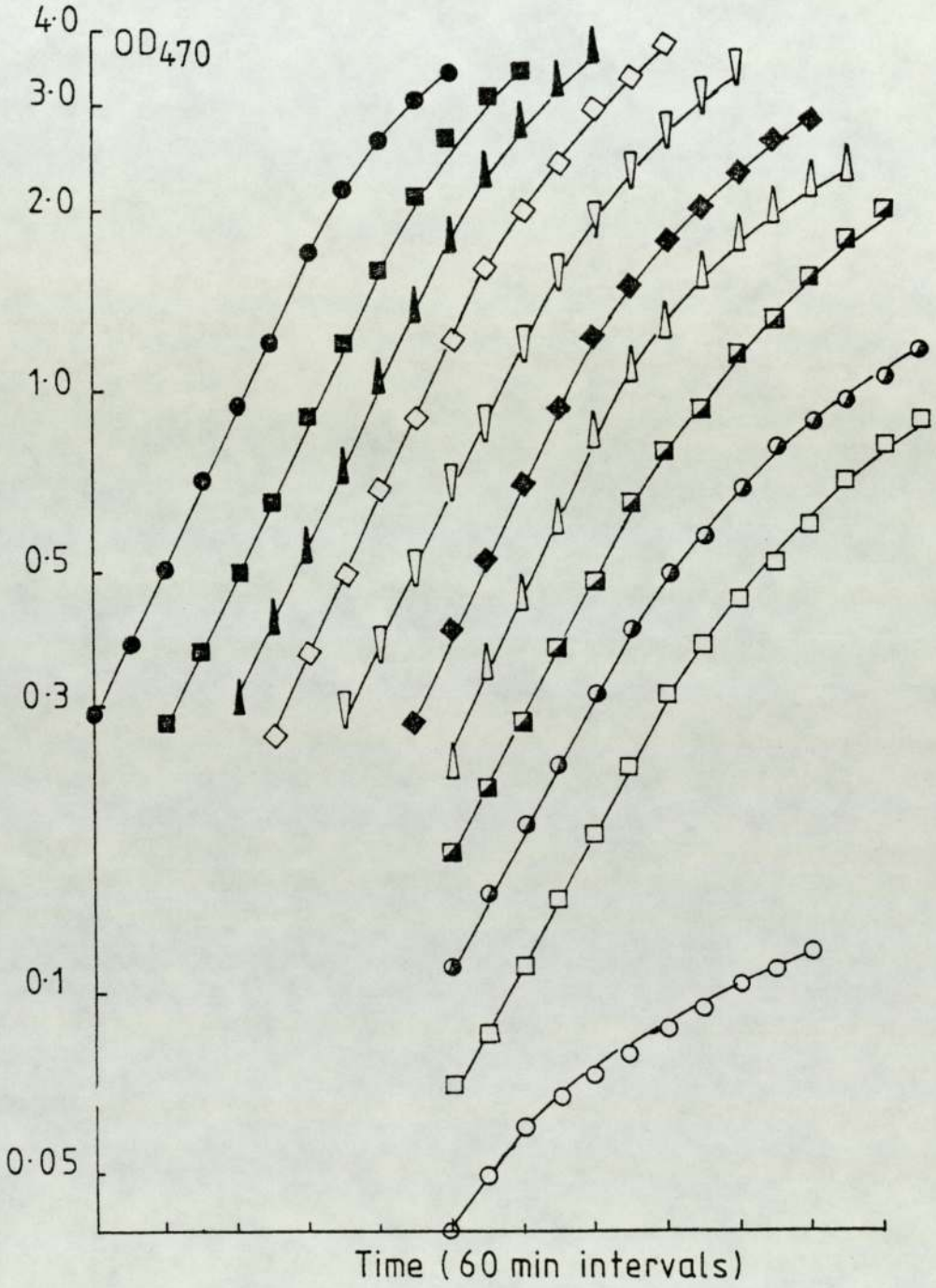
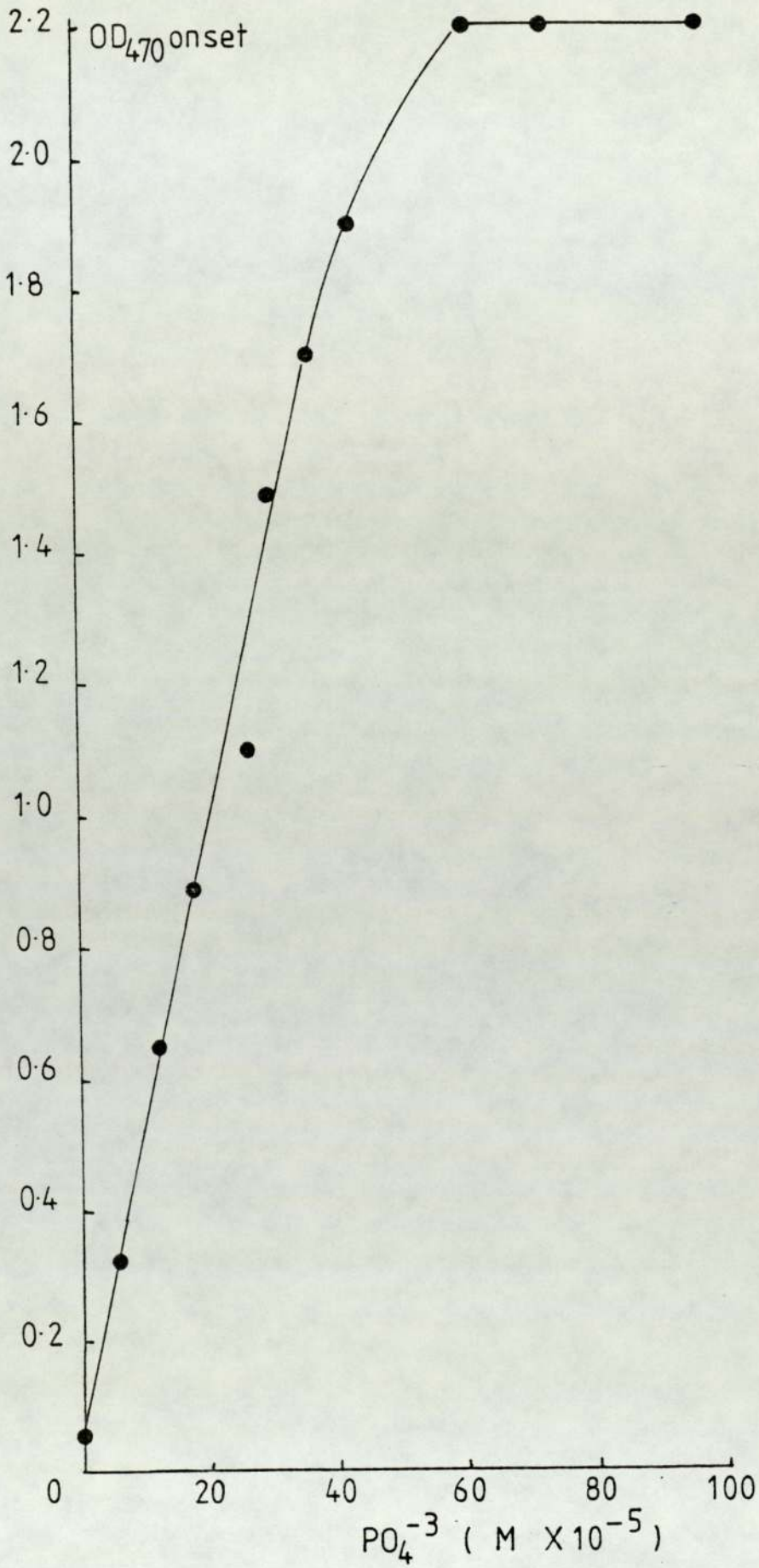

4·0
OD470
3·0
2·0
1·0
0·5
0·3
0·1
0·05
Time (60 min intervals)

Fig. 29 Relation between onset of non-linear growth of *P. cepacia* and initial phosphate concentration.


2·2
2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
OD470 onset
20
40
60
80
100
PO4-3 (M X10-5)

limitations, exhibiting a progressive decrease in growth rate after the onset of limitation. Fig. 31 shows the relationship between onset of non-linear growth and added sulphate concentration in the medium. Double reciprocal plots of the growth rate versus sulphate concentration are shown in Fig. 32.

3.1.8 Saturation constant and maximum growth rate

The saturation constant, K_s, and the maximum growth rate, μ_{max}, for iron, magnesium and sulphate 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 $\frac{K_s}{u_m}$ and intercept on the Y axis of $\frac{1}{u_m}$. Using figures 22, 25 and 32, the two constants were calculated and are summarized in Table 9.

Fig. 30 Growth of P. cepacia in CDM with graded concentrations of sulphate.

Sulphate concentration (M)
0.0
1.2×10^{-5}
2.4×10^{-5}
3.6×10^{-5}
4.8×10^{-5}
6.0×10^{-5}
8.4×10^{-5}
1.2×10^{-4}
2.4×10^{-4}
3.0×10^{-4}
3.6×10^{-4}
4.8×10^{-4}

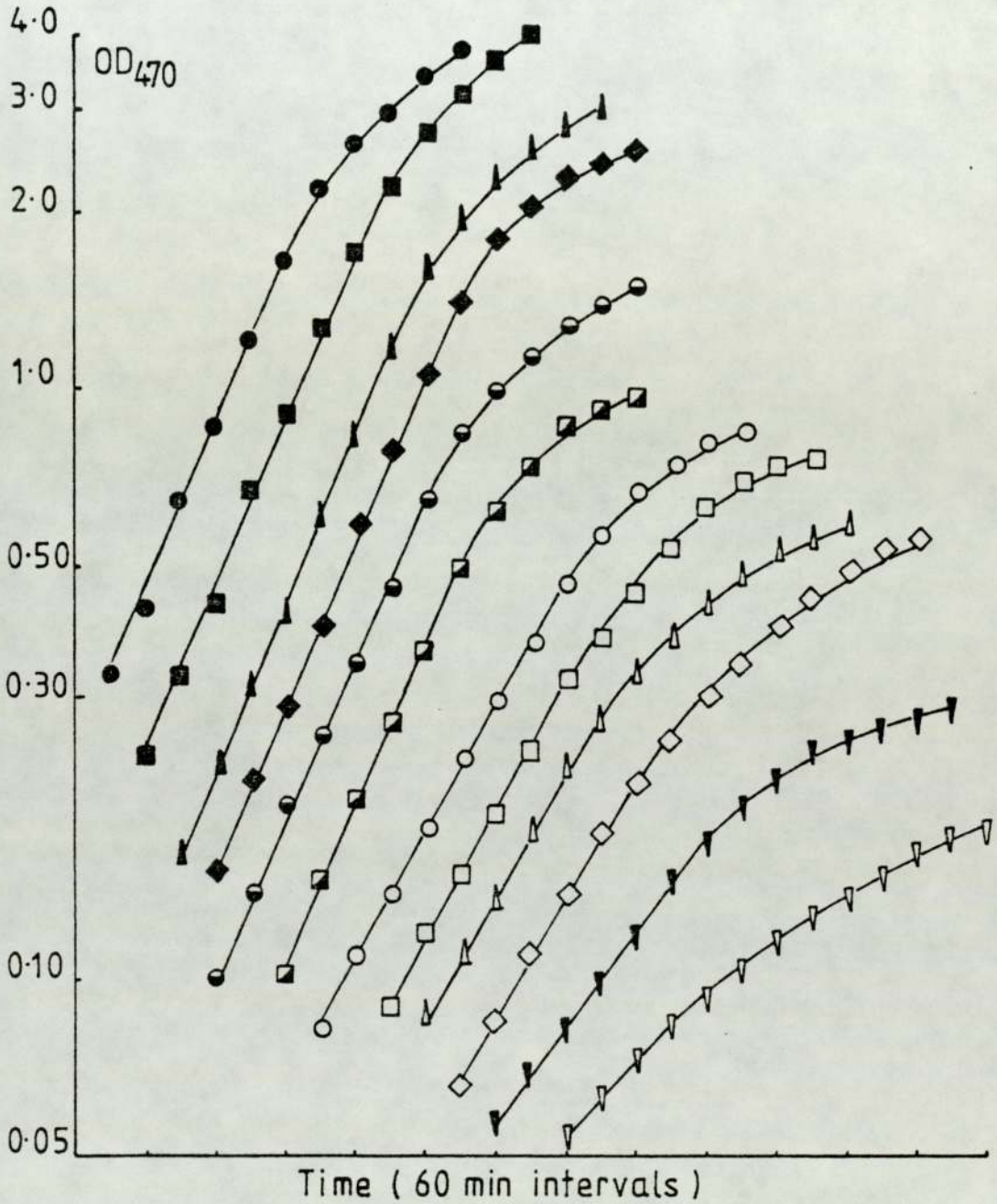

4·0
3·0
2·0
1·0
0·50
0·30
0·10
0·05
OD470
Time (60 min intervals)

Fig. 31 Relation between onset of non-linear growth of *P. cepacia* and initial sulphate concentration.

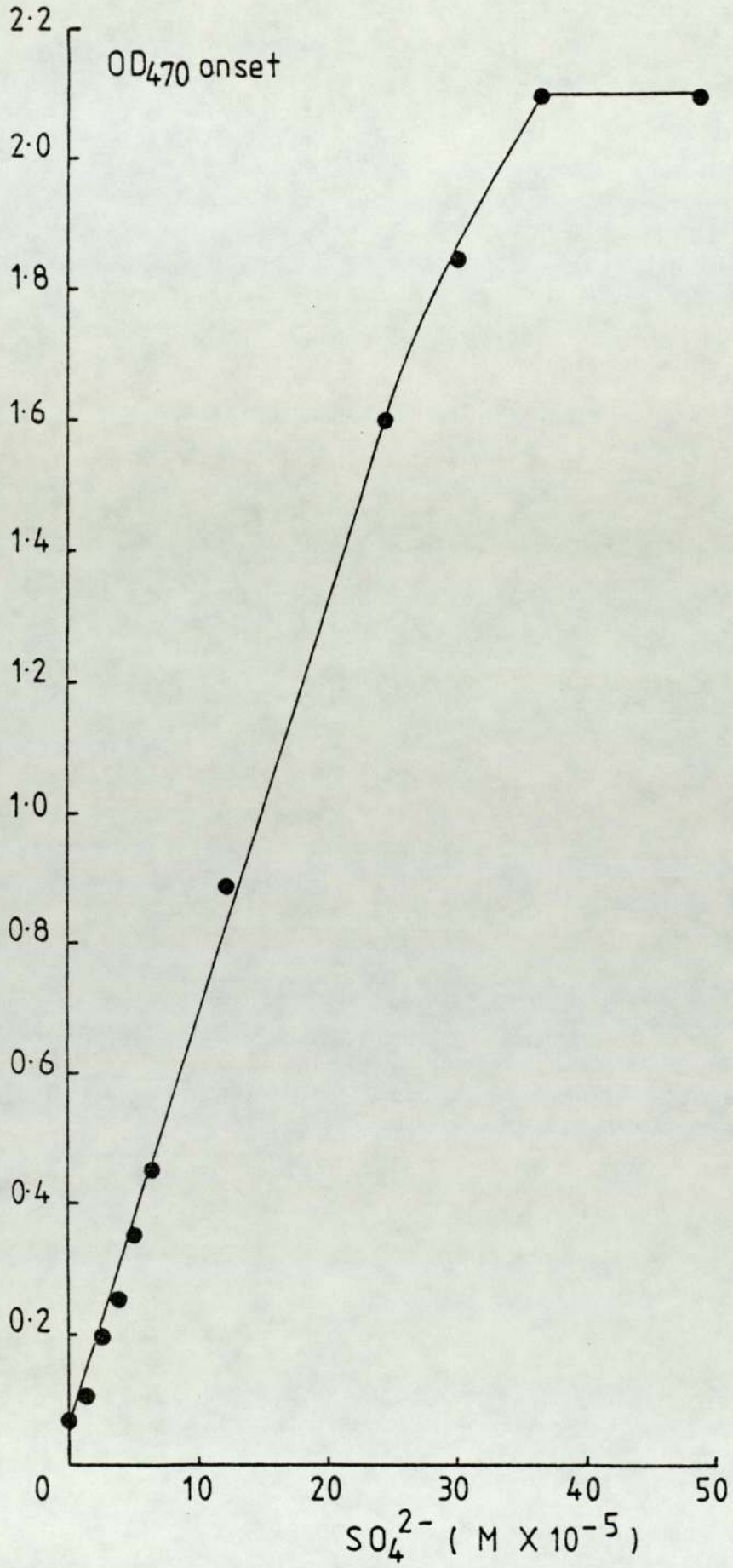

2·2
2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
10
20
30
40
50
OD470 onset
SO4 2- (M X 10-5)

Fig. 32 Double reciprocal plot of *P. cepacia* growth rate versus medium sulphate concentration.

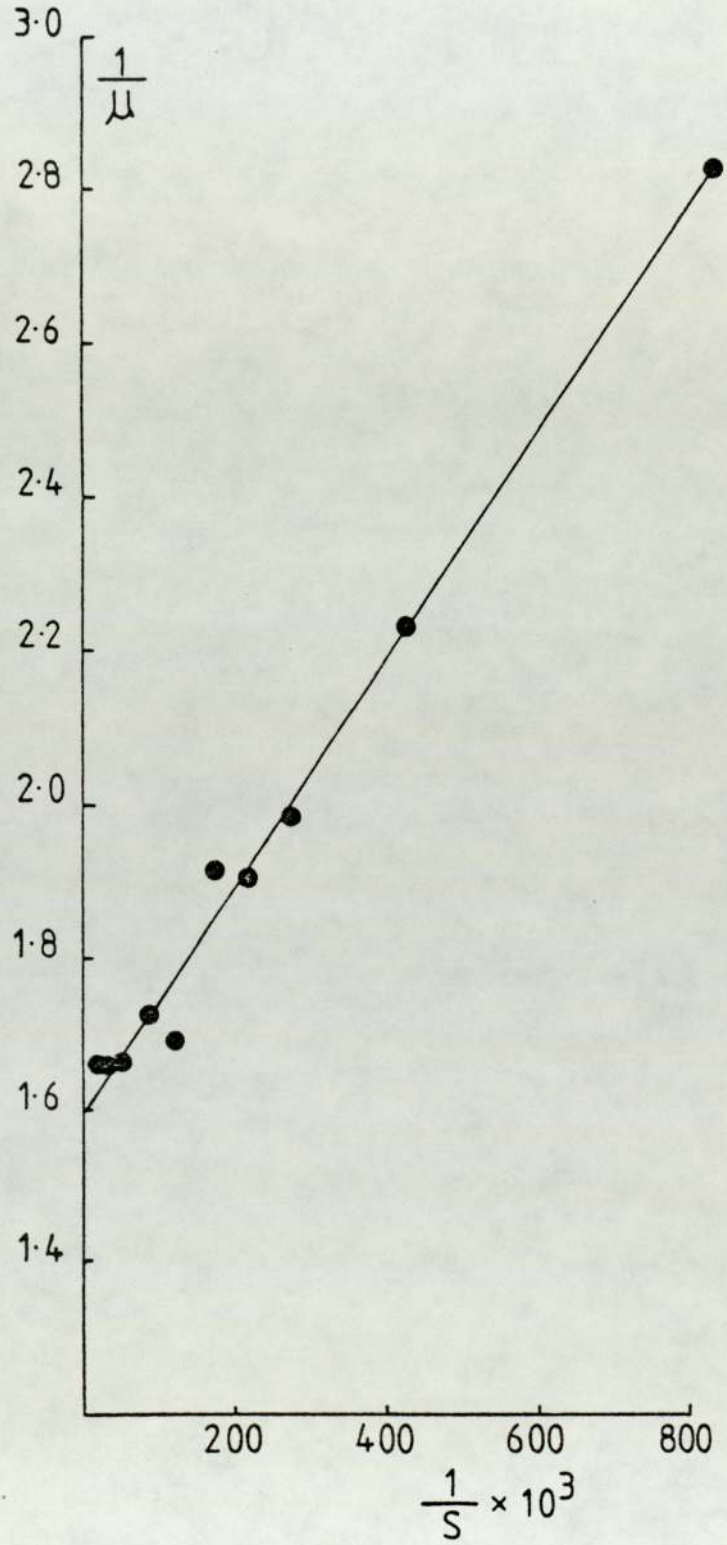

3·0
2·8
2·6
2·4
2·2
2·0
1·8
1·6
1·4
1/μ
200
400
600
800
1/S × 10³

Table 9

Growth rate constant, μ_m, and saturation constant, K_s, of P. cepacia grown under different nutrient limitations

Limiting Nutrient	μ_m	K_s
Iron	0.658	0.6×10^{-7}
Magnesium	0.685	8×10^{-7}
Sulphate	0.625	1.25×10^{-6}

Knowledge is like a garden, if it is not cultivated, it can not be harvested.

Guinean Proverb.

3.2 Some Physical Investigations of The Behaviour of Bacterial Surface

3.2.1 Surface charge as studied by particle microelectrophoresis

The surface charge density of a particle can be studied by measuring its electrophoretic mobility. The equipment used for electrophoretic measurement was the Rank Particle Microelectrophoretic Apparatus Mark II (Rank Bros.,Bottisham, Cambridge). It was described in detail by Kayes (1975).

3.2.1.1 Effect of nutrient depletion on electrophoretic mobility of P. cepacia

P.cepacia NCTC 10661 was grown in an appropriate medium (see Table 8),which would support the cells to growth to an OD_{470} of 1.0 with certain nutrient becoming depleted (for oxygen limitation, OD_{470} reached 4.5). The cells were grown in an orbital shaker, at 37°, with a shaking rate of 140 rpm for 24-26 h. The cells were harvested by centrifugation at 8,000x g for 10 min, washed once with phosphate buffer,pH 7.0 and ionic strength 0.013 (Hill et al.,1963),and resuspended in the medium to give OD_{470} of 0.2.

The above cell suspension was transferred to the electrophoretic cell by means of a dropper, the electrodes were slowly inserted and the velocity measurements made. The cell was washed with distilled water between samples and care was taken to fill completely the tube excluding air bubbles. Particle velocities were determined by timing

individual particles over a fixed distance on the eyespiece scale. Only light spots in sharp focus were timed. The current was adjusted to give a transit time of 5 to 10 seconds over the measured distance, where possible, as timings of this magnitude are optimum with respect to Brownian motion error and operator timing error. Particles were timed in alternate directions, by use of the reversing switch, so that the effect of drift can be largely eliminated. The velocity was calculated from an everage of about 50 timings. The results of the measurement are shown in Table 10.

From the results, it showed that the electrophoretic mobilities of P.cepacia grown under different nutrient limitations were in the following order: Logarithmic-phase CDM and Carbon-depleted cells > stationary-phase cells > sulphate-depleted and phosphate-depleted cells > nitrogen-depleted cells > iron-depleted and magnesium-depleted cells.

Table 10

Electrophoretic mobilities of P. cepacia NCTC 10661 grown under different nutrient limitations

Limiting nutrient	Electrophoretic mobility *
Carbon-depleted	2.73 ± 0.30
Iron-depleted	0.85 ± 0.07
Phosphate-depleted	1.23 ± 0.10
Magnesium-depleted	0.83 ± 0.08
Nitrogen-depleted	1.00 ± 0.09
Sulphate-depleted	1.48 ± 0.14
Stationary-phase CDM	1.93 ± 0.15
Log-phase CDM	2.97 ± 0.22

* It is expressed in 10^{-8} m^2 V^{-1} S^{-1}

3.2.1.2 Effect of CC_5 or NPN on the electrophoretic mobility of P. cepacia NCTC 10661

P.cepacia NCTC 10661 was grown in complete CDM in an orbital shaker at 37° with shaking rate of 140 rpm. The cells were harvested at the mid-logarithmic phase (OD of 0.8) by centrifugation at 8,000 x g for 10 min, washed once with phosphate buffer, pH 7.0 and ionic strength 0.013, and resuspended in this buffer to give OD_{470} of 0.2. 25 ml of the cell suspension was transferred to a conical flask and 0.1 ml of either methanol or CC_5 in methanol or NPN in methanol (final concentration 1 x 10^{-5}M). They were incubated in shaking water bath for 15 min, then the cells were centrifuged at 8,000 x g for 15 min, the supernatant was discarded and the pellet was resuspended in 25 ml of phosphate buffer. The electrophoretic mobilities of these cells were then measured as described in section 3.2.1.1. The results are shown in Table 11.

