The use of cells frozen at -196° for the bioassay of preservatives

A thesis submitted by

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for the degree of

MASTER OF PHILOSOPHY

of the University of Aston in Birmingham

May 1984

To my husband
and my children

The University of Aston in Birmingham

The use of cells frozen at -196° for the bioassay of preservatives.

by Wedad Abdul Razak Mahdi

Submitted for the degree of Master of Philosophy (1984).

By growing Pseudomonas cepacia in a chemically-defined medium, stationary phase cells depleted of different nutrients could be prepared.

Cells depleted of various nutrients showed marked variation in sensitivity to benzalkonium chloride (BKC) and to damage associated with freezing and thawing. In both cases, cells depleted of magnesium were the most resistant. These observations may be due to alterations in the envelope of P. cepacia in response to changes in the growth environment. Mg^{2+}-depleted cells had lower concentrations of cyclopropane fatty acids than the more sensitive glucose-depleted cells, particularly in the cytoplasmic membrane.

PO_4^{3-}-depleted cells were intermediate in resistance to BKC and preliminary results suggested their resistance to freezing damage was similar to that of Mg^{2+}-depleted cells. The cyclopropane fatty acid content was also low.

Different n-alkyl chain lengths of BKC were tested. Both glucose- and Mg^{2+}-depleted cells showed increased sensitivity with increase in chain length up to 14 and 18.

Using different cooling rates, survival of P. cepacia increased as the cooling rate increased, reaching an optimum at $6°min^{-1}$, a minimum at $144°min^{-1}$, and a high level again at an ultra rapid cooling rate.

A cooling rate of $6°min^{-1}$ was chosen to assess the sensitivity of frozen cells to BKC. The sensitivity of Mg^{2+}-depleted cells to BKC was unchanged by freezing. PO_4^{3-} and glucose-depleted cells became more sensitive to BKC following freezing.

Key words: Pseudomonas cepacia, freezing, resistance, cell envelope, nutrient depletion, BKC-resistance.

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ACKNOWLEDGEMENTS

I am gratefully indebted to Dr Ruth Klemperer for her encouragement, tolerance and advice.

My sincere thanks to Dr Hosmin Anwar for his valuable advice.

My sincere thanks to all the staff of the Microbiology Research Laboratory for their help and advice.

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ABBREVIATIONS

BKC	benzalkonium chloride
CDM	chemically defined media
CMC	critical micelle concentration
MIC	minimum inhibitory concentration
CM	cytoplasmic membrane
OM	outer membrane
pL	phospholipid
LPS	lipopolysaccharide
FA	fatty acid
KDO	2-keto-3-deoxy octonic acid
PE	phosphatidyl ethanolamine
PG	phosphatidyl glycerol
DPG	diphosphatidyl glycerol
SDS	sodium dodecyl sulphate
EDTA	ethylene diamine tetraacetic acid
DNA	deoxyribonucleic aicd
MOPS	3-N-morpholino sulphonic acid
PAGE	polyacrylamide gel electrophoresis
Tris	tris (hydroxymethyl)-amino methane buffer
TEMED	N,N,N^{I},N^{I}-tetramethylethylene diamine
PB	polymyxin
OMP	outer membrane protein

1. Introduction

1.1 Benzalkonium chloride

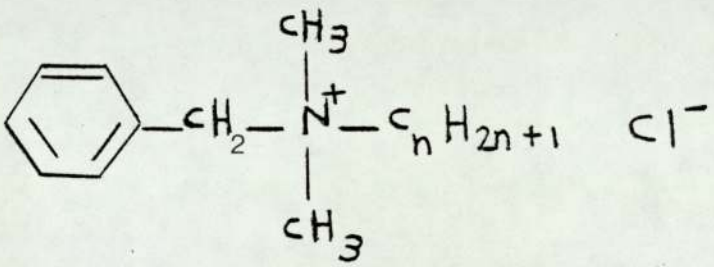
In some pharmaceutical formulations, the concentration of available preservative is best assayed microbiologically. One well-known preservative is benzalkonium chloride (BKC). Commercially available BKC products are a mixture of alkyl benzyldimethylammonium salts comprised of the C_8-C_{16} homologues (Mukhayer et al., 1975). The British Pharmacopeia (1980) defines BKC as being a mixture of alkyl-benzyldimethylammonium chlorides, with an average empirical formula of $C_{22}H_{40}NCl$. The alkyl groups contain 8 to 18 carbon atoms. These criteria could be satisfied by a pure compound with n-alkyl groups equal to C_{11}, by an equimolar mixture of C_{18} and C_4 or by an almost infinite permutation of mixtures of compounds with n-alkyl C_4 to C_{20} (Daoud et al., 1983). It is apparent that BKC can have a very wide range of composition; furthermore, there is not a satisfactory chemical assay (see page 4). Therefore, it was the intention of this study to investigate methods of assaying BKC microbiologically.

The bioassay of BKC requires the use of inocula of reproducible sensitivity, but variations in the cell envelope following nutrient depletion (Ismail et al., 1980) lead to changes in the cell envelope and in resistance, therefore, Pseudomonas cepacia was grown in batch cultures in a chemically-defined medium (CDM) and its sensitivity to BKC following different nutrient depletions was investigated. It is desirable to store inocula. Different cooling rates affect cell envelope properties, so the best cooling rate was chosen which gave maximum survival. Cells cooled at this rate were used for the bioassay of BKC.

1.1.1 Properties of benzalkonium chloride

BKC is one of the quaternary ammonium compounds (QACs) which contain both hydrophilic and hydrophobic groups and consequently they tend to migrate to surfaces as cationic surfactants according to the ionisation of the hydrophilic group. Quaternaries are so named because they may be thought of as derivatives of an ammonium halide in which four hydrogen atoms are variously substituted. It appears that for useful bactericidal activity, it is necessary for at least one of the substituents on the nitrogen atom to have an effective chain length of 8-18 carbon atoms (Hugo, 1980).

The structure of BKC is :-



$n = 8$ to 18

Surface-active agents, are characterised by two distinct regions of opposing solution affinities within the same molecule or ion. When present in a liquid medium at low concentrations, the amphiphiles exist separately and are of such a size as to be subcollodial. As the concentration is increased, aggregation occurs over a narrow range of concentration. These aggregates, which may contain 50 or more monomers, are called micelles (Martin et al., 1969). The concentration at which micelles form is termed the critical micelle concentration (CMC). In the case of amphiphiles in waters the hydrocarbon chains face inwards into the micelle. Surrounding this hydrocarbon core are the polar portions of the amphiphiles associated

with the water molecules of the continuous phase. Aggregation also occurs in nonpolar liquids. In this case the polar heads face inwards while the hydrocarbon chains are associated with the continuous nonpolar phase. It seems likely that spherical micelles exist at concentrations relatively close to the CMC. At higher concentrations there is an increased tendency for laminar micelles to form and exist in equilibrium with spherical micelles.

Micellisation is, therefore, an alternative mechanism to adsorption by which the interfacial energy of a surfactant solution might decrease. Thermal agitation and electrostatic repulsion between the charged head groups on the surface of the micelle oppose this aggregation tendency. Consequently, a low CMC would be expected to be favoured by increasing the hydrophobic part of the surfactant molecules (for a homologous series each additional CH_2 group approximately halves the CMC), lowering the temperature and the addition of simple salts (e.g. kCl) which reduce the repulsive forces by their screening action (Shaw, 1970). The addition of salts decreases the CMC of ionized detergents, presumably because the screening action of the simple electrolytes lowers the repulsive forces between the polar head groups, and less electrical work is required in micelle formation (Elworthy et al., 1968).

The micelle size increases with increased salt concentration, due to the reduction in electrical repulsion affecting the balance of forces upon which the size of the micelle depends. The effect of salts may also be due to a reduction in the hydration of the monomers, which increases their hydrophobicity, and consequently their tendency to micellize.

1.1.2 Chemical assay of benzalkonium chloride

The chemical assay of BKC as described in British Pharmacopeia (1980) is determined by titrating with potassium iodate and calculating as $C_{22}H_{40}ClN$.

It is apparent that the chemical assay of BKC is not satisfactory because it measures the quaternary ammonium and produces no information about the length of alkyl side-chains.

1.1.3 Importance of chain lengths

On comparing the minimum inhibitory concentration (MIC) and the kinetics of sterilization in deionized water, Tomlinson et al., (1977) found that there was a log-linear relationship between rate of sterilization and carbon number up to C_{18}, but when activity was measured by MIC there was a log-linear relationship up to C_{14}, and then there was a turndown in activity. Comparison of the colloidal association of BKC in deionized water and in a simple salts growth medium led to the suggestion that the use of high concentrations of nutrient salts in MIC tests will lower the effective concentration of surface active agents and that in such circumstances the MIC test does not give a true reflection of the intrinsic activity of these compounds. Daoud et al. (1983) investigated the antimicrobial activity of a homologous series of BKC against Gram-positive and Gram-negative bacteria. They found that generally Gram-negative bacteria were more resistnat than Gram-positive bacteria. C_{16} was the most active chain length against Gram-negative bacteria and C_{14} was the most active against Gram-positive ones.

The importance of the cell envelope in sensitivity was confirmed by Brown and Tomlinson (1979) who used a series of step-wise polymyxin (PB)-resistant envelope mutants of Pseudomonas aeruginosa to test the activity of a homologous series (C_{10}-C_{18}) of BKC. A log-linear relation was found between the rate of death and the concentration of BKC and for all mutants there was also a log-linear relationship between alkyl chain length and the concentration required to reduce the colony count to 10% in 2hr. Lambert (1974) found that the antimicrobial activity in homologous series of N-alkyldiethanol amines is exhibited by those compounds bearing more than six carbon atoms in the alkyl chain. Baker et al. (1941) studied the effect of homologous series of straight chain alkyl sulfates and sulfoctates (C_8-C_{18}) and demonstrated that maximum inhibition was exerted by the 12, 14 and 16 carbon compounds. These results demonstrated that other cationic detergents can exhibit an inhibitory activity comparable to QACs.

1.1.4 Mode of action of benzalkonium chloride

The membrane-active antibacterial agents, which include BKC have as their characteristic mode of action the ability to promote the leakage of small molecular weight material from the cytoplasm of the microbial cell (Hugo, 1978); purines, pyrimidine, ribose, amino acids and potassium are detected in this reaction. This phenomenon was attributed to a change in the integrity of the cytoplasmic membrane (CM) (Hamilton, 1968; Longworth, 1971; Lambert and Hammond, 1973). Miller and Baker (1940) suggested that detergents disorganise the cell membrane and denature certain proteins essential to metabolism and growth. Hotchkiss (1946) found that treatment of bacterial cells with certain surface-active compounds in bactericidal concentrations

inhibited bacterial metabolism and caused a release of nitrogenous and phosphorus-containing substances. Release of cellular constituents from Staph. aureus treated with cetyltrimethyl ammonium bromide was reported by Salton (1951).

The inhibition of bacterial metabolism by surface-active compounds was interpreted by Hotchkiss (1946) as evidence against the idea that an essential detergent-sensitive enzyme exists. On the other hand, Knox et al. (1949) showed that cell-free preparations of certain enzymes from Escherichia coli inhibited at detergent/protein ratios which suggested that the specific inhibition of detergent-sensitive enzymes, such as the lactic acid oxidase, could account for metabolic inhibition, cell death and increased permeability observed in bacteria treated with bactericidal amounts of the cationic detergents.

Since the primary site of action of surface-active bactericides is generally considered to be the cell membrane, which is a relatively large cellular component (Hugo, 1967) the compounds must be adsorbed by the cells in relatively large quantities in order to affect cellular metabolism (Sykes, 1965).

To be effective, antimicrobial agents must be capable of achieving effective concentration at their biochemical sites of action. Direct action upon the outermost surface of bacteria is rare; more commonly, lesions within the cell envelope or cell itself are implicated (Brown, 1980). Antimicrobial agents must therefore be able to interact with and penetrate the cell envelope in order to exert their inhibitory action.

Electron micrographs of P. cepacia cells grown in nutrient broth containing BKC showed an external membrane which was distinctly wavy. The main site of damage to the visibly affected cells appeared to be the CM (Richard and Cavill, 1980). Cells of P. aeruginosa resistant to BKC underwent unique ultrastructural reorganisations when they were grown in the present of BKC. The resistant cells usually contained a single centrally positioned pseudovacuole in the CM.

Cationic bactericidal agents stimulated membrane-bound ATPase activity of Bacillus subtilis in the absence of Mg^{2+} (Rosenthal and Buchanan, 1974). It was suggested that activation of membrane ATPase by these agents is a secondary phenomenon, dependent upon their interaction with acidic membrane compounds such as phospholid (pL) which alters the membrane environment of the enzyme.

QACs reduce the electronegativity of bacterial suspensions (McQuillen, 1950), and Chaplin (1952) noted that a resistant culture of Serratia marcescens was less negatively charged than its more sensitive counterpart. The amount absorbed at saturation levels, is, however, much more than the amount corresponding to a closely-packed monolayer, suggesting binding sites additional to those on the cell surface (Salton, 1951).

1.1.5 Mechanism of resistance

Resistance to antimicrobial agents is generally thought to be associated with changes in cell envelope chemistry which result in prevention of the compounds from reacting with active sites on the cell membrane (Hamilton, 1968). Blois and Swarbrick (1972) reported

that Gram-negative bacteria are more resistance to QACs than Gram-positive bacteria. This difference was attributed to the presence of cephalin (phosphatidylethanolamine) in the cell wall of Gram-negative bacteria. Several observations tend to strengthen this theory. It was observed that prior addition of pL to media containing Gram-positive cells can prevent the inhibition of metabolism by cationic surfactants (Baker et al., 1941). It was postulated that the pL was adsorbed onto the bacterial surface, where it subsequently interacted with the bactericide, rendering it inactive. Such experimental evidence suggests that the pL portion of the complex cell wall is responsible for the greater degree of protection afforded the Gram-negative bacteria. Changes in bacterial cell envelope lipid composition are also associated with resistance of Gram-negative bacteria to pB and tetracyclines (Dunnick and O'Leary, 1970).

Hamilton (1968) proposed that the mechanism of resistance to membrane-active drugs in Gram-negative bacteria was exclusion of the drug from the sensitive CM by the impermeable outer membrane (OM). Spheroplasts prepared from two strains of P. cepacia were more susceptible than the whole cells to BKC (Maneillo et al., 1978). It was suggested that lysozyme caused a disruption of the OM layer of P. cepacia which rendered it sensitive to BKC. Treatment of QAC-resistant P. aeruginosa with ethylenediamine tetraacetic acid (EDTA) (which disrupts the OM permeability barrier) renders the cell sensitive to QACs (MacGregor and Elliker, 1958).

1.2 Properties of Pseudomonas cepacia

P. cepacia previously known as a plant pathogen (Ballard et al., 1970), has in the last decade been implicated in nosocomial infections with increasing frequency (Basset et al., 1970). It is appearing more frequently in the medical literature as a causative agent of human disease (Philipsand Eykyn 1971; Speller et al., 1971). It has been isolated from hospital water supplies (Basset 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 antimicrobial agents. It is capable of survival and even multiplication in QACs (Adair et al., 1969; Basset et al., 1970). It showed high resistance to PB, another cationic agent that is effective against Gram-negative bacteria (Maniello et al., 1978). P. cepacia survived in an organic salt solution which contained commercial 0.05% BKC for fourteen years (Geftic et al., 1979). It can also survive easily in distilled water in a temperature range 12°C-48°C (Carson et al., 1973). P. cepacia isolated from an oil-in water emulsion containing preservative showed the ability to destroy it (Close and Nelsen, 1976). It showed also the ability to utilize penicillin G as the source of carbon (Beckman and Lessie, 1979). Borovian (1983) has shown recently the adaptability of P. cepacia to increase its resistance to increased preservative concentrations.

1.3 The Gram-negative bacterial cell envelope

The cell envelope of Gram-negative bacteria is a complex structure, through which a bacterium reacts with its environment. It includes the CM, the peptidoglycan (or murein lipoprotein complex), the periplasmic space, the OM and those structures external to the OM.

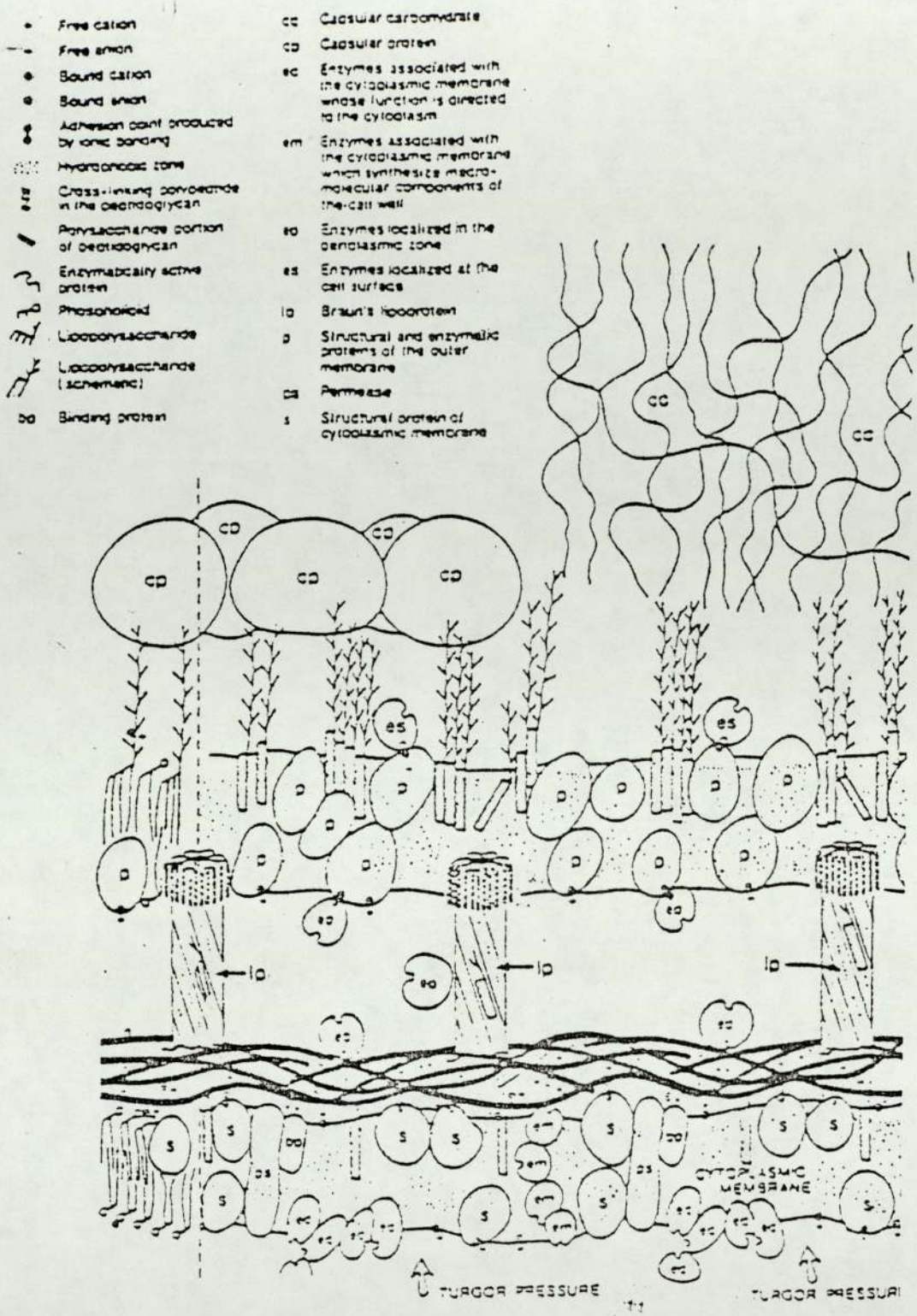


Fig. 1 The Gram-negative cell envelope (Costerton & Cheng, 1975)

The chemical composition of these layers differsmarkedly. While the walls of Salmonella typhimurium, E. coli and P. aeruginosa have been well characterized relatively little is known about the wall of P. cepacia. However, the wall of P. cepacia was found to be grossly similar to that of other Gram-negative organisms of which P. aeruginosa is considered representative (Maneillo et al., 1979). The envelope structure shown in Fig.1 described by Costerton and Cheng (1975), although somewhat outdated is still useful in outline.

1.3.1 The cytoplasmic membrane

The CM, also known as inner membrane, is located between the cell wall and the cytoplasm. Singer's liquid crystal model described the structure of the CM (Singer, 1972, 1974) (See Fig. 2). The phospholipids of the membrane are arranged in the form of a liquid hydrophobic bimolecular layer with the globular proteins partially embedded in the phospholipid matrix and partially extended from it (Fig. 3). These membrane proteins have been classified in two types, peripheral and integral proteins based on their ease of dissociation from the membrane (Singer, 1972). The penetrability of this membrane is affected by alterations in the chain length and degree of saturation of its component fatty acids(McElhaney et al., 1973). The CM of P. cepacia consists of 80% pL and 20% protein (Anwar et al., 1982).

