ROLE OF THE *KLEBSIELLA AEROGENES* ENVELOPE

IN RESISTANCE TO ANTIBIOTICS AND HOST DEFENCES

A thesis submitted by

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for the degree of

DOCTOR OF PHILOSOPHY

of the University of Aston in Birmingham

December 1983

TO MY WIFE

AND

MY PARENTS

The University of Aston in Birmingham

ROLE OF THE *KLEBSIELLA AEROGENES* CELL ENVELOPE IN RESISTANCE TO ANTIBIOTICS AND HOST DEFENCES

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The nutritional requirements of *Klebsiella aerogenes* DL1 were quantitatively defined using a chemically defined medium (CDM).

Antibiotic sensitivity patterns were determined using nutrient-depleted cultures. Iron-depleted (CDM-Fe) *K.aerogenes* DL1 was more sensitive to polymyxin than magnesium-depleted or log phase DL1. Loss by mutation of the K and O antigens failed to increase the sensitivity of *K.aerogenes* to a range of hydrophilic and hydrophobic antibiotics.

CDM-Fe *K.aerogenes* synthesized at least six new OM proteins (IRMP) in the 69,000 to 83,000 M_r range. Enterochelin was detected in the supernatants of CDM-Fe cells. An 18,500 M_r OM protein was present in iron replete cells but repressed in iron-depleted cells. The IRMP and enterochelin were produced within two generations of inoculating iron replete cells into a CDM containing $< 17 \times 10^{-7}$ M Fe^{3+} and before any change in growth rate was apparent. Iron-depleted cells required four generations in iron replete CDM for the relative level of IRMP to return to the low levels always detected in the OM of stationary phase iron replete cells. The appearance of the IRMP was unaffected by prior growth in a gross excess of iron suggesting that whether or not *K.aerogenes* could store iron, it rapidly responded to the extracellular iron concentration. Serum grown DL1 also synthesized the same IRMP unless saturated with added iron.

The presence of both the O and K antigens was necessary to protect *Klebsiella* from serum-killing or phagocytosis in the absence of specific antisera. Optimal phagocytic ingestion of NCTC 5055 ($K2^+$ $O1^+$) required either anti K or anti O sera. DL1 was not opsonised by anti O sera unless its capsule was first physically reduced. Serum was bacteriostatic for M10 (K^+ $O1^+$) but rapidly bactericidal for M10B (K^- O^-). Chelation of serum with MgEGTA delayed killing of M10B but enhanced killing of M10. The O antigen, whilst not antiphagocytic, conferred protection against serum-killing.

Culture filtrate vaccines containing LPS, protein and polysaccharide prepared from virulent *Klebsiella* strains grown in iron depleted CDM, protected mice against lethal i.p. challenge with heterologous and homologous *Klebsiella* strains.

Key Words: *Klebsiella aerogenes*; iron ; phagocytosis; serum-killing ; antibiotics.

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Acknowledgements

I am gratefully indebted to my supervisor Professor M.R.W. Brown for all his help, encouragement and advice throughout the course of my research.

My thanks are due to Dr. P.A. Lambert for many helpful discussions. Also to Drs. R.J. Jones and E.A. Roe for their help and permission to include the immunological data concerning the *Klebsiella* vaccine.

I also wish to thank my colleagues in the Microbiology Research Group, particularly Mr. R. Tilling for his expert technical assistance.

I would like to gratefully acknowledge the University of Aston for financing this research project.

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ABBREVIATIONS

A_{470}	Absorbance at 470nm
AAA	atomic absorption spectrophotometry
AS	ankylosing spondylitis
CDM	chemically defined medium
CM	cytoplasmic membrane
°C	degrees centigrade
EDTA	ethylene diamine tetra-acetic acid
EGTA	ethylene glycol-bis-(β-aminoethylether)-N:N'-tetra-acetic acid
g	gramme
hr	hour
i.p.	intraperitoneal
IRMP	iron-regulated membrane proteins
KDO	2-keto-3-deoxyoctonic acid
L	litre
LPS	lipopolysaccharide
M	moles per litre
µg	microgramme
µg/ml	micrograms per millilitre
µl	microlitre
µm	micrometre
mm	millimetre
mg	milligramme
mg/ml	milligrammes per millilitre
min	minute
MIC	minimum inhibitory concentration
MLD	minimum lethal dose

M_r	molecular weight
nm	nanometres
OM	outer membrane
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PMN	polymorphonuclear leucocyte
rpm	revolutions per minute
s	seconds
SDS	sodium dodecyl sulphate
v/v	volume by volume
w/v	weight by volume

1. INTRODUCTION

1.1. *Klebsiella* - Taxonomy and Nomenclature

The genus *Klebsiella* was created in 1852 to honour the German bacteriologist Edwin Klebs (1834-1913). It contains non-motile gram-negative bacilli that conform to the definitions of the family Enterobacteriaceae and tribe Klebsiellae (Richard,1982; Edwards & Ewing,1972). A notable characteristic of this genus is the production of large moist colonies on solid media which are often very mucoid. *Klebsiella* species are opportunistic pathogens which are found in the gastro-intestinal tract of man and in a wide variety of environmental situations such as soil, vegetation and water (Edmondson, Cooke, Wilcock & Shinebaum, 1980)

There is some disagreement about the nomenclature of the species which form this genus. Cowan (1974) in a brief review of the taxonomy of Klebsiella recognized seven named species: *K.aerogenes; K.pneumoniae (sensu stricto); K.oxytoca; K.atlantae; K.edwardsii; K.ozanae* and *K.rhinoscleromatis*. Orskov (1974) however, described only three species including *K.pneumoniae (sensu lato), K.ozanae* and *K.rhinoscleromatis*. Thus publications in the United Kingdom tend to refer to *K.aerogenes* (encompassing *K.aerogenes, K.oxytoca, K,atlantae* and *K. edwardsii*) and *K.pneumoniae (sensu stricto)* in conflict with the single species *K.pneumoniae (sensu lato)* described in most American and Western European literature (Barr,1977). More recently

two new *Klebsiella* species have been identified, *K.planticola* and *K.terrigena* (Richard,1982), both of which are found predominantly in non clinical environments.

Throughout this thesis the preferred nomenclature is that used in the United Kingdom.

1.2. Epidemiology and Clinical features of *Klebsiella* Infections

Infections with Klebsiella species have characteristic epidemiological and clinical features and the importance of this genus in nosocomial infections, particularly in the hospital environment, has been stressed in a number of reports (S.Young, 1982; L.S.Young, 1982; O'Callaghan,Rousset & Harkess, 1978; Rennie & Duncan, 1974; Price & Sleigh, 1970; Smith, Digori & Eng, 1982; Ullmann, 1983).

Multi-drug resistant bacteria complicate the clinical treatment of infections by reducing the number of potentially useful chemotherapeutic agents. A large number of human *Klebsiella* isolates are resistant to a wide range of antibiotics and up to 80% of these resistant strains carry transmissible "R" plasmids (Talbot,Yamamoto,Smith & Siedler,1980; Richards,Datta & Hughes,1981).

The urinary tract is the most common site of nosocomial infection caused by *Klebsiella* species (Rennie & Duncan,1974) but major life threatening infections such as septicaemia, meningitis and bronchopneumonia are also encountered (Montgomery & Ota,1980; L.S.Young,1982). Patients particularly at risk to these more serious conditions are those with respiratory damage, renal or hepatic diseases or those who have been compromised by burning, immunosuppression or other invasive or manipulative procedures (Montgomerie & Ota, 1980; Casewell,Dalton,Webster

& Philips,1977; Montgomerie,1970; Price & Sleigh 1970). *Klebsiella* is causing infections in cancer patients with increasing frequency and its emergence has been associated with effective control of *Staphylococcus* and *Pseudomonas* in those patients whose immunological defences have been compromised by radiotherapy or cytotoxic chemotherapy. The incidence and severity of infection in patients with acute leukaemia is inversely related to the number of circulating neutrophils. The prognosis in such neutropenic patients is poor despite effective antibiotics (Umsawadi,Middleman,Luna & Body,1973). Montgomerie (1980) has shown that mortality due to Klebsiella bacteraemia was influenced by the underlying disease, age of the patient and the site of initial infection. In patients with non fatal underlying diseases, deaths only occurred in patients with pulmonary or abdominal infections and did not occur in patients in whom the portal of entry was the urinary tract or an intravenous site. Therapy with an aminoglycoside antibiotic was adequate in this group. In patients suffering chest or abdominal infections, the mortality rate was 45% despite antibiotic treatment.

Klebsiella has also been implicated in the aetiology of the inflammatory arthritic disease ankylosing spondylitis (AS). It was shown that certain *Klebsiella* antigens cross react with an HLA-B27 associated cell surface marker on the lymphocytes of about 80% of patients with AS (Sullivan, Upfold, Geczy, Bashir & Edmonds, 1982).

Despite the availability of good typing methods such as capsular serotyping (Palfreyman, 1978) and bacteriocine typing (Bauernfeind, Petermuller & Schneider, 1981), the epidemiology of *Klebsiella* is not fully understood. Also, little is known of the significance of environmentally derived strains in causing infections in hospital patients (Edmondson,Cooke,Wilcock & Shinebaum, 1980). A number of workers reported a correlation between capsular type and the site of isolation from the body (Riser & Noone,1981; Smith *et al* ,1982) but there appears to be no correlation between site of isolation and antibiogram for a given capsular type (Smith *et al* ,1982). In United Kingdom hospitals, *Klebsiella* types 2,9 and 21 appear to predominate. Type 2 strains have been responsible for major epidemics in both the United Kingdom and the United States of America (Riser & Noone, 1981).

1.3. Composition and Structure of the Cell Envelope and Capsule of Gram-negative Bacteria.

The bacterial cell envelope is a complex organelle interposed between the cytoplasm and the environment, serving both to protect the cell and to regulate the uptake of essential nutrients. The cell envelope of gram-negative bacteria consists of two cell membranes separated by a periplasmic space and a monolayer of peptidoglycan (Fig.1). It is capable of undergoing dramatic compositional alterations in response to environmental changes (Nikaido & Nakae, 1979; Lugtenburg & van Alphen, 1983; Inouye, 1979).

The inner or cytoplasmic membrane (CM) contains phospholipids and proteins. It contains all the known active transport systems and many of the cell envelope enzymes and it acts as an anchor for DNA at least during replication.

The peptidoglycan layer which surrounds the cytoplasmic membrane is a rigid cross linked polymer which enables the cell to withstand the osmotic pressure of the cytoplasm and is partly responsible for the rod shape of gram-negative bacteria.

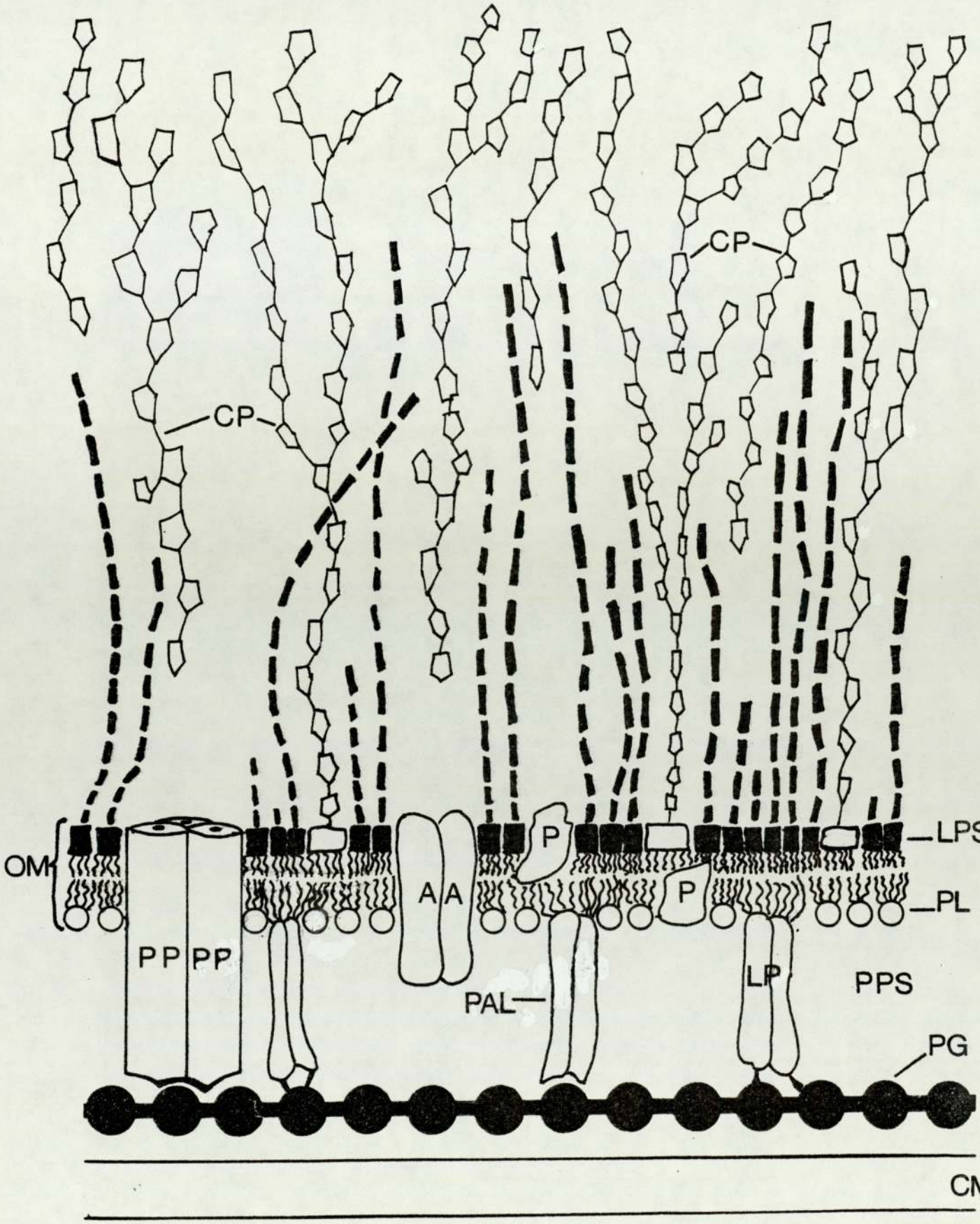
The outer membrane (OM) which contains lipopolysaccharide (LPS) in addition to phospholipid and protein is covalently linked to the peptidoglycan via a lipoprotein.

Figure 1. Diagrammatic Representation of the Cell Envelope of an Encapsulated Gram-Negative Bacterium

The postulated anchorage of the capsular polysaccharide to the OM by means of the lipid components described by Jann and Jann (1983) and by Gotschlich *et al* (1981) has been incorporated into the diagram.

Key:

OM	Outer Membrane
CM	Cytoplasmic Membrane
PPS	Periplasmic Space
PG	Peptidoglycan
CP	Capsular Polysaccharide
LPS	Lipopolysaccharide
PL	Phospholipid
PP	Pore Protein
LP	Lipoprotein
PAL	Peptidoglycan-Associated Lipoprotein
A	Omp A Type Protein


CP
CP
CP
OM
PP PP
A A
P
P
PAL
LP
PPS
LPS
PL
PG
CM

The two membranes are interconnected by the so called "zones of adhesion", about 200-400 of these sites are present per cell covering some 5% of the membrane surface (Bayer, 1979).

The OM is not always the outer layer as the cell envelope is often covered with a polysaccharide and/or protein layer.

The outer membrane and capsule of the Enterobacteriaceae play important roles in conferring protection against antimicrobial agents and host defence mechanisms such as serum complement and phagocytosis. Their molecular composition and supramolecular organisation with particular reference to *Klebsiella* and the Enterobacteriaceae will therefore be discussed in detail.

1.3.1 Molecular Composition of the Outer Membrane

The outer membrane has a thickness of 7.5nm as determined by electron microscopy (Glauert & Thornley,1969). It contains protein (9-12% of the total cellular protein), LPS and phospholipids as its major constituents. Enterobacterial Common Antigen (ECA) which is shared by almost all the Enterobacteriaceae, is a minor component (Wicken & Knox, 1980). ECA is a linear polymer of 1,4 linked N-acetyl-D-glucosamine and N-acetyl-D-mannosaminuronic acid, esterified to a small extent by palmitic and acetic acids (Wicken & Knox, 1980).

1.3.1.1. Phospholipids

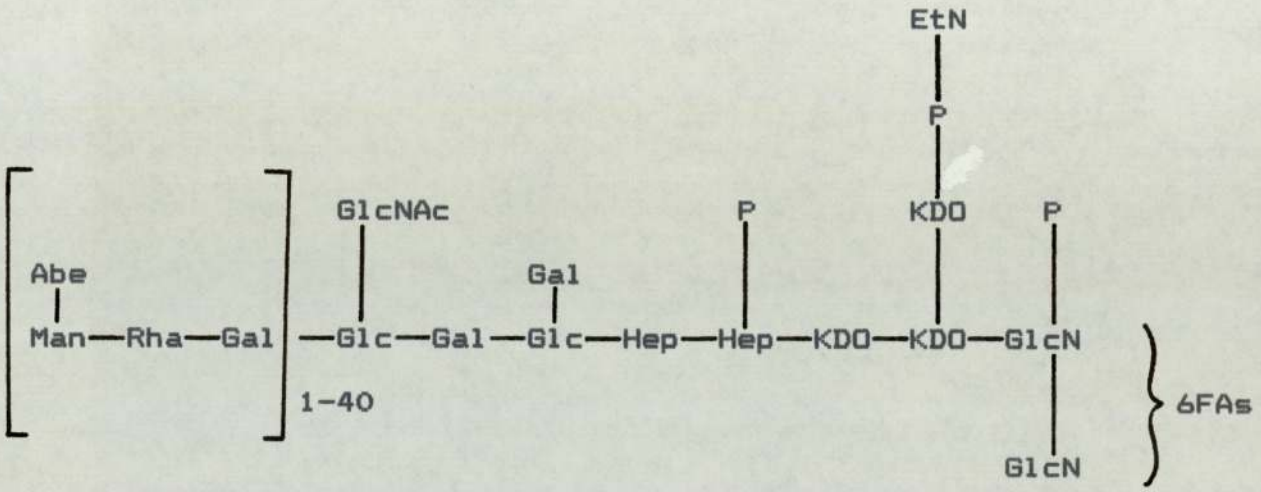
The phospholipids of the Enterobacteriaceae are all located in the cell envelope and in general are closely related to those of *E.coli* (Cronan, 1979). Phosphatidylethanolamine (PE) is the major species but substantial amounts of phosphatidylglycerol (PG) and diphosphatidylglycerol (cardiolipin) are also found. The qualitative phospholipid composition of the two membranes is very similar but the OM is enriched with PE when compared with the CM (Osborn, Gander, Parisi, & Carson, 1972; Lugtenburg & van Alphen, 1983). This may be explained by the ability of PE to form stable bilayers with LPS (Fried & Rothfield, 1978). It is important to realise that the phospholipid composition of bacterial membranes can vary with the growth conditions. In particular, phosphate limitation of growth results in the replacement of phospholipids by neutral lipids and fatty acids (Minnikin *et al*, 1974; Noy, 1982).

1.3.1.2. Lipopolysaccharide

The OM of gram-negative bacteria contains LPS, a unique component which is located exclusively in the outer leaflet of the OM. LPS is an amphipathic molecule consisting of three distinct regions covalently linked, namely: the O specific polsaccharide sidechain, the R specific polysaccharide core, and lipid A (Westphal, Jann & Himmelsbach, 1983; Wicken & Knox, 1980) (Fig.2a).

Figure 2 (a). Structure of Lipopolysaccharides from strains of *Salmonella typhimurium* (Nikaido & Nakae, 1979).

Key:		
	Abe	Abequose
	Man	D-Mannose
	Rha	Rhamnose
	Glu	Glucose
	Gal	Galactose
	GlcNAc	N-acetyl-D-glucosamine
	Hep	L-glycero-D-mannoheptose
	P	Phosphate
	KDO	2-keto-3-deoxyoctonic acid
	EtN	Ethanolamine
	FA	Fatty acid
	Ra-Re	Strains of *Salmonella typhimurium*


EtN
P
GlcNAc
P
KDO
P
Abe
Gal
Man—Rha—Gal
—Glc—Gal—Glc—Hep—Hep—KDO—KDO—GlcN
1-40
6FAs
GlcN

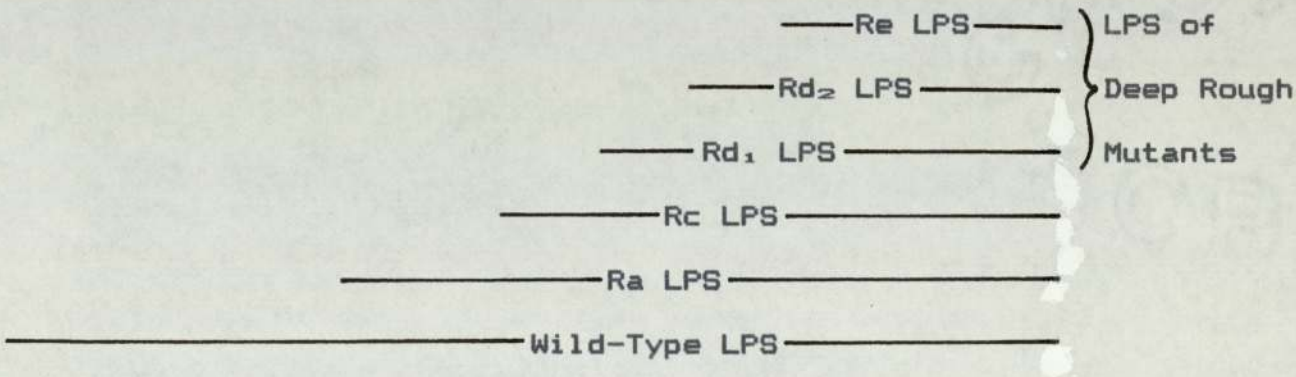
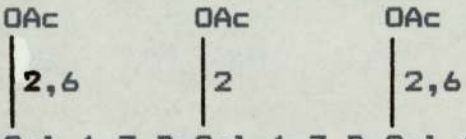
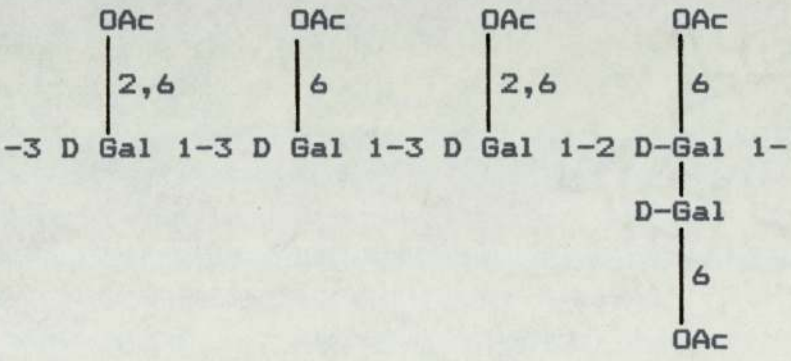

Re LPS
Rd₂ LPS
Rd₁ LPS
LPS of
Deep Rough
Mutants
Rc LPS
Ra LPS
Wild-Type LPS

Figure 2(b). The Chemical Composition of the *Klebsiella* O Antigens.

The eight *Klebsiella* lipopolysaccharides so far characterised (Mizuta *et al*,1983) consist of a common core containing KDO, hexoses and heptose although the amount of hexose relative to KDO can be relatively low (Wilkinson, S.G.,1977). *Klebsiella aerogenes* NCTC 5055 contains the O1 type O antigen which is unusual in that in contrast to *Salmonella species and E.coli*, its core polysaccharide does not contain galactose (Poxton & Sutherland, 1976).

Key: Gal Galactose
Man Mannose
Rib Ribose
Rha Rhamnose
OAc O Acetyl Group
GlcNAc N-acetyl-D-glucosamine

O Antigen Type	Partial Structure of the Repeating Unit
O1	-3 D-Gal 1-
O3	-2 D-Man 1-3 D-Man 1-3 D-Man 1-2 D-Man 1-2 D Man 1-
O4	-4 D-Gal 1-2 D-Rib 1-
O5	-3 D-Man 1- 2 D-Man 1-3 D Man 1-2 D-Man 1-2 D-Man 1-
O7	-2 L-Rha 1-2 D-Rib 1-3 L-Rha 1-3 L-Rha 1-
O8	
O9	
O12	-3 D GlcNAc 1-3 L Rha 1-

The O antigen is a long chain polysaccharide built up from repeating units of oligosaccharides containing between 1 and 7 sugars per unit. Enterobacterial O antigens can be homopolysaccharides or heteropolysaccharides with or without short chain branches (Westphal *et al* ,1983). Twelve *Klebsiella* O antigens have been proposed (Nimmich & Korten, 1970) but further structural and immunological studies revealed that some were identical (Mizuta *et al*, 1983). Eight *Klebsiella* O antigens have now been identified (O1;O3;O4;O5;O7;O8;O9;O12) (Mizuta *et al*, 1983). O specific polysaccharides were initially thought to contain about 10-15 repeating units. However it has been shown by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of LPS preparations that in most smooth (S) *E.coli* strains, part of the LPS core is not substituted with an O specific chain and LPS preparations from a given smooth strain are heterogeneous with respect to the length of the O specific chains. Thus there always appears to be a population of LPS entities differing by molecular weight increments equivalent to the number of repeating units (Goldman & Leive, 1980; Munford *et al* 1980; Tsai & Frasch, 1982; Palva & Makela, 1980). In SDS-PAGE this gives rise to a ladder-like pattern. Most LPS preparations are therefore mixtures of molecules with O specific polysaccharides ranging from 1 up to 40 repeating units.

It has been known for some time that wild type Enterobacteriaceae growing as smooth colonies on agar may undergo a spontaneous change to rough mutants (Westphal *et*

al 1983). The morphological smooth to rough mutation is combined with the disappearance of the O antigen leaving an LPS consisting of only core oligosaccharide bound to lipid A. This core polysaccharide consists of an inner and outer region. The inner core contains heptose and keto-deoxyoctonic acid (KDO) while the outer core contains glucose, galactose and in some cases N-acetyl glucosamine. KDO links the core polysaccharide with lipid A via an acid labile bond (Wicken & Knox,1980). This core region is generally regarded as being of constant composition for smooth strains from a particular genus although differences occur in the core structures of different genera.

The lipid A regions of LPS from a wide range of gram-negative bacteria share a general structural similarity in which there are phosphatidyl glucosamine residues forming a dissaccharide to which are attached fatty acids by ester and amine bonds (Westphal *et al* 1983). The lipid A moeity of LPS is responsible for many of the endotoxic properties of LPS (Wicken & Knox, 1980).

1.3.1.3. Outer membrane proteins.

A comparison of the CM and OM preparations by SDS-PAGE shows relatively few, but often heavy, bands in the case of the OM. This does not necessarily mean that there are only a few OM proteins as some may be present in amounts just below the level of detection whereas others are inducible and only

appear in detectable amounts under certain growth conditions. The OM of *E.coli* K12 is regarded as characteristic in that it contains 3 major classes of proteins: the porin proteins, Omp A protein and lipoprotein. The definition "major protein" is rather arbitrary as a minor protein may become a major protein when it is fully induced (Lugtenburg & van Alphen,1983).

1.3.1.3.1. Lipoprotein.

Braun and his co-worker (1969) purified an *E.coli* OM lipoprotein which was covalently bound to peptidoglycan and of M_r 7,200. This bound form of the molecule is present in approximately 2.4 x 10^5 copies per cell (Braun, 1975). However it was subseqently observed that twice as many copies of the same lipoprotein exist in a free form i.e. not bound to peptidoglycan (Inouye, Shaw & Shen, 1972). The lipoprotein in terms of number of copies is by far the most abundant cell protein. Lipoproteins have also been found in all the Enterobacteriaceae so far studied as well as in *P.aeruginosa* (Lugtenburg & van Alphen,1983). Its presence is not essential for survival of the cell, although a lipoprotein deletion mutant has severe defects such as an increased production of OM vesicles, increased sensitivity to EDTA and leakage of periplasmic enzymes. When in addition to the lipoprotein, OmpA is also missing, cells are unable to grow in the rod form and require high concentrations of calcium and magnesium for growth (Sonntag, Schwartz, Hirota & Henning,1978). Moreover abundant blebbing was observed in

the double mutant and the peptidoglycan layer was no longer connected with the outer membrane (Sonntag *et al* 1978). Lipoprotein and Omp A therefore appear to be involved in the maintenance of the rod shape, in stabilization of the OM structure and in anchoring the OM to the peptidoglycan (Lugtenberg & Van Alphen, 1983).

Several other classes of lipoprotein have now been identified (Lugtenburg & van Alphen 1983) for example, the peptidoglycan associated lipoproteins (PAL), which are present in various gram-negative bacteria including *Klebsiella* (Mizuno,1981). PAL is closely but not covalently linked to peptidoglycan, it does not immunologically cross react with Brauns lipoprotein and has an M_r in *E.coli* and *Klebsiella* of about 21,000.

1.3.1.3.2. Omp A protein.

The Omp A protein of *E.coli* is present in approximately 10^5 copies per cell. It is rich in beta structure (Nakamura & Mizushima,1976) and is heat modifiable i.e. its M_r on SDS polyacrylamide gels is higher (35,000) after denaturing in SDS by heating than in its non-denatured form (28,000). A protein cross reacting with Omp A was detected in all strains of *E.coli* and in all the Enterobacteriaceae tested including *Klebsiella* (Hofstra & Dankert,1979; Lugtenburg & van Alphen,1983). Omp A does not form tight complexes with peptidoglycan but some Omp A is covalently linked to the diaminopimelic acid residues of the peptidoglycan layer in

stationary phase cells (Diedrich & Schnaitman,1978). Omp A plays a role in F pilus mediated conjugation apparently by stabilizing mating aggregates (Lugtenburg & van Alphen, 1983). Together with Brauns lipoprotein, it is also involved in maintaining both the structural integrity of the OM as well as the rod shape of the cell (Lugtenburg & van Alphen,1983).

1.3.1.3.3. Peptidoglycan-associated pore proteins.

Porin proteins are characterised by their tight but non-covalent association with peptidoglycan and high beta structure content (Inouye,1979). Electron micrographs of negatively stained porin protein peptidoglycan complexes show that the porin protein molecules are arranged as a hexagonal lattice layer with a 7.7nm repeat (Rosenbusch,1974; Steven *et al* 1977). There are approximately 1.5×10^5 molecules of porin protein per cell and the hexagonal layer covers some 60% of the outer surface of the peptidoglycan layer (Steven *et al* 1977). *E.coli* K12 contains two peptidoglycan associated proteins known as Omp C (M_r 36,000) and Omp F (M_r 37,000) which are immunologically related. All strains of the Enterobacteriaceae so far tested produce at least one peptidoglycan associated protein which cross reacts with the *E.coli* proteins (Lugtenburg & van Alphen, 1983). The family of peptidoglycan associated pore proteins is even larger if one takes into account that not all proteins are constitutively present but some are induced under certain

growth conditions only (Overbeeke & Lugtenburg,1980; Hancock & Carey,1980) or are coded for by a phage (Reeve & Shaw,1979) or by a plasmid (Iyer,1979; Achtman,Kennedy & Skurray,1977).

To explain the impermeability of the enterobacterial OM to bile salts and its excellent permeability for nutrients and other molecules with an M_r of up to 600, Nikaido and Nakae (1979) developed the concept of water-filled transmembrane pores. By incorporating porin proteins into artificial LPS-phospholipid bilayers, they showed that these artificial vesicles had the same molecular sieving properties as does the intact OM. Their results indicate that the function of the pore proteins is to form passive diffusion pores and because of this property they are called "porins"(Nikaido, 1979).

The diffusion rate of solutes is determined by the difference in concentration at the two sides of the membrane and by factors such as solute size, charge and hydrophobicity. In the Enterobacteriaceae, oligosaccharides of M_r 600-700 are the largest molecules which can diffuse through a pore channel. This size limit corresponds to a pore diameter of 1nm (Nikaido,1979). Pore diameters have also been calculated from conductivity measurements through black lipid films and shown to be of 0.9-1.4nm in diameter (Nikaido,1979; Schindler & Rosenbusch,1978; Benz & Hancock,1981). An important feature of the porin protein channels is that they exist as trimers associated with LPS

in their biologically active forms. in E.coli they have been shown to be homologous and heterologous combinations of Omp F and Omp C (Ichihara & Mizushima, 1979) which are sufficiently large to span the thickness of the OM (Tokunuga *et al* ,1979)

These porins exist in either the open or closed state and are in equilibrium with each other. A functional pore consists of 3 monomers and it appears that each monomer can form a pore and that the opening of the 3 channels within a trimer is a highly co-operative phenomenon (Lugtenburg & van Alphen,1983; Nakae,Ishii & Tokunaga,1979).

1.3.1.3.4. Other outer membrane proteins

When grown under conditions where iron is limiting gram-negative bacteria de-repress the synthesis of several OM proteins which in *E.coli* and *S.typhimurium* have M_r between 74,000 and 83,000 (Griffiths,1983). *In vivo* during an infection with *E.coli* these proteins are present in quantities equal to or greater than the so called major OM proteins (Griffiths *et al*,1983). Their function and regulation will be discussed in sections 1.5.2.1.and 5.1.

The bacteriophage lambda receptor protein (M_r 47,000 in *E.coli*) is induced in the presence of maltose and is an essential component for the lambda phage (Konisky,1979). It is involved in the uptake of maltose at low ($<1\mu M$) but not high (1mM) substrate concentrations (Szmelcman & Hofnung,

1975). After induction it becomes a major OM protein comparable in abundance with the pore proteins (Braun, Krieger & Brauer, 1977). It is peptidoglycan associated and appears to facilitate the diffusion of other nutrients (Braun *et al*,1977; Nakae & Ishii,1980). A similar protein of M_r 44,000 which cross reacts with the *E.coli* lambda protein has been identified in *Salmonella* (Palva,1979).

Vitamin B12 (M_r 1,327) is too large to pass through the general diffusion pores and requires a specific OM protein (M_r60,000 in *E.coli*) to facilitate its translocation across the OM. This protein is also the receptor for phage BF23 and the E. colicins. The cell can be protected from killing by phage and colicin by the binding of vitamin B12 to the receptor protein (Konisky,1979).

Several other OM proteins with as yet unidentified functions have also been reported present in *E.coli*. Examples include protein III (Hindenach & Henning,1975; Lugtenburg & van Alphen,1983); LPS binding protein (Geyer,Galanos,Westphal & Golecki,1979);proteins induced by sulphate limitation (Lugtenburg & van Alphen,1983); various phage and plasmid coded OM proteins (Lugtenburg & van Alphen,1983).

The OM unlike the CM is poor in enzymic activity. The first OM enzyme detected was a phospholipase A1 of M_r 28,000 (Bell,Mavis,Osborn & Vagelos, 1971; Nishijima,Nakaike,Tamori & Nojima,1977). Other OM enzymes have since been detected (Lugtenburg & van Alphen,1983) including one which modifies

the ferric enterochelin receptor (Fiss, Hollifield & Neilands, 1979).

1.3.2. Molecular Organization of the Outer Membrane.

Nikaido and Nakae (1979) provisionally estimated that the outer surface of the OM of gram-negative bacteria consisted of 41% LPS and 59% protein and the inner surface, 58% phospholipid and 47% protein. However, Lugtenburg and van Alphen (1983) calculated that the outer layer of the OM consisted of 68% LPS and 32% protein with the inner layer containing 46% phospholipid, 50% protein and 4% lipoprotein. Differences in the calculated surface area occupied by LPS, the LPS:protein:phospholipid ratio and in the strain and growth conditions used, were held responsible for the discrepancies. It is therefore important to realise the profound effect that the prevailing growth conditions will exert on the composition of the OM, making it difficult to make any meaningful generalisations concerning its overall composition.

Studies utilizing ferritin labelled antibodies (Muhlradt & Golecki, 1975) and exogenous galactose oxidase (Funahara & Nikaido,1980) directed against the O antigen of LPS showed that LPS is indeed located exclusively on the outer leaflet of the OM. A substantial proportion of the LPS molecules in

the OM form tight complexes with OM proteins, the *in vitro* biological activity of which, is often dependent on the presence of the LPS (Lugtenburg & van Alphen, 1983). On the other hand, the phospholipids of the wild type cells are located only on the inner surface of the OM where they are inaccessible to detergents and bile salts. "Re" mutants (which lack the polysaccharide moeity of LPS (Fig.2a)) and pore protein deficient mutants are sensitive to these and other hydrophobic agents. They possess increased amounts of phospholipid which are probably located in the outer leaflet of the OM (Tamaki & Matsuhashi,1973; Wilkinson,Gemski & Stocker, 1972; Smit, Kamio & Nikaido,1975). Additional evidence has been provided by electron-spin resonance studies which have clearly shown that for both OMs and model bilayers, phospholipids and LPS are segregated into separate domains with the vast majority of phospholipid molecules localized in the inner layer of the OM (Nikaido, Takeuchi, Ohnishi & Nakae, 1977; Takeuchi & Nikaido, 1981).

Between 40-75% of the surface area of gram-negative bacteria is covered by protein. There are some 6.5 x 10^5 molecules of porin protein per cell (Steven *et al* ,1977) and about 10^5 molecules per cell of Omp A (Lugtenburg & van Alphen,1983). Both types of protein have sites exposed on the surface of the OM (Kamio & Nikaido,1977). Neither the bound or the free form of lipoprotein appears to be present on the outer surface of the OM (Lugtenburg & van Alphen,1983).

Freeze-etch electron microscopy of the surface of *E.coli* showed numerous randomly spaced depressions of about 4.5nm in diameter which were suggested to be the entrances to the aqueous pores (van Alphen *et al*,1978; Bayer & Leive,1977). Freeze-fracture methods applied to the interior of the OM showed that it could be cleaved into two halves suggesting a lipid bilayer structure (Smit,Kamio & Nikaido,1975; Verkleij,Lugtenburg & Ververgaert,1976). The two leaflets differ clearly in that the concave or outer fracture face is covered with particles 4-8nm in diameter whereas the convex or inner fracture face contains pits which are probably complementary to the particles. Particles and pits are considered to be reflections of complexes between LPS and proteins such as Omp C, Omp F, Omp A and Lam B (Lugtenberg & van Alphen,1983).

Divalent cations play an important role in maintaining the integrity of the OM. Some of the proteins present in large numbers exposed on the outer surface of the OM are strongly acidic (Schmitges & Henning,1976) and the hydrophilic portion of LPS is anionic. There would therefore be powerful electrostatic repulsion between these compounds providing a large potentially destabilising force in the OM. Divalent cations, particularly calcium and magnesium are known to be necessary to maintain the integrity of the OM as EDTA treatment of *E.coli* (Leive,1965) and *P.aeruginosa* (Rogers *et al* ,1969) releases predominantly LPS and LPS-protein complexes from the two organisms respectively. These

divalent cations probably act by neutralising and bridging the anionic groups. Boggis *et al* (1979) and Kenward *et al* (1979) produced evidence to show that these cationic bridges stabilising the OM of *P.aeruginosa* are the common initial site of action of EDTA and polymyxin B. However, their effect is not complete as under a variety of growth conditions, the phenomenon of "blebbing" has been observed (de Petris, 1976; Smit *et al*, 1975). This may be caused by the strong electro-static repulsion at the outer surface between membrane components and it has been suggested that the Braun lipoprotein serves as an anchor pulling the OM down to the underlying peptidoglycan (Nikaido & Nakae, 1979). *E.coli* mutants lacking the Braun lipoprotein and Omp A produce very large blebs in the outer membrane. They are unable to grow in the rod form and required high concentrations of magnesium and calcium in order to multiply (Sonntag, Schwartz, Hirota & Henning, 1978). Thus it appears that gram-negative bacteria maintain their highly asymmetric OM depending, at least in part, on the divalent cations and the interaction between lipoprotein and peptidoglycan.

It is important to note that the loss of an OM constituent brings about a re-organisation of the molecular make-up of the OM. The lack of a major protein or a defect in LPS structure will result in alterations in the protein, LPS and

phospholipid composition of the OM (Lugtenburg & van Alphen,1983).

1.3.3. Structure and Function of Extra-cellular Polysaccharides.

Many gram-negative and gram-positive bacteria produce extra-cellular polysaccharides (exopolysaccharides). Exopolysaccharides together with the O antigens constitute the primary immunogens and antigens of bacteria because of their location on the extreme outer surface of the cell. They will therefore influence the way in which the bacterial cell recognises and interacts with its environment and thus cell-host, cell-virus, cell-cell or cell-effector (e.g. antibody) interactions are determined to a major extent by the presence of a capsule (Troy,1979).

Exopolysaccharides may exist in the form of a discrete capsule surrounding the bacterial cell or in the form of a loose slime, unattached to the cell surface. Capsules can be visualised by the India ink staining technique (Wilkinson,1958) or by electron microscopy after reaction with specific antibody (Bayer & Thurow,1977) or ferritin conjugated antibody (Kasper & Baker,1979).

Klebsiellae are predominantly encapsulated organisms surrounded by a firmly adhering polysaccharide coat, more than 80% of which sediments with the cell on centrifugation (Troy,Frerman & Heath, 1971). This capsular material or K antigen exhibits specific antigenic properties and the classification of *Klebsiella* is based on the serological

reactions of the capsule (Palfreyman,1978). 77 capsular types have been identified (1-72,74,79-82) (Richard,1982). Many of these capsular polysaccharides have been completely characterised chemically in the quest to correlate primary molecular structure with immunological activity.

The polysaccharides of *Klebsiella* are complex heteropolysaccharides made up of repeating units which may be linear structures (with from 2-6 sugars), single and double branched units with from mono- to tetra-saccharide side chains. Some examples are given in fig.3.

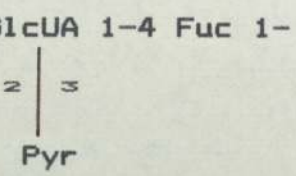
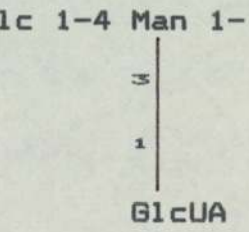
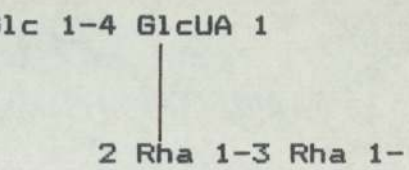
Many *Klebsiella* polysaccharides are acidic and therefore negatively charged mainly through the uronic acid and pyruvic substituents which provide additional complexity to their molecular structure (Powell,1979).

It has recently been reported that the capsular polysacharides of *E.coli* and *N.meningitidis* are linked to a lipid which was shown to be a phosphatidic acid (Jann,Jann,1983; Gotschlichet al ,1981). In the K12,K13 and K14 polysaccharides of *E.coli* the phosphatidic acid is linked to the polysaccharide through KDO whilst those which do not contain KDO in the repeating unit are also substituted with a phosphatidic acid at the reducing end of the polysaccharide chain. There is evidence that the reducing sugar constituents of all these polysaccharides is KDO (Schmidt & Jann,1982). The functional role of the lipid is not yet clear but it has been suggested to be involved in

Figure 3. The Composition of some *Klebsiella* Exopolysaccharides

Key:

Glc	Glucose
GlcUA	Glucuronic Acid
Pyr	Pyruvate
Man	Mannose
Rha	Rhamnose

Capsular Type	Components	Structure
1	Fucose, Glucose, Glucuronic Acid, Pyruvate	
2	Mannose, Glucose, Glucuronic Acid, (Various acyl groups)	
44	Glucose, Rhamnose, Glucuronic Acid	

anchoring the capsule to the OM (Jann & Jann,1983).

Although the chemical composition of many *Klebsiella* K antigens is well known numerous attempts to demonstrate their fine structure by electron microscopy have been disappointing (Roth,1977). Schmid *et al* (1981) used osmium tetroxide and glutaraldehyde/osmium tetroxide to preserve the capsular fine structure of a type 1 *Klebsiella* strain. They showed that log phase cells possessed well preserved capsules with thicknesses from 280 - 350nm. consisting of filaments with diameters between 3 and 5nm. The filaments appeared to extend from the OM almost parallel to each other and appear to interlace after the first third of their extension. In contrast, stationary phase cells exhibited shrunken capsules with coarse and short filaments projecting for approximately 100 - 180nm from the outer surface of the OM and with diameters of up to 25nm. No remarkable disconnection of the capsule at the site of presumptive cell division could be observed, the contour of the capsule running paralell to the OM i.e. the filaments have constant lengths (Schmid et al ,1981). Comparable structures have also been found in *in vivo (Nakao et al*,1981).

Little is known of the function of microbial extracellular polysaccharides. They do not appear to be essential to the viability of the cell producing them. The great majority of polysaccharide producing organisms appear to be unable to utilize their own extracellular polysaccharides as carbon sources and thus these compounds do not serve as reserve

sources of carbon and energy (Dudman,1977). Capsular polysaccharides may protect the organism from dessication although there is little direct experimental evidence for this. They are involved in the the adhesion of bacteria to surfaces. (Costerton, Irvin & Cheng,1981). As most capsules are composed of acidic polysaccharides, they must exert a profound influence on the traffic of charged molecules into and out of the cells by ion-exchange and salt formation as well as chelation and co-ordination complexes (Dudman,1977; Costerton *et al*,1981; Ombaka *et al*,1983). Extracellular polysaccharides bind metal ions and will act as barriers to prevent their entry into cells (Friedman & Dugan,1968). In addition to ion exchange functions extracellular polysaccharides whether neutral or acidic can be expected to act in varying degrees as diffusion barriers, molecular sieves and adsorbents. Dugan et al (1971) showed that bacterial polysaccharides could absorb dissolved organic materials such as proteins and amino acids and suggested that such polysaccharides may serve as a mechanism for concentrating nutrients for micro-organisms in aqueous environments especially where the level of nutrients is low.

Capsular polysaccharides may also contribute to the virulence of an organism by confering protection against phagocytic cells and serum bactericidal factors (see section 1.5.3.).

1.3.4. Effect of the Environment on the OM and Capsule of Gram-negative Bacteria.

The prevailing environmental conditions exert a profound effect on the OM and capsular polysaccharides of gram-negative bacteria. Growth temperature affects fatty acid synthesis (Garwin & Cronan,1980), oxygen metabolism (Mainzer & Hempfling,1976) and protein synthesis (Yamamaori & Yura,1980) in *E.coli*. Investigations of the effect of temperature on capsular polysaccharide production in *Klebsiella, Salmonella* and *Escherichia* species concluded that capsule production was enhanced or unaffected by temperatures below 37°C (Duguid & Wilkinson,1953). However strains of *E.coli* expressing the K1 capsule (which is frequently the causative agent of neonatal meningitis) do not synthesize their capsular polysaccharides at 22°C. Significant amounts of capsule are only observed when *E.coli* K1 is grown at temperatures of 30°C or above (Bortolussi,Ferrieri & Quie,1983).

Lugtenburg *et al* , (1976) studied the effect of growth temperature and growth medium on the production of porins in *E.coli* K12. The ratio of Omp F to Omp C was greatly influenced by the growth medium and although no clear pattern was observed, in nutritionally rich media there appeared to be far more Omp C and less Omp F present in the OM than in cells grown in glucose minimal medium. Although the amounts of the two proteins varied, the total amount

altered far less in any one strain suggesting that the system regulating porin concentration was geared to maintaining an approximately constant total amount. The effect of growth temperature on OM protein composition was less dramatic but at higher temperatures there was an increase in the concentration of the Omp C protein and a related decrease in Omp F. Again the more significant finding was the maintenance of the *status quo* in terms of the overall OM porin protein concentration (Lugtenburg *et al*,1976).

The concentration of porin proteins in the OM is also affected by the growth medium osmolarity (van Alphen *et al* ,1977; Kawaji,Mizuno & Mizushima,1979). Supplementation of the growth medium with high concentrations of NaCl, KCl, sugars or low molecular weight dextrans resulted in changes in the ratio of Omp F to Omp C leading to a reduction in the amount of Omp F and a cocommitant increase in Omp C. The reasons for these changes are not yet fully understood.

Nutrient limitation may result in the induction of new OM proteins. In *E.coli* phosphate limitation results in the expression of a 36,000 M_r protein (Phoe E) which shares many of the properties of Omp F and Omp C and is immunologically related (Overbeeke,van Scharrenburg & Lugtenburg,1980). Sulphate limitation of *E.coli* K12 resulted in the induction of 2 OM proteins of M_r 15,000 and 19,000 whose functions are as yet unidentified (Lugtenburg & van Alphen,1983). In the presence of glucose *P.aeruginosa* induced protein D1 (Hancock

& Carey,1980) whilst under magnesium limited conditions OM protein H1 is induced. The presence of H1 in the *Pseudomonas* OM appears to be correlated with a decreased susceptibility to aminoglycosides, polymyxin and EDTA (Nicas & Hancock,1983).

When confronted with an iron deficient environment enteric bacteria synthesize several new OM proteins with apparent M_rs in the 74,000-83,000 range together with low molecular weight iron chelating compounds (or siderophores) such as enterochelin (Klebba et al,1982). In *E.coli* four OM proteins of M_r 74,000, 78,000, 81,000 and 83,000 are induced. The 81,000 M_r functions as the ferric enterochelin receptor (Ichihara & Mizushima,1978), the 78,000 M_r protein is involved in the uptake of iron from the fungal siderophore, ferrichrome (Wayne & Neilands,197). The role of the 74,000 and 83,000 M_r proteins in iron transport is as yet undefined. Hancock *et al* (1976) showed that *E.coli* strains grown in the presence of 1mM citrate contain a polypeptide of M_r 81,000 that appears to be involved in citrate mediated iron uptake. The involvement of low molecular proteins in *E.coli* (Klebba *et al*,1982) and *P.aeruginosa* (Sokol & Woods,1983) has recently been reported.