Table 11

Effect of CC_5 or NPN on the electrophoretic mobility of P. cepacia NCTC 10661

Probe treated	E.M ($X\ 10^{-8}\ m^2\ V^{-1}\ S^{-1}$
Control (methanol)	2.85 ± 0.20
CC_5	2.79 ± 0.19
NPN	2.76 ± 0.23

3.2.1.3 Electrophoretic mobility of P.aeruginosa grown under iron limitation in the chemostat

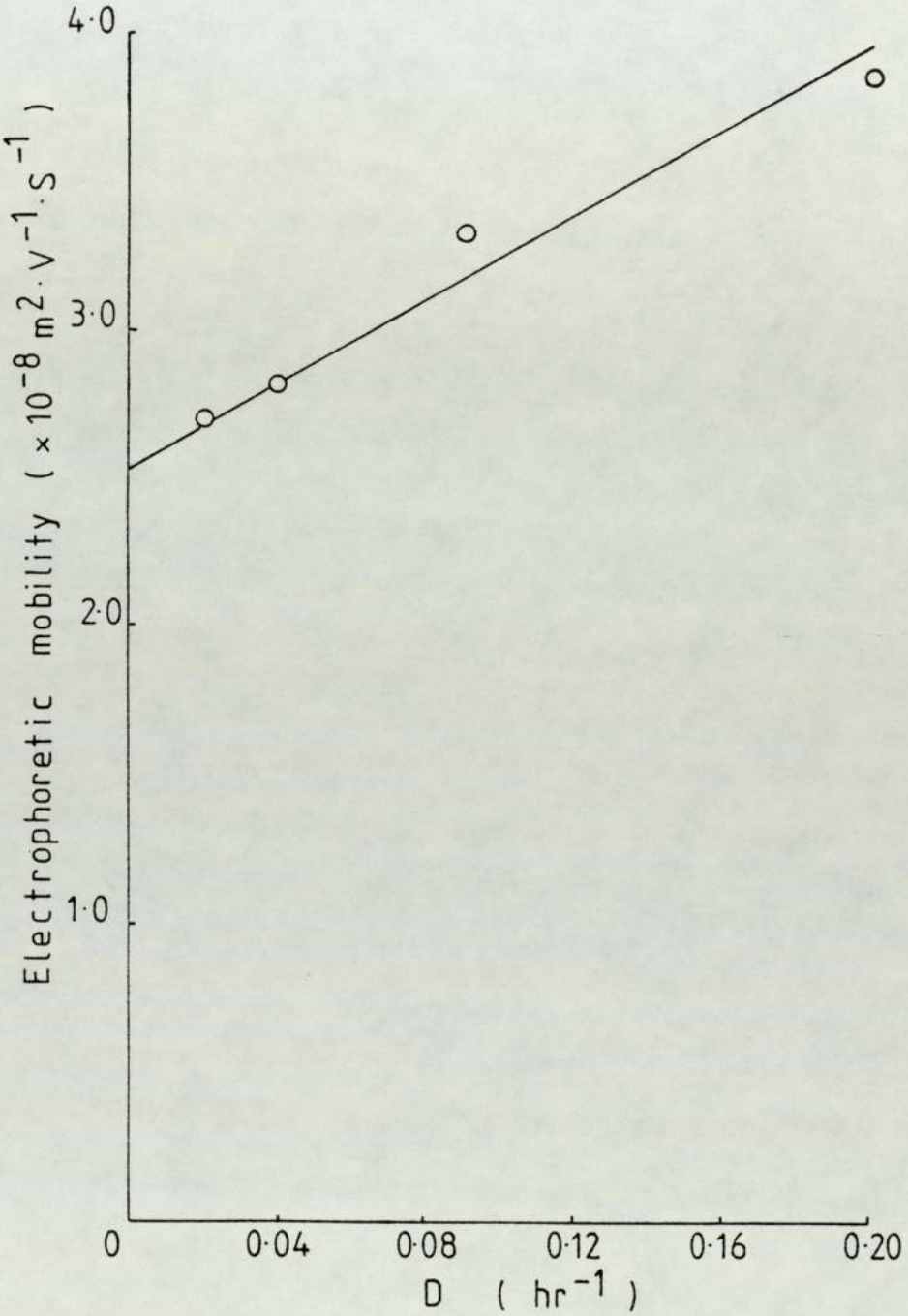
P.aeruginosa NCTC 6750 (wild type) was grown in a chemostat under iron limitation with different dilution rates by Dr. E.A. Ombaka (Ombaka,1980). The cells were harvested by centrifugation at 8,000 x g for 10 min,washed once with phosphate buffer,pH 7.0 and ionic strength 0.013, and resuspended in this buffer to give OD_{470} of 0.2. The electrophoretic mobilities of these cells were then determined as described in section 3.2.1.1. The results are shown in Fig. 33.

3.2.2 Surface hydrophobicity as studied by contact angle measurement

The method used for the measurement of the contact angle of bacteria was described by Van Oss et al. (1975).

P.cepacia NCTC 10661 was grown in an appropriate medium (see Table 8), which would support the cells to growth to an OD_{470} of 1.0 when a specific nutrient became depleted (for oxygen-depleted cells, OD_{470} reached 4.5), in an orbital shaker at 37°,with the shaking rate of 140 rpm for 24 h. P. aeruginosa NCTC 6750 (wild type) and P. aeruginosa M7 were grown in an appropriate medium for 36 h as described by Dr. E.A. Ombaka (1980). The cells were harvested by centrifugation at 8,000 x g for 10 min,washed once with sterilised physiological saline, then resuspended in this medium to give OD_{470} of 2.0 to 3.0. 25 to 30 ml of this bacterial suspension was filtered through a 0.4 μm pore

Fig. 33 The electrophoretic mobilities of iron-limited P.aeruginosa NCTC 6750 grown in chemostats at different dilution rates.


4·0
3·0
2·0
1·0
0
0·04
0·08
0·12
0·16
0·20
Electrophoretic mobility ($\times 10^{-8}$ $m^2 \cdot V^{-1} \cdot S^{-1}$)
D (hr^{-1})

diameter cellulose acetate membrane filter (diameter 47 mm), which had previously been soaked three times in boiled distilled water to remove the detergent from the membrane filter. It was then taken off the filter apparatus by using a spatula, and stuck on a glass slide (50 x 60 mm) by using a water based adhesive ("Gloy"). It was allowed to dry in air to remove excess moisture. The length of time of drying can be judged by disappearance of surface glossiness and the appearance of a more matted surface aspect. A contact angle is an angle between a solid surface (bacterial layer) and a liquid drop (physiological saline). It was determined by measuring a saline drop of a standard size (10 ul) with a goniometer. At least ten drops of physiological saline should be measured and the average taken. The results are presented in Table 12.

Table 12

Contact angle of *P. cepacia* and *P. aeruginosa* grown under different nutrient limitations

Organism	Limiting nutrient	Contact angle*
P.cepacia		
NCTC 10661	Phosphate	21.80 ± 0.91
	Stationary-phase	21.13 ± 0.91
	Nitrogen	19.89 ± 0.88
	Carbon	19.74 ± 0.85
	Magnesium	18.95 ± 0.67
	Sulphate	18.28 ± 0.78
	Logarithmic-phase	17.0 ± 0.69
	Iron	15.69 ± 0.66
P. aeruginosa		
NCTC 6750	Nitrogen	24.13 ± 0.72
	Sulphate	21.18 ± 0.63
	Carbon	20.85 ± 0.60
	Iron	19.77 ± 0.61
	Magnesium	16.16 ± 0.56
P. aeruginosa		
M_7 (mucoid)	Nitrogen	23.31 ± 0.85
	Sulphate	20.46 ± 0.71
	Carbon	18.1 ± 0.62
	Magnesium	16.16 ± 0.61
	Iron	14.80 ± 0.62
	Phosphate	13.72 ± 0.67

* non-opsonized cells

3.3 Phagocytosis and Killing of P. cepacia by Blood

3.3.1 Method

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

P.cepacia NCTC 10661 was grown in an appropriate medium (see Table 8) and harvested by centrifugation at 8,000 x g for 10 min, washed once and resuspended in sterile 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 bacterial suspension were mixed together in a sterile plastic tube. The blood/bacteria mixtures (in triplicates) were incubated in a shaking water bath at 37°. The blood was used in the study within an hour of collection. At the start of incubation (0 min) 0.1 ml sample was removed and transferred into 0.9 ml of sterile distilled water, mixed well and allowed to stand for 10 min at room temperature. The time interval allowed lysis of blood cells without killing of bacteria. At timed intervals, 0.1 ml samples were removed from the phagocytic mixture. These were transferred to 0.9 ml of sterile distilled water at room temperature. Samples were further diluted to 10^5 in half strength nutrient broth. Viable counts were performed by using the Miles and Misra method described in section 2.2.1. Plates were incubated for 18 hr at 37°. 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 leukocytes and also the number of PMN leukocytes without bacteria.

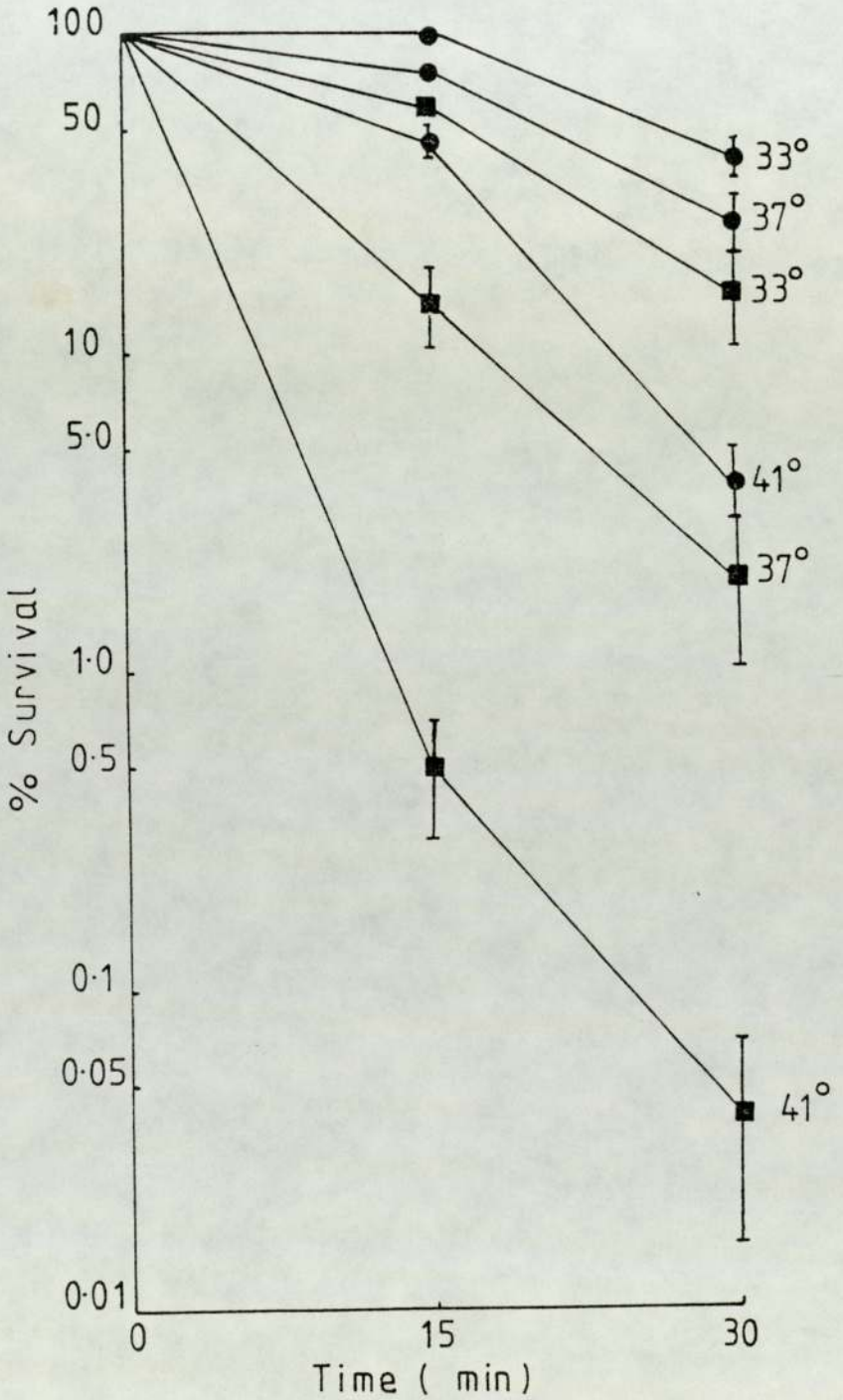
3.3.2 Effects of temperature and nutrient limitation on killing by blood

The effect of temperature on killing by blood was carried out by using cells grown under different nutrient limitations for 24 hr at 37°. The blood/bacteria mixtures were incubated at 33°, 37° and 41°C and samples removed at 15 and 30 min. In Fig. 34, log-phase cells showed a decrease in survival as the temperature was increased from 33° to 37° and then to 41°. Decrease in survival was also observed in the stationary-phase cells as the temperature was increased from 37° to 41°, but there was apparently little difference between 33° and 37°. Comparison of the killing of the log-phase and stationary-phase cells by blood at the same incubation temperature showed that stationary-phase cells were much more resistant than the log-phase cells to chemical and enzymatic attacks by the phagocytes. However, it was found that the stationary-phase cells were more easily engulfed than the log-phase cells by PMN leukocytes as shown in Table 13. Temperature seems to have little effect on the engulfment of the stationary-phase cells, as it may be due to

Fig. 34 Effect of temperature on killing of P. cepacia by whole blood.

■ Logarithmic-phase cells

● Stationary-phase cells


100
50
10
5·0
1·0
0·5
0·1
0·05
0·01
% Survival
0
15
30
Time (min)
33°
37°
33°
41°
37°
41°

the fact that they had been engulfed to the greatest extent even at lowest temperature. However, increase in the number of bacteria taken per PMN leukocyte was observed in the log-phase cells as the temperature of incubation was increased.

Figures 35 to 37 show the killing by blood of P.cepacia grown under six different nutrient limitations at three different temperatures. Carbon-depleted cells were found to be most sensitive at three different temperatures used in the study. There was no significant effect of temperature on killing of these very sensitive carbon-depleted cells. The other nutrient-depleted cells seems to be affected by the temperature at which phagocytosis was performed. Increase in survival occurred when the phagocytosis was performed at lower temperatures. Increase in resistance to killing by blood at any of the temperatures used was found to be in the following order: carbon, iron, sulphate, nitrogen, phosphate and magnesium-depleted cells. Carbon-depleted cells were also found to be most easily engulfed by PMN leukocytes (Table 13). There was a slight effect of temperature on the number of carbon-depleted cells taken per PMN leukocyte. Magnesium-depleted cells were not only the most resistant to killing but also engulfed with great difficulty among the nutrient limitations being studied. The other nutrient-limited cells were found to lie in sensitivity between carbon and magnesium-depleted cells.

Fig. 35 Killing of *P. cepacia* by whole blood at 41°.

- ● Magnesium-depleted cells
- ■ Phosphate and nitrogen-depleted cells
- ◪ Sulphate-depleted cells
- □ Iron-depleted cells
- ○ Carbon-depleted cells

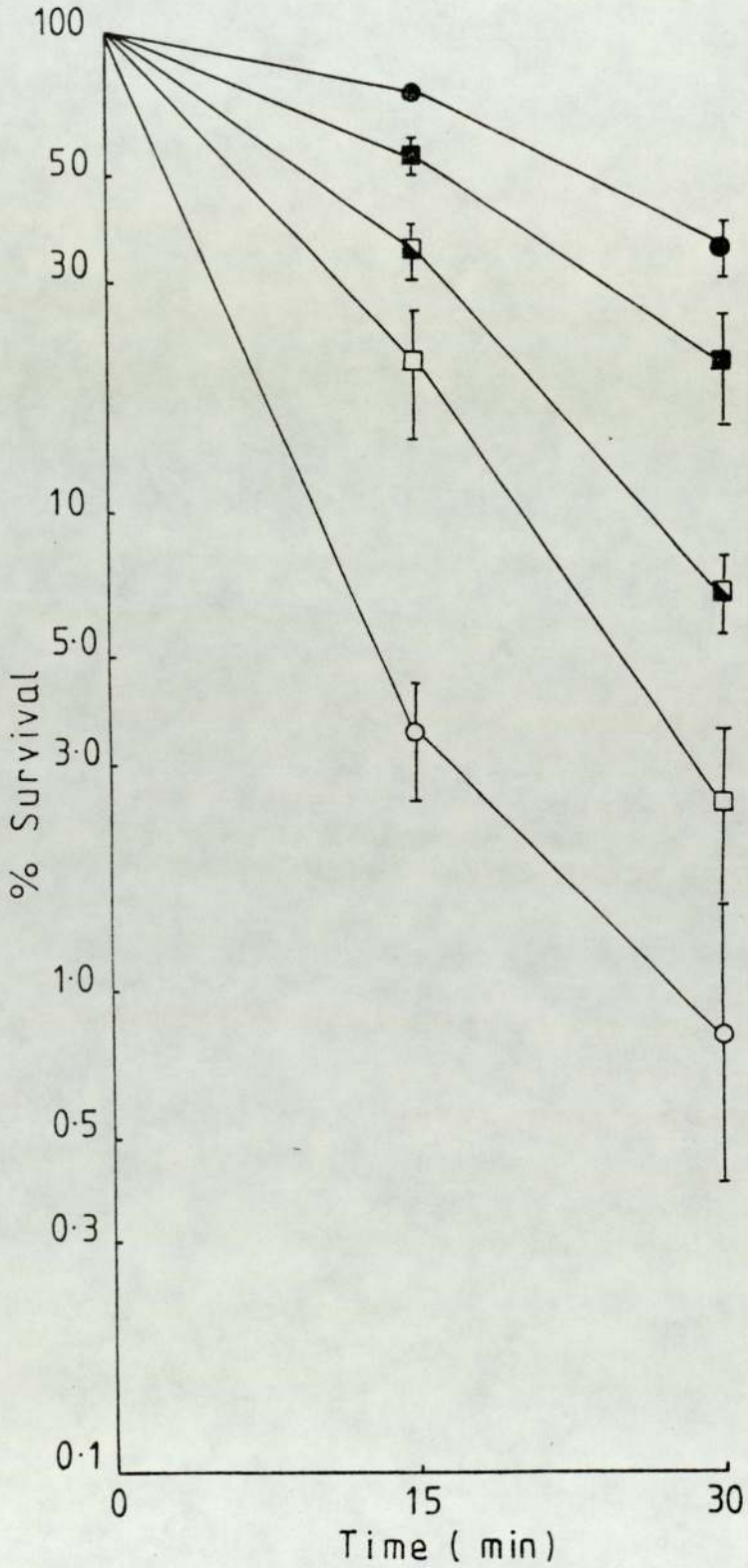

100
50
30
10
5·0
3·0
1·0
0·5
0·3
0·1
% Survival
0
15
30
Time (min)

Fig. 36 Killing of *P. cepacia* by whole blood at 37°.

- ● Magnesium-depleted cells
- ■ Phosphate and nitrogen-depleted cells
- ◪ Sulphate-depleted cells
- □ Iron-depleted cells
- ○ Carbon-depleted cells

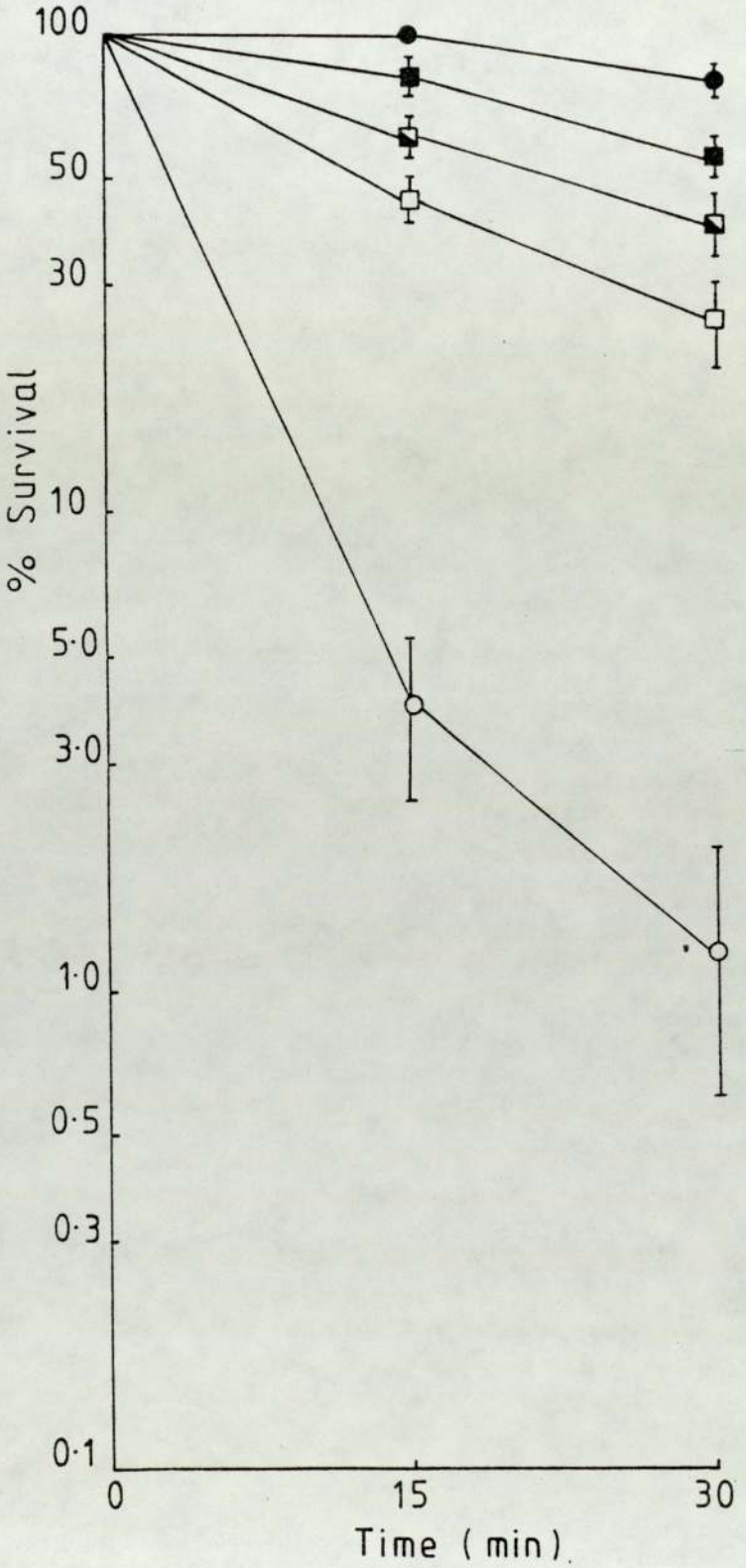

100
50
30
10
5·0
3·0
1·0
0·5
0·3
0·1
% Survival
0
15
30
Time (min)

Fig. 37 Killing of *P. cepacia* by whole blood at 33°.