1.3.2 The peptidoglycan layer or murein lipoprotein complex

The peptidoglycan layer consists of a net-work in which linear amino sugar chains, containing alternating residues of N-acetyl glucosamine and N-acetyl muramic acid, are covalently linked to each other by glycosidic bonds. These rigid chains are cross-linked by peptide

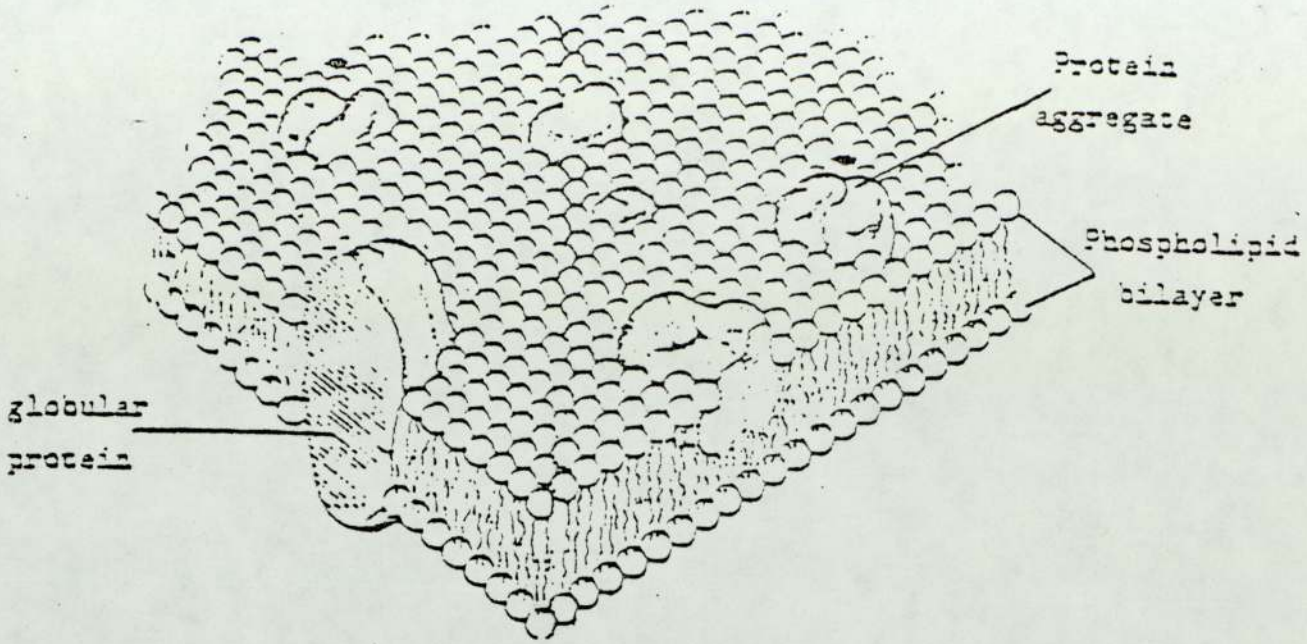


Fig. 2. A schematic representation of the three-dimensional organization of the cytoplasmic membrane (Singer, 1972)

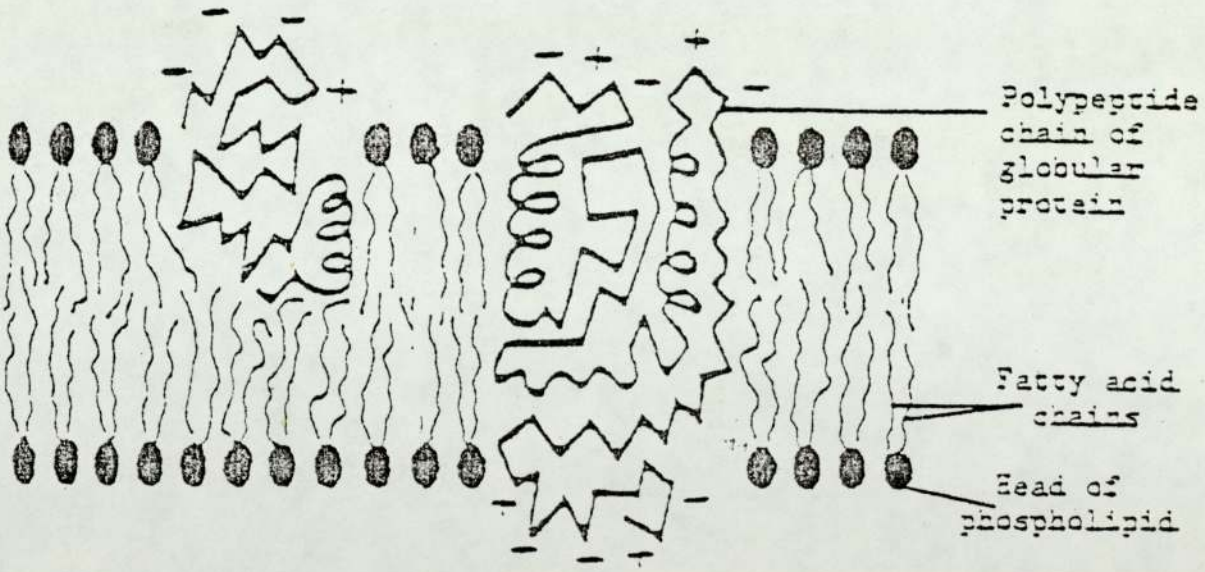


Fig. 3 A diagrammatic representation of a cross section of the cytoplasmic membrane (Singer,1972)

bonds between the meso-diamino pimelic acid and the D-alanine of the neighbouring peptide side chains . The main function of the peptidoglycan layer is to maintain cell rigidity. Besides its function in the maintenance of cell rigidity, it is believed to act as a barrier to the passage of certain drugs (Tsang and Bryan, 1974). It is known to be the target of the action of several antibiotics (Blumberg and Strominger, 1974).

1.3.3 The periplasmic space

The periplasmic space is located between the two membranes. It has been reported to comprise as much as 20-40% of the total cell volume (Stock et al., 1977). The space contains proteins and oligosaccharides. Proteins comprise approximately 4% of the total cell protein (Nossal and Heppel, 1966). With respect to their functions three classes of periplasmic proteins can be distinguished: proteins with a catabolic function (e.g 5'-nucleotidase and alkaline phosphatase) convert solutes for which no transport system exists to a form that can be transported through the CM. Another class of periplasmic proteins are the binding proteins which have affinity for nutrients like sugars, amino acids or ions. A third class of periplasmic proteins is involved in the degradation or modification of harmful compounds such as antibiotics and heavy metals .

1.3.4 The outer membrane

The OM of Gram-negative bacteria has been shown to contain lipopolysaccharides (LPS), pL and proteins. Nikaido and Nakae (1979) proposed an arrangement of the OM based on current knowledge of its properties (Fig. 4). They suggest that the outer half of the OM is almost exclusively occupied by protein and LPS, the pL molecules being mostly found in the inner half of the OM.

Fig. 4. Outer membrane of Gram-negative bacteria (Brown et al., 1979) adapted from Nikaido & Nakae (1979)

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

TPP, Trimer of porin protein.

STP, Specific transport protein.

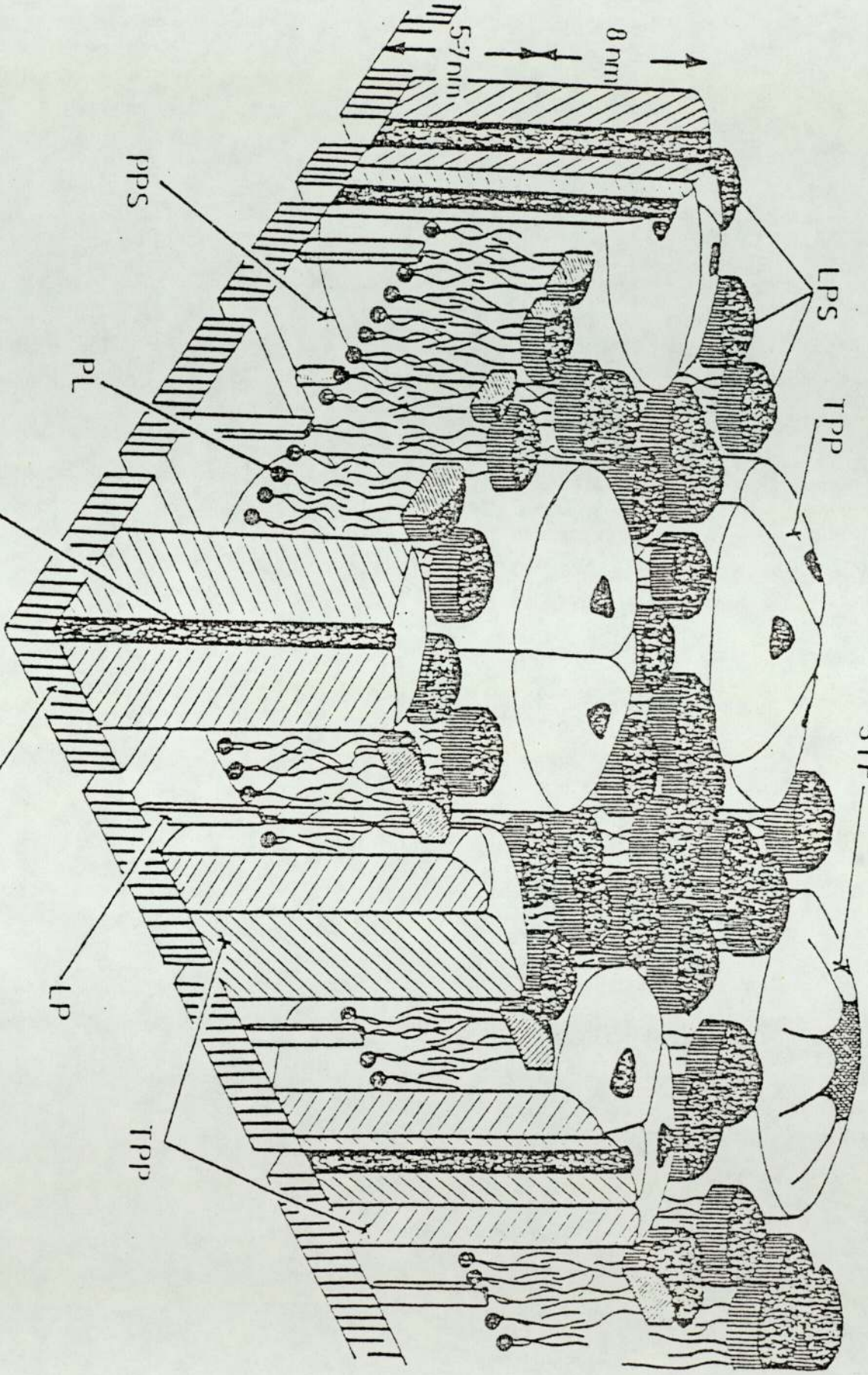
PPS, Periplamsic space,

PL, Phospholipid.

P, Hydrophilic pore.

PG, Peptidoglycan

LP, Braun's Lipoprotein


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

1.3.4.1 Lipopolysaccharide

Fig. 5 shows the structure of LPS of *P. aeruginosa* proposed by Kropinski *et al.* (1979). Three regions are recognisable; these are Lipid A, the core and the O-antigen. LPS is an amphipathic molecule with a hydrophilic polysaccharide portion and a hydrophobic portion, Lipid A. Although these three regions of LPS are known to occur in all the enterobacteria and Pseudomonads (Nikaido and Nakae, 1979) care should be taken in equating LPS of different genera.

Lipid A is generally a glycolipid with a D-glucosaminyl-BI-6-D-glucosamine backbone to which fatty acid (FA) residues are attached through amide and ester linkages (Nikaido and Nakae 1979).

The 'core' region is usually linked to lipid A via 2-keto-3-deoxy octonic acid (KDO) (Luderitz *et al.*, 1974); however, Galanose *et al.* (1977) and Maniello *et al.* (1979) found that LPS extracted from *P. cepacia*, although biologically active, contained no detectable KDO. Maniello *et al.* (1979) found that the core region of LPS extracted from *P. cepacia* contained phosphate, rhamnose, glucose, heptose and hexosamine in concentrations comparable to those found in *P. aeruginosa*, but *P. cepacia* had less phosphorus and more heptose.

The peripheral portion of LPS, the 'O' antigen, consists of oligosaccharide repeating units and shows extreme variability even within a single species (Munford *et al.*, 1980). For this reason the 'O' antigen has been used in serological typing especially in *Enterobacteriaceae* (Nikaido and Nakae, 1979).

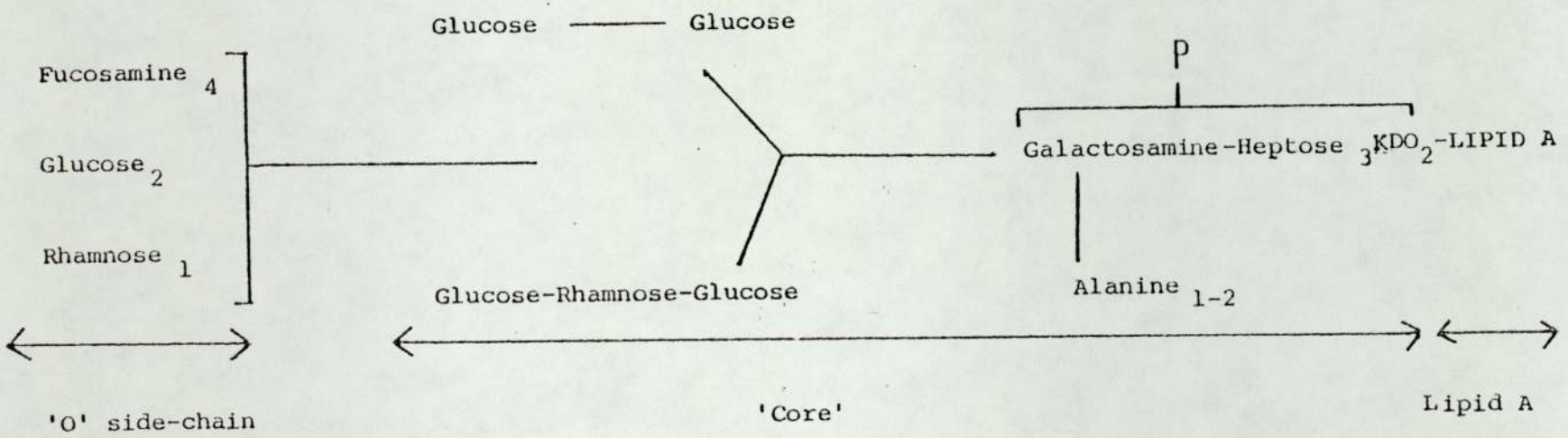


Fig.5 Structure of P. aeruginosa lipoplysaccharide

(Kropinski et al., 1979)

1.3.4.2 Phospholipids

pL are part of the lipid bilayer of the OM (Lugtenberg and van Alphen, 1983). As it has been calculated that the OM contains hardly enough pL to cover one monolayer (Smit et al., 1975), it seems likely that pL are mainly or completely located in the inner monolayer. The fact that, despite serious attempts, investigators have not succeeded in showing accessibility of pL to exogenous agents in intact cells of E. coli and of S. typhimurium, supports this idea (Lugtenberg and van Alphen 1983).

Little attention has been paid to the distribution of the pL between the two individual membranes (Lugtenberg and van Alphen, 1983). Osborn et al. (1972b) reported that the two membranes of S. typhimurium contain equal amounts of pL and the OM is enriched in phosphatidyl ethanolamine (pE) whereas the CM is enriched in the other two major species, phosphatidyl glycerol (PG) and diphosphatidyl glycerol (DPG). Enrichment of the OM in PE was also observed in P. cepacia (Anwar, 1981).

1.3.4.3 Proteins

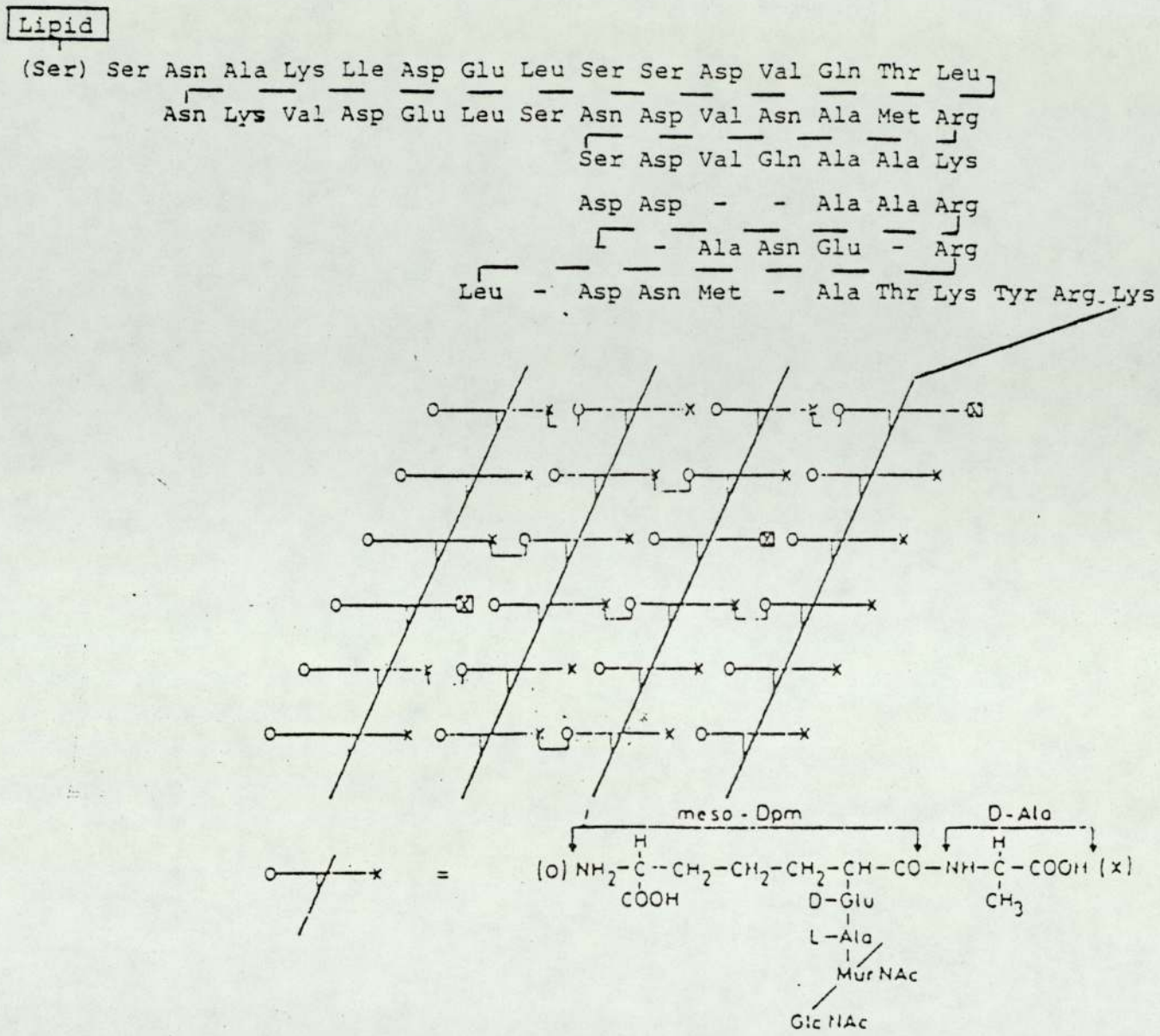
The identification of protein components is mainly dependent on separation of proteins in bands using SDS-polyacrylamide gel electrophoresis (Schnaitman, 1970). Compared with the present methods which give a high resolution (Lugtenberg et al., 1975, Pugsley and Schnaitman, 1979) the systems used earlier were rather poor.

Improved resolution has enabled a clearer identification of difficult-to-resolve protein components. Initially, OM protein

patterns obtained in various laboratories differed drastically, partly because different solubilization temperatures were used during the preparation of the sample and partly because different gel systems were applied.

1.3.4.3.1 Properties of proteins

Murein lipoprotein known also as Braun's lipoprotein, by whom it was discovered, has been characterised and reviewed in detail (Braun, 1975). It is one of the major OM proteins in Gram-negative bacteria. E. coli has been calculated to have approximately 250,000 lipoprotein molecules evenly distributed over a one-layered murein-net (Braun, 1975). Fig 6 shows the lipoprotein-peptidoglycan complex of E. coli. It has been found to have fifty eight amino-acid residues and is covalently bound to the carboxyl group in every tenth to twelfth diamino-pimelic acid residue of the peptidoglycan layer through the ε-NH_2 group of its C-terminal lysine residue (Braun, 1975). It was subsequently discovered that twice as many copies of exactly the same lipoprotein molecule exist in the free form, not covalenty attached to the peptidoglycan (Inouye et al., 1972). Both forms of the lipoprotein have been purified and the free form has been crystallized (Inouye et al., 1976). The free form is thought to be chemically identical with the bound form (Braun et al., 1975). It was speculated that the lipoprotein evolved from a 15 amino acid long peptide by gene multiplication and subsequent mutations (Inouye et al., 1976). The N-terminal cysteine residue is substituted both with a diglyceride moiety in a thioether linkage as well as with an amide-linked fatty acid.