In *Salmonella typhimurium*, three iron regulated OM proteins are expressed of M_r 82,000, 79,000 and 77,000 and have been designated OM1, OM2 and OM3 (Bennett & Rothfield,1976; Ernst *et al*,1978)

The yield of exopolysaccharide from microbial cultures is also dependent on the cultural conditions. The highest polysaccharide yields obtained in *E.coli* and *Klebsiella* were obtained when carbohydrate was present in excess and growth was limited by the available nitrogen,phosphorous or sulphur source (Duguid & Wilkinson,1953; Wilkinson *et al*,1954). The ratio of polysaccharide produced :cell nitrogen was highest for nitrogen limitation and lowest for sulphur limitation (Sutherland,1977). Little stimulus of polysaccharide production occured under potassium limited conditions because of antagonism between potassium ion and ammonium ion uptake under conditions of potassium limitation (Dicks & Tempest,1967). Ombaka *et al* (1983) reported that extracellular polysaccharide production by a mucoid P.aeruginosa strain varied in a complex manner with the growth conditions. Potassium limitation produced most extracellular polysaccharide whilst sulphate limitation resulted in the production of relatively low levels.

1.4. The Role of the Outer Membrane and Capsule in Drug Resistance.

The unique structure of the OM, whilst permitting the uptake of nutrients, functions as a permeability barrier preventing the penetration of many antimicrobial agents (Nikaido,1979; Lambert,1983). Wild type strains of *E.coli* and *Salmonella typhimurium* are naturally sensitive to low molecular weight hydrophilic compounds such as ampicillin ,cycloserine, neomycin and cephalothin. They are much more resistant to hydrophobic compounds e.g. novobiocin, rifampicin, actinomycin D and erythromycin, to dyes e.g. crystal violet, and to detergents e.g. bile salts and SDS. Their sensitivity to these agents is dramatically increased in strains that produce extremely defective LPS i.e. deep rough mutants (Nikaido,1979). In addition, treatment of the wild types with EDTA to remove LPS (Leive,1974) renders them sensitive to novobiocin, rifampicin and actinomycin D.

Nikaido (1979) concluded that there were two pathways by which agents can cross the OM, a hydrophilic pathway via the aqueous porin channels and a hydrophobic pathway involving diffusion across the OM bilayer. The porin mediated pathway for small hydrophilic molecules is available in rough and smooth strains, as the complete O side chains do not impede access of such molecules to the hydrophilic pores. The hydrophobic pathway is not available in wild type smooth strains either because the LPS O side chains prevent access

of the hydrophobic molecule to the outer face of the OM or because of a lack of hydrophobic patches on the OM which could act as receptor surfaces. Only in the deep rough mutants can the hydrophobic molecule approach the OM, bind and cross by a diffusion process. Apart from a lack of protecting O polysaccharide chains on the surface of rough strains, it is thought that phospholipid molecules present on the outer face might provide sites of access for hydrophobic molecules (Kamio & Nikaido,1976). The occurence of phospholipids on the outer surface of rough strains has been questioned (Shales & Chopra,1982) but it seems likely that the excision of 50% of the LPS of smooth strains by EDTA must result in a re-orientation of OM lipids and that under these conditions the cells become sensitive to large hydrophobic molecules like actinomycin D (Leive,1974).

Small hydrophilic molecules presumably traverse the OM via the aqueous porin channels. Three factors control the rate of passage of molecules through porin channels: size, hydrophobicity and charge (Lambert,1983). The exclusion limit of porin channels is around M_r 650, equivalent to a diameter of around 1.2nm and which is large enough to permit the passage of the majority of beta-lactam and aminoglycoside antibiotics (Nakae & Nakae, 1982; Lambert,1982). Small hydrophobic compounds may fail to pass through the porins as a result of the highly structured nature of the water in the pores through hydrogen bonding to ionic groups lining the channel. The passage of hydrophobic species would therefore reqire an energetically unfavourable

disturbance of the hydrogen bonding (Nikaido,1979). Charge may also affect the passage of molecules through the porins channels. Zimmerman and Rosselet, (1977) showed that the zwitterionic compound cephaloridine penetrated the OM faster than the more hydrophilic anionic compounds cephacetrile and cephazolin. Acidic groups lining the porin channel were suggested to repel the passage of the anionic cephalosporins.

As the majority of antibiotics used in the treatment of gram-negative bacterial infections are small hydrophilic molecules, their effectiveness will depend largely on the availability of the porin channels (Lambert,1983). Gram-negative bacteria require an adequate supply of nutrients for growth and cannot dispense entirely with their porins. However, porin deficient mutants derived from bacteria producing multiple porin species have been shown to aquire an increased resistance to carbenicillin,ticarillin and sulbenicillin (associated with decreased levels of Omp F porins in *E.coli*) and to cephalosporins (Sawai ,1982)(associated with the loss of proteins assumed to be porins in *Proteus mirabilis* and *Enterobacter cloace*)

P.aeruginosa provides an interesting example of intrinsic resistance due to the permeability barrier of the OM. The reason for its resistance has not been completey explained particularly in the light of the apparently large exclusion limit of its porin channels (M_r 4000-6000) (Hancock & Nikaido,1978). Zimmerman (1979,1980) has produced a mutant

strain of *P.aeruginosa* which is sensitive to a wide range of antibiotics to which its parent is resistant. It is presumed to be a permeability mutant although analysis of its OM has so far failed to reveal any major compositional differences (Angus *et al* ,1982; Kropinski *et al*,1982)

The composition of the gram-negative cell envelope is profoundly influenced by the prevailing growth conditions and consequently environmental parameters may affect the sensitivity to antimicrobial agents (Brown,1977; Dean,Ellwood,Melling & Robinson,1976). The susceptibility of *K.aerogenes* to cycloserine and to penicillin G (Sterkenburg,Flaner,Buijsman & Wouters,1983) varies with the growth rate and nutrient depletion. Chemostat grown ammonium limited cultures were much more susceptible to cycloserine than phosphate limited cells and increasing the growth rate increased the sensitivity of both phosphate and ammonium limited cultures. The sensitivity of glucose limited cells appeared to be independent of growth rate. The authors correlated the phenotypic changes in the susceptibility of *K.aerogenes* to cycloserine with variations in its uptake system for the antibiotic.. Iron limited *K.aerogenes* was much more resistant to penicillin G than when ammonium limited. However, since there was no de-repression of beta-lactamase, no major alterations in the penicillin binding protein pattern and the altered OM protein profile appeared to have no effect on the OM permeability, the factors involved in the resistance of iron limited

K.aerogenes to penicillin G were unclear (Sterkenburg,Flaner,Buijsman & Wouters,1983).

The resistance of magnesium deficient *P.aeruginosa* to gentamicin, polymyxin B and EDTA has been associated with the replacement of magnesium ions by protein H1 (Nicas & Hancock,1983). However, other mutants which are resistant to polymyxin B do not display elevated levels of H1 so different mechanisms of resistance must be involved (Gilleland & Lyle,1979; Conrad & Gilleland,1981).

The capsule and exopolysaccharides of many gram-negative bacteria may constitute a further potential barrier to the penetration of antibiotics. These polysaccharides have been shown to figure prominantly when cells are grown *in vivo* or isolated from natural environments and viewed under the electron microscope (Costerton *et al*,1981; Costerton & Marie, 1983). A number of workers have speculated whether the presence of these polysaccharides interferes with antibiotic penetration (Costerton,Brown & Sturgess,1979, Slack & Nichols,1981; Marks,1981) by acting as a static layer through which antibiotics must diffuse before reaching the cell wall, and by providing some frictional resistance to diffusion. Many bacterial extra-cellular polysaccharides are negatively charged and therefore incoming positively charged molecules including antibiotics would have to saturate the binding sites of the matrix before penetrating to the bacterial surface (Slack & Nichols,1982) The

diffusion of a charged antibiotic may be reduced with an oppositively charged matrix although the mechanism of this is uncertain (Slack & Nichols,1982). The positively charged aminoglycosides are potentially subject to this effect within hydrated *P.aeruginosa* exopolysaccharides (Slack & Nichols,1981). However the trapping of antibiotics within the capsule could lead to a high local concentration around the cells which would then be available for transport into the cells if any subsequent dissociation of bound antibiotic occurred. Capsular polysaccharides and exopolysaccharides do not appear to restrict the uptake of nutrients since encapsulated strains are capable of multiplying as rapidly as unencapsulated strains. They do not present a physical barrier against the penetration of low molecular weight hydrophilic compounds and therefore much uncertainty still surrounds the contribution of polysaccharide matrices to antibiotic resistance.

1.5. Role of the Outer Membrane and Capsular Polysaccharides in Infection

To cause infection a pathogen must enter the host, multiply in host tissues, resist or stimulate host defences and damage the host. The microbial products responsible for these processes are the determinants of pathogenicity and many of them are surface components. Since pathogens must accomplish all four processes to produce disease, and as each stage is complex, several determinants are usually involved in the overall effect, and the lack of one member of the full complement may result in the considerable attenuation of the strain concerned (Smith,1976).

1.5.1. Attachment of Bacteria to Mucosal Membranes.

The first step in animal or human infection by virulent bacteria is usually attachment at the site of an injury or to a mucous membrane and it is therefore logical that specific structures on the OM should play a part. The mucosal membranes usually invaded by micro-organisms are those in the oro-pharyngeal area and the respiratory, gastro-intestinal and urinary tracts (van Furth,1981). Many studies of the specific interactions between surface components and host tissues have been made to elucidate the mechanisms of selective adherence of some species in certain areas of the host e.g. in the human oral cavity,

Streptococcus mutans adheres to the teeth whilst *Streptococcus salivarus* adheres to the tongue (Gibbons & van Hauter,1975). Attempts have been made to correlate *Klebsiella* capsular type with the site of infection or isolation (Riser & Noone,1981). A predilection of certain types for particular sites of infection has been demonstrated leading to the conclusion that the specific site where the infection occurs may also be a factor contributing to the virulence of a *Klebsiella* strain.

Filamentous bacterial appendages known as "pili" which are anchored in the OM of gram-negative bacteria play a role in a variety of infectious diseases. The K88 antigen of an *E.coli* which causes a diarrhoeal disease in swine, is a pilus-like structure (Jones,G.W. *et al*,1980) and is required for the adherence of *E.coli* to gut epithelial cells. Its loss renders the organism avirulent.

Type 1 pili found on members of the Enterobacteriaceae act as haemagglutinins of various mammalian erythrocytes (Duguid,Clegg & Wilson,1979). They also serve as attachment mechanisms of *E.coli* and *Klebsiella* in the urinary tract and *Salmonella typhimurium* in the gastro-intestinal tract (Fader *et al*,1982).

1.5.2. Iron and Infection

Iron availability is of major importance in bacterial pathogenesis as nearly all bacteria require this essential nutrient which is involved in many biological reactions crucial to life (Neilands,1974). The body iron of vertebrate hosts is mainly located intracellularly in compounds such as haemoglobin, haemosiderin and ferritin. In extracellular fluids, the iron binding glycoproteins, transferrin and lactoferrin with their high Fe^{3+} association constants (approximately 10^{36}) ensure that very little free ionic iron is available to the invading micro-organism. Transferrin is found in blood and lymph whilst lactoferrin is present in external secretions such as milk and bronchial mucus and also in the secretory granules of polymorphonuclear leucocytes (PMNs)(Bullen,1981; Griffiths,1983). An important function of lactoferrin and transferrin is to form soluble iron-protein complexes as simple ferric salts are hydrolysed at neutral pH to form extremely insoluble $Fe(OH)_3$. Both lactoferrin and transferrin are capable of reversibly binding two ferric ions with the simultaneous incorporation of two bicarbonate ions (Griffiths, 1983). Under normal conditions they are relatively unsaturated(25-30%) with iron. The amount of free ionic iron in human plasma in equilibrium with transferrin and lactoferrin has been reported to be approximately $10^{-18}M$ which is far to low for normal bacterial growth (Weinberg,1978; Bullen ,1981).In addition, during infection, vertebrate hosts reduce the

amount of iron present in the circulating transferrin-iron pool. The secretion of lactoferrin from PMNs lowers serum iron by removing iron from transferrin and the Fe^{3+}-lactoferrin complex is then taken up by macrophages and removed rapidly from the circulation by the reticulo-endothelial system (van Snick *et al*,1974).

The virulence of many micro-organisms including the Enterobacteriaceae can be enhanced in experimental animals by the injection of iron compounds (Miles,Khimji & Maskell,1979; Bullen,Leigh & Rogers,1968; Fosberg & Bullen,1972; Kaye,Gill & Hooke,1967). *E.coli* and haemoglobin together make a lethal combination in the peritoneal cavity due to the stimulation of bacterial growth by the iron in haem (Bornside,Bovis & Cohn,1968; Bullen,1981).

Increased susceptibility to infectious diseases in man has been observed in iron overloaded states such as beta-thalassaemia major and in haemolytic states such as sickle cell disease or Bartonellosis (Griffiths,1983). The availability of free ionic iron is therefore of major importance in predisposing both man and animals to infection.

1.5.2.1. Microbial Aquisition of Iron at Low Concentrations.

In aerobic conditions at neutral pH and therefore under most biological conditions, iron is present either in the form of very insoluble colloidal hydroxides or bound to organic

ligands (Spiro & Saltman,1969). To satisfy their iron requirements *in vivo* where the extremely low concentration of ferric iron is in equilibrium with ferric hydroxide, transferrin and lactoferrin, bacteria produce low molecular weight iron chelating compounds (Neilands,1981).

The best studied of these is produced by bacteria in the genera *Escherichia, Salmonella* and *Klebsiella* which secrete a cyclic trimer of 2,3 dihydroxybenzoyl serine (DBS) known as enterochelin or enterobactin (Fig.4). In *E.coli*, the biochemistry and genetics of enterochelin synthesis and transport have been determined in great detail (Griffiths,1983;) At physiological pH i.e.pH 7.4, enterochelin is thermodynamically and kinetically the most effective siderophore yet characterised. It has an association constant for ferric iron of 10^{52} and is capable of removing iron from transferrin and lactoferrin (Neilands,1981).

When the iron concentration becomes limiting, enterochelin is synthesized and secreted into the growth milieu where it forms the ferric-enterochelin complex. This complex is transported back into the cell and iron is released for cellular metabolism after the esterolytic hydrolysis of ferric-enterochelin to release free DBS (Fig.5) (Rosenberg & Young,1974).

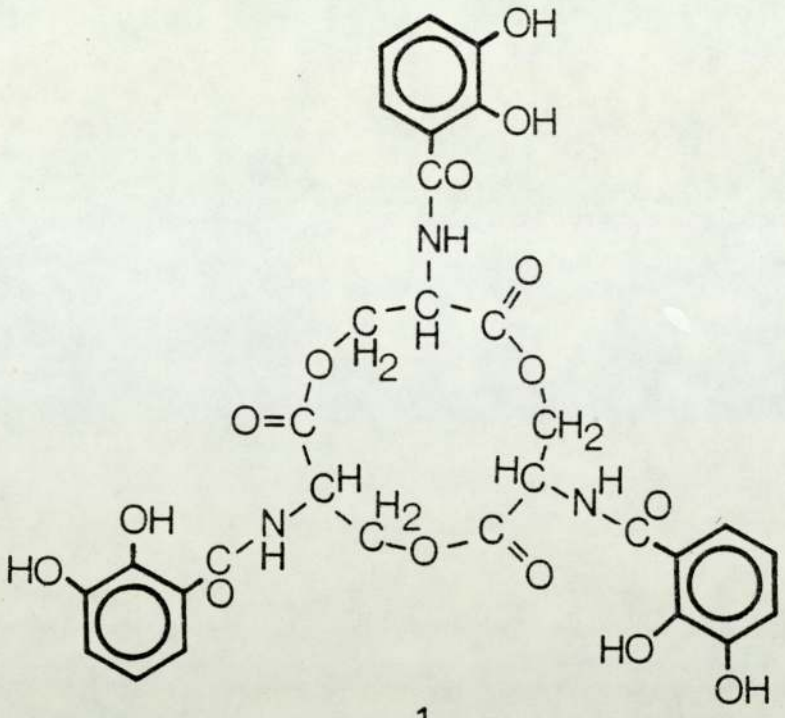
Although enterochelin appears to be the primary mechanism by which the Enterobacteriaceae aquire iron in low

Figure 4. Structures of Enterochelin and Aerobactin

(Griffiths, 1983)

Key: 1 Enterochelin

2 Aerobactin



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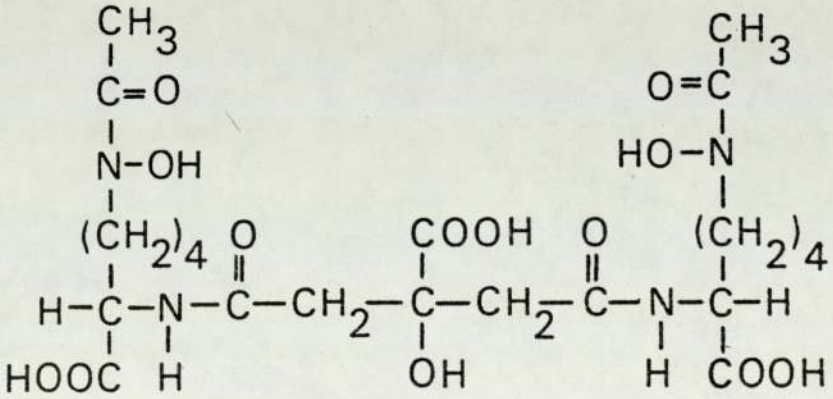

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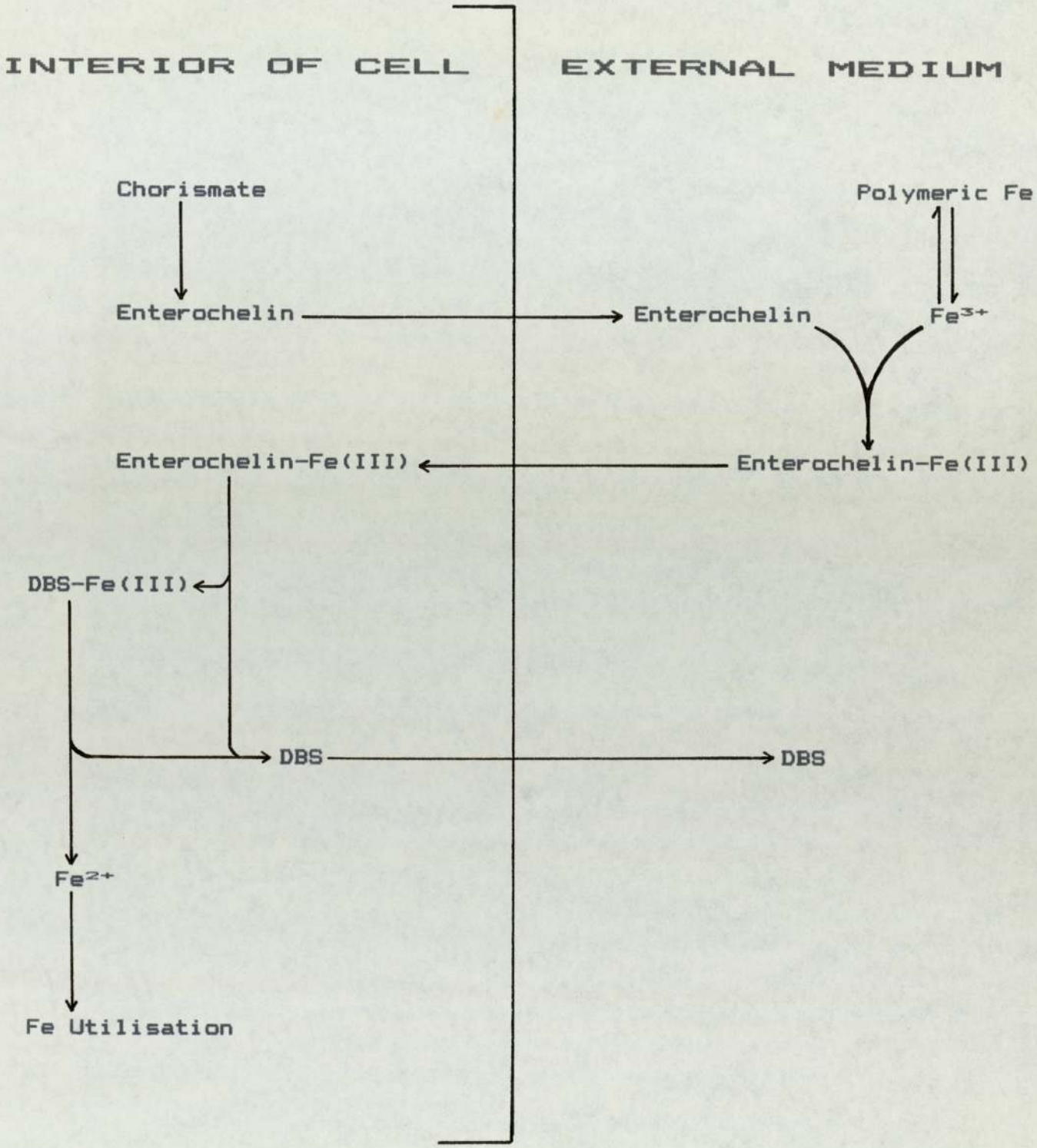
Figure 5. Schematic Representation of the Operation of the Enterochelin System of Iron Transport

Key: DBS 2,3 Dihydroxy-N-benzoyl-L-serine


INTERIOR OF CELL
EXTERNAL MEDIUM
Chorismate
Polymeric Fe
Enterochelin
Enterochelin
Fe3+
Enterochelin-Fe(III)
Enterochelin-Fe(III)
DBS-Fe(III)
DBS
DBS
Fe2+
Fe Utilisation

iron environments, they are capable of utilising exogenously supplied chelators such as ferrioxime B and ferrichrome produced by other micro-organisms. *E.coli* and *P.aeruginosa* also possess citrate inducible iron uptake systems utilising ferric citrate as the iron source (Griffiths,1983). Whilst it is not known whether these systems operate *in vivo*, exogenously supplied desferrioxime B enhances both *Klebsiella* (Miles & Khimji,1978) and *Salmonella* (Jones *et al*,1977) infections.

In addition to the enterochelin system several enteric species produce aerobactin , a dihydroxamate derivative of citric acid (Neilands,1981)(Fig.4). The secretion of this siderophore is thought to play an important role in the virulence of invasive *E.coli* strains (Williams & Warner,1980). How it confers a selective advantage on organisms already capable of synthesizing entrochelin is not clear particulary since the stability constant for ferric-enterochelin is much higher than that of ferric-aerobactin which approximates to that of transferrin (Harris *et al*,1979). Konopka *et al* (1982) found that aerobactin ,despite its relatively low affinity constant ($10^{22.9}$)(Raymond & Carrano,1979) could remove iron from transferrin in the presence of receptor bearing cells of *E.coli* acting as a thermodynamic sink for the iron. An important difference between the mode of action of aerobactin and enterochelin is that enterochelin is enzymatically destroyed following its iron transport

function whereas aerobactin can be re-used (Griffiths,1983). Ferric-enterochelin is therefore an energetically expensive way of assimilating iron. Cooper *et al* (1978) suggested that it is easier to remove Fe^{3+} from the hydroxamate derivative than from enterochelin. Enterochelin has low aqueous solubility and its aromatic character causes it to adhere to proteins and to act as a hapten for antibody formation. Antibodies to enterochelin do in fact occur in normal human serum and are thought to act together with transferrin to hinder iron assimilation by enterochelin producing pathogens (Moore *et al* 1980; Moore & Earhart,1981). In a situation where the enterochelin system is inhibited, the ability of an invading micro-organism to secrete aerobactin may therefore confer a selective advantage.

A concommitant response to iron stress is the synthesis of several new OM receptor proteins and enzymes involved in the uptake and release of iron from the chelators. *E.coli* produces four high molecular weight iron regulated membrane proteins (IRMP) of M_r 83,000; 81,000; 78,000 and 74,000. Four genes (fep A, fep B, fes and ton B) have been identified for ferric-enterochelin transport (Griffiths,1983). Fep A specifies an 81,000 M_r protein which is the ferric-enterochelin receptor (Ichihara & Mizushima,1978). Fep B probably controls a function required to translocate the iron complex across the cytoplasmic membrane whilst the fes product controls the hydrolysis of enterochelin after delivery of iron into the cell (Konisky,1979). The mode of action of the ton B gene product

is at present unknown but its function appears to be required in all high affinity iron transport systems. The 78,000 M_r protein is the ferrichrome receptor and is the product of the ton A gene (Konisky,1979). The function of the 74,000 and 83,000 M_r proteins has not been elucidated. In the presence of 1mM citrate, Hancock *et al* (1976) showed that *E.coli* induced another OM protein with an M_r of 81,000(fec A protein) which is thought to be part of the citrate dependent iron transport system (Hussein *et al* 1981; Wagegg & Braun,1981).

An OM protein of M_r 74,000 specified by the col V plasmid appears to be involved in aerobactin mediated iron transport (Grewal *et al*,1982).

Three OM proteins designated OM1, OM2 and OM3 (M_rs 82,000, 79,000 and 77,000 respectively) are induced in *Salmonella typhimurium* under iron limited conditions (Ernst *et al* ,1978).

Although most of the work on the IRMP has been carried out *in vitro* with laboratory strains of *E.coli*, Griffiths *et al* (1983) found that pathogenic *E.coli* obtained from lethally infected guinea pigs express their IRMP. These proteins were present in the OM of *E.coli* O111 grown *in vitro* in broth containing ovotransferrin and *in vivo* during an infection in amounts at least equal to that of the so called major OM proteins. Of considerable significance is the recent finding of Brown, Anwar & Lambert (1983) that the OM of a *P.*

aeruginosa strain isolated directly without subculture from the respiratory tract of a cystic fibrosis patient contained IRMP.

Apart from the phenotypic changes that occur in iron deficient bacteria, iron restricted *E.coli* contains altered tRNA species. These changes play an important part in regulating the expression of the operons of the aromatic biosynthetic pathway. Since enterochelin is synthesized from chorismic acid via a branch of this biosynthetic pathway, multiple control by amino acids and iron is therefore important in maintaining a balanced metabolism during growth under iron restricted conditions (Griffths,1983). The finding that these changes occur in the tRNAs of *E.coli* recovered directly from the site of a lethal infection (Griffiths *et al*,1978) stresses the importance of iron aquisition to the invading pathogen.

1.5.2.2. Inhibition of Microbial Acquistion of Iron.

The ability to produce an iron chelator does not necessarily determine the virulence of an organism. Miles *et al* (1975) studied a number of virulent and avirulent *Klebsiella*, *Escherichia* and *Salmonella* strains and found an equal competence in the synthesis of chelators when grown in the laboratory under iron deficient conditions. Virulence as far as the uptake of iron is concerned appears to be determined by microbial features confering resistance to those host defences which prevent adequate expression of *in vivo* uptake

by avirulent forms (Fitzgerald & Rogers,1980).

Co-operation between antibody and transferrin will exert a bacteriostatic effect on certain pathogenic *E.coli*. Enterochelin specific antibodies found in normal human serum act together with transferrin to exert a bacteriostatic effect on *E.coli*. The evidence suggested that antibody blocked enterochelin mediated Fe^{3+} uptake but not that from ferrichrome or citrate (Moore *et al*,1980; Moore & Earhart,1981). An alternative immune mechanism was described by Fitzgerald and Rogers (1980) . Serotype specific horse serum antibodies and secretory IgA from human milk, in conjunction with transferrin or lactoferrin were bacteriostatic, apparently by interfering with enterochelin synthesis or secretion. These antibodies recognise the monosaccharide colitose which is the terminal sugar of the O antigen of *E.coli* O111 LPS. The addition of colitose abolished the bacteriostatic effect of both whole serum and mixtures of antibody and iron binding proteins (Fitzgerald & Rogers,1980).

Fever may exert a beneficial effect during infections by enabling the host to interfere with microbial iron aquisition as siderophore synthesis is inhibited by elevated temperatures (Garibaldi,1972; Weinberg,1978; Grieger & Kluger,1978; Kluger & Rothenburg,1979). Efficient iron chelator production in *Salmonella typhimurium* takes place at 37°C but not at 41°C where additional iron or preformed siderophores are required to obtain growth (Garibaldi,1972).

1.5.3. Role of the Bacterial Surface in Complement-Mediated Serum Killing and Phagocytosis

1.5.3.1. Complement Mediated Serum Killing.

Complement mediated serum killing constitutes an important component of the host's defence in combating infections with gram-negative bacteria (Taylor,1983; Frank,1980). The acute stage of many gram-negative bacterial infections is characterised by the increased biosynthesis and turnover of complement proteins (Atkinson & Frank,1980), and in a reduction in the serum level of natural complement inhibitors (Whaley,Schur,McCabe & Ruddy,1980). Furthermore, strains causing severe infections involving tissue penetration and damage are often serum resistant, whilst inherited complement deficiencies in humans have been associated with a predisposition to infection with gram-negative bacteria (Taylor,1983).

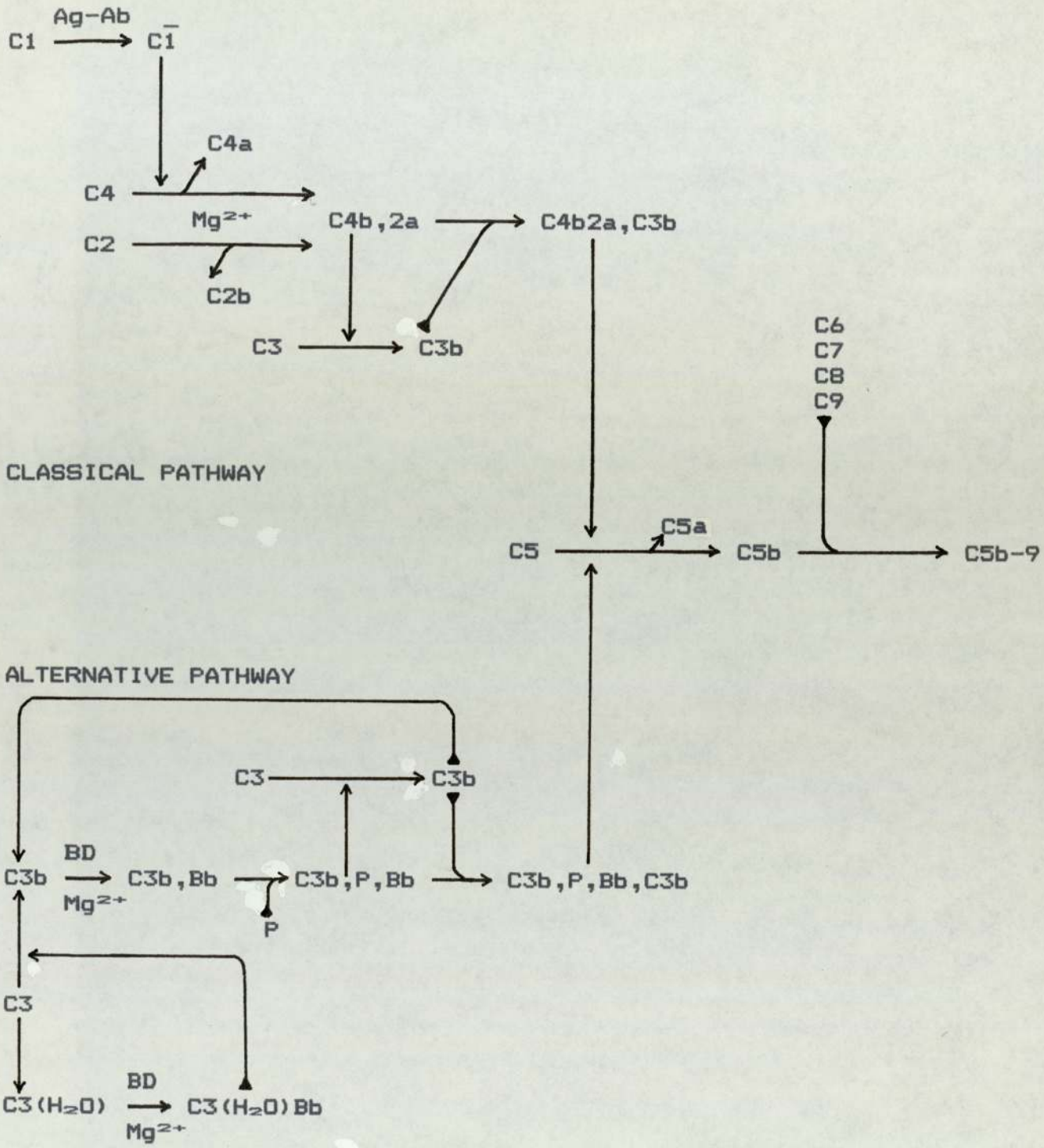
The killing of susceptible bacteria by complement involves the activation of a cascade sequence of enzymic reactions controlled by a complex set of inhibition and amplification factors (Fig.6). There are two pathways of activation, the classical pathway (Inoue *et al*,1959) and the alternative pathway (Schreiber *et al*,1979). The classical pathway may be activated by the interaction of antibody and bacterial surface antigens (Lachmann,1979) or directly by the lipid A

Figure 6. Schematic Representation of the Classical and Alternative Pathways of Complement Activation

Key: Ag Antigen

Ab Antibody

P Properdin


Ag-Ab
C1
C1̄
C4a
C4
C2
Mg2+
C4b,2a
C4b2a,C3b
C2b
C3
C3b
C6
C7
C8
C9
CLASSICAL PATHWAY
C5a
C5
C5b
C5b-9
ALTERNATIVE PATHWAY
C3
C3b
BD
C3b
C3b,Bb
C3b,P,Bb
C3b,P,Bb,C3b
Mg2+
P
C3
BD
C3(H2O)
C3(H2O)Bb
Mg2+

moeity of LPS (Loos *et al*, 1978). The alternative pathway may be activated in the absence of specific antibody by a variety of polysaccharides (Lachmann,1979). The two pathways interact and whenever classical pathway initiation has occurred, some degree of alternative pathway involvement is found due to the feedback of common intermediary components. Classical and alternative pathway activity can be differentiated by their requirements for divalent metal cations. Activation of the former is dependent upon the presence of both Mg^{2+} and Ca^{2+} whilst the latter requires Mg^{2+} only. Chelation of serum with ethylene glycol tetra-acetic acid (EGTA) which selectively removes Ca^{2+} will prevent activation of the classical pathway leaving the alternative pathway functional (Fine *et al*,1972). Serum killing is often but not invariably accompied by bacteriolysis, which depends on the presence of adequate amounts of lysozyme which degrades the bacterial peptidoglycan (Taylor,1983).

Initiation of the complement cascade results in an ordered sequential activation of circulating proteolytic enzyme precursors, each activated enzyme catalysing the next step of the cascade causing molecular amplification at each step. Evidence that the killing of gram-negative bacteria required all 9 complement components was provided by Inoue *et al*, (1968). This pathway is usually activated by the interaction between antigen and antibody complexes involving IgM or IgG and C1 . C1 circulates as a Ca^{2+} dependent complex composed of 3 sub-units; C1q, C1s, and C1r. C1q, has 6 spatially

separated binding sites and binds to Fc portions of antigen complexed antibody. More than one Fc must bind before activation of C1r and C1s takes place. This accounts for the far greater efficiency of pentameter IgM over monomer IgG (which has only one Fc portion) in activating complement: two molecules of the latter would have to be bound to antigen in close proximity, whereas every molecule of IgM would be effective on its own. After binding of C1q to the Fc regions of the immune complex, C1s aquires esterase activity and brings about the activation and transfer (in the presence of Mg^{2+}) to sites on the membrane (or immune complex) of first C4 and then C2. The proteolytic cleavage of C4 and C2 results in the generation of the C4b2a complex which constitutes the C3 convertase. This enzyme cleaves C3 in solution into 2 fragments, C3a (an anaphylatoxin) which is released, and C3b which binds to the activating surface and a new C4b2a3b complex is generated. This constitutes the C5 convertase. C5 is split into a chemotactic factor (C5a) and C5b. Cleavage of C5 results in the formation of the "membrane attack complex" (MAC) which is a stable transmembrane complex incorporating C5b,C6,C7,C8 and C9, and is responsible for the destruction of susceptible cells (Taylor,1983; Penn,1983).

Initiation of the alternative pathway depends on amplification (in the presence of suitable surfaces provided by activating substances) of the slow spontaneous activation of C3. This amplification resulting from the reaction of spontaneously formed C3b with Bb (produced from factor B by

the action of factor D which exists in serum in an activated form) to the C3 convertase. C3Bb is normally inhibited by the control proteins beta-1H and C3b inactivator but on binding of spontaneously formed C3b to an activating substance e.g. LPS, the action of these inhibitors is retarded. Factor P (Properdin) stabilises C3bB in the presence of Mg^{2+} so that it forms a stable convertase which may then bind an additional C3b fragment to be converted to C3b,P,Bb,C3b, the C5 convertase of the alternative pathway The interaction of proteins C3b,B and D is inefficient in that alternative pathway activation in the absence of antibody is kinetically slow and highly serum concentration dependent, requiring high concentrations of serum to produce effective C3 and B cleavage *in vitro*. A schematic representation of the complement cascade is presented in fig.6.

The exact nature of the lesion formed by the MAC and its physiological effect on bacterial cells is poorly understood (Taylor,1983). It appears that once functional lesions have been assembled, loss of viability rapidly ensues in a logarithmic fashion as reflected in the steep killing kinetics of susceptible organisms (Davis &Wedgewood, 1965; Taylor & Kroll,1983). Damage to the OM is unlikely to be responsible for the lethal activity of complement alone in killing gram-negative bacteria. Integration of the MAC into the phospholipid bilayer areas of the OM disrupts its integrity and removes the barrier to further attack on the peptidoglycan layer by lysozyme and the CM to further attack

by complement (Taylor,1983). However, there is some controversy over the exact molecular processes by which the OM and CM are damaged. One explanation is that polyC9 disrupts the OM by a detergent like action (Taylor,1983) since exposure of susceptible gram-negative bacteria to serum freqently results in the release of OM fragments as protein-LPS-phospholipid complexes into the surrounding environment (Feingold *et al*,1968; Wilson & Spitznagel,1968; De Monty & Graeve,1982). This detergent-like disruption may then allow C5b-8 to be translocated into active sites at the CM subsequent to polyC9 generation. Such a translocation has been suggested by the observation that the serum bactericidal reaction requires an input of bacterially generated energy (Griffiths,1971; Taylor & Kroll,1983). The precise nature of the lethal event after interaction of MAC proteins with fluid bacterial membranes is unknown. However, the lethal effect of complement may involve the dissipation of the energised state of the CM together with the formation of ion channels by the MAC which results in a rapid efflux of intracellular cations (Taylor,1983).

1.5.3.2. Resistance to Serum Bactericidal Activity

Some gram-negative bacteria are rapidly killed by human serum whilst others are completely refractory even in the presence of adequate amounts of sensitising antibody. Prolonged incubation of these strains may even result in a considerable increase in the viable cell numbers (Hughes *et al*,1982; Taylor,1978). Between these two extremes are strains which only show a significant reduction in viable count after lengthy periods of incubation in serum (Hughes *et al*,1982; Taylor,1974; Traub & Kleber,1976). However, these strains should be regarded as serum susceptible or at least different from strains that are capable of replicating in serum (Taylor,1983).

The OM constitutes a major barrier to the serum killing of gram-negative bacteria as protoplasts derived from serum resistant enterobacteria are rapidly lysed by complement (Reynolds & Rowley,1969). Resistant cells may be sensitized to the bactericidal action of serum by treatment with Tris, EDTA or polymyxin; agents which disrupt the integrity of the OM (Reynolds & Pruul,1971; Fierer & Finlay,1979).

Serum resistance does not result from a block in the activation of the complement cascade. MACs are formed on the surface of serum resistant strains but are not effectively inserted into the bacterial membranes and are released without causing lethal damage (Joiner,Hammer,Brown,Cole &

Frank,1982; Joiner,Hammer,Brown & Frank,1982). One parameter that appears to be intimately related to the ability of MACs to integrate into membranes is the degree of fluidity of these structures. Consequently, macromolecules which affect the fluidity of the OM and CM such as phospholipids,LPS and proteins may be crucial in determining serum resistance, as a reduction in membrane fluidity may exclude integration of the MACs (Taylor,1983).

LPS plays an important role in determining the susceptibility of enterobacteria to serum. Mutations from the S to R forms usually but not invariably associated with the loss of the ability to synthesize LPS O antigen side chains (Orskov,Orskov,Jann & Jann,1977; Wilkinson,1977) are accompanied by a drastic increase in serum sensitivity (Nelson & Roantree,1967; Rowley,1968; Taylor,1975)However, the serum resistance of gram-negative bacteria is not determined solely by the length and number of O antigens as many strains carrying a full complement of O side chains may be susceptible to serum via the delayed sensitive response (Taylor,1983). The mechanism by which a high degree of substitution of LPS core stubs by long O side chains is responsible for these delayed killing characteristics of smooth strains is unclear. Taylor (1983) has proposed that low membrane fluidity resulting from synthesis of a complete LPS, reduces the rate of insertion of functional MACs into the cell envelope. Antibody mediated activation of complement at some distance from the site of lesion formation because of the presence of long O antigens may

result in the decay of complement intermediates before they can be incorporated into functional MACs (Muschel & Larsen,1970; Rowley,1973). Such a concept can be extended to include antibody independent activation of the alternative pathway as the polysaccharide moeity of LPS is known to activate complement (Morrison & Kline,1977).

Bacterial exopolysaccharides were thought to influence the serum bactericidal reaction by impeding antibody binding and the subsequent attachment of complement components to the bacterial surface (Glyn & Howard,1970). More recent evidence has tended to suggest that capsules do not represent a diffusion, permeability or adsorption barrier to macromolecules such as IgG (King & Wilkinson,1981) or other proteins (King,Biel & Wilkinson,1980). However, this work was concerned exclusively with staphylococcal capsular polysaccharides and the same may not be true of gram-negative exopolysaccharides.

Other workers have failed to find any correlation between exopolysaccharide production and serum resistance (Taylor,1976;McCabe,Kaijser,Olling,Uwaydah & Hanson,1978;Van Dijk,Verbrugh,Peters,van der Tol, Peterson & Verhoef,1979). *Klebsiella* strains are frequently serum susceptible (Frierer,Finlay & Braude,1972; Gower *et al*,1972) even though most members of this genus produce copious amounts of acidic polysaccharide (Sutherland,1977). Mucoid strains of *P.aeruginosa* isolated from cystic fibrosis patients are in general more sensitive to serum than isolates from other

sources (Hoiby & Olling,1977). There therefore appears to be substantial evidence against a major role for acidic polysaccharides as mediators of serum resistance, at least in the general sense that can be discerned by comparative epidemiological investigations (Taylor,1983).

Several OM proteins have also been implicated in serum resistance. A serum resistant mutant derived from a smooth delayed susceptible urinary isolate of *E.coli* produced greater amounts of an envelope protein of M_r 46,000 than the parent (Taylor & Parton,1977). Alterations in the amount of this protein correlated well with changes in the response of this organism to serum (Taylor,Messner & Parton,1981). Other OM proteins have been shown to modify the serum sensitivity of *Neisseria gonorrhoea* (Hildebrandt et al,1978) and *Aeromonas salmonicida* (Munn *et al*,1982).

The growth environment may exert a profound effect on the serum susceptibility of gram-negative bacteria partly by its influence on bacterial surface structures (Taylor,1978) and partly by its effect on the metabolic state of the cell (Griffiths,1974). Gram-negative bacteria are more readily killed by serum when harvested in the early log phase than in lag or stationary phase (Dematteo, Hammer, Baltch, Smith, Sutphen & Michelson, 1981) and when grown in simple salts media rather than in nutritionally complex media (Melching & Vas,1971). Nutrient limitation and to a lesser extent growth rate modified the serum sensitivity of chemostat grown *E.coli* isolates (Taylor, 1978; Taylor,Messner &

Parton,1981). Anwar, Brown and Lambert (1983), showed that oxygen depleted and iron depleted *Pseudomonas cepacia* were more resistant to serum killing than were log phase or carbon depleted cells.

1.5.3.3. Phagocytosis

The ingestion and killing of invading micro-organisms by phagocytic cells is a critical determinant of resistance to infectious disease. The polymorphonuclear leucocytes (PMNs) comprising some 65% of the human white blood cell population, are the major phagocytic cells defending the host against micro-organisms which have elicited an acute inflammatory response (De Chatelet, 1979; Karnovsky, Robinson, Briggs & Karnovsky, 1981; Quie, Scott, Gebink & Peterson, 1981). It is important to realise that phagocytosis, whilst it is initially a surface phenomenon is a multi-step process involving:

1. Recognition and attachment of the bacterium via an energy independent process involving either non specific or specific ligand receptor interactions.

2. A burst of metabolic activity leading to generation of oxygen derived metabolites (superoxide,hydrogen peroxide and hydroxyl radicals) and induction of pseudopod formation.

3. Envelopment of the bacterium by pseudopodia.

4. Closure of the pseudopodia such that the bacterium is completely surrounded by the phagosome membrane.

5. Fusion of the cytoplasmic granules. Two sets of granules are recognised, the azurophil granules and the specific granules. Lysozyme, several cationic proteins and myeloperoxidase are associated with the azurophil granules; lactoferrin and lysozyme are found in the specific granules.

6. Discharge of the granule contents into the phagosome.

7. Killing of the bacterium: this is mediated by both oxygen dependent mechanisms i.e. the myeloperoxidase-hydrogen peroxide-halide system and oxygen independent mechanisms i.e. low pH, lysozyme, lactoferrin and the cationic proteins.

8. Digestion of the bacterium by proteolytic and hydrolytic enzymes.

The mechanisms by which PMNs recognise and ingest invading bacteria involve surface components (Stendahl,1983). The phagocytic process is therefore initially a surface phenomenon in which two main recognition mechanisms are generally considered to be operative on the phagocyte plasma membrane. One is mediated by specific receptors such as those for the Fc moeity of IgG and the activated forms of complement C3(C3b,C3b') termed CR1 and CR3. The other is mediated by more general physicochemical surface properties such as hydrophobicity and surface charge (Stendahl,1983).

Several investigators have demonstrated a correlation between bacterial surface hydrophobicity and susceptibility to phagocytosis; the more hydrophobic the bacterial surface relative to the phagocyte, then the more easily is that organism phagocytosed (Mudd *et al*,1934; van Oss,1978; Edebo *et al*,1980).

Gram-negative bacteria may resist phagocytosis by means of envelope components such as pili, capsular polysaccharides and the O specific polysaccharide of LPS.

Pili and other surface adhesins may facilitate bacterial adhesion to phagocytic cells leading to their subsequent ingestion (BarShavitt *et al*,1977; Silverblatt *et al*,1979) or, as in the case of the gonococcus, may protect the cell from ingestion (Ofek *et al*,1977; Jones & Buchanan,1978). Most unencapsulated *Klebsiella* strains recovered from clinical specimens are capable of adhering to epithelial cells via mannose inhibitable adhesins (MIAT). These adhesins are also receptors for the coliphages T3 and T7 (Pruzzo *et al*,1982). *Klebsiella* strains bearing these adhesins were found to be much more resistant to phagocytosis and intracellular killing than non-MIAT carrying strains (Pruzzo *et al*,1982).

In *E.coli* and *Salmonella* species, the presence of the complete O antigen protects these bacteria from phagocytic ingestion and killing (Stendahl,Norman & Edebo,1979). Smooth to rough mutations leading to various defects in LPS

structure diminish resistance to phagocytosis; deep rough mutants are phagocytosed to a considerable extent even in the absence of antibody and complement (Stendahl, Edebo, Magnusson, Tageson & Hjerten, 1977).

In encapsulated gram-negative bacteria , the polysaccharide K antigen may contribute to the virulence of the organism by enabling it to resist phagocytosis (Howard & Glyn,1971). The capsule shields the bacterium, an effect which is directly proportional to the amount of capsular polysaccharide present (Howard & Glyn,1971; Robbins *et al*,1980). K antigens protect gram-negative bacteria only in the absence of specific antibodies. The antiphagocytic effect can be overcome by opsonisation i.e. coating of the bacterium with antibodies *in vivo* and *in vitro*. Most encapsulated bacteria require specific anticapsular antibodies for effective opsonisation to occur (Robbins *et al*,1980). However much controversy still surrounds the ability of anti O and anti core glycolipid antibodies to opsonise encapsulated gram-negative bacteria (van Dijk *et al*,1981). Anticapsular antibodies react with complement and also bind to the Fc receptors of phagocytes leading to the rapid ingestion and intracellular killing of the opsonised bacterium (Kaijser & Ahlstedt,1977; Welch & Stevens,1979; Julianelle,1926; Fukutome *et al*,1980).

Apart from the specific ligand-receptor interactions, it has become evident that the binding of IgG antibodies to the surface of certain bacteria alters their general surface

properties. Opsonisation of smooth *Salmonella typhimurium* with IgG, has been shown to mimic a smooth to rough mutation, rendering the bacterial surface more hydrophobic (Stendahl, 1983). Complement also enhances the physicochemical effects and thus the phagocytosis promoting effects of IgG (Stendahl,1983).

1.6. Vaccines and Antisera Against Gram-negative Bacteria.

The limited success of control measures and of antibiotics in the treatment of highly drug resistant bacteria has led to an interest in immunoprophylaxis as an alternative or complementary form of treatment. However, the diversity of the antigenic types found amongst opportunistic gram-negative bacteria has made the task seem daunting. There are approximately 80 capsular types of both *Klebsiella aerogenes* and *Streptococcus pneumoniae* whilst known strains of *E.coli* exhibit 92 K antigens and 148 O antigens. It is not surprising therefore that research workers have looked for protective antigens common to more than one species of gram-negative bacteria to try and reduce the number of vaccines needed to achieve wide protective cover (Jones,1981; Hambleton & Melling,1983). Many attempts to prepare vaccines and antisera against these organisms have centered upon cell envelope components such as LPS and capsular polysaccharides, whilst a more novel approach has been that of the ribosomal vaccine.