● Magnesium-depleted cells

■ Phosphate and nitrogen-depleted cells

◪ Sulphate-depleted cells

□ Iron-depleted cells

○ Carbon-depleted cells

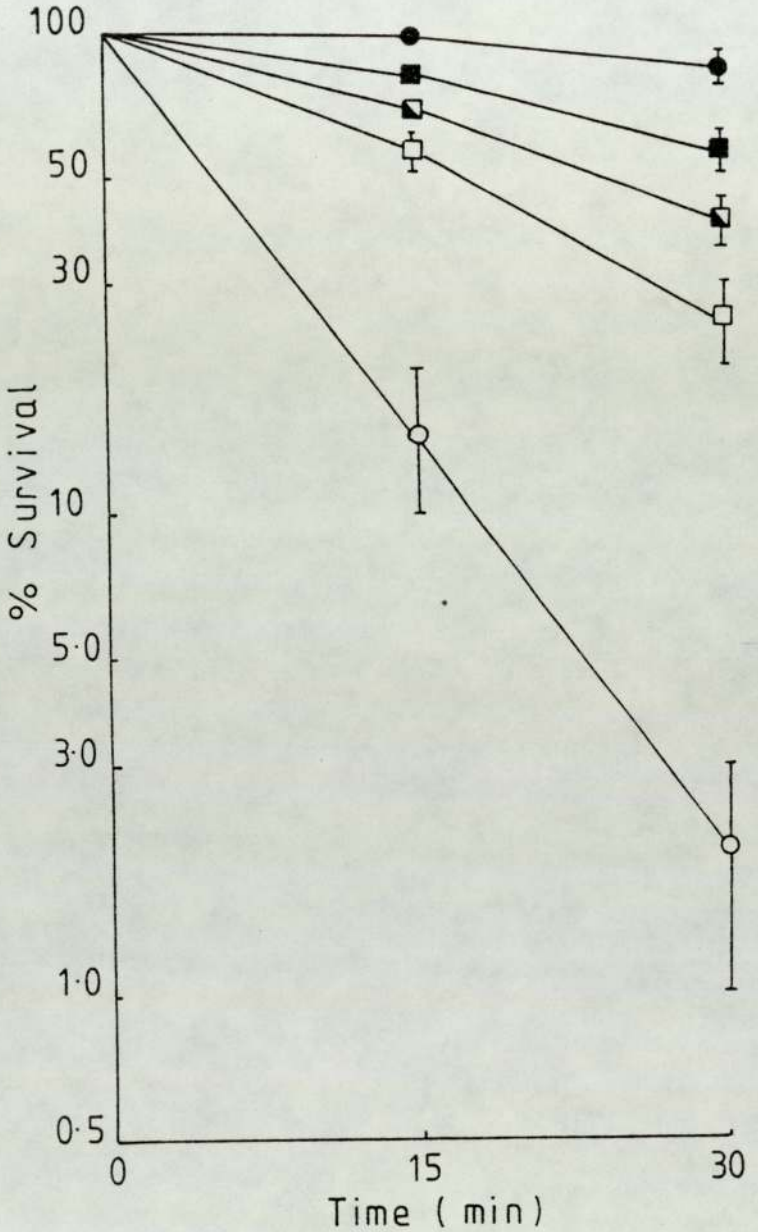

100
50
30
10
5·0
3·0
1·0
0·5
% Survival
0
15
30
Time (min)

Table 13

Engulfment of P. cepacia NCTC 10661 by PMN leukocytes at three different temperatures

Limiting nutrient	Factors investigated	Temperature		
		41°	37°	33°
Iron	% active PMN	94	90	74
	No. bacteria/active PMN	7.8	5.5	4.1
	No. bacteria/ PMN	6.4	4.2	3.3
Carbon	% active PMN	98	90	98
	No. bacteria/active PMN	10.3	9.8	8.0
	No. bacteria/PMN	10.1	8.8	7.7
Nitrogen	% active PMN	96	92	90
	No. bacteria/active PMN	7.0	4.7	4.8
	No. bacteria/PMN	6.7	4.4	4.2
Phosphate	% active PMN	90	76	74
	No. bacteria/active PMN	6.3	4.7	4.1
	No. bacteria/PMN	4.6	2.3	1.9
Sulphate	% active PMN	95	94	92
	No. bacteria/active PMN	8.5	7.7	5.9
	No. bacteria/PMN	8.2	7.3	5.3
Magnesium	% active PMN	86	68	55
	No. bacteria/active PMN	3.7	2.8	2.2
	No. bacteria/PMN	3.1	2.0	1.3
Log-phase cells	% active PMN	92	90	90
	No. bacteria/active PMN	7.5	5.6	5.0
	No. bacteria/PMN	6.6	5.1	4.6
Stationary-phase cells	% active PMN	100	98	100
	No. bacteria/active PMN	10.6	9.8	9.3
	No. bacteria/ PMN	10.6	9.5	9.3

Means of duplicate determinations.

3.4 Studies of The Properties of The Membranes of P. cepacia NCTC 10661 With Fluorescent Probes

3.4.1 Introduction

Fluorescent probing techniques have been suggested by several workers (Radda, 1975 ; Azzi, 1975) in the study of the structure and functions of biological membranes.

In this study, several fluorescent probes which are commercially available were used to investigate the effects of growth environment on the membranes of P. cepacia. Fig. 38 shows the chemical formula of the probes used in the study. Their excitation and emission wavelengths are given in Table 14.

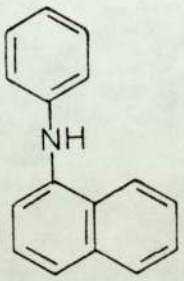
Table 14

Excitation and emission wavelengths of the fluorescent probes used in the study

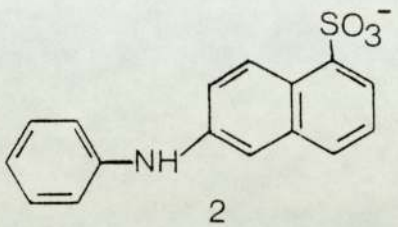
Probe	Excitation wavelength	Emission wavelength
ANS	355 nm	530 nm
TNS	365 nm	430 nm
CC_5	470 nm	505 nm
NPN	340 nm	405 nm
2-methyl anthracene	375 nm	435 nm
DPH	365 nm	430 nm

Fig. 38 Structural formula of the fluorescent probes used in the study.

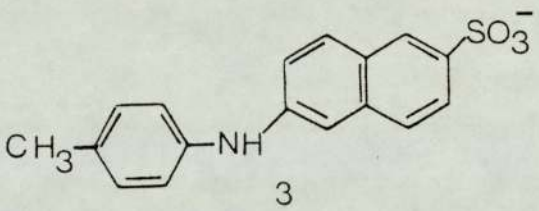
1. N-Phenyl-1-naphthylamine (NPN).
2. 6-Anilino-1-naphthalene-sulphonic acid (ANS).
3. 2p-Toluidinyl-naphthalene-6-sulphonate (TNS).
4. 2-methyl anthracene.
5. 1,6-Diphenyl-1,3,5-hexatriene (DPH).
6. 3,3'-Dipentyloxacarbocyanine iodide (CC_5).


NH

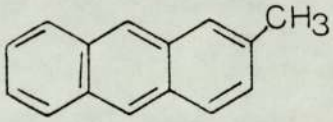
1


SO3-
NH

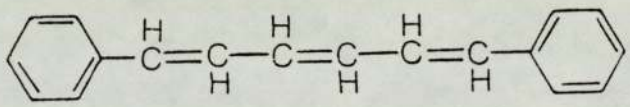
2


SO3-
CH3
NH

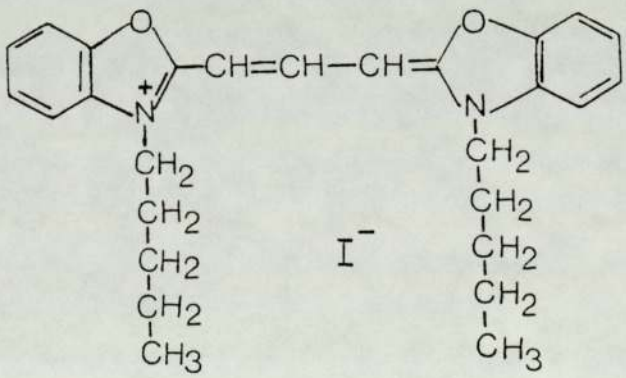
3


CH3

4



5


O
CH=CH—CH=
O
+
N
N
CH2
CH2
CH2
CH2
CH3
I-
CH2
CH2
CH2
CH2
CH3

6

3.4.2 Studies of P. cepacia with TNS or ANS

It was shown by Newton (1954) that TNS fluoresced weakly in water; addition of bacterial cells (P. aeruginosa) did not show much increase in fluorescence intensity. However, upon addition of polymyxin or treatment of the cells by boiling, an immediate increase in fluorescence intensity was observed. His interpretation was that polymyxin or boiling damaged the membrane and increased penetration of the probe into the cells. Ballard et al. (1972) found that addition of ANS to E. coli suspensions showed very weak fluorescence at neutral pH. However, as the pH was lowered, an increase in fluorescence intensity was observed. It was intended to study these effects in P. cepacia with TNS and ANS.

3.4.2.1 Effect of boiling on the fluorescence of TNS or ANS in P. cepacia grown under different nutrient depletions

ANS or TNS was dissolved in methanol (5×10^{-4} M). It was prepared daily and discarded after use.

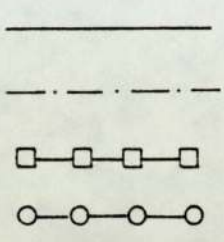
P. cepacia NCTC 10661 grown overnight in iron-deficient CDM was used as an inoculum. It was washed twice with sterilised normal saline, and resuspended in this medium to give an OD_{470} of 1.0. 0.25 ml of this cell suspension was used to inoculate 24.75 ml of the appropriate medium (see Table 8). The medium was designed so that P. cepacia could grow linearly up to an

OD_{470} of 1.0 before a specific nutrient became limited in the medium. The growth was followed as mentioned in section 2.2.3. The cells were separately harvested 3 h after the turn-off point from logarithmic growth, by centrifugation and resuspended to an OD_{470} of 1.0. They were heated in a boiling water bath for 10 min, centrifuged at 1,500 x g for 15 min, washed twice with normal saline and resuspended in this solution to give an OD_{470} of 0.25. 4.9 ml of this bacterial suspension was transferred to a test tube and 0.1 ml of TNS or ANS (final concentration, 10 µM) in methanol was added. They were then incubated for 10 min at 37^{o} and fluorescence spectra taken. No fluorescence was observed in unheated cells treated with ANS or TNS (data not shown). Fluorescence was observed in cells heated in a boiling water bath for 10 min. The results are shown in Figures 39 and 40. Both probes showed decrease of fluorescence intensity in the following order: glucose, iron, phosphate and nitrogen-depleted cells.

3.4.2.2 Effects of pH and buffer systems on the fluorescence of TNS or ANS in P. cepacia

ANS or TNS is almost non-fluorescent in water or in the presence of bacterial cells at neutral pH. However, Ballard et al. (1972) found that fluorescence of ANS in the presence of E. coli was pH dependent and fluorescence intensity increased as the pH was lowered. Several buffer

Fig. 39 Fluorescence of TNS in suspensions of boiled P. cepacia NCTC 10661



Glucose-depleted cells.

Iron-depleted cells.

Phosphate-depleted cells.

Nitrogen-depleted cells.

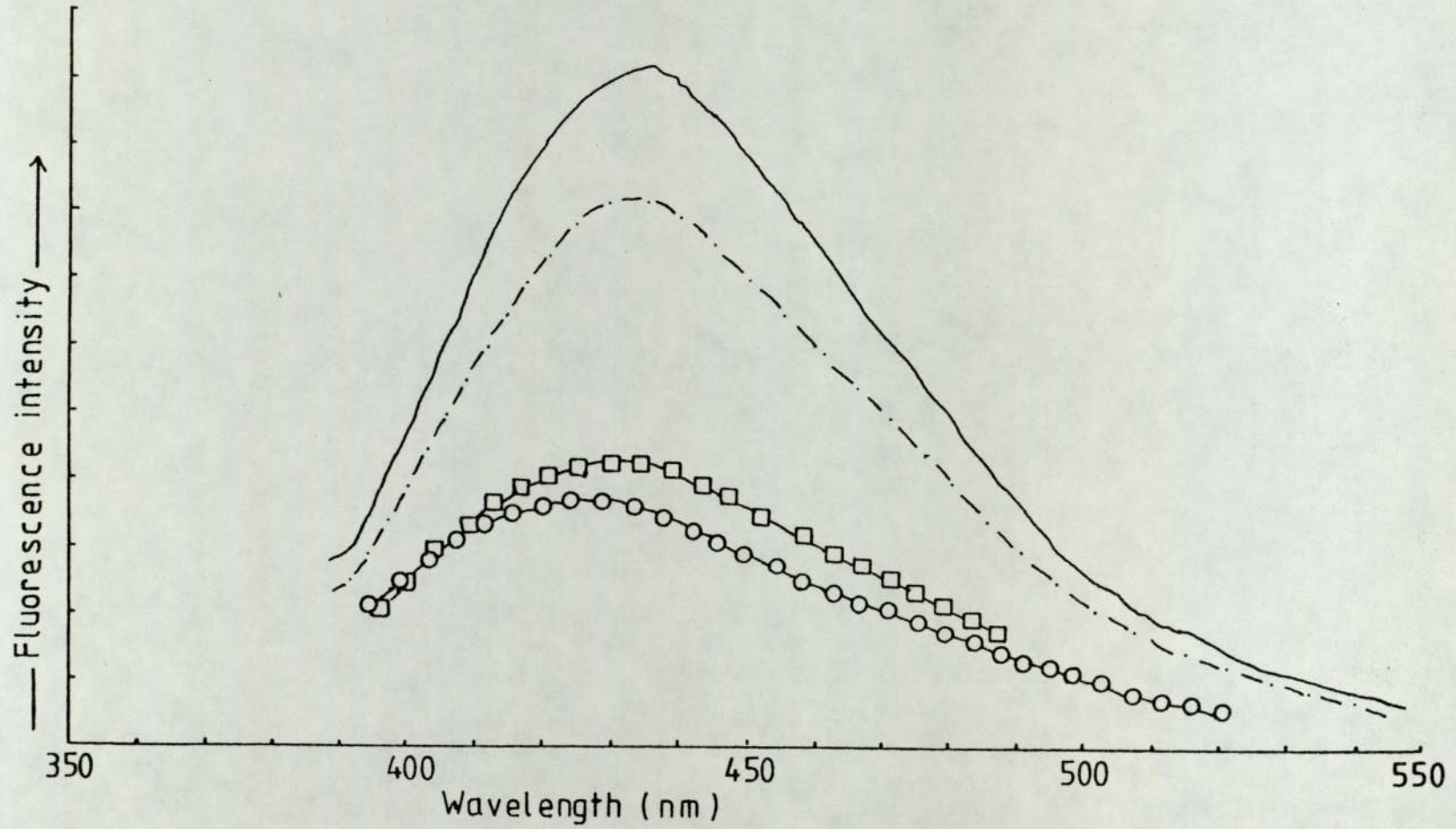
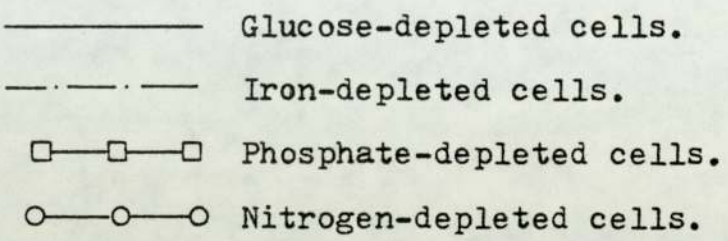

Fluorescence intensity
350
400
450
500
550
Wavelength (nm)

Fig. 40 Fluorescence of ANS in suspensions of boiled *P. cepacia* NCTC 10661.



Fluorescence intensity
350
400
450
500
550
Wavelength (nm)

systems were employed in this study including tris(hydroxymethyl) amino methane (Tris).

It is now well documented that Tris-buffer may damage bacterial envelopes (Irvin et al., 1981). Tris has been shown to break the salt bridges formed by metal cations with structural components such as LPS and release these components from the cell walls (Voss,1967).

Buffer systems such as Tris-buffer, citrate-phosphate buffer and MOPS buffer are prepared as following:

(a) Tris(hydroxymethyl) aminomethane - maleate buffer.

Stock solutions:

A: 0.2 M solution of Tris acid maleate (24.2 g of tris(hydroxymethyl) amino methane + 23.2 g of maleic acid or 19.6 g of maleic anhydride in 100 ml).

B: 0.2 N NaOH.

50 ml of A + x ml of B, then diluted to a total of 200 ml.

x (ml)	pH
7.0	5.2
31.5	6.2
42.5	6.6
48.0	7.0
54.0	7.4

(b) Citrate-phosphate buffer.

Stock solutions:

A: 0.1 M solution of citric acid (19.21 g in 1000 ml).

B: 0.2 M solution of dibasic sodium phosphate (53.65 g of $Na_2HPO_4.7H_2O$ or 71.7 g of $Na_2HPO_4.12H_2O$ in 1000 ml).

X ml of A + Y ml of B. diluted to a total of 100 ml.

X (ml)	Y (ml)	pH
23.3	26.7	5.2
16.9	33.1	6.2
13.6	36.4	6.6
6.5	43.6	7.0

(c) MOPS buffer.

3-(N-morpholino)propanesulphonic acid buffer (MOPS) solution was adjusted to give appropriate pH with concentrated NaOH solution (final concentration 0.05 M).

P. cepacia NCTC 10661 was grown in complete CDM (initial OD_{470} about 0.04) and harvested at an OD_{470} of 0.5. The cells were harvested by centrifugation at 1,500 x g for 15 min, washed twice with normal saline, then resuspended in an appropriate buffer described above to give an OD_{470} of 0.25. 4.9 ml of each cell suspension was transferred to a test tube and 0.1 ml of ANS or TNS was added (final concentration, 10 uM). They were then incubated at 37° for 15 min and the fluorescent spectra were taken. The results are presented from Figures 41 to

44.

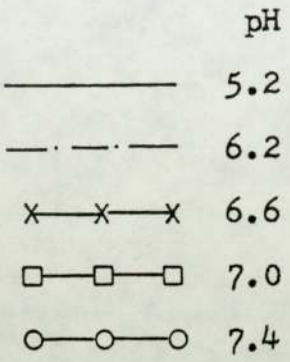
In these experiments, we found that fluorescence intensity of TNS or ANS was increased consistantly, when pH was lowered. However, great enhancement could only be observed when Tris-buffer was used; much less increase in fluorescence occurred when MOPS or citrate-phosphate buffer was used. These results confirmed previous observations by other workers (Voss, 1967; Irvin et al., 1981) that Tris-buffer has some toxic effects on the bacterial envelope and it seems that the toxic effects are enhanced when pH is lowered.

3.4.3 Fluorescence of 3,3'-dipentyloxacarbocyanine iodide (CC_5) in P. cepacia

When CC_5 is added to normal saline, it displays a characteristic fluorescence emission spectrum (Fig. 45), with excitation and emission maxima at 470 nm and 505 nm respectively. If the solvent is methanol, the spectrum is qualitatively the same, but the dye fluoresces more strongly in organic solvents than it does in aqueous phase. If the dye is added to a suspension of cells, there is an immediate increase in the fluorescence intensity (Fig. 45). The result suggests that the dye is moving to the hydrophobic area of the cell membrane from the aqueous medium, and is, as a consequence, showing a more intense emission intensity.

P. cepacia NCTC 10661 grown overnight in complete CDM

Fig. 41 The effects of pH and Tris-buffer on fluorescence of ANS in P. cepacia NCTC 10661.



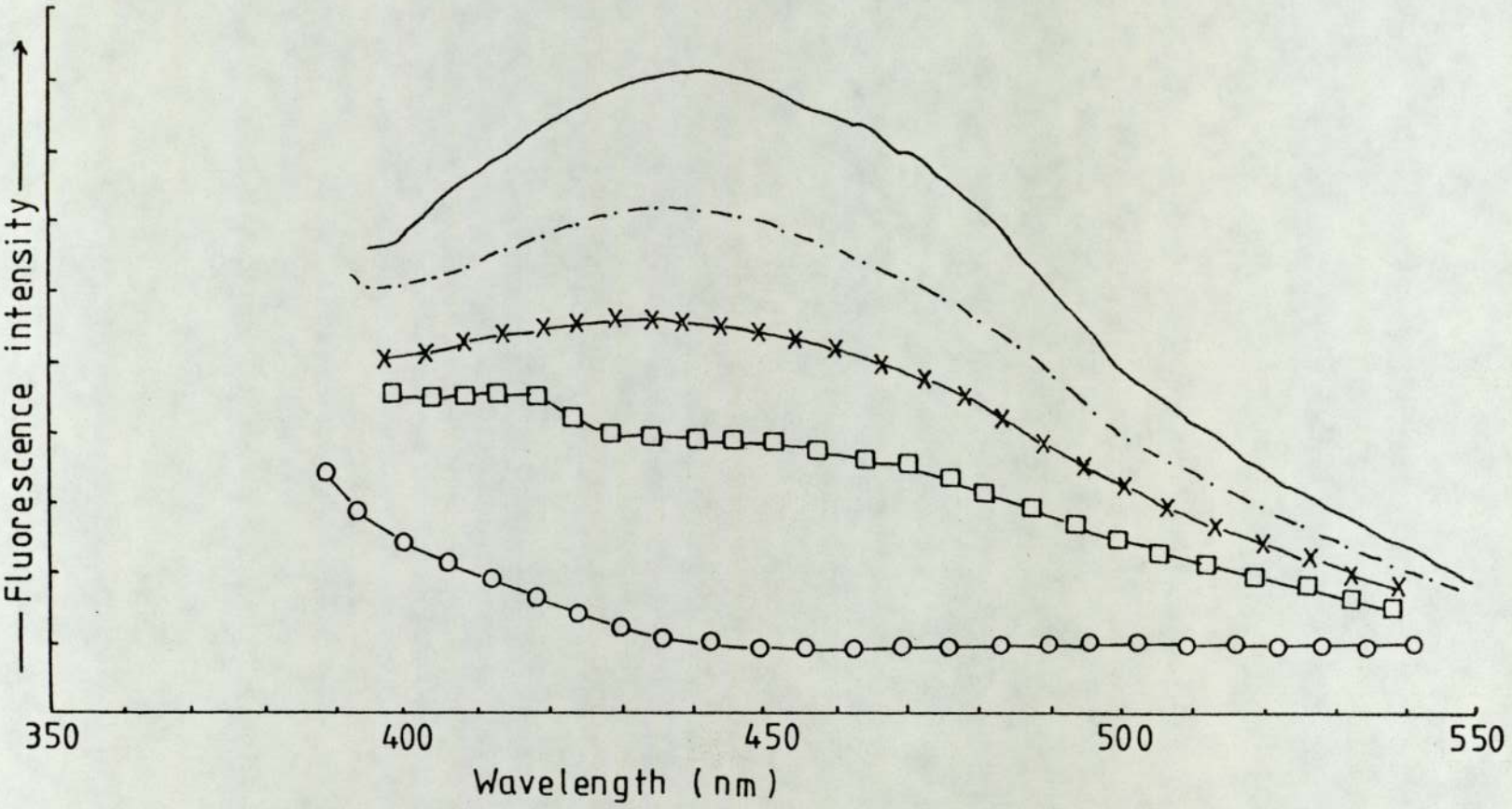
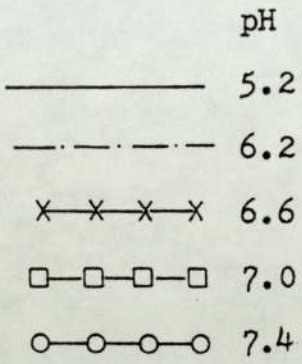

Fluorescence intensity
350
400
450
500
550
Wavelength (nm)

Fig. 42 The effects of pH and Tris-buffer on fluorescence of TNS in P. cepacia NCTC 10661.