⊠ Attachment sites of Lipoprotein replacing D-alanine

Fig 6 The lipoprotein-peptidoglycan complex of E. coli (Braun, 1973). The parallel heavy lines symbolize the polysaccharide chains. They are crossed-linked by the T-like peptide side chains which are drawn to allow a long-range covalent fixation of the peptidoglycan, which is a necessity in Gram-negative bacteria.

Heat-modifiable proteins

They were initially identified in E. coli and S. typhimurium as OM proteins with altered electrophoretic mobilities after heating in SDS prior to electrophoresis (Nakamura and Mizushima, 1976; Nikaido and Nakae, 1979). Anwar (1981) found 3 heat-modifiable proteins in P. cepacia. These proteins had molecular weight of 40,000, 36,000 and 24,000 daltons when they were solubilised at 100°C for 5 min. However, they moved with apparent molecular weights greater than 70,00 daltons when they were solublised at 60°C for 30 min. The addition of LPS to these heat-modifiable proteins in P. aeruginosa caused the modification of these proteins to be reversed except for protein F. It was suggested that protein F belongs to a different class of heat-modifiable proteins.

Peptidoglycan-associated proteins

Several OM proteins are designated as peptidoglycan-associated proteins, namely as proteins which following treatment of the cell envelope with SDS at temperatures below 70°C, are the only proteins which remain strongly but non-covalently bound to the peptidoglycan (Rosenbusch, 1974).

The successful resolution of protein HI and H2 of P. aeruginosa by Hancock and Cary (1979) has shown that they are in fact two quite distinct proteins; protein H2 is a peptidoglycan-associated protein unlike HI (Hancock et al., 1981). The proteins in the OM involved in the formation of hydrophilic pores are also peptidoglycan-associated (Nikaido, 1979). They exist in large numbers in the bacterial cell (Rosenbusch, 1974). Smit and Nikaido (1979) found that S. typhimurium contains three major porins of molecular weight 34K,

35K and 36K in the OM. They also found that newly inserted porins appeared as discrete patches uniformly distributed over the surface of the cell and that the sites of adhesion between the CM and the OM were most probably the pathway by which the newly made porin molecules appeared on the surface.

Protein F is the major pore-forming protein in P. aeruginosa (Benz and Hancock, 1981) and P. cepacia (Anwar, 1981).

Porins of E. coli solubilised by SDS exist as trimers (Palva and Randall, 1978) and also seem to exist as trimers in the OM (Nikaido, 1979). When the cell envelope of E. coli is extracted with SDS at room temperature, peptidoglycan sheets with hexagonally arranged oligomers of porin are left behind (Rosenbusch, 1974). As a result of this regular arrangement Steven et al.(1977) were able to use image-enhancement techniques in electron microscopy. The porins were clearly seen to exist as trimers, and a triplet of holes is produced by each monomeric unit.

Ueki et al.(1979) examined the small-angle X-ray scattering produced by oriented layers of the intact OM of S. typhimurium. A most interesting feature was the presence of a central electron-transport area of a "hole" of 5-6nm in diameter. This could correspond to a single channel in the centre of the porin trimer, but it is also possible that the triplet of holes observed by Steven et al.(1977) appeared as in one large hole in this study.

1.3.4.3.2. Functions of proteins

A possible role for lipoprotein in the cell envelope has been

elucidated by use of an E. coli mutant having an altered peptidoglycan-lipoprotein structure (Yem and Wu, 1978). The mutant had an altered OM and increased sensitivity to chelating agents, and it leaked periplasmic enzymes. The authors concluded that lipoprotein plays an important role in the maintenance of the rod shape, in stabilization of the OM structure and in anchoring the OM to the peptidoglycan. Additional evidence for the latter function comes from recent experiments of Wensink and Witholt (1981) who showed the OM vesicles released by growing E. coli cells contain only a small amount of free lipoprotein, hardly any bound lipoprotein and reduced amounts of OMP A. The vesicles also contained reduced amounts of protein V, a protein assumed to be identical to a newly discovered lipoprotein (Wensink and Witholt, 1981).

The most important conclusions from the work done on the functioning of pores can be summarized as follows, the simplest interpretation of a pore is a non specific molecular-sieving channel through which hydrophilic solutes diffuse. The diffusion rate is determined by the difference in concentration at the two sides of the OM and it is influenced by factors like size, charge and hydrophobicity, resulting in large differences in permeability coefficients among solutes (Benz and Hancock 1981).

Early studies using oligosaccharides and plasmolysed cells (Decad and Nikaido, 1976) with S. typhimurium, E. coli and P. aeruginosa (Hancock et al., 1979) showed that the pore size probably varies with organisms. P. aeruginosa was shown to allow the passage of molecules up to 9000 daltons while E. coli and S. typhimurium allow only molecules of less than 600 daltons. However, P. aeruginosa is more

resistant and less permeable to antibiotics in general than many other Gram-negative bacteria (Brown, 1975). The relatively large pores have been explained on the basis of a high proportion of non-functional porin protein (Benz and Hancock, 1981). Recent data reported by Caulcott et al. (1984) suggested that P. aeruginosa has a far lower exclusion limit than previously reported.

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

1.3.5 External layers and structures

Some Gram-negative bacteria have a protein layer attached externally to the LPS layer (Thornley et al., 1974). It has been shown that this protein layer can be removed from the external layer without affecting bacterial survival. Many Gram-negative bacteria have a loosely associated capsule or slimelayer composed of carbohydrate. The open fibrillar structure of capsular materials does not represent a simple penetrating barrier to antibiotics, but the fact that they consist of predominantly anionic sugar molecules (Brown, 1975) bound in long, insouble polymers can cause extensive adsorbtion of antibiotic molecules. Thus heavily encapsulated cells of P. aeruginosa are more resistant to polymyxin (Brown, 1975), gentamicin and carbenicillin than are non-mucoid cells.

1.3.6 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) and regulating the activity of several enzyme systems. The role of magnesium in the maintenance of the stability as well as permeability of the membrane, especially the OM, has been observed by treating cells with EDTA, which chelates magnesium in the OM. It may then cause damage to the membrane and hence induces change in the permeability of the membrane (Leive, 1965).

1.4. Effect of freezing and thawing on microbial cells

1.4.1. Introduction

Although microbial cryosurvival has been studied extensively, generalization and comparison between studies has been difficult to make, because of the diversity of techniques and conditions used and the lack of information presented in the studies. The inconsistencies in the earlier literature have been attributed by Mazur (1966) to many factors such as variation in strain, type of organism and cooling and warming rates.

1.4.2. Nature of freezing damage

When bacterial populations are frozen and thawed, the bacteria exhibit many types of damage including loss of viability (Calcott and Macleod, 1974 ; Macleod and Calcott, 1976), outer and cytoplasmic membrane damage (Calcott and Macleod, 1975; Beuchat, 1978) and deoxyribonucleic acid damage (Swartz, 1971; Calcott and Garget, 1981).

Freezing damage to the OM and CM of *E. coli* has been extensively studied. Part of the injury sustained is assumed to be the result of

structural changes of membrane lipid occurring at subzero temperature (Souzu, 1980). Freeze-thawing of E. coli caused a release of cell membrane components such as proteins pL and LPS (Souzu, 1980). Conclusions regarding membrane damage arise from the finding that if microbes are frozen and thawed, leakage of materials occurs from the cells. In several cases a correlation has been reported between the extent of loss of internal solutes and percentage survival of the cells (Lindeberg and Lode, 1963). Most of the lost materials are low molecular weight materials including K^+, inorganic phosphate, phosphorylated sugars, sugars, fatty acids, esters and amino compounds (Sakagami, 1959).

Other evidence of membrane damage arises from the increased penetrability of certain compounds into cells after freezing and thawing. Strange and Postgate (1964) showed that a molecule as large as RNase can enter A. aerogenes after freezing. E. coli became more sensitive to the toxic action of Cu^{2+} (Macleod et al., 1967). Kohn (1960) showed that if E. coli was frozen to -80°C and thawed the cells could be converted to spheroplasts by treatment by lysozyme. This indicated that freezing and thawing increased the penetrability of the outer cell wall membrane of this bacterium to lysozyme, permitting access of the lysozyme to the underlying peptidoglycan layer. Ray et al. (1972) confirmed these findings using S. typhimurium; E. coli cells exposed to freezing-thawing were sensitised to several antibiotics such as vancomycin, novobiocin and bacitracin (Mackey, 1983). It was suggested that freezing-thawing increased the penetrability of the OM of this bacterium to these antibiotics. Morichi (1969) observed that freezing and thawing of E. coli caused the release of the periplasmic enzyme,

cyclicphosphodiesterase, which was released almost quantitatively from the cells of another strain of E. coli that had been frozen and thawed (Calcott and Macleod, 1975). The release of periplasmic enzymes from the cells provides further evidence that freezing damage leads to an increase in the penetrability of the OM of this organism.

A study has been made of the effect of cooling and warming rates during freezing and thawing on CM damage in E. coli (Calcott and Macleod, 1975). The effect was found to vary depending on whether the cells were frozen in water or 0.85% NaCl. When cells were frozen in water there was no release of U.V.-absorbing material from the cells at cooling rates below the optimum for survival in the low cooling rates range (6°C min^{-1}). Above the optimum cooling rate for survival the release of U.V.-absorbing material was inversely proportional to the survival of the population. For cells frozen in saline, death can be related to CM damage even at cooling rates below the optimum for survival in the low cooling rate range.

1.4.3 Mutagenicity of freezing and thawing

The possibility that DNA might be damaged by freezing and thawing has been proposed by a number of workers. (Calcott and Thomas, 1981, Calcott and Gargett, 1981). Single-stranded and double-stranded breaks have been detected in the macromolecule following freeze-thawing and frozen storage (Alur and Greeze, 1975). These may result in mutations (Postgate and Hunter, 1961; Calcott and Gargett, 1981). The latter have also shown that the damage of DNA which happened during freezing-thawing can be repaired by the post replication repair mechanism. Calcott and Gargett (1981) reinforced the idea that freezing-thawing is a mutagenic process by examining

the effect of frozen storage at -20°C and -80°C on the production of mutants in E. coli. Storage of rapidly or slowly frozen preparations at both temperatures for up to 14 days did not increase the frequency of mutants in the populations above that of frozen and immediately thawed preparations, although further cells died, indicating that the mutational event was not due to the time spent frozen but more likely that the events of freezing and thawing were responsible.

1.4.4 Consideration of factors contributing to death by freezing

During freezing and thawing, a cell is exposed to alteration in temperature, water content, state of water and solute concentration. The first event to occur is ice formation in the extracellular medium; the water within the cells remains initially supercooled and because of its high vapour pressure begins to flow out of the cell and freeze extracellularly (Mazur and Schmidt, 1968). If the cooling rate is slow, the cell will dehydrate sufficiently to remain in vapour pressure equilibrium with the external ice and will therefore be incapable of freezing internally, but if cooling is rapid, a cell will become increasingly supercooled, eventually freezing internally (Mazur and Schmidt, 1968). A number of electron microscope studies support the conclusion that only at cooling rates greater than the optimum for survival does ice form intracellularly (Mazur, 1967; Bank and Mazur, 1973). Mazur thus concluded that death by freezing at cooling rates greater than the optimum was due to intracellular ice formation. Death by freezing at cooling rates below the optimum, since it occurred in cells which had become dehydrated before ice could form internally, was believed to be due to slowly concentrating solutes acting on the cells both

internally and externally during the freezing process. Failure of cells to die when cooling and warming rates were ultra-rapid was attributed to the formation of small and hence non-lethal ice crystals.

1.4.5 Cryoprotectants

It has been known for many years that the presence of certain compounds in aqueous suspensions of microbial cells can drastically alter the extent of survival on freezing and thawing. These compounds serve as cryoprotectants. The two groups of cryoprotectants can be distinguished, those which are penetrating, such as glycerol and dimethyl sulphoxide and those which are non-penetrating (Meryman, 1971). Among the latter are sucrose, Tween 80 and polyvinyl-pyrrolidone. These cryoprotectants modify the pattern of response to different rates of cooling and warming (Macleod and Calcott, 1976). It has been proposed that they protect primarily against solution effects. They might do this by reduction in the electrolyte concentration in the residual unfrozen solution in and around a cell at any given concentration (Mazur, 1970). Calcott and Thomas (1981) found recently that *E. coli* can be protected from slow freezing and thawing by using ionophores such as gramicidin or valinomycin. In combination, gramicidin and valinomycin were detrimental to cryosurvival.

1.4.6 Thawing

The rate of thawing is not important except after very rapid freezing, when rapid thawing results in greater survival than slow thawing. One explanation of this is that, during slow thawing, the very small intracellular ice crystals formed as a result of rapid freezing grow in size and doing so cause greater damage to the

cell. Rapid thawing prevents this crystal growth and also minimizes the time spent in contact with concentrated solutes.

After thawing, the interval before plating may be important. Postgate and Hunter (1963) found with A. aerogenes that fewer survivors were recovered when the period of plating after thawing was increased. Ray and Speck (1973) reported that plate counts on non-selective medium were reduced by 40% within 2hr after thawing cells of S. anatum in water, but not with E. coli.

1.4.7 Time/temperature relations during frozen storage

After freezing, death occasionally follows a roughly exponential course with the passage of time, at constant temperature (Kiser, 1943). More usually, death is initially more rapid, but gradually becomes relatively slow until in the later stages viable numbers remain almost constant. The progressive decline in viability is thought to be due mainly to continued exposure to concentrated solutes, and so might present an extension of the processes responsible for immediate death on freezing (at low freezing rates). The nature of the solutes may however change with time, owing to differential precipitation (Van den Berg, 1968). Alternatively, the change in size and distribution of the ice crystals, known to occur through grain growth (Luyet, 1966; Kent, 1975) might be important.

Generally, the lower the storage temperature, the lower the rates of inactivation during storage. In particular, death at -2 to -5° is often much more rapid than at -10°C to -20°C. Mazur (1966) notes various exceptions to this generalization. Evidence obtained with a number of species of bacteria shows that at storage temperatures of below -60°, death rates are low or zero (Mazur, 1966).

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Organism

The organism used throughout the study was Pseudomonas cepacia NCTC 10661. It was maintained on nutrient agar slopes. Fresh cultures were prepared from single colony isolates at intervals of one month. These were kept at 4°C. Identification of the organism was done using API 20E test.

2.1.2 Complex media

Nutrient agar (Oxoid CM_3) and nutrient broth (Oxoid CM_1) were from Oxoid Ltd, London SE1 9HF.

Letheen agar and Letheen broth were from Difco Laboratories, Detroit, Michigan, U.S.A.

2.1.3 Chemicals

The water used in preparing chemically-defined medium (CDM) was double distilled in a glass still. All chemicals used in preparing CDM were of Analar grade and were obtained from British Drug Houses, Chemicals Ltd, Poole, Dorset.

Other chemicals obtained as follows:- MOPS (3-N-morpholino, propane sulphonic acid) and dimethyl sulphoxide, Sigma Chemical Company, London.

Methanol (SLR) and glycerol (Analar), British Drug Houses.

Benzalkonium chloride B.P. (BKC) was supplied as 50% w/v, Lot. M5146, Macarthy's and stored in screw-capped bottles. Dilutions were prepared in distilled water and autoclaved.

Samples of BKC of individual chain lengths were a gift from Dr P Gilbert, University of Manchester. These were stored in a desiccator over P_2O_5.

Tris (hydroxy methyl) aminomethane (Tris), Sigma.

Acrylamide, British Drug Houses.

N,N - methylene;bisacrylamide (Bis), Sigma.

Ammonium persulphate, British Drug Houses.

2-mercaptoethanol, Sigma.

N,N,N^1,N^1 - Tetra methylethylene diamine (TEMED), British Drug Houses.

Coomassie brilliant blue R-250, Sigma.

Sucrose:- Analar grade, British Drug Houses.

Lysozyme:- Crystalline, from egg white, British Drug Houses.

Ribonuclease and Deoxyribonuclease, Sigma.

Gas-liquid chromatography fatty acid standard Lot No-4-5436: Supelco Inc. Bellefonte, Pennsylvania, U.S.A.

2.1.4 Sterilisation of media

All complex media were sterilised by autoclaving at 121°C for 20 minutes. Chemically defined media were also sterilised by autoclaving, except for phosphates which were sterilised by filtration through millipore membranes (pore size 0.2 μm) (Millipore UK Ltd, Wembley, Middx).

2.1.5 Apparatus

Spectrophotometer:- Unicam S.p 600. Pye Unicam Instruments Ltd, Cambridge.

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

Water bath:- Mickle reciprocating shaker bath, Camlab Ltd, Cambridge, England.

Orbital Incubator:- Gallenkamp, England.

Iron constantan thermocouple:- obtained from Physics Department, Aston University.

Chart Recorder:- Bryans, Southern Instruments, Ltd.

2ml plastic ampoules, Jencons Scientific Ltd, Leighton Buzzard, Beds.

Liquid nitrogen container and biological freezer (ampoule holder), Jencons Scientific Ltd.

Automatic M.L.A pipettes with 0.02 ml and 0.25 ml tips (sterilised by autoclaving):- Frost Instrument, Ltd, Wokingham, Berks.

Whirlimixer:- Fisons Scientific Apparatus, Ltd, Loughborough.

Glassware:- Corning Glass Ltd (Pyrex), Laboratory Division. Sunderland.

Universal Temperature Measuring Instrument:-

KG Dr. Ing, Herbet Knauer, 1 Berlin 37 (West), Holstweg 18.

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

Centrifuges:- 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.

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

French Press:- Model j4-3398A, Aminco, American Instruments Company., Inc., Silver Spring, Maryland, U.S.A.

2.1.6 Preparation of glassware used with CDM

Pyrex glassware was totally immersed in 5% Extran 300 (British Drug Houses, Poole, Dorset) for 18 hours. It was rinsed once in distilled water, once in 1% v/v hydrochloric acid, then six times in distilled water and finally twice in double distilled water. After rinsing, the glassware was dried at 60°C then covered with aluminium foil and sterilised by dry heat (160°C for 3 hours).

2.2 Experimental methods

2.2.1 Preparation of inocula for experiments

Prior to an experiment, a sample from an agar slope was inoculated into a complete chemically defined medium (CDM) (Table 1) and incubated overnight in an orbital shaker at 140 oscillations min^{-1} at 37°C. The culture was harvested by centrifugation at 8000g for 10 min and resuspended in the appropriate CDM.

2.2.2 Measurement of bacterial cell concentration

The concentration of a bacterial suspension is usually determined by optical density measurement with a spectrophotometer or by total count with a microscope. The photometric determination of bacterial concentration depends primarily on light scattering rather than light absorbtion. At relatively low concentrations, the light scattered by the bacterial cells is directly proportional to the concentration of the cells in the suspension. A useful relationship similar to the

Table 1

Chemically defined media used for the study of colony counts and nutrient requirements of P. cepacia.

Ingredients (molar)	Nutrient depletion	
	Carbon	K^+
Glucose	*	0.04
NaCl	0.003	0.003
$MgSO_4.7H_2O$	0.002	0.002
$(NH_4)_2SO_4$	0.024	0.024
K_2HPO_4	0.003	-
$FeSO_4.7H_2O$	0.0003	0.0003
MOPS	0.05	0.05
KCl	0.0002	*
Na_2HPO_4	-	0.003

Media pH = 7.4

* Data as shown in the growth experiments.

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 = Extinction coefficient

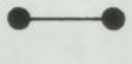
This relation holds at low concentrations. However, at high concentrations of bacteria it is no longer valid. It has been suggested that this is due to secondary scattering of the light by the cells (Meynell and Meynell, 1976). Hence it is necessary to determine the optical density (OD) at which the Beer-Lambert law is still applicable. An overnight culture in CDM was resuspended in CDM lacking glucose. From it a series of dilutions of the bacterial suspension were prepared using distilled water as diluent. The OD was measured. These suspensions were diluted a further 10 times, the OD measured and then multiplied by the dilution.

From the two plots (Fig 7.), it can be seen that the relation between cell concentration and OD complies with the Beer-Lambert law up to OD 0.3. Above that OD, suspensions have to be diluted before measuring OD.