LPS is a particularly potent immunogen common to the envelopes of all gram-negative bacteria. As such, LPS could theoretically form the basis of a broad spectrum gram-negative vaccine. Unfortunately LPS is toxic to man and animals, causing a wide range of systemic effects including intravascular coagulation, hypotension, renal cortical necrosis and lethal endotoxic shock (Braude,Douglas

& David,1973). However, antisera raised against LPS from one species of bacteria will passively immunise rabbits against LPS from another bacterial species (Freedman,1959). This heterologous protection can be explained by the possession of a common antigenic core shared by endotoxins from antigenically unrelated species of gram-negative bacteria (Luderitz,Staub & Westphal,1966). Antisera against this core glycolipid gave protection against both endotoxin (Tate, Douglas & Braude,1966) and lethal gram-negative bacterial challenges (Chedid, Parant, Parant & Boyer,1968). McCabe *et al* (1977) found that the core glycolipid from a mutant of *Salmonella minnesota* that contained only KDO and lipid A, protected against *Klebsiella, Proteus* and *Salmonella* challenge, whereas the lipid A alone gave no protection. Braude *et al* (1981) used a mutant *E.coli* O111 (J5) which was unable to attach O side chains to the LPS core. Broad spectrum protection against lethal infections by different gram-negative bacteria in immunosuppressed animals could be achieved by passive immunisation with antisera to this LPS core glycolipid. Encouraged by these results, they conducted a double blind clinical trial in patients with gram-negative bacteraemia. They reported that the survival rate from deep shock increased from 28% to 82%, whilst the mortality rate was cut in half in patients passively immunised with the anti J5 antiserum.

In addition to providing antiserum, core glycolipid vaccines may prove to be of value for prophylaxis with hospital patients identified as being at risk to gram-negative

infection (Hambleton & Melling,1983).

The need to prevent infection particularly with *P.aeruginosa* in burn patients and cystic fibrosis patients has led to the development of a crude polyvalent *P.aeruginosa* vaccine (Miler *et al*,1977). This vaccine which consists of a mixture of LPS and outer envelope antigens has been used with some success in clinical trials to combat *P.aeruginosa* infections in burn patients (Jones *et al*,1976); Jones *et al*;1980). Attempts to develop an antipseudomonal vaccine based on purified OM proteins and free of LPS have not yet achieved the same degree of success (Fernandes *et al*,1981).

The potential value of capsular vaccines for preventing disease caused by encapsulated bacteria is being widely investigated (Gotschlich, Austrian, Cvjetanovic & Robbins, 1980). Polysaccharide capsular antigens are shared by many species and extensive cross reactions have been found notably amongst *Streptococcus pneumoniae, K.aerogenes* and *E.coli* (Lee & Fraser,1980). At least 12 of the 15 antigens present in contemporary pneumococcal vaccines are similar to capsular antigens of *K.aerogenes* and *E.coli* (Heidelberger & Nimmich,1976) and may confer some protection against these organisms as well.

The usefulness of polysaccharide vaccines is limited by the poor immunogenicity of polysaccharides in children under two years old and by the phenomenon of "immune paralysis", whereby an immune response may be poor or absent following

immunisation with large amounts of polysaccharide antigens (Robbins *et al*,1980; Jann & Jann,1983). To overcome these deficiences, several investigators have coupled bacterial poysaccharides to appropriate protein carriers to render them immunogenic. Some positive results have so far only been obtained in animal experiments (Schneerson, Robbins, Chu, Sutton, Schiffman & Vann,1983; Geyer, Schlect & Himmelspach,1982).

Protective vaccines have also been prepared from the ribosomes or ribosomal extracts of many different micro-organisms including *Klebsiella* (Youmans & Youmans,1966; Riottot,Fournier & Jouin,1981; Lieberman,1978; Venneman,1972; Gonggrijp *et al*,1980). However, several investigators (Riottot *et al*,1981; Liberman,1978), have concluded that the protective activity of these ribosomal vaccines is due to contaminating cell envelope antigens, and only a few experiments have yielded evidence to support the idea that RNA is required for the protective activity (Venneman,1972; Gonggrijp *et al*,1980). Current opinion has opted for LPS as the main immunogenic ingredient of ribosomal vaccines as pure ribosomal fractions are only weakly immunogenic, whereas addition of LPS to the ribosomal fraction gives a strong immunogen (Liebermann,1978). From a practical viewpoint, ribosomal vaccines suffer from a major disadvantage in that they only induce protection against the bacterial strain from which they were made, although a recent report (Gonggrijp,Mullers & van Boven,1981) claimed that a ribosomal pseudomonas vaccine could protect mice

against challenge with a heterologous strain.

Much of the work with vaccines and antisera against gram-negative bacteria is at an experimental stage. Future progress in the rational design of vaccines will depend upon the futher elucidation of pathogenic mechanisms and in particular, a better understanding of the way in which bacteria adapt to growth *in vivo* (Hambleton & Melling, 1983; Griffiths, 1983).

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Bacteria

Klebsiella aerogenes DL1 was a clinical isolate from Safdajhang hospital, New Delhi, India, and together with the strains used in development of the anti-klebsiella vaccine was kindly donated by Drs. R.J. Jones and E.Roe (M.R.C. Vaccines Research Laboratories, Clinical Research Block, Medical School, University of Birmingham, Birmingham B15 2TJ). DL1 was typed by counter-current immunoelectrophoresis at the Public Health Laboratory, Coventry (Palfreyman,1978). It produced a serotype K1 capsular polysaccharide. This strain was chosen as a representative strain for the design of a chemically defined simple salts medium.

K.aerogenes NCTC 5055 (capsular type K2, O antigen type, O1) was obtained from the National Collection of Typed Cultures, Central Public Health Laboratory, Colindale Av., London NW9 5HT. Mutants M10(K^-,O^+) and M10B(K^-,O^-) isolated after nitrosoguanidine mutagenesis of NCTC 5055, were kindly donated by Drs. I.R. Poxton and I.W. Sutherland (Poxton & Sutherland,1976). DL1 and NCTC 5055 share immunologically cross reacting LPS.

All strains were maintained by monthly subculture on nutrient agar and stored at 4°C.

2.1.2. Chemicals

All chemicals used were of Analar grade (BDH Chemicals, Poole,Dorset) or equivalent. All solutions were made up in double, glass distilled, water.

Antibiotic sensitivity testing of DL1 was undertaken using Neosensitabs antibiotic discs, manufactured by A.S. Rosco, Tarstrup, Denmark.

Polymyxin B sulphate was obtained from Burroughs Wellcome, London; gentamicin sulphate fron Roussel, Wembley Park Middlesex; tetracycline hydrochloride from Lederle, Gosport, Hants; novobiocin from Upjohn Ltd., Crawley, W.Sussex; azlocillin from Bayer U.K., Haywards Heath, W.Sussex; rifampicin from Ciba, Horsham, Surrey and cefuroxime from Glaxo, Greenford, Middlesex.

2.1.3. Glassware

All glassware was immersed overnight in 5% w/v Extran 300 (BDH), rinsed once in distilled water, once in 1% v/v HCl and finally rinsed thoroughly in double distilled water before drying at 60°C and sterilising by dry heat at 160°C for 3 hours.

Glassware used in iron depletion studies was subjected to the additional step of soaking overnight in 0.01% w/v EDTA at room temperature before the final rinsing.

2.1.4.Complex Media

Nutrient agar was obtained from Oxoid, London. Meuller-Hinton agar used for the antibiotic disc tests, was obtained from Difco Laboratories, Detroit, Michigan, U.S.A. Both were prepared according to the manufacturer's instructions and sterilised by autoclaving at 121°C for 15 min.

Heat inactivated horse serum was obtained from Gibco (Paisley,Glasgow) and diluted to 50% v/v in sterile 0.9% w/v NaCl before use. 80 μM $FeSO_4$ was added in some experiments to alleviate the iron restriction imposed by serum transferrin.

2.1.5. Chemically Defined Media

K.aerogenes was grown initially in a simple salts medium based on that devised for *E.coli* W3110 by Klemperer *et al* (1979) but modified as the requirement for each nutrient became known.

The individual media constituents were prepared as sterile concentrated stock solutions, sterilised by autoclaving at 121°C for 15 min. The phosphate buffer required clarification by filtration through a 0.22 μm membrane filter before sterilisation. 1ml of concentrated HCl was

added to 1L of 0.05M $FeSO_4.7H_2O$ to prevent precipitation of the ferrous iron as ferric hydroxide during autoclaving. The complete chemically defined medium (CDM) was aseptically reconstituted immediately prior to inoculation.

For iron depletion experiments, the contaminating level of iron was reduced by passing the phosphate buffer down a column of the cation exchange resin Chelex 100 (Bio-Rad, Watford).

2.1.6. Equipment

Spectrophometers

Optical density measurements were made using a CE 292 Digital U.V. Spectrophotometer (Cecil Instruments, Cambridge). Spent culture supernatants were scanned for absorption maxima with a Unicam 8000 Scanning U.V. Spectrophometer. Growth medium iron content was estimated by flameless atomic absorption spectrophotometry on a Perkin Elmer Model 360 spectrophometer fitted with a deuterium background corrector and an HGA-74 Graphite Furnace (Bodenseewerk, Perkin Elmer)

Centrifuges

Bacterial cultures were harvested in either a J2 (Beckmann) Centrifuge or an I.E.C. B20 centrifuge (International Equipment Company, Needham Heights, Massachusetts, U.S.A). An M.S.E. Superspeed 50 (Measuring and Scientific Equipment, Crawley, Sussex) was used to isolate outer membranes and LPS. Serum was separated from coagulated blood by centrifugation in an M.S.E. Bench Centrifuge.

Incubators

Batch cultures were grown in either a Mickle Reciprocating Water Bath (Cam Lab Ltd., Cambridge) or in an Orbital Shaking Incubator (Gallenkamp, London).

Microscope

Microsopic counts of bacteria and PMNs were made using a Wild Model B20 Binocular Phase Contrast Microscope obtained from Micro Instruments, Oxford.

Freeze Dryer

Freeze drying apparatus was supplied by Edwards High Vacuum Ltd., Crawley, Sussex.

pH Meter

pH measurements were carried out on a Pye 290 pH Meter (Pye-Unicam, Cambridge).

Sonicator

Bacterial cells were broken by sonication with a Dawe Soniprobe.

Densitometer

Coomassie blue stained SDS polyacrylamide gels were scanned by a Bio-Rad Model 1650 Scanning Densitometer (Bio-Rad, Watford) coupled to a chart recorder (Model 28000, Bryans Southern Instruments Ltd., Mitcham, Surrey).

Osmometer

Osmolarity was determined by the measurement of freezing point depression using a Knauer Osmometer.

2.2. Experimental Methods

2.2.1. Measurement of Bacterial Cell Concentration

The three main methods of estimating bacterial cell concentrations are as follows:

1. Total counts, by direct counting of the bacteria when viewed under the microscope.

2. Viable Counts, a technique which seeks to measure the living population of a bacterial by adding samples to solid culture media and counting the colonies assumed to be produced by the growth of each original cell in the sample.

3. Indirect counts, which employ a spectrophotometer and make use of the ability of a bacterial suspension to scatter or absorb incident light. Thus, by measuring the turbidity or light scattering properties of such a suspension, an indirect count can be made. This method is most appropriate for following changes in cell concentration throughout the bacterial growth cycle in growth depletion experiments.

2.2.1.1. Spectrophotometric Measurements

2.2.1.1.1. Choice of Wavelength for the Measurement of Turbidity

The turbidity of a culture is determined by measuring either the intensity of refracted light emerging from a culture or the reduction in intensity of the undeviated light emerging from the culture.

Certain basic conditions should be considered when selecting a suitable wavelength for the measurement of turbidity. Ideally the absorption of cell pigments, media constituents and metabolic products should be minimal and the wavelength chosen should show the maximum sensitivity to changes in absorbance (A).

Koch (1961) found that the total amount of light scattered is directly proportional to the ratio of cell size to the wavelength of incident light. Thus, the shorter the wavelength, the more sensitive will the instrument be to changes in absorbance.

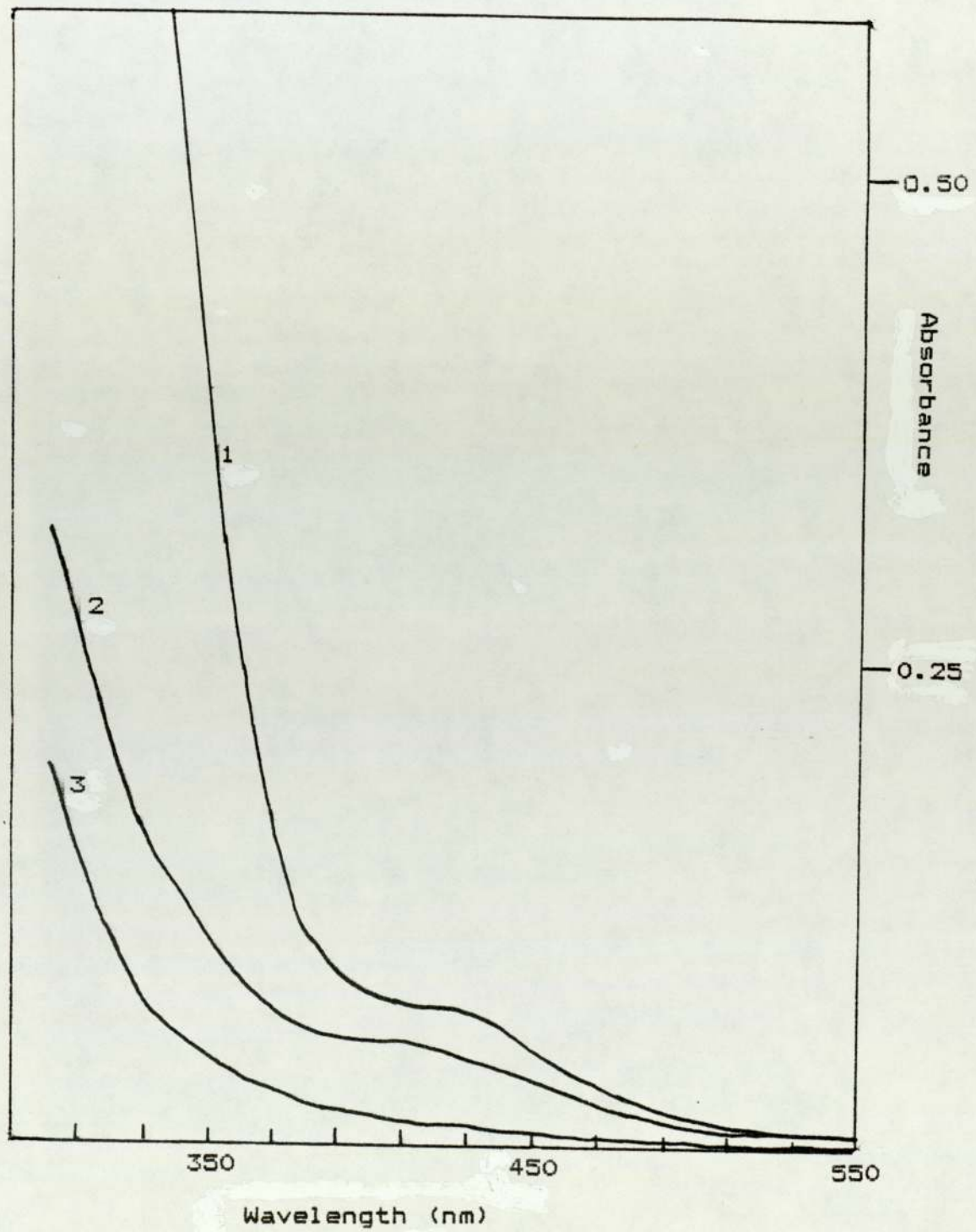
K.aerogenes is a non pigmented organism although when grown under iron depletion, it secreted a yellow compound into the growth medium. Supernatants recovered from mid log phase, iron-depleted and magnesium-depleted cultures were scanned between 550nm and 290nm (fig.7). An absorbance maximum at 440nm which tailed off to 470nm is apparent from the scan of

Figure 7. Visible Spectra of Nutrient Depleted *Klebsiella aerogenes* Culture Supernatants

Key: 1 Iron Depleted Supernatant

2 Magnesium Depleted Supernatant

3 Oxygen Depleted Supernatant


0.50
Absorbance
0.25
1
2
3
350
450
550
Wavelength (nm)

the iron depleted-culture. Therefore, the shortest and most sensitive wavelength which could be used to monitor changes in bacterial cell density was 470nm.

2.2.1.1.2. Measurement of Absorbance

At relatively low concentrations, the light scattered by a bacterial cell suspension is directly proportional to the concentration of cells in that suspension. This is expressed by the Beer-Lambert Law:

$$A \propto \frac{I_o}{I}$$

where I_o= the intensity of the incident light

I = the intensity of the emergent light

provided that the light path is of constant length

However, at high cell concentrations this relationship does not hold because of the secondary scattering of the light (Meynell & Meynell,1970). It was therefore necessary to determine the absorbance at 470nm (A_{470nm}) at which the Beer-Lambert law was no longer obeyed.

A series of dilutions in distilled water were made, in duplicate, of a dense stationary phase culture of DL1. Each dilution was thoroughly mixed and its A_{470nm} determined. The relationship between absorbance and cell concentration

obeyed the Beer-Lambert Law up to A_{470nm} 0.3 (fig.8). At higher concentrations, the increase in A_{470nm} was less than anticipated in proportion to the increase in the bacterial population. Linearity was restored upon dilution to an A_{470nm} less than 0.3. The lowest cell concentration which could be measured, at the limit of the spectrophotometer sensitivity was A_{470nm} 0.03. In all experiments requiring the measurement of the absorbance of a bacterial suspension, any suspension with an A_{470nm} of more than 0.25 was diluted with distilled water.

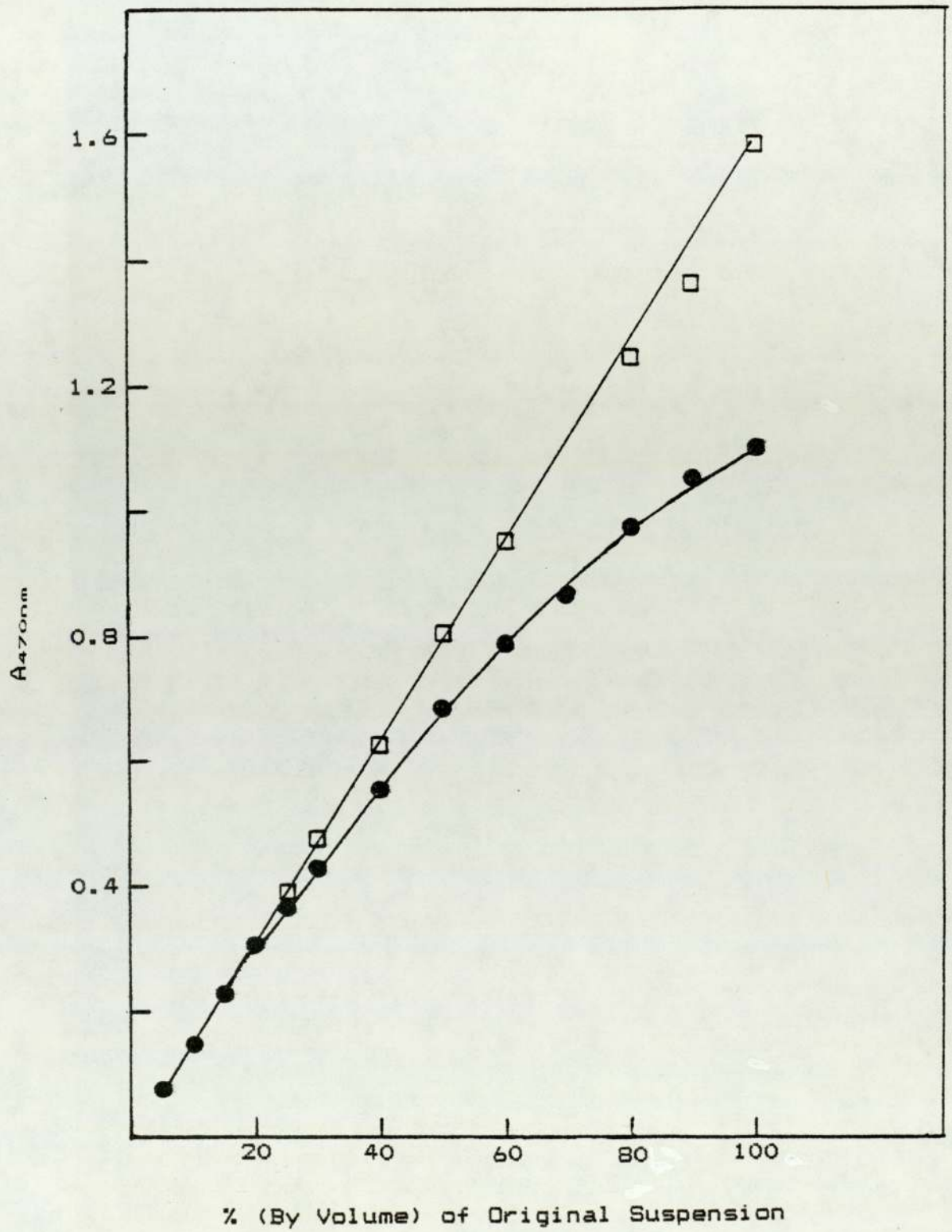
2.2.1.2. Total counts

Total bacterial or human white blood cell counts were estimated using haemocytometers with a chamber depth of 0.1mm and "improved Neubauer" rulings. Bacterial suspensions were diluted in formalin (final concentration 1% v/v) to give approximately 10 cells per small square. White blood cell counts were made by first diluting whole fresh blood 1 in 20 in 1% v/v glacial acetic acid containing a few drops of methylene blue, to lyse the erythrocytes. The chamber was allowed to stand at room temperature for 20 min in a petri dish containing water saturated filter paper, before counting.This was to prevent loss of sample volume by evaporation from the counting chamber. The haemocytometer was viewed under phase contrast (bacterial cells) or bright field (white blood cells) with a x40 objective and a x10 binocular eyepiece. All counts were made in duplicate at least twice.

Figure 8. Relationship between the Real and Observed Absorbance of a *Klebsiella aerogenes* DL1 Cell Suspension

Key:

- ● Observed A_{470nm} of Undiluted Suspension
- □ Corrected A_{470nm} of Diluted Suspension (Water as Diluent)


1.6
1.2
0.8
0.4
A470nm
20
40
60
80
100
% (By Volume) of Original Suspension

2.2.1.3. Viable Counts

Viable counts for serum and whole blood killing experiments were made by the method of Miles & Misra, (1938). The procedure was as follows: bacterial suspensions of A_{470nm} 0.2 were diluted 1:1 with blood,serum or 0.9% saline. 5, tenfold serial dilutions were made in distilled water to give approximately 20 colonies per 20 μl of the final dilution. 8, 20 μl drops were then pipetted onto each overdried nutrient agar plate, incubated overnight for 18 hours and the viable count calculated from the mean number of colonies per 20 μl drop per plate.

2.2.1.4. Dry Weight Determination

Weighed 50ml centrifuge tubes were dried to constant weight over phosphorous pentoxide in an evacuated dessicator. 20mls of the sample bacterial suspension in 1% v/v formalin were pipetted into each tube and centrifuged at 20,000 rpm (8x50ml head;J2 centrifuge) at 4°C for 40min. The cell pellet obtained was washed once in distilled water before drying to constant weight as before. Each dry weight was determined in triplicate.

2.2.2. Growth Experiments in Chemically Defined Media.

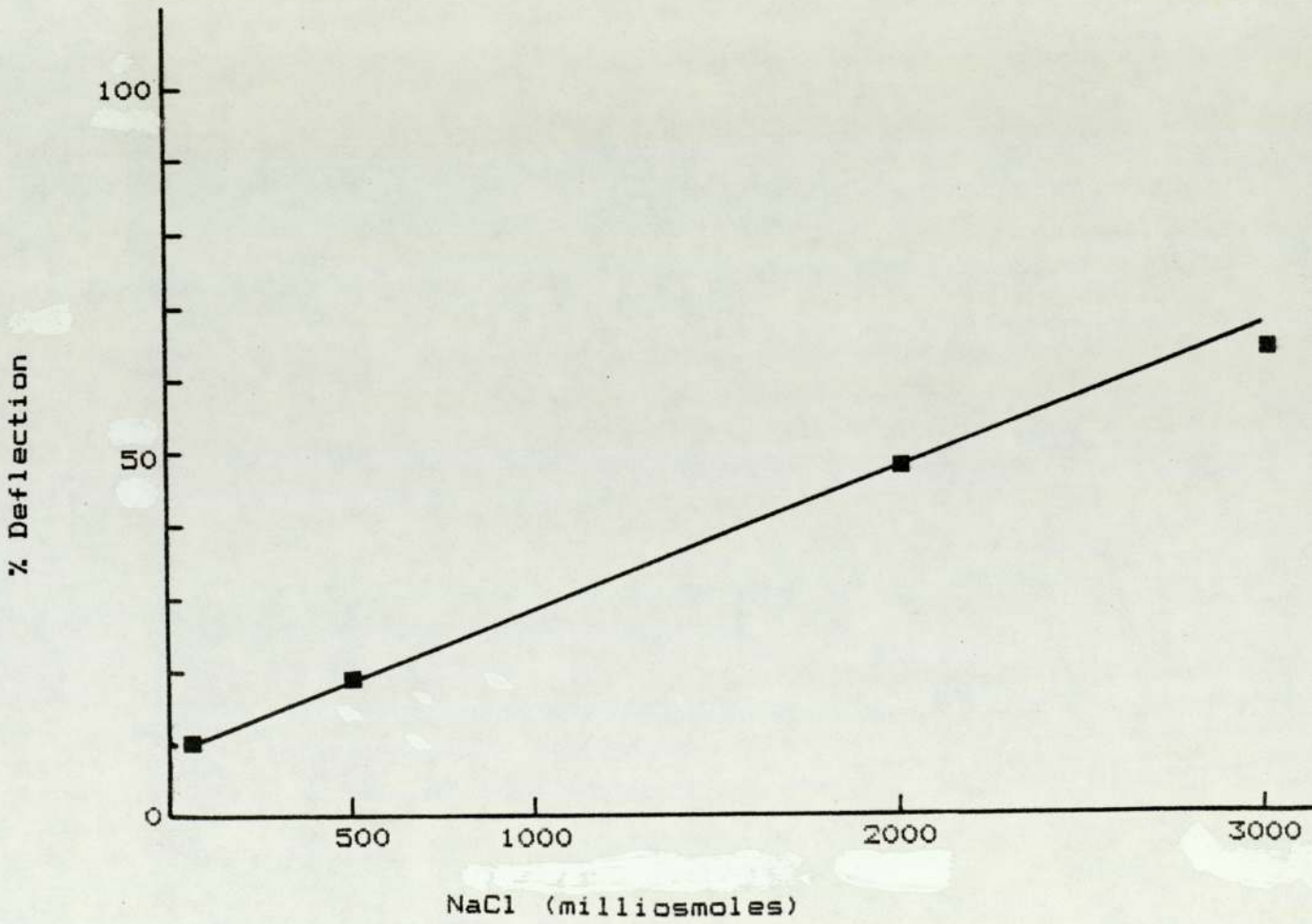
2.2.2.1. Growth Depletion Experiments

Growth depletion experiment inocula were prepared from a culture of DL1 grown overnight in a simple salts medium in which all the ingredients were present in excess (CDM1+Fe). 5 mls of the overnight culture were centrifuged at 18,000 rpm for 15min at room temperature and resuspended to an A_{470nm} of 1.0 in CDM1 lacking glucose and the nutrient being investigated. 0.25 mls of this suspension was added to 24.75 mls of the appropriate prewarmed medium in 100 ml Ehrlenmeyer flasks. Each flask was agitated to provide adequate oxygenation at 37°C in a shaking water bath at 120 rpm. The first A_{470nm} measurement was taken about 1 hour after inoculation, and thereafter samples were removed at 30 min intervals and diluted when necessary. Undiluted samples were returned to the flask to prevent undue reduction of culture volumes, whilst diluted samples were discarded.

2.2.2.2. Determination of Medium Osmolarity

Growth medium osmolarity was determined by the measurement of freezing point depression (Wallwork & Grant,1977). Sodium chloride solutions of known osmolarity were used to construct a calibration curve (fig.9) from which the osmolarity of the CDM could be calculated.

Figure 9. Calibration Curve for the Estimation of Medium Osmolarity



2.2.2.3. Growth Phase and Iron Deprivation Experiments

250 ml suspensions of DL1 in CDM2+Fe (see section 3.1.9) were incubated overnight at 37°C and 200 rpm in 1000 ml Ehrlenmeyer flasks in an orbital shaking incubator. The culture was harvested as in section 2.2.2.1., its A_{470nm} determined, washed twice in 0.9% w/v saline unless otherwise stated and inoculated into fresh prewarmed CDM2 with or without added iron to an A_{470nm} of 0.2 or 0.02. Bacteria were then harvested at intervals along the growth curve by a replicate flask method to obtain sufficient cell mass for OM preparation. At each time point, flasks were removed from the incubator, cooled rapidly in ice, the cells harvested and the supernatants retained.

2.2.3. Preparative Techniques

2.2.3.1. Removal of Contaminating Iron from Sodium Phosphate Buffer

The contaminating level of iron in CDM2-Fe was reduced by passing the sodium phosphate buffer (the major contributor) down a column containing the ion exchange resin, Chelex 100, prepared as follows: a slurry of the resin in distilled water was poured into a glass gel filtration column (Height 600mm; Diameter 25mm) clamped vertically. The slurry was allowed to settle to a column height of at least 200mm and then regenerated using the following sequence: 2 bed volumes of 1M HCl, 5 bed volumes of water, 2 bed volumes of 1M NaOH and finally rinsed with 5 bed volumes of water. Sodium phosphate buffer pH 7.4 at a concentration of 0.667M (ten times the concentration required to buffer CDM2) was passed down the column at a flow rate of 5ml/min/cm^2 until the pH of the Chelex resin was reduced to pH 7.4. Following this, the first 100ml of the same buffer was discarded after passing through the column and the remainder collected, diluted to 0.333M, filtered through a 0.22 μm membrane filter and sterilised by autoclaving (121°C for 15min). The regeneration of iron loaded Chelex 100 resin was accomplished using the sequence described above but with the additional step of heating the resin in 1M HCl at 80-100°C for one hour.

2.2.3.2. Preparation of Culture Filtrate Vaccines

Each *K.aerogenes* strain was subcultured in 400mls of CDM1-Fe in 1L Ehrlenmeyer flasks, and shaken at 170rpm and 37°C in an orbital incubator. After 5 days, 1.2mls of 40% v/v formaldehyde was added to each culture. Bacteria were harvested by centrifugation at 10,000rpm (6x250ml head, I.E.C. centrifuge) for 60min at 12°C. The supernatants were filtered through a 0.22 μm Millipore membrane filter under positive pressure and concentrated by vacuum dialysis through standard dialysis tubing. The concentrates were then dialysed against running tap water for 36 hours and freeze dried.

2.2.3.3. Preparation of Outer Membranes

A minimum bacterial population equivalent to 100mls of A_{470nm} 1.0 was required for the preparation of OMs for SDS-PAGE. After harvesting, the cells were resuspended in 10mls of distilled water, broken by sonication (3x60s) at 4°C and 1ml of 20% w/v sodium N-lauroyl sarcosinate (sarkosyl,Sigma Chemical Co.) added. Sarkosyl solubilises the CM but leaves the OM essentially intact (Filip *et al*,1973). After 30min at room temperature, OMs were pelleted by centrifugation at 20,000rpm (10x12.5ml head, I.E.C.centrifuge) for 60 min at 4°C, washed once in distilled water, resuspended in 0.5ml of distilled water and

stored at -20°C.

2.2.3.4. Peptidoglycan Associated Proteins

The method used to isolate those OM proteins of DL1 which are bound tightly but not covalently to peptidoglycan was essentially that of Mizuno and Kageyama (1979).

Approximately $5x10^9$ cells were resuspended in 50mls of 10mM sodium phosphate buffer pH 7.2. Bacteria were broken by sonication (3x60secs) and the resulting cell envelopes pelleted by centrifugation at 40,000 rpm (8x25ml head, MSE Superspeed 50) for 60min at 4°C and washed once in the same buffer. The envelope pellet obtained was extracted with a solution containing 2% w/v SDS, 10% v/v glycerol and 10mM Tris-HCl buffer pH 7.8 in 0.1M NaCl for 30min at 30°C followed by centrifugation at 40,000rpm for 60min as before. This procedure was repeated twice and the peptidoglycan protein complex obtained washed once in distilled water, resuspended in 0.5mls of distilled water and stored at -20°C.

2.2.3.5. Preparation of LPS and Capsular Polysaccharide

LPS was prepared from K.aerogenes DL1 and M10 by the method of Westphal and Jann (1965).

A bacterial wet weight of approximately 10g was suspended in 175mls of distilled water and warmed to 65°C. An equal

volume of 90% w/v aqueous phenol at the same temperature was added and the mixture stirred vigorously for 5min, cooled rapidly and centrifuged at 3000rpm for 30min to permit phase separation. The upper aqueous phase was carefully removed and dialysed against running tap water for 48 hours to remove the phenol. LPS was isolated as a clear viscous gel after centrifugation at 40,000rpm (8x25ml head, MSE Superspeed 50) for 4 hours.

A crude mixture of LPS and capsular polysaccharide was obtained by ethanol precipitation of the supernatants from bacteria grown in CDM1+Fe. The precipitate was collected by centrifugation at 20,000rpm (6x250ml head,J2) for 30min, dialysed against running tap water for 24 hours and freeze dried.

2.2.3.6. Preparation of Antiserum

Antiserum against *K.aerogenes* (whole bacterial cells) DL1, NCTC 5055, M10 and M10B was raised in male New Zealand white rabbits (4.5kg) in collaboration with Dr.R.J.Jones (MRC Vaccine Reasearch Laboratories, Clinical Research Block, Medical School, University of Birmingham, Birmingham). Bacterial suspensions containing 10^{8} organisms/ml in 0.9% w/v NaCl containing 0.5% v/v of formaldehyde were prepared from overnight, nutrient agar grown cultures. Intravenous injections of suspensions of 0.1, 0.2, 0.4, 0.4, 0.6, 0.8, 0.8, 1.0 and 1.0 ml were given on days 1, 2, 3, 9, 10, 11, 17, 18 and 19 respectively. Blood (10ml) was taken before

immunisation, before the final injection and 10 days after the last injection, from the marginal ear vein. Serum was separated and filtered through a 0.45 μm Millipore membrane filter into sterile bottles and stored at 4°C.

2.2.3.7. Absorption of Antiserum

Antiserum to *K.aerogenes* NCTC 5055 was absorbed with whole live M10 grown overnight on nutrient agar and resuspended in 0.9% w/v NaCl. To 1.0ml of antiserum, 1.0ml of a suspension of M10 containing 10^{10} organisms/ml was added and the mixture allowed to stand at 4°C for 1 hour. The suspension was filtered through a 0.45 μm Millipore membrane filter and the whole procedure repeated until no agglutination could be observed by slide agglutination against a saline suspension of *K.aerogenes* M10 (10^{10} organisms/ml).

2.2.3.8. Preparation and Inactivation of Human Serum

Fresh venous blood was obtained from a single healthy volunteer. The blood was clotted for 1 hour at room temperature and the serum separated by centrifugation at 3000 rpm for 5 min. Serum was heat inactivated at 55°C for 30min. To isolate functional alternative complement pathway activity, samples of serum were treated with 10mM EGTA (ethylene glycol tetra-acetic acid) and 10mM Mg^{2+} in 0.9% w/v NaCl, prepared according to the method of Fine *et al* (1972).

2.2.3.9. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

OM protein profiles of *K.aerogenes* were obtained using the method of Lugtenberg *et al* (1975). Gels were composed of a separating gel containing either 15% or 10% acrylamide, and a stacking gel, and were made up as follows:

	15% Gel	10% Gel	Stacking Gel
1.Stock I	18.5ml	12.5ml	-
2.Stock II	-	-	5.0ml
3.SDS 10%w/v	1.5ml	1.5ml	0.3ml
4.Tris 1.5M pH8.8	18.5ml	18.5ml	-
5.Tris 0.5M pH6.8	-	-	7.5ml
6.Distilled Water	20.0ml	26.0ml	16.0ml
7.TEMED	0.14ml	0.14ml	0.08ml
8.AMPS 10%w/v	0.20ml	0.20ml	0.10ml

where TEMED is NNNN' Tetramethylethylene diamine and AMPS is Ammonium Persulphate.

Stock I consisted of 44% w/v acrylamide and 0.8% w/v N,N'-methylene-bis acrylamide (BIS); Stock II consisted of 30% w/v acrylamide and 0.8% w/v BIS. AMPS was freshly made for each gel. All other solutions were stable for 2 months.

Specially purified SDS (BDH Chemicals) was used for all PAGE.

Solutions were mixed in a 250ml beaker and stirred with a magnetic stirrer. Polymerisation was started with the addition of 0.14ml or 0.08ml of TEMED.

Protein samples were mixed in a 1:1 ratio with the sample buffer (see below) and boiled for 10min at 100°C. 20-80 µl of sample solution was loaded onto the stacking gel. 10% gels were used to investigate the relationship between iron depletion and growth phase on the OM protein profile. Comparable protein concentrations were achieved by resuspending bacteria harvested at different stages of the growth cycle to the same A_{470nm} before isolating their OMs.

Sample Buffer pH 6.8

Tris 0.5M pH6.8	5.0ml
SDS 10% w/v	10.0ml
Mercaptoethanol	0.5ml
Glycerol	5.0ml
Distilled Water	10.0ml
Bromophenol Blue Solution	0.1ml

Electrode Buffer pH 8.3

Tris	0.025M
Glycine	0.190M
SDS 10%w/v	20.0ml
Distilled Water to	2,000ml

The electrophoresis was performed at room temperature at a constant current of 40mA and was stopped when the tracking dye had moved approximately 12cm.

Gels were fixed and stained with 0.1%w/v Coomassie Brilliant Blue R250 in 50% v/v methanol, 10% v/v glacial acetic acid in water, scanned with a BioRad Scanning Densitometer (Model 1650) coupled to a chart recorder, and photographed.

2.2.4. Chemical Assays

2.2.4.1. Phenol Sulphuric Acid Assay for Total Carbohydrate Estimation

The carbohydrate content of the culture filtrate fractions, cell suspensions and supernatants was determined by the method of Dubois *et al* (1956).

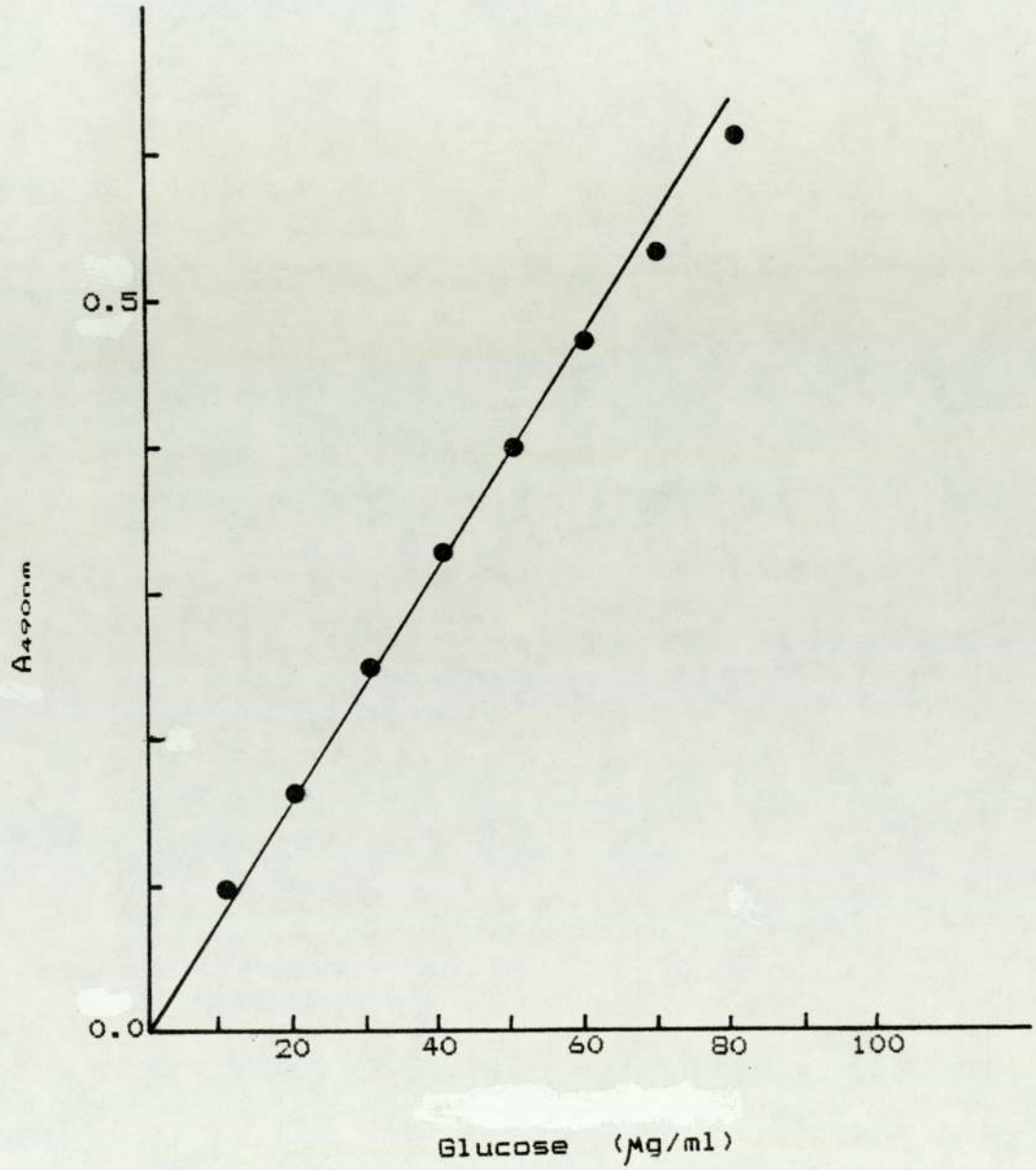
2.0ml samples of glucose containing 10 to 80 µg glucose/ml were prepared from a 1.0 mg/ml D-glucose standard solution in distilled water. Samples of unknown carbohydrate content were also made up to 2.0ml in distilled water. To each sample 1.0ml of 5% w/v aqueous phenol was added followed by the rapid addition of 5.0ml of 95.5%w/v H_2SO_4. After cooling to room temperature for 30min, the absorbance of each solution at 490nm was recorded. A standard calibration curve of glucose concentration against A_{490nm} is shown in fig.10.

2.2.4.2. Folin-Ciocalteau Protein Assay

Culture filtrate fractions were assayed for total protein by the method of Lowry *et al* (1951).

Bovine serum albumin standards in the range 10 to 100 µg/ml were prepared. Both samples and standards were made up to

Figure 10. Calibration Curve for the Estimation of Total Carbohydrate by the Phenol-Sulphuric Acid Assay



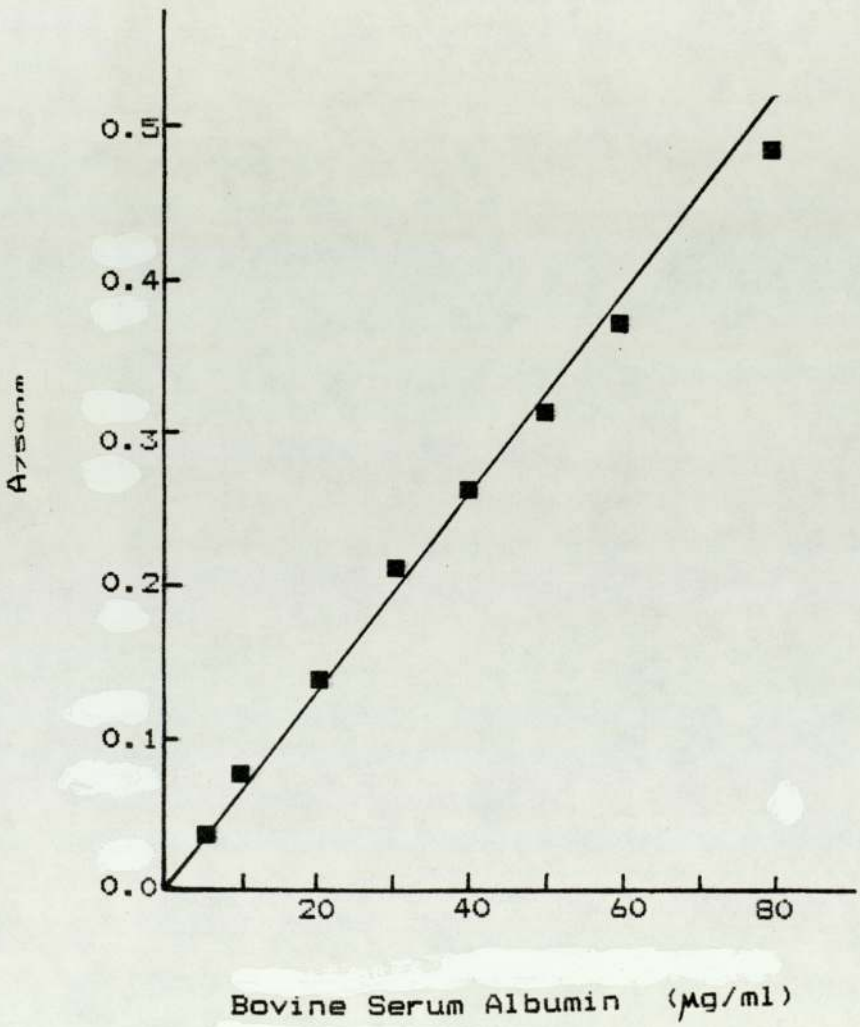
0.5ml with distilled water, 0.5ml of 1M NaOH added and the solutions boiled at 100°C for 5 min. After cooling to room temperature, 2.5ml of a solution made by mixing 2.0ml of 0.5% w/v copper sulphate in 1% w/v potassium tartrate and 50ml of 5% w/v sodium carbonate, was added to samples and standards. After 10min at room temperature, 0.5ml of the Folin-Ciocalteau reagent was added and the resulting blue colour developed for 30min before reading the absorbance at 750nm. The total protein content of the culture filtrate fractions was estimated from a calibration curve of albumin concentration against A_{750nm} (Fig.11).

2.2.4.3. KDO (2 keto-3 deoxyoctonic acid) Assay

A method based on that of Osborn (1963) was used.

Samples of KDO standard (10 to 100 μg/ml), freeze dried culture filtrate or culture supernatant were hydroylsed in 1.25ml of 0.05M H_2SO_4 by heating in sealed tubes at 100°C for 30min. After cooling, 1.25ml of 0.025M periodic acid (or sodium periodate) in 0.025M H_2SO_4 was added. After 20min at 55°C, 2.5ml of 2% w/v sodium arsenate in 0.5M HCl were pipetted into each tube and the contents thoroughly mixed. Three min later, 2.0ml of a 3.0% w/v thiobarbituric acid solution in distilled water were added to each sample , the sample tube sealed and then heated at 100°C for 20min. After cooling to room temperature any precipitation was removed by centrifugation at 10,000rpm (10x12.5ml head, I.E.C.

Figure 11. Calibration Curve for the Estimation of Total Protein by the Folin-Ciocalteau Assay



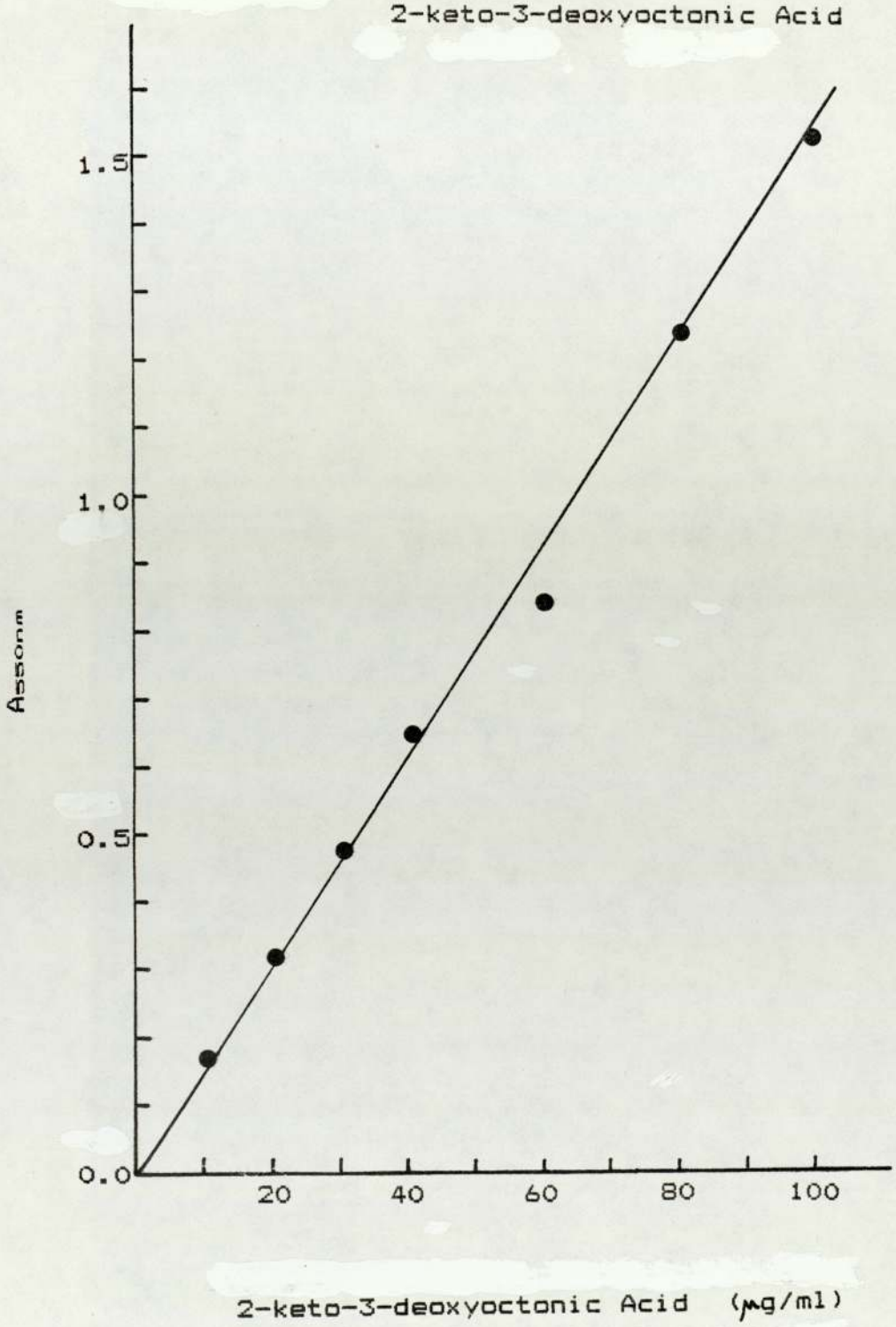
centrifuge) for 20min. The absorbance of each solution at 550nm was recorded and a standard calibration curve constructed (Fig.12).