Fluorescence intensity
350
400
450
500
550
Wavelength (nm)

Fig. 43 The effects of pH and citrate-phosphate buffer on the fluorescence of TNS in P. cepacia NCTC 10661.

pH

———— 5.2, 6.2, 6.6

—·—·— 7.0

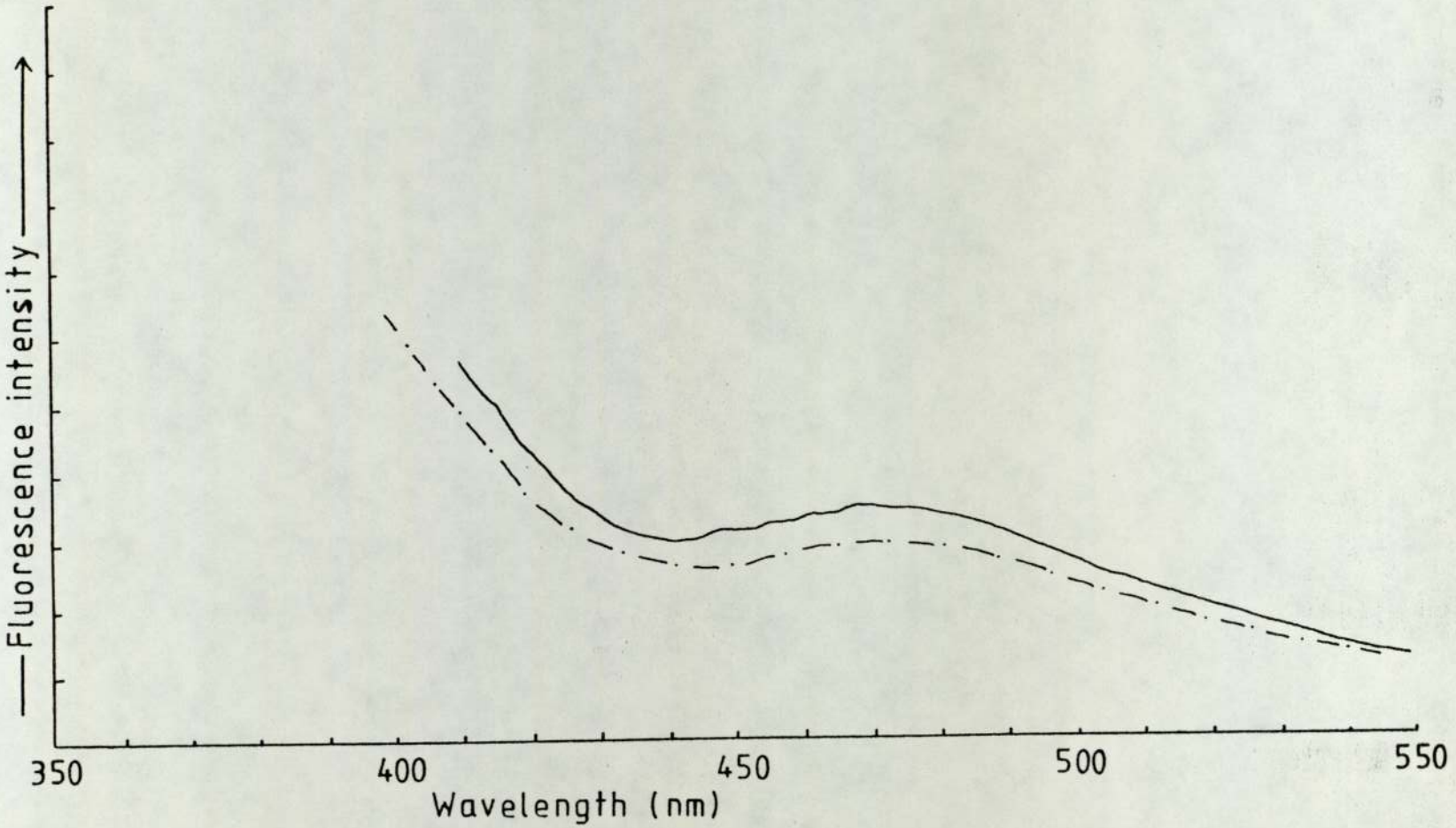
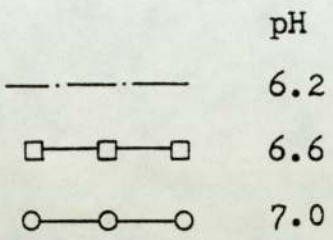

Fluorescence intensity
350
400
450
500
550
Wavelength (nm)

Fig. 44 The effects of pH and MOPS buffer on the fluorescence of ANS in P. cepacia NCTC 10661.



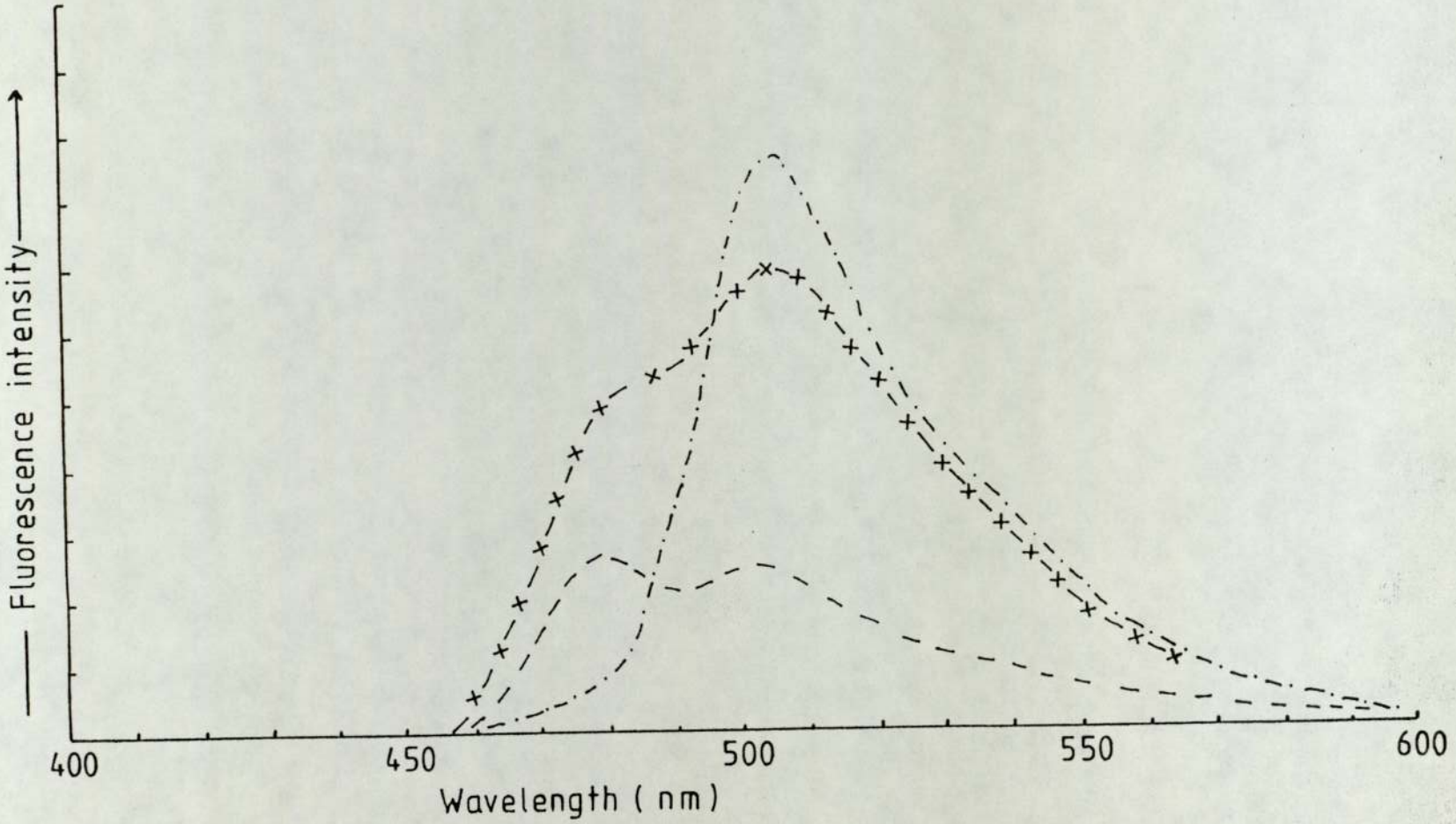
Fluorescence intensity
350
400
450
500
550
Wavelength (nm)

Fig. 45 CC_5 emission spectra in different systems.

—·—·— in methanol.

X—X—X in suspension of P. cepacia (saline).

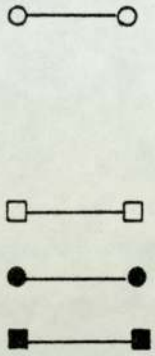
— — — — in normal saline.


Fluorescence intensity
400
450
500
550
600
Wavelength (nm)

was used as an inoculum. It was washed twice with sterilised normal saline and resuspended in this medium to give an OD_{470} of 1.0. 1 ml of this cell suspension was used to inoculate 125 ml of an appropriate medium (see Table 8). The medium was designed so that P. cepacia could grow linearly up to an OD_{470} of 1.0 before a specific nutrient became limited in the medium.

The flasks were incubated at 37° in a water bath with a shaking rate of 120 throws per min. Growth was followed by taking the OD_{470} of these cultures at 30 min intervals. Cells were harvested at different stages along the growth curve (Fig. 46), and centrifuged at 1,500 x g for 15 min, washed twice with normal saline and resuspended to give an OD_{470} of 0.25. 4.9 ml of each cell suspension was transferred to a test tube and 0.1 ml of CC_5 in methanol (final concentration, 2×10^{-6} M) was added. The cell suspensions were then incubated at 37° for 10 min and the fluorescence spectra were taken. The results are shown in Fig. 47. The results indicate the dynamic changes of fluorescence of CC_5 along the growth curve. It is surprising that the fluorescence reaches its highest intensity around the turn-off points in the growth curves in all cases and then starts to decrease. The extent of the decrease in fluorescence intensity seems to be dependent on the nutrient being depleted. Little change in fluorescence was observed with nitrogen and phosphate-depleted cells after the turn-off points. However, a large

Fig. 46 Growth of P. cepacia under different nutrient limitations.



Magnesium-depleted cells (the number corresponds to the time at which the cells were harvested).

Nitrogen-depleted cells.

Phosphate-depleted cells.

Glucose-depleted cells.

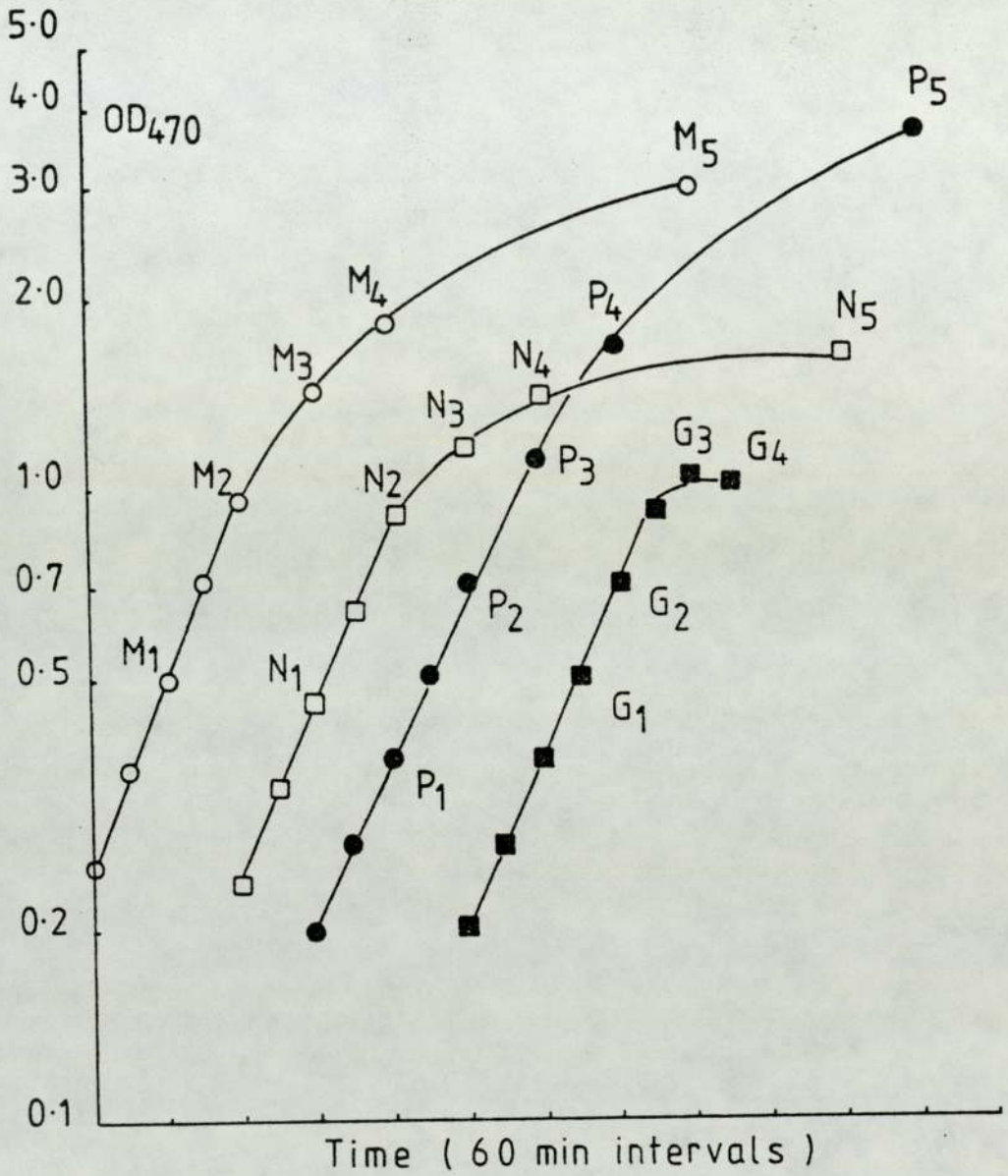

5·0
4·0
3·0
2·0
1·0
0·7
0·5
0·2
0·1
OD470
M1
M2
M3
M4
M5
N1
N2
N3
N4
N5
P1
P2
P3
P4
P5
G1
G2
G3
G4
Time (60 min intervals)

Fig. 47 Fluorescence of CC_5 in P. cepacia grown under different nutrient limitations at different stages of the growth curves.

(Note: Growth curves in Fig. 46).

●——● Magnesium-depleted cells.

○——○ Nitrogen-depleted cells.

□——□ Phosphate-depleted cells.

■——■ Glucose-depleted cells.

Fluorescence intensity
-2
-1
0
+1
+2
+3
+4
+5
Hours from turn-off points

decrease in fluorescence intensity was observed after the turn-off point in magnesium-depleted cells, which probably indicates structural changes of the cell envelope, so that it becomes less permeable to the probe.

3.4.4 Effects of probes on the viable count of *P. cepacia*

P. cepacia NCTC 10661 was grown in complete CDM and harvested in the exponential phase. The cells were centrifuged at 1,500 x g for 10 min, washed twice with sterilised normal saline and resuspended to give an OD_{470} of 0.25. 4.8 ml of this cell suspension was transferred to a test tube and 0.2 ml of NPN (final concentration 4×10^{-6}M) or CC_5 (4×10^{-6}M) or methanol was added. 0.2 ml sterilised distilled water was used as a control. The cells treated with the probes were incubated at 37° for 15 min and colony counts were done by using the spread plate method as described in section 2.2.1. The results are shown in Table 15. The results clearly indicate that the presence of the probes in the cell envelope of *P. cepacia* does not have any significant effects on the viability of *P. cepacia*, even when the concentrations of the probes were twice those used in other studies.

Table 15

The effects of fluorescent probes on the viability of P. cepacia NCTC 10661.(Dilution factor 1 x 10^6).

Count / Replicate plate	Treatment			
	Control	methanol	NPN	CC_5
1	186	159	172	158
2	171	188	153	181
3	208	194	181	165
4	164	183	177	151
Means	182.2	181	170.8	163.8
Standard deviation	16.9	13.3	10.7	11.1
Coeff. of variation	9.3%	7.3%	6.3%	6.8%

3.4.5 Location of the probes in the outer and cytoplasmic membranes of P. cepacia

Fluorescent probes have been employed in the study of the membranes of Gram-negative bacteria by several workers (Ballard et al., 1972; Trauble and Overath, 1973; Helgerson et al., 1974). However, no information is available regarding the location of the probes in the envelope of Gram-negative bacteria which is known to have the outer and cytoplasmic membranes.

In this experiment, attempts were made to study the possible location of the probes in the outer and

cytoplasmic membranes. The probes used were NPN, CC_5, 2-methyl anthracene and DPH.

P. cepacia NCTC 10661 was grown in double strength nutrient broth at 37° overnight in an orbital shaker. The cells were harvested by centrifugation at 8,000 x g for 10 min, washed twice with normal saline, then resuspended to an OD_{470} of 0.5. 200 ml of the cell suspension was transferred to a 500 ml conical flask, then 1 ml of NPN or other probes in methanol (except that DPH was dissolved in tetrahydrofuran) was added. The final concentration of each probe was 5 μM. The cells treated with the probes were incubated at 37° in an orbital shaker for 15 min, then centrifuged at 8,000 x g for 10 min, washed once with normal saline. The cells were resuspended in 20% sucrose- 30 mM Tris-buffer (pH 8.0) and the outer (OM) and cytoplasmic (CM) membranes were separated by sucrose density gradient centrifugation as described in section 2.2.6. Separation of the OM from the CM is shown in Fig. 48. The OM and CM were resuspended in distilled water and adjusted to an OD_{600} of 0.2, incubated at 37° for 15 min and fluorescence spectra were taken. Absorbance at 600 nm was chosen, because the probes used in the study have the excitation and emission wavelengths in the range of 350 nm to 510 nm. The results are shown from Figs. 49 to 52. For CC_5 and 2-methyl anthracene, a higher fluorescence intensity was observed in the OM than in the CM while a higher fluorescence intensity was

Fig. 48 Separation of the outer membrane (bottom fraction) from the cytoplasmic membrane (top fraction) labelled with fluorescent probes.

A : Cells labelled with NPN.

B : Cells labelled with CC_5.


A
A
B
B

Fig. 49 Fluorescence of NPN in the OM and CM of *P. cepacia*.

Note: NPN was labelled to the whole cells, the OM and CM were then separated as described in section 2.2.6.

———————— The cytoplasmic membrane (CM).

— — — — The outer membrane (OM).

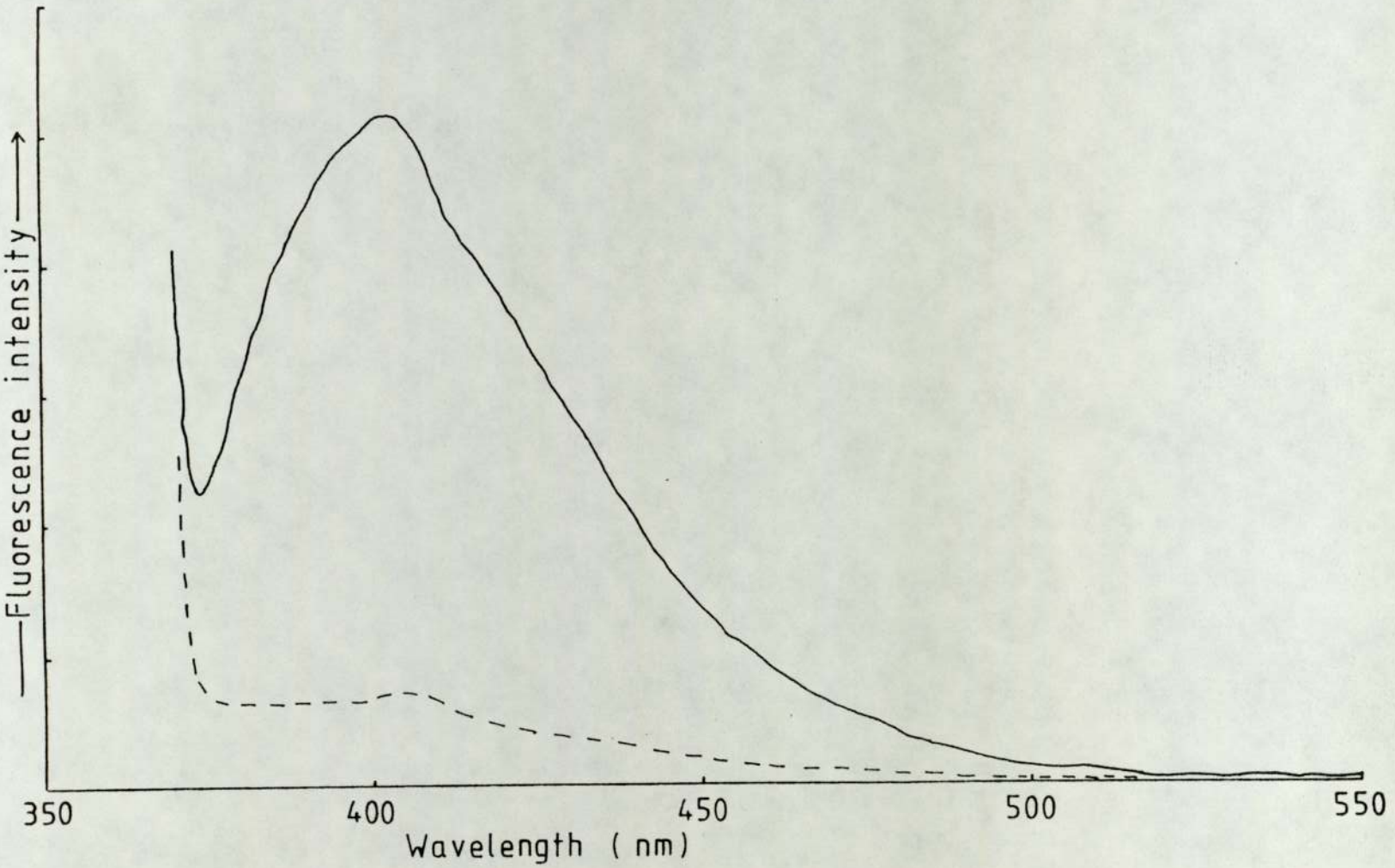

Fluorescence intensity
350
400
450
500
550
Wavelength (nm)

Fig. 50 Fluorescence of DPH in the OM and CM of P. cepacia

Note: DPH was labelled to the whole cells, the OM and CM were then separated as desribed in section 2.2.6.

———— The cytoplasmic membrane (CM).

— — — — The outer membrane (OM).

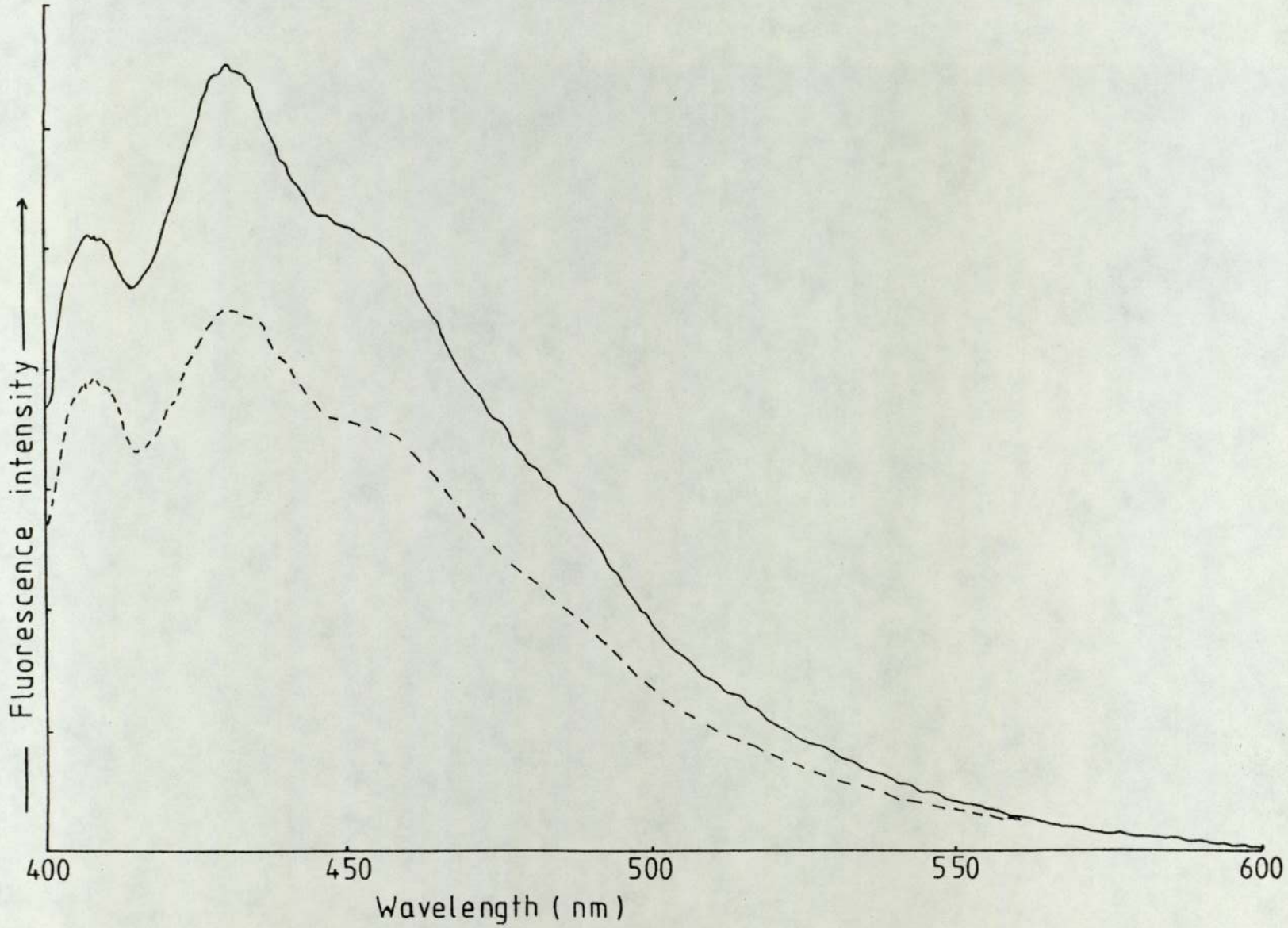

Fluorescence intensity
400
450
500
550
600
Wavelength (nm)

Fig. 51 Fluorescence of CC_5 in the OM and CM of *P. cepacia*

Note: CC_5 was labelled to the whole cells, the OM and CM were then separated as described in section 2.2.6.