2.2.3 Colony count

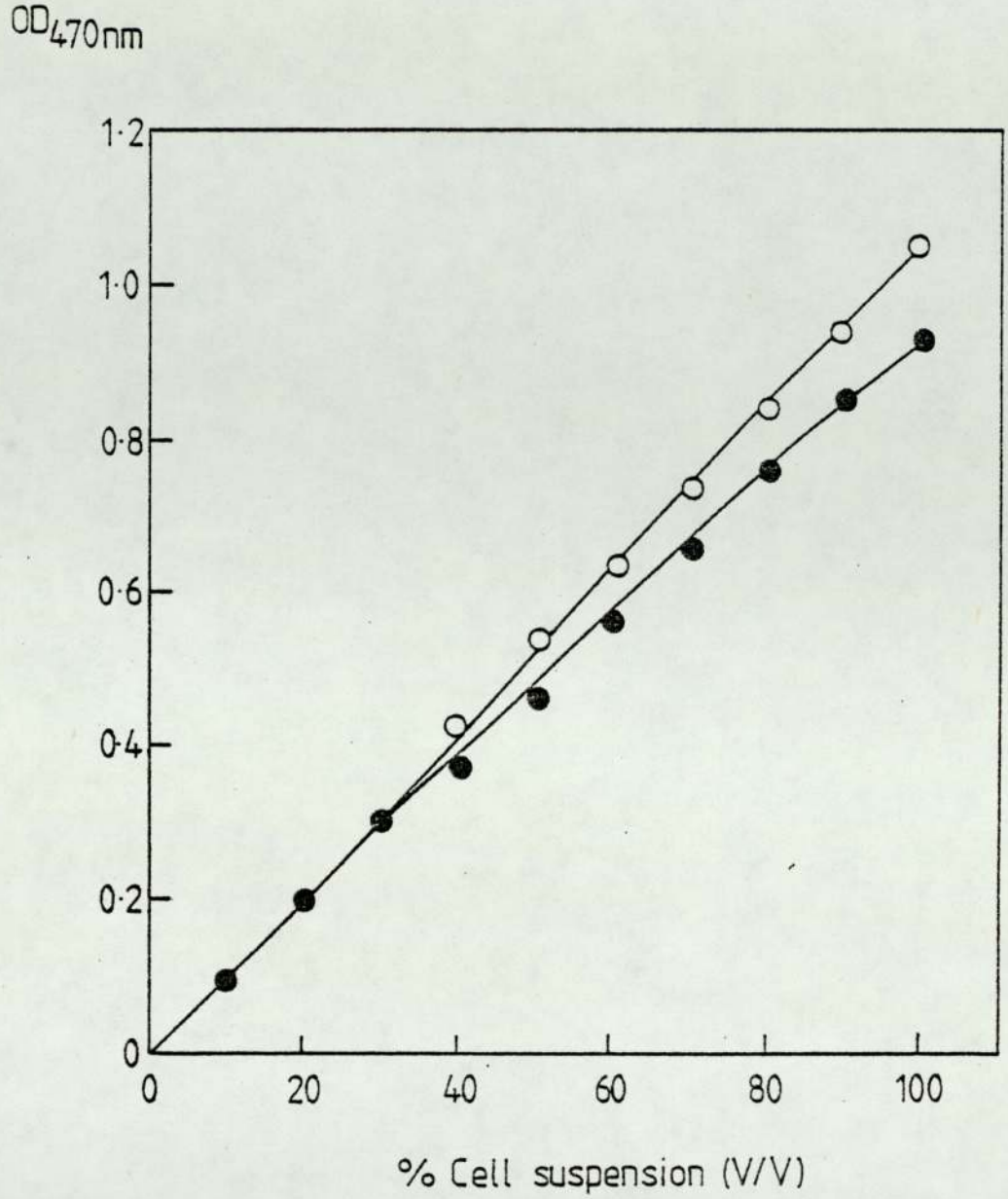
Viable counts of cultures were made by either the spread plate method (Crone, 1948; Roberts, 1961) or by the drop method (Miles and Misra, 1938). To check the accuracy of the technique P. cepacia was grown in a complete chemically defined medium (CDM) (Table 1) to OD470nm≈

Fig 7. **The relation between optical density and cell concentration for P.Cepacia.**



Undiluted suspension

Suspension diluted and the result multiplied appropriately


OD470nm
1·2
1·0
0·8
0·6
0·4
0·2
0
0
20
40
60
80
100
% Cell suspension (V/V)

0.2. Five sets of dilutions were made in nutrient broth (NB) at room temperature. From each of the five final dilutions, five aliquots of 0.1 ml were spread on over-dried nutrient agar plates. The plates were incubated at 37°C for about 30 hours (hr) and the resulting colonies counted. Results are shown in Table 2.

In the Miles and Misra method drops of 20µl of diluted culture were deposited on overdried nutrient agar plates. One plate had up to eight drops consisting of four dilutions. After incubation for 18 hr at 37°, counts were made for every drop. Results are shown in Table 3.

Table 2

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

	Counts				
	A	B	C	D	E
Replicate plates					
1	100	107	98	99	109
2	104	109	102	103	112
3	97	95	100	104	99
4	98	104	99	106	99
5	102	102	104	111	95
Mean	99.75	103.75	99.75	103	103
Standard deviation		4.4	5.1	2.4	2.9	4.9
Coeff. of variation		4.4%	4.9%	2.4%	2.8%	4.8%
Mean of population				101	
Standard deviation				2.1	
Coeff. of variation				2%	

Table 3

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

	Counts			
Drops	A	B	C	D
1	20	17	18	24
2	18	23	22	22
3	17	24	17	19
4	20	20	20	17
5	19	21	23	18
Mean	18.7	19.57	19.68	19.7
Standard deviation		1.2	2.1	2.1	2.3
Coeff. of variation		6.4%	10.7%	11.1%	11.8%
Mean of population				19.7
Standard deviation				2.2
Coeff. of variation				11.3%

2.2.4 Determination of growth requirements

Flasks of CDM (see Table 1) (25 ml in 100 ml flasks) containing different glucose or K^+ concentrations were inoculated with 0.1 ml of an overnight culture as described in Section 2.2.1. Flasks were incubated in a shaking water bath (120 throws min^{-1}) at 37°C and optical densities measured every 1/2 hr.

2.2.5 Measurement of osmolarity

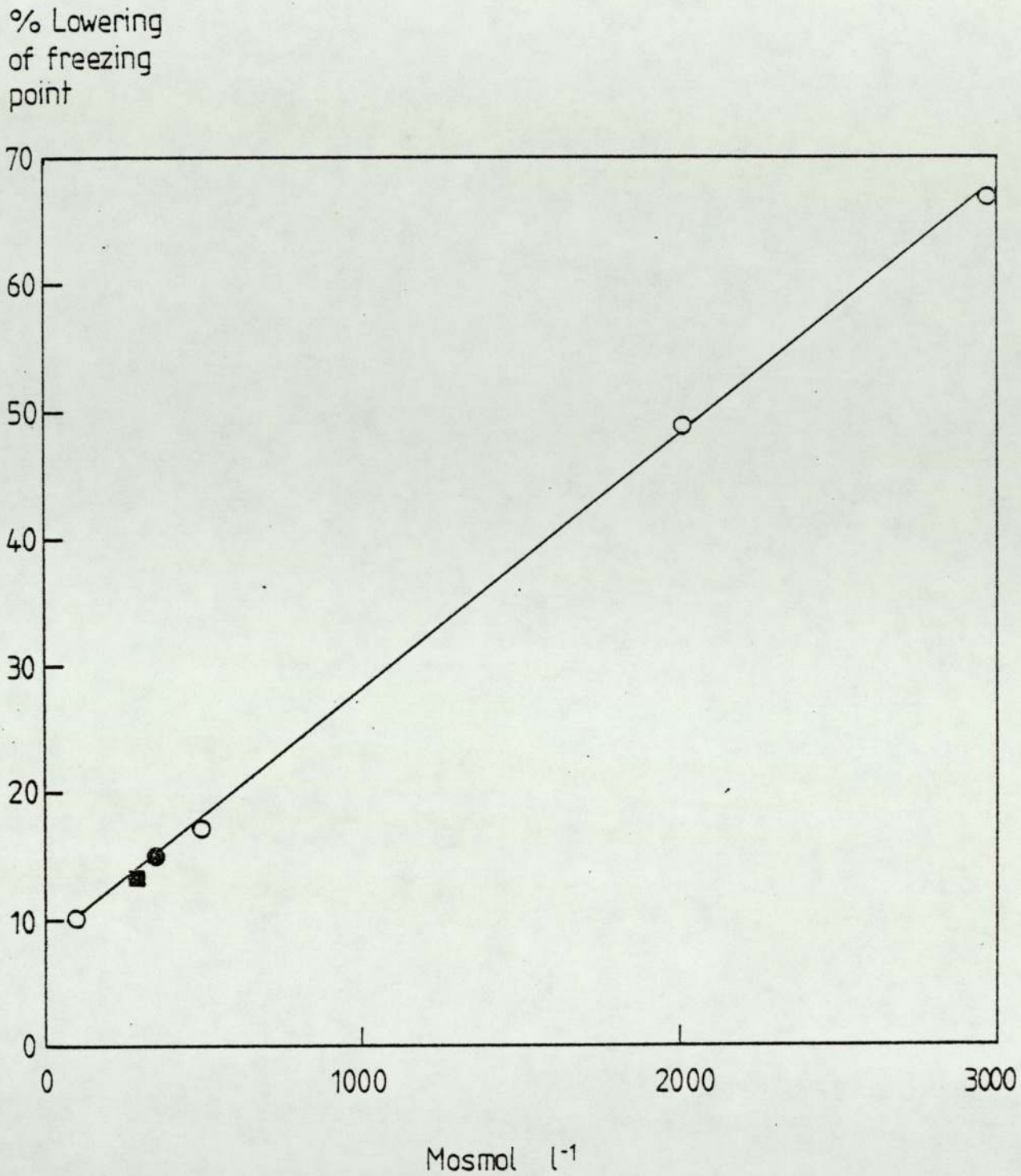
The osmolarity of the medium was determined from the depression of the freezing point compared with standard concentrations of NaCl, using a Universal Temperature Measuring Instrument. Results are shown in Fig 8.

Fig 8. The determination of the osmolarity of the chemically - defined medium .

○ Standard solutions

● Chemically-defined medium

■ 0.85% saline


% Lowering of freezing point
70
60
50
40
30
20
10
0
0
1000
2000
3000
Mosmol l-1

2.2.6 To determine the effect of nutrient depletion on the survival of *P. cepacia* in benzalkonium chloride.

The basic CDM used is shown in Table 4. The concentration of limiting nutrients was such that exponential growth ceased at an OD_{470nm} of approximately 1.0 (glucose) or 0.5 for (Mg^{2+}, Po_4^{3-}, NH_4^+, Fe^{2+} and K^+). To obtain depleted cells, inocula were prepared as described in Section 2.2.1 and resuspended at an OD of 1.0. 0.25 ml was added to 24.75 ml appropriate CDM in 100 ml flask and the growth followed by OD measurements until an OD of 1.0 was reached. To ensure that all cells tested had been stationary for the same period of time cells were harvested by centrifugation, resuspended in CDM to OD 1.0 lacking the nutrient under study and incubated for 60 min at 37°C. They were then diluted with distilled water or glycerol 5% to an OD_{470} of 0.2 ≈ $2x10^8$ cells ml^{-1}. 0.1 ml diluted cell suspension was inoculated in 9.9 ml BKC prewarmed at 37° (final cell conc ≈ $2x10^6$ cell ml^{-1}) and left for 15 min at 37°C. The action of BKC was stopped immediately by diluting the cells in letheen broth. Triplicate samples from the BkC mixture were diluted in letheen broth. The control used was peptone water inoculated in the same way. Viable counts were done using the drop method on letheen agar plates. Each plate received 5-7 drops. Plates were incubated at 37°C over-night.

2.2.7 Methods for obtaining differing cooling rates.

In order to obtain a variety of cooling rates, 2 ml plastic ampoules containing 0.1 ml of cell suspension, were held in various positions in the N_2 vapour. The rate of cooling depended on the distance of the ampoules from the liquid N_2 and from the external atmosphere, and also upon the number of ampoules. The level of liquid N_2 was always

Table 4 Chemically defined media used for the study of the effect of nutrient depletion on the sensitivity of *P. cepacia* to the BKC and further study.

CDM 20

Ingredients (molar)	Glucose	Nutrient depletion under study NH_4^+	PO_4^{3-}	Mg^{2+}	Fe^{2+}	K^+
D-Glucose	0.005	0.1	0.1	0.1	0.1	0.1
NaCl	0.08	0.08	0.08	0.08	0.08	0.08
$MgSO_4.7H_2O$	2.6×10^{-4}	2.6×10^{-4}	2.6×10^{-4}	6.2×10^{-6}	2.6×10^{-4}	2.6×10^{-4}
$(NH_4)_2SO_4$	3.6×10^{-4}	8.6×10^{-6}	3.6×10^{-4}	3.6×10^{-4}	3.6×10^{-4}	3.6×10^{-4}
Na_2HPO_4	0.006	0.006	1.5×10^{-4}	0.006	0.006	0.006
MOPS	0.05	0.05	0.05	0.05	0.05	0.05
KCl	2×10^{-5}	2×10^{-5}	2×10^{-5}	2×10^{-5}	2×10^{-5}	5×10^{-7}
$FeSO_4.7H_2O$	6×10^{-6}	6×10^{-6}	6×10^{-6}	6×10^{-6}	1.4×10^{-7}	6×10^{-6}

pH Media = 7.4

checked by its relation to the storage cans in the container, being kept approximately 1" below the top of the cans. Two devices were used. The ampoule holder supplied by Jencons Scientific Ltd (Fig. 9) was inserted into the stopper of the liquid N_2 container (Fig. 10) and kept at various distances above the liquid N_2 by an adjustable rubber ring, or used without the ring. To obtain faster cooling rates, a holder devised by Mr R Tilling (Dept. of Pharmacy, Aston University) was used to lower the ampoules nearer the N_2 (Fig. 11), in 3 different positions, A, B & C (Fig. 12). In an alternative method for fast cooling rates the ampoule was held in the top position of a storage cane (Fig. 13) suspended beneath the Jencon holder (see table 5). In addition, ultrarapid cooling was obtained by dropping the cells directly into liquid N_2 in an ampoule. This was held by means of a cane in a small vacuum flask containing liquid N_2 (Fig. 14).

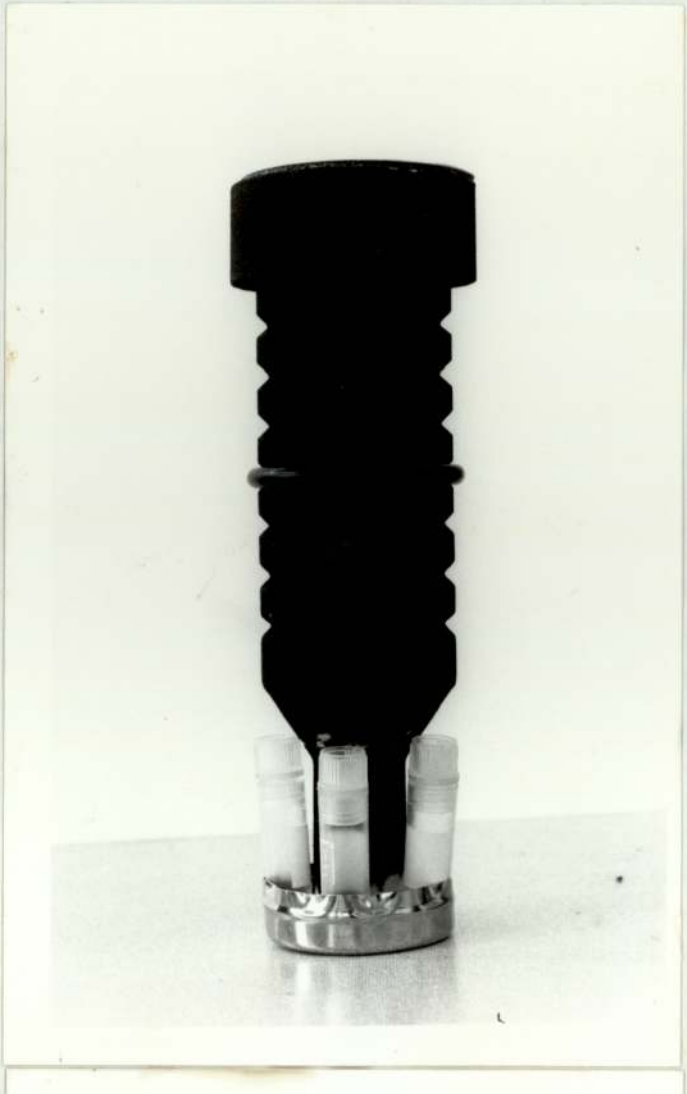
2.2.8. Measurement of cooling rates.

To measure cooling rates (except ultrarapid cooling) a copper constantan thermocouple was used with chart recorder and speed adjusted as necessary.

Typical cooling curves are shown in Fig 15. Table 5 shows cooling rates calculated from tangents to the curves over the ranges 25°C to 0°C, 0°C to -72°C and 25°C -72°C. As most damage to cells probably occurs between 0°C and -72°C (Mazur and Schmidt, 1968; Souzu 1980), the rates quoted in all experiments refer to this range. All results in Table 5 are the mean of two experiments.

Fig. 9. Ampoule holder supplied by jencons scientific Ltd.

Fig. 10. Liquid nitrogen container.



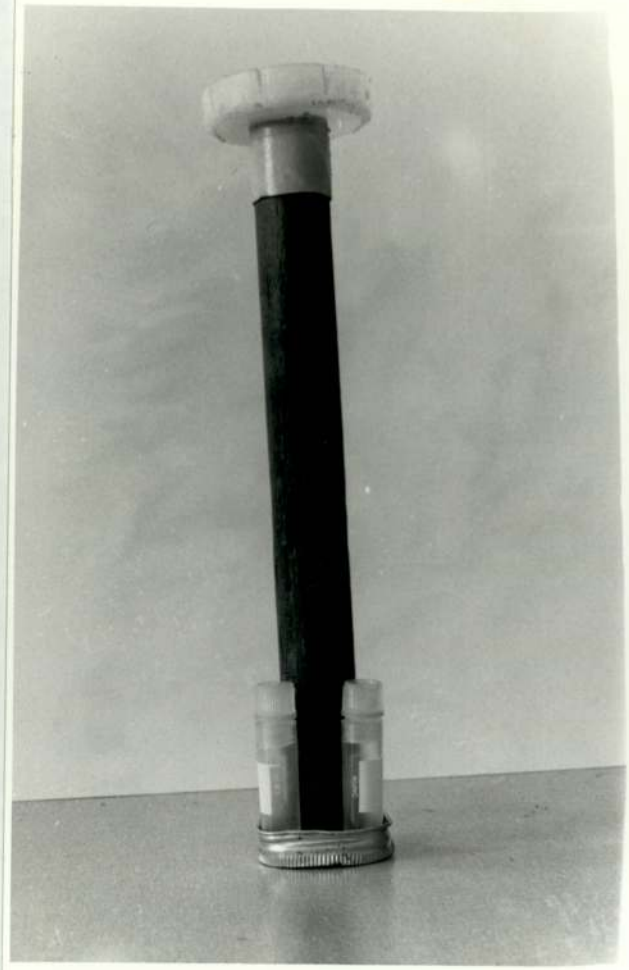

LIQUID NITROGEN
REFRIGERATOR

Fig. 11. Tilling holder.

This holder is inserted into the stopper of the liquid N_2 container, in place of Jencon's holder.

Fig. 12. Different positions of Tilling holder.

The stopper was inserted into the liquid N_2 container in three different positions, A, B and C (fully inserted).




CT
B
A

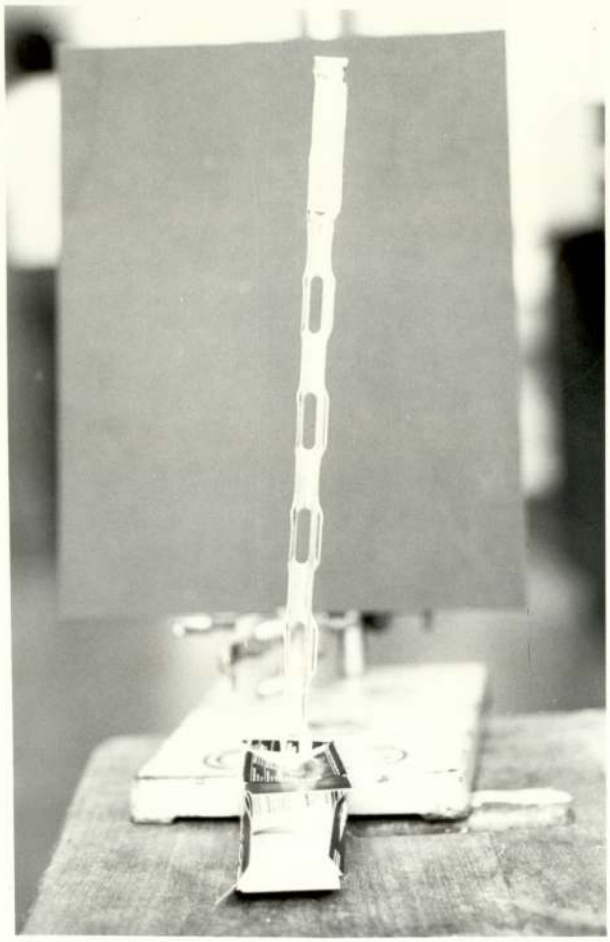
Fig. 13. Storage cane with ampoule in the top position

Fig. 14. Vacuum flask designed for obtaining rapid cooling rates.