2.2.4.4. Hydrolysis and Paper Chromatography of Klebsiella Capsular Polysaccharides

50mg of freeze dried culture filtrate was dissolved in 1ml of 2M H_2SO_4 and the solution heated on a boiling water bath for 6 hrs. The solution was neutralised with barium carbonate, centrifuged at 3000rpm (M.S.E.bench centrifuge) for 30min to sediment the barium carbonate before removing the supernatant, which was then chromatographed as follows:

1 to 4 spots of the supernatant solution were applied to the paper chromatogram and dried with a hot air blower. The chromatogram was developed with butanol, ethanol, water (50:30:20 parts by volume) for 24hrs. The paper was dried, dipped in a solution of aniline hydrogen phthalate (1.66g phthalic acid and 0.93g of aniline dissolved in 100ml of butanol saturated with water). After heating the paper chromatogram at 105°C for 10min, hexoses were visible as reddish brown spots and deoxy sugars as dark brown spots. It was practicable to compare the sugars present in 6 polysaccharide samples on the same chromatogram.

Figure 12. Calibration Curve for the Estimation of 2-keto-3-deoxyoctonic Acid



2.2.4.5. Detection and Assay of Enterochelin

Enterochelin and related phenolates were assayed by the method described by Arnow (1937). Fig.13a compares the visible spectra of 3,4, dihydroxyphenylalanine and spent culture supernants obtained from iron depleted DL1 after treatment with the Arnow reagent. Absorption maxima at around 515nm are clearly apparent. No absorption maximum is observed after treatment of the spent culture supernatant with ethyl acetate (which is used to selectively extract enterochelin from aqueous solutions (Young,I.G., 1976)) followed by treatment with the Arnow reagent.

1.0ml standard solutions of L-DOPA (3,4 dihydroxyphenylalanine) containing 1 to 50 μg/ml were prepared. To 1.0ml of spent culture supernatant or 1.0ml of standard, 1.0ml of 0.5M HCl was added followed by 1.0ml of the nitrite-molybdate reagent (10g of sodium nitrite and 10g of sodium molybdate in 100ml of distilled water). After the addition of 1.0ml of 1M NaOH, the absorbance of each solution at 515nm was recorded and a standard calibration curve constructed (Fig.13b) from which the enterochelin content of the supernatants was estimated.

2.2.4.6. Detection of Hydroxamate Siderophores

Hydroxamate type siderophores were assayed by the method developed by Csaky (1948) and modified by Gibson and

Figure 13(a). Visible Spectra of *K.aerogenes* DL1 Spent Culture Supernatants and 3,4 Dihydroxyphenylalanine after Treatment with the Arnow Reagent

Key:

1. 3,4 Dihydroxyphenylalanine
2. Supernatant from DL1 grown to stationary phase in CDM2-Fe
3. Supernatant from DL1 grown to stationary phase in CDM2-Fe after treatment with ethyl acetate to extract enterochelin and related phenolates

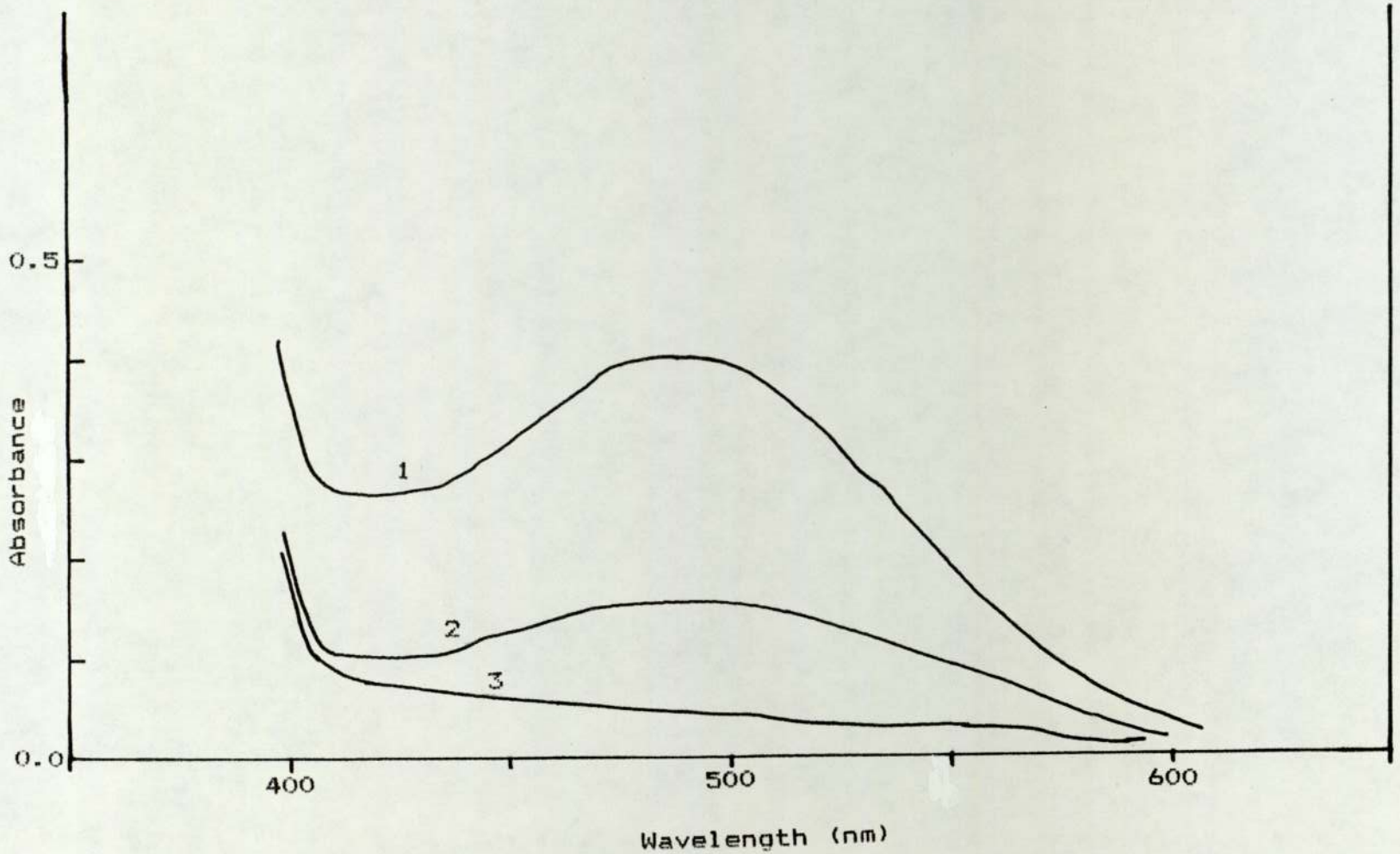
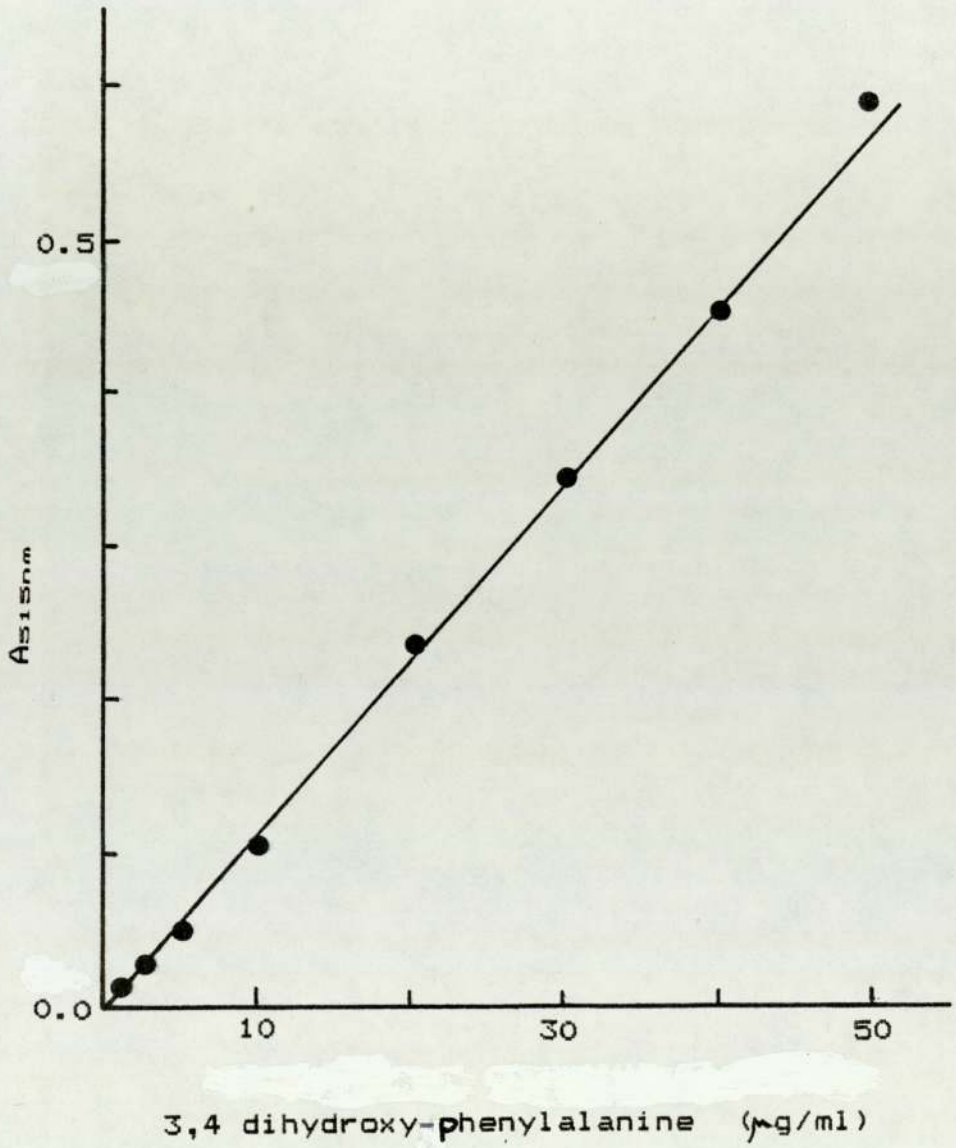

0.5
0.0
Absorbance
1
2
3
400
500
600
Wavelength (nm)

Figure 13(b). Calibration Curve for the Estimation of Enterochelin



Macgrath (1969).

0.5ml of culture supernatant was autoclaved at 130°C for 30min in a sealed tube in the presence of 0.5M H_2SO_4 and cooled to room temperature. 1.0ml of 1% w/v sulphanilic acid in 30% v/v glacial acetic acid and 0.5ml of 1.3% w/v iodine in 30% v/v glacial acetic acid were added to each sample. After 5min at room temperature, the excess iodine was destroyed by 1.0ml of 2% w/v sodium arsenate solution. A solution of 0.3% w/v naphthylamine (1.0ml) was mixed with each sample and the total volume made up to 10ml with distilled water. After 30min at room temperature the absorbance at 526nm was recorded.

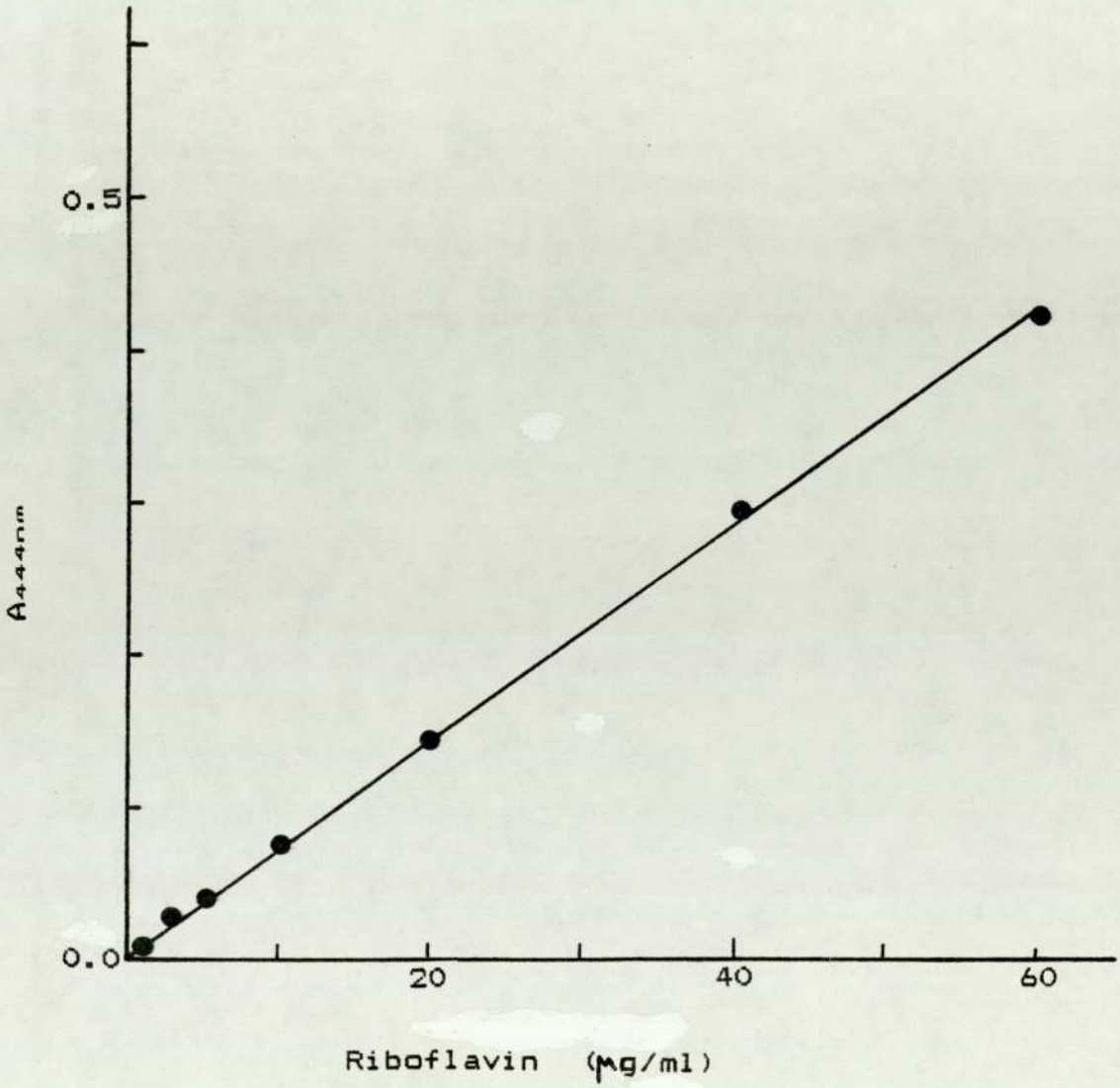
2.2.4.7. Estimation of Riboflavin

The concentration of riboflavin produced during the growth cycle of DL1 in CDM2+Fe and CDM2-Fe was estimated from the absorbance maximum of riboflavin at 444nm after the reduction of supernatant pH to pH 4.8 with concentrated HCl. A standard calibration curve of riboflavin concentration against A_{444nm} is shown in fig.14.

2.2.4.8. Determination of Iron by Atomic Absorption Spectrophotometry

Samples of Fe-CDM were analysed for their contaminating iron content by flameless atomic absorption spectrophotometry

Figure 14. Calibration Curve for the Estimation of Riboflavin



using a Perkin Elmer Model 360 Spectrophotometer fditted with a deuterium background corrector and coupled to a chart recorder. Instrumental conditions are summarised in table 1. A standard 1000ppm $FeCl_3$ solution (BDH) was used to prepare the calibration curve (Fig.15.).

2.2.4.9. Agar Gel Immunodiffusion

The Ouchterlony double diffusion technique (Ouchterlony, 1958) was used to investigate the immunological cross reactions between the LPSs and capsular polysaccharides of DL1, NCTC 5055, M10 and M10B.

5ml of 1% w/v agarose in Tris-barbitone buffer pH 8.6 was poured onto 5x7cm glass plates and allowed to solidify at room temperature. A pattern consisting of a central and 8 laterally surrounding wells were cut with a No.2 cork borer. Up to 20 μl of antibody or antigen were pipetted into each well and the plates stored in a sealed moist box at room temperature for 24hrs or until no further development of immunoprecipitin lines occurred. The results were recorded photographically.

Figure 15. Calibration Curve for the Estimation of Iron by Atomic Absorption Spectrophotometry

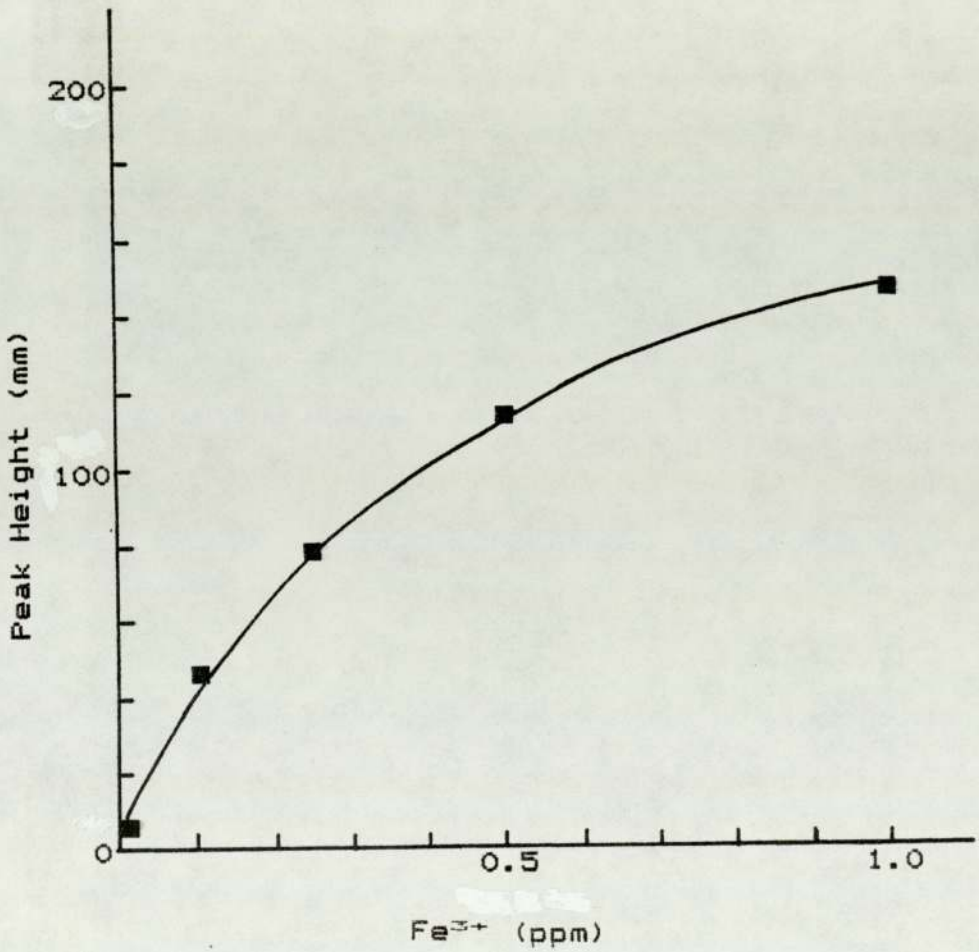


Table 1. Instrumental Conditions for the Estimation of Iron by Atomic Absorption Spectrophotometry

Wavelength	248.3 nm
Spectral Slit Width	0.7 nm
Sample Size	20.0 µl
Source	Electrodeless Discharge Lamp (Perkin-Elmer)

Time and Temperature Settings

	Temp (°C)	Time (secs)
Drying	154	30
Charring	480	15
Atomising	908	5

2.2.5. Biological Assays

2.2.5.1. Immunogenicity of Culture Filtrate Vaccines

Preliminary experiments were undertaken in collaboration with Dr.E.Roe (M.R.C. Vaccines Research Laboratories, Clinical Research Block, Medical School, University of Birmingham, Birmingham) to ascertain the immunogenicity of each culture filtrate vaccines in terms of the ability to protect mice against a lethal dose of the challenge *Klebsiella* strain.

The minimum lethal dose (MLD) for each of 77 capsular types was determined as follows: a saline suspension containing approximately $2x10^9$ organisms/ml was made from a culture grown on blood agar for 18 hours at 37°C. A range of dilutions was prepared from this suspension and 1ml of each dilution inoculated intraperitoneally (i.p.) into 25g adult, BK (strain W2) female mice. The MLD is the lowest concentration of bacteria required to kill a mouse. This concentration was used as the challenge dose in the mouse protection tests. The MLD also served as an index of the virulence of a particular *Klebsiella* capsular type.

Culture filtrate vaccines were tested for the ability to protect mice against challenge by the same capsular type (homologous) or by each of the other 76 capsular types (heterologous) challenge. 1mg of the vaccine to be tested

was dissolved in 10mls of sterile distilled water and then 1 in 100 in sterile 0.9% w/v NaCl. Where vaccines made from different capsular types were pooled, each component was dissolved in distilled water as before and 1ml aliquots of each solution were pooled and diluted to 100ml in 0.9% w/v NaCl. 1ml of either the single or multi-component vaccine was injected i.p. into a 16g mouse (day 0). This was repeated on day 4. On day 8 the mouse was given an i.p. injection of a 1ml saline suspension containing 1MLD of the challenge organism. In these experiments 2 mice were vaccinated, 2 were not (controls) and all 4 challenged with the same *Klebsiella* strain. The mice were observed for at least 48 hours and the number of deaths in each group recorded. The mice were deemed to have been protected if the vaccinated mice survived.

2.2.5.2. Serum Bactericidal Assays

Bacteria for serum bactericidal assays and phagocytosis, unless otherwise stated, were grown on nutrient agar plates at 37°C to optimise capsule production in the encapsulated strains. Cells were resuspended in 0.9% w/v NaCl before addition to blood or serum. Resuspension directly from nutrient agar plates reduced the loss of capsular material that resulted from growth in liquid media and subsequent centrifugation at the high speed required to sediment these encapsulated bacteria.

To 0.6ml of human serum containing the appropriate rabbit serum or pre-immune serum together with 0.9% w/v NaCl or MgEGTA in a capped polypropylene tube, 0.6ml of the appropriate bacterial suspension (A_{470nm} 0.2) prepared as described above was added. The serum-bacteria mixture was incubated at 37°C in a reciprocal shaking water bath shaking at 120rpm. At timed intervals, 100 μl samples were taken from the mixture, diluted with sterile distilled water and viable counts made according to the method of Miles & Misra (1938)(see section 2.2.1.3.) after overnight growth for 18 hours at 37°C. Each experiment was carried out in triplicate.

2.2.5.3. Phagocytosis in Whole Blood

Phagocytosis was carried out in heparinised whole blood by means of the method described by Jones *et al* (1979). Fresh venous blood (10ml) drawn from a single healthy volunteer was placed in a sterile, heparinised plastic tube (New Brunswick LH/10) and used within 1 hour of withdrawal. To 0.5ml of blood in a capped polypropylene tube, 40 μl of the appropriate rabbit antiserum or pre-immune rabbit serum was added followed by 0.5ml of a bacterial suspension (A_{470nm} 0.2) prepared as in section 2.2.5.2. The volume of antiserum added was chosen to give optimum opsonisation without detectable agglutination of the bacteria. A direct microscopic count showed that the bacteria:PMN ratio in this incubation mixture was 15:1. This ratio was chosen to

optimise the level of ingestion; in some experiments, the ratio was reduced to 1.5:1 by reducing the number of bacteria. Pre-opsonisation of the bacteria before addition of the blood did not affect phagocytosis. The blood-bacteria mixture was incubated in a shaking water bath at 120rpm and 37°C. 100 μl samples were removed at intervals, added to 0.9ml of sterile distilled water at room temperature and allowed to stand for 5min before further dilution to lyse the blood cells without killing the bacteria, before making a viable count. 30min after the start of each experiment, a small sample was removed from the blood-bacteria mixture and spread on a glass microscope slide. The blood smear was dried in air, fixed with methanol and stained with Giemsa's stain. The number of bacteria associated with 50 PMNs randomly counted under the microscope was recorded, to provide an estimate of the number of bacteria ingested per PMN. Each experiment was repeated in duplicate twice. To retain some continuity, a single blood donor was used. However, the results obtained were confirmed by repeating some of the experiments with blood from 3 other volunteers.

2.2.6. Antibiotic Assays

2.2.6.1. Determination of the Antibiotic Sensitivity of *K.aerogenes* DL1 using "Neosensitab" Antibiotic Discs

Stationary phase DL1 grown in CDM1+Fe were harvested and resuspended in CDM1+Fe lacking glucose to give a suspension of approximately 10^5organisms/ml. Well dried Meuller-Hinton agar plates were flooded with 2.0ml of this suspension and excess liquid removed with a pasteur pipette. 28 different antibiotic discs were dispensed onto the plates (maximum of 6 discs per plate - see fig.30). Each plate was allowed to stand at room temperature for 1 hour to enable the antibiotic to diffuse into the agar, and then incubated for 18 hours at 37°C. The resulting zones of inhibition were measured to the nearest millimetre and recorded.

2.2.6.2. Antibiotic Agar Assays

A range of concentrations of the antibiotic to be tested, in nutrient agar, at 40°C were prepared, poured into petri dishes and allowed to set. Bacteria were diluted in 0.9% w/v NaCl (for bacteria grown on nutrient agar) or in CDM1 lacking glucose and the nutrient being investigated (for bacteria grown in simple salts media) to give approximately 10^3 organisms/ml. 4x100 μl samples and 3x100 μl samples were spread over nutrient agar and antibiotic agar plates respectively and incubated for 18 hours at 37°C. Each experiment was performed in duplicate.

3. - 7. RESULTS AND DISCUSSIONS

3. Nutritional Requirements of *K.aerogenes* DL1 in Chemically Defined Media

3.1. Growth Depletion Experiments

Attempts to grow *Klebsiella aerogenes* DL1 in the CDM designed for *E.coli* W3110 by Klemperer *et al* (1979) revealed that the buffering power of 25mM MOPS (3-(N-morpholino) propanesulphonic acid) was insufficient to maintain pH at physiological values. A MOPS concentration in excess of 100mM was required to prevent pH falling below pH 6.5 (Table 2). To avoid the use of a high MOPS concentration, Sorensens phosphate buffer was used for all growth depletion experiments except phosphate depletion where a 100mM solution of MOPS was used.

DL1 grew exponentially to A_{470} 1.5 in the CDM of Klemperer *et al*(1979) buffered with Sorensons' phospate buffer pH7.4. The pH after overnight growth to stationary phase in this medium was pH 6.9. Addition of 20×10^{-6} M $FeSO_4.7H_2O$ to this medium enabled DL1 to grow exponentially to A_{470} 4.0 after which the carbon and nitrogen sources were later found to be limiting.

Table 2. Effect of MOPS concentration on pH after growth of DL1 for 16 hrs in CDM1+Fe buffered with MOPS

MOPS pH7.2 (mM)	25	50	100	150	200
pH(after 16hrs growth)	3.5	4.7	6.63	6.75	6.82
A_{470nm} (after 16hrs growth)	2.95	2.75	3.05	2.8	3.0

MOPS = 3-(N-morpholino) propanesulphonic acid, adjusted to pH 7.2 with concentrated NaOH.

3.1.1. Carbon Depletion

The addition of increasing concentrations of D-glucose as the carbon source to CDM1+Fe (Table 4) in which all other ingredients were present in excess resulted in an extended log phase and a greater cell mass as determined by absorbance measurements at 470nm (Fig.16). At the lower concentrations, growth ceased abruptly whilst at higher concentrations, a more gradual slowing of the growth rate was observed. A linear relationship was found to exist between the concentration of added glucose and the end of the log phase up to A_{470}8.0 (Fig.17). This line extrapolated through the origin showing that DL1 has an absolute requirement for glucose in this medium. Addition of more than 28mM of glucose did not enable the organism to grow exponentially above A_{470} 8.0 (Fig.17). The doubling time (or mean generation time) is defined as the time required for all components of the culture to increase by a factor of two (Stanier *et al*,1977). For added glucose concentrations of between 2 mM and 35 mM, the doubling time of DL1 during the log phase was calculated to be 33 min. The growth rate (μ) of DL1 can now be calculated from the following equation:

$$\mu = \frac{\ln 2}{g}$$

Therefore the growth rate of *K.aerogenes* during the log phase was 1.26 hr^{-1}

3.1.2. Magnesium Depletion

In contrast to growth in carbon depleted CDM1+Fe, the growth of DL1 in CDM1+Fe containing increasing magnesium ion concentrations did not cease abruptly but slowed progressively (Fig.18). The absorbance at 470nm at which the organism stopped growing exponentially was designated A_{onset} and a plot of A_{onset} against the added magnesium concentration was constructed (Fig.19). A linear relationship was observed up to an A_{470} of 7.25 (Fig.19). Extrapolation of this plot to the x axis revealed a contaminating magnesium level of approximately 1×10^{-6}M. The growth rate of DL1 during the log phase was 0.63 hr^{-1} (doubling time 66min) in the absence of added magnesium and was 1.26 hr^{-1} (doubling time 33 min) in the presence of added magnesium concentrations of between 0.5 and 25×10^{-5}M.

3.1.3. Nitrogen Depletion

The nitrogen (supplied as ammonium ions (NH_4^+)) depletion growth curves were similar in shape to those of carbon depletion (Fig.20). A linear relationship, passing through the origin, between the maximum absorbance achieved and the added ammonium concentration was observed up to an A_{470} of 8.0 (Fig.21).

3.1.4. Sulphur Depletion

The sulphur (supplied as sulphate) depletion growth curves (Fig.22) resembled those obtained under conditions of magnesium depletion (Fig.23). A linear relationship existed between A_{onset} and added sulphate up to A_{470} 6.6. A contaminating level of approximately 2.4 x 10^{-5}M sulphate was present permitting exponential growth to A_{470} 0.65

3.1.5. Phosphate Depletion

The phosphate depletion growth curves shown in fig.24 were comparable to those obtained for magnesium and sulphate depletions inasmuch that a gradual slowing in growth rate was apparent at the end of the log phase. A plot of A_{onset} against the added phosphate concentration was linear up to A_{470} 3.75 (Fig.25). The low growth yield obtained in phosphate depleted media was later discovered to be due to a low medium potassium concentration. Phosphate contamination of CDM1+Fe in the absence of added phosphate was estimated to be 3.0×10^{-5}M.

3.1.6. Iron Depletion

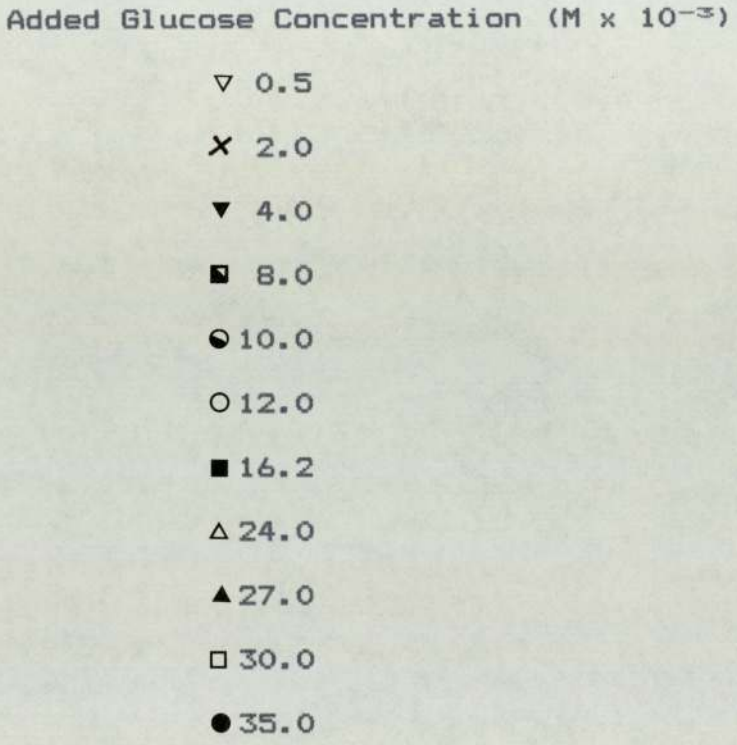
The addition of increasing concentrations of ferrous iron to CDM1 in which all other ingredients were present in excess resulted in an extended log phase and a greater cell mass (Fig.26). A linear relationship was found to exist between the concentration of added iron and the end of the log phase

up to A_{470} 6.2. (Fig.27). If no iron was added to the medium, the organism grew exponentially to an A_{470} of 2.1. The mean doubling time for DL1 in CDM1 both in the presence and absence of added Fe^{2+} during the log phase was 33min which is equivalent to a growth rate of $1.26hr^{-1}$. In 50% v/v heat inactivated horse serum, DL1 doubled at the same rate as in CDM1 up to an A_{470} of 0.5 (Fig.28). The reduction in the contaminating level of iron obtained by passage of the sodium phosphate buffer down a column of the cation exchange resin Chelex 100 resulted in a CDM (CDM2 - see 3.1.9.) which supported exponential growth up an A_{470} of 0.7 (Fig.28). Atomic absorption analysis (AAA) revealed that this medium contained $8 \times 10^{-7}M$ Fe^{3+}. The results show a discrepancy between the value for the contaminating concentration of iron ($5 \times 10^{-6}M$) in CDM1-Fe determined by extrapolation of fig.27 to the x-axis and the value of $1.4 \times 10^{-6}M$, obtained directly by AAA of CDM2-Fe (before treatment with Chelex 100), both media enabling DL1 to grow exponentially to A_{470} 2.1. One possible explanation for this discrepancy can be derived from the data described in 5.1.3. This data showed that the cells induced the formation of their IRMP and enterochelin several generations before A_{onset}. It therefore seems likely that the extrapolation method cannot be applied to estimate the concentration of contaminating iron.

3.1.7. Potassium Depletion

Replacement of the potassium hydrogen phosphate salt of Sorensons buffer by the corresponding sodium salt and the omission of added potassium chloride gave the growth curve shown in fig.29a. Therefore, DL1 has a definite requirement for potassium ions. Addition of $3x10^{-4}M$ potassium chloride enabled DL1 to grow exponentially to $A_{470}4.0$ (Fig.29b). CDM1+Fe buffered with the sodium/potassium phosphate buffer supplies $13.1x10^{-3}M$ potassium, which is sufficient (Fig.29).

Figure 16. Effect of Glucose Concentration on the Growth of *K.aerogenes* DL1



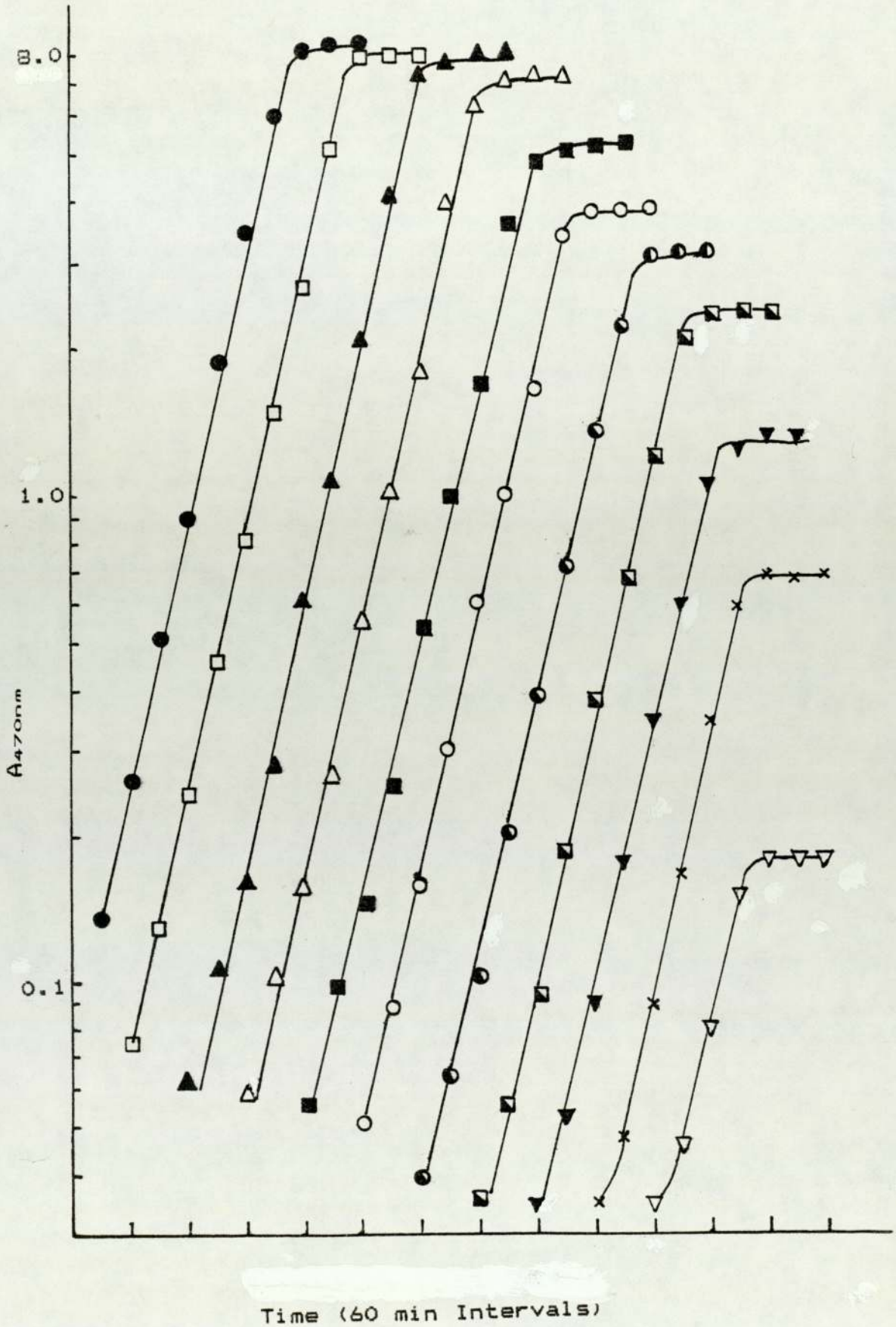

8.0
1.0
0.1
A470nm
Time (60 min Intervals)

Figure 17. Relationship between Maximum Exponential Growth of *K.aerogenes* DL1 and the Added Glucose Concentration

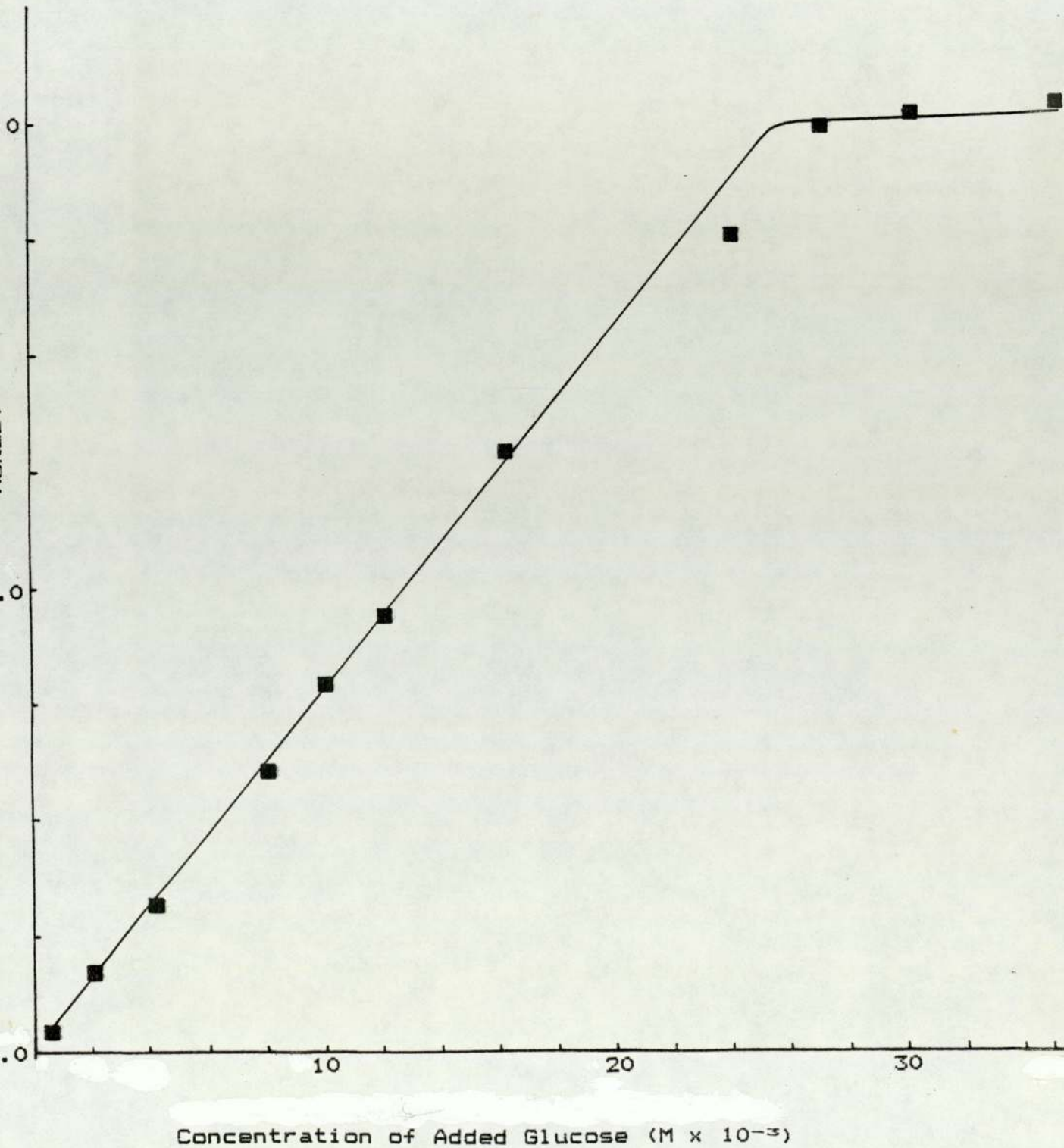
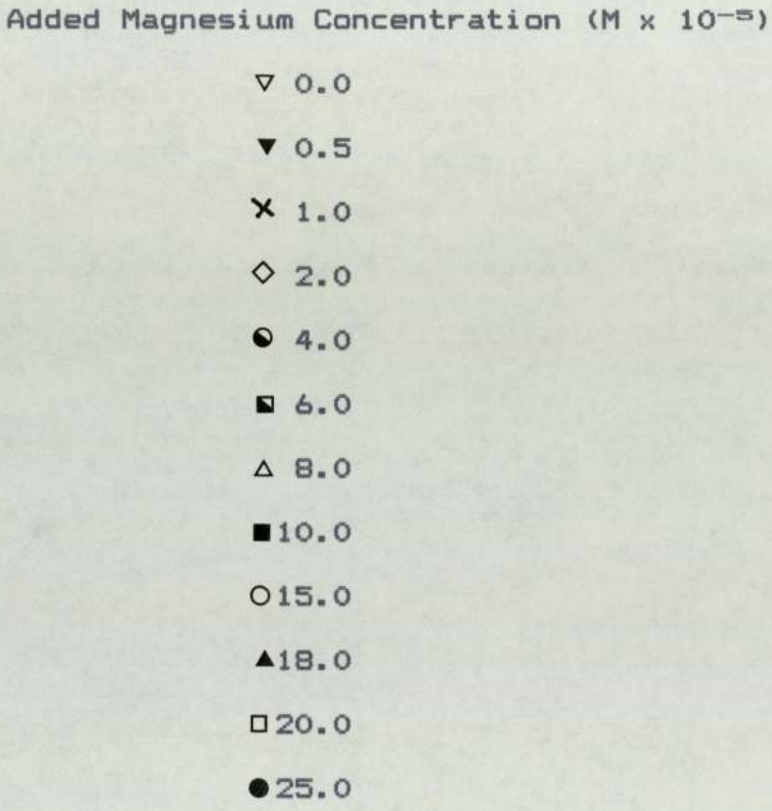


Figure 18. Effect of Magnesium Concentration on the Growth of *K.aerogenes* DL1



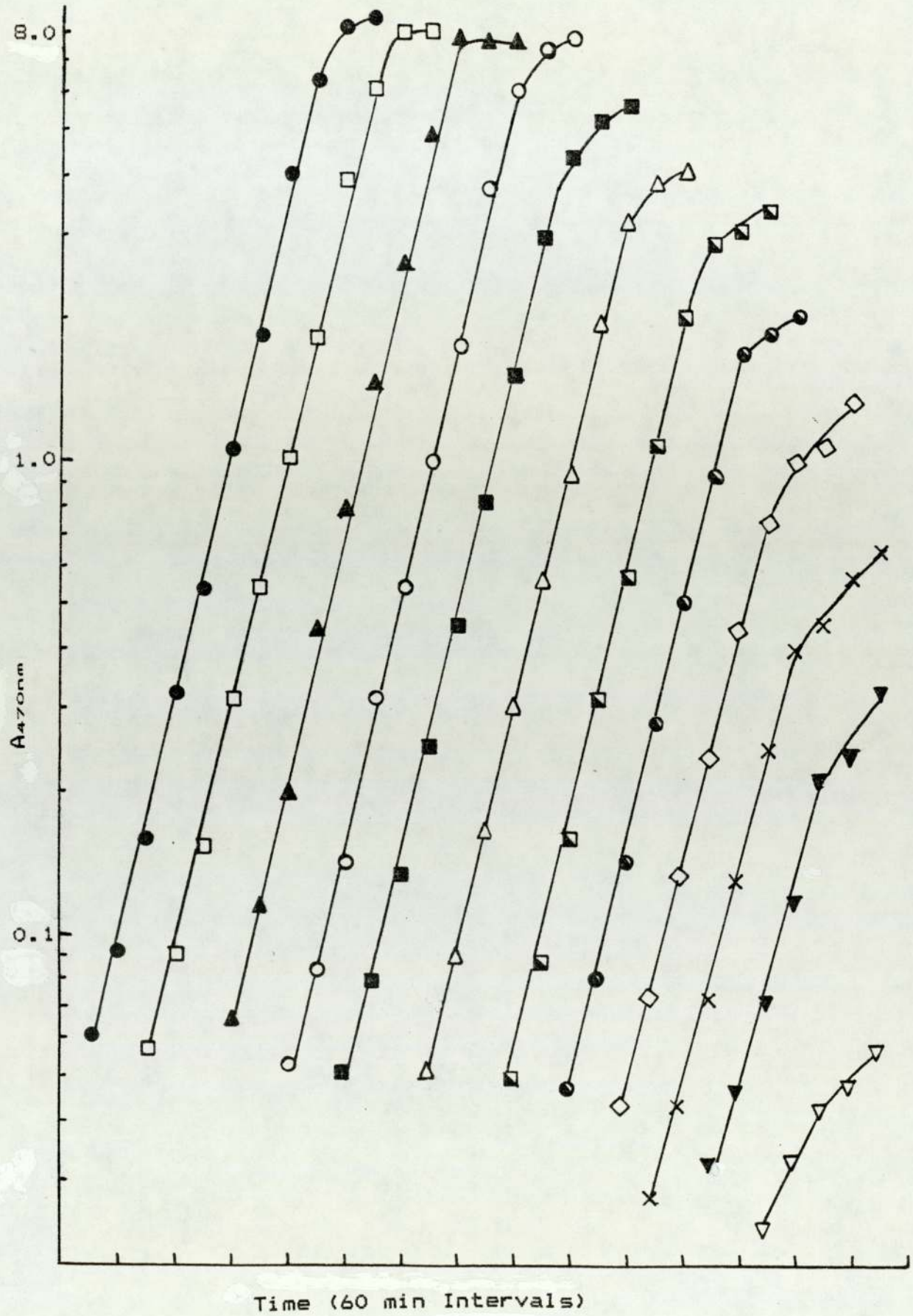

8.0
1.0
0.1
A470nm
Time (60 min Intervals)

Figure 19. Relationship between Maximum Exponential Growth of *K.aerogenes* DL1 and the Added Magnesium Concentration