———— The cytoplasmic membrane (CM).

— — — — The outer membrane (OM).

Fluorescence intensity
400
450
500
550
600
Wavelength (nm)

Fig. 52 Fluorescence of 2-methyl anthracene in the OM and CM of P. cepacia.

Note: 2-methyl anthracene was labelled to the whole cells, the OM and CM were then separated as described in section 2.2.6.

———— The cytoplasmic membrane (CM).

— — — — The outer membrane (OM).

Fluorescence intensity
400
450
500
550
600
Wavelength (nm)

observed in the CM than in the OM for cells treated with NPN or DPH. It is surprising that a much higher fluorescence intensity was observed in the CM and only trace levels detected in the OM for cells treated with NPN. The results clearly indicate that NPN has a higher affinity towards the CM than the OM, although it was also possible that the probe was first located in the OM and then distributed to the CM during the separation of the OM and CM. If NPN was added to isolated OM and CM, a higher fluorescence intensity was still observed in the CM (see Fig. 53). However, a significant increase in fluorescence intensity was observed in the OM (compare Fig. 49). This indicates that larger amount of the probe exist in the OM, probably due to increased permeability to the probe in isolated OM or the competitive factor (CM) has been removed.

3.4.6 Effects of Sarkosyl on the fluorescence of CC_5 in the OM and whole cells of P. cepacia

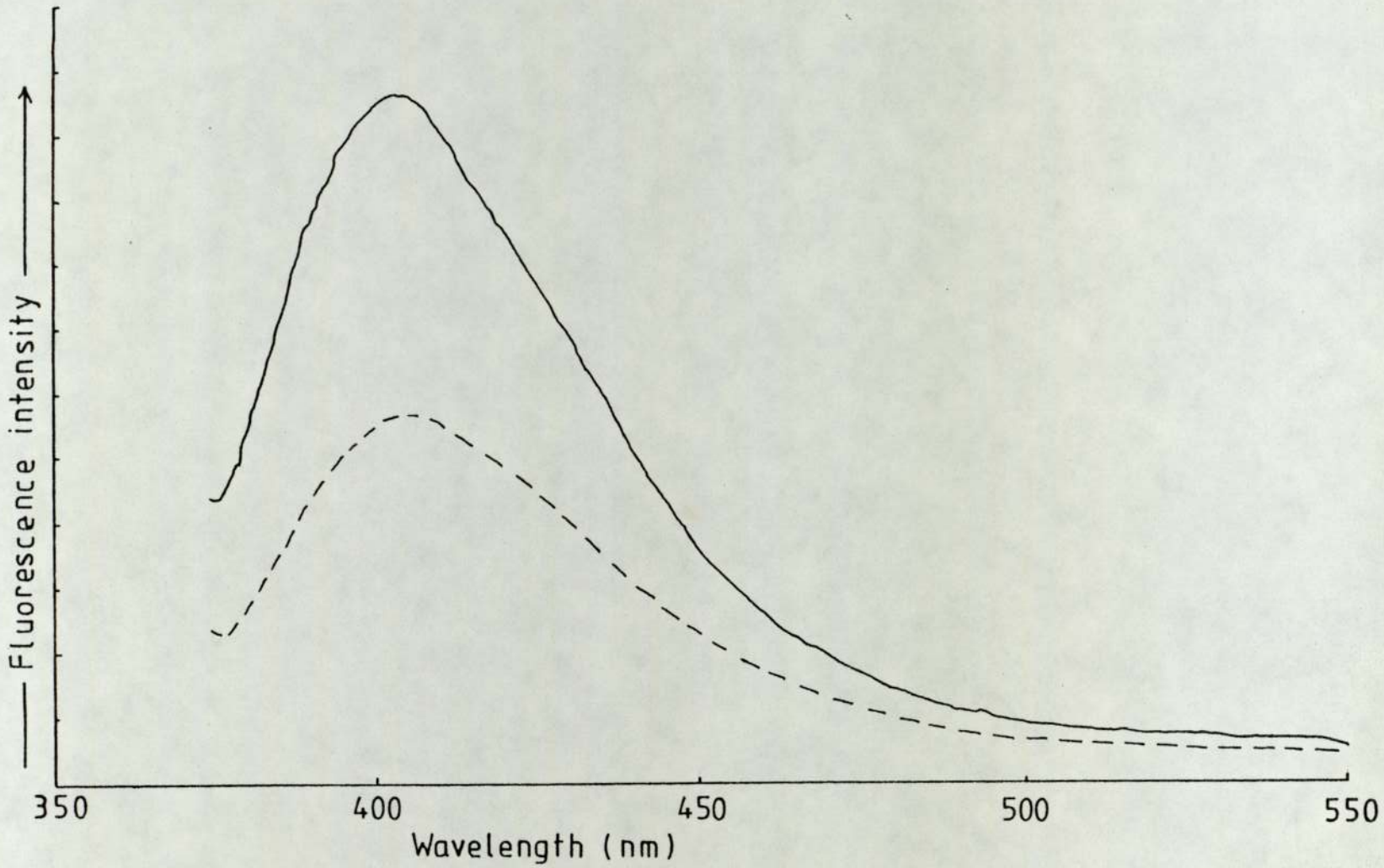
The OM isolated by the technique described in section 2.2.6 or whole cells (grown in nutrient broth) were treated with CC_5 (final concentration 2×10^{-6}M), incubated for 15 min, centrifuged at 38,000 x g for 1 h, washed once with distilled water. They were then treated with sarkosyl (final concentration 2%) or without (control) for 30 min, centrifuged at 38,000 x g for 1 h. The supernatant was collected and the pellet was resuspended

Fig. 53 Fluorescence of NPN in the isolated OM and CM of *P. cepacia*.

Note: NPN was labelled to the isolated OM and CM.

———— The cytoplasmic membrane (CM).

— — — — The outer membrane (OM).


Fluorescence intensity
350
400
450
500
550
Wavelength (nm)

in the same volume of distilled water (10 ml). Fluorescence spectra of the supernatant (Sarkosyl soluble materials) and Sarkosyl insoluble materials from both samples were taken.

Fig. 54 shows the fluorescence of CC_5 in the OM before and after Sarkosyl treatment and Fig. 55 shows the fluorescence of CC_5 in the whole cells. Both indicated that CC_5 had been extracted from the OM and whole cells by Sarkosyl as can be seen that no fluorescence was observed in the Sarkosyl insoluble fraction. In other words, the site where CC_5 located was accessible to Sarkosyl.

3.4.7 Fluorescence polarization studies of P. cepacia

Fluorescence polarization is the technique most frequently used in the study of membrane fluidity (Shinitzky and Barenholz, 1978). It was employed in this study to investigate the effect of nutrient limitation on fluorescence polarization of the whole cells, OM and CM of P. cepacia. DPH, CC_5 and 2-methyl anthracene were used. The microviscosity of the membranes was also calculated from fluorescence polarization measurements of DPH as described by Shinitzky and Barenholz (1978).

P. cepacia NCTC 10661 was grown under iron and magnesium-depletions (Table 7) or in double strength nutrient broth for 24 h at 37° as described in section 2.2.5. The cells were harvested and membranes were prepared

Fig. 54 Effects of Sarkosyl on fluorescence of CC_5 in the OM of P. cepacia NCTC 10661.

———— The outer membrane.

— — — — Sarkosyl soluble materials.

—·—·—·— Sarkosyl insoluble materials.

Fluorescence intensity
400
450
500
550
600
Wavelength (nm)

Fig. 55 Effects of Sarkosyl on fluorescence of CC_5 in the whole cells of <u>P. cepacia</u> NCTC 10661.

———— Whole cells.

— — — — Sarkosyl soluble materials.

—·—·—·— Sarkosyl insoluble materials.

Fluorescence intensity
400
450
500
550
600
Wavelength (nm)

as described in section 2.2.2. Whole cells, OM and CM were resuspended in distilled water and adjusted to an OD_{470} of 0.2. 0.25 ml of the probe solution was added to 25 ml of the cell suspension (final concentration 5 x 10^{-6} M), incubated for 15 min at 37°, centrifuged at 38,000 x g for 60 min, washed once with distilled water and resuspended in 25 ml of distilled water. Fluorescence polarization was then measured as described by Gratzel and Thomas (1973) in the following equation:

$$P = \frac{I_{EE} - I_{EB} (I_{BE} / I_{BB})}{I_{EE} + I_{EB} (I_{BE} / I_{BB})}$$

The reproducibility of the fluorescence polarization and microviscosity measurements was tested by performing four replicate measurements of the OM of P. cepacia grown in nutrient broth treated with DPH and the results subjected to an analysis of variance (Tables 16 and 17).

Table 16

Variation of replicate fluorescence polarization measurements of the OM of P. cepacia (DPH)

Replicate	Fluorescence polarization
1	0.31
2	0.29
3	0.31
4	0.32
Means	0.31
Standard deviation	0.013
Coeff. of variation	4%

Table 17

Variation of replicate microviscosity measurements of the OM of P. cepacia (DPH)

Replicate	microviscosity (in poise)
1	1.46
2	1.49
3	1.65
4	1.59
Means	1.55
Standard deviation	0.09
Coeff. of variation	5.7%

Several probes were employed in the study of the fluorescence polarization of P. cepacia grown under iron and magnesium-depletions, since probes with different physical properties will locate at different parts of the membrane and they may also have different emission properties in response to their environment. The results are shown in Tables 18 and 19.

From the results, the fluorescence polarization of the OM was significantly higher than that of the CM in magnesium and iron-depleted cells while the fluorescence polarization of both membranes was little different in nutrient broth grown cells. The microviscosity measurements with DPH were also in agreement with fluorescence polarization data (Table 19). In magnesium and iron-depleted cells, the microviscosity of the OM was approximately three times as large as that of the CM. However, only a slight difference was observed in the membranes of nutrient broth grown cells. In other words, when a bacterium is faced with the lack of magnesium or iron, it manufactures an OM which is approximately three times as viscous as the OM occurring when grown in nutrient broth.

Table 18

Fluorescence polarization of the membranes of P.cepacia studied with 2-methyl anthracene

System investigated	Cultural condition	Fluorescence polarization
Whole cells	Mg-depleted	0.34
	Fe-depleted	0.20
	Nutrient broth grown	0.19
OM	Mg-depleted*	0.4 ± 0.04
	Fe-depleted*	0.21 ± 0.01
	Nutrient broth grown**	0.13 ± 0.01
CM	Mg-depleted*	0.11 ± 0.02
	Fe-depleted*	0.12 ± 0.01
	Nutrient broth grown**	0.11 ± 0.02

* Means of two determinations.

** Means of four determinations.

Table 19

Fluorescence polarization and microviscosity of the membranes of P. cepacia studied with DPH

System investigated	Cultural conditions	fluorescence polarization	microviscosity
Whole cells	Mg-depleted	0.32	2.21
	Fe-depleted	0.46	4
	Nutrient broth grown	0.32	2.21
OM	Mg-depleted*	0.36 ± 0.02	2.66 ± 0.33
	Fe-depleted*	0.37 ± 0.01	3.03 ± 0.19
	Nutrient broth grown**	0.30 ± 0.02	1.55 ± 0.09
CM	Mg-depleted*	0.24 ± 0.03	1.0
	Fe-depleted*	0.26 ± 0.01	1.38 ± 0.25
	Nutrient broth grown**	0.26 ± 0.02	1.18 ± 0.12

* Means of two determinations.

** Means of four determinations.

Microviscosity expressed in poise.

3.4.8 Effects of Sarkosyl on the OM of P. cepacia as studied by fluorescence polarization

Sarkosyl was found to extract CC_5 from the OM of P. cepacia as described in section 3.4.1.6. In this study, the effects of Sarkosyl on the properties of the OM were studied by monitoring the fluorescence polarization. P. cepacia was grown in double strength nutrient broth and the OM prepared as described in section 2.2.6 and resuspended in distilled water. Sarkosyl was added to the membrane suspension (final concentration 2%), incubated for 30 min at room temperature, centrifuged at 38,000 x g for 1 h, washed twice with distilled water and resuspended in the same volume of distilled water (10 ml). The fluorescent probe was then added to the suspension (final concentration 5×10^{-6} M), incubated at 37^{o} for 15 min, then centrifuged at 38,000 x g for 1 h, washed once with distilled water and resuspended in the same volume of distilled water (10 ml). Fluorescence polarization was then measured as described in section 3.4.2.7. The results are shown in Table 20. The results indicate that fluorecence polarization as well as microviscosity as measured with DPH was increased following treatment of the OM with Sarkosyl.

Table 20

Effects of Sarkosyl on fluorescence polarization and microviscosity of the OM from P. cepacia NCTC 10661 grown in nutrient broth

Probe	Sarkosyl treated OM		OM	
	Fluorescence polarization	Microviscosity	Fluorescence polarization	Microviscosity
CC_5	0.22 ± 0.01	-	0.12 ± 0.02	-
DPH	0.37 ± 0.02	2.06 ± 0.07	0.30 ± 0.02	1.55 ± 0.09
2-methyl anthracene	0.22 ± 0.03	-	0.13 ± 0.01	-

Data are means of three determinations.

Microviscosity expressed in poise.

3.5 Separation of The Outer and Cytoplasmic Membranes of Pseudomonas cepacia

3.5.1 Separation of the outer and cytoplasmic membranes by sucrose density gradient centrifugation

The cell envelope of Gram-negative bacteria is known to include two membranes, the outer membrane (OM) and cytoplasmic membrane (CM). The separation of these two membranes was successfully achieved in E. coli by Miura and Mizushima (1968) by a technique involving isopycnic sucrose density gradient centrifugation of membranes obtained from a spheroplast lysate. Modifications of the technique was made by Osborn et al. (1972) for S. typhimurium. The field is expanding fast and has also been achieved in P. aeruginosa (Mizuno and Kageyama,1978), P. fluorescens, P. chlororaphis and P. putida (Meyer et al., 1979). In this study, the separation of the OM from the CM of P. cepacia NCTC 10661 was attempted by the method described by Hancock and Nikaido (1978) with some modifications. To date, separation of the OM and CM has been done on fast-growing cells (Miura and Mizushima, 1968; Osborn et al., 1972). The difficulties in the separation of these two membranes in slow-growing cells (stationary-phase cells) may be due to (1) These two membranes have similar density that they can not be separated by sucrose density gradient centrifugation. (2) They are tightly associated, possibly through the cross-linkage of the murein. The success in the separation

of the OM and CM in this study could be attributed to plasmolysing the cells (see section 2.2.6). Hence it seems that the latter assumption is probably true.

Table 21 shows the results of the activity of several enzymes in the OM and CM of P. cepacia. These enzymes have been reported to be markers of the CM, since they are found to be located only in the CM (Osborn et al., 1972). Indeed, they were detected in significantly higher activity in the CM fraction than in the OM fraction. However, it was found that only succinate dehydrogenase was the enzyme present in abundance in the CM, the others were sometimes difficult to detect. KDO is usually a marker of the OM. However, it was not detected in this study. It confirms the published data (Manniello et al., 1979) that P. cepacia does not have KDO in its LPS.

Table 21

Localization of the activity of several enzymes in the OM and CM of P. cepacia NCTC 10661

Enzyme	Specific Activity					
	Mg^{2+}dep		Fe^{2+}dep		NB grown	
	OM	CM	OM	CM	OM	CM
Succinate dehydrogenase	19.2	315	11.4	187	9.5	218
D-Lactate dehydrogenase	ND	82	ND	ND	-	-
Gluconate dehydrogenase	ND	21	ND	ND	-	-
NADH oxidase	ND	34	-	-	-	-

Specific activity — expressed as nanomoles per min per mg protein.

ND — not detectable.

3.5.2 Studies of the OM protein pattern of P. cepacia

The OM and CM proteins of P. cepacia NCTC 10661 were verified by using SDS-polyacrylamide gel electrophoresis. The system described by Lugtenberg et al. (1975) was adopted with slight modification and has been discussed in section 2.2.10.

Fig. 56 shows the relationship between logarithm of the known polypeptide chain molecular weights and the electrophoretic mobilities. Linear relationship were observed and confirmed the observations by Weber and Osborn (1969). This was used to calculate the molecular weights of the OM proteins of P. cepacia.

Analysis of the proteins by SDS-polyacrylamide gel electrophoresis showed that the proteins of the CM (slots A and D) and the OM (slots C and F) were significantly different (see Fig. 57). There was only a slight difference between the unseparated fraction (slots B and E) and the OM fraction (slots C and F), but to reduce cross contamination of the CM in the OM or vice versa, those between the CM and OM were designated as unseparated fraction.

Fig. 58 shows that the OM protein patterns of P. cepacia grown in three different media and the effects of heating and 2-mercaptoethanol on the electrophoretic mobilities of these proteins. The OM protein profile of magnesium-depleted cells was much simpler than that of iron-depleted cells and nutrient broth grown cells. Several

Fig. 56 The relationship between logarithm of the known polypeptide chain molecular weights and the electrophoretic mobilities.

	M.W.
Albumin, bovine	66,000 daltons
Egg albumin	45,000 daltons
Pepsin (hog stomach mucosa)	34,700 daltons
Trypsinogen (bovine pancreas)	24,000 daltons
B-lactoglobulin, bovine milk (subunit)	18,400 daltons
Lysozyme (egg white)	14,300 daltons

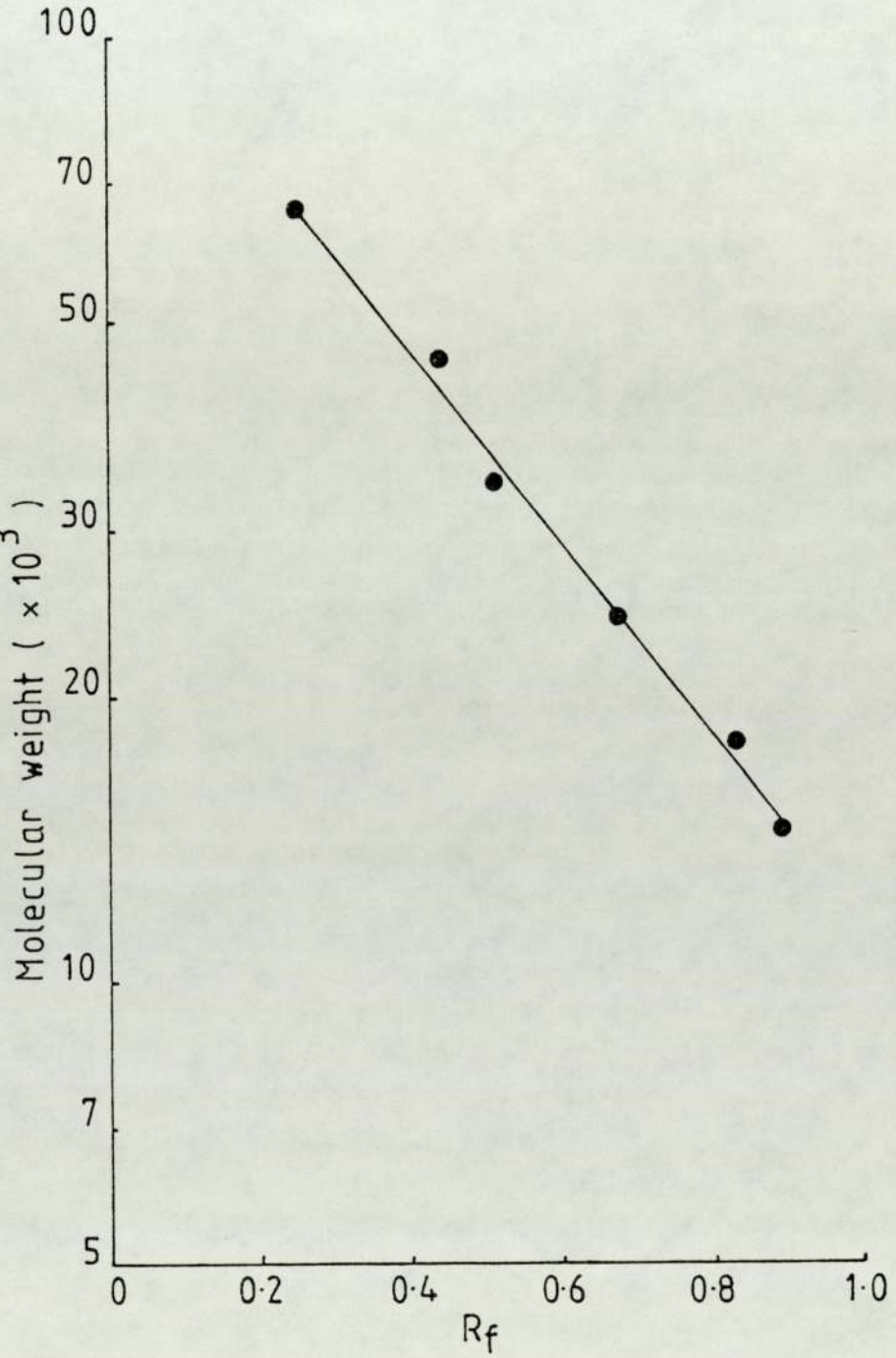

100
70
50
30
20
10
7
5
Molecular weight ($\times 10^3$)
0
0·2
0·4
0·6
0·8
1·0
R_f

Fig. 57 SDS-polyacrylamide gel electrophoresis of the OM and CM of P. cepacia NCTC 10661.

Slot A : The CM fraction of nutrient broth grown cells.

Slot B : The unseparated of nutrient broth grown cells.

Slot C : The OM fraction of nutrient broth grown cells.

Slot D : The CM fraction of magnesium-depleted cells.

Slot E : The unseparated fraction of magnesium-depleted cells.

Slot F : The OM fraction of magnesium-depleted cells.

Slot G : The OM fraction of iron-depleted cells.

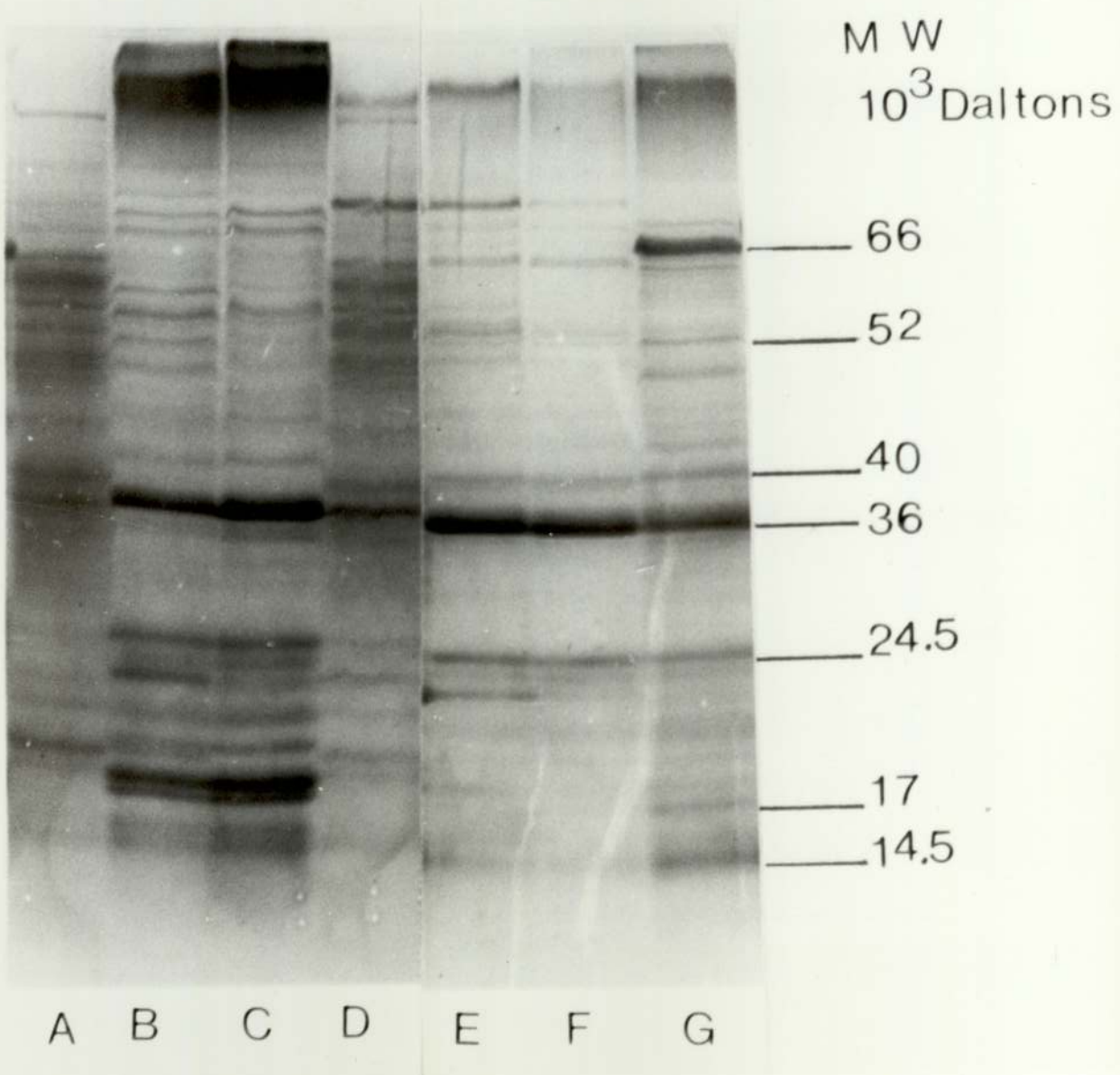

M W
10³ Daltons
66
52
40
36
24.5
17
14.5
A
B
C
D
E
F
G

Fig. 58 The effects of heating and 2-mercaptoethanol on the electrophoretic mobilities of the OM proteins of P. cepacia grown under different nutrient limitations.