Approximate dimensions of flask

external 60 x 100 mm

internal 40 x 75 mm



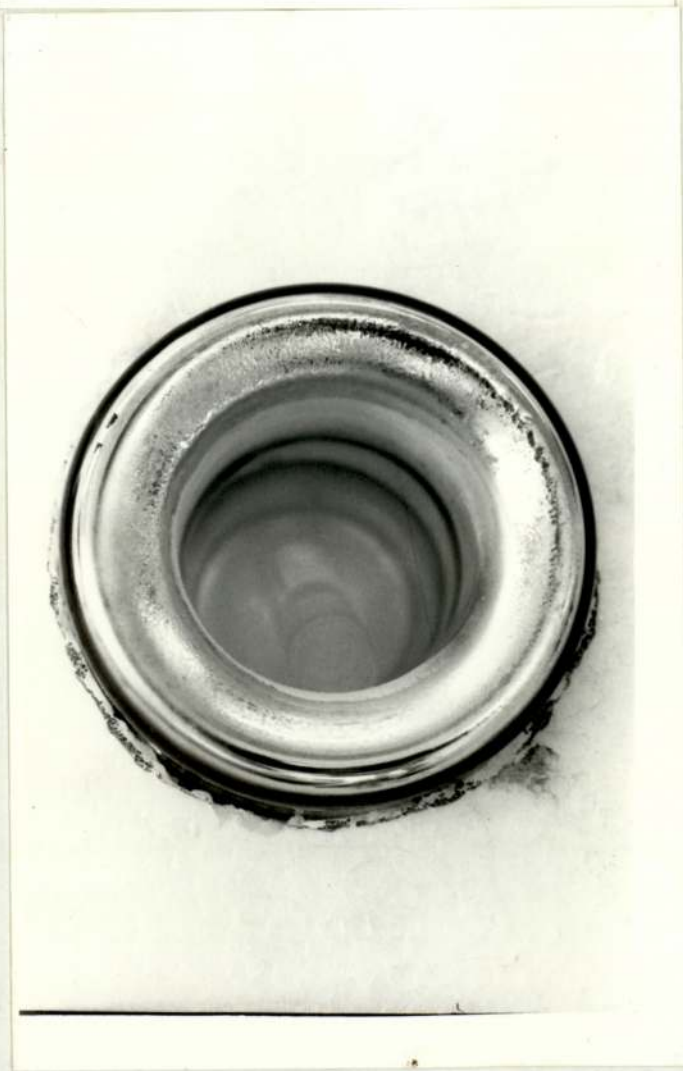


Table 5 Cooling rates measured by thermocouple and chart recorder

Ampoule holder	Ampoules		Cooling rates		
	position	number	25-0 ($°min^{-1}$)	0- -72 ($°min^{-1}$)	25- -72 ($°min^{-1}$)
Jencons	D	5	3.1	0.7	0.9
"	C	7	3.5	1.5	1.8
"	A	9	6.2	6	6
"	A	4	8	9	7.5
"	A	2	8	10	9.7
"	without rubber ring	1	12.2	14.5	14
"	Top position of the storage cane suspended below the holder	2	125	48	57
"	"	1	250	72	97
Tilling	A	2	125	37.8	48.5
	B	2	125	48	57
	C	2	250	144	197

Fig 15. Typical cooling rates measured by thermocouple and chart recorder.

	1	2	3	4
No of ampoules	7	9	2	2
Ampoule holder	Manufacture	Manufacture	Tilling	Tilling
Position	G	A	B	C
Chart speed	1mm min^{-1}	1mm min^{-1}	10mm min^{-1}	10mm min^{-1}
Cooling rate:-				
25^{o}-0°	3.5° min^{-1}	6.2° min^{-1}	125° min^{-1}	250° min^{-1}
0° - -72°	1.5° min^{-1}	6° min^{-1}	48° min^{-1}	144° min^{-1}
25° - -72°	1.8° min^{-1}	6° min^{-1}	57° min^{-1}	197° min^{-1}

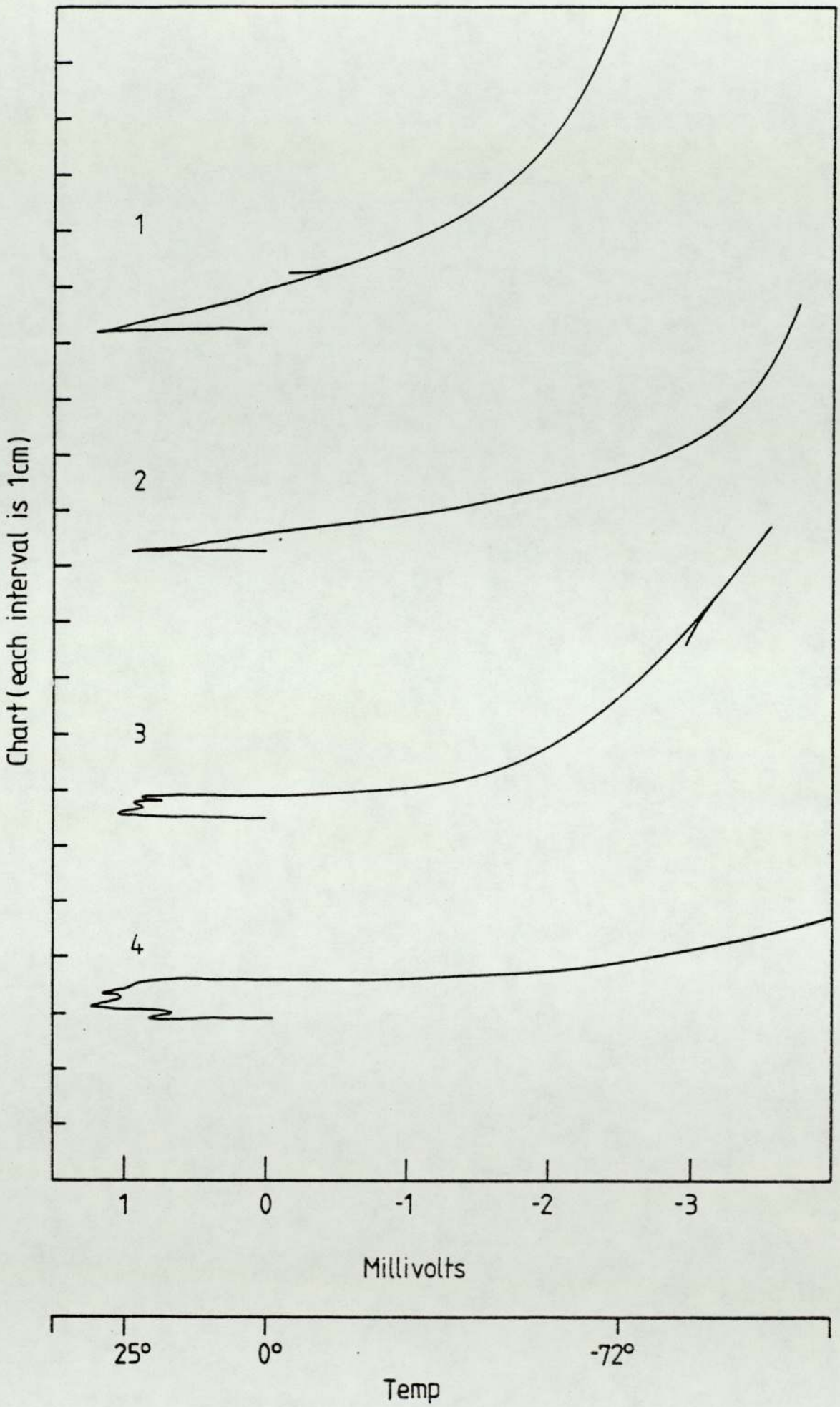


Chart (each interval is 1cm)
1
2
3
4
1
0
-1
-2
-3
Millivolts
25°
0°
-72°
Temp

2.2.9 The effect of freeze-thawing on survival

Cells were prepared as described in section 2.2.6 except that after harvesting they were resuspended to OD 8.0. They were mixed with an equal volume of cryoprotectant and 0.1 ml was put in each of the required number of 2 ml plastic ampoules. These were frozen at the cooling rate wanted and left overnight in liquid N_2. Next day cells were rewarmed rapidly by plunging the ampoules into a water bath at 37°C and immediately adding 1.9 ml distilled water or 0.85% saline prewarmed to 37°. Cells were counted in initial experiments on letheen agar plates and nutrient agar plates for comparison. No difference was found. Subsequent counts were made on letheen agar only. Control counts were done before freezing by adding 1.9 ml distilled water or saline and counting.

2.2.10 Effect of freezing on surival of *P. cepacia* in benzalkonium chloride

To test the effect of BKC on freeze-thawed cells, Mg^{2+}, PO_4^{3-} and carbon-depleted cells were prepared as in Section 2.2.9 and frozen at a rate of 6° min^{-1}.

After freezing two controls were done, one as soon as the frozen cells had been rewarmed and the other after 15 min at 37°C for a comparison with cells treated with BKC.

2.2.11 Growth of 8 litre batch cultures for preparation of cell envelopes.

The composition of the media are shown in Table 4. The components of the media, except glucose, were autoclaved at 121°C for 30 min. At the time of inoculation sterile glucose was added. The final volume was 4 x 2 litres in 4 litre flasks. An overnight glucose-depleted culture was used as inoculum. The flasks were incubated in the orbital shaker incubator at 37°C overnight. The maximum OD reached by glucose-depleted cultures was 1.0, Mg^{2+} - depleted cultures was 1.1 and PO_4^{3-} – depleted cultures was 1.0.

2.2.12 Preparation of the cell envelopes for the separation of the outer and cytoplasmic membrane (Sucrose density gradient centrifugation).

The method used was a modification of one described by Hancock and Nikaido (1978).

Eight litres of cells grown as described in Section 2.2.11 were harvested by centrifugation at 8,000 g for 10 min. This and all subsequent operations were done at 4°C. The reagents and French press were pre-cooled by keeping in the refrigerator 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 deoxy-ribonuclease 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 this 4 mg egg white lysozyme was added and incubated in the ice-bath for 20 min. The preparation was then diluted with 40 ml 30 mM Tris-buffer, pH 8.0. Cell debris was removed by centrifugation at 1,500g for 10 min. Usually it was

necessary to repeat this centrifugation once. The supernatant was then centrifuged at 38,000 g for 60 min. The pellet was resuspended in 25 ml 20% (w/v) sucrose in 30 mM Tris-buffers, pH 8.0. Two ml were layered onto a sucrose step gradient containing 9 ml each of 60% (w/v) sucrose and 70% (w/v) sucrose in 30 mM Tris-buffer, pH 8.0. The gradient was centrifuged at 100,000 g in a MSE 20° angle rotor for 14 to 17 hrs. Two 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 g for 60 min and washed twice with distilled water to remove traces of sucrose. The pellets were resuspended in a small amount of distilled water and frozen at -20°C.

2.2.13 Total protein assay

The total protein content of the OM and CM membranes was estimated by the method of Lowry et al. (1951). A standard curve was obtained using a solution of bovine serum albumin containing 0-200 µg/ml protein and reading the OD at 750 nm (Fig. 16). A linear relationship was obtained for concentrations of 0 to 200 µg/ml. Sample determinations were performed in duplicates.

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

The method used for the demonstration of the protein pattern of inner and outer membrane fractions was that of Lugtenberg et al., (1975). Two stock acrylamide solutions were used: Stock I(44% w/v acrylamide/0.8% w/v N,N' - methylene-bisacrylamide (BIS)).

Stock 2 (30% w/v acrylamide/0.8% w/v BIS).

Fig 16. Protein assay calibration curve.

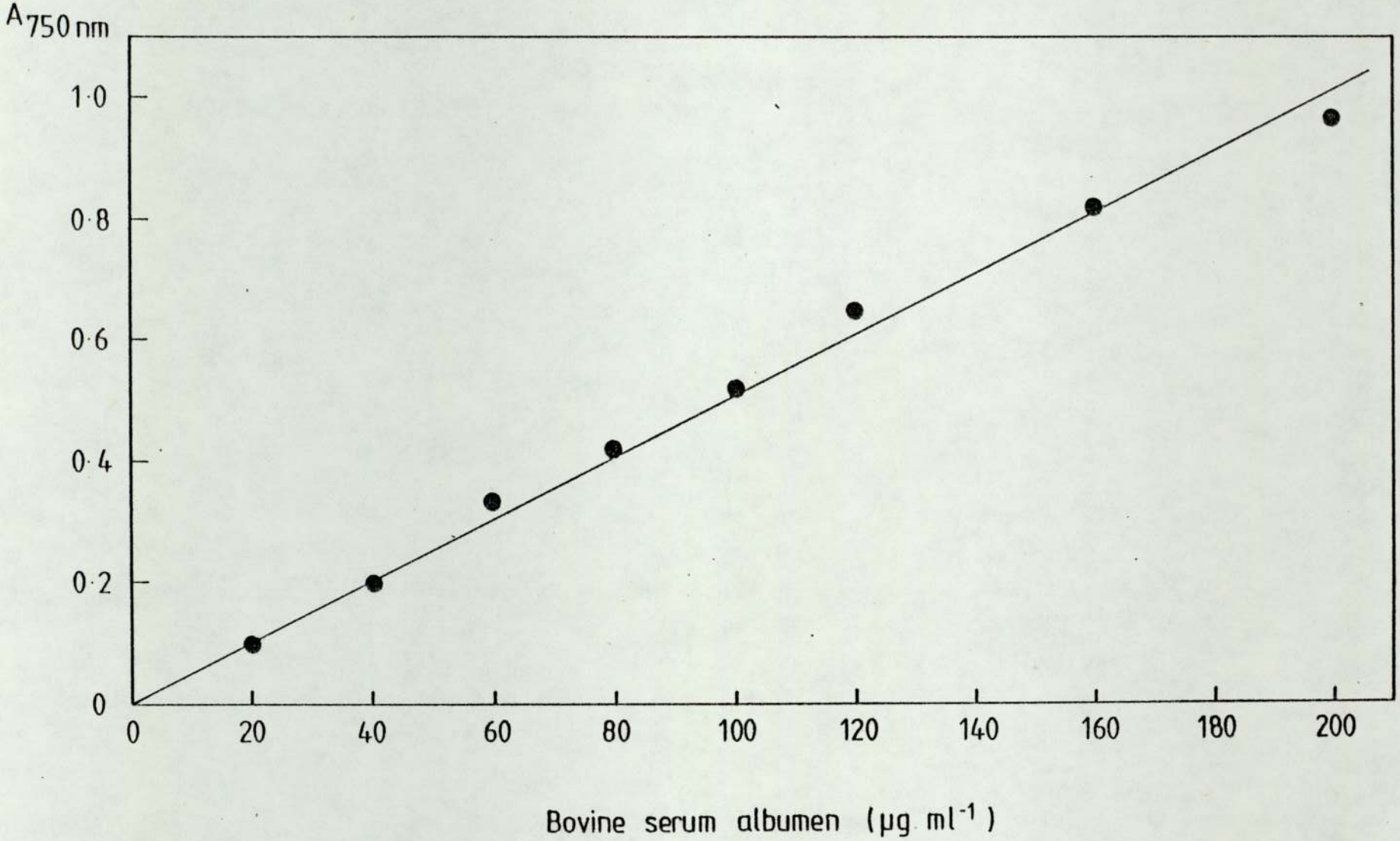

A750 nm
1·0
0·8
0·6
0·4
0·2
0
0
20
40
60
80
100
120
140
160
180
200
Bovine serum albumen (µg ml-1)

Table 6 shows the composition of the running gel and stacking gel, Table 7 the sample buffer. 50 µl of sample buffer was diluted with 50 µl of sample and 10 µl of bromophenol blue. The solution was boiled for 10 min; after cooling approximately 75 µl of solution was applied to each slot. Electrode buffer contained 0.025 M Tris, 0.19 M glycine and 0.1% SDS. The pH of this buffer was 8.3.

Table 6

Composition of gels for electrophoresis

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

APS = Ammonium persulphate

TEMED = N N N^{I} N^{1} Tetramethylethylene diamine

Table 7

Composition of sample buffer

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

2.2.15 Fatty acid extraction

0.5 ml samples of OM or CM were put in test tubes containing 2.5 ml 5% NaOH in 50% methanol. The tubes were sealed with Teflon-lined caps, and the samples were saponified for 30 min at 100°C. After cooling to room temperature, their pHs were lowered to 2.0 with conc HCl. The methyl esters of the free fatty acids were formed by adding 2.5 ml 14% boron trifluoride/methanol complex (14% BF w/v) and the mixture heated for 5 min at 80°C. The fatty acid (FA) methyl esters were then extracted from the cooled mixture with 5 ml chloroform/petroleum ether (1:4). The upper layer was evaporated to dryness using a rotary evaporator. The samples were stored at -20°C.

2.2.16 Fatty acid analysis

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

Column: 10ft x 2 mm ID x 1/4 in OD glass, packed with 3% SP-2100 DOH on 100/200 Supelcoport (Supelco Inc.).
Column temperature: 150°C -233°C at 2°C min^{-1} increases.
Gas pressure: Hydrogen 14.5 psi
Air 6.5 psi
Sample size 2 µl

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

3. RESULTS

3.1 Determination of growth requirements of P. cepacia

Most of the nutrient requirements for this organism had been determined by Anwar (1981). To assess that its properties had not varied, its glucose requirement was checked. Its requirement for K^+ was also determined.

3.1.1 Growth of P. cepacia in different glucose concentrations

The growth curves for glucose requirement are shown in Fig. 17. Glucose was the only carbon source used in this study.

The relation between initial glucose concentration and the onset of non-linear growth is shown in Fig. 18.

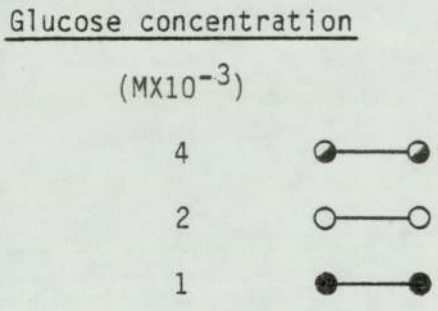
3.1.2 Growth of P. cepacia in different potassium concentrations

Typical results were presented in Fig. 19. It will be noticed that the CDM is contaminated with approximately 10^{-5} M K^+. The relation between onset of limitation and added potassium is shown in Fig. 20. The relation was linear to an OD_{470} of 2.75. Fig. 20 represents the mean of two experiments.

3.2 The survival of nutrient depleted P. cepacia in benzalkonium chloride

Fig. 21 shows considerable differences in the sensitivity of P. cepacia to BkC, depending on the depleting nutrient. Resistance of P. cepacia was greatest following Mg^{2+} - depletion > PO_4^{3-} depletion > NH_4^+, Fe^{2+} or carbon-depletion>K^+ depletion.

Fig 17. The effect of glucose concentration on the growth of P. cepacia .