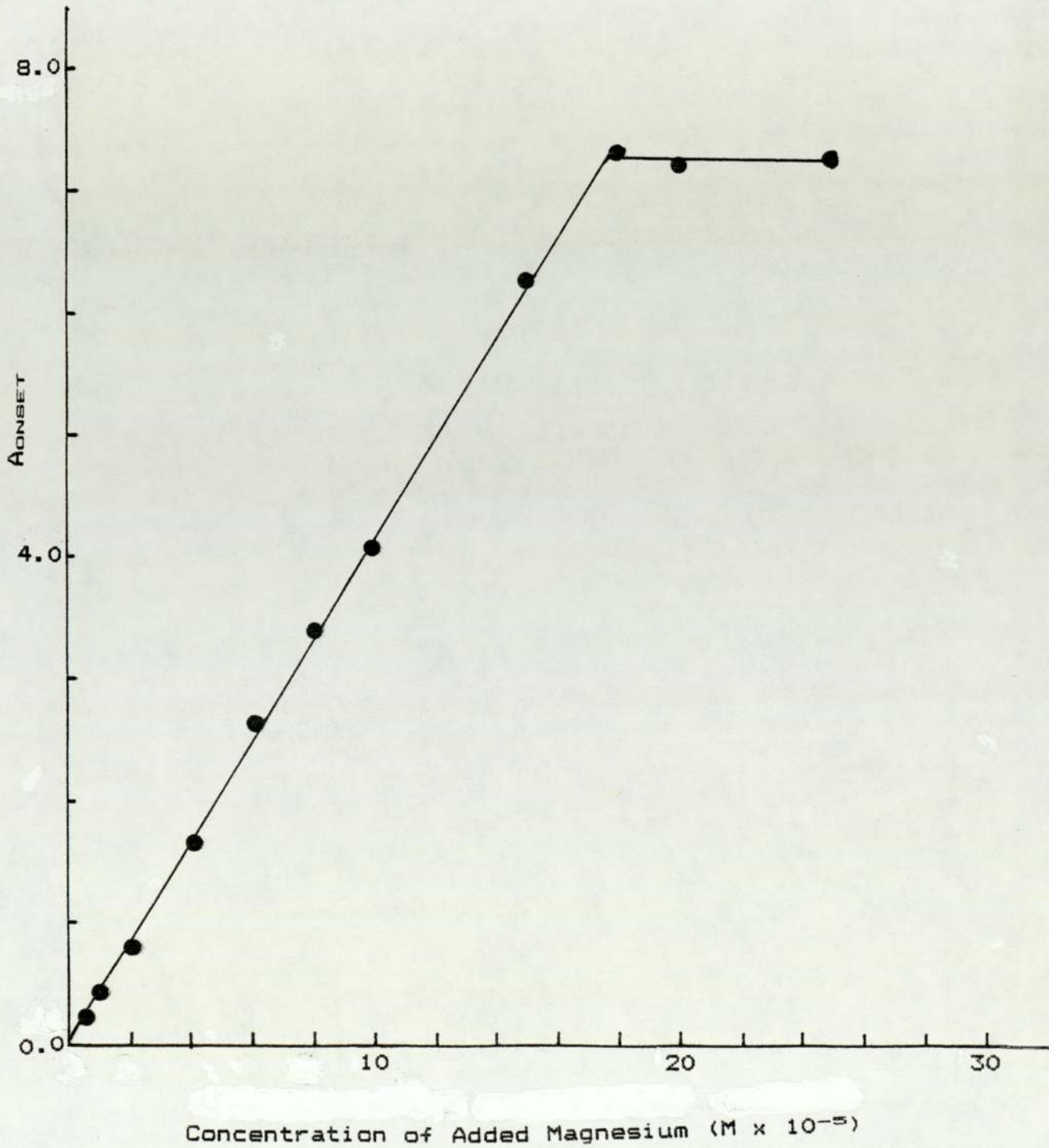
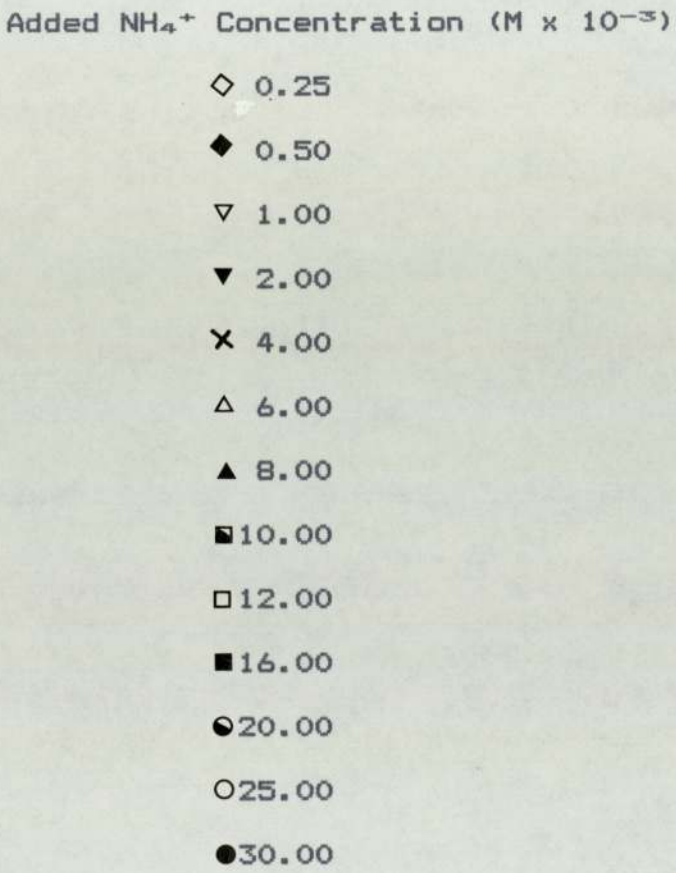


Figure 20. Effect of Nitrogen Concentration on the Growth of *K.aerogenes* DL1



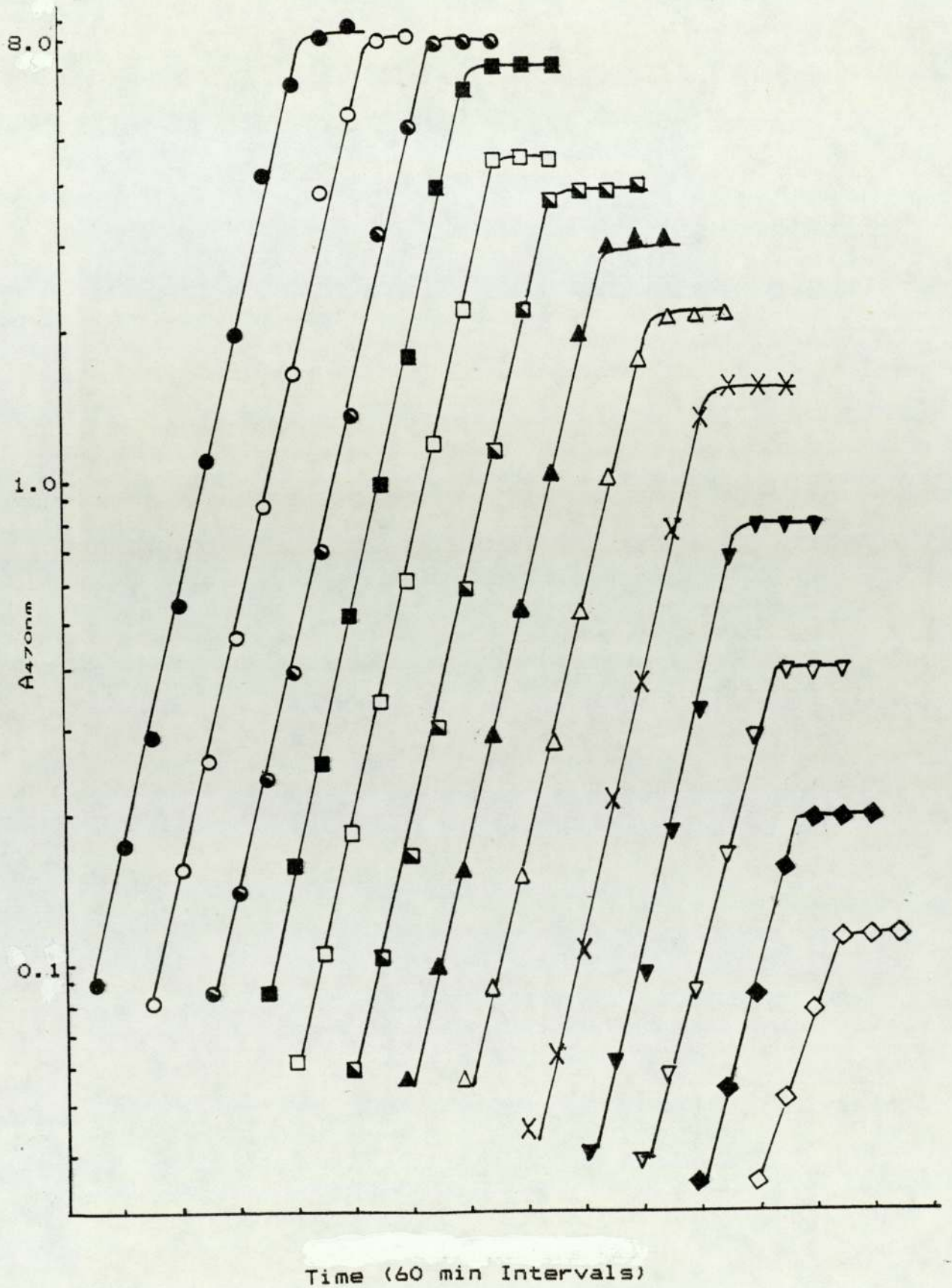

8.0
1.0
0.1
A470nm
Time (60 min Intervals)

Figure 21. Relationship between Maximum Exponential Growth of *K.aerogenes* DL1 and the Added Nitrogen Concentration

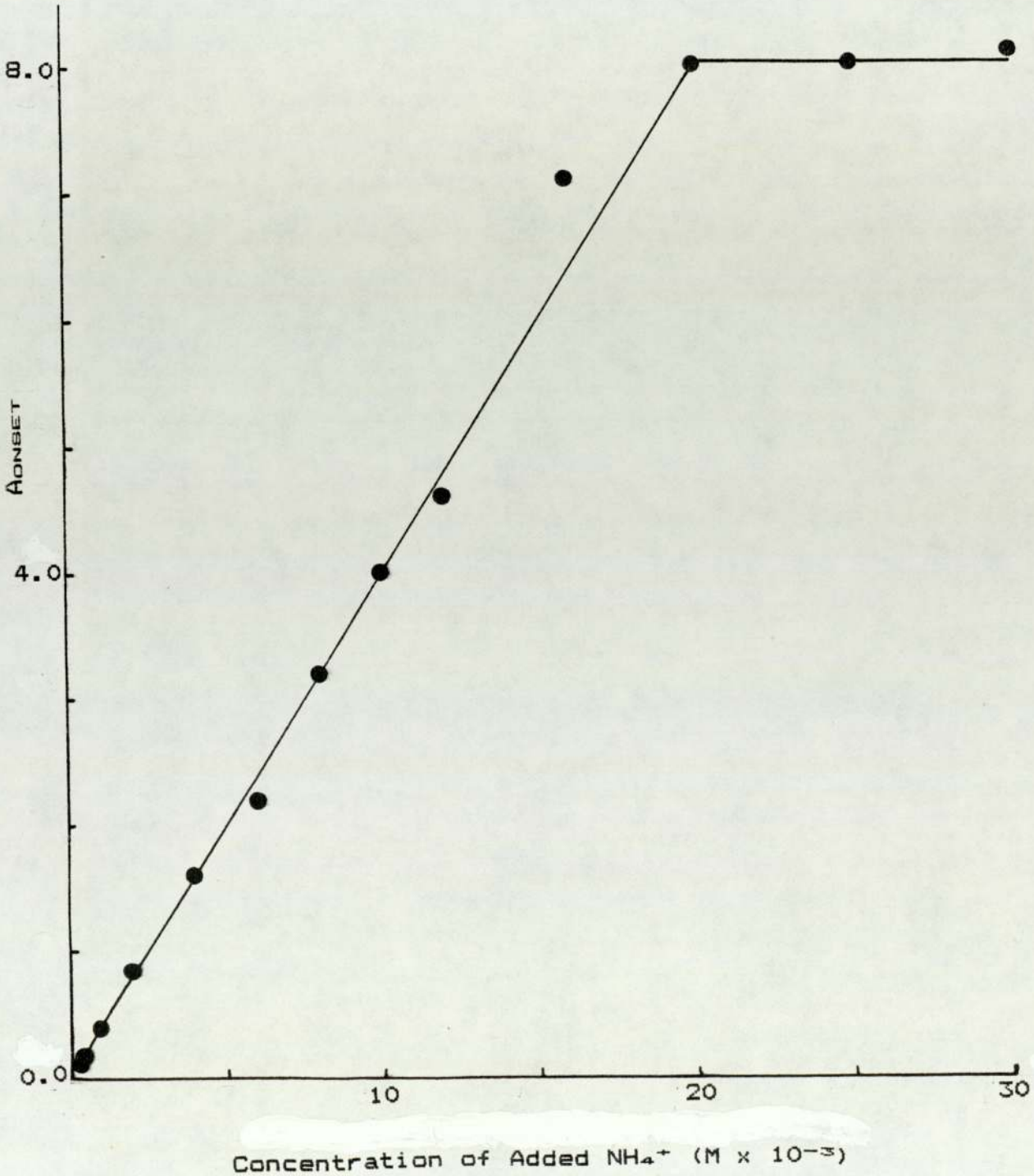
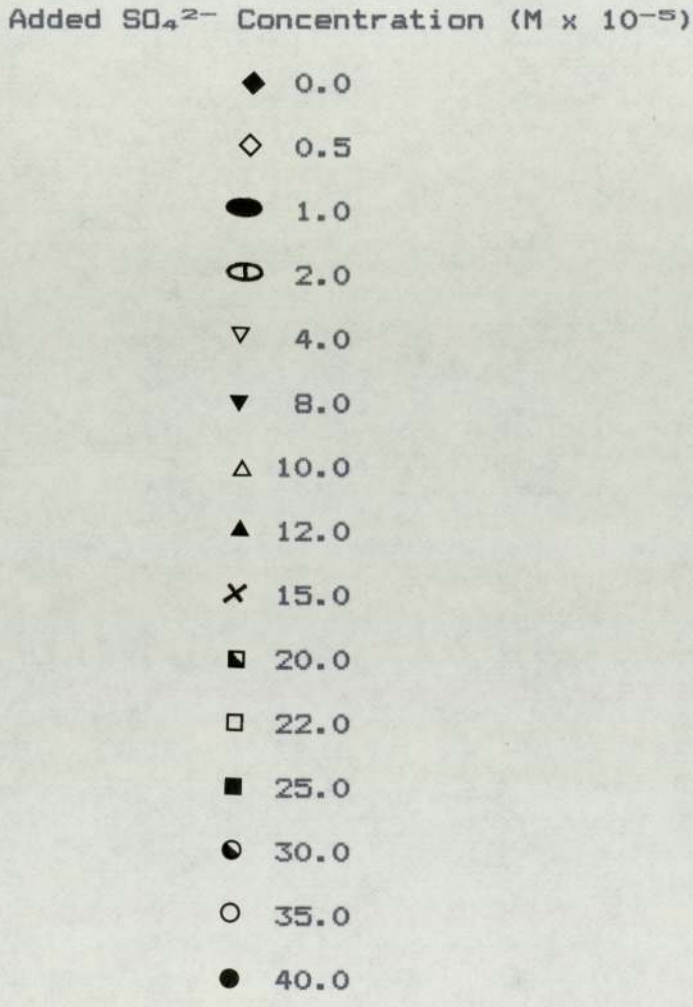


Figure 22. Effect of Sulphur Concentration on the Growth of *K.aerogenes* DL1



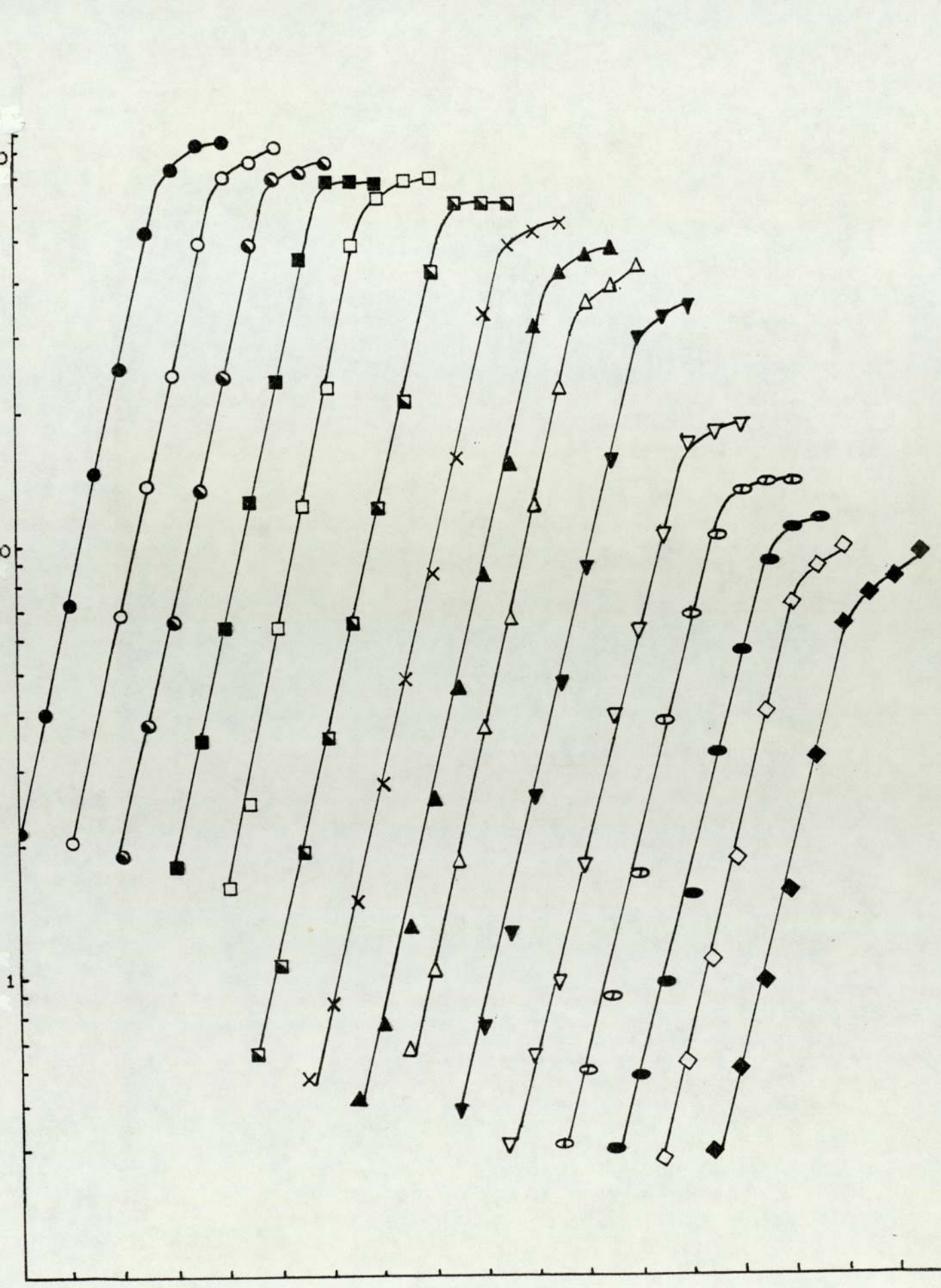

Time (60 min Intervals)

Figure 23. Relationship between Maximum Exponential Growth of *K.aerogenes* DL1 and the Added Sulphur Concentration

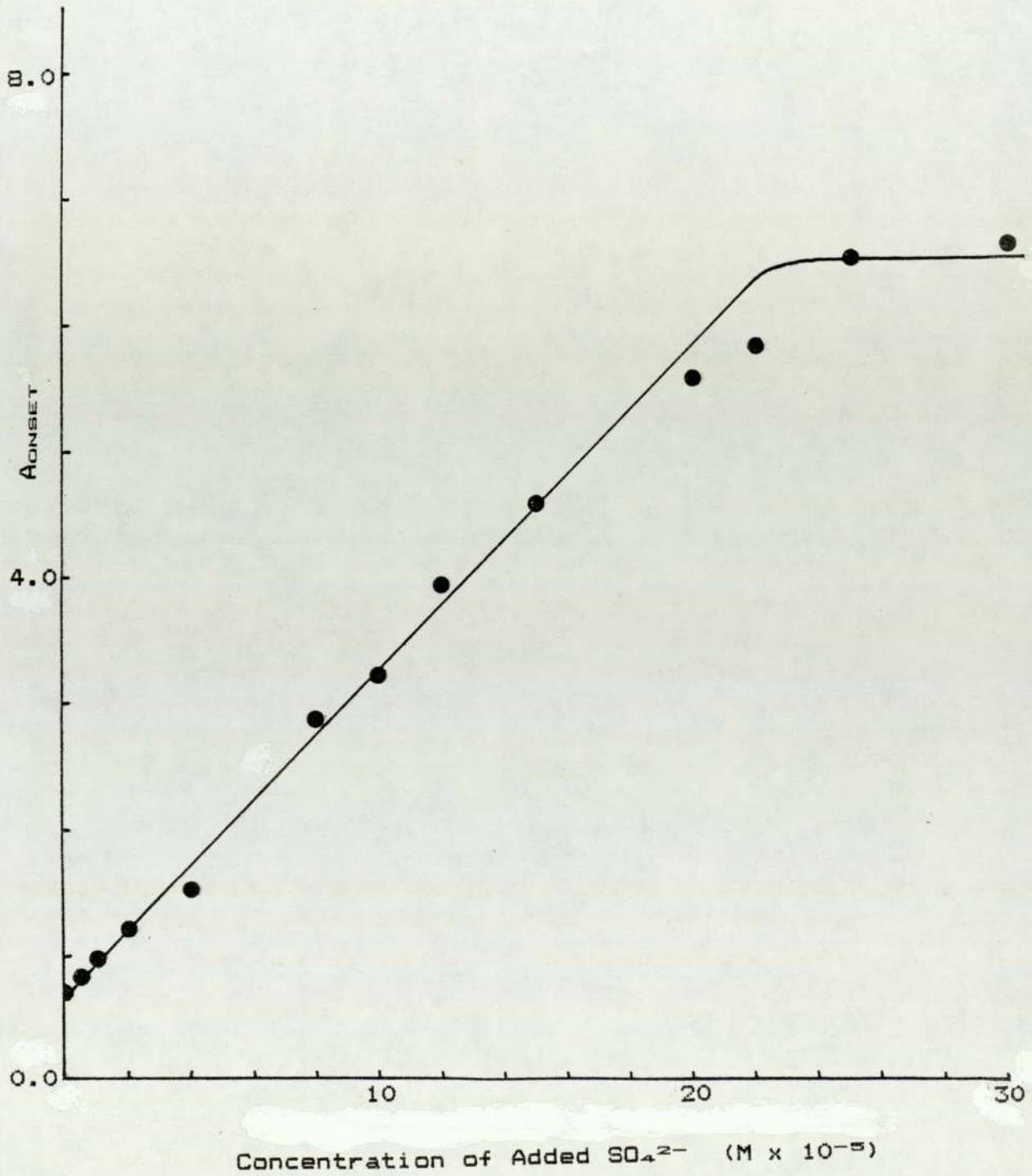


Figure 24. Effect of Phosphate Concentration on the Growth of *K.aerogenes* DL1

Added PO_4^{3-} Concentration (M x 10^{-4})

▼ 0.5

△ 1.0

▲ 2.0

◪ 4.0

□ 6.0

■ 8.0

◔ 10.0

○ 12.0

● 16.0

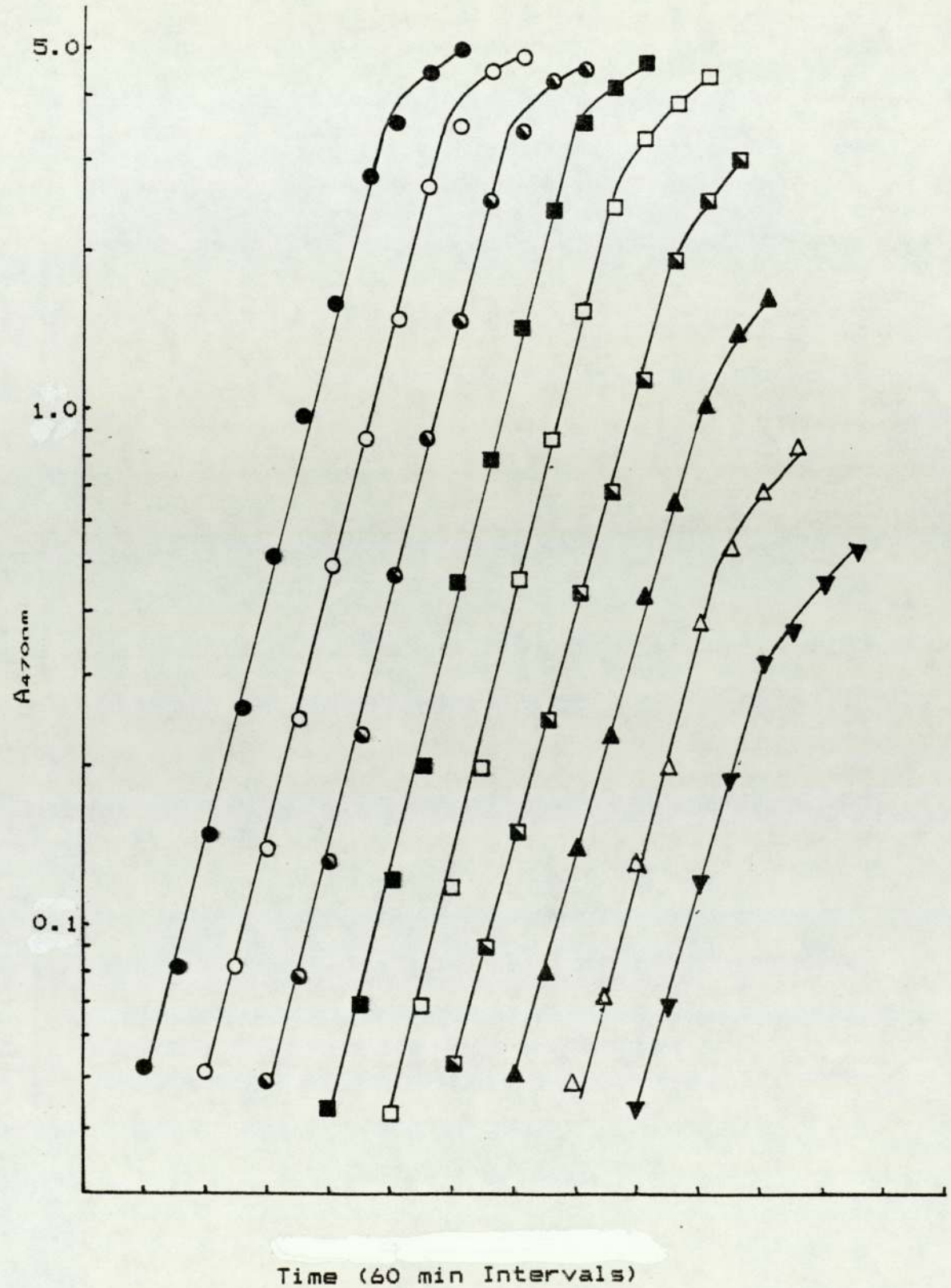

5.0
1.0
0.1
A470nm
Time (60 min Intervals)

Figure 25. Relationship between Maximum Exponential Growth of *K.aerogenes* DL1 and the Added Phosphorous Concentration

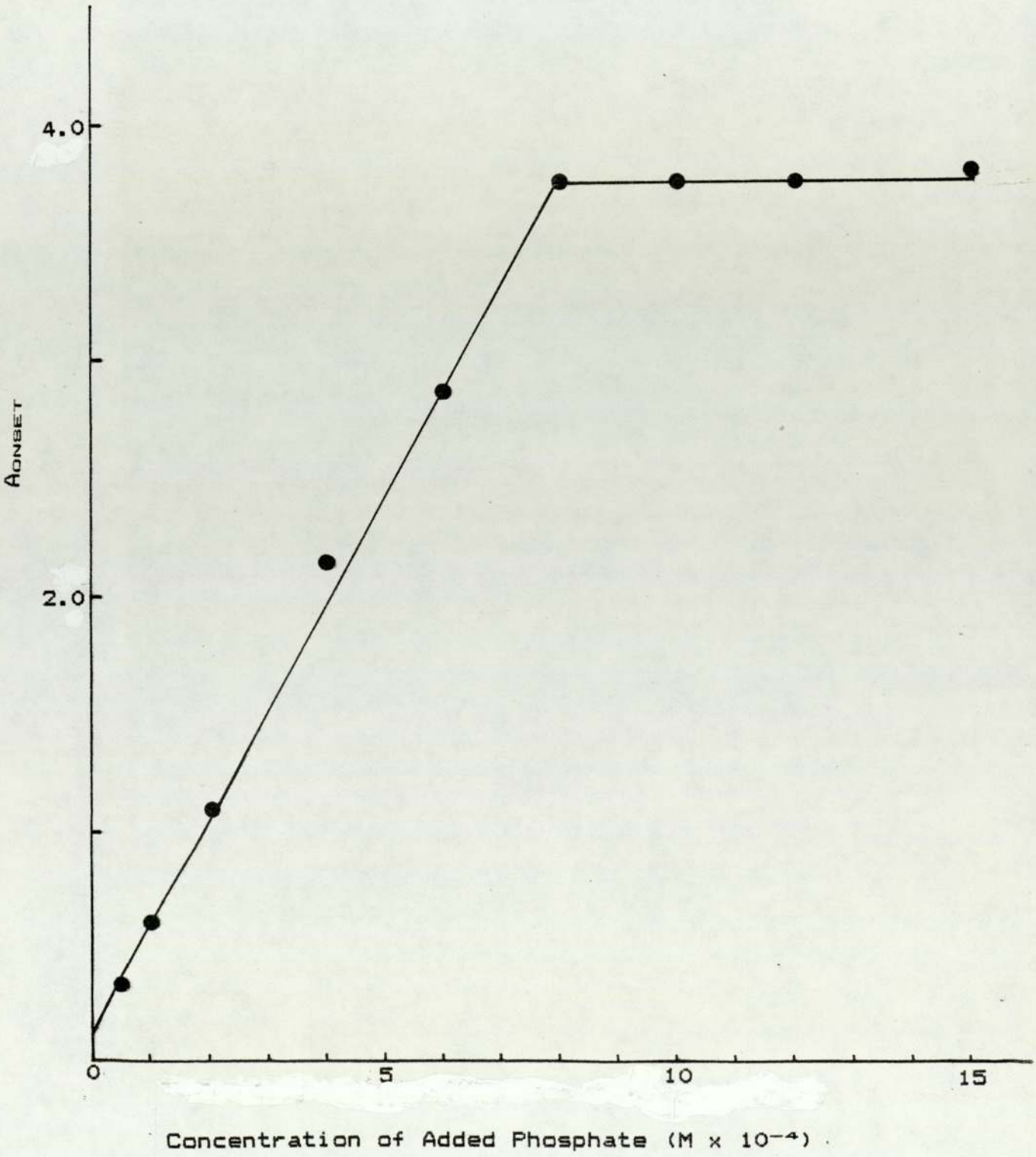


Figure 26. Effect of Iron Concentration on the Growth of *K.aerogenes* DL1

Added Fe^{2+} Concentration ($M \times 10^{-6}$)

△ 0.0

▲ 1.0

⊖ 2.0

● 4.0

◪ 5.0

□ 7.5

■ 10.0

◔ 15.0

○ 20.0

● 25.0

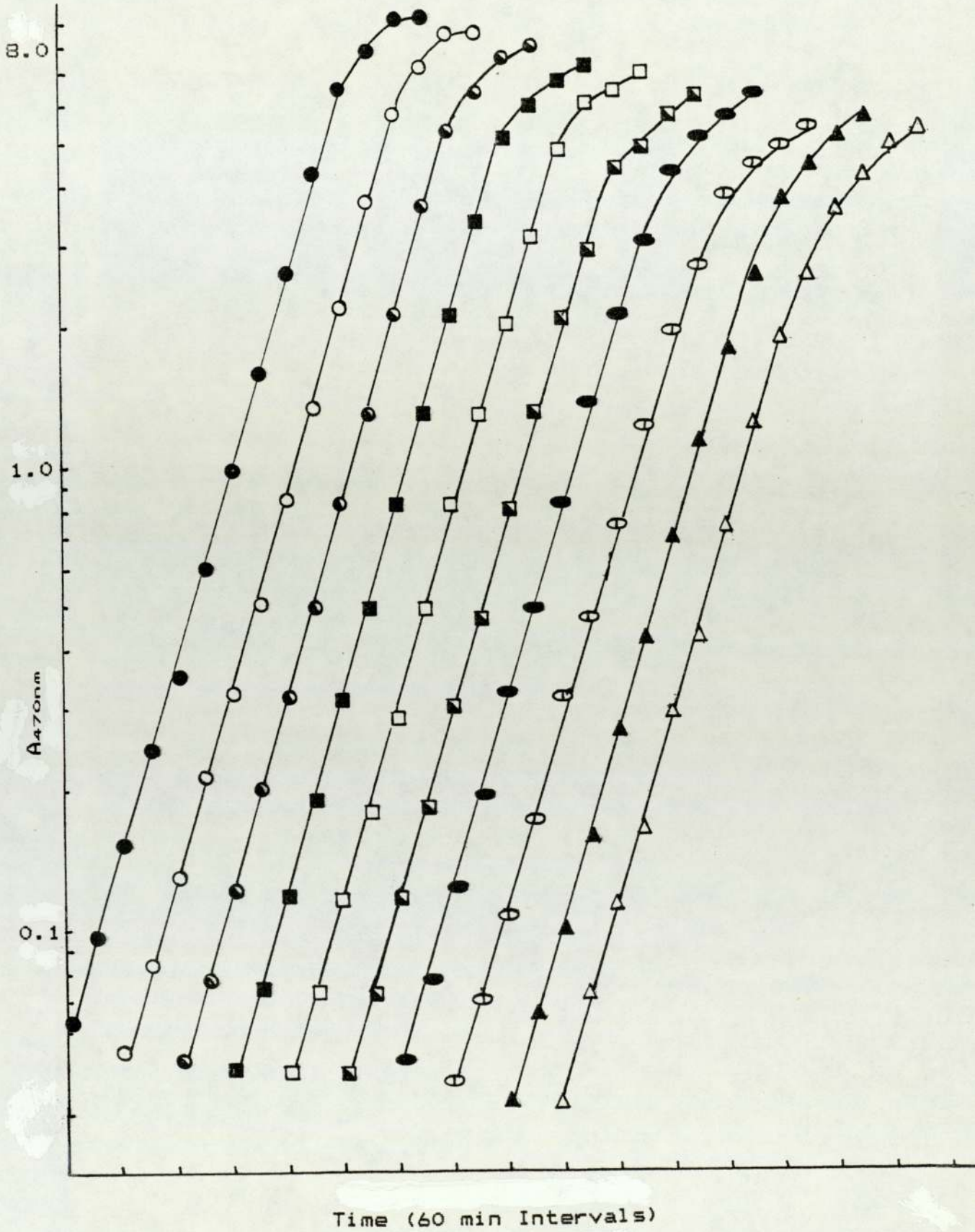

8.0
1.0
0.1
A470nm
Time (60 min Intervals)

Figure 27. Relationship between Maximum Exponential Growth of *K.aerogenes* DL1 and the Added Iron Concentration

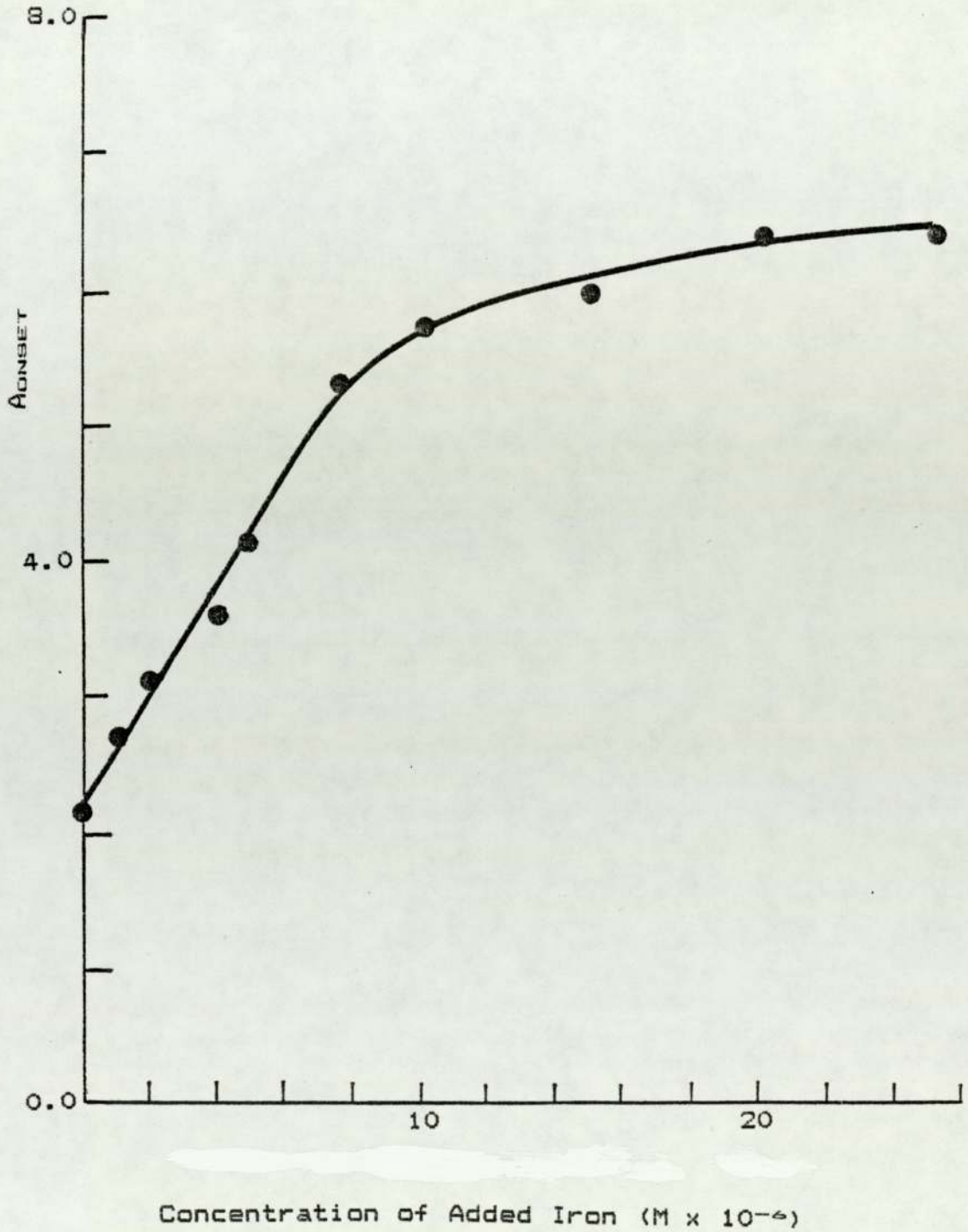


Figure 28. Growth of *K.aerogenes* DL1 in Iron Depleted and Iron Restricted Media

Key:

- ■ CDM2-Fe (Before Treatment with Chelex 100)
- ▲ CDM2-Fe (After Treatment with Chelex 100)
- ● Serum

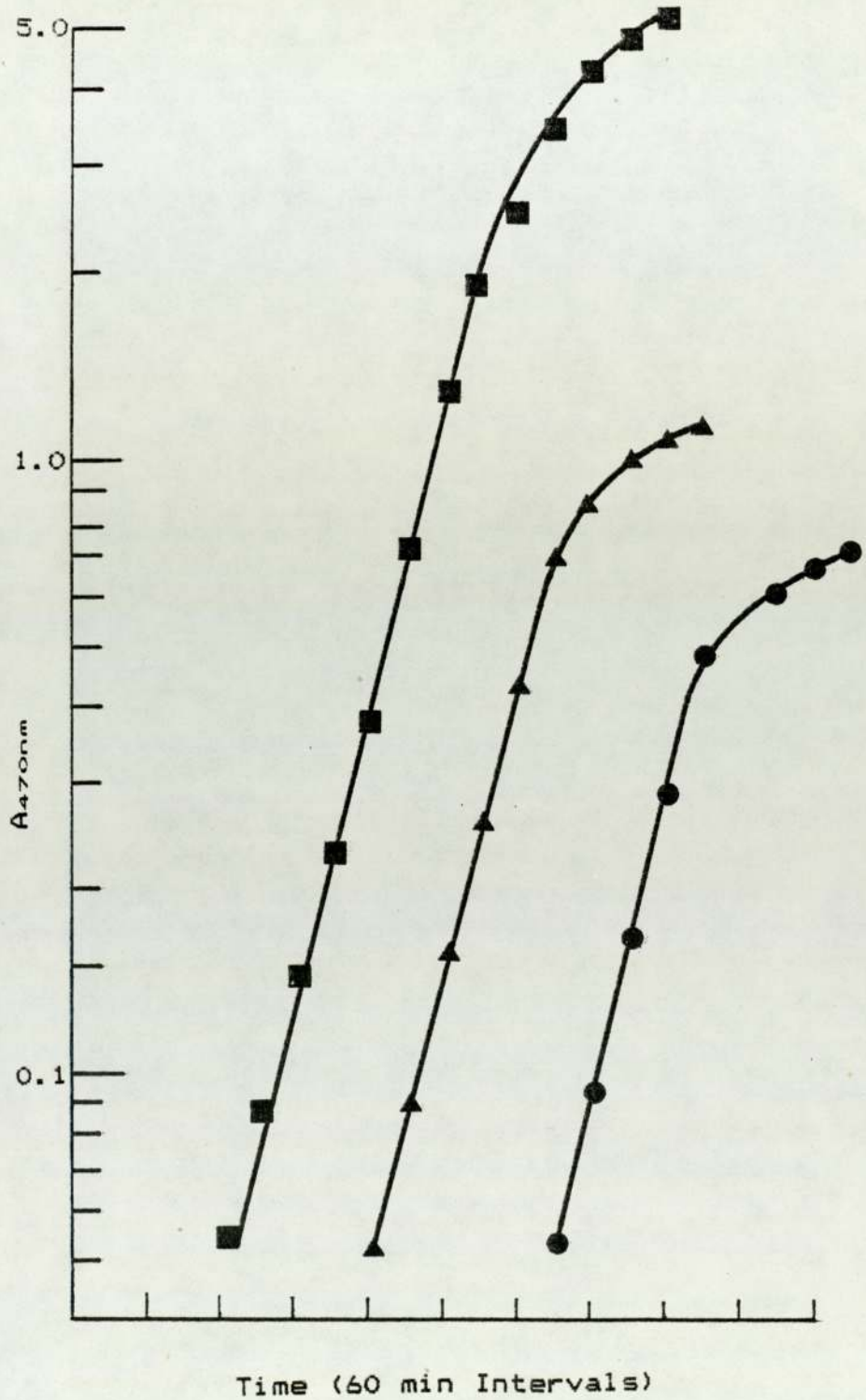

5.0
1.0
0.1
A470nm
Time (60 min Intervals)

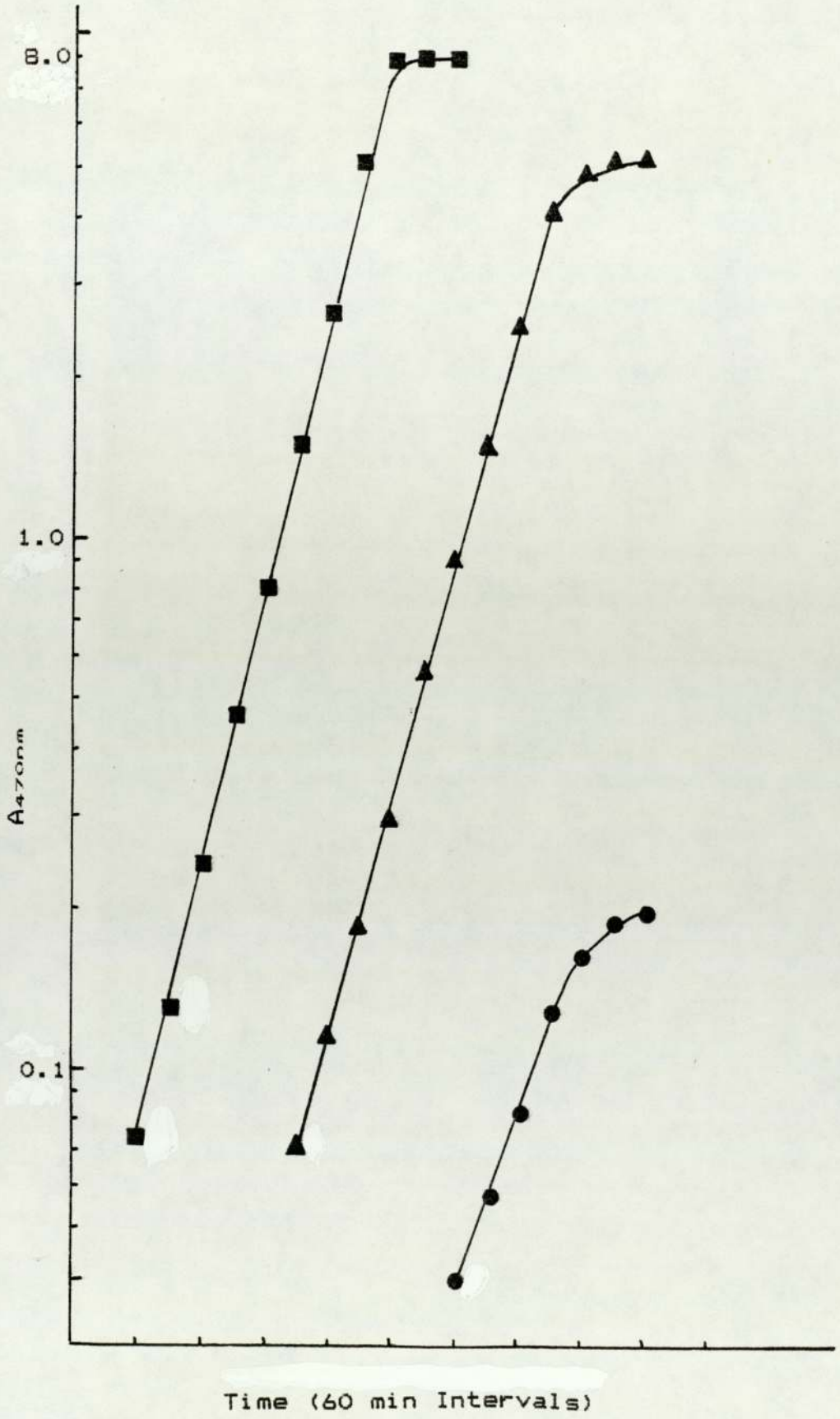
Figure 29. Effect of Potassium Concentration on the Growth of *K.aerogenes* DL1

Added Potassium Concentration ($M \times 10^{-4}$)

● 0.0

▲ 3.0

■134.0


8.0
1.0
0.1
A470nm
Time (60 min Intervals)

3.1.8. Summary of Media Requirements

The composition of the media used to define the requirements of *K.aerogenes* DL1 for each nutrient is summarised in table 3. Table 4 outlines the composition of the CDM1+Fe (CDM1) which will permit the maximum growth of *K.aerogenes* in batch culture.

3.1.9. Composition of the Medium used to Investigate the effect of Iron Deprivation on the Production of Outer Membrane Proteins and Siderophores by *Klebsiella aerogenes*

To investigate the effects of iron depletion on *K.aerogenes* DL1, the composition of CDM1+Fe was amended as shown in table 5. In CDM2 potassium dihydrogen phosphate was replaced by sodium dihydrogen phosphate and the medium supplemented with potassium chloride. The disodium hydrogen phosphate/sodium dihydrogen phosphate buffer was then treated with the Chelex 100 (sodium form) cation exchange resin to reduce the concentration of contaminating iron. As Chelex 100 also removes other trace metals, CDM2 was supplemented with the trace elements shown in table 5. $FeSO_4.7H_2O$ (20×10^{-6} M) was added to obtain an iron enriched medium (CDM2+Fe). The osmolarity of CDM2 was calculated by freezing point depression measurements to be 230 milliosmoles. Sodium chloride (45.2 mM) was therefore

added to both CDM2+Fe and CDM2-Fe to render them isotonic with serum (approximately 300 milliosmoles).

Table 3. Summary of the Medium Composition Used for Each Nutrient Depletion

Nutrient	Depletion (mM)						
	C	Mg	N	S	P	Fe	K
D-Glucose	*	35	35	35	35	35	35
KCl	0.3	0.3	0.3	0.3	0.3	0.3	0.3
$MgCl_2$	-	-	-	0.2	-	-	-
NH_4Cl	25	25	*	25	25	25	25
$MgSO_4.7H_2O$	0.2	*	0.2	0.2	0.2	0.2	0.2
$(NH_4)_2SO_4$	0.2	0.2	-	*	-	0.2	-
Na_2SO_4	-	-	0.2	-	-	-	-
Na_2HPO_4 /KH_2PO_4 (pH 7.4)	66.7	66.7	66.7	66.7	-	66.7	-
Na_2HPO_4 /NaH_2PO_4 (pH 7.4)	-	-	-	-	-	-	66.7
**MOPS (pH 7.4)	-	-	-	-	100	-	-
$FeSO_4.7H_2O$	0.02	0.02	0.02	-	0.02	*	0.02
$FeCl_2.4H_2O$	-	-	-	0.02	-	-	-
Na_2HPO_4 .$12H_2O$	-	-	-	-	*	-	-

* Nutrient being studied

**MOPS = 3-(N-morpholino) propanesulphonic acid adjusted to pH 7.4 with concentrated NaOH

Table 4. Composition of CDM1+Fe, a Simple Salts Medium Designed to obtain Maximum Growth of *K.aerogenes* DL1 in Batch Culture

Nutrient	Concentration (M)
Glucose	35×10^{-3}
$MgSO_4.7H_2O$	20×10^{-5}
NH_4Cl	25×10^{-3}
Na_2HPO_4 / KH_2PO_4 (pH 7.4)	66.7×10^{-3}
KCl	30×10^{-5}
$(NH_4)_2\ SO_4$	20×10^{-5}
$FeSO_4.7H_2O$	2×10^{-5}

CDM1+Fe will enable *K.aerogenes* DL1 to attain an A_{ONSET} of between 6.0 and 8.0. In the absence of added iron sulphate (i.e. CDM1-Fe), DL1 will grow to an A_{ONSET} of about 2.0.