Slot A : The OM proteins of magnesium-depleted cells (denatured at 100°C for 5 min with 5% 2-mercaptoethanol, condition I).

Slot B : The OM proteins of magnesium-depleted cells (denatured at 100°C for 5 min without 2-mercaptoethanol, condition II).

Slot C : The OM proteins of magnesium-depleted cells (denatured at 60°C for 30 min with 5% 2-mercaptoethanol, condition III).

Slot D : The OM proteins of iron-depleted cells (condition I).

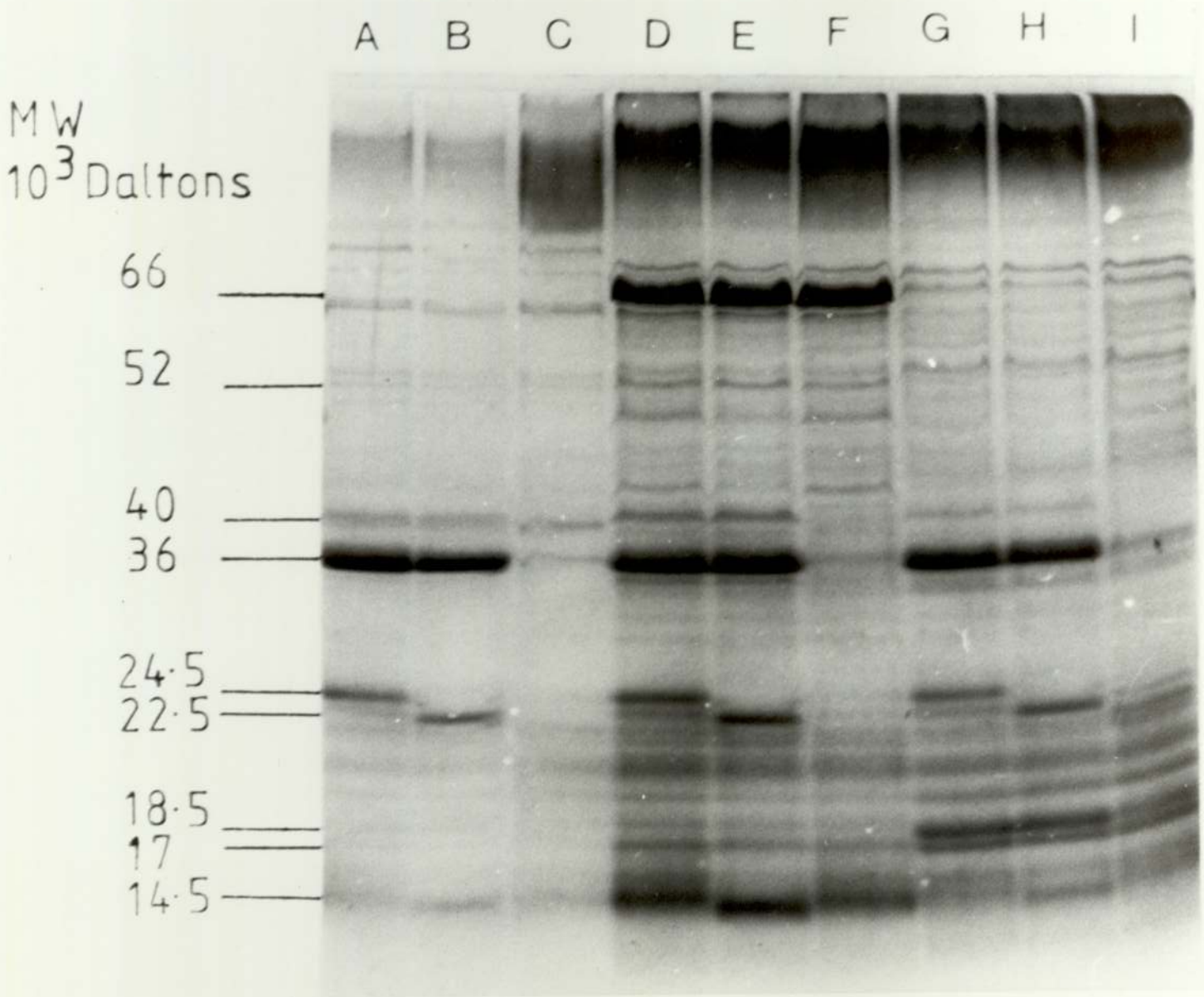
Slot E : The OM proteins of iron-depleted cells (condition II).

Slot F : The OM proteins of iron-depleted cells (condition III).

Slot G : The OM proteins of nutrient broth grown cells (condition I).

Slot H : The OM proteins of nutrient broth grown cells (condition II).

Slot I : The OM proteins of nutrient broth grown cells (condition III).


A
B
C
D
E
F
G
H
I
MW
10³ Daltons
66
52
40
36
24·5
22·5
18·5
17
14·5

proteins with apparent molecular weights lower than 20,000 daltons were missing (Fig. 58). The apparent molecular weights of the OM proteins of Mg^{2+}-depleted cells were: 40,000, 36,000, 24,500 and 14,500 daltons. The OM protein with an apparent molecular weight of 36,000 daltons was present in large quantity and might correspond to the pore-forming proteins as observed in other organisms to be in the range of 33,000 to 40,000 daltons (Nikaido and Nakae, 1979). The synthesis of the OM protein with an apparent molecular weight of 66,000 daltons was induced when P. cepacia was grown under iron-depletion. It appeared as a major band in this condition (Fig 58, slots D to F). The synthesis of several OM proteins with apparent molecular weights in the range of 60,000 to 85,000 daltons were induced when P. aeruginosa was grown under iron-depleted condition (Mizuno and Kageyama, 1978) and these proteins might involve in the translocation of iron through the OM (Meyer et al., 1979). The synthesis of two OM proteins with apparent molecular weights of 17,000 and 18,500 daltons were induced when P. cepacia was grown in nutrient broth in this study.

The electrophoretic mobilities of several major OM proteins of P. aeruginosa were found to be affected by the solubilization temperature and 2-mercaptoethanol by Hancock and Carey (1979). It is interesting that the mobility of protein F (33,000 daltons) is dependent on the concentration of 2-mercaptoethanol in the denaturing

buffer. At low concentrations of 2-mercaptoethanol (0 - 0.01%), it moves with an apparent molecular weight of 33,000 daltons (protein F). However, at higher concentrations of 2-mercaptoethanol (0.1 - 0.5%), it moves with an apparent molecular weight of 42,000 daltons (its F^* form). Protein F was also found to be heat-modifiable. When it was solubilised at low temperature, it moved with an apparent molecular weight of 39,000 daltons. However, it moved with an apparent molecular weight of 41,000 daltons when it was denatured at high temperature (100°C).

In this study, the electrophoretic mobility of one OM protein was found to be affected by the presence of 2-mercaptoethanol (Fig. 58). In the presence of 2-mercaptoethanol, it moved with an apparent molecular weight of 24,500 daltons. However, when 2-mercaptoethanol was excluded from the denaturing buffer, it moved with an apparent molecular weight of 22,500 daltons in all three growth conditions used in the study.

Several major OM proteins of P. cepacia were found to be heat-modifiable. These proteins had apparent molecular weights of 40,000, 36,000 and 24,000 daltons when they were denatured at 100° for 5 min in the presence of 2-mercaptoethanol. However, they moved with apparent molecular weights greater than 70,000 daltons when they were solubilised at 60° for 30 min. They apparently moved as a single diffuse band (Fig. 58). One OM protein with

an apparent molecular weight of 66,000 daltons, in which its synthesis was induced when iron was excluded from the growth medium was not heat-modifiable. Its electrophoretic mibility was also not affected by 2-mercaptoethanol.

PAS (Periodic acid, Schiff's reagent) is used to stain glycoproteins, heparin, chondroitin sulphate, polysaccharides and other materials with a high carbohydrate content. In this study, PAS was used to stain the LPS or any molecule associated with carbohydrate. The gel was stained with PAS according to the method described by Steck et al. (1971). The results are shown in Fig. 59. In iron-depleted cells, three bands were found to be PAS positive (slot A), two bands were observed in magnesium-depleted cells, while 5 to 6 bands were observed in nutrient broth grown cells. Interestingly, the OM proteins with apparent molecular weights of 36,000 and 66,000 daltons were found to be PAS positive. At present , we have no evidence to propose that these proteins are glycoproteins or associated with LPS.

Sarkosyl is often used to prepare OM. It was first introduced by Filip et al. (1973) that Sarkosyl specifically solubilised the CM and the OM remained intact after the treatment. Fig. 60 shows the OM protein patterns of P. aeruginosa PAO-1, E. coli NCIB 8277 and P. cepacia NCTC 10661 prepared by Sarkosyl method (section 2.2.7). The OM protein patterns of these three organisms were different which suggested that this might be a useful

Fig. 59 SDS - polyacrylamide gel electrophoresis of the OM of P. cepacia NCTC 10661 (stained with Periodic acid, Schiff's reagent).

Slot A : The OM of iron-depleted cells.

Slot B : The OM of magnesium-depleted cells.

Slot C : The OM of nutrient broth grown cells (oxygen-depleted).

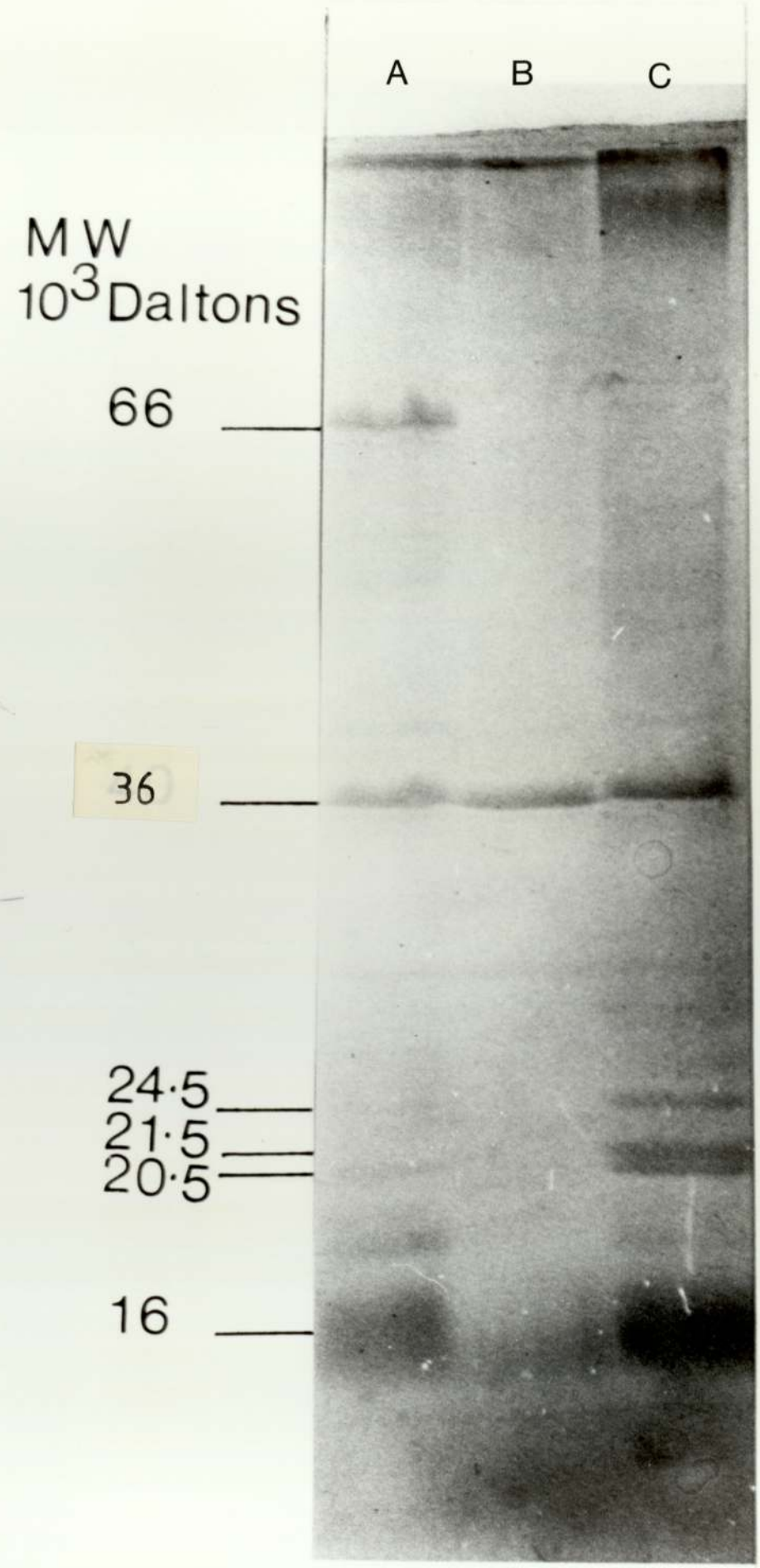

A
B
C
MW
10³ Daltons
66
36
24·5
21·5
20·5
16

Fig. 60. SDS - polyacrylamide gel electrophoresis of the OM of several Gram-negative bacteria prepared by Sarkosyl method.

Slot A : The OM of *P. aeruginosa* PA01.

Slot B : The OM of *E. coli* NCIB 8277.

Slot C : The OM of *P. cepacia* NCTC 10661 (iron-depleted).

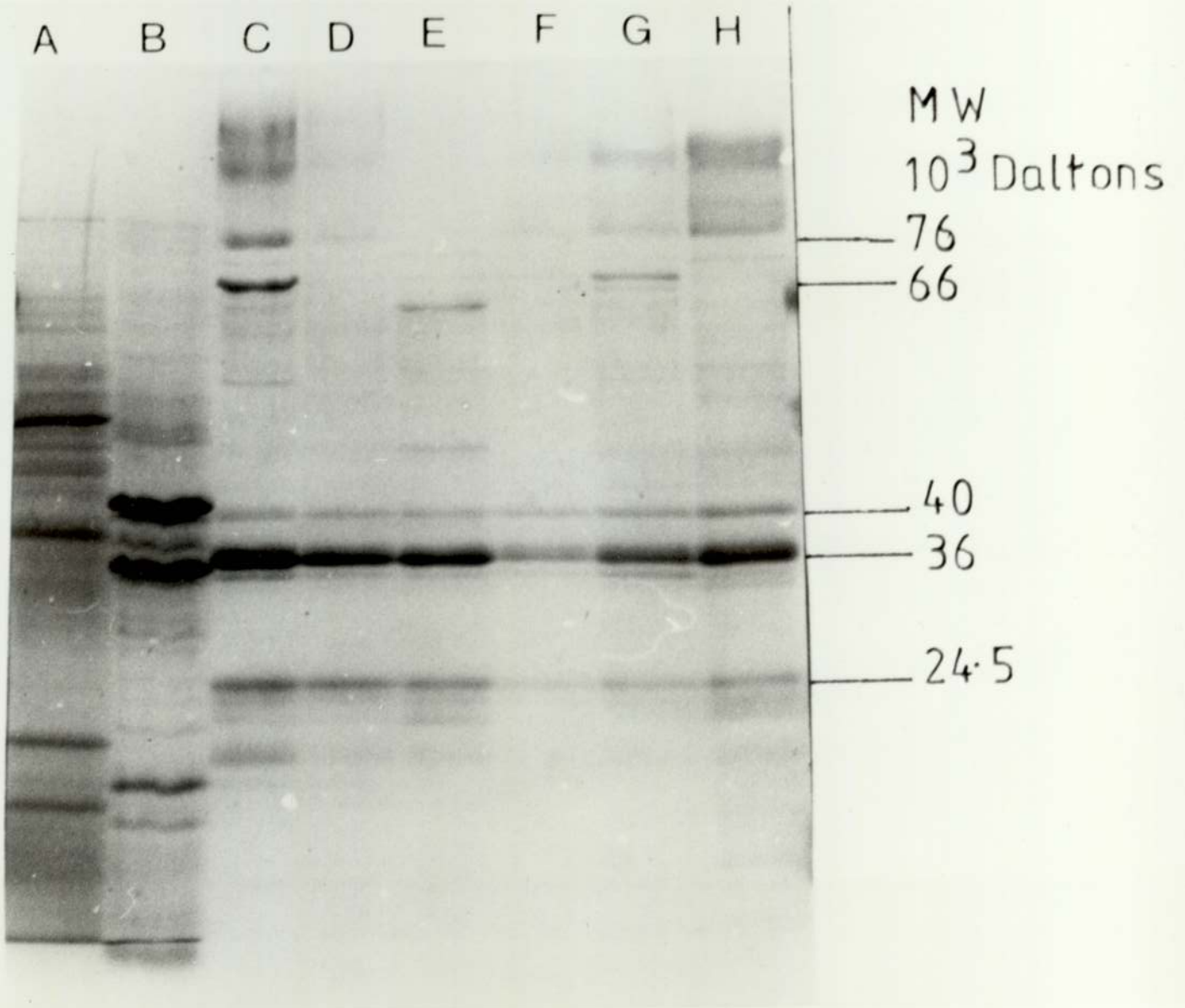
Slot D : The OM of *P. cepacia* NCTC 10661 (magnesium-depleted).

Slot E : The OM of *P. cepacia* NCTC 10661 (phosphate-depleted).

Slot F : The OM of *P. cepacia* NCTC 10661 (sulphate-depleted).

Slot G : The OM of *P. cepacia* NCTC 10661 (nitrogen-depleted).

Slot H : The OM of *P. cepacia* NCTC 10661 (nutrient broth grown cells).


A
B
C
D
E
F
G
H
MW
10³ Daltons
76
66
40
36
24·5

diagnostic method. However, the OM protein patterns prepared by Sarkosyl method (Fig. 60) were found to be simpler than those prepared by sucrose density gradient centrifugation method (Fig. 58). It probably indicated that some minor OM proteins had been removed by Sarkosyl. Chopra and Shales (1980) found that Sarkosyl removed several minor proteins from the OM of E. coli. Sarkosyl was found to remove CC_5 from the OM or whole cells labelled with CC_5 in this study (Fig. 54 and Fig.55). Fluorescence polarization of several probes was increased after Sarkosyl treatment (Table 20).

3.5.3 Protein and phospholipid contents of the OM and CM of P. cepacia NCTC 10661

The protein contents in the OM and CM were assayed by the method described in section 2.2.8 and the lipids were extracted by the method described in section 2.2.11. Phospholipids were fractionated into individual PL by thin-layer chromatography as described in section 2.2.12.

The results are shown in Table 22. In all cases, the OM was found to be enriched in protein while the CM was enriched in PL. The OM was found to consist of approximately 50% of protein and 20% of PL and presumably the rest was LPS while the CM was found to consist of 80% PL and 20% protein. Phospholipids detected in the study were mainly PE and PG. Trace amount of PC was also detected. DPG was not detected in the study, even after

charring at 160° for 2 h. The ratio of PE/PG+PC was calculated. The ratio was higher for the OM than that of the CM in three growth conditions used which meant that the OM had higher content of PE than that of the CM. It is in agreement with the findings by Peters and Lugtenberg (1976). The ratio of PE/PG+PC was found to vary dramatically depending on the growth conditions (Table 22). The ratio was found to be highest in iron-depleted cells, then followed by nutrient broth grown cells and magnesium-depleted cells.

To doubt everything or to believe everything are two equally convenient solutions; both dispense with the necessity of reflection.

French mathematician and physicist,
Poincari.

Table 22

Protein and lipid composition of the OM and CM of P. cepacia NCTC 10661 grown under different nutrient depletions

Limiting nutrient	membrane fraction	% dry weight				
		REL	PL	FAN	PE/PG + PC	Protein
Nutrient broth grown	OM	20.8	18.8	2	6.7	50.7
	CM	79.9	67.9	12	4.5	14.9
Fe-depleted	OM	26.3	23.7	2.6	13.3	44.5
	CM	85.4	78.3	7.1	7.3	12.9
Mg-depleted	OM	19.8	17.2	2.4	2.7	51.2
	CM	76.6	68.7	8.0	1.1	15.9

Data are means of two duplicate determinations.

REL - readily extractable lipid.

PL - phospholipid.

FAN - free fatty acid and neutral lipid.

PE - phosphatidylethanolamine.

PG - phosphatidylglycerol.

4. Discussion

4.1 Nutrient Requirements of P. cepacia NCTC 10661

4.1.1 General

P. cepacia, previously known mainly as a plant pathogen (Ballard et al., 1970), is appearing more frequently in the medical literature as a causative agent of human disease (Philips and Eykyn, 1971; Speller et al., 1971). It has been isolated from hospital water supplies (Bassett et al., 1970), physiological saline and disinfectant solutions (Burdon and Whitby, 1967; Speller et al., 1971). It is known to be more resistant than most other Gram-negative organisms to the commonly used anti-microbial agent, benzalkonium chloride, and also showed high resistance to polymyxin B, another cationic agent that is effective against most Gram-negative bacteria (Manniello et al., 1978). P. cepacia can survive in an inorganic salt solution which contained commercial 0.05% benzalkonium chloride for fourteen years (Geftic et al., 1979). LPS of this strain of P. cepacia contained no detectable 2-keto-3-deoxyoctonic acid (Manniello et al., 1979).

Minimal nutritional requirements have been quantitatively determined in E. coli (Klemperer et al., 1979), P. aeruginosa (Ombaka, 1980) and Proteus mirabilis (Al-Dujaili, 1979). Natural environments rarely contain all the nutrients required for cell systhesis in an amount sufficient to permit microbial growth to proceed at the maximum rate. This has very

important implications, since it has been established that when a dividing bacterium is faced with the lack of an essential nutrient, it manufactures an envelope characteristic of that particular depletion which probably has an important role in resistance to drugs and body defense mechanism (Brown, 1975; 1977). The minimal requirements of several essential nutrients of P. cepacia NCTC 10661 are discussed below and compared with the requirements of other Gram-negative bacteria.

4.1.2 Glucose

Glucose provides the cell carbon and energy for growth. When all nutrients required for the growth of P. cepacia in CDM were present in excess except glucose, growth ceased abruptly when the latter became depleted (Fig. 18) in cultures with low initial glucose concentrations. However, cultures with high initial glucose concentrations eventually showed a progressive decline in the growth rate. This happened when the concentration of glucose was above 9×10^{-3}M. It indicates that above this concentration, glucose was no longer the limiting nutrient (Fig. 18) and probably oxygen became the limiting nutrient. The exponential growth rates of the cultures were independent of the concentrations used in this study. A linear relationship existed between optical density at the end of exponential

growth and glucose concentration in the medium up to an OD_{470} of 2.3 as shown in Fig. 19. This line extrapolates back through the origin which indicates that there was no other carbon source present in the medium which can be used to produce an increase in the cell mass more than that produced by the added glucose. P. cepacia has been reported to be able to utilize penicillin G as the carbon source (Beckman and Lessie, 1979).