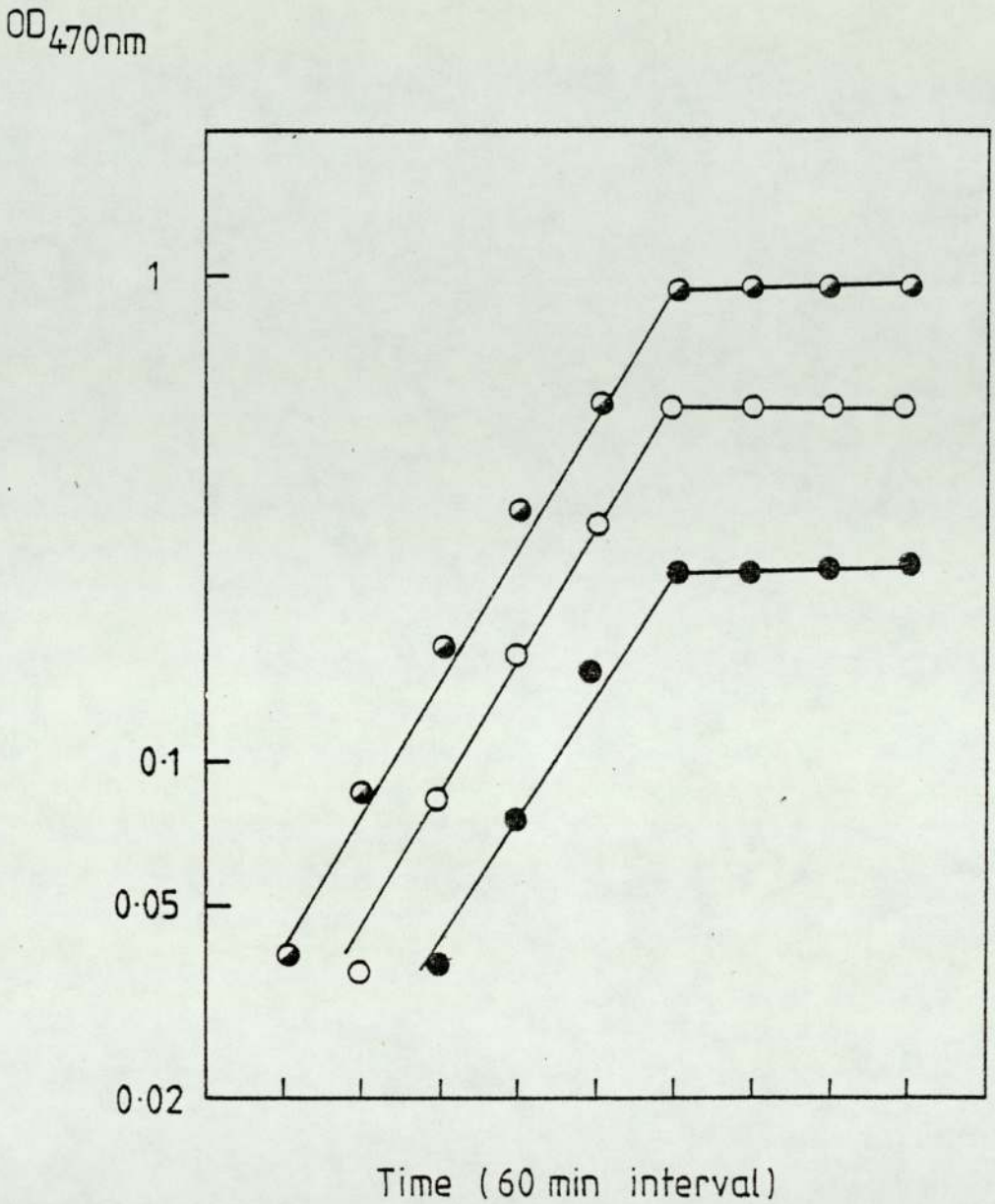

OD470nm
1
0·1
0·05
0·02
Time (60 min interval)

Fig 18. The effect of glucose concentration on the maximum absorbance reached during exponential growth.

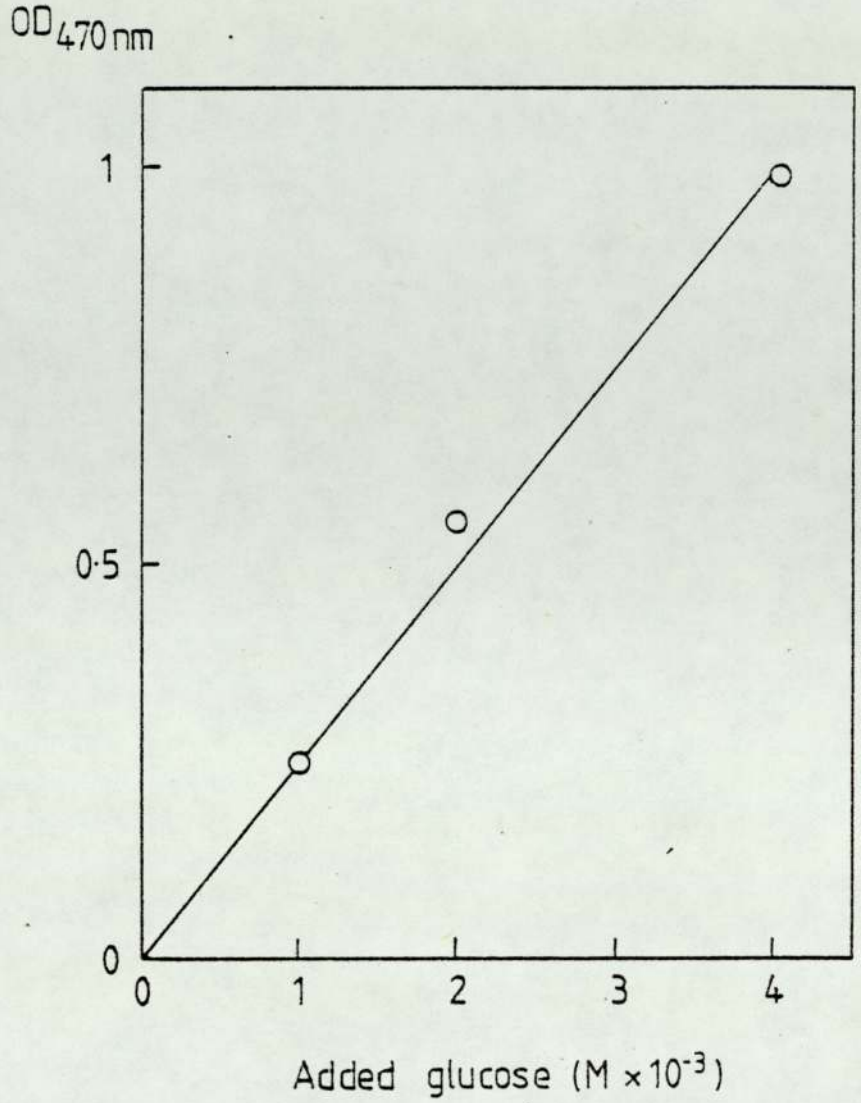
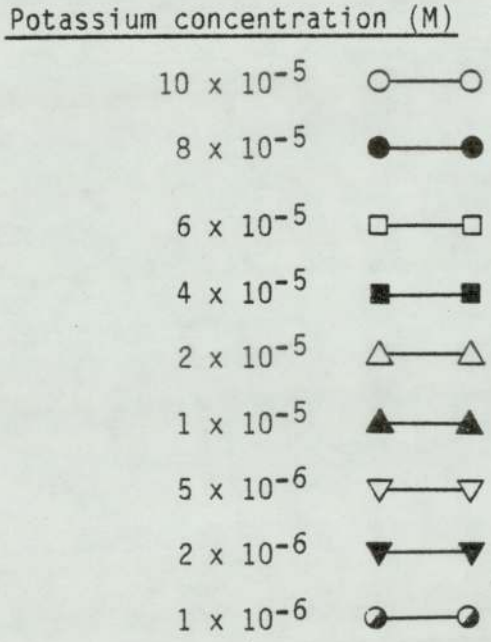

OD 470 nm
1
0·5
0
0
1
2
3
4
Added glucose (M × 10⁻³)

Fig 19. The effect of potassium concentration on the growth of P. cepacia



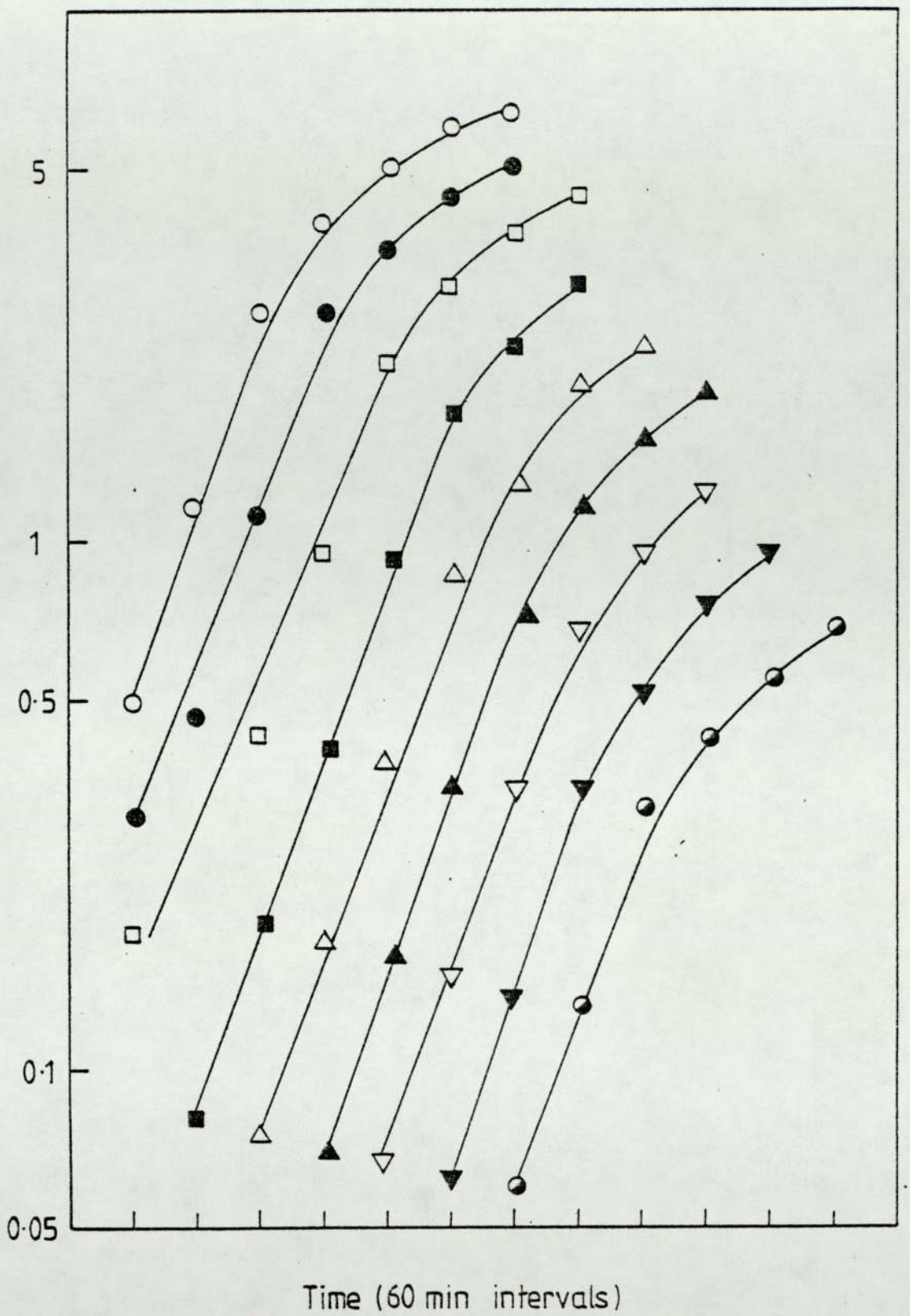

OD470nm
5
1
0·5
0·1
0·05
Time (60 min intervals)

Fig 20. The effect of potassium concentration on the maximum absorbance reached during exponential growth.

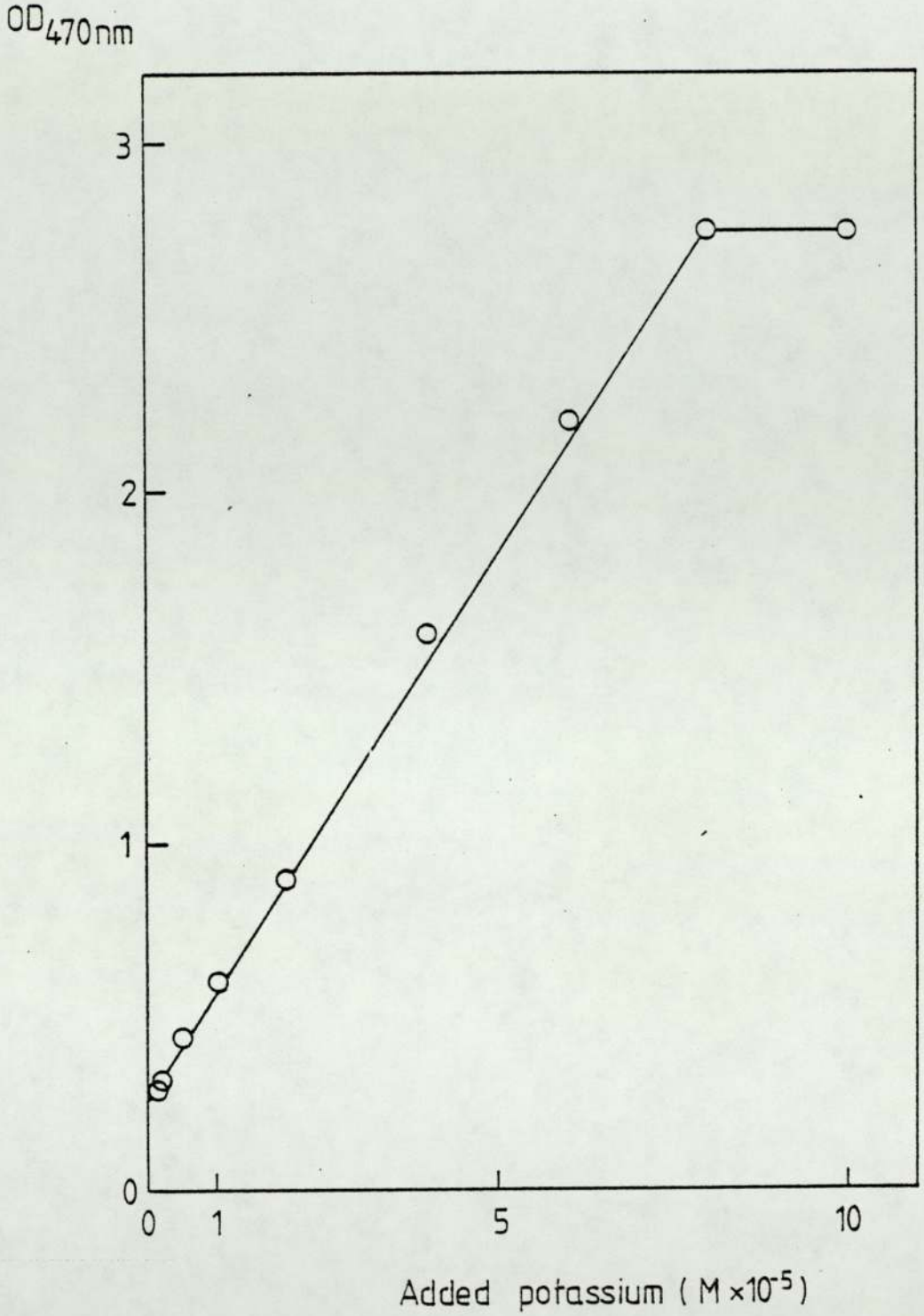
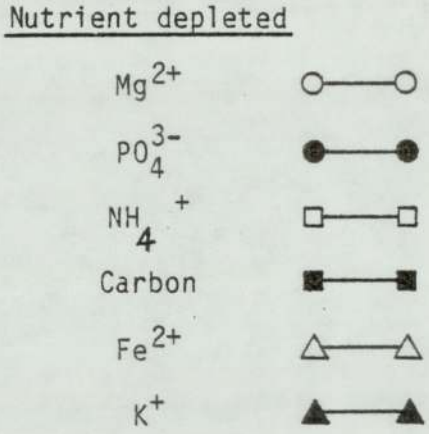
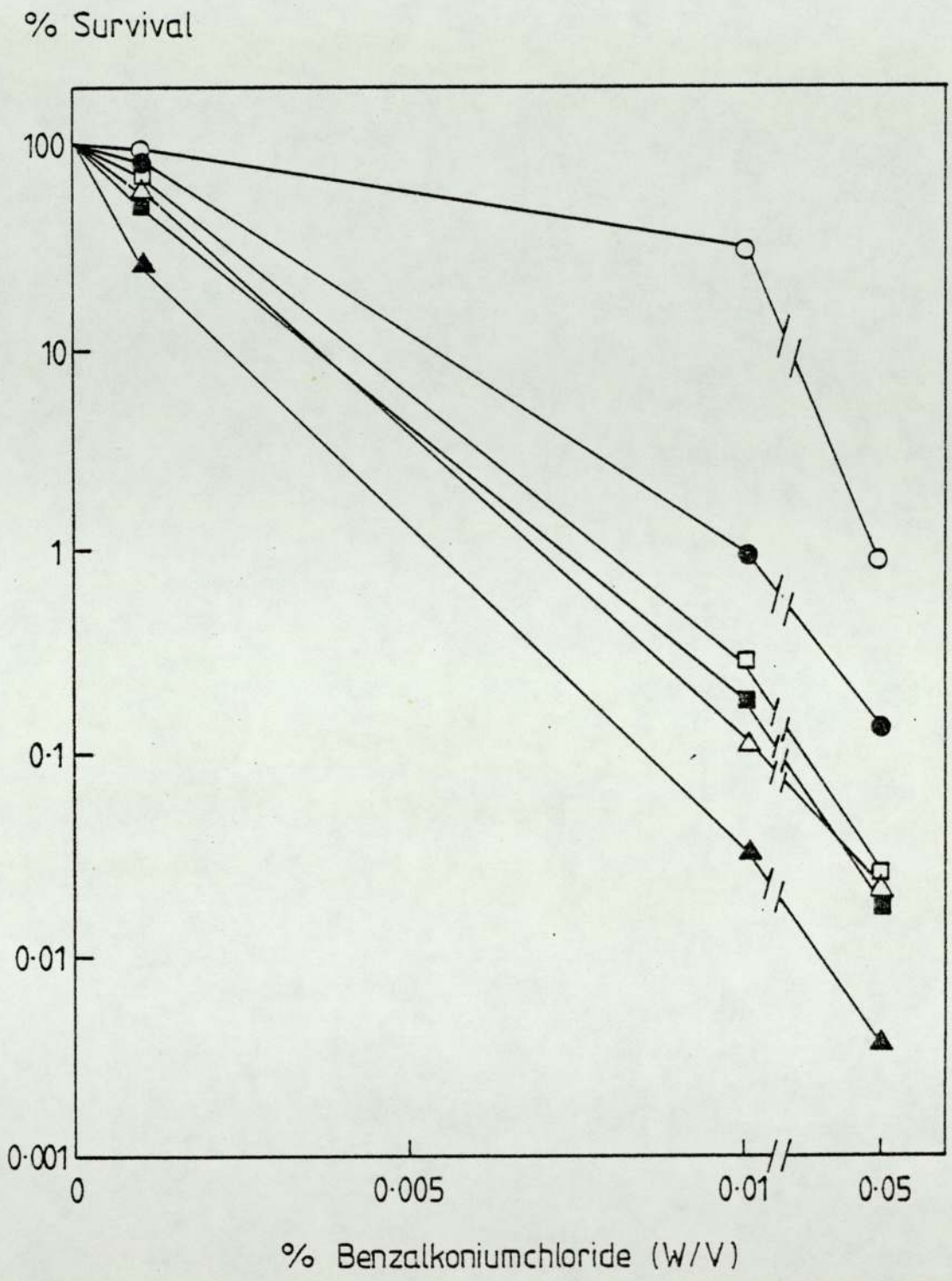

OD470nm
3
2
1
0
0
1
5
10
Added potassium (M ×10⁻⁵)

Fig 21. The effect of nutrient depletion on the survival of P. cepacia in benzalkonium chloride.




% Survival
100
10
1
0·1
0·01
0·001
0
0·005
0·01
0·05
% Benzalkoniumchloride (W/V)

3.3 Effect of chain length of benzalkonium chloride on the survival of *P. cepacia* following nutrient depletion.

Fig. 22 shows that *P. cepacia* was most sensitive to chain lengths of 14 and 18. At all chain lengths tested, carbon-depleted cells were more sensitive than Mg^{2+} - depleted ones (Fig. 23) but the relative sensitivities at different chain lengths were independent of the method of depletion. Most of the points are the mean of 2-5 experiments.

3.4 Effect of freeze-thawing on survival of *P. cepacia*

3.4.1 Effect of cryoprotectant

Table 8 shows the difference between two cryoprotectants used in the freezing study-glycerol and dimethyl sulphoxide. It can be noticed from the results that there was no significant difference between them.

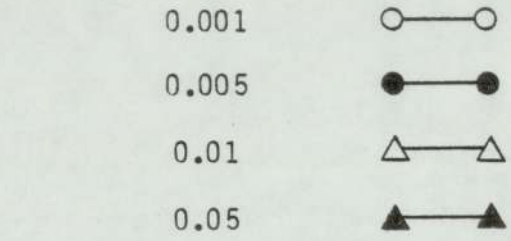
3.4.2 Media for warming

Table 9 shows the effect of two warming media. Following carbon depletion, survival of the organism rewarmed with water was greater than its survival in saline. Following magnesium depletion no difference was detectable following warming in water or saline.

3.4.3 Effect of nutrient depletion on survival of *P. cepacia* at different cooling rates.

Fig. 24 shows that at all cooling rates tests, Mg^{2+} - depleted cells were more resistant than carbon-depleted ones. The variation in resistance with cooling rates was the same for both kinds of cell whether warmed in water or saline. Survival increased as the cooling rate increased reaching a maximum at a cooling rate of $6°\ min^{-1}$.

Fig 22. The effect of the chain length of benzalkonium chloride on the survival of carbon-depleted *P. cepacia*.