Table 5. Composition of CDM2-Fe, a Simple Salts Medium used to Investigate the Effect of Iron Deprivation on the Production of Outer Membrane Proteins and Siderophores by *Klebsiella aerogenes*

Nutrient	Concentration(mM)	A*
Glucose	35.0	10.0
NH_4^+	25.0	10.0
Mg^{2+}	0.4	18.0
Na_2HPO_4 / NaH_2PO_4 (pH 7.4)	66.7	-
K^+	15.0	20.0
SO_4^{2-}	0.4	13.3
NaCl	45.2	-

Where A* is the maximum theoretical A_{ONSET} obtainable. The simple salts medium described above was supplemented with the following trace elements: $CaCl_2.6H_2O$, 0.5 μM; H_3BO_3, 0.5 μM; $CoCl_2.6H_2O$, 0.05 μM; $CuSO_4.7H_2O$, 0.05 μM; $ZnSO_4.7H_2O$, 0.05 μM; $MnSO_4$, 0.1 μM; $(NH_4)_6Mo_7O_{24}.4H_2O$, 0.005 μM. This medium which is isotonic with serum was called CDM2-Fe. $FeSO_4.7H_2O$ (20×10^{-6}M) was added to obtain an iron rich medium (CDM2+Fe). Atomic absorption analysis showed that after treatment of the sodium phosphate buffer with Chelex 100, CDM2-Fe contained 8×10^{-7}M Fe^{3+}.

3.1.10. The Effect of Nutrient Depletion on the Relationship between Absorbance, Dry Weight and Total Count

Triplicate samples of log phase and magnesium-depleted (Mg-) *K.aerogenes* DL1 were harvested at A_{470} 2.0 and dry weight estimations and total counts performed as in section 2.2.

Table 6. The Effect of Nutrient Depletion on the Relationship between Absorbance, Dry Weight and Total Count

	A_{470}	Dry Weight (µg/ml)	Total Count (orgs/ml)	Dry Weight (µg/10^8orgs/ml)
Log phase	1.0	233	3.4×10^8	68.5
Mg-	1.0	337	4.2×10^8	80.2

Table 6 shows that the dry weights of log phase and magnesium-depleted cells are different when based on µg/ml at A_{470} 1.0. This difference is not apparent if they are expressed as µg/10^8orgs/ml. Stationary phase magnesium-depleted cells are therefore smaller than log phase cells and so scatter less light.

3.2. Discussion

Besides water, all living cells consist mainly of carbon, nitrogen, phosphorous, sulphur, potassium, magnesium and to a lesser extent, iron. Hence in order to grow, all of these elements must be present in the medium in a useable form. In the natural environment, essential nutrients will frequently be depleted as a consequence of the metabolic activity of indigenous microbial populations and therefore growth will often be nutrient limited. When nutrient limited, a dividing bacterium will manufacture an envelope characteristic of the particular depletion (Brown,1977). The effect of different nutrient depletions in addition to effects on general biochemistry is to give rise to microbial populations with radically different envelopes. This phenomenon is particularly evident with respect to the gram negative outer membrane and has been shown to influence their response to antimicrobial agents (Finch & Brown,1975; Noy,1982; Miveld,1983; Sterkenburg & Wouters,1981) and host defences (Finch & Brown,1978; Anwar *et al*,1983).

When the growth of a culture is limited by a particular nutrient, the growth yield obtained is directly related to the initial concentration of the nutrient and will depend on the way the nutrient is utilised by the cell and the structures into which it is incorporated. The quantitative formulation of a simple salts medium enables each constituent to be included in concentrations leading to cell

depletion at a predetermined absorbance or in known excess. The nutritional requirements of *K.aerogenes* DL1 are such that it can be grown in a simple salts medium without the addition of complex growth factors. DL1 is therefore a non-exacting heterotroph and in CDM1+Fe i.e. all ingredients present in excess, achieved a maximum absorbance at 470nm of between 6.0 and 8.0. The addition of trace elements did not increase the growth yield. Thus, further growth is presumably prevented either by the accumulation of toxic metabolites or because oxygen became limiting, as all other nutrients were still present in excess.

The minimal nutritional requirements of DL1 can usefully be compared with those quantitatively determined for *E.coli* (Klemperer *et al*,1977) and *P.aeruginosa* (Noy, 1982) (Table 7). These requirements not only reflect an organism's metabolism but also its chemical composition. Therefore a difference in the nutrition of gram-negative bacteria may be due to variation in chemical composition or to a more or less efficient system of metabolising a particular nutrient. It should, however, also be noted that changes in cell size accompanying nutrient depletion (see Table 6 or Tempest *et al*,1965) affect the light scattering properties of a culture, so that the same absorbance measurement for different cultures does not necessarily reflect the same dry weights or total cell numbers.

Table 7. Growth Requirements in Simple Salts Media of *K.aerogenes*, *E.coli* and *P.aeruginosa*.

	Concentration Required for Exponential Growth to A 1.0			
Organism	*K.aerogenes* DL1	*E.coli* RP1$^-$	*E.coli* RP1+	*P.aeruginosa* NCTC 6750
Absorbance (nm)	470	420	420	470
Nutrient				
Glucose (Mx10^{-3})	3.50	2.50	2.80	4.00
NH_4^+ (Mx10^{-3})	2.50	1.85	1.75	4.00
PO_4^{3-} (Mx10^{-4})	2.20	1.70	5.60	3.20
SO_4^{2-} (MX10^{-5})	3.80	2.60	1.90	5.20
K^+ (Mx10^{-5})	7.50	2.60	4.20	6.20
Mg^{2+} (Mx10^{-5})	2.40	1.30	2.50	4.00
Fe^{2+} (Mx10^{-7})	2.40	-	1.00	6.20

The concentration of each nutrient required to enable the organism to reach an absorbance of 1.0 was obtained from plots of A_{ONSET} against added nutrient concentration.

3.2.1. Glucose

Bacteria utilise glucose both as a carbon source and as an energy source. Carbon substrate limitation is characterised by a high carbon conversion efficiency with minimum diversion of substrate carbon into exocellular products. When relieved of carbon limitation, organisms frequently catabolise excess substrate at a high rate but because their anabolic processes do not increase at a similar rate, growth proceeds with a markedly decreased efficiency (Tempest & Wouters,1981).

When all the nutrients required for the growth of *K.aerogenes* DL1 except glucose were present in excess, growth ceased abruptly when the latter became exhausted. The shapes of the growth curves for a range of glucose concentrations are comparable with those observed for *E.coli* (Ismail,1977) and *P.aeruginosa* (Noy, 1982) in that a sharp cut off point is observed at the lower glucose concentrations (Fig.16). At glucose concentrations of 28mM and above, no further increase in growth yield was obtained presumably because oxygen became limiting. The exponential growth rate (1.26 hr^{-1}) which is equivalent to a doubling time of 33 min) was independent of the initial glucose concentration except at 0.5mM when an exponential growth rate of 1.15 hr^{-1} (doubling time 36 min) was observed. A linear relationship was found to exist between A_{470} at the end of exponential growth and glucose concentration up to

A_{470} 8.0 (fig.17). This line extrapolates through the origin, which indicates that the added glucose was the only utilisable carbon source. *K.aerogenes* DL1 in common with most other extracellular polysaccharide producing micro-organisms is unable to metabolise its own polysaccharide capsule (Dudman,1977) and therefore its capsule does not act as a reserve source of carbon and energy.

3.2.2. Magnesium

The growth curves of magnesium depleted DL1 differ markedly to those of glucose depleted DL1 (Fig.18). Growth did not cease abruptly upon exhaustion of the magnesium but progressively slowed. This gradual decrease in growth rate may arise as a result of a reduction in the synthesis of RNA and protein (McCarthy,1962). Magnesium is essential for the activity of many enzymes including those involved in the synthesis of cell wall components such as fatty acids (Knivett & Cullen,1967), peptidoglycan (Garrett,1969) and phospholipids (White *et al*,1971). It plays a role in maintaining the stability and permeability of cell membranes (Brock,1962) and in ribosmal and RNA synthesis (Cohn & Ennis,1967). There is much evidence that magnesium is a structural component of the OM of gram negative bacteria especially *P.aeruginosa* (Kenward *et al*,1979). The

magnesium content of bacterial cells has been shown to be greatly influenced by the prevailing growth conditions (Tempest & Sykes, 1966). Magnesium deficiency exerts a profound effect on ribosomal structure and function. Kennell and Magasanik (1962) observed changes in the ribosomal content of various bacteria when incubated in magnesium depleted media. In a magnesium limited culture of *Aerobacter aerogenes* (synonym:*Klebsiella aerogenes*), it was found that on increasing the growth rate, the ribosome, RNA and magnesium concentrations in the cells were increased (Tempest *et al*,1965). Also, the RNA:Mg ratio was found to be constant in magnesium limited *A.aerogenes* at several dilution rates. Similar findings for *P.putida* (Sykes & Tempest, 1965) supported the hypothesis that the ribosome and RNA content and hence rate of protein synthesis may be controlled by the amount of available magnesium.

In the absence of added magnesium, the exponential growth rate of DL1 was approximately half that observed in the presence of initial magnesium concentrations between 0.5 and 25 x 10^{-5}M i.e. 0.63 hr^{-1} as opposed to 1.26 hr^{-1} (Fig.18).

3.2.3. Nitrogen

Nitrogen, supplied as ammonium ions is an essential constituent of nucleic acids, proteins and some phospholipids. A rapid cessation of growth at the end of the exponential phase, similar to that observed for carbon

depletion, follows the exhaustion of medium nitrogen. Under conditions of nitrogen limitation, *K.aerogenes* and *E.coli* de-repress the synthesis not only of high ammonium uptake systems but also of analogous systems for the uptake of amino acids (Tempest & Wouters,1981). This may render cells particularly sensitive to those antibiotics like cycloserine that enter the cell by means of specific amino acid transport enzymes. Thus, ammonium limited cultures of *K.aerogenes* were found to be at least 10 times more susceptible to cycloserine than were potassium limited cells (Sterkenberg & Wouters,1981). Exopolysaccharide formation in *K.aerogenes* is favoured by nitrogen limitation particularly in the presence of excess carbohydrate (Sutherland,1977).

3.2.4. Sulphate

Sulphate is an essential component of all living cells. It is present in two important amino acids (methionine and cysteine) and in a number of coenzymes (e.g. coenzyme A, biotin, ferredoxin)(Stanier,1977). *K.aerogenes* can utilise sulphate as the sole source of sulphur. The growth curves of sulphate depleted *K.aerogenes* (Fig.22) resembled those obtained under magnesium, phosphate and iron depletions.

Robinson and Tempest (1973) reported that the sulphate limited growth of *K.aerogenes* caused a lowering of the

protein content of the cell envelope fraction and in the sulphur content of the soluble protein fraction as compared with glucose limited fractions but they found no differences in the sulphur content of ribosomal fractions or in the ribosomal protein content of the cells. Minor differences in outer membrane proteins, lipid and phosphate contents of have been shown to occur in sulphate depleted *P.aeruginosa* (Miveld,1983).

3.2.5. Phosphorous

Growth curves of phosphate depleted *K.aerogenes* DL1 were similar in shape to those of magnesium and sulphate depletions; as phosphate became limiting there was a progressive slowing of the growth rate. Dicks and Tempest (1965) reported the existence of a growth rate independent molar stoichiometry between magnesium, potassium, phosphorous and RNA in *A.aerogenes*. Changes in RNA were accompanied by corresponding changes in magnesium, phosphorous and potassium so that the stoichiometry was maintained. The gradual decrease in growth rate as phosphorous became limiting may therefore be explained by the reduction in protein synthesis as a result of ribosomal degradation.

The energy required to drive a biosynthetic reaction often

involves the mobilisation of chemical energy which is stored in the phosphoanhydride bonds of adenosine triphosphate (ATP). Phosphorous therefore plays an important role in biosynthesis as a component of ATP. It is also a constituent of the LPS and phospholipids of gram negative bacteria. Phosphate limitation of growth has been shown to result in changes in the phospholipid content of bacterial membranes (the phospholipids being functionally replaced by neutral lipids and fatty acids (Minnikin *et al*,1974; Noy,1982)) ,LPS, divalent cations and proteins (Noy,1982). The derepression of synthesis and excretion of a number of phosphate mobilising hydrolases also occurs (Tempest & Wouters,1981).

3.2.6. Potassium

Potassium may be present in bacteria in amounts equal to or greater than that of phosphorous (Tempest & Wouters, 1981). It is unique among the major nutrients in that it is contained within the cell in an unmodified and largely unbound state and can be exceedingly mobile. The concentration of potassium present in the cytoplasm is frequently much in excess of that present in the growth medium and hence under many conditions the organism will have to expend energy in order to concentrate the potassium within the cell against a large transmembrane potassium

gradient (Tempest & Wouters,1981). the potassium content of bacteria has been found to vary with growth rate and the osmolarity of the medium (Tempest & Wouters,1981). Potassium limitation of *K.aerogenes* unlike phosphorous sulphur or nitrogen limitation in the presence of excess carbohydrate does not stimulate exopolysaccharide production (Duguid & Wilkinson,1954). It has been suggested that under potassium deprivation, antagonism between ammonium and potassium ions occurs (Dicks & Tempest,1967), resulting in reduced uptake of the carbon substrate.

The growth curves of potassium depleted DL1 (Fig.29) resemble those of magnesium and phosphorous depletion. This may be correlated with the stoichiometry between RNA, phosphorous, magnesium and potassium in *A.aerogenes* (Dicks & Tempest,1966) which would explain the gradual decrease in growth rate as potassium became depleted. The potassium requirements of DL1 although not investigated in detail, were estimated to be 7.5×10^{-5}M to achieve an absorbance of 1.0.

3.2.6. Iron

The growth curves of *K.aerogenes* DL1 are illustrated in fig.26. They resemble those of magnesium depletion although the change in growth rate as iron became depleted appeared

to be more gradual.

Cells starved of magnesium show a progressive and often complete degradation of ribosomes. This is not the case when cells are deprived of iron (Iorio & Plocke,1981). However, the *in vitro* activity of ribosomes of iron depleted cells is much less than those derived from cells grown in iron rich conditions.

The effect of iron depletion on the OM proteins and siderophores of DL1 will be discussed in detail in section 5.2.

4. Resistance of *Klebsiella aerogenes* to Antibiotics

4.1. Antibiotic Sensitivity of *K.aerogenes* DL1

The "Neosensitab" antibiotic disc system described in Section 2.2.6.1. was used to determine the sensitivity of DL1 to 27 different antibiotics. The results are shown in Table 8. The criteria upon which the organism was designated sensitive or resistant were based on the breaking points described by Casals & Pedersen (1979). The zones of inhibition recorded in Table 8 were calculated from the mean diameter of three separate zones measured to the nearest whole mm, of the regular circular section of the zone. Fig.30 illustrates the zones of inhibition observed using this technique.

An estimation of the minimum inhibitory concentration (MIC) for each antibiotic to which the organism was sensitive, was obtained by extrapolation of the linear regression curves provided by the manufacturers, of MIC against zone of inhibition.

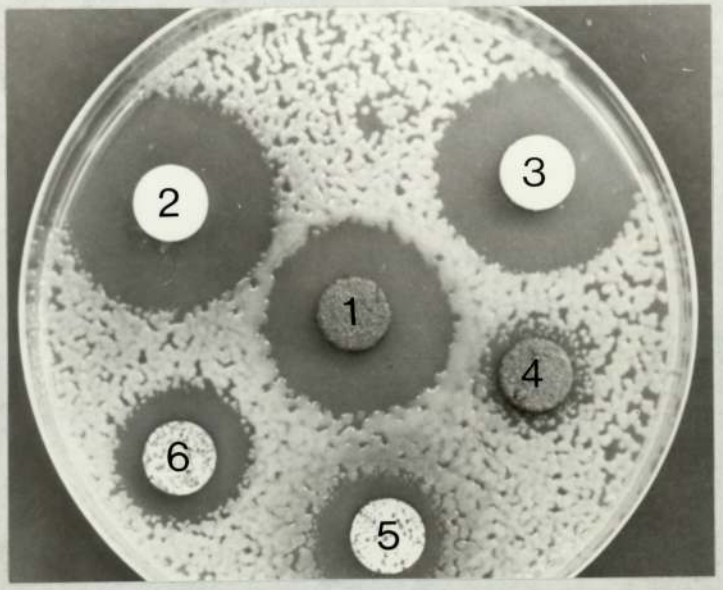
DL1 was resistant to all but six of the antibiotics tested. These were amikacin, cephalothin, mecillinam, nalidixic acid, nitrofurantoin and polymyxin. The ability of DL1 to hydrolyse nitrocefin indicated that it possessed a beta lactamase enzyme which would account, at least in part, for the resistance of DL1 to penicillin, ampicillin and

Table 8. Antibiotic Sensitivity of *K.aerogenes* DL1 Determined Using the Neosensitab Disc Method

Antibiotic	Diffusible Amount of Antibiotic (µg)	Mean Zone of Inhibition (mm)	Sensitivity	MIC (µg/ml)
Ampicillin	33	0	R	-
Amikacin	40	28	S	2.0
Bacitracin	60 i.u.	0	R	-
Carbenicillin	115	0	R	-
Chloramphenicol	60	0	R	-
Cotrimoxazole	5.2+240	0	R	-
Cephalothin	66	25	S	4.0
Erythromycin	78	17	R	-
Fucidin	400	0	R	-
Gentamicin	40	18	R	-
Kanamycin	100	0	R	-
Lincomycin	19	0	R	-
Mecillinam	33	26	S	1.5
Methicillin	29	0	R	-
Metronidazole	16	0	R	-
Nalidixic Acid	130	31	S	3.0
Nitrofurantoin	260	29	S	1.3mg%
Novobiocin	100	16	R	-
Penicillin (Low)	8 i.u.	0	R	-
Penicillin (High)	100 i.u.	0	R	-
Polymyxin	150	22	S	1.8
Rifamycins	30	14	R	-
Spectinomycin	200	0	R	-
Streptomycin	160	0	R	-
Sulphonamides	240	0	R	-
Tetracyclines	80	0	R	-
Tobramycin	40	19	R	-
Vancomycin	70	0	R	-

K.aerogenes DL1 was grown in CDM1+Fe. Well dried Meuller Hinton agar plates were flooded with a suspension of DL1 containing approximately 10^5orgs/ml. After overnight incubation at 37°C, each zone of inhibition was measured to the nearest mm. The criteria upon which the organism was designated sensitive (S) or resistant (R) were based on the breaking points described by Casals & Pedersen (1979).

Figure 30. Antibiotic Sensitivity Testing of *K.aerogenes* DL1 using the Neosensitab Antibiotic Disc Method.



Key:

	Antibiotic
1.	Polymyxin
2.	Nitrofurantoin
3.	Mecillinam
4.	Fucidin
5.	Erythromycin
6.	Gentamicin

carbenicillin.

4.2. Nutrient Depletion and Antibiotic Susceptibility

4.2.1. Resistance of Nutrient Depleted *K.aerogenes* DL1 to Antibiotics

Neosensitab antibiotic discs were used to screen nutrient depleted DL1 for any major changes in drug sensitivity (Table 9). No statisically significant changes in sensitivity were observed for any of the antibiotics chosen from those listed in 4.1. which had showed some activity against DL1.

4.2.2. Effect of Polymyxin and Cephalothin on Nutrient Depleted *K.aerogenes*

Figs.31 and 32 illustrate the effect of increasing concentrations (in nutrient agar) of polymyxin and cephalothin respectively on *K.aerogenes* DL1. Iron depleted cells showed a small increase in sensitivity to polymyxin when compared with magnesium depleted or mid log phase cells (Fig.31). There was no difference in the susceptibility of iron depleted, magnesium depleted or mid log phase DL1 to cephalothin (Fig.32).

Table 9. Antibiotic Sensitivities of Nutrient Depleted *K.aerogenes* DL1 Determined Using the Neosensitab Antibiotic Disc Method

Mean Zone of Inhibition (mm)

Depletion	CDM1+Fe	Fe	Mg	P	C	NC	S
Antibiotic							
Amikacin	28	27	28	27	28	27	27
Cephalothin	25	25	26	26	25	25	25
Erythromycin	17	17	16	17	19	17	18
Gentamicin	18	18	19	19	18	18	19
Mecillinam	27	26	27	26	26	26	26
Nalidixic Acid	32	30	31	31	30	31	31
Nitrofurantoin	29	28	29	29	29	28	28
Novobiocin	15	16	15	15	15	15	14
Tobramycin	19	18	20	19	19	19	18
Rifamycins	13	14	14	13	13	15	13
Polymyxin	27	25	25	26	25	25	25

The antibiotic sensitivities of nutrient depleted DL1 were determined as described in 2.2.6.1. Stationary phase bacteria were obtained from cultures grown in CDM1+Fe and in CDM1+Fe containing sufficient of the nutrient being studied to reach a maximum exponential growth of A_{470} 0.5. For iron depletion studies, CDM1-Fe was the growth medium used. The contaminating level of iron in CDM1-Fe enabled DL1 to grow exponentially to A_{470} 2.1. NC represents DL1 depleted by both carbon and nitrogen.

Figure 31. Effect of Nutrient Depletion on the Susceptibility of *K.aerogenes* DL1 to Polymyxin

Key:

- ■ Log Phase
- ● Iron Depleted
- ▲ Magnesium Depleted

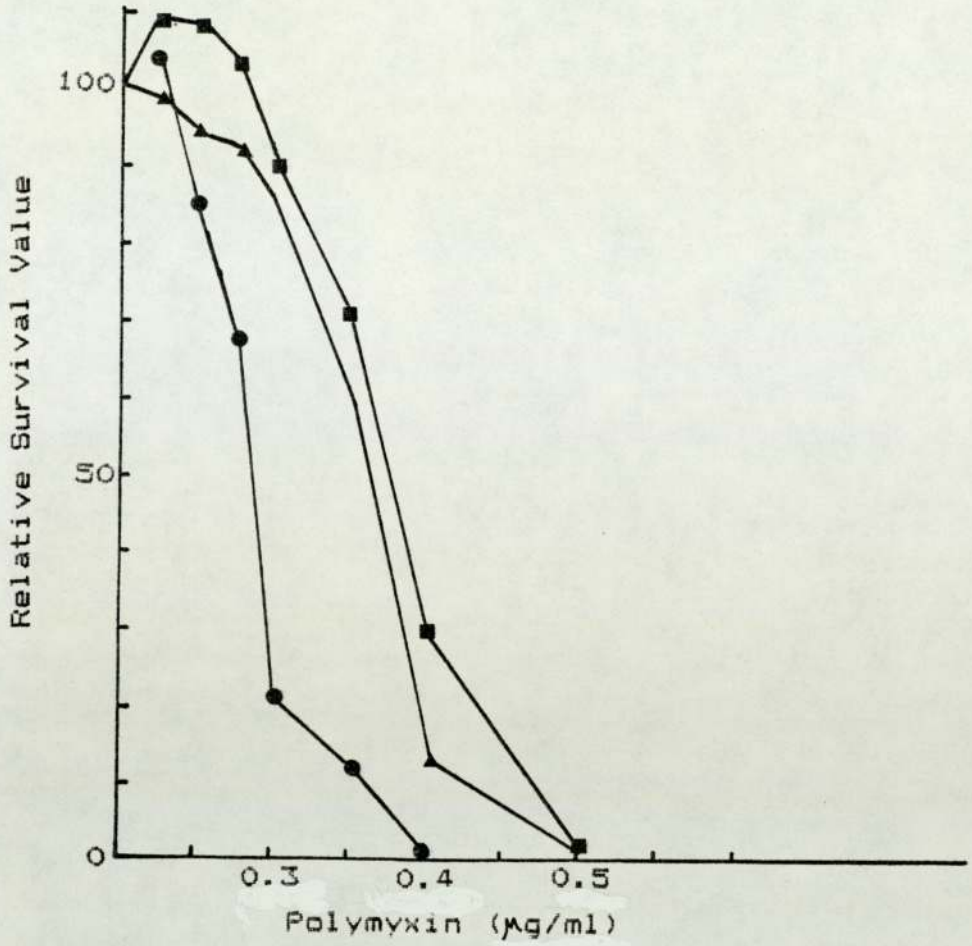
The survival values indicate viable counts relative to those obtained in the absence of drug.

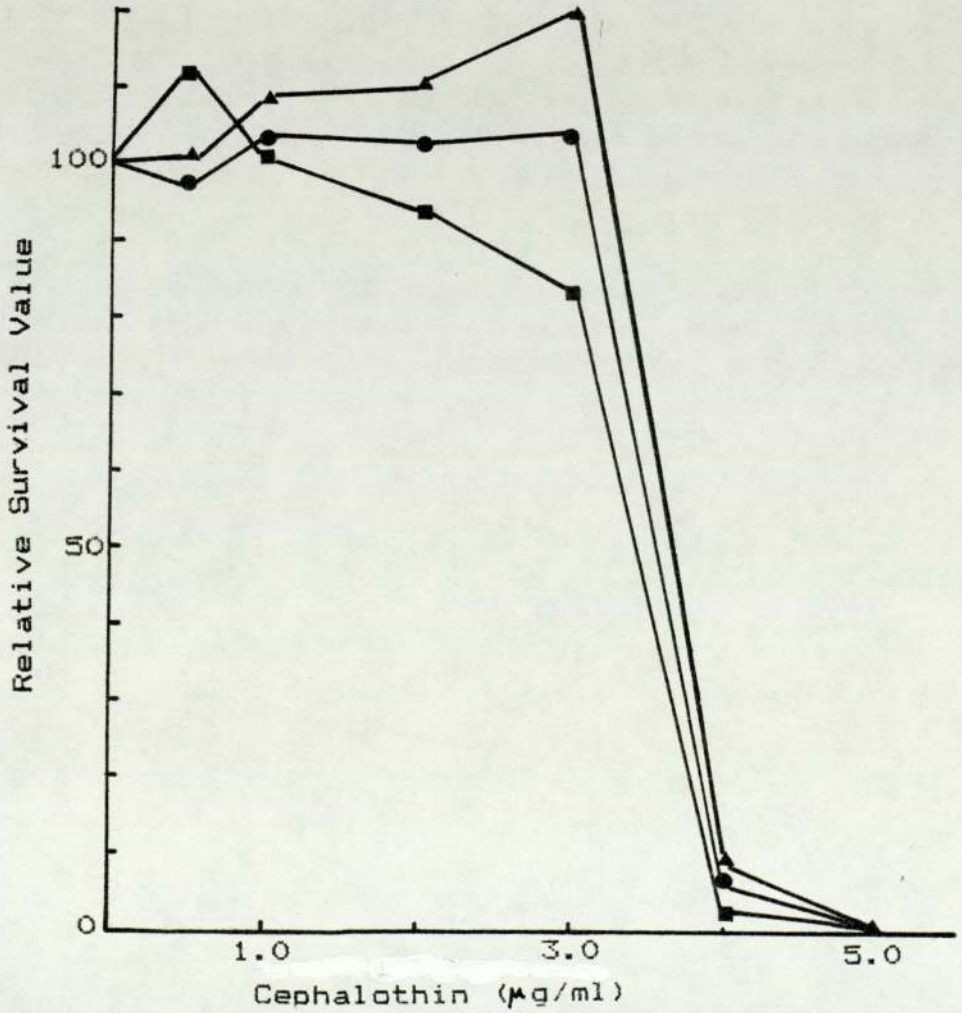
Figure 32. Effect of nutrient depletion on the Susceptibility of *K.aerogenes* DL1 to Cephalothin

Key:

- ▲ Log Phase
- ■ Iron Depleted
- ● Magnesium Depleted

The survival values are expressed as for figure 31.


100
50
0
Relative Survival Value
0.3
0.4
0.5
Polymyxin (μg/ml)


100
50
0
Relative Survival Value
1.0
3.0
5.0
Cephalothin (μg/ml)

4.3. Role of the O and K antigens of *K.aerogenes* in Antibiotic Resistance

The response of the isogenic *K.aerogenes* mutants M10 and M10B together with the parent strain NCTC 5055 to a range of hydrophilic and hydrophobic antibiotics in nutrient agar is shown in Figs.33 to 39.

There was little difference betweeen the sensitivities of NCTC 5055, M10 and M10B to the hydrophobic antibiotics rifampicin (Fig.33) and tetracycline (Fig.35) or to the hydrophilic antibiotics cefuroxime (Fig.36), azlocillin (Fig.34), polymyxin (Fig.37) and gentamicin (Fig.39).

However, M10 was much more sensitive to the hydrophobic antibiotic novobiocin than NCTC 5055. M10B was completely resistant to this antibiotic in the concentrations used (Fig.38).

Figure 33. Effect of the Loss of Capsule and O Antigen on the Susceptibility of *K.aerogenes* to Rifampicin

Key:

- ● *K.aerogenes* NCTC 5055 (K^+ O^+)
- ■ *K.aerogenes* M10 (K^- O^+)
- ▲ *K.aerogenes* M10B (K^- O^-)

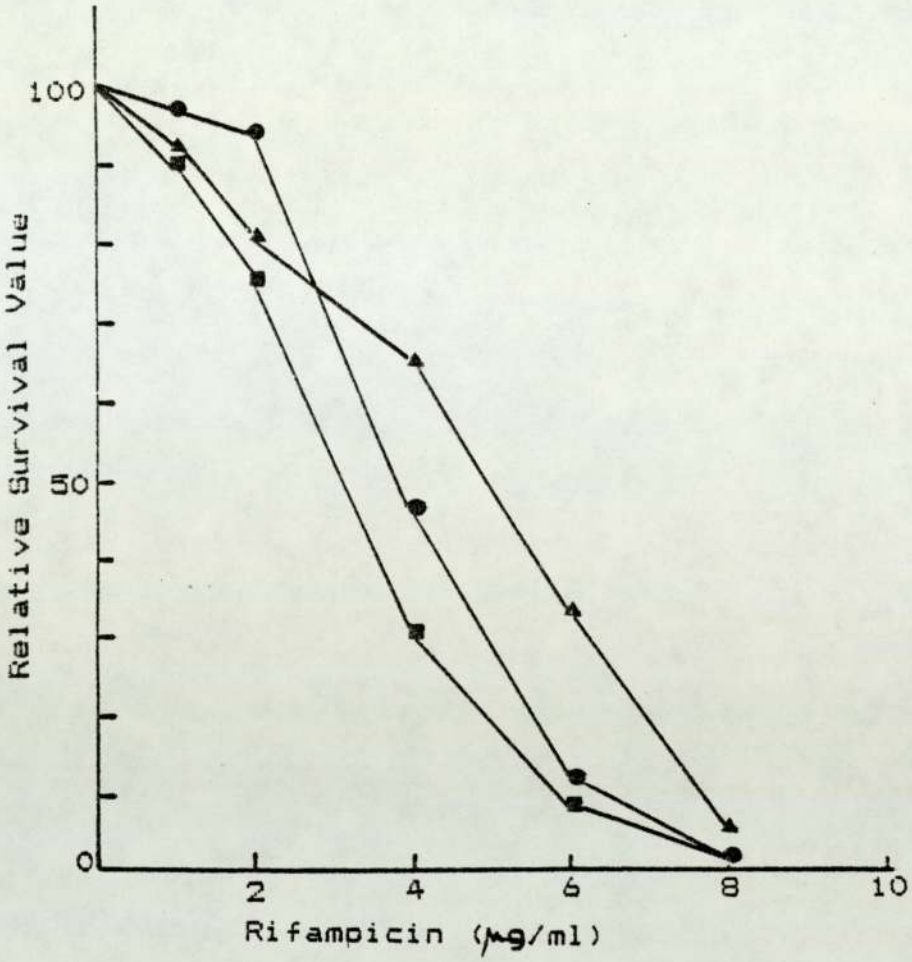
The survival values are expressed as for figure 31.

Figure 34. Effect of the Loss of Capsule and O Antigen on the Susceptibility of *K.aerogenes* to Azlocillin

Key:

- ● *K.aerogenes* NCTC 5055 (K^+ O^+)
- ■ *K.aerogenes* M10 (K^- O^+)
- ▲ *K.aerogenes* M10B (K^- O^-)

The survival values are expressed as for figure 31.


100
50
0
Relative Survival Value
2
4
6
8
10
Rifampicin (μg/ml)

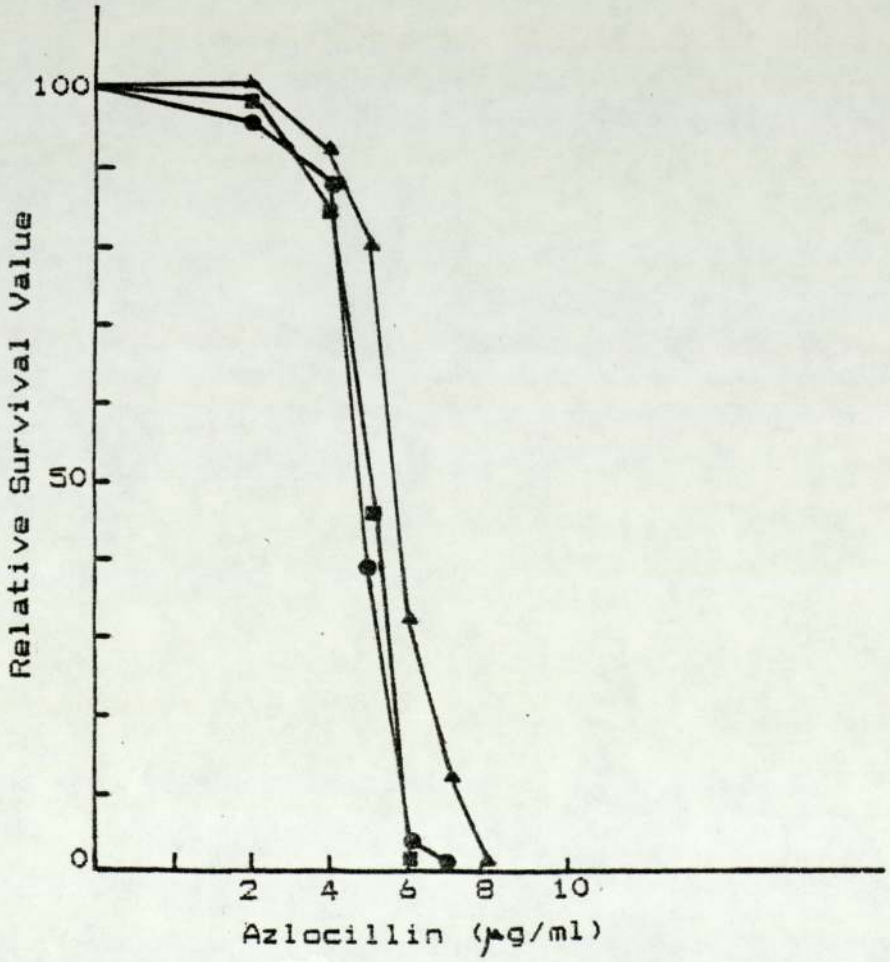

100
50
0
Relative Survival Value
2
4
6
8
10
Azlocillin (μg/ml)

Figure 35. Effect of the Loss of Capsule and O Antigen on the Susceptibility of *K.aerogenes* to Tetracycline

Key:

- ● *K.aerogenes* NCTC 5055 (K^+ O^+)
- ■ *K.aerogenes* M10 (K^- O^+)
- ▲ *K.aerogenes* M10B (K^- O^-)

The survival values are expressed as for figure 31.

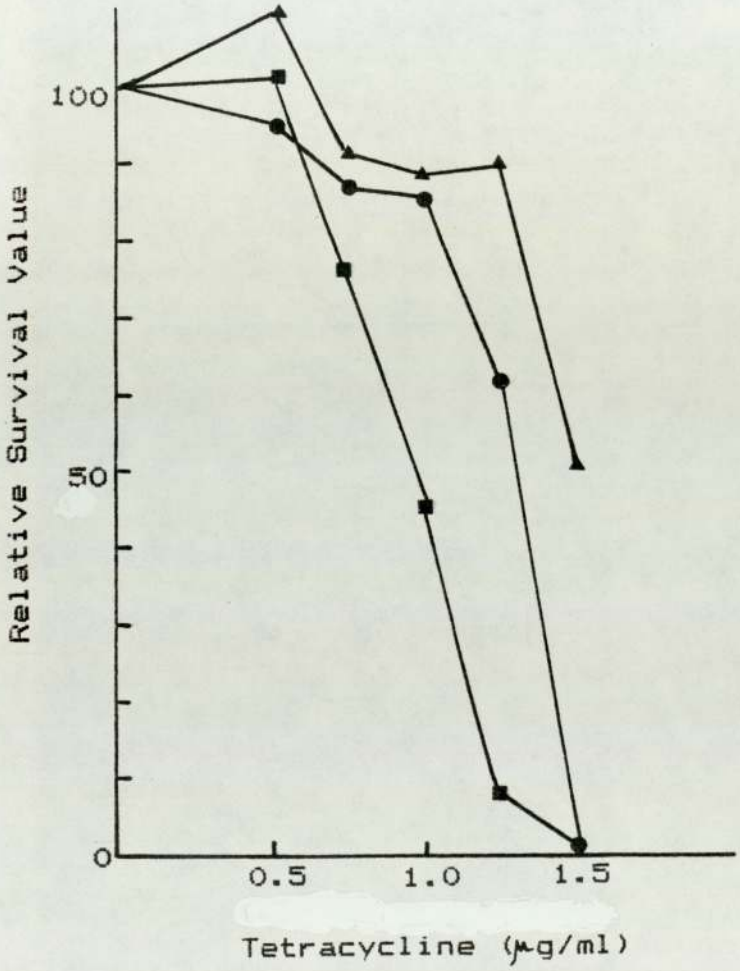

Relative Survival Value
100
50
0
0.5
1.0
1.5
Tetracycline (μg/ml)

Figure 36. Effect of the Loss of Capsule and O Antigen on the Susceptibility of *K.aerogenes* to Cefuroxime

Key:

- ● *K.aerogenes* NCTC 5055 (K^+ O^+)
- ■ *K.aerogenes* M10 (K^- O^+)
- ▲ *K.aerogenes* M10B (K^- O^-)

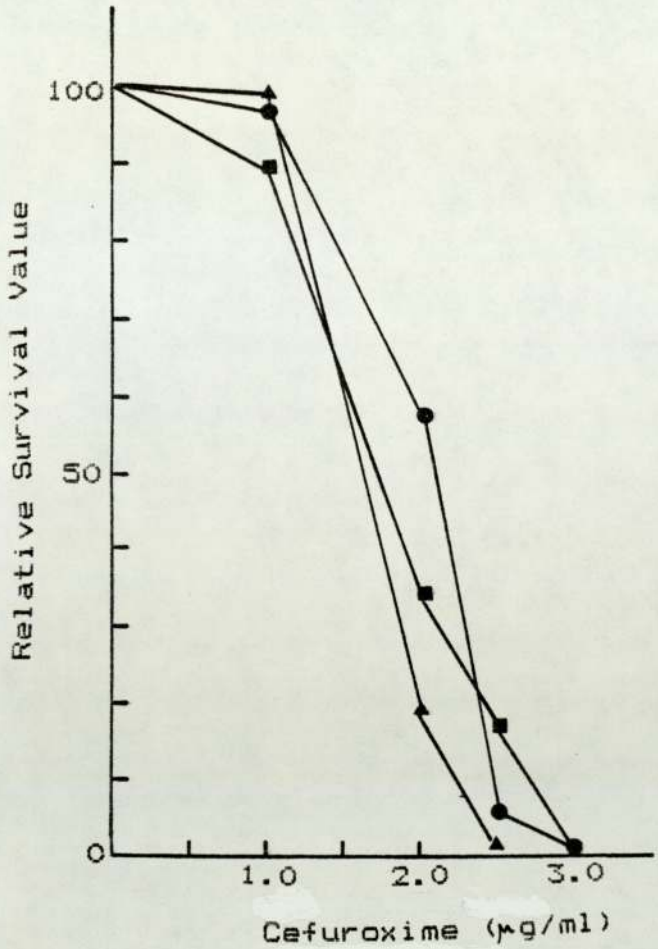
The survival values are expressed as for figure 31.

Figure 37. Effect of the Loss of Capsule and O Antigen on the Susceptibility of *K.aerogenes* to Polymyxin

Key:

- ● *K.aerogenes* NCTC 5055 (K^+ O^+)
- ■ *K.aerogenes* M10 (K^- O^+)
- ▲ *K.aerogenes* M10B (K^- O^-)

The survival values are expressed as for figure 31.


100
50
0
Relative Survival Value
1.0
2.0
3.0
Cefuroxime (μg/ml)

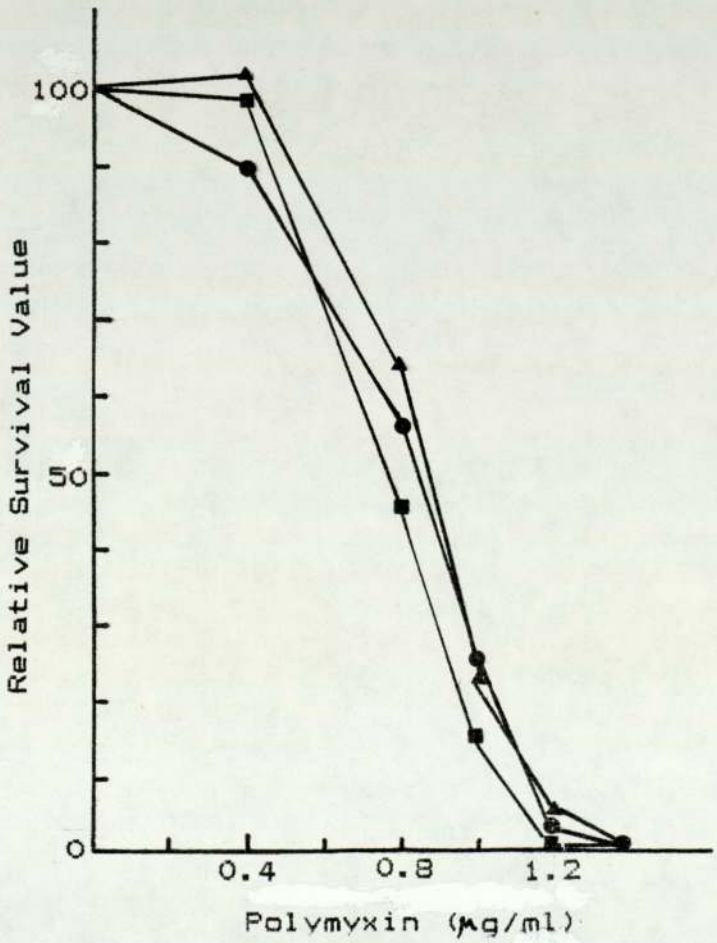

100
50
0
Relative Survival Value
0.4
0.8
1.2
Polymyxin (μg/ml)

Figure 38. Effect of the Loss of Capsule and O Antigen on the Susceptibility of *K.aerogenes* to Novobiocin

Key:

- ● *K.aerogenes* NCTC 5055 (K^+ O^+)
- ■ *K.aerogenes* M10 (K^- O^+)
- ▲ *K.aerogenes* M10B (K^- O^-)

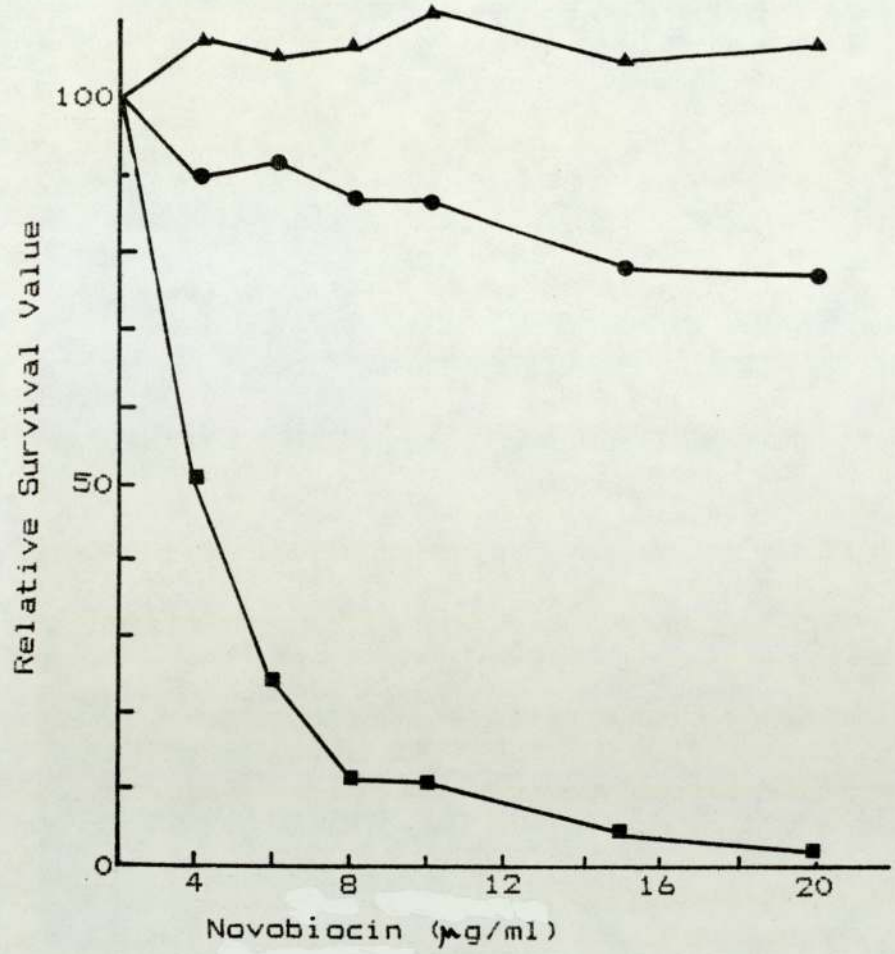
The survival values are expressed as for figure 31.

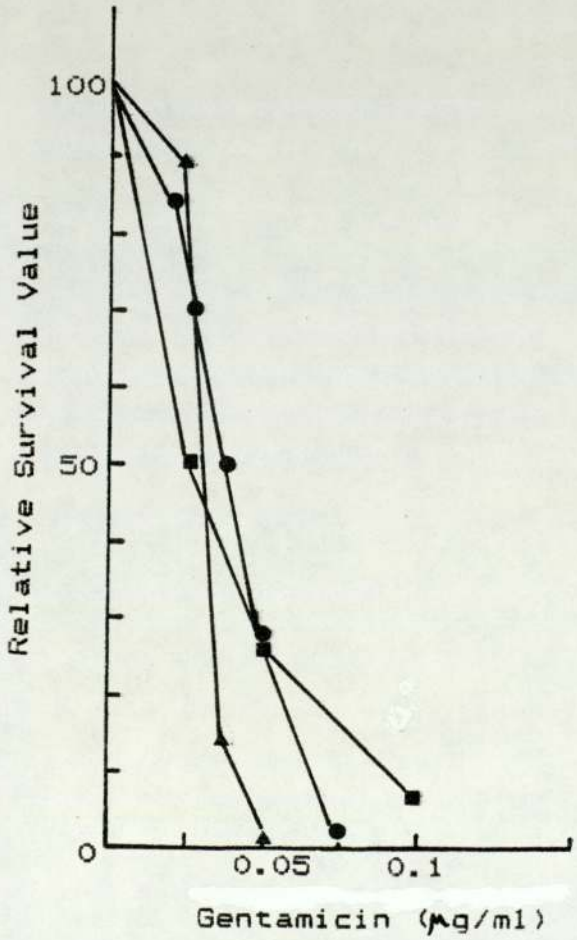
Figure 39. Effect of the Loss of Capsule and O Antigen on the Susceptibility of *K.aerogenes* to Gentamicin

Key:

- ● *K.aerogenes* NCTC 5055 (K^+ O^+)
- ■ *K.aerogenes* M10 (K^- O^+)
- ▲ *K.aerogenes* M10B (K^- O^-)

The survival values are expressed as for figure 31.


100
50
0
Relative Survival Value
4
8
12
16
20
Novobiocin (μg/ml)


100
50
0
Relative Survival Value
0.05
0.1
Gentamicin (μg/ml)

4.4. Discussion

Multidrug resistant *Klebsiella* strains have become an increasing problem because they are often the cause of outbreaks of hospital infection (Richards *et al*,1981; S.J.Young,1982; L.S.Young, 1982; Smith *et al*,1982; Ullmann,1983). *K.aerogenes* DL1 isolated from a patient at Safdajhang Hospital, New Delhi, India proved to be no exception. DL1 was resistant to all but 6 of the 27 antibiotics tested, as judged by "Neosensitab" antibiotic disc sensitivities (Table 8). The resistance of *Klebsiella* species to antibiotics is frequently related to the presence of transmissible R plasmids (Richards *et al*,1981) as well as to the presence of chromosomally and R plasmid mediated beta lactamase enzymes (Ogawara,1981). The ability of DL1 to hydrolyse nitrocefin pointed to the presence of a beta lactamase enzyme which is presumably responsible for its resistance to those beta lactam antibiotics tested. In a clinical situation, whilst the aminoglycosides remain the most active drugs against *Klebsiella* (Smith *et al*,1982), their use carries a significant risk of nephrotoxicity. Therefore, the susceptibility of *Klebsiella* including gentamicin resistant strains to the new cephalosporins (e.g. cefoxitin, cefotaxime and ceftazidime)(Ullmann,1983) may result in the replacement of the nephrotoxic aminoglycosides by these agents in the treatment of life threatening *Klebsiella* infections.