The OD_{470} reached by P. cepacia for the same amount of glucose is similar to that by P. aeruginosa (Ombaka, 1980), slightly less than P. mirabilis (Al-Dujaili, 1979) or E. coli (Ismail, 1977).

4.1.3 Iron

Iron is absolutely required for the growth of bacteria. It is a constituent of cytochromes and other heme or non-heme proteins and is a cofactor of a number of enzymes (Stanier et al., 1977). A prime function of iron in microbial species is in respiration — the reduction of oxygen by means of the cytochrome chain and the concomitant generation of chemical energy.

Since iron is an essential trace element for microorganisms and is involved in a variety of vital cellular functions (Stanier et al., 1977), microorganisms must secure a sufficient amount of this element for their growth. In nature, however, iron is virtually unavailable to microorganisms, as under conditions suitable for their

growth, it forms a variety of polynuclear complexes of extremely low solubility (Neilands, 1974). Microorganisms are faced with the problem of the solubilization of such complexes. Research has revealed that microorganisms are able to synthesize a wide spectrum of powerful chelators, called siderophores, which have high iron affinity and enable them to scavenge the iron they required from the medium, even when it is present in low concentration. Synthesis of siderophores is regulated by negative feedback mechanisms, which shut off their synthesis when iron is abundant. In this situation, one may ask whether the microorganisms will synthesize some sorts of molecules which enable them to store iron when they are grown in a medium which has excess of iron. Indeed, it was reported by Harrison (1979) that bacteria synthesize a group of ferritin-like molecules, which enable them to store iron in large amounts when they are grown in a medium rich in iron.

The role of iron in bacterial infection has received much attention in the last decade (Weinberg, 1978). In the living body, iron is not freely available to the bacteria. The bulk of the metal is locked up in ferritin, hemosiderin, myoglobin and in the hemoglobulin in red cells (Lanzkowsky, 1976). The iron-binding proteins, transferrin and lactoferrin, which possess only a minute fraction of the total body iron, are normally only partly saturated with iron and have an exceptional high

association constant of about 10^{36} for the metal (Weinberg, 1978). This means that the amount of free iron in equilibrium with these proteins is only about 10^{-18} M, which is far too low for normal bacterial growth. To obtain iron from normal tissue, bacteria must therefore synthesize iron-chelating agents with association constants similar to those of transferrin and lactoferrin, which has been described above. In bacterial infections, further reduction in availability of iron to bacteria has been observed (Sussman, 1974). Weinberg (1978) proposed several possible mechanisms whereby an infected host could deprive microbial invaders of growth essential iron. Implication of iron in bacterial infection has inspired us to study the nutritional requirement of iron in P. cepacia and possibly ways of getting iron-depleted cells which will enable us to study the properties of their envelopes.

Al-Dujaili (1979) found that maximum growth could be attained by P. mirabilis without any added ferrous ion in the medium. A similar phenomenon was also observed by Ismail (1977) in E. coli. However, a demonstrable iron requirement was observed in E. coli with R plasmid RP1. In this preliminary study, it was not possible to limit the growth of P. cepacia with iron. This might be due to iron contamination from other ingredients in the medium, which is approximately 0.4 μM (Klemperer et al., 1979) or storage of iron in P. cepacia, since P. cepacia grown

in an iron rich medium was used as an inoculum. Iron depletion was observed when P. cepacia grown in an iron deficient medium was used as an inoculum. This procedure was subsequently used routinely.

Fig. 14 shows the comparison of the growth of P. cepacia, from an inoculum grown either in an iron-depleted culture or also in a complete CDM (iron-rich). The supernatant of an iron-depleted medium was used as the growth medium. The doubling time in iron-depleted culture was 110 min and was 60 min for complete CDM culture. Addition of 2×10^{-5}M ferrous sulphate to the media of both types of cells, resulted in an immediate decrease in the doubling time for the cells from iron-depleted culture. There was no observable lag-phase before the cells started dividing for iron-depleted cells when ferrous sulphate was added. This was presumably due to the presence of a large amount of siderophores in the medium which helped the cells in getting iron required for growth without delay. However, the doubling time remained unchanged for the cells from complete CDM. This result favours the observations of iron storage in bacteria and also indicates that iron is absolutely required for the growth of P. cepacia.

Figures 20 and 21 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 logarithmic-

linear growth was maintained to an OD_{470} of 2.1 corresponding to 1 uM Fe^{2+}. The line in Fig. 21 did not pass through the origin indicating the presence of iron even in the pre-used medium which was approximately 0.2 uM. P. aeruginosa required 1 uM of Fe^{2+} to grow to an OD_{470} of 1.0 (Ombaka, 1980). However, P. cepacia only required half that amount of added iron to reach the same OD.

4.1.4 Magnesium

Fig. 23 shows the growth curves of magnesium-depleted cultures. They are markedly different from those of glucose-depleted ones (Fig. 18). Magnesium depletion showed a progressive decrease in growth rate after the end of the exponential growth while growth ceased abruptly in glucose depletion. This gradual decrease in growth rate may be due to a decrease in synthesis of RNA and protein. Magnesium is essential for the activity of many enzymes including those involved in the synthesis of the cell wall components such as fatty acids (Knivett and Cullen, 1967), LPS (Edstrom and Heath, 1967), peptidoglycan (Garrett, 1969) and phospholipids (White et al., 1971). It is also essential for the integrity of ribosomes and their activity (McCarthy, 1962; Stanier et al., 1977) and in the synthesis of ribosomes and RNA (Cohn and Ennis, 1967) and in the stability and permeability control in membranes (Brock, 1962). There is much evidence that magnesium is a structural component of the outer membrane of Gram-negative bacteria

(Costerton et al., 1974) especially in P. aeruginosa (Brown and Melling, 1969 a;b ; Boggis et al., 1979).

The OD reached by P. cepacia was slightly higher than that by P. mirabilis (Al-Dujaili, 1979), wild-type and mucoid-strain P. aeruginosa (Ombaka, 1980) for the same amount of magnesium added to the medium. It is possible that these differences in magnesium requirement can be attributed to the metabolic and structural differences between them.

Fig. 25 shows a linear relationship between the reciprocal of the specific growth rate of exponential-phase cells and the reciprocal of magnesium concentration for P. cepacia. A similar relationship was also observed by Finch (1976) in P. aeruginosa. However, Al-Dujaili (1979) observed a biphasic curve relation in P.mirabilis. He suggested that it was probably due to the presence of a dual transport system for magnesium of high and low affinity, as was previously suggested for phosphate by Shehata and Marr (1971).

4.1.5 Nitrogen

Nitrogen is a constituent of proteins, nucleic acids, some phospholipids such as phosphatidylethanolamine and coenzymes. Fig. 26 shows the growth curves of nitrogen-depleted cultures. A linear relationship was maintained between the optical density at the end of exponential growth and nitrogen concentration up to an OD_{470} of 2.2 (Fig. 27).

The intercept of the line showed a contamination level of 1×10^{-3}M, equivalent to utilizable nitrogeneous materials from other medium ingredients. Over the range of ammonium sulphate concentrations used in the study, it can be seen that the growth rate was independent of ammonium concentration added to the medium (Fig. 26).

The optical density attained by P. cepacia was similar to those by wild-type and mucoid-strain P. aeruginosa (Ombaka, 1980) and E. coli (Ismail, 1977), but much higher than that by Proteus mirabilis (Al-Dujaili, 1979) for the same amount of ammonium sulphate.

4.1.6 Phosphate

The shape of growth curves for phosphate-depleted cultures shown in Fig. 28 is similar to that of magnesium, as phosphate became limiting, there was a progressive decrease in growth rate. There is a stoichiometry between the amount of magnesium, potassium, phosphate and RNA in bacteria (Tempest, 1969). The gradual decrease of growth rate may be explained by the reduction in the rate of protein synthesis due to decrease of phosphorus content in the ribosomal RNA.

Phosphorus is also a constituent of high energy compounds such as adenosine triphosphate (ATP) in which the energy is stored in the form of phosphoanhydride bonds. Apart from its many metabolic roles, phosphorus is present in the cell walls of Gram-negative bacteria as a constituent

of the phospholipid and LPS.

Fig. 29 shows the relationship between the onset of non-linear growth and initial phosphate concentration. The relationship was linear to a concentration of 3.5×10^{-4}M, corresponding to an OD_{470} of 1.7. Over the range of phosphate concentrations used in the study, it can be seen that the growth rate was independent of phosphate concentration added to the medium (Fig. 28).

The phosphate requirement of P. cepacia is very much less than that of P. mirabilis (Al-Dujaili, 1979), slightly less than wild-type P. aeruginosa (Ombaka, 1980) and slightly higher than E. coli (Ismail, 1977). A phosphate concentration of 2×10^{-4}M supported exponential growth of P. cepacia, P. aeruginosa and E. coli up to an OD of 1.0, 0.8 and 1.2 respectively, while 2×10^{-2}M of phosphate was required to support exponential growth of P. mirabilis to an OD of 1.0.

4.1.7 Sulphate

Sulphur is required for the synthesis of amino acids such as cysteine and methionine and as a constituent of some coenzymes such as coenzyme A, cocarboxylate, biotin, ferredoxin and thiamine (Stanier et al., 1977).

Fig. 30 shows the growth characteristics of P. cepacia under sulphate depletion. The shape of the growth curves is similar to those of magnesium, phosphate and iron depletion, as sulphate became limiting, there was a progressive

decrease in growth rate.

Fig. 31 shows the relationship between onset of non-linear growth and initial sulphate concentration. The relationship was linear to a concentration of 2.5×10^{-4}M, corresponding to an OD_{470} of 2.1. P. cepacia seems to require about 3 times as much sulphate as E. coli (Klemperer et al., 1979) and P. mirabilis (Al-Dujaili, 1979). Similarly P. aeruginosa requires only half the amount of sulphate required by P. cepacia (Ombaka, 1980). This probably indicates that P. cepacia contains larger amount of sulphate-containing compounds in the cells than other Gram-negative organisms.

4.1.8 Conclusion

P. cepacia like other Gram-negative organisms such as P. aeruginosa (Ombaka, 1980), P. mirabilis (Al-Dujaili, 1979) and E. coli (Ismail, 1977) can be grown in a simple CDM containing glucose as carbon source, an inorganic source of nitrogen (NH_4^+) and phosphate and small quantities of iron, magnesium, potassium and sulphate. Other ions such as sodium, chloride, zinc, manganese, calcium may be required in quantities minute enough to be satisfied by contaminants in other components of the medium.

In common with P. aeruginosa (Ombaka, 1980), P. cepacia can grow to quite high densities even when no ferrous ion is added to the medium. Fig. 14 shows the results obtained when ferrous-depleted cells and ferrous-

sufficient cells were grown in a medium that had already been used to produce ferrous-depleted cells. Ferrous-depleted cells had nearly trebled before exponential growth ended, this was presumably due to the introduction of new sources of iron on 'clean' glassware etc., possibly being made available by siderophores in the supernatant. Ferrous-sufficient cells showed an approximately 10 fold increase before exponential growth ceased. This was presumably due either to the use of endogeneous iron or possibly to the use of iron actively stored by the cells. Recently, ferritin-like iron storage compounds have been described in some species (Harrison, 1979).

P. cepacia, like P. aeruginosa, had a higher requirement for glucose than P. mirabilis and E. coli. It also had a higher requirement for sulphate than P. mirabilis, P. aeruginosa and E.coli. However, it had a lower requirement for magnesium and phosphate than P. mirabilis and P. aeruginosa

For all required nutrients, the relation between the optical density at the end of exponential growth and nutrient concentration was linear up to an OD_{470} of 2.1, although a sufficient concentration of each nutrient was tested to theoretically maintain linearity above 6.0. This may indicate that some additional limitations had been superimposed at ODs greater than 2.1. In most of the growth studies, the pH was found to have dropped from 7.4 to 7.0-7.2 after maximum growth of OD_{470} of 4.5 had been reached. Al-Dujaili (1979)

found that initial growth rate was the same between pH 6.2 and 7.3 in P. mirabilis, so this drop in pH on its own would be unlikely to affect the growth rate. It seems probable that departure from linearity above the optical density of 2.1 in this study was mainly due to oxygen depletion and/or accumulation of toxic metabolites.

It should also be noted that a comparison of the yield of different organisms using OD readings does not necessarily reflect similar cell dry weights or even total number of cells, since they probably have different light scattering properties. Different nutrient-depleted cells may have different sizes. Tempest et al. (1965) found that the cell size of chemostat-grown A. aerogenes cultures decreased with increasing magnesium limitation.

4.2 Physical Characteristics of Bacterial Surfaces

4.2.1 Introduction

The physicochemical surface properties of the outermost interface of bacteria may play an important role in their behaviour. Surface adsorption of antibiotics or immunogens is obviously important. The surface is also important in the ingestion of microorganisms by phagocytes (Van Oss *et al.*, 1978) as well as adhesion of microorganisms on solid surfaces (Rogers, 1979).

The structure and composition of the bacterial surface is not fixed in a stoichiometric way. Gene expression may be modified by responses of the surface to changes in environment. The surface of the microorganisms may also be altered by the composition of the ions, ionic strength and pH of the medium in which they are suspended. This is due to their effects on ionization of their surface groups and adsorption of ions. The physicochemical surface properties of the outermost surface of bacteria can essentially be of two kinds: (a) Electrical surface potential (electric charge), and (b) Interfacial tension (hydrophobicity).

4.2.2 Surface charge as studied by particle microelectrophoresis

Particle microelectrophoresis is a useful technique for giving information about the electric charge properties

of the outermost surface layer of bacteria. One of the advantages of the method is that it is possible to make determinations on healthy living cells. Individual particles can be selected for measurement, their size and shape can be observed. Very dilute dispersions can be studied and under these conditions interactions between particles is negligible.

The theoretical background and general principles of microelectrophoresis have been authoritatively reviewed by Shaw (1969). Only a brief discussion is given here.

Most particles acquire an electric charge in aqueous suspension due to the ionization of their surface groups and adsorption of ions. The surface charge attracts ions of opposite charge in the medium and results in the formation of an electric double layer. If a tangential electric field is applied along the charged surface, the particles tend to move in one direction while the ions in the mobile part of the double layer show an equivalent motion in the opposite direction carrying solvent with them. Thus, when carried out in a closed system, electrophoresis and electro-osmosis at the chamber wall take place simultaneously. The electrophoretic mobility of the particle depends on the zeta potential at the plane of shear between the charged surface and the electrolyte solution. Smoluchowski (1914) regarded electrophoresis as the opposite of electro-osmosis and derived the following equation:

$$m = \frac{D \zeta}{4 \pi \eta}$$

Where m is the electrophoretic mobility of the particle, D is the dielectric constant of the medium, η is the viscosity of the medium and ζ is the potential at the surface of shear. This equation is applicable to a particle of any size, shape or orientation provided the radius of curvature of the surface is at all points much greater than the thickness of the electric double layer. More precise treatments have been given by Shaw (1969) but, because of theoretical difficulties and ambiguities, most workers have preferred to expressed their results as electrophoretic mobilities, the measured values. As zeta potential is sensitive to changes in ionic strength of the suspending medium, the ionic strength must be clearly defined (Barry and James, 1952). In this study, phosphate buffer (pH 7.0 and ionic strength 0.013) described by Hill et al. (1963) was adopted.

Table 10 shows the electrophoretic mobilities of P. cepacia grown under different nutrient depletions. The electrophoretic mobilities of P. cepacia were in the following order: Log-phase = carbon-depleted cells > stationary-phase cells > sulphate-depleted = phosphate-depleted cells > nitrogen-depleted cells > iron-depleted = magnesium-depleted cells. Interestingly, log-phase cells and carbon-depleted cells had the highest negative charges

of any of the nutrient-depleted cells.

From the results, it seems that when the cells were depleted of an essential nutrient, they would response to the new environment by changing the cell envelope in terms of surface charge and hydrophobicity. The negative charge of the outermost surface layer was found to decrease in most cases, possibly through the decrease in the number of negatively charged groups such as carboxyl groups or phosphate groups or increase in the number of positively charged groups such as amino groups of the proteins exposed on the outermost surface layer or neutralization of the negatively charged groups through increase adsorption of cations such as Mg^{2+} on the bacterial surface. Carbon-depleted cells had similar negative charge properties as the exponential-phase cells. It possibly indicated that the change of the surface charge required metabolic energy which had to be supplied by the carbon source. When the cells were depleted of a carbon source, such an alteration may be unlikely to take place.

Comparison of the electrophoretic mobility of P.cepacia with the results on ingestion of bacteria by PMN leukocytes (Table 13) showed no significant correlation. The electrophoretic mobilities in this study were obtained by using non-opsonized cells and the engulfment was performed by using whole blood in which opsonization might have occurred prior to ingestion by PMN leukocytes, so the discrepancy might be due to their difference in the degree

of opsonization. However, Van Oss (1978) suggested that the strength of the electrical surface potential of particles did not appear to be directly linked to the facility with which those particles became engulfed, since the pleomorphic and irregular shape of phagocytic cells played a much important role in surmounting electrostatic repulsion forces than did the absolute values of the electrokinetic potentials.

Microelectrophoresis technique can be used to detect surface charge changes due to chemical and enzymatic modifications. Modification of the surface groups such as carboxyl group could be done with chemical reagents like ethanolic diazomethane (Gitten and James, 1963) which resulted in the alteration of the electrophoretic mobilities of Aerobacter aerogenes. Fluorescent probes such as CC_5 are positively charged, if they bind to the outermost surface layer by reacting with the negatively charged groups located at the surface, then the electrophoretic mobilities of the cells will be altered. Two probes, namely, CC_5 and NPN, were tested. Mid-logarithmic-phase cells were chosen, since they had the highest negative charge on the surface. The results are shown in Table 11. It indicated that neither CC_5 nor NPN could alter the electrophoretic mobilities of the cells. It meant at least that the probes were not located at the outermost surface layer. NPN is known to be located at the hydrocarbon core of the membrane (Radda, 1975) while little information is available concerning the location of

CC_5 in the membrane. Cyanine dyes such as CC_5 are known to be amphipathic; oxygen atoms and electric charge produce hydrophilic forces, while alkyl groups produce lipophilic forces (Fig. 38). The location of cyanine dyes in a bilayer membrane seems to be affected by the alkyl groups attached on the molecule. If only one carbon is attached to the molecule, then the dye is impermeable and occupies on the membrane surface (For Gram-negative bacteria, the situation is probably different, since the dye is quite hydrophilic it probably penetrates the OM through the porin channels). However, if the carbon chains of the alkyl groups are increased to 5 or 6, then the dyes are found to be located at the hydrophobic core of the bilayer membrane (Huebner, 1977). CC_5 has 5 carbon atoms in the alkyl groups, so it is most likely to occupy the hydrophobic core of the membrane. Our results are in agreement with their findings.

The effect of growth rate on the electrophoretic mobility was also investigated by using P. aeruginosa NCTC 6750 grown under iron limitation at different dilution rates in the chemostats. The results are shown in Fig. 33. The results indicate that an increase in growth rate will follow by an increase in negative charge on the bacterial surface.

4.2.3 Surface hydrophobicity as studied by contact angle measurements

The hydrophobicity of the surface of bacteria has received much attention in the last decade, because it probably plays an important role in the determination of the ingestion by phagocytes. The surface hydrophobicity of the bacteria can be determined by measuring the angle formed between a thick layer of bacteria and a saline drop (10 µl). Van Oss and his associates (Van Oss et al.,1975) determined the contact angles of a large number of bacteria and found a striking correlation between hydrophobicity and engulfment. Those bacteria with angles greater than 18^{o} (more hydrophobic than the neutrophils) were readily engulfed while those with angles less than 18^{o} were phagocytized with difficulty. Furthermore, both antibodies 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 (Cunningham et al., 1975).

Some antibiotics were found to enhance or depress ingestion of bacteria by phagocytes (Van Oss et al., 1975). Gentamicin was found to make phagocytes more hydrophilic and bacteria more hydrophobic, thereby resulting in much enhanced ingestion by phagocytes, while ampicillin made phagocytes somewhat more hydrophilic, but it made bacteria much more hydrophilic, which resulted in an overall depression of phagocytosis.

Van Oss and his associates have done the most interesting work on the physicochemical properties of bacterial surfaces and found a striking correlation between hydrophobicity and the degree of engulfment. However, most of the organisms used in their studies were grown in complex media and even in some cases that the cells grown in solid media then washed off with saline were used (Van Oss et al.,1975). It has been known that radical changes in envelope composition occurs as a response to environmental changes. In particular, when a dividing bacterium is faced with the lack of an essential nutrient, it makes an envelope characteristic of the particular depletion (Brown and Watkins, 1970; Brown, 1975; 1977; Kenward et al.,1978).

In this study, the effects of nutrient depletion on the contact angles of P. cepacia and P.aeruginosa were investigated. The results are shown in Table 12. No significant correlation between the electrophoretic mobilities (Table 10) and hydrophobicity (Table 12) of the bacterial surface was observed in the study. Presumably the reduction of the uncharged carbohydrate chain length does not have any significant effect on the surface charge but it will significantly increase the hydrophobicity of the bacterial surface or the adsorption of different ions on the bacterial surface will probably affect mainly the surface charge.

Comparison of the contact angles with the engulfment of P. cepacia by PMN leukocytes (Table 13) showed that

there was no significant correlation between the contact angles and the degree of engulfment by PMN leukocytes. Again the contact angles were obtained by using non-opsonized cells and the engulfment was performed by using whole blood in which opsonization might have occurred prior to ingestion by PMN leukocytes, so the discrepancy might be due to their difference in the degree of opsonization. Van Oss et al.(1975) found that the role of opsonization was to increase the hydrophobicity of the bacteria and therefore enhanced ingestion of bacteria by PMN leukocytes. Divalent cations such as Mg^{2+} were found to affect the contact angle of bacteria (Brown, M.R.W. , unpublished data), so the suspending medium used in the study should be clearly defined.

In this study, the effect of growth environment on the hydrophobicity of bacteria was investigated. The results confirmed the observations by previous workers (Brown, 1977; Kenward et al., 1978) that when a bacterium is faced with the lack of an essential nutrient, it makes an envelope characteristic of the particular depletion which has particular physicochemical properties of the outermost surface in this study.

An increased bactericidal capacity of PMN leukocytes at 40^{o} relative to 37^{o} has been observed with E. coli, S. typhimurium and Listeria monocytogenes, but not with Staph. aureus (Mandell, 1975; Roberts and Steigbigel, 1977). In infection there is usually an elevated temperature and

host resistance may be increased by enhancement of the bactericidal action of blood against certain organisms (Roberts, 1979). Finch and Brown (1978) found that growth environment affected the killing of P. aeruginosa by rabbit PMN leukocytes and by cationic proteins. In this study, the effects of growth environment on the engulfment and killing of P. cepacia in whole blood at three different temperatures were investigated (Figs 34 to 37).

In Fig. 34, log-phase cells showed a decrease in survival as the temperature was increased from 33° to 37° and then to 41°. Decrease in survival was also observed in the stationary-phase cells as the temperature was increased from 37° to 41°, but there was apparently little difference between 33° and 37°. The stationary-phase cells were much more resistant than the log-phase cells to chemical and enzymatic attacks by phagocytes at the same temperature. However, the stationary-phase cells were more easily engulfed than the log-phase cells by PMN leukocytes as shown in Table 13. Engulfment and killing should be considered as two separate events. Cells can be easily engulfed and easily killed by PMN leukocytes. On the other hand, they can also be engulfed easily and killed with great difficulty, possibly due to alteration in the envelope that makes them less vulnerable to chemical and enzymatic attacks by PMN leukocytes. This may well explain the above observation. Temperature seems to have little effect on the engulfment of the stationary-phase cells, as it may be due

to the fact that they had been engulfed to the greatest extent even at the lowest temperature (33°). However, increase in the number of bacteria taken per PMN leukocyte was observed in the log-phase cells as the temperature of incubation was increased.

Figures 35 to 37 show the killing by blood of P.cepacia grown under six different nutrient limitations at three different temperatures. Carbon-depleted cells were found to be most sensitive at three different temperatures used in this study. There was no significant effect of temperature on killing of these very sensitive carbon-depleted cells. The other nutrient-depleted cells seems to be affected by the temperature at which phagocytosis was performed. Increase in survival was observed when the phagocytosis was performed at lower temperatures. Increase in resistance to killing by blood at any of the temperatures used was found to be in the following order: carbon, iron, sulphate, nitrogen , phosphate and magnesium-depleted cells. Carbon-depleted cells were found to be most easily engulfed by PMN leukocytes. There was a slight effect of temperature on the number of carbon-depleted cells taken per PMN leukocyte. Magnesium-depleted cells were not only the most resistant to killing but also engulfed with great difficulty among the nutrient depletions being studied. The other nutrient-depleted cells were found to lie in sensitivity between carbon and magnesium-depleted cells. The results confirmed the observations by Finch and Brown

(1978) that growth environment had significant effects on the killing of microorganisms by PMN leukocytes.