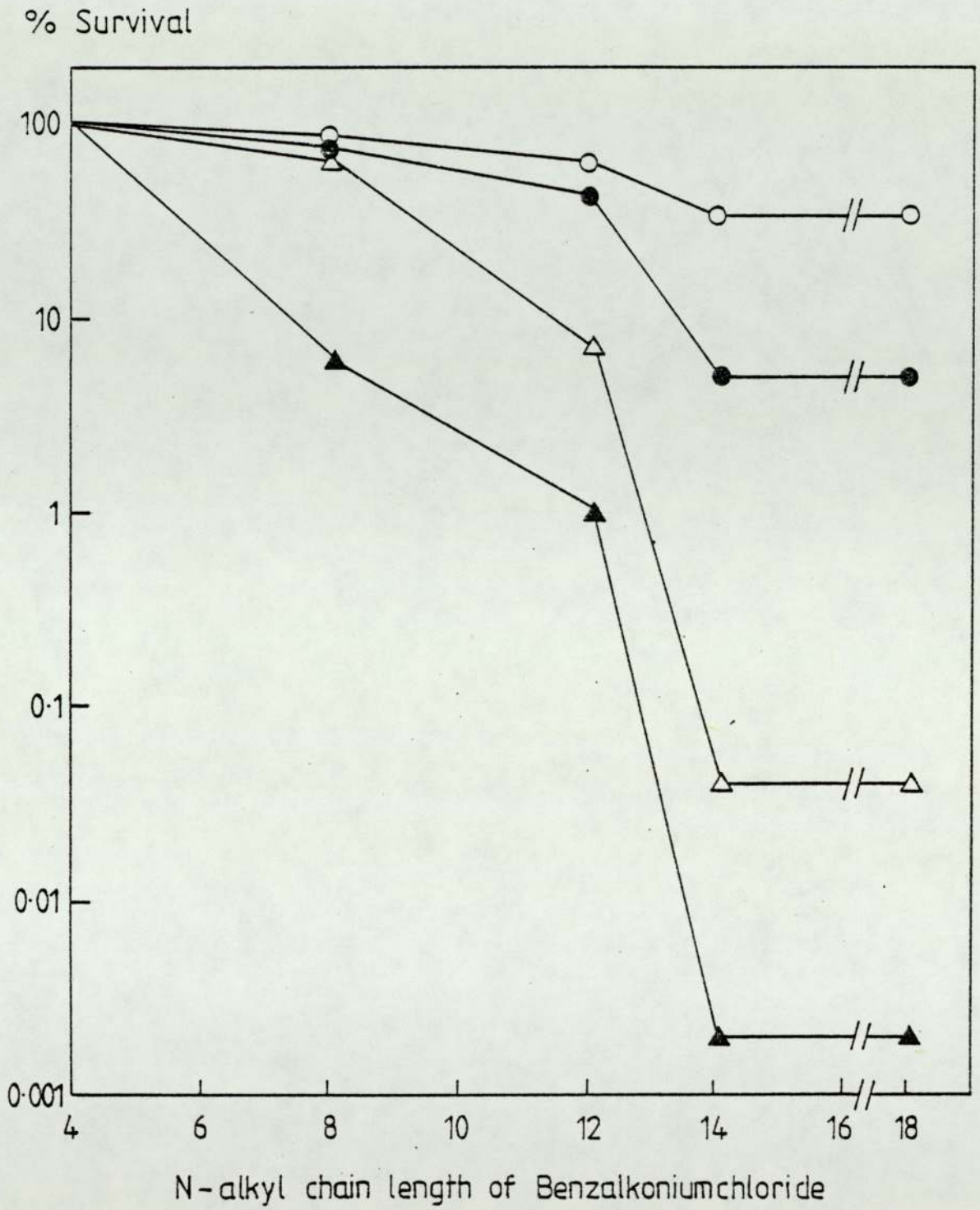
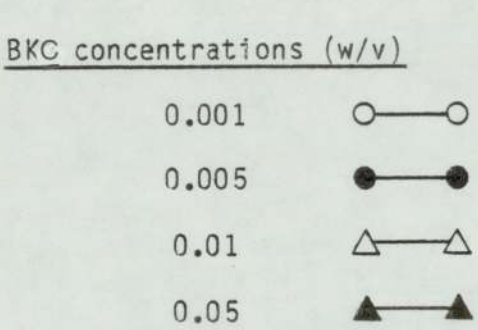

% Survival
100
10
1
0·1
0·01
0·001
4
6
8
10
12
14
16
18
N-alkyl chain length of Benzalkoniumchloride

Fig 23. The effect of the chain length of benzalkonium chloride on the survival of Mg^{2+}-depleted *P. cepacia*.



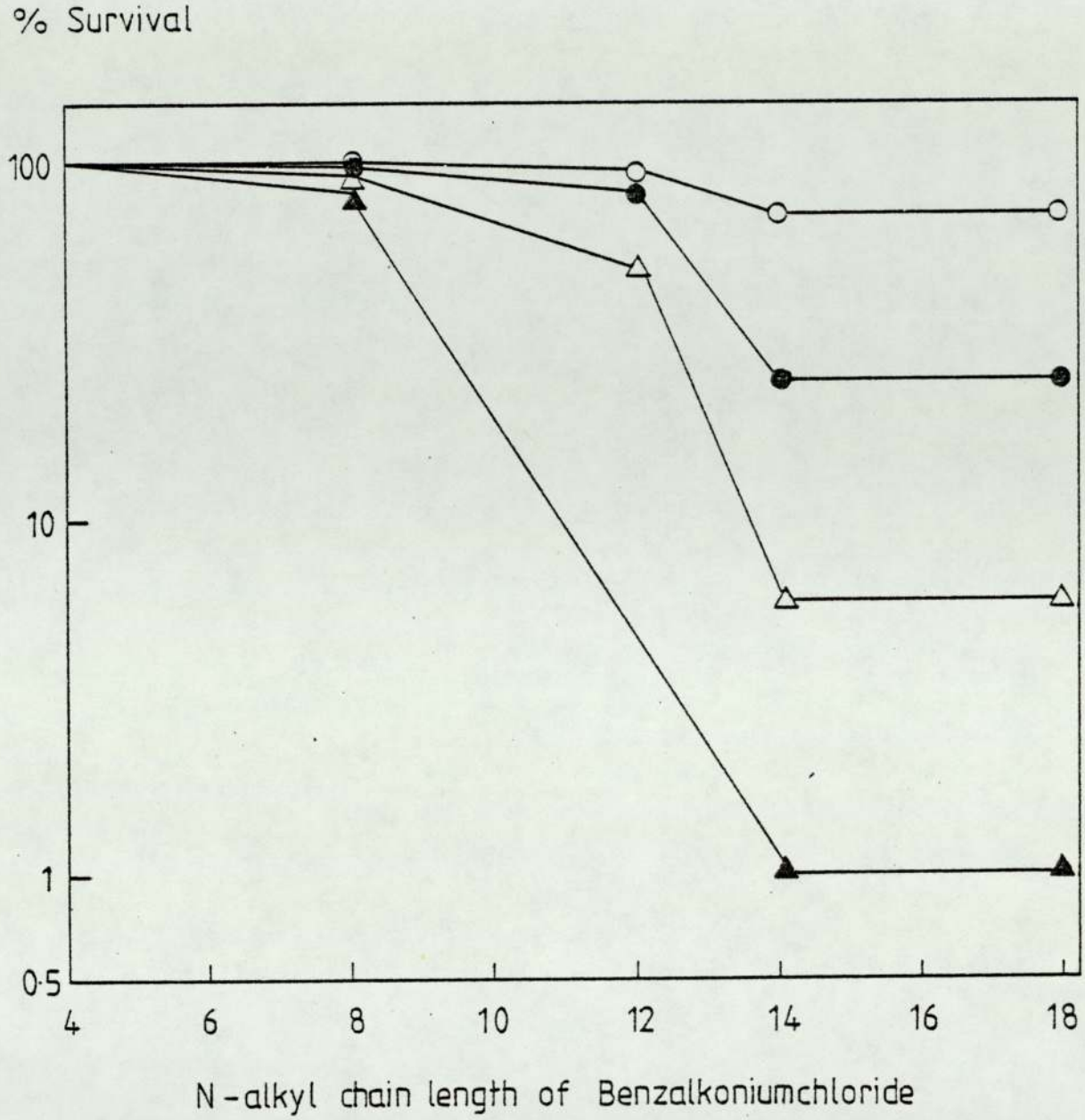

% Survival
100
10
1
0·5
4
6
8
10
12
14
16
18
N-alkyl chain length of Benzalkoniumchloride

Table 8

Effect of cryoprotectants on survival of P. cepacia

Cooling rates ($°min^{-1}$)	% Survival*	
	Cryoprotectants	
	Glycerol	DMSO
1	79	83
6	100	96
10	80	80

*Mean of 2 experiments

Cells rewarmed by addition of water

Table 9

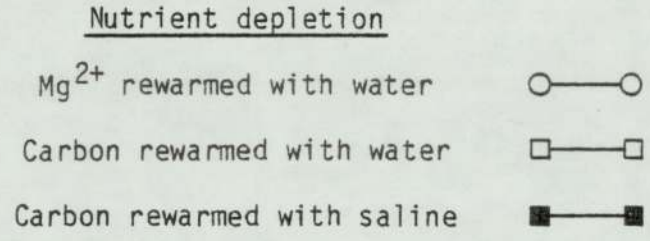
Effect of medium used to warm P. cepacia after freezing

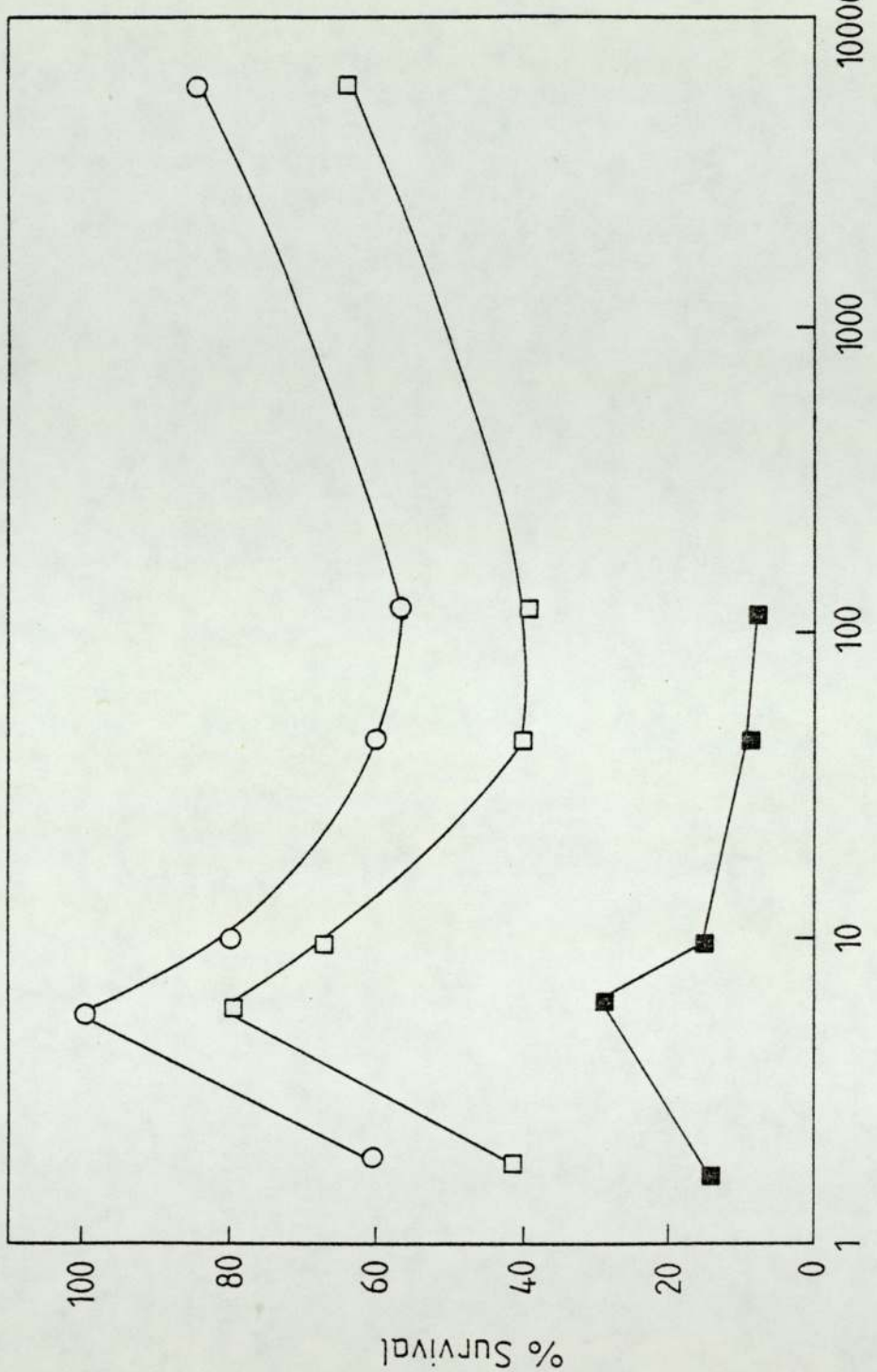
Cooling rates ($°min^{-1}$)	% Survival*			
	Nutrient depletion			
	Carbon		Mg^{2+}	
	Water	Saline	Water	Saline
1	57	15	79	82
6	80	23	100	98
10	58	15	80	85
48	42	11	60	59

*Mean of 2 experiments

Cryoprotectant used was glycerol.

Fig 24. The effect of nutrient depletion on survival of *P. cepacia* at different cooling rates.




% Survival
100
80
60
40
20
0
1
10
100
1000
10000
Cooling rates (°min⁻¹)

Then it decreased as the cooling rates increased to 144° min^{-1}. Survival again reached a high level at the ultrarapid cooling rate. In these experiments the cryoprotectant used was glycerol. Points presented in Fig. 24 are the mean of two experiments.

3.5 Effect of freezing at a cooling rate of $6°min^{-1}$ on the subsequent survival of *P. cepacia* in benzalkonium chloride

To assay BKC the cells used must have suffered as little damage as possible during freezing. The cooling rate chosen was $6°min^{-1}$ because it gave maximum survival. Tables 10, 11 and 12 show the effect of BKC on cells frozen at a cooling rate of $6°min^{-1}$. Freezing had little effect on the sensitivity of Mg^{2+} - and PO_4^{3-} - depleted *P. cepacia* to BKC, but carbon-depleted cells were more sensitive after freezing.

Table 10

Effect of freezing at $6°min^{-1}$ on subsequent survival of Mg^{2+}-depleted *P. cepacia* in benzalkonium chloride.

BKC %	% Survival*		
	Before freezing	Rewarming 0'	Rewarming 15'
0	100	97 ± 2	100 ± 2
0.001	79 ± 7	—	74 ± 5
0.005	25 ± 4	—	27 ± 6
0.01	2 ± 0.5	—	3 ± 3
0.05	0.7	—	0.6 ± 2

* All calculations based on no. of cells before freezing. No. of experimentswere 3 ± SD.

Table 11

Effect of freezing at $6°min^{-1}$ on subsequent survival of PO_4^{3-} depleted *P. cepacia* in benzalkonium chloride.

BKC %	% Survival *		
	Before freezing	Rewarming 0'	Rewarming 15'
0	100	79 ± 8	86 ± 6
0.00025	100	—	100
0.001	100	—	83 ± 3
0.005	17 ± 8	—	12 ± 5
0.01	3 ± 3	—	1 ± 5
0.05	0.7 ± 2	—	0

*All calculations based on the no. of cells before freezing. No. of experiments were 3 ± SD.

Table 12

Effect of freezing at $6°min^{-1}$ on subsequent survival of carbon-depleted P. cepacia in benzalkonium chloride.

BKC %	% Survival *		
	Before freezing	Rewarming 0'	Rewarming 15'
0	100	70 ± 2	84 ± 6
0.00025	82 ± 6	—	62 ± 7
0.001	46 ± 12	—	19 ± 0
0.01	0.2 ± 5	—	0
0.05	0	—	0

*All calculations based on no. of cells before freezing. No. of experiments were 3 ± SD.

3.6 Chemistry of cell envelopes

3.6.1 Protein profiles of outer and cytoplasmic membranes

The molecular weight of the OM proteins of P. cepacia were determined by Anwar (1981). The OM and CM proteins of P. cepacia were verified using (SDS-PAGE) as described in section 2.2.14. Analysis of the proteins of the CM (slots D,E,F) and the OM (slots A,B,C) showed that they were significantly different (see Fig. 25). The salient feature of all the CM fractions examined is the lack of any major proteins and the presence of a wide variety of minor proteins. The OM had several major proteins. There is little difference in the OM proteins of Mg^{2+} - depleted and PO_4^{3-} — depleted cells. In both cases, there may be an increased amount of proteins of molecular weight 36,000 and 40,000 daltons, in addition Mg^{2+} - depleted cells appear to have a protein of molecular weight 66,000 dalton. The OM protein profile of carbon-depleted cells differs from that of Mg^{2+} and PO_4^{3-} — depleted cells by the apparent replacement of the protein of molecular weight 40,000 dalton by two proteins of similar molecular weight.

3.6.2 Fatty acid composition of outer and cytoplasmic membranes

Fig. 26 shows a typical gas-liquid-chromatogram recording. Tables 13 and 14 show the individual fatty acids (FA) present in the OM and CM of P. cepacia. Tables 15 and 16 categorise the FA into various types.

Fig 25. **Protein profile of P. cepacia.**

Slot A: The OM fraction of PO_4^{3-} - depleted cells.

Slot B: The OM fraction of Mg^{2+} - depleted cells.

Slot C: The OM fraction of carbon-depleted cells.

Slot D: The CM fraction of PO_4^{3-} - depleted cells.

Slot E: The CM fraction of Mg^{2+} - depleted cells.

Slot F: The CM fraction of carbon-depleted cells.

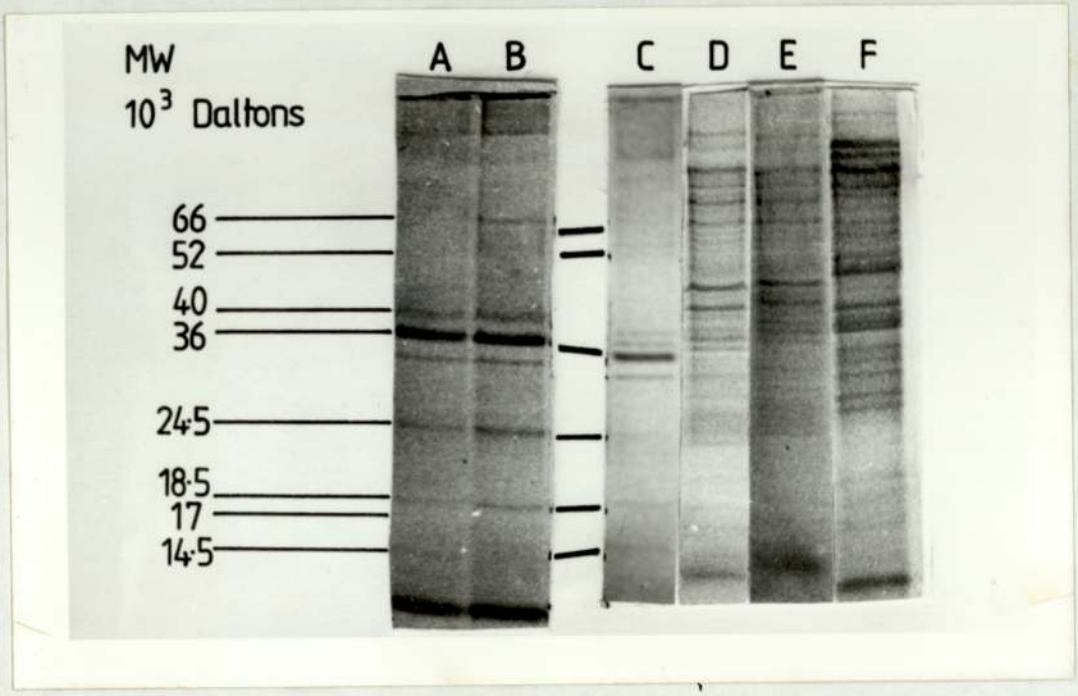

MW
10³ Daltons
66
52
40
36
24·5
18·5
17
14·5
A
B
C
D
E
F

Fig 26. Gas liquid chromatogram of fatty acid of the outer membrane of PO_4^{3-}—depleted cells.