4.4.1. Nutrient Depletion and the Susceptibility of DL1 to Cephalothin and Polymyxin

No difference in the response of log phase, magnesium depleted or iron depleted DL1 to cephalothin was observed. Whilst this may be the case, it must be noted that the result may also reflect the insensitivity of the assay procedure for nutrient depleted bacteria. Cephalothin acts by interfering with peptidoglycan biosynthesis exerting its effect primarily on growing and dividing cells (Gale *et al*,1981). Following inoculation onto nutrient agar, nutrient depleted bacteria will only retain their particular envelope characteristics for a few generations. The assay is therefore dependent upon the action of the antibiotic on those first few generations and differences in sensitivity between nutrient depleted and log phase cells, if any, may not become apparent.

Polymyxin on the other hand, does not require bacteria to be actively multiplying in order to exert its effect. Iron depleted *K.aerogenes* DL1 showed a small increase in sensitivity to this peptide antibiotic when compared with magnesium depleted and log phase DL1. There is much evidence to suggest that polymyxin disrupts the structure and function of the cytoplasmic and outer membranes by interaction with phospholipids and LPS with the concommitant leakage of cytoplasmic constituents (Klemperer *et al*,1979; Sixl & Galla, 1981). Magnesium, phosphate and sulphate depletions have all been shown to modify the response of

P.aeruginosa to polymyxin (Finch & Brown,1975; Noy,1982; Miveld,1983). In particular, batch cultures of *P.aeruginosa* were shown to become progressively more resistant to polymyxin as they became magnesium depleted (Brown & Melling,1969). In contrast, magnesium depleted DL1 were as sensitive as log phase DL1 suggesting that the role of magnesium ions in the cell wall of *Klebsiella* may be different to that in *P.aeruginosa*.

Iron depleted DL1 showed a small increase in sensitivity to polymyxin. Iron deprivation is known to depress the synthesis of the cytochromes and iron sulphur proteins of the electron transport chain (Haddock,1977). Polymyxin has been reported to interact with acidic phospholipids leading to the disorganisation of bacterial membranes (Sixl & Galla,1981). Therefore, if an increase in the CM phospholipid content occurs as a result of the loss of cytochromes and iron sulphur proteins, a partial explanation of this increased sensitivity may involve alterations in the phospholipid content of the CM. However, the contribution of changes in OM components including the derepression of the high affinity iron uptake systems is unknown. Substantiation of the role of envelope changes in the increased susceptibility of iron depleted DL1 would require the quantitative estimation of individual envelope components.

4.4.2. Role of the O and K Antigens of *K.aerogenes* in Antibiotic Resistance

Wild type Enterobacteriaceae are naturally sensitive to many low molecular weight hydrophilic compounds such as ampicillin and cephalothin. These agents are presumably taken up via the general diffusion pores, a mechanism which does not appear to be much affected by alterations in LPS structure (Nikaido, 1979). In contrast, wild type Enterobacteriaceae are often resistant to hydrophobic compounds e.g. novobiocin and rifampicin but are rendered sensitive by mutations involving LPS structure (Nikaido, 1979) or following treatment with EDTA, which removes up to 50% of the LPS present in the OM (Leive,1974). Deep rough mutations of *S.typhimurium* or *E.coli* show drastically increased susceptibilities to these hydrophobic agents (Nikaido,1979).

In the light of these observations, an investigation was made of the effect that loss by mutation of the O and K antigens of *K.aerogenes* NCTC 5055 has on the sensitivity of this organism to a range of hydrophilic and hydrophobic agents. No major differences in the sensitivities of NCTC 5055 (K^+ O^+), M10 (K^- O^+) or M10B (K^- O^-) to the hydrophilic antibiotics azlocillin, cefuroxime, gentamicin and polymyxin or to the hydrophobic antibiotics rifampicin and tetracycline were observed. The negatively charged *Klebsiella* polysaccharide does not appear to interfere

through ionic interactions with the penetration of positively charged agents such as gentamicin. However, the hydrophilicity of the capsule may restrict penetration of the hydrophobic compound, novobiocin, as NCTC 5055 was more resistant to this antibiotic than was M10. An unexpected finding was that M10B, which has no capsule or O antigen, and which has the most hydrophobic surface, was more resistant than M10 or NCTC 5055 to novobiocin. This result may have arisen as a consequence of a modification to the target site, DNA gyrase (an enzyme which is essential for DNA replication (Gale *et al*,1981)), by the nitrosoguanidine mutagenesis. Partial confirmation of such a modification could be obtained from an investigation of the sensitivity of the three strains to the hydrophilic antibiotic nalidixic acid, whose site of action is also DNA gyrase (Gale *et al*,1981).

5. Effect of Iron Deprivation on the Production of Outer Membrane Proteins and Siderophores by *K.aerogenes*

5.1. Effect of Iron Deprivation on the Outer Membrane Protein Profile of *K.aerogenes*

The pattern of the major OM proteins obtained from sarkosyl insoluble membranes of DL1 grown to stationary phase in CDM2+Fe, CDM2-Fe, serum+Fe and CDM2+Fe+1mM citrate is shown in Fig.40a. Figures 40b and 40c are densitometer tracings of the OM protein profiles of DL1 grown in CDM2+Fe (Fig.40b) and CDM2-Fe (Fig.40c).

The OM protein profile of *K.aerogenes* DL1 is closely related to *E.coli* in that three major bands between 30,000 and 42,000 M_r are observed (Lugtenberg & van Alphen, 1983). Their M_r values were 32,500, 35,500 and 39,000 respectively. They were always present and appeared to be independent of the growth conditions employed. The 35,500 and 39,000 M_r proteins remained bound to peptidoglycan after incubation at 60°C in 2% w/v SDS but were released after heating in denaturing buffer (containing 2% w/v SDS) for 10min at 100°C prior to electrophoresis (Fig.40a). These proteins are therefore bound tightly although not covalently to peptidoglycan (Mizuno & Kageyama,1979) and are probably related to the Omp F and Omp C porin proteins of *E.coli*.

Fig.41(Tracks A,C,E, & G) compares the OM protein profiles

Figure 40(a). SDS-PAGE of the Outer Membrane Proteins of *K.aerogenes* DL1 after Growth in Iron Depleted and Iron Restricted Media

Key:

Track	Medium
(A)	CDM2+Fe
(B)	CDM2-Fe
(C)	Serum + 80 μM Fe^{2+}
(D)	Serum
(E)	CDM2+Fe + 1mM Citrate
(F)	Peptidoglycan-Associated proteins of DL1 after growth in CDM2-Fe

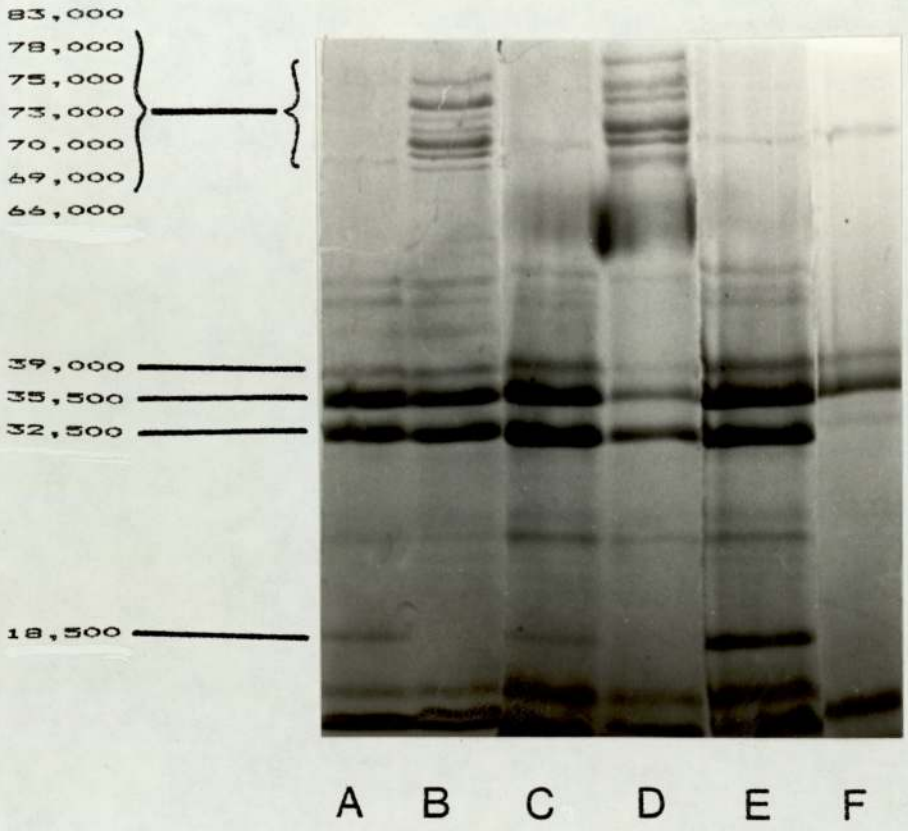

83,000
78,000
75,000
73,000
70,000
69,000
66,000
39,000
35,500
32,500
18,500
A
B
C
D
E
F

Figure 40(b). Densitometer Scan of a Coomassie Blue Stained Polyacrylamide Gel showing OM Proteins of *Klebsiella aerogenes* DL1 Grown in CDM2+Fe (Lane A from Figure 40a).

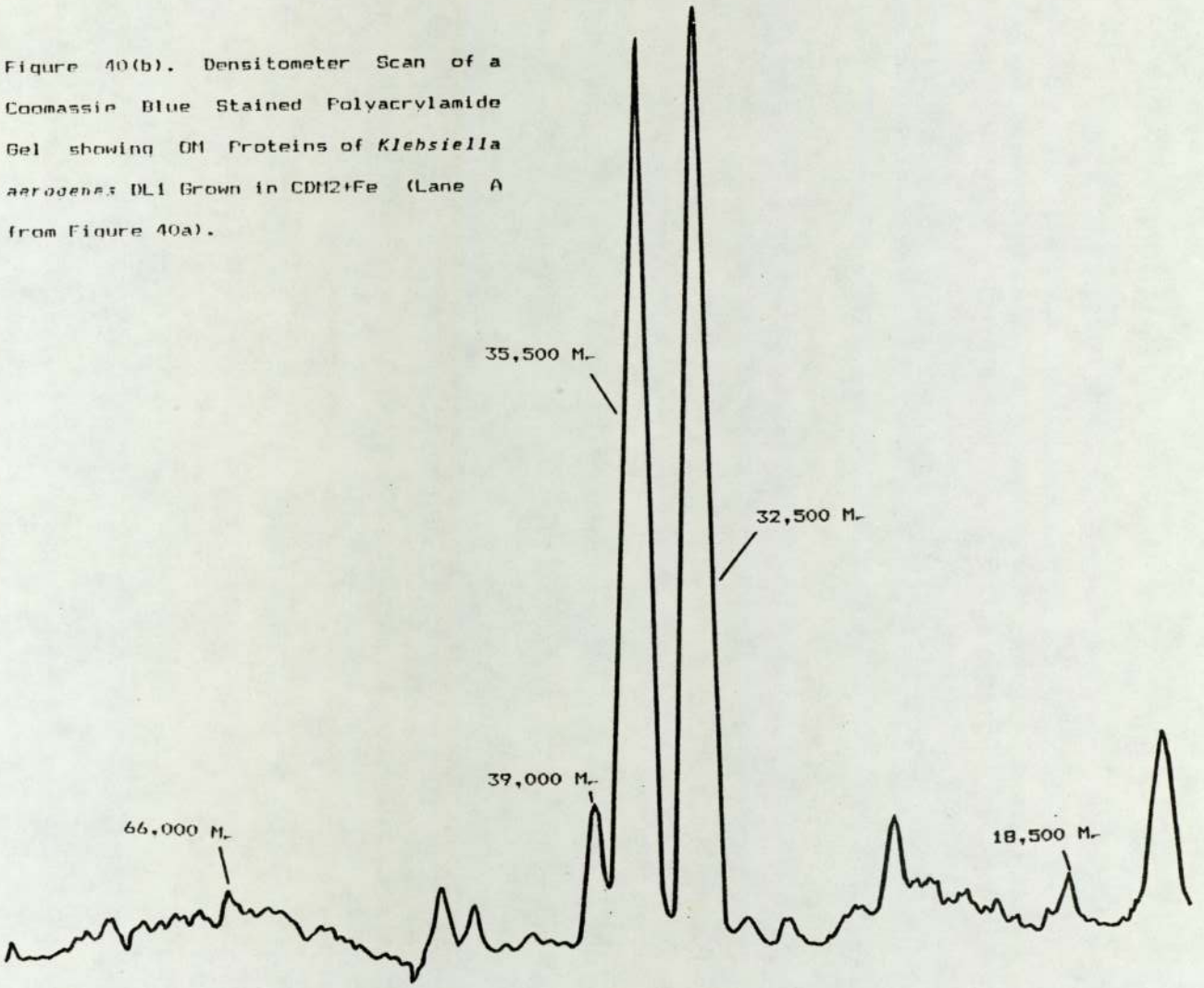


Figure 40(c). Densitometer Scan of a Coomassie Blue Stained Polyacrylamide Gel showing OM Proteins of *Klebsiella aerogenes* DL1 Grown in CDM2-Fe (Lane B from Figure 40a).

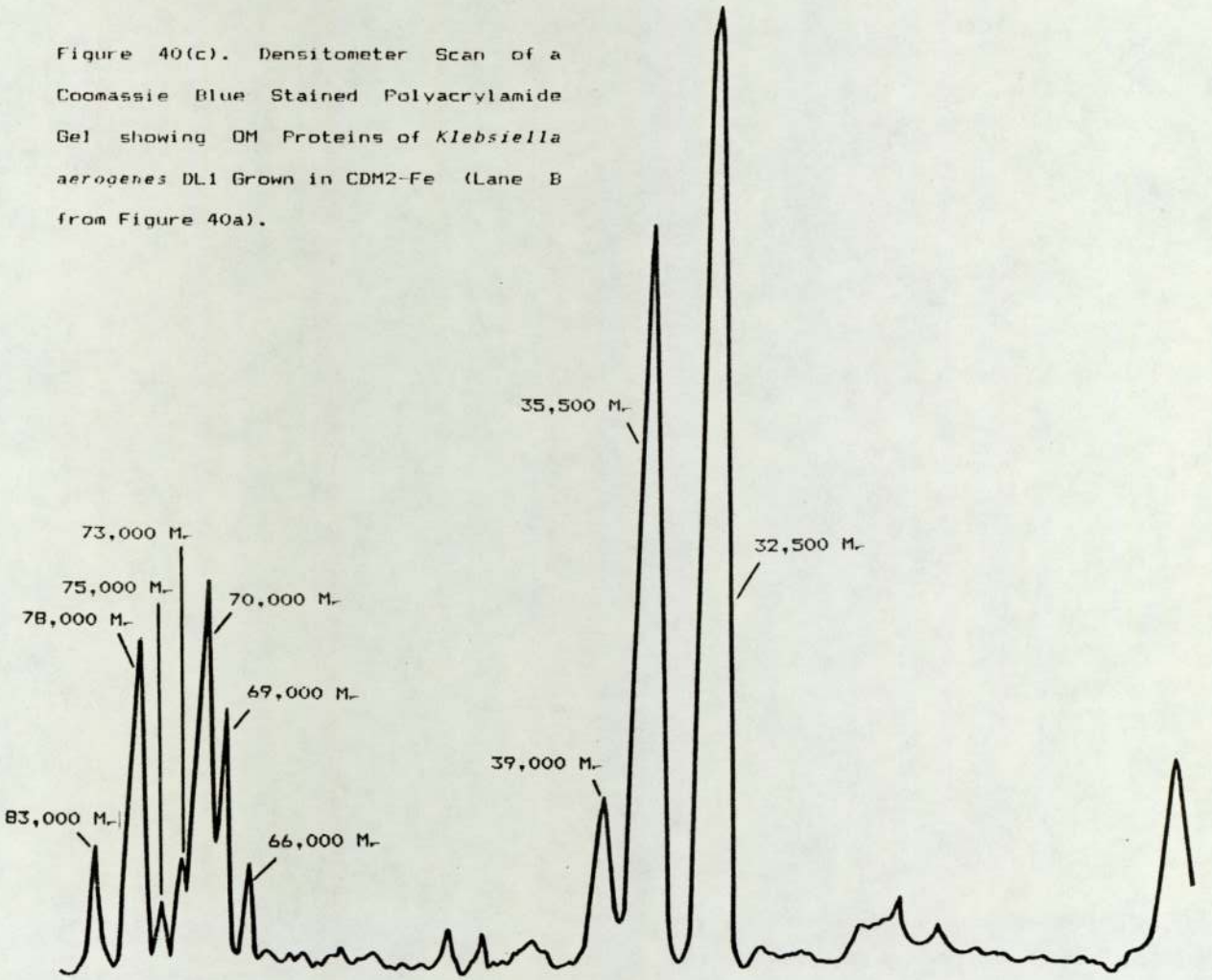
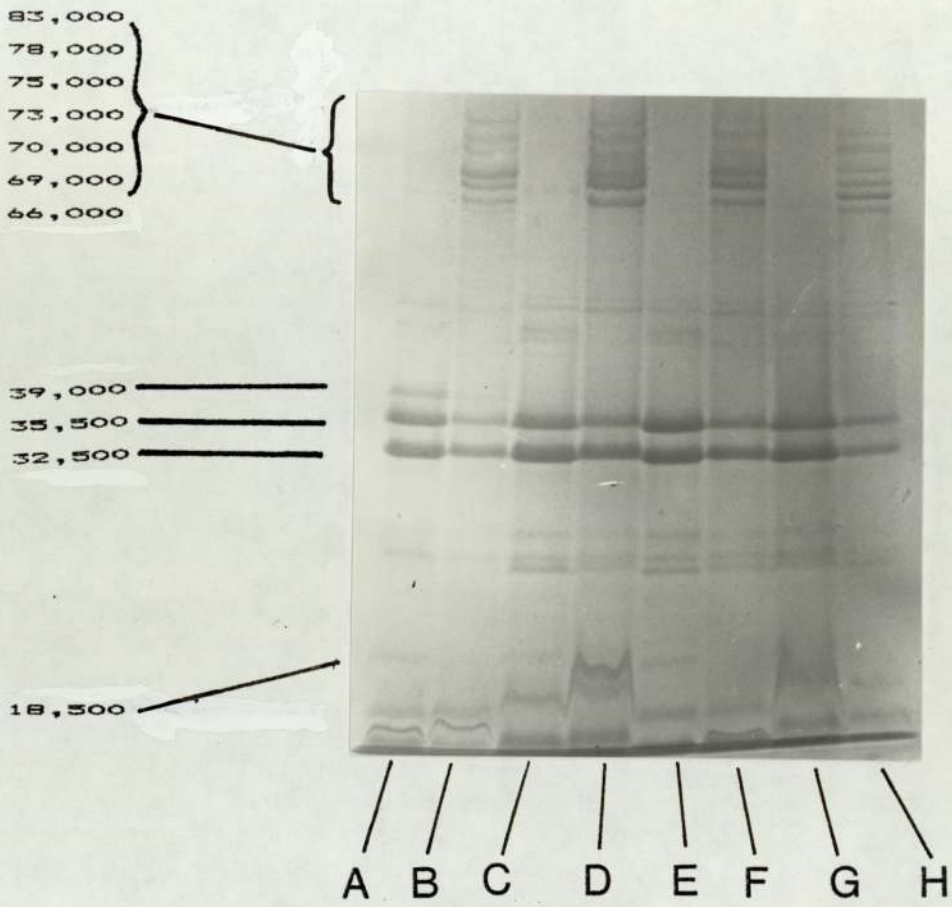


Figure 41. SDS-PAGE of the Outer Membrane Proteins of *K.aerogenes* after Growth in Iron Enriched and in Iron Depleted CDM2

Key:

Track	Organism	Medium
(A)	DL1	CDM2+Fe
(B)	DL1	CDM2-Fe
(C)	NCTC 5055	CDM2+Fe
(D)	NCTC 5055	CDM2-Fe
(E)	M10	CDM2+Fe
(F)	M10	CDM2-Fe
(G)	M10B	CDM2+Fe
(H)	M10B	CDM2-Fe


83,000
78,000
75,000
73,000
70,000
69,000
66,000
39,000
35,500
32,500
18,500
A
B
C
D
E
F
G
H

of *K.aerogenes* DL1, NCTC 5055, M10 and M10B grown in CDM2+Fe. All of these strains express the 32,500 and 35,500 M_r proteins but only DL1 expresses the 39,000 M_r protein.

Other workers have reported the presence of two major OM proteins in *Klebsiella* of M_r 35,000 and 38,000; the 38,000 M_r protein being peptidoglycan associated (Lugtenberg *et al* ,1977; Hofstra & Dankert,1979). The 32,500 M_r protein was not peptidoglycan associated and is therefore akin to the *E.coli* Omp A protein which plays a role in F pilus mediated conjugation (van Alphen *et al*,1977).

In iron restricted (serum) or iron deficient (CDM2-Fe) conditions, at least six new *K.aerogenes* DL1 OM proteins, which were not bound to peptidoglycan and of M_r 69,000, 70,000, 73,000, 75,000, 78,000 and 83,000 were apparent. They were only just visible in the OM of DL1 grown in CDM2+Fe or in iron saturated serum. These high molecular weight iron inducible proteins were also derepressed in the OMs of NCTC 5055, M10 and M10B grown in CDM2-Fe (see Fig.41, Tracks B,D,F & H) and will therefore be referred to as the iron regulated membrane proteins (IRMP). *E.coli* has been reported to produce four such proteins of M_r 83,000, 81,000 78,000 and 74,000 (Klebba *et al*,1982). The 81,000 M_r protein functions as the ferric enterochelin receptor and the 78,000 M_r protein is involved in the uptake of iron from the fungal siderophore, ferrichrome. The role of the 74,000 and 83,000 M_r proteins in iron transport is as yet undefined. As *Klebsiella* and *E.coli* are closely related members of the

Enterobacteriaceae it is reasonable to assume that their IRMP will therefore be related. Hancock *et al* (1976) have shown that the OMs of *E.coli* strains grown in the presence of 1mM citrate contained a polypeptide of M_r 81,000 that appeared to play a role in citrate mediated iron uptake. *K.aerogenes* DL1 however, did not induce the formation of a similar protein in CDM supplemented with 1mM citrate.

The OM of stationary phase DL1 contained a protein of M_r 18,500 after growth in CDM2+Fe, iron saturated serum and in citrate supplemented CDM2+Fe (Fig.40a). It was also visible in the OMs of NCTC 5055 and M10 following growth to stationary phase in CDM2+Fe (Fig.41). This protein was not peptidoglycan associated and was repressed after growth in CDM2-Fe or in serum. Its function is unknown, although the involvement of low molecular weight OM proteins in iron metabolism in both *E.coli* (Klebba *et al*, 1982) and *P.aeruginosa* (Sokol & Woods, 1983) has recently been reported.

5.2. Siderophore Production by *K.aerogenes* DL1

Under conditions of iron stress *K.aerogenes* DL1 secreted enterochelin but not aerobactin. The supernatants obtained from iron deficient cultures of DL1 were yellow in colour. This pigment was subjected to further investigation to establish any role in iron metabolism. It was found to be a low molecular weight (dialysable), non ethyl acetate extractable compound which fluoresced under u.v. light. It

was identified by its fluorescence excitation maximum (375nm) and emission maximum (444nm) as riboflavin (see Fig.42).

To follow the appearance of enterochelin during the growth cycle, cells grown overnight in CDM2+Fe were washed and resuspended to A_{470} 0.02 in CDM2-Fe. The growth at 37°C was followed by the change in absorbance at fixed time intervals. Bacteria were harvested after cooling to 4°C, OMs prepared and supernatants assayed for riboflavin and enterochelin. The results in fig.43 show that enterochelin biosynthesis paralleled the growth of the organism and a maximum of 27μg/ml was detected in late stationary phase. Thus the synthesis of enterochelin takes place several generations before any change in growth rate occurs. Stationary phase iron depleted DL1 produced 1.6 μg/ml of riboflavin as opposed to stationary phase iron replete cells which produced 0.6 μg/ml. *K.aerogenes* DL1 grown in CDM2-Fe secreted approximately three times more riboflavin than in CDM2+Fe indicating that iron is involved in the control of riboflavin synthesis (Demain, 1972).

5.3. Effect of Growth Phase on the Appearance of the IRMP

To follow the rate at which the IRMP accumulated in the OM, DL1 grown in CDM2+Fe was resuspended to A_{470} 0.2 in CDM2-Fe. Outer membranes could then be prepared from cells harvested at approximately half generation intervals. The

Figure 42. Fluorescence Spectrum of Riboflavin from the Culture Supernatant of *K.aerogenes* DL1 grown in CDM2-Fe

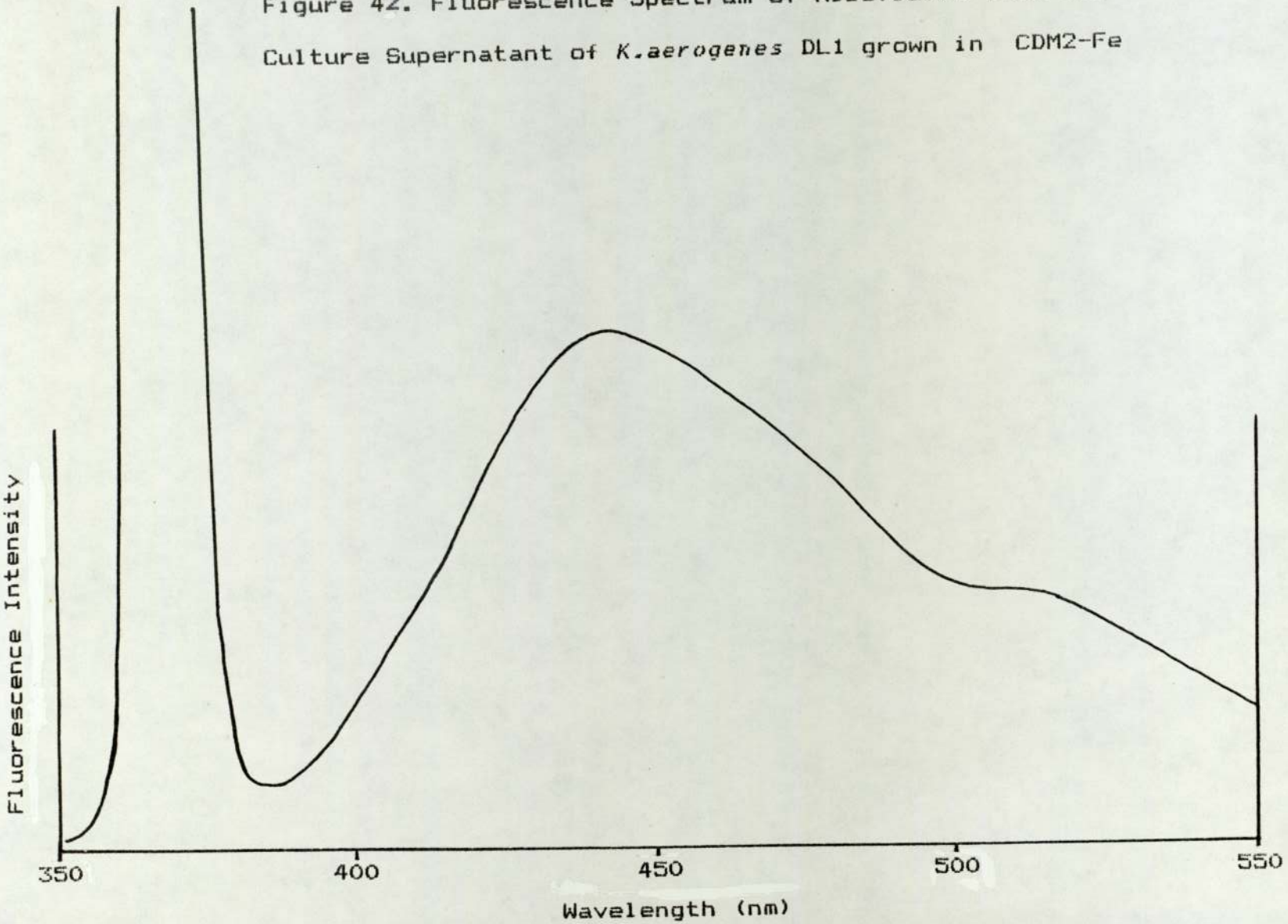


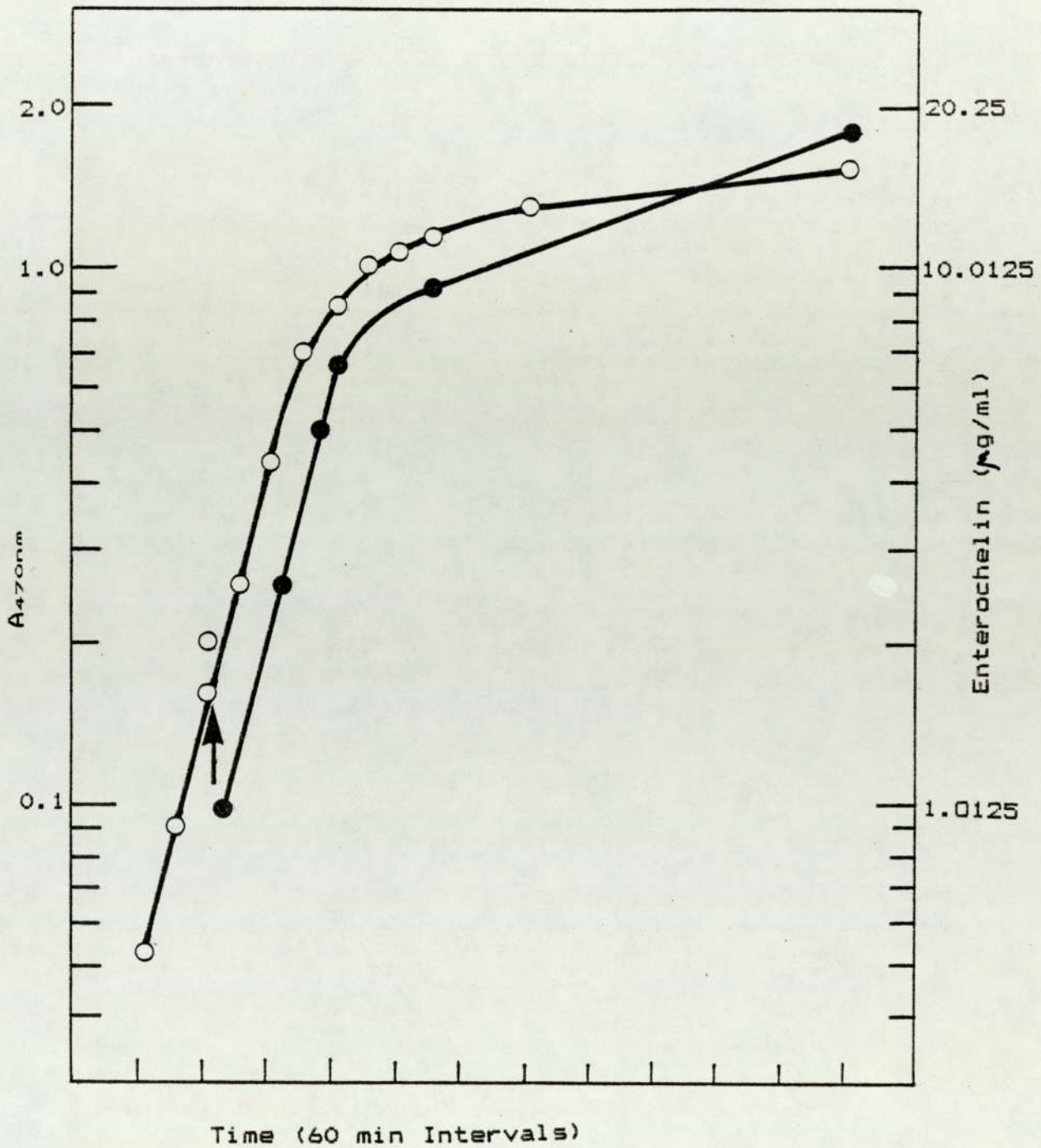
Figure 43. Appearance of the Iron Regulated Membrane Proteins and Enterochelin during the Growth Cycle

Key:

- ○ Growth of iron replete DL1 in CDM2-Fe measured by absorbance at 470nm.

- ● Concentration of enterochelin and related phenolates in the culture medium.

At the point marked by an arrow, the IRMP were fully induced.


2.0
1.0
0.1
A470nm
20.25
10.0125
1.0125
Enterochelin (μg/ml)
Time (60 min Intervals)

IRMP were clearly visible 64min (1.95 generations) after inoculation into Fe-CDM2 (Fig.44). The same data is presented in Fig.45 as the ratio of the IRMP to the 39,000 M_r protein, the concentration of which did not vary under the conditions used. A sharp increase in the ratio occurs some 53min (1.8 generations) after inoculation, corresponding to the accumulation of the IRMP in the OM. Thus, in the presence of a low extracellular iron concentration, the IRMP were rapidly derepressed and appeared in the OM within two generations. Therefore, both the siderophore enterochelin and the IRMP, which presumably include the enterochelin receptor are produced several generations before any change in growth rate is apparent.

5.4. Growth of Iron Deprived DL1 in CDM2+Fe

The effect of inoculating late log phase CDM2-Fe grown *K.aerogenes* DL1 into CDM2+Fe on the OM protein profile is shown in Figs.45 and 46. The time required for the relative level of the IRMP to decrease by one half was estimated to be 49.5 min which is close to one and a half cell divisions. After 4 generations (approximately 132min) the ratio was reduced to that observed after growth to stationary phase in CDM2+Fe. Therefore, the IRMP appeared to be diluted exponentially rather than actively excreted from the OM as the iron depleted organism multiplied in an iron replete environment. The reduction in the level of IRMP following restoration of iron to the medium is analogous to the loss of protein H1 from magnesium depleted *P.aeruginosa* upon

Figure 44. SDS-PAGE of the Outer Membrane Proteins of *K.aerogenes* showing the Time Course for the Appearance of the IRMP following Subculture of Iron Replete Cells into Iron Deficient Media (CDM2-Fe)

Key:

Track	Time (min)
(A)	22.4
(B)	37.4
(C)	52.8
(D)	64.4
(E)	89.1
(F)	117.2

(G) OM proteins of DL1 grown in CDM2+Fe and used to inoculate CDM2-Fe (Time 0)

(H) OM proteins of DL1 grown in CDM2-Fe (Control)

The mean generation time throughout was 33 min.


83,000
78,000
75,000
73,000
70,000
69,000
66,000
39,000
35,500
32,500
18,500
A B C D E F G H

Figure 45. Alterations in the Relative Level of the IRMP after Inoculation of Iron Replete DL1 into CDM2-Fe, CDM2+Fe and after Inoculation of Iron Deficient DL1 into CDM2+Fe

Key:

- ● Iron replete DL1 into CDM2-Fe
- ■ Iron replete DL1 into CDM2+Fe
- ▲ Iron deficient DL1 into CDM2+Fe

The ratio of the IRMP to the 39,000 M_r protein which was present at constant levels throughout the growth curve, was obtained as described in 2.2.3.9.

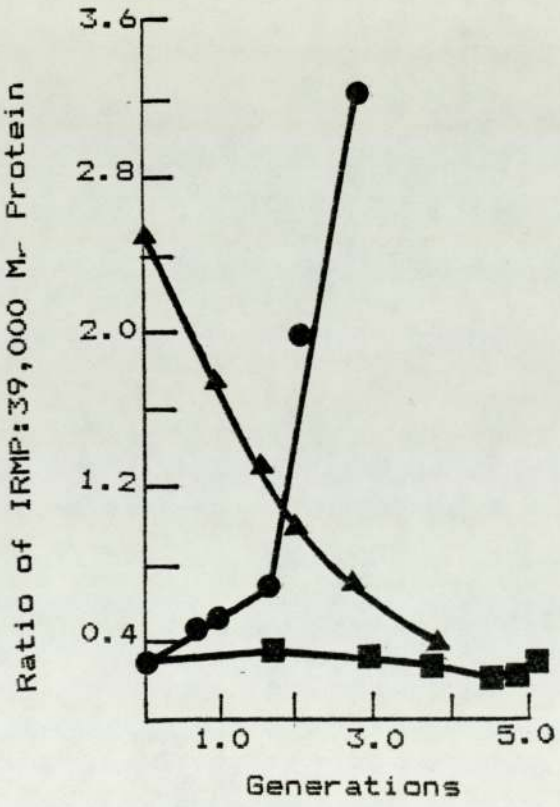

Ratio of IRMP:39,000 M- Protein
3.6
2.8
2.0
1.2
0.4
1.0
3.0
5.0
Generations

Figure 46. SDS-PAGE of the Outer Membrane Proteins of *K.aerogenes* showing the Time Course for the Loss of the IRMP following Subculture of Iron Deficient Cells into Iron Replete Media (CDM2+Fe)

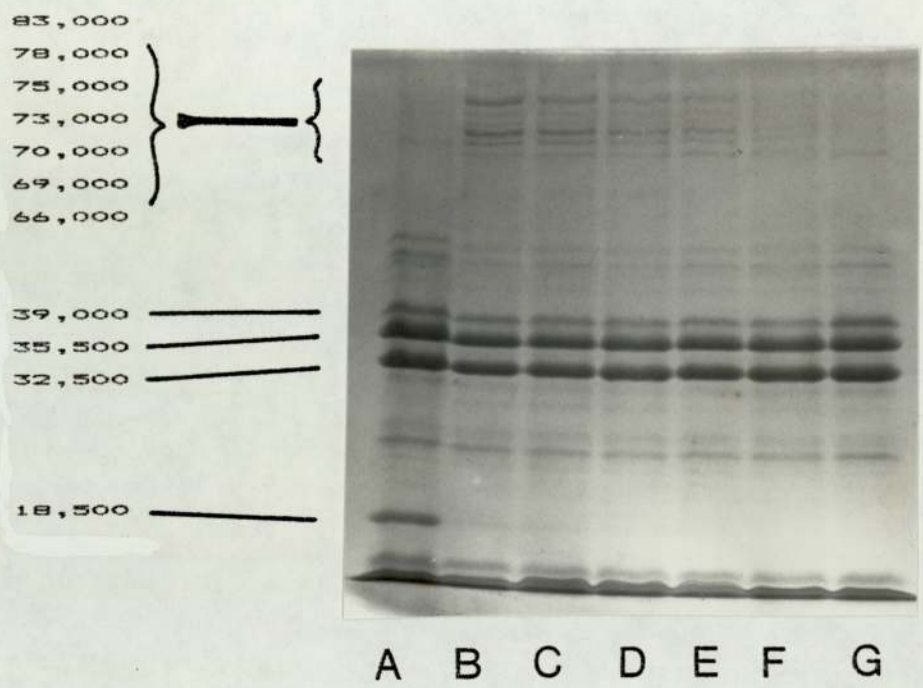
Key:

Track	Time (min)
(C)	29.7
(D)	51.0
(E)	64.7
(F)	90.1
(G)	126.1

(A) DL1 grown to stationary phase in CDM2+Fe

(B) Log phase DL1 grown in CDM2-Fe

The mean generation time throughout was 33 min.


83,000
78,000
75,000
73,000
70,000
69,000
66,000
39,000
35,500
32,500
18,500
A B C D E F G

addition of magnesium to the growth medium (Nicas & Hancock,1983). However, since it took one and a half rather than one cell division after inoculating late log phase iron depleted DL1 into CDM2+Fe, to reduce the relative level of IRMP by half, it appears that DL1 continues to manufacture IRMP for half a generation after its subculture into an iron replete environment. This may reflect the relatively long half lives of the mRNAs for OM proteins (Inouye,1979).

5.5. Growth of Iron Replete DL1 in CDM2+Fe

The changes occuring in the OM protein composition of *K.aerogenes* DL1 throughout the growth cycle of iron replete cells in CDM2+Fe are shown in Figs.45 and 47. They reveal that the IRMP never completely disappear but remain in the OM in amounts just detectable by Coomassie Blue staining of the SDS polyacrylamide gels. To rule out the possibility that the presence of low levels of IRMP in iron replete conditions was due to the treatment of the inoculum during harvesting, the organism was washed and centrifuged in 0.9% w/v NaCl $\pm$ 20×10^{-6}M $FeSO_4.7H_2O$; or in CDM $\pm$ 20×10^{-6}M $FeSO_4.7H_2O$ $\pm$ glucose; or not washed or centrifuged before inoculation into fresh CDM2+Fe. The IRMP were still detectable at low levels after subculture into CDM2+Fe.

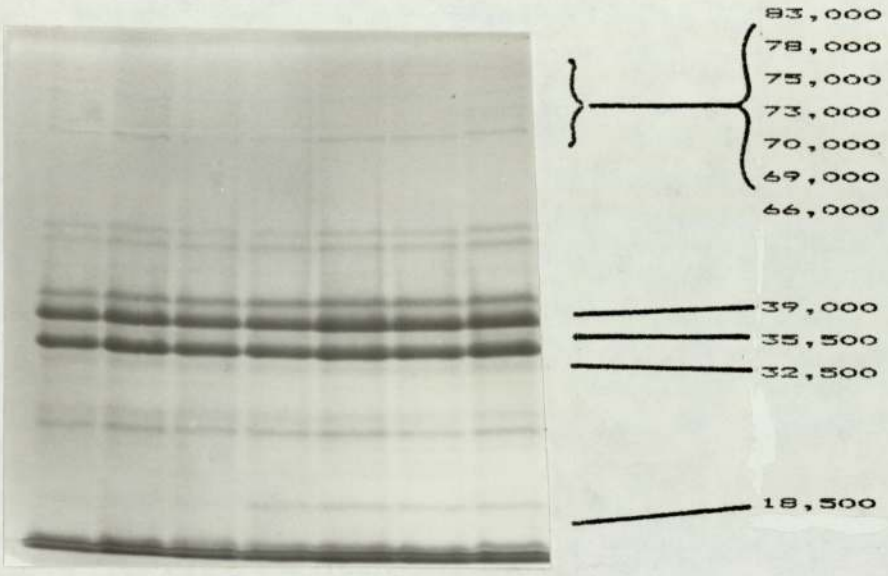
An additional observation made from Fig.47 was that the 18,500 M_r protein which was virtually absent from CDM2-Fe and serum grown DL1, was induced in the log phase in CDM2+Fe and was retained throughout the remainder of the growth cycle.

Figure 47. SDS-PAGE of the Outer Membrane Proteins of *K.aerogenes* DL1 throughout the Growth Cycle of Iron Replete Cells in Iron Replete Media (CDM2+Fe)

Key:

Track	Time (min)
(A)	56.1
(B)	95.7
(C)	122.1
(D)	148.5
(E)	158.4
(F)	163.4

(G) OM proteins of DL1 grown to stationary phase in CDM2+Fe. These cells formed the inoculum at time 0 for subculture into CDM2+Fe.


93,000
78,000
75,000
73,000
70,000
69,000
66,000
39,000
35,500
32,500
18,500
A B C D E F G

5.6. Relationship between the Extracellular Iron Concentration and Derepression of the IRMP

DL1 manufactured an OM complete with its high affinity iron transport systems within two generations of subculture into CDM2-Fe even after growth in a gross excess of iron (20 x 10^{-6}M). The inoculation of DL1 grown in a range of iron concentrations between 20 and 100 x 10^{-6}M into CDM2-Fe and its subsequent effect on the appearance of the IRMP was investigated (Fig.48). The derepression of the IRMP and their incorporation into the OM still occurred within two generations in CDM2-Fe despite prior growth in up to 100 x 10^{-6}M of Fe^{2+}.

The relationship between the initial extracellular iron concentration and the rate at which the IRMP appeared in the OM is shown in Fig.49. After growth in CDM2+Fe, DL1 was subcultured in fresh CDM to which a range of iron concentrations between 0 and 200 x 10^{-7}M $FeSO_4.7H_2O$ had been added. After two generations of growth, only the OM from cells grown in CDM2-Fe (no added iron) expressed the IRMP. The iron content of this CDM2-Fe was calculated to be 12 x 10^{-7}M Fe^{3+}. The experiment was repeated with a narrower range of added iron concentrations (0 to 5 x 10^{-7}M). An initial total medium iron content of <17 x 10^{-7}M Fe^{3+} enabled DL1, after growth to stationary phase in CDM2+Fe, to induce the formation of its high affinity iron transport systems so that the IRMP were clearly visible within two generations.

Figure 48. SDS-PAGE Showing the Outer Membrane Proteins of *K.aerogenes* DL1 Grown to Stationary Phase in Increasing Concentrations of Iron and then Subcultured for Two Generations in Iron Depleted Media (CDM2-Fe)

K.aerogenes DL1 was first grown to stationary phase in:

(A) $20 \times 10^{-6}M\ Fe^{2+}$

(B) $40 \times 10^{-6}M\ Fe^{2+}$

(C) $70 \times 10^{-6}M\ Fe^{2+}$

(D) $100 \times 10^{-6}M\ Fe^{2+}$

Cells were then subcultured for 2 generations in CDM2-Fe (Tracks B,D,F, and H corresponding with tracks A,C,E and G respectively).

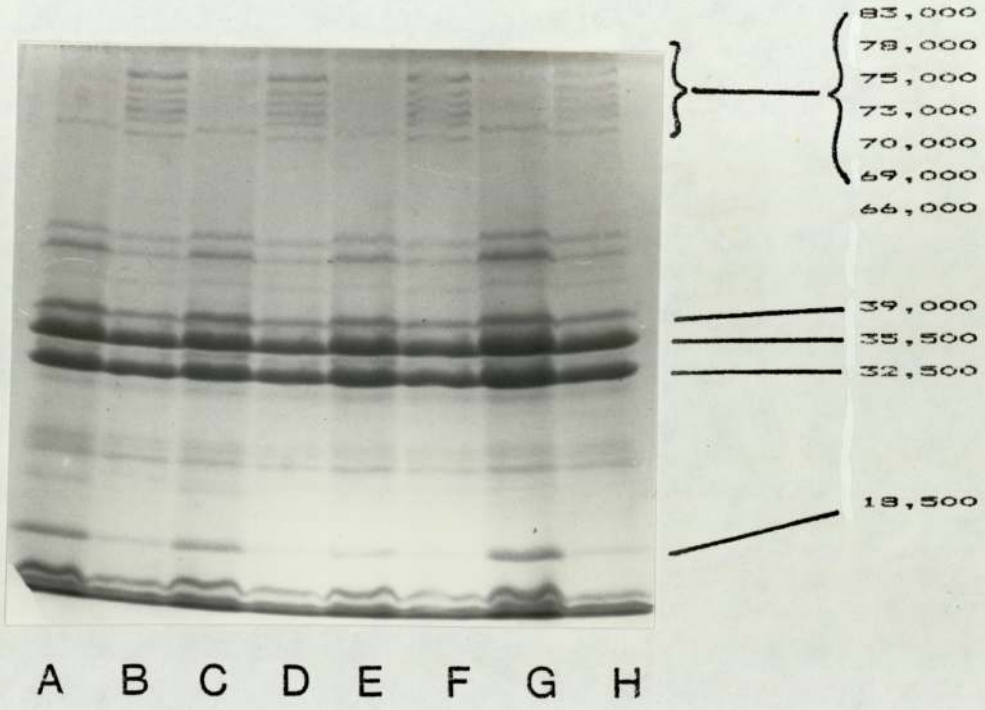

83,000
78,000
75,000
73,000
70,000
69,000
66,000
39,000
35,500
32,500
18,500
A B C D E F G H

Figure 49. SDS-PAGE Showing the Appearance of the IRMP of *K.aerogenes* DL1 following Subculture of Stationary Phase Cells harvested from CDM2+Fe into CDM2-Fe Containing Increasing Concentrations of Iron

Stationary phase CDM2+Fe grown cells (OM protein profile shown in track A) were grown for two generations in CDM2-Fe containing:

(B) $12 \times 10^{-7}M\ Fe^{2+}$

(C) $17 \times 10^{-7}M\ Fe^{2+}$

(D) $22 \times 10^{-7}M\ Fe^{2+}$

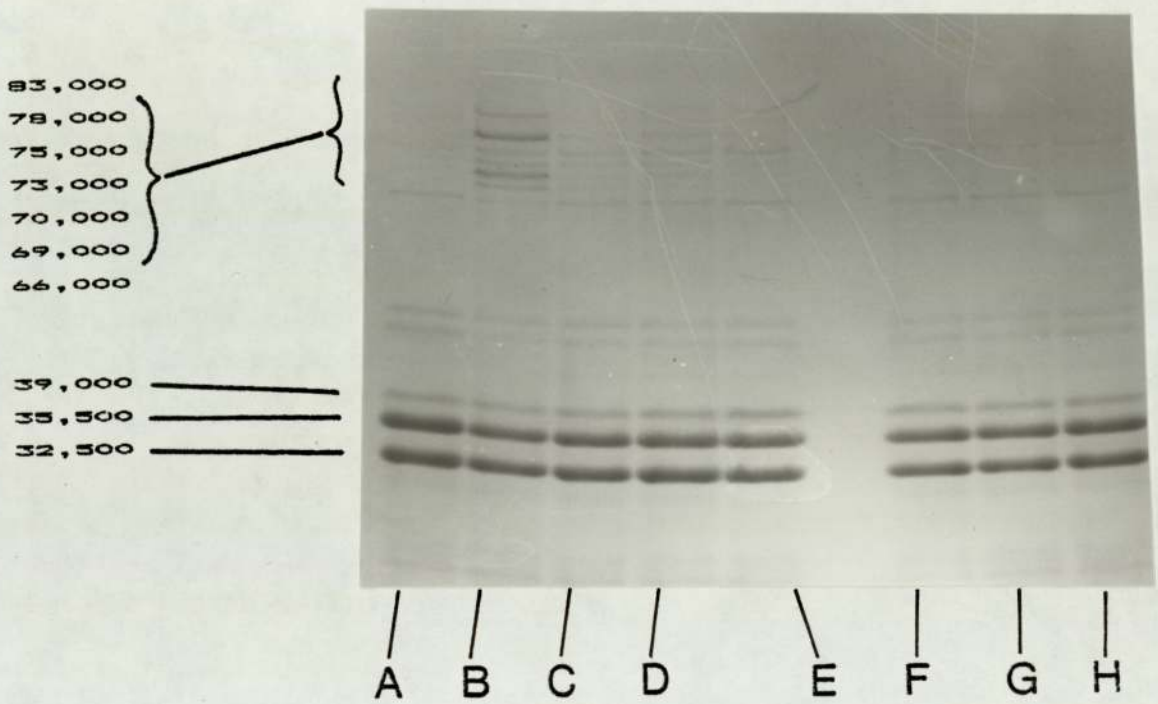
(E) $32 \times 10^{-7}M\ Fe^{2+}$

(F) $52 \times 10^{-7}M\ Fe^{2+}$

(G) $92 \times 10^{-7}M\ Fe^{2+}$

(H) $112 \times 10^{-7}M\ Fe^{2+}$

All cultures grew at the same rate. No lag phase was observed.