4.3 Studies of The Membranes of P. cepacia With Fluorescent Probes

4.3.1 Introduction

Fluorescent probing techniques were first used by Newton (1954) to investigate the mechanism of action of polymyxin. Unfortunately, there was a gap of more than a decade before it was re-introduced by Stryer (1968) to the study of biological systems. The application of the fluorescence technique to biological systems has progressed parallel to the development of a theoretical basis for fluorescence data interpretation and the synthesis of a large number of fluorescent probes. These were organic molecules having fluorescence characteristics that are dependent on their environment. A number of excellent reviews on the topics have been published in the recent years (Radda, 1971; 1975; Azzi, 1975; Shinitzky and Barenholz, 1978). Several probes which have often been used in the study of natural and artificial membranes were employed in this study to investigate the effects of growth environment on the membranes of P. cepacia.

4.3.2 Effects of boiling on the fluorescence of TNS or ANS in P. cepacia grown under different nutrient limitations

TNS or ANS is almost non-fluorescent in water or in

the presence of bacterial cells at neutral pH. The structure of the OM of Gram-negative bacteria may probably act as a barrier to prevent the entry of these probes. This barrier can be disrupted by several means such as boiling the cells for a short period or treatment with EDTA. Newton (1954) found that fluorescence of TNS was increased several hundred fold when P. aeruginosa was boiled. The explanation could be that boiling cause damage of the OM and that penetration of TNS to the OM could proceed. Therefore increased fluorescence was observed.

P. cepacia grown under different nutrient depletions was boiled for 10 min, then treated with ANS or TNS as described in section 3.4.2.1 The results are shown in Figures 39 and 40. Both probes showed the decrease of fluorescence intensity in the following order: glucose, iron, phosphate and nitrogen-depleted cells. No fluorescence was observed in unheated cells of all nutrient depletions (data not shown). The results indicated that glucose-depleted cells were most permeable to TNS or ANS after heating in a boiling water bath, then followed by iron, phosphate and nitrogen-depleted cells. Another explanation is that glucose-depleted cells were disrupted to the greatest extent, then followed by iron, phosphate and nitrogen-depleted cells. The results probably reflect the rigidity of the OM which may possibly be influenced by the growth environment.

4.3.3 Effects of pH and buffer systems on fluorescence of TNS or ANS in P. cepacia

Ballard et al. (1972) found that fluorescence intensity increased as the pH was lowered. Several buffer systems including tris(hydroxymethyl) amino methane were used in this study. It is well documented that there is a toxic effect of Tris-buffer on bacterial cells. Tris has been found to break the salt bridges formed by metal cations with structural components such as LPS and release these components from the cell wall (Voss, 1967). Irvin et al. (1981) recently found that Tris-buffer increased OM permeability on the basis of an increased V_o of whole cell alkaline phosphatase activity and on the basis of sensitivity to lysozyme and altered localization pattern of alkaline phosphatase. Several buffer systems were employed to study the effects of pH on the fluorescence of TNS or ANS. The results are shown from Figures 41 to 44. The results indicated that Tris-buffer increased the permeability of the OM so that ANS or TNS could penetrate as observed in an increase of fluorescence intensity. Acidic conditions might also enhance the toxic effect of Tris. The change of permeability was observed at concentrations above 0.1 M by Irvin et al. (1981). However, the concentration of Tris used in this study was 0.05 M. This is not surprising since the fluorescent technique is usually a thousand fold more sensitive than the absorption technique. Therefore, our results provide

confirmation of the toxic effect which Tris-buffer exerts on the OM.

4.3.4 Fluorescence of CC_5 in P. cepacia

Dyes of carbocyanine family are generally used in the study of the dynamic properties of membranes. CC_5 was employed to monitor trans-membrane potential by Bramhall *et al.* (1976). CC_5 has an affinity for the hydrophobic environment of the plasma membrane in which it fluoresces more intensely than in an aqueous milieu (Sims *et al.*, 1974). CC_5 was used in this study to investigate the change of cell envelope at different stages of the growth curve. The results (Fig. 47) indicate the dynamic change of fluorescence of CC_5 along the growth curve. It is surprising that fluorescence was highest around the turn-off points in the growth curves of the nutrient limitations being studied and then fluorescence of CC_5 decreased. The greatest decrease after the turn-off point was observed in magnesium-depleted cells. The results may be explained from the points of permeability of the cell envelope. The permeability of the envelope of *P. cepacia* to certain molecules is highest at the turn-off point and then decreases when certain nutrients are no longer available in the medium. In other words, a change of growth environment will reflect dynamic changes of the physiology of the membranes.

Recently, Brown (M.R.W. Brown, unpublished data)

investigated the contact angles of P. aeruginosa at different stages of the growth curve. He found that the contact angle of lag-phase cells was around 20°, then increased to about 60° at the turn-off point then dropped to about 20° after 24 h in the culture. Highest fluorescence intensity observed in this study at the turn-off points may be due to high hydrophobicity of the bacterial surface, therefore a larger amount of the probe penetrates the OM or it fluoresced more intensely due to the hydrophobic environment of the cell envelope.

4.3.5 Location of the probes in the outer and cytoplasmic membranes of P. cepacia

Fluorescent probing techniques can provide information about the physical properties of membranes. However, Gram-negative bacteria are known to have two membranes, CM and OM. When a certain probe is added to a suspension of bacterial cells, how it will be distributed in these membranes. In this study, the distribution of several probes in the OM and CM of P. cepacia was investigated. The results are shown from Fig. 49 to Fig. 52. For CC_5 and 2-methyl anthracene, a higher fluorescence intensity was observed in the OM than in the CM while a higher fluorescence intensity was observed in the CM than in the OM for cells treated with NPN or DPH. It is surprising that a much higher fluorescence intensity was observed in the CM and only trace level was detected in the OM for

cells treated with NPN. The results clearly indicate that NPN has a higher affinity towards the CM than the OM although it was possible that the probe was first located in the OM and then distributed to the CM during the separation of the OM and CM. If NPN was added to isolated OM and CM, higher fluorescence intensity was still observed in the CM (see Fig. 53). However, a significant increase in fluorescence intensity was observed in the OM (compare with Fig. 49). This indicates that larger amount of the probe exists in the OM, probably due to increased permeability to the probe in isolated OM or the competitive factor (CM) has been removed. The higher fluorescence intensity observed in the CM may be due to the fact that NPN fluoresces more efficiently in the CM than in the OM or else a higher permeability of the CM to the probe.

4.3.6 Effects of Sarkosyl on the fluorescence of CC_5 in the outer membrane and whole cells of P. cepacia

Sarkosyl is often used in the preparation of the OM. Filip et al (1973) found that Sarkosyl specifically solubilised the CM of E. coli leaving the OM relatively intact. However, their conclusion was based on the similarity of the OM protein patterns obtained by sucrose density gradient centrifugation and Sarkosyl methods. Chopra and Shales (1980) found that several minor OM proteins had been extracted on Sarkosyl treatment. In this study, isolated OM or whole cells were treated with CC_5

then treated with Sarkosyl (Figs. 54 and 55). The results indicate that Sarkosyl removes CC_5 from the OM or whole cells. In other words, the location of CC_5 in the OM or whole cells is accessible to Sarkosyl. The mechanism probably involves squeezing of Sarkosyl into the OM or whole cells, then followed by partitioning of CC_5 into Sarkosyl. Other constituents of the OM has also been found to be extracted by Sarkosyl in P. aeruginosa (P.A. Lambert, personal communication). The effects of Sarkosyl on the properties of the OM has also been investigated by fluorescence polarization (section 3.4.7). The results indicate that the fluorescence polarization of three probes as well as the microviscosity (studied with DPH) were significantly increased after Sarkosyl treatment (Table 20).

4.3.7 Fluorescence polarization studies of the membranes of P. cepacia NCTC 10661

Fluorescence polarization is known to be applicable in the study of the fluidity of biological membranes. It was employed by Cheng et al. (1974) to investigate the properties of the membranes of E. coli. They found that the OM of E. coli was twice as viscous as the CM. It has also been used in the study of the mechanism of action of colicin (Helgerson et al., 1974). An excellent review on the subject has been published recently (Shinitzky and Barenholz, 1978). The method of calculation of

microviscosity using DPH in this study has in fact been discussed by them in the following equation:

$$\bar{\eta} = \frac{I_{\parallel}/I_{\perp} - 1}{0.73 - 0.27\ (\ I_{\parallel}/I_{\perp})}$$

The results are shown in Tables 18 and 19. From these results, the fluorescence polarization of the OM was significantly higher than that of the CM in magnesium and iron-depleted cells while the fluorescence polarization of both membranes was similar in nutrient broth grown cells. The microviscosity measurement with DPH was also in agreement with fluorescence polarization data (Table 19). In magnesium and iron-depleted cells, the microviscosity of the OM was approximately three times as large as that of the CM. However, only a slight difference was observed in the membranes of nutrient broth grown cells. In other words, when a bacterium is faced with the lack of magnesium or iron in the medium, it manufactures the OM which is three times as viscous as the OM occurring when grown in nutrient broth. There was slight variation in the order of fluorescence polarization of the two probes (Tables 18 and 19) in measuring the fluidity of the membranes. This is probably due to the difference in their emission properties which are determined by their chemical structures and their location in the membrane. There was also a slight variation in the fluorescence polarization of the whole cells and membranes.

The fluorescence polarization of the whole cells presumably represents the average fluorescence polarization of the probe, since it is difficult to determine exactly the percentage of distribution of the probe in the membranes. The discrepancy could be due to treatments that had been done in preparing the OM and CM.

4.4 Separation of The Outer and Cytoplasmic Membranes of P. cepacia NCTC 10661

4.4.1 Separation of the OM and CM by sucrose density gradient centrifugation

Morphological studies showed that the cell wall of Gram-negative bacteria was composed of the OM and underlying murein layer (Murray et al., 1965). However, cell disruption followed by differential centrifugation usually produced 'cell envelopes' containing not only the OM and murein, but also the CM. Miura and Mizushima (1968; 1969) first successfully devised a technique for separating the OM of E. coli from other components of the envelope. Thus detailed biochemical studies of the OM and CM are now possible. Various procedures and modifications in order to be applicable to specific organism and special purposes have since been published (see section 1.1.5).

The success of the separation of the OM away from the CM is often judged by examining the activities of several enzymes which are found to be located exclusively in the CM (Osborn et al., 1972). The activities of several enzymes were investigated in this study. The results are shown in Table 21. From the results, it was found that succinate dehydrogenase was the enzyme to be present in large quantity in the CM while the others were sometimes hardly detected. From the study, it is suggested that succinate dehydrogenase is the best marker of the CM. High enzyme activities were also detected in the

supernatant after centrifugation of the whole cell envelope in the preparation of the membrane (data not shown) which suggested that these enzymes were loosely bound to the CM, probably attached on the inner leaflet of the CM and were released during the breakage of the cells. KDO is often used as a marker of the OM. Unfortunately, it was not detected in P. cepacia in this study which confirmed the observations by Manniello et al. (1979) that P. cepacia did not have KDO in its LPS. The success of the separation was also verified by examining the protein patterns of the OM and CM in SDS-polyacrylamide gel electrophoresis.

4.4.2 The protein patterns of the OM and CM of P.cepacia

Weber and Osborn (1969) investigated forty proteins with polypeptide chains of well characterized molecular weights in SDS- polyacrylamide gel electrophoresis. When the electrophoretic mobilities of these proteins were plotted against the logarithm of their molecular weights, a smooth curve was obtained. The electrophoretic mobilities of several polypeptides of known molecular weights were investigated in the study. Linear relationship was obtained (see Fig. 56).

The system described by Lugtenberg et al. (1975) was adopted in this study. This system resulted in a good resolution of the major OM protein (44,000 daltons) of E. coli K-12. It was resolved into four bands using this

system. However, only two bands were observed when the system described by Laemmli (1970) was used (Lugtenberg et al., 1975).

The synthesis of several OM proteins of Gram-negative bacteria was found to be affected by the growth environment. Synthesis of protein D1 was induced when P. aeruginosa was grown in the presence of glucose (Hancock and Carey, 1980). Iron limitation induced the synthesis of several OM proteins with apparent molecular weights in the range of 60,000 to 85,000 daltons in P. aeruginosa (Mizuno and Kageyama, 1978) and they might be involved in the translocation of iron across the OM (Meyer et al., 1979). Magnesium limitation induced the synthesis of protein H1 in P. aeruginosa PAO1 (Nicas and Hancock, 1980) and was suggested to be involved in the resistance to aminoglycosides such as gentamicin and streptomycin (Hancock et al., 1981) and also to polymyxin (Nicas and Hancock, 1980). Molecular size and osmolarity of sugars and dextrans in the growth medium could affect the synthesis of the OM proteins O-8 (34,000 daltons) and O-9 (35,000 daltons) of E. coli K-12 (Kawaji et al., 1979). Supplementation of the growth medium with high concentrations of sugar or low-molecular weight dextrans resulted in the induction of O-8 synthesis and suppression of O-9 synthesis while induction of O-9 synthesis and suppression of O-8 synthesis occurred when E. coli K-12 was grown in a medium with sugars or dextrans of molecular

weights greater than 600 daltons. Expression of protein e of *E. coli* K-12 was induced when the cells were grown under phosphate limitation (Overbeeke and Lugtenberg, 1980).

The protein patterns of the OM and CM were analysed by SDS- polyacrylamide gel electrophoresis. The results are shown in Fig. 57. The proteins patterns of the CM (slots A and D) were significantly different from those of the OM (slots C and F). Fewer protein bands were observed in the OM but they were present in large quantities. There was only a slight difference between the unseparated fraction (slots B and E) and the OM fraction (slots C and F), but to reduce cross contamination of the CM in the OM or *vice versa* , those between the CM and OM were designated as unseparated fraction.

Fig. 58 shows the OM protein patterns of *P. cepacia* grown under three different media and the effects of heating and 2-mercaptoethanol on the electrophoretic mobilities of these OM proteins. The OM protein profile of magnesium-depleted cells was much simpler than those of the iron-depleted and nutrient broth grown cells (oxygen-depleted cells). Several proteins with apparent molecular weights lower than 20,000 daltons were missing in the OM of magnesium-depleted cells. Magnesium is known to be essential for the integrity of ribosomes and their activity (Stanier *et al.*, 1977) and in the synthesis of ribosomes and RNA (Cohn and Ennis, 1967).

Therefore, deficiency of magnesium in the growth medium will result in the reduction of protein synthesis. The apparent molecular weights of the OM proteins of Mg^{2+}-depleted cells were 40,000, 36,000, 24,500 and 14,500 daltons. The OM protein with an apparent molecular weight of 36,000 daltons was present in large quantities in all three growth conditions and might correspond to the pore-forming protein (porins) as observed in other Gram-negative organisms to be in the range of 33,000 to 40,000 daltons (Nikaido and Nakae, 1979). The synthesis of the OM protein with an apparent molecular weight of 66,000 daltons was induced when P. cepacia was grown under iron depletion. The result is in agreement with the published data in other organisms that iron depletion induces the synthesis of OM proteins with apparent molecular weights in the range of 60,000 to 85,000 daltons (Mizuno and Kageyama, 1978 ; Meyer et al., 1979). The synthesis of two OM proteins with apparent molecular weights of 17,000 and 18,500 daltons were induced when the cells were grown in nutrient broth (oxygen-depleted). They were missing when the cells were grown under magnesium depletion (Fig. 58).

Several factors such as the choice of detergents, buffer systems and the presence of urea in the SDS - polyacrylamide gel were found to affect the relative migration of the OM proteins (Pugsley and Schnaitman, 1979). This was probably due to the effects on the charge and/or

the configuration of the detergent-protein complexes. The electrophoretic mobilities of several major OM proteins of P. aeruginosa were found to be affected by the solubilization temperature and 2-mercaptoethanol by Hancock and Carey (1979). It is interesting that the mobility of protein F (33,000 daltons) which is a pore - forming protein is dependent on 2-mercaptoethanol concentration. At low concentration of 2-mercaptoethanol (0 - 0.01%), it moved with an apparent molecular weight of 33,000 daltons (protein F). However, at higher concentrations of 2-mercaptoethanol (0.1 - 0.5%), it moved with an apparent molecular weight of 42,000 daltons (its F^* form). Their explanation for the effects of 2-mercaptoethanol was that it was affecting one or possibly two cysteine disulphide bonds. Since the mobility of protein F (or its F^* form) was decreased by 2-mercaptoethanol treatment, the alteration was probably due to the breaking of intrachain (rather than interchain) disulphide bridges. This would cause an alteration in the conformation of the protein and thus increase the binding of SDS of the molecule. Protein F was also found to be heat-modifiable. At low temperature (below 88°C), it moved with an apparent molecular weight of 39,000 daltons. However, at high temperature (100°C), it moved with an apparent molecular weight of 41,000 daltons.

In this study, the electrophoretic mobility of one OM protein was found to be affected by the presence of

2-mercaptoethanol and the electrophoretic mobilities of several OM proteins were affected by the solubilization temperature. In the presence of 2-mercaptoethanol, it moved with an apparent molecular weight of 24,500 daltons. However, when 2-mercaptoethanol was excluded from the denaturing buffer, it moved with an apparent molecular weight of 22,500 daltons in all three growth conditions used in the study. The electrophoretic mobility of one major OM protein with an apparent molecular weight of 36,000 daltons in P. cepacia was not affected by the presence of 2-mercaptoethanol. This is an indication that this protein behaves differently from the protein F of P. aeruginosa. The results presumably indicate that this protein (36,000 daltons) does not have cysteine as a component of the polypeptide or the disulphide bonds (if any) are hindered from the reduction by 2-mercaptoethanol.

Several major OM proteins of P. cepacia were found to be heat-modifiable (Fig. 58). These proteins had apparent molecular weights of 40,000, 36,000 and 24,000 daltons when they were solubilised at 100° for 5 min. However, they moved with apparent molecular weights greater than 70,000 daltons when they were solubilised at 60° for 30 min. They apparently moved as a single diffused band (Fig. 58).

The effects of solubilization temperature on the electrophoretic mobilities of the 'heat-modifiable'

proteins of P. cepacia appeared to behave differently from those of P. aeruginosa (Hancock and Carey, 1979) and E. coli (Reithmeier and Bragg, 1977). In P.aeruginosa and E. coli, they move slower (higher apparent molecular weights) when they are denatured at high temperature and move faster (smaller apparent molecular weights) when they are denatured at low temperature. It is untenable to beleive that a protein should have two different molecular weights; therefore, the behaviour of this protein must be explained in terms of two different stable conformational states in SDS solution. Studies had been done to demonstrate that this was the case (Nakamura and Mizushima, 1976; Reithmeier and Bragg, 1977). However, the 'heat-modifiable' proteins of P. cepacia moved faster (higher apparent molecular weights) when they were denatured at low temperature and moved slower (smaller molecular weights) when they were denatured at high temperature (Fig. 58). This is just the opposite as observed in P. aeruginosa and E. coli. The explanation can still be due to two different stable conformational states of the protein molecule in SDS solution or the proteins probably occur as dimer or trimer in nature and when they are denatured at high temperature (100^{o}C), they are split into monomers, therefore they move faster in the SDS - polyacrylamide gel.

PAS (Periodic acid, Schiff's reagent) is used to stain glycoproteins, heparin, chondroitin sulphate,

polysaccharides and other materials with a high carbohydrate content. It was employed to investigate the properties of LPS of S. typhimurium (Munford et al.,1980). In this study, PAS was used to stain the LPS or any molecules associated with carbohydrate. The results are shown in Fig. 59. In iron-depleted cells, three bands were found to be stained by PAS (slot A), two bands were observed in magnesium-depleted cells while 5 to 6 bands were observed in nutrient broth grown cells. Interestingly, the OM proteins with apparent molecular weights of 36,000 and 66,000 daltons were PAS positive. Schweizer et al. (1978) found that protein II (33,000 daltons) of E.coli was associated with its LPS. At present, we have no evidence to suggest that PAS positive proteins of P.cepacia are glycoproteins or associated with LPS.

Sarkosyl (sodium lauryl sarcosinate) is often used in the preparation of the OM, because it is a simpler and quicker way of getting the OM. It was first reported by Filip et al. (1973) that Sarkosyl specifically solubilised the CM of E. coli and the OM remained intact after the treatment. The OM protein patterns of P. aeruginosa, E. coli and P. cepacia prepared by this method are shown in Fig. 60. The OM protein patterns of these three organisms were significantly different and might be used as fingerprints for diagnotic purposes. However, we found that the OM protein pattern obtained by this method was much simpler than that prepared by a

sucrose density gradient centrifugation method (Fig.60). It indicated that some minor OM proteins had been extracted by Sarkosyl. In fact, Chopra and Shales (1980) found that Sarkosyl removed several minor proteins from the OM of E. coli. Sarkosyl was found to removed CC_5 form the OM or whole cells of P. cepacia labelled with CC_5 in this study (see Figs. 54 and 55) and fluorescence polarization of several probes was increased after Sarkosyl Sarkosyl treatment which indicated that OM treated with Sarkosyl had different physical properties to the untreated untreated one. It is concluded that the Sarkosyl method is useful in the preparation of the OM for diagnotic purposes and should not be encouraged in the preparation of the OM for the studies of the physical and chemical nature of the OM.

4.4.3 Protein and phospholipid contents of the OM and CM of P. cepacia

Protein and phospholipid compositions of the OM and CM of P. cepacia have been determined. The results are shown in Table 22. In all the growth conditions used in the study, the OM was found to be enriched in protein while the CM was enriched in phospholipid (PL). The OM was found to consist of approximately 50% protein, 20% PL and the rest was probably LPS while the CM was found to consist of 80% PL and 20% protein. The relatively low content of protein in the CM might be due to the release

of the protein from the CM during the breakage of the cells, as high enzyme activity such as succinate dehydrogenase was detected in the supernatant after centrifugation of the whole envelope in the preparation of the membrane (data not shown). These enzymes such as succinate dehydrogenase were presumably loosely bound to the inner leaflet of the CM and released when the cells were broken by passing through the French press. The loss of the protein could possibly be estimated by measuring the protein content in the supernatant and also in the whole cells. The results could then be compared with the protein contents in the OM and CM.

Phospholipids were fractionated into individual PL by thin-layer chromatography. They were found to be mainly phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) with trace amount of phosphatidylcholine (PC). Diphosphatidylglycerol (DPG) was not detected in the study, even after charring at 160° for 2 h. Pseudomonas BAL-31 was also lacked of DPG (Diedrich and Cota-Robles, 1974). The ratio of PE/PG + PC was calculated as described in section 2.2.12. It was found that the ratio was higher in the OM than in the CM in all three growth conditions used in the study which meant that the OM had higher content of PE than that of the CM. Enrichment of the OM in PE has also been observed in Pseudomonas BAL-31 (Diedrich and Cota-Robles, 1974), P. mirabilis (Rottem et al., 1975), S. typhimurium (Osborn et al., 1972)

and E. coli K-12 (Lugtenberg and Peters, 1976). However, Kenward et al. (1979) found that the walls of P. aeruginosa NCTC 6750 to be enriched in PG and DPG. This probably indicates that P. aeruginosa has the OM significantly different from those organisms mentioned above.

The protein and total phospholipid contents of the OM and CM of different nutrient-depleted cells were found to vary only slightly (Table 22). However, dramatic variation of the ratio of PE/PG+PC was observed among nutrient-depleted cells. The ratio was found to be highest in iron-depleted cells, followed by nutrient broth grown cells (oxygen-depleted) and magnesium-depleted cells. This variation could be attributed to their difference in fatty acid composition as a response to the changes of growth environment. Lugtenberg and Peters (1976) investigated the effects of fatty acid composition on the phospholipid composition of the membranes of E. coli by growing the cells at different growth temperatures or using mutants which have an altered fatty acid metabolism. They concluded that E. coli possessed a mechanism that caused the enrichment of the CM in PG plus DPG and in unsaturated plus cyclopropane carbocylic fatty acids, accompanied by the enrichment of the OM in PE and in saturated fatty acids.

In conclusion, the studies illustrate the importance of defined cultural conditions in studying the behaviour of microorganisms.

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