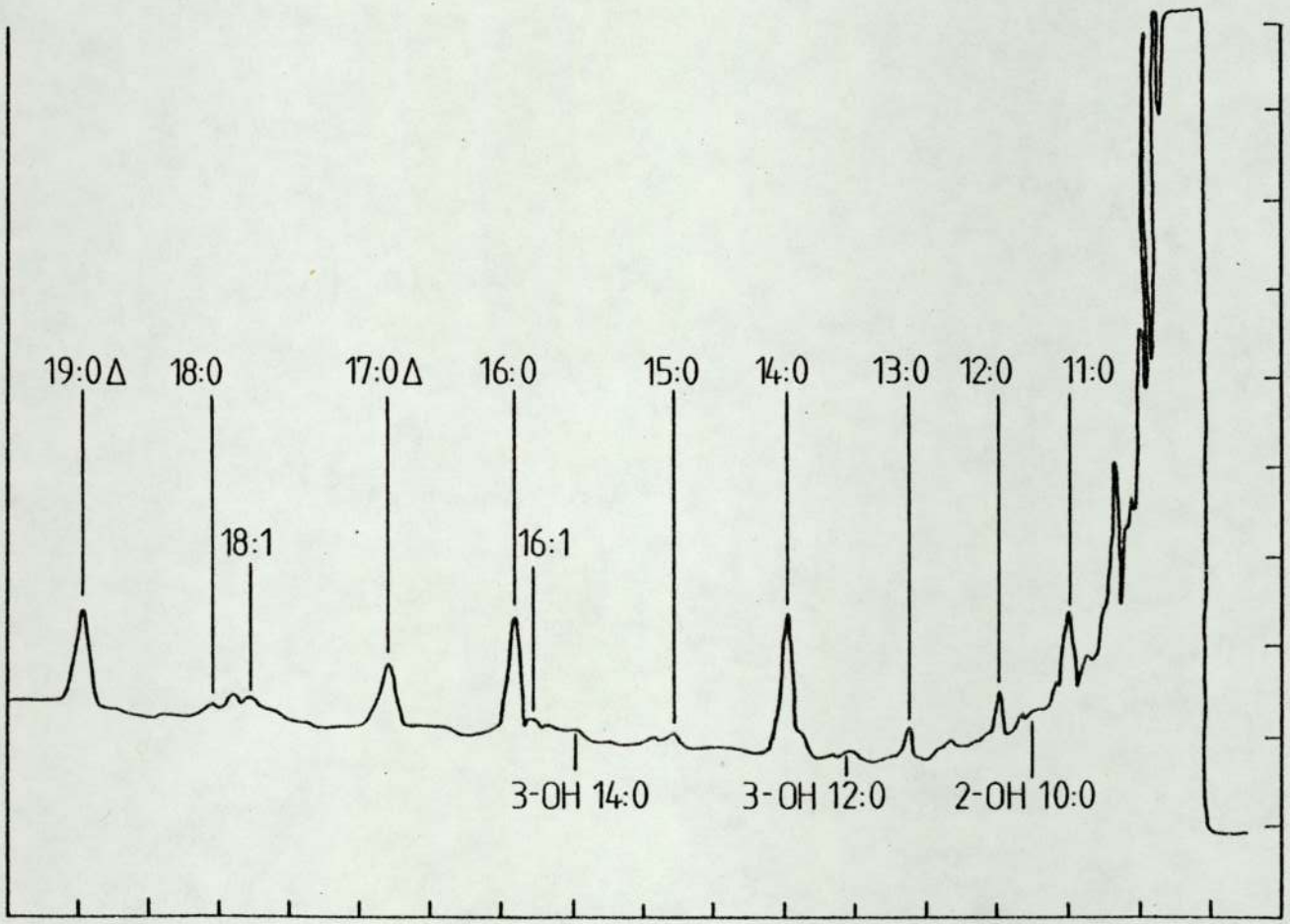

19:0Δ
18:0
18:1
17:0Δ
16:0
16:1
15:0
14:0
13:0
12:0
11:0
3-OH 14:0
3-OH 12:0
2-OH 10:0
Detector response
Time intervals (2mins/div.)

Table 13

Outer membrane fatty acids of P. cepacia

% Fatty acids	Cells depleted of Mg^{2+}	PO_4^{3-}	Carbon
11:0	7.9	8.6	2.9
2.0H 10:0	2.3	2.0	0.9
12:0	6.6	4.7	2.4
13:0	4.4	3.8	1.8
3.0H 12:0	3.9	3.9	1.5
14:0	30.9	27.9	11.6
15:0	1.6	0.5	4.9
3.0h 14:0	0.8	1.6	2.3
16:1	1.1	0.5	6.9
16:0	11.1	16.5	9.5
a17:0	N.D	N.D	0.3
Δ17:0	5.5	8.9	7.4
17:0	N.D	N.D	0.2
3.0H 16:0	N.D	N.D	1.7
18:1	1.1	1.6	1.3
18:0	1.6	0.8	1.5
Δ19:0	13.9	12.6	17
19:0	7.3	6.1	25.9

1. Results are from 1 sample
2. All readings are the average of two runs.
3. N.D. = not detectable

Table 14

Cytoplasmic membrane fatty acids of P. cepacia

% Fatty acids	Cells depleted of Mg^{2+}	Cells depleted of PO_4^{3-}	Cells depleted of Carbon
11:0	7.4	9.4	1.8
2.0H 10:0	0.2	0.3	0.1
12:0	5.0	7.6	0.1
13:0	8.0	5.5	1.0
3.0H 12:0	0.1	0.1	N.D
14:0	28.6	34.6	7.1
15:0	3.8	1.4	0.1
3.0H 14:0	0.05	0.05	0.05
16:1	2.5	2.6	N.D
16:0	9.5	6.8	23.2
a17:0	N.D	N.D	N.D
Δ17:0	6.6	5.5	20.7
17:0	N.D	N.D	N.D
3.0H 16:0	0.05	0.05	0.05
18:1	1.0	1.0	N.D
18:0	1.2	2.1	1.3
Δ19:0	24.6	21.7	31.1
19:0	1.4	1.3	13.4

1. Results are from I sample
2. All readings are the average of two runs
3. N.D = not detectable

Table 15

Outer membrane fatty acid types of *P. cepacia*

Type of fatty acid	% total fatty acid cells depleted of Mg^{2+}	% total fatty acid cells depleted of PO_4^{3-}	% total fatty acid cells depleted of Carbon
Unsaturated	2.2	2.1	8.2
Saturated	78.4	76.4	67.4
Cyclopropane	19.4	21.5	24.4

Table 16

Cytoplasmic membrane fatty acid types of *P. cepacia*

Type of fatty acid	% total fatty acids cells depleted of Mg^{2+}	% total fatty acids cells depleted of PO_4^{3-}	% total fatty acids cells depleted of Carbon
Unsaturated	3.5	3.6	N.D
Saturated	65.3	69.2	48.2
Cyclopropane	31.2	27.2	51.8

N.D. = not detectable

4. DISCUSSION

4.1 Chemistry of cell envelope

4.1.1 Proteins

The protein patterns of the OM and CM were analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (see section 2.2.14). The results are shown in Fig 25. The protein patterns of the CM (Slots D,E,F) were significantly different from those observed in the OM (Slots A,B,C). Fewer bands were observed in the OM but the proteins were present in large quantities.

The synthesis of several OM proteins of Gram-negative bacteria has been found to be affected by the growth environment. Synthesis of protein DI was induced when P. aeruginosa was grown in the presence of glucose (Hancock and Carey, 1980). Magnesium limitation induced the synthesis of protein HI in P. aeruginosa (Nicas and Hancock, 1980) and it was suggested to be involved in the resistance to aminoglycosides such as gentamycin and polymyxin (PB) and also EDTA (Nicas and Hancock, 1980). Expression of protein e of E. coli was induced when the cells were grown under phosphate limitation (Overbeeke and Lugtenberg, 1980). Fig 25 shows the OM protein pattern of P. cepacia when depleted of glucose, phosphate or magnesium. The protein of apparent molecular weight 36,000 dalton was present in large quantities in all three growth conditions and might correspond to the pore-forming protein (porin) observed in other Gram-negative bacteria to be in the range of 33,000 to 40,000 daltons (Nikaido and Nakae, 1979). Two protein bands with similar molecular weight (40,000 daltons) were observed when P. cepacia was grown under carbon limitation only. However, this might result from the low loading of OM in the gel, but it seems more likely that these bands induced in carbon-depleted

P. cepacia correspond to protein D1. It was found by Hancock and Carey (1980) in P. aeruginosa when it was grown in the presence of glucose as mentioned previously. No special proteins were induced in Mg^{2+} or PO_4^{3-} - depleted P. cepacia, unlike results with P. aeruginosa and E. coli (Nicas and Hancock, 1980; Overbeeke and Lugtenber 1980).

4.1.2 Fatty acids

The fatty acid (FA) composition of the cell is drastically affected by the growth medium (El-Khani and Stretton, 1981 Conrad et al., 1981) and incubation temperature (Marr and Ingraham, 1962), and significant differences also occur between strains of P. cepacia (Oyaizu and Komagata, 1982). Major FA of P. cepacia found by Oyazin and Komagata (1982) in general are 14:0, 16:0, 16:1, 18:1, 17:0Δ, 19:0Δ and 19:0. These findings agree with those of Moss, 1978 and our findings.

It was found in this study that the OM and CM of both Mg^{2+}- and PO_4^{3-} - depleted P. cepacia were composed of FA which were very similar to each other in overall composition. They were significantly different from the OM and CM of carbon-depleted cells. However, the proportion of cyclopropane FA (CFA) varies between cells tested. Both OM and CM of carbon-depleted cells have a higher level of CFA than those of Mg^{2+} and PO_4^{3-} - depleted cells (Tables 15 and 16). Although CFA are a major component of the membrane phospholipids (pL) in a large variety of bacteria (Coldfine, 1972) the function of these acids is unclear.

In transition from exponential to stationary phase, bacteria undergo

a dramatic change in metabolism and cell composition. One such change is in FA composition, with the appearance in some bacteria of CFA and the disappearance of unsaturated FA (Cronan et al., 1974; Cronan and Gelmann, 1975). This is due to methylation of the unsaturated moieties of the membrane pL in a reaction catalyzed by the enzyme CFA synthetase (Law, 1971). This may explain the low proportions of unsaturated FA in all cells tested especially the carbon-depleted cells which have the highest proportion of CFA. The conversion of pL unsaturated FA moieties to their cyclopropane derivatives could increase the temperature of the order to disorder transition of the membrane pL (Kito et al., 1973). A large alteration in the transition temperature would have a very significant effect on membrane function (Jackson and Sturtevant, 1977).

The fluid character of the membrane is largely determined by the relative proportion of FA, increasing concentrations of CFA increasing membrane fluidity. Nikaido (1979) suggested that the low fluidity of LPS FA will hinder the dissolution of hydrophobic molecules into the membrane interior. PB resistance in Klebsiella has been correlated with a drastic reduction in total CFA composition. This may explain the resistance of Mg^{2+} and PO_4^{3-}-depleted P. cepacia to BKC in this study.

It has been found that P. cepacia has a different set of hydroxy FA from those commonly found in the outer species of the genus Pseudomonas (Ikemoto et al., 1978; Oyaizu and Komagata, 1983). The major hydroxy FA in P. cepacia are 2.OH 10:0 , 3.OH 12:0, 3.OH 14:0 and 3.OH 16:0 but these varied between cells tested. The outer

membrane was found to have a higher percentage of hydroxy FA than in the CM. This might be due to the presence of hydroxy FA from the LPS in addition.

4.2 Benzalkonium chloride

4.2.1 Effect of nutrient depletion on bactericidal action of benzalkonium chloride

As discussed in section 1.1.4 BKC causes extensive disruption of the CM with concomitant loss of cytoplasmic contents leading to cell death. Increasing concentrations of BKC reduce the number of survivors of all cells tested (Fig. 21,22 and 23), but there is a higher initial rate of killing in the NH_4^+, Fe^{2+} and PO_4^{3-}-depleted cells. This may reflect differing CM composition. Fig. 24 shows the CM protein profiles of the different cells tested. The large number of proteins present make analysis difficult, though individual proteins do vary in concentration. It was noticed that P. cepacia exhibits similar changes in sensitivity to other antimicrobial agents such as chlorhexidine and cetrimide, following depletion of different nutrients (Cozens and Brown, 1983).

The cell envelope, and particularly the OM of Gram-negative bacteria, is known to change in response to changes in the growth environment (Melling and Brown, 1975; Ellwood and Tempest, 1972; Gilbert and Brown, 1978). It is therefore likely that these changes in resistance may reflect changes in the cell envelope which either prevent access of the drug to the site of action or alter the site of action such that the drugs show decreased activity. Little is known about the cell envelope of P. cepacia. The lipopolysaccharide (LPS) is atypical, not containing 2-keto-3-deoxy octonic acid (KDO) (Maneillo et al., 1979). Protein and FA were discussed in section

4.1.1 and 4.1.2.

4.2.2 Effect of different chain lengths of benzalkonium chloride on the survival of P. cepacia.

As discussed in section 1.1.3 there is an increase in activity with increase in n-alkyl chain lengths of BKC up to chain length 16 for Gram-positive and Gram-negative bacteria and yeasts (Daoud et al., 1983). Fig 22 and 23 show the same pattern of response with P. cepacia.

A parabolic relationship exists between the antimicrobial properties of cationic surface active agents and their hydrophobic character (Ross et al., 1953). That is, there exists a direct relationship between activity and alkyl chain length with increased carbon number up to a maximum. However, it was noticed that the response to P. cepacia to chain length 18 was similar to that found with chain length 14. These results are different than those found by Tomlinson et al. (1977) with P. aeruginosa. They found that there was a turndown in activity in chain lengths greater than 14. This might be due to the difference in the growth environment of both organisms in the two studies. However, Brown and Tomlinson (1979) noticed that chain lengths with optimal activity varied with different strains of P. aeruginosa.

Blois and Swarbrick (1972) suggested that the turn down in activity is probably related to more than one physical property of the compounds, the longer the hydrocarbon chain the greater the tendency would be for the molecule to be adsorbed at the surface of a bacterium and also there would be a reduction in aqueous solubility of the molecule as the carbon number is increased. However it was

noticed during the study that BKC chain length 18 was less soluble in water than the other chain lengths.

Hansch and Fujita (1964) and Lien et al. (1968) argued that the differences in biological activity of chemically related antimicobials, acting at similar sites within the cell, would reflect their relative case of penetration through the various liquid barriers of the cells and their ability to react at that site. The outercomponents of the cells were considered as a series of aqueous and lipophilic layers. Substances of low water solubility would be unable to penetrate these aqueous layers and would accumulate within the lipid region (Parker and McRobbie, 1974), similarly those of low oil solubility would be unable to cross the lipophilic barriers. Compounds between these extremes must exist which possess the optimum balance between hydrophilicity and lipophilicity for traversing the cell barriers. These observations led Daoud et al. (1983) to suggest that high chain lengths are able to interact at targets inaccessible to the lower chain length, and the opposite is also possible.

4.3 Effect of freezing on survival of P. cepacia

4.3.1 Cooling rates

In the introduction (Section 1.5), it was suggested that freezing injury depends on the interaction of two types of factors, firstly, the intracellular freezing of intracellular water, an event that becomes more probable as the cooling velocity is raised, and, secondly, the chemical injury from dehydration or solute concentration, injury that should be augmented by the long exposure produced by slow cooling. Results of the effect of different cooling rates on survival of P. cepacia is shown in Fig. 24. Survival of

P. cepacia increased as the cooling rate increased reaching maximum at cooling rate 6°min^{-1}. Then survival decreased as cooling rates decreased reaching minimum at 144°C min^{-1}. Survival increased again at ultra-rapid rates of cooling. Most bacteria examined respond to cooling rates in a manner similar to P. cepacia. Studies with P. aeruginosa (Davies, 1970), E. coli (Calcott and Macleod, 1974), Azotobacter chroococcum, Klebsiella aerogenes, Salmonella typhimurium and Streptococcus faecalis (Calcott et al., 1975) show a peak of survival in the low cooling rate range and increased survival again at ultra-rapid rates of cooling.

It has been suggested that the factor responsible for death at cooling rates below the optimum for survival in the low cooling rate range is related to high solute concentrations (Mazur, 1966).

Maximum survival at about 7°C min^{-1} is associated with reduced amount of intracellular ice (Mazur and Schmidt, 1968).

Several lines of evidence indicate that the sharply lowered survivals accompanying cooling velocities above 6°min^{-1} are due to intracellular freezing (Mazur and Schmidt, 1968). The major drop in viability occurs when the cooling velocity rises from 6° to 144° min^{-1}. The rate of cooling was shown to be in the same range of velocities as the rate at which the water inside the cells supercooled. Following supercooling, intracellular ice was likely to form (Mazur and Schmidt, 1968).

4.3.2 Nutrient depletion

As discussed in section 1.5.2 damage to membrane may be an important

factor in death of cells during freezing. It was therefore of interest that the changes in the membranes following nutrient-depletion could be correlated with cryosurvival.

P. aeruginosa grown under Mg^{2+}-depletion showed a similar cooling rate-survival profile to a carbon-depleted culture (See Fig. 24). However, at all cooling rates tested it was noted that survival of Mg^{2+}- depleted P. cepacia was higher than that of a carbon-depleted culture.

Calcott and Macleod (1974) investigated the influence of nutritional status and growth rate on the cryosurvival of E. coli grown under carbon and nitrogen limiting conditions in a chemostat. Results obtained by them are similar to those obtained in this study. It is noticeable that in no circumstances was the survival of carbon-limited E. coli as great as that obtained with carbon-depleted P.cepacia. This might be due to their use of glycerol as a carbon source instead of glucose, but it seems more likely that it could be due to a difference between log phase and stationary phase cells as reported by Gorill and McNeil (1960) for P. aeruginosa, Serratia marcescens (Strange and Ness, 1963) and Aerobacter aerogenes (Strange and Dark, 1962). P. cepacia was only tested in the stationary phase in this study. Davies (1970) presented evidence that not only did phase of growth or organisms influence the survival pattern during freezing-thawing but also the growth medium. He showed an optimum cooling rate increase for cryosurvival for Pseudomonas in the staionary phase.

It is well documented that differences in response of bacterial populations to freezing-thawing were related to their different

chemical compositions (Strange, 1968). He presented evidence that for nitrogen limited Aerobacter aerogenes, grown at varying rates in a chemostat, the carbohydrate content influenced their survival during freezing-thawing. The organisms containing higher levels survived longer than those with lower ones, However, Calcott and Macleod (1974) reported that it is impossible to explain why the possession of high levels of carbohydrate make organisms more resistant to freezing-thawing. If it is the carbohydrate directly which contributes to this response, is it intracellular as granules associated with LPS, or extracellular as a slime capsule? It is of interest that Bennet et al. (1981) found that deep rough mutants of S. typhimurium with decreased chain length and core defects were more susceptible to slow and rapid freeze-thawing in water and saline. These strains exhibited more OM damage than the wild type. Hwever it is known that LPS of P. cepacia has no KDO in its core (Maniello et al., 1979).

The OM protein profile of Mg^{2+}-depleted P. cepacia was similar to that of PO_4^{3-} -depleted cells and different from that of carbon-depleted cells. Proteins may be important in cryosurvival because OM and CM proteins are released during freezing-thawing damage (Souzu, 1980).

CFA has been implicated recently in resistance to freezing-thawing damage (Calcott et al., 1984). It was found that the mutant population of E. coli which synthesized much less CFA was more resistant than the wild type to freezing-thawing damage whether it was frozen in water or saline. It was proposed that the responses were due to altered membrane fluidity of the mutant. Similarly, it

was noticed in this study that FA composition of carbon-depleted P. cepacia differed significantly from Mg^{2+} and PO_4^{3-} -depleted ones. The high rate of CFA in carbon-depleted P. cepacia can be correlated with its highest sensitivity to freeze-thaw damage whether it was rewarmed with water or saline. The effect of 0.85% NaCl on the survival of organisms during freezing-thawing was discussed in section 1.5.2. Calcott et al. (1975) found that many species of bacteria were sensitive to salt on freezing and thawing. They found that survival in saline was always less (by at least 20%) than comparable cells frozen and thawed in water, except for S. faecalis which is Gram-positive and which was resistant to the presence of NaCl.

4.4 Sensitivity of frozen P. cepacia at cooling rate 6°min^{-1} to benzalkonium chloride.

A cooling rate of 6°min^{-1} gave maximum survival for all cultures of nutrient-depleted P. cepacia tested (See Fig. 24). This cooling rate was found to be the optimum for many bacteria tested (Calcott et al., 1975; Calcott and Macleod, 1974 ; Davies, 1970). It eliminates or greatly reduces lethal events during freezing-thawing (Mazur and Schmidt, 1968).

Mg^{2+} and PO_4^{3-} -depleted P. cepacia were more resistant at this cooling rate than carbon-depleted cultures and sensitivity of Mg^{2+} and PO_4^{3-} -depleted P. cepacia to BKC before and after freezing remained rather stable. Carbon-depleted cultures were sensitive to freeze-thaw injury at this cooling rate, and their sensitivity to BKC increased after freezing at this cooling rate. It is of interest that culture conditions which favour cryosurvival also appear to increase resistance to BKC.

It can be seen that it is possible to prepare cells of different sensitivity to BKC and store them by freezing. These cells could then be used for the assay of BKC. Preliminary tests of preserved pharmaceutical products using such standard cells indicate a wide variation in biological activity of the BKC used (Lee and Klemperer, 1984).

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