83,000
78,000
75,000
73,000
70,000
69,000
66,000
39,000
35,500
32,500
A
B
C
D
E
F
G
H

5.7. Discussion

Two major problems arise in formulating a CDM to study iron deprivation. Firstly, the level of contaminating iron from glassware and chemicals may be sufficiently high to enable the organism to reach a cell density at which another nutrient (often oxygen) becomes limiting. Secondly the poor solubility of iron salts at pH 7.4 may make it difficult to obtain an iron enriched CDM. Reduction in the contaminating level of iron was achieved by treatment of the sodium phosphate buffer (which was the major contributor) with the cationic exchange resin, Chelex 100. CDM2+Fe was prepared by the addition of iron (II) sulphate; by increasing the concentration of this salt, a longer log phase and greater cell mass was obtained.

In highly aerated conditions in aqueous solution at physiological pH, Fe^{2+} is oxidised to Fe^{3+}, the process being driven by the hydration of Fe^{3+} to form insoluble ferric hydroxide polymers (Fenchal & Blackburn, 1979). Therefore, the amount of free ionic iron in solution will be extremely low. The solubility of iron in simple salts medium, however, may also depend on other factors, for example, the Na^+ : K^+ ratio (Hartmann & Braun, 1981) and on the presence of other negatively charged species such as phosphates, and in the case of *Klebsiella*, its acidic polysaccharide. DL1 produces a serotype 1 polysaccharide capsule which has been reported (Sutherland,1977) to consist

of negatively charged repeating units of glucose, fucose, glucuronic acid and pyruvate. This capsular material is present in solution, as a colloid and in a form closely associated with the cell surface in liquid cultures of *K.aerogenes* (Rudd *et al*,1982). It may therefore act as a cation sink, influencing the traffic of charged molecules into and out of cells by ion exchange and salt formation as well as chelation and co-ordination complexes (Dudman, 1977; Costerton *et al*,1981). Despite the complex equilibria which exist in liquid culture in CDM2+Fe, the end result is that the organism acquires iron presumably via some low affinity iron uptake mechanism as the high affinity transport system is not induced. In iron poor conditions, enterochelin ensures that whilst iron is present, it can be transported into the bacterium, against the concentration gradient via the high affinity uptake mechanism.

In an iron restricted environment e.g. serum, the doubling times of gram-negative bacteria may be increased (Griffiths & Humphreys, 1978; Bullen *et al*,1974). In contrast, DL1 doubled in serum at the same rate as in CDM2+Fe. As differences in doubling time will affect the attainment of critical bacterial populations, it may be crucial in determining the outcome of an infection. the ability of DL1 to multiply rapidly in serum without a long lag phase or an increased doubling time, may contribute to the virulence of the organism *in vivo* during an infection.

In CDM2+Fe, the OM protein profile of DL1 resembled that of

E.coli in that three proteins in the 30,000 to 40,000 M$_r$ range were predominant (Lugtenberg & van Alphen, 1983). When subjected to conditions of iron stress, DL1 synthesized at least six new OM proteins together with the iron chelator, enterochelin. *K.aerogenes* DL1 unlike *E.coli* did not induce a high molecular weight protein in the presence of 1mM citrate (Hancock *et al*, 1976). At least one of the new OM proteins of DL1 is presumably involved in ferric enterochelin uptake (Ichihara & Mizushima, 1978). The function of the others requires further investigation. As *E.coli* can utilise exogenous chelators such as ferrichrome (Wayne & Neilands, 1975), it is conceivable that the DL1 IRMP may also act as receptors for exogenous siderophores. Some evidence in support of this was obtained by Khimji and Miles (1978) who reported that *Klebsiella* infections could be enhanced by supplying the *Streptomyces* iron chelator, desferrioxime B.

Iron depleted *K.aerogenes* DL1 overproduces riboflavin (vitamin B2). this vitamin is generally present in cells as a component of the protein bound coenzymes flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) which play important roles in the oxidative processes of living cells. The control of riboflavin biosynthesis involves iron. Ferrous iron severely inhibits riboflavin production by "low overproducers" (Demain,1972). The mechanism by which this occurs appears to involve an iron flavoprotein which suppresses riboflavin synthesis. Growth in iron depleted media, however, results in the production of cells with little or no repressor and thus riboflavin synthesis is

derepressed (Demain,1972).

Much is known about the enterobacterial iron chelatores and IRMP although little is known of the mechanisms by which these high affinity transport systems are regulated. In *E.coli* the biosynthesis of the 83,000, 81,000 and 74,000 M$_r$ IRMP and enterochelin are co-ordinately regulated by the intracellular iron concentration (McIntosh & Earhart,1977). There also appears to be a regulatory mechanism which covers all iron uptake systems and is superimposed on the regulation of individual systems (Hantke,1981). The synthesis of enterochelin and the IRMP of *K.aerogenes* is repressed in the presence of an excess of free iron in both CDM2+Fe and in serum. The effect of iron deprivation on the regulation of these iron uptake systems was followed throughout the growth cycle. The high affinity iron uptake system was expressed at least two generations before the onset of stationary phase due to iron depletion. *Klebsiella* is therefore capable of rapidly responding to low iron levels in the extracellular environment by the co-ordinate synthesis of enterochelin and the IRMP, an ability which may contribute to the virulence of the organism.

Inoculation of iron depleted DL1 into fresh CDM2+Fe resulted in the exponential dilution of the IRMP from the OM as the cells divided. However, a few copies of these proteins appeared to be present throughout the growth cycle in CDM2+Fe, a phenomenon which may arise as a result of the methods employed or which may reflect the biochemical basis

of regulation of the IRMP.

According to the conditions employed, *E.coli* can store iron intracellularly during periods of iron repletion and use it during periods of iron stress (Klebba *et al*, 1982). The appearance of the IRMP of *K.aerogenes* DL1 was unaffected by the prior growth of the organism in high concentrations of iron. A number of possible explanations can be made: DL1 may be unable to store iron; derepression may depend on the organism's ability to sense the extracellular iron concentration independently of the stored or free intracellular concentration; derepression may be dependent on the free as opposed to the stored intracellular iron concentration. For *E.coli* it has been suggested that once the intracellular levels of iron fall below a specific level, synthesis of iron transport components becomes derepressed and, in iron replete cells, the stored iron becomes accessible during the induction sequence, increasing the concentration of free intracellular iron and therefore retarding induction kinetics (Klebba *et al*, 1982; McIntosh & Earhart, 1977).

6. The Role of the O and K Antigens of *K.aerogenes* in Resistance to Serum Killing and Phagocytosis

The role of the O and K antigens of *K.aerogenes* in protecting the organism from complement-mediated serum killing and phagocytosis was investigated using *K.aerogenes* DL1 ($K1^+$ O^+), NCTC 5055 ($K2^+$ $O1^+$) and Mutants M10 (K^- O^+) and M10B (K^- O^-). M10 and M10B were derived from NCTC 5055 after nitrosoguanidine mutagenesis (Poxton & Sutherland, 1976). Interpretation of the results of this study assumes that the mutations only affected the O and K antigens, other outer envelope constituents remaining unchanged. Outer membrane protein profiles of *K.aerogenes* NCTC 5055, M10 and M10B above M_r 18,000 revealed by sodium dodecyl sulphate polyacrylamide gel electrophoresis of sarkosyl insoluble membranes failed to show significant differences (see Fig.50).

6.1. Effect of Serum on Encapsulated *K.aerogenes*

K.aerogenes NCTC 5055 and DL1 were found to be resistant to serum (Fig.51), their viable counts increasing by some 600-800% over the 3 hour incubation period. Their viability was unaffected by the addition of a range of homologous and heterologous antisera raised against each of the *Klebsiella*

Figure 50. SDS-PAGE of the Outer Membrane Proteins of Nutrient Agar-Grown *K.aerogenes*

Key:

Track	Organism
(A)	NCTC 5055
(B)	M10
(C)	M10B
(D)	DL1

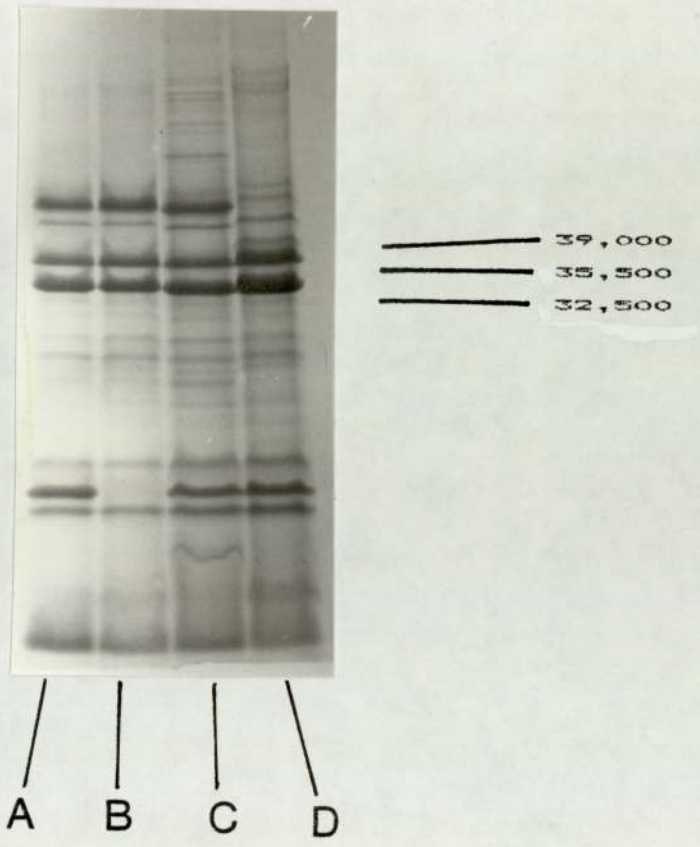
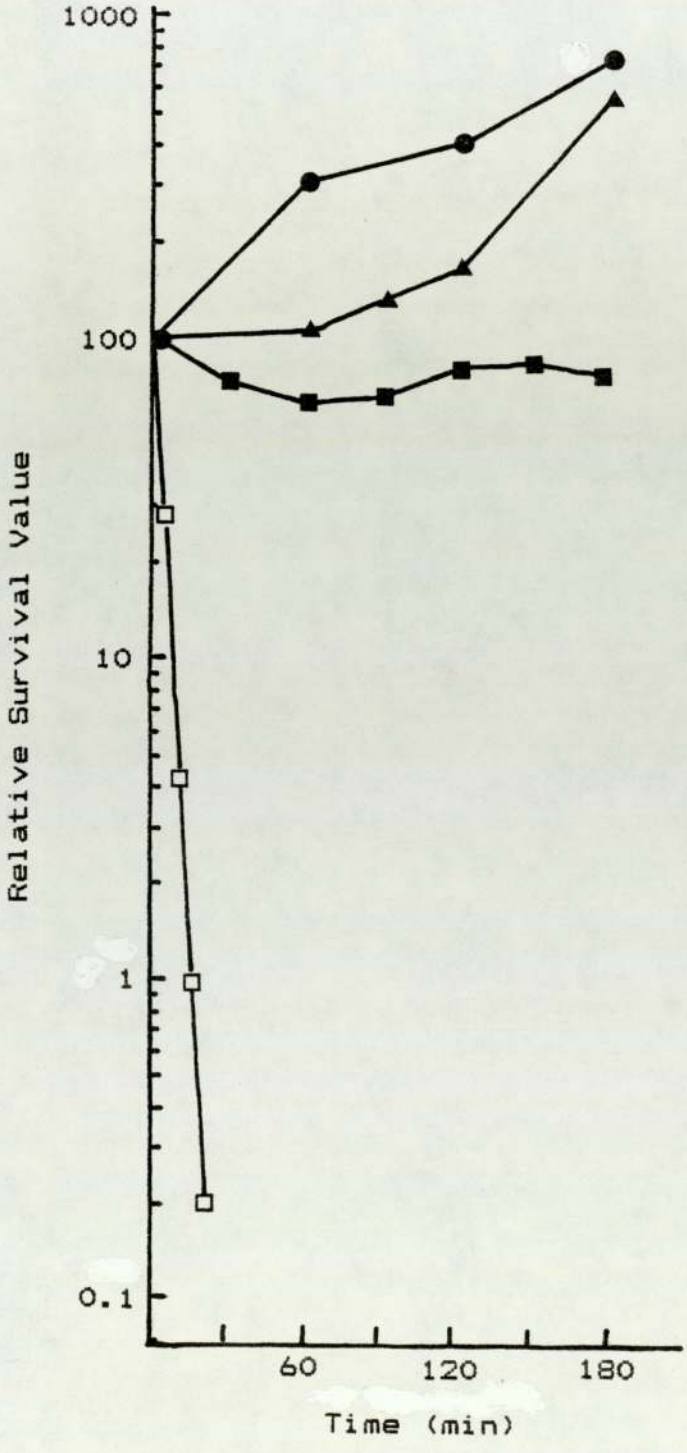

39,000
35,500
32,500
A
B
C
D

Figure 51. Kinetics of Killing of *K.aerogenes* by Normal Human Serum

Key:

- ● DL1 ($K1^+$ O^+)
- ▲ NCTC 5055 ($K2^+$ $O1^+$)
- ■ M10 (K^- $O1^+$)
- □ M10B (K^- O^-)

The survival values indicate viable counts relative to time zero (100%).


1000
100
10
1
0.1
Relative Survival Value
60
120
180
Time (min)

strains used in the study or by chelation of the serum with MgEGTA to prevent activation of the classical complement pathway whilst leaving the alternative pathway functional. Both strains grew in heat inactivated serum

6.2. Effect of Serum on Non-Encapsulated *K.aerogenes*

Normal human serum was bacteriostatic for M10 whilst the rough mutant M10B was rapidly killed (Fig.51). Both strains grew in heat inactivated serum.

The results of further investigation of the effect of serum on M10B are shown in fig.52. The viable count was reduced by some 3 log scales in 20 minutes in fresh serum, whilst chelation with MgEGTA delayed the rapid kill by some 15 minutes. Thus, both classical and alternative pathway activity is rapidly bactericidal for M10B. The kinetics of the alternative pathway are such that its activation and subsequent effect is slower than the more efficient classical complement pathway (Root *et al*,1972)

During the 3 hour incubation period in fresh serum at 37°C no increase in the viable count of M10 was observed. Addition of a range of concentrations of anti M10 serum (to a final concentration of 1 in 100,000 and 1 in 20), had no effect on the bacteriostasis at the higher dilutions but at a concentration of 1 in 20, the antiserum appeared to

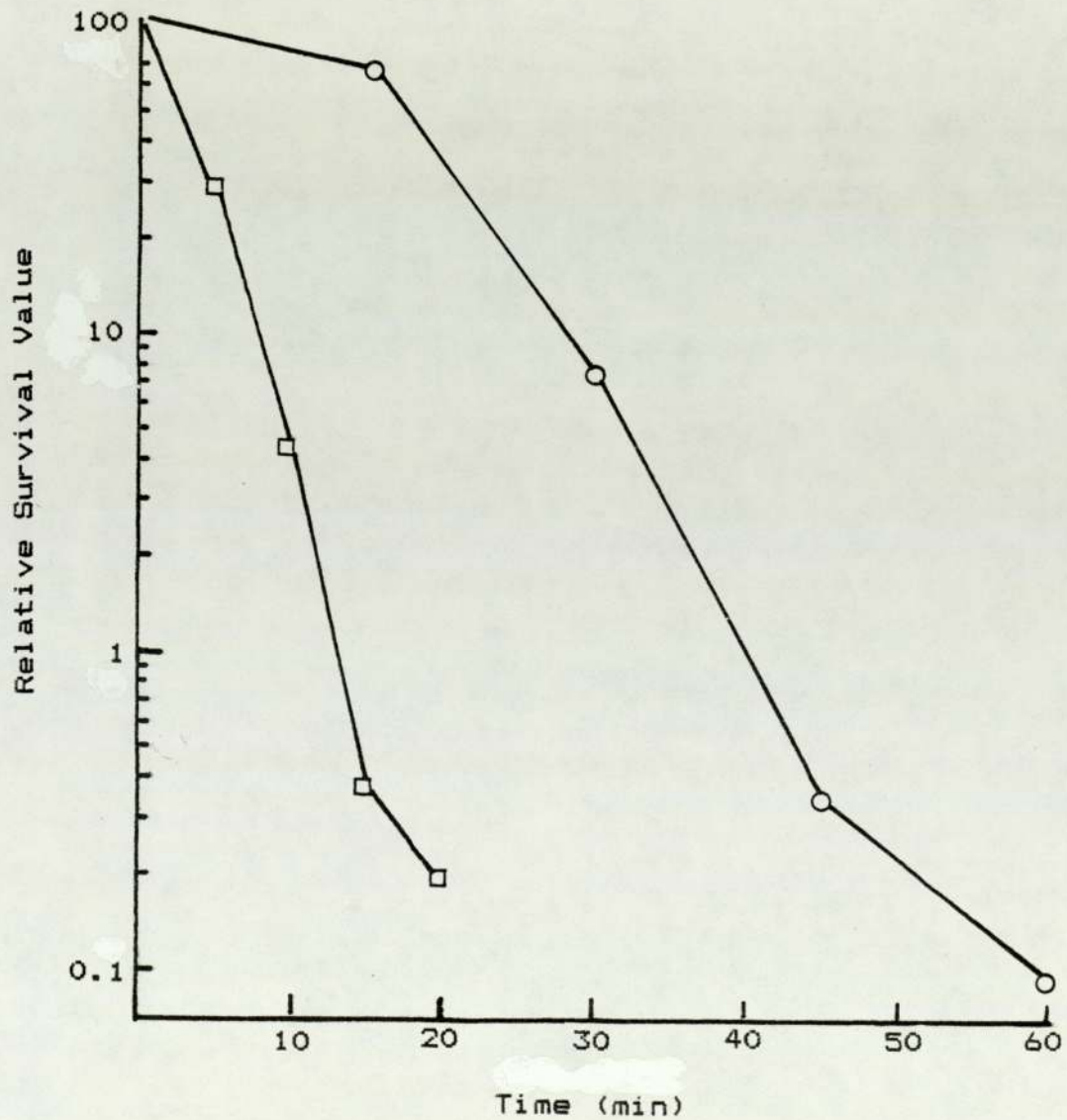
Figure 52. Kinetics of Killing of *K.aerogenes* M10B by Normal Human Serum

Key:

□ Normal Human Serum Alone

○ Normal Human Serum Chelated with 10mM-MgEGTA

The survival values are expressed as in figure 51.


100
10
1
0.1
Relative Survival Value
10
20
30
40
50
60
Time (min)

antagonise the bacteriostatic effect thus allowing the organism to proliferate (Fig.53). Chelation of the serum to isolate functional alternative pathway activity resulted in a fall in viable count over the 3 hour incubation whilst a similar effect was observed upon addition of anti M10B serum (which lacks anti O antibodies) to the assay system (Fig.53). However, the organism multiplied in the presence of MgEGTA in heat inactivated serum and therefore the effect was not due to the MgEGTA alone (Fig.53). Addition of homologous anti M10B serum to M10B did not antagonise the bactericdal effect of normal human serum for this strain.

6.3. Whole Blood Killing of *K.aerogenes*

A similar bactericidal response to that obtained in serum in the absence of added specific antibody was found to occur in blood (Fig.54). Cell association rather than ingestion is the preferred term as the assay system employed does not differentiate completely between those bacteria adhering to the neutrophil outer surface and those enveloped by the phagosome.

Analysis of the bacteria/PMN association profile revealed that whilst M10 was phagocytosed to the extent of 4.2±0.5 bacteria per PMN, the encapsulated strains NCTC 5055 and DL1 effectively resisted phagocytosis (Fig.55). Thus the O antigens of these *Klebsiella* strains are not in themselves

Figure 53. Kinetics of Killing of *K.aerogenes* M10 by Normal Human Serum

Key:

■ Normal Human Serum Alone

○ Normal Human Serum plus Antiserum to M10 (1 in 20 dilution)

□ Heat-Inactivated Normal Human Serum Chelated with 10mM-MgEGTA

△ Normal Human Serum Chelated with 10mM-MgEGTA

● Normal Human Serum plus Antiserum to M10B (1 in 20 dilution)

The survival values are expressed as in figure 51.

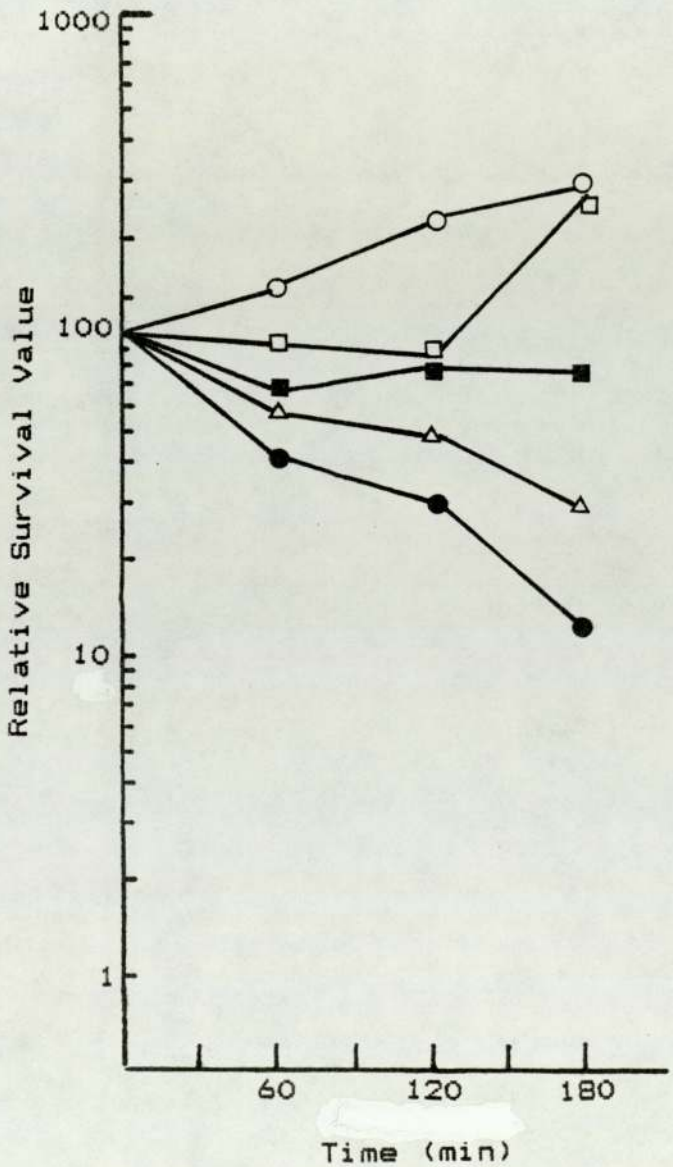
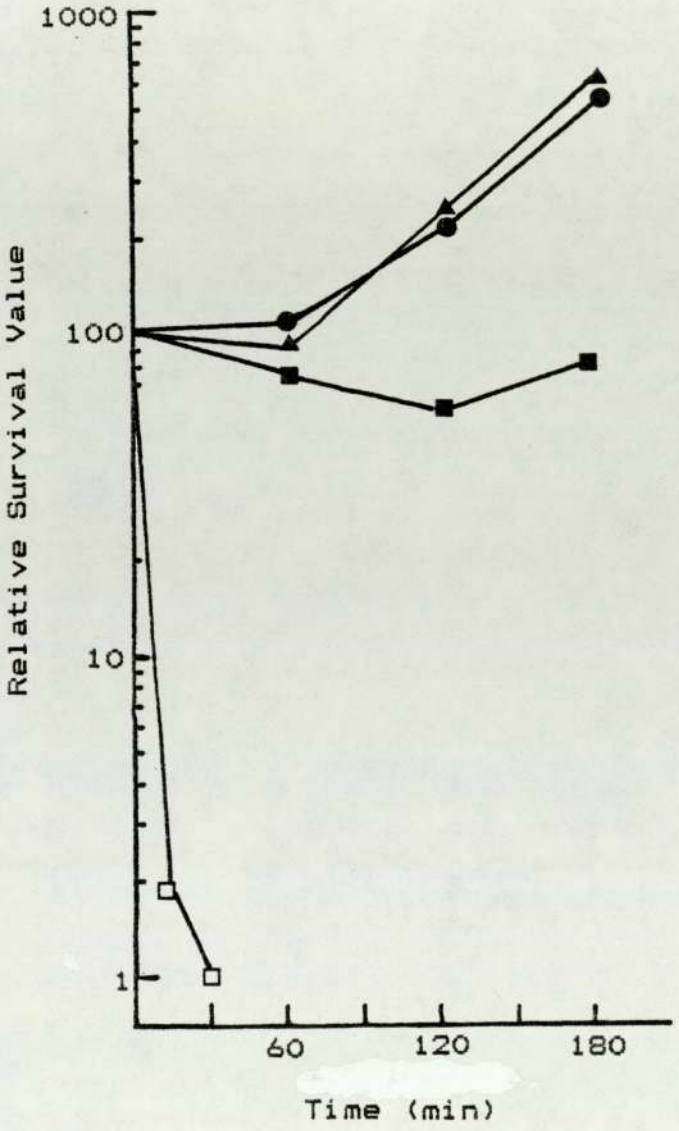

1000
100
10
1
Relative Survival Value
60
120
180
Time (min)

Figure 54. Kinetics of Killing of *K.aerogenes* by Whole Human Blood

Key:

- ● DL1 ($K1^+$ O^+)
- ▲ NCTC 5055 ($K2^+$ $O1^+$)
- ■ M10 (K^- $O1^+$)
- □ M10B (K^- O^-)

The survival values are expressed as in figure 51.


1000
100
10
1
Relative Survival Value
60
120
180
Time (min)

antiphagocytic.

The further investigation of the effects of specific antisera on phagocytic ingestion in terms of cell association of *K.aerogenes* NCTC 5055, DL1 and M10 is summarised in fig.56. Antisera to M10, M10B, 5055 and DL1 each increased the number of M10 associated with PMN compared to the control of pre-immune serum (Fig.56c).

Uptake of NCTC 5055 occurred not only in the presence of anti 5055 serum but also in the presence of anti M10 serum. As anti M10 serum did not contain anticapsular antibodies, it appeared that antibodies directed against components other than the polysaccharide capsule were responsible for opsonisation. By contrast, anti M10B gave only a small increase in the number of PMN associated NCTC 5055.

These findings indicate that the capsule and O antigens were the main surface structures involved in opsonisation and that the presence of the K antigen does not completely mask access of antibody directed to O antigen. Confirmation that anti K antibodies alone could opsonise NCTC 5055 was obtained by absorbing anti 5055 with whole live M10 organisms. The immunodiffusion plate shown in fig.57 shows the absence of anti O antibodies in the absorbed antiserum.

K.aerogenes DL1 has a type 1 polysaccharide capsule but possesses an O antigen that immunologically cross reacts with M10 LPS (Fig.58a;Fig.58b). DL1 resisted phagocytosis in

Figure 55. Phagocytosis of *K.aerogenes* in the Absence of Added Specific Antisera

Key:

DL1	($K1^+$ O^+)
NCTC 5055	($K2^+$ $O1^+$)
M10	(K^- $O1^+$)

The histograms show bacteria/PMN cell association profiles.The bars represent standard deviation.

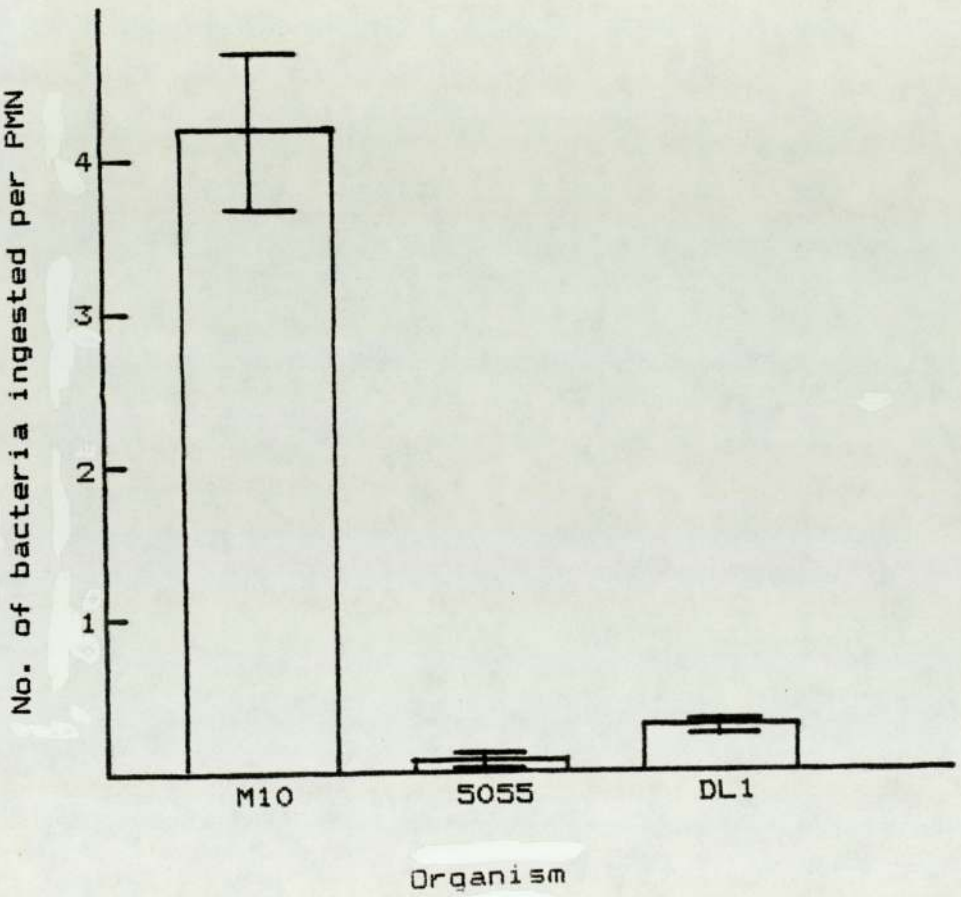

No. of bacteria ingested per PMN
4
3
2
1
M10
5055
DL1
Organism

Figure 56. Phagocytosis of *K.aerogenes* in the Presence or Absence of Specific Antisera

Key:

(a) NCTC 5055 ($K2^+$ $O1^+$)

(b) DL1 ($K1^+$ O^+)

(c) M10 (K^- $O1^+$)

The histograms show bacteria/PMN cell association profiles. Ingestion of *K.aerogenes* NCTC 5055 (a), DL1 (b) and M10 (c) in the presence or absence of antisera prepared against whole cells of *K.aerogenes* NCTC 5055, M10, M10B and DL1 as indicated. 5055abs indicates the addition of NCTC 5055 antiserum pre-absorbed with whole live cells of M10. M10* (panel b) represents the association profile obtained after homogenizing and washing the cells (DL1) before subjecting them to the whole human blood phagocytic ingestion assay in the presence of antiserum to M10. The bars represent standard deviation.

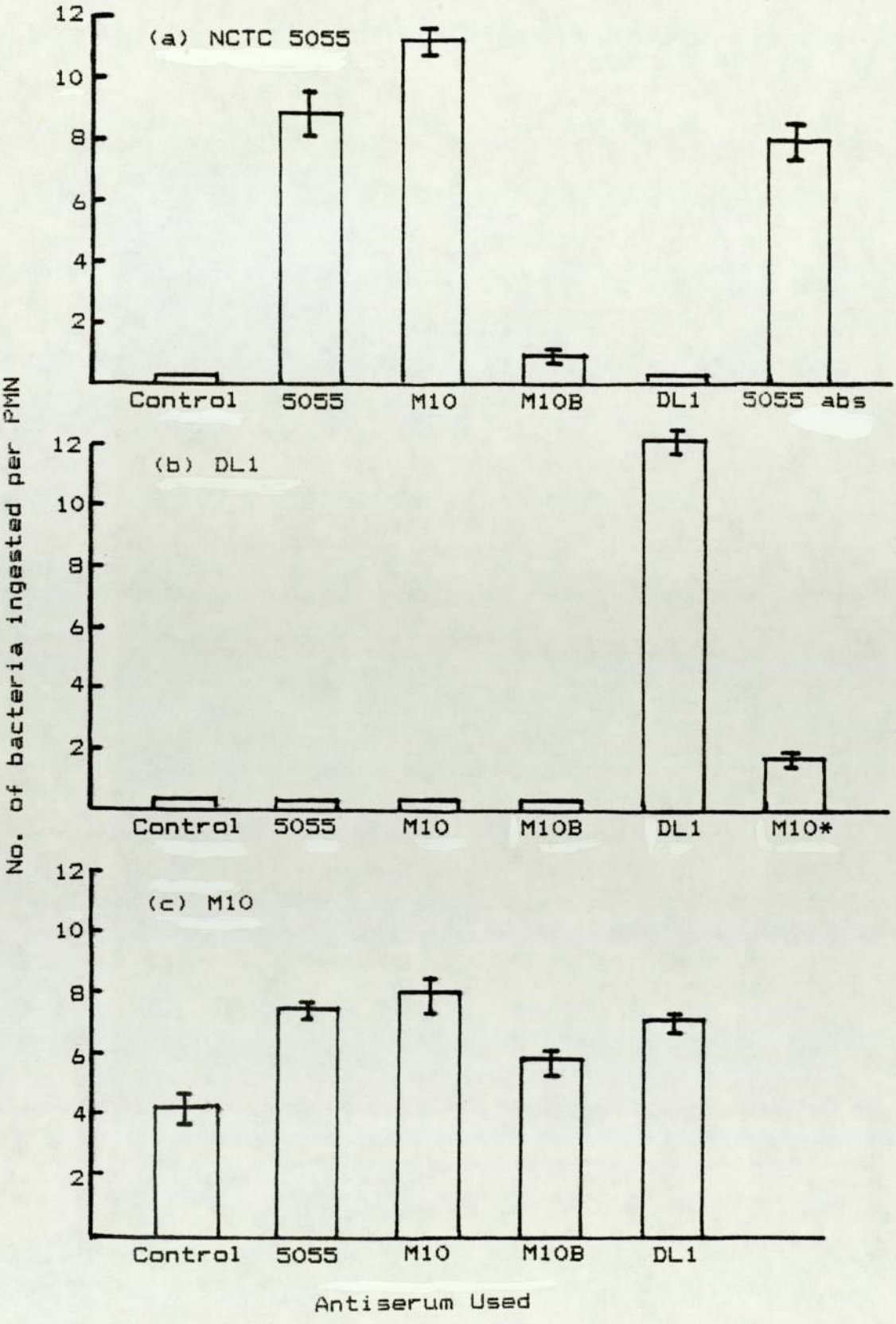
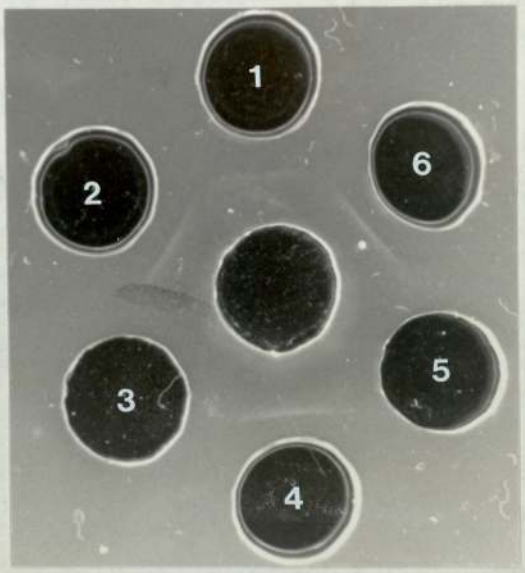

(a) NCTC 5055
(b) DL1
(c) M10
No. of bacteria ingested per PMN
12
10
8
6
4
2
Control
5055
M10
M10B
DL1
5055 abs
M10*
Antiserum Used

Figure 57. Double Diffusion Immunoprecipitation



The immunodiffusion was carried out in 1% w/v agarose, Tris/Barbital buffer, pH 8.6. The centre well contained antiserum to NCTC 5055 pre-absorbed with whole live cells of *K.aerogenes* M10. The outer wells contained purified LPS from M10 prepared by hot phenol extraction of whole cells (wells 3,5) or a crude mixture of capsular polysaccharide and LPS from NCTC 5055 prepared by ethanol precipitation of cell culture supernatant (wells 1,2,4,6).

the absence of homologous antiserum (0.3±0.05 bacteria/PMN as opposed to 12±0.4 bacteria/PMN in the presence of anti DL1)(Fig.56b). In this case, addition of anti M10 or anti M10B to the blood bacteria mixture did not result in opsonisation of DL1. On the basis of colonial morphology, DL1 is much more mucoid than NCTC 5055; a greater covering of acidic polysaccharide may shield the O antigen or prevent access of the anti O antibodies to their target receptor. Physical reduction of the amount of capsule surrounding DL1 was achieved by gently homogenizing the organism before centrifuging at 18,000rpm and resuspension in fresh saline. After this treatment DL1, was opsonised to a greater extent by anti M10 serum (Fig.56b) (1.64±0.08 as compared with 0.3±0.05 bacteria/PMN).

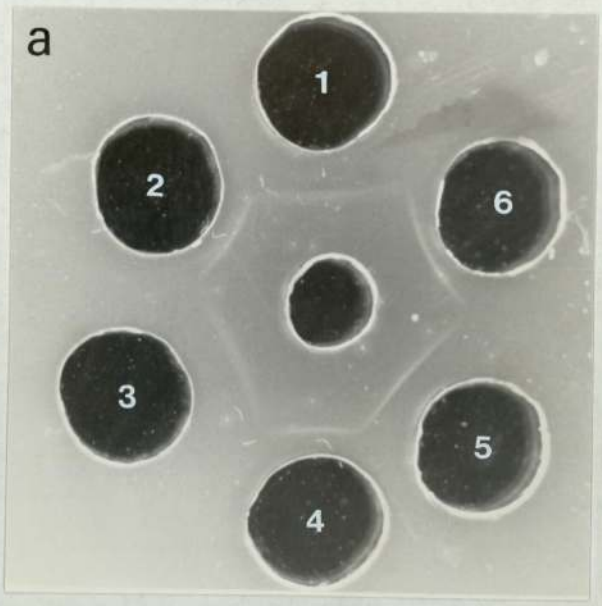
The phagocytic system employed was optimised to study the effect of various antisera in promoting PMN association and ingestion. The ratio of bacteria to PMNs was approximately 15:1. Little of the bactericidal capability of the PMNs was apparent at this bacterial cell density. Fig.59 shows the result of reducing the ratio 10-fold i.e. 1.5:1 which makes clearly apparent the killing ability of the PMNs in the presence of added antisera.

Figure 58 (a). Double Diffusion Immunoprecipitation

Centre well contained antiserum to DL1, the outer wells contained purified LPS from M10.

Figure 58 (b). Double Diffusion Immunoprecipitation

Centre well contained antiserum to M10, the outer wells contained purified LPS from M10 (1,3,5) or purified LPS from DL1 (2,4,6).


a
1
2
6
3
5
4

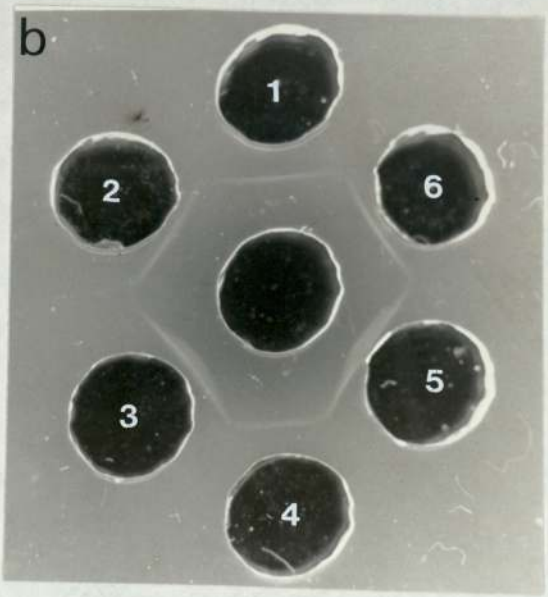

b
1
2
6
5
3
4

Figure 59. Kinetics of Killing of *K.aerogenes* NCTC 5055 by Whole Human Blood

Key:

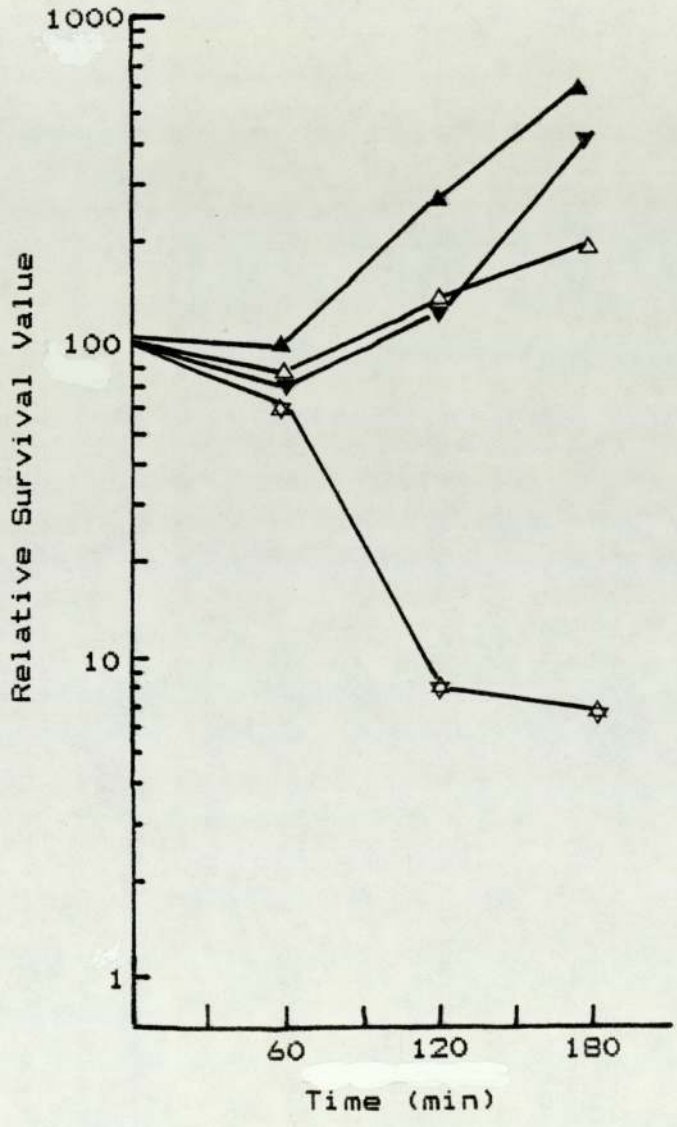
▲ NCTC 5055 ($K2^+$ $O1^+$) - Bacteria:PMN ratio 15:1

△ NCTC 5055 ($K2^+$ $O1^+$) - Bacteria:PMN ratio 1.5:1

▼ NCTC 5055 ($K2^+$ $O1^+$) plus antiserum to NCTC 5055 - Bacteria:PMN ratio 15:1

✡ NCTC 5055 ($K2^+$ $O1^+$) plus antiserum to NCTC 5055 - Bacteria:PMN ratio 1.5:1

The survival values are expressed as for figure 51.


1000
100
10
1
Relative Survival Value
60
120
180
Time (min)

6.4. Effect of Iron Depletion on the Whole Blood Killing of *K.aerogenes*

Whole blood killing of DL1 grown in CDM1-Fe and to mid log phase in CDM1+Fe is shown in fig.60. The mouse virulent strain DL1 is compared with a mouse avirulent capsular type 1 strain, DL33. In the absence of added antisera, log phase DL1 and log phase DL33 rapidly multiplied during the 60min incubation period. However, whilst there was no reduction in the viable count of iron depleted DL1 or iron depleted DL33, they did not multiply. Whether log phase or iron depleted, both strains effectively resisted phagocytic ingestion in the absence of added antiserum (Table 10).

Figure 60. Kinetics of Killing of Iron Depleted *K.aerogenes* by Whole Human Blood

Key:

▲ Iron-depleted DL1 ($K1^+$ O^+)

● Log phase DL1 ($K1^+$ O^+)

□ Iron-depleted DL33 ($K1^+$ O^+)

■ Log phase DL33 ($K1^+$ O^+)

DL1 and DL33 were both grown in CDM1-Fe (to obtain iron-depleted cells) and in CDM1+Fe (to obtain log phase cells). The survival values are expressed as for figure 51.

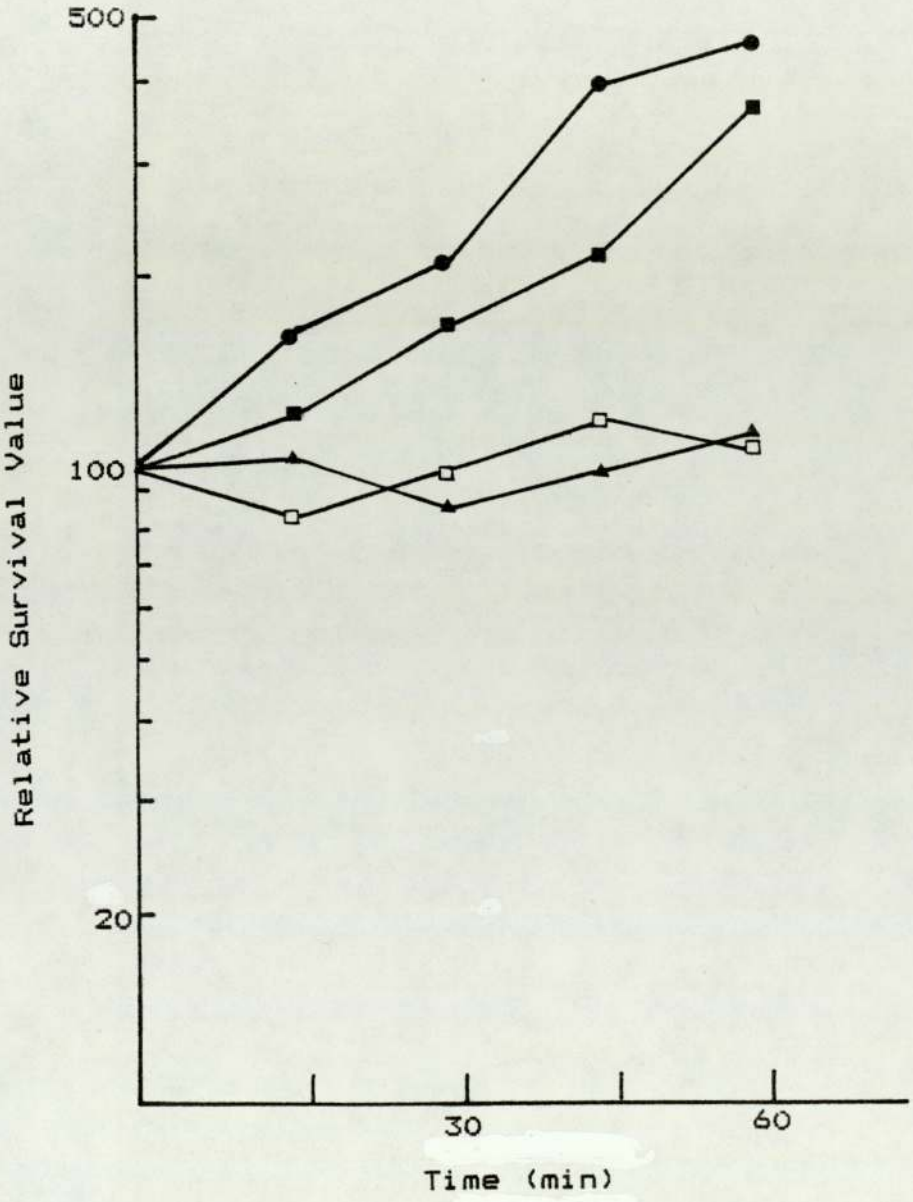

500
100
20
Relative Survival Value
30
60
Time (min)

Table 10. Phagocytosis of Log Phase and Iron Depleted *K.aerogenes*

	Log Phase	CDM2-Fe
DL1	0.12±0.000	0.12±0.003
DL33	0.22±0.005	0.21±0.004

K.aerogenes DL1 is a mouse virulent strain, DL33 is avirulent.

6.5. Discussion

The O and K antigens of *K.aerogenes* were both found to be involved in determining the resistance of the organism to complement mediated serum killing and to phagocytic ingestion by human PMNs. The O antigen of M10 was not antiphagocytic but it was found to protect M10 from the rapid bactericidal action of serum. This protection was not complete since, unlike the encapsulated parent strain, M10 could not grow in fresh serum. This bacteriostatic effect could however be reversed since the cells grew upon addition of anti M10 serum. This phenomenon is presumably due to the blocking effect of anti O IgG since it did not occur upon addition of anti M10B (lacking anti O IgG) to the assay system. A reversal from serum sensitivity to complete resistance and growth has been reported for other gram negative bacteria (Taylor,1972; Traub,1981; Guttman & Waibron,1975) as a result of the presence of, or following the addition of immunoglobulins. This was interpreted in terms of the prevention of access of complement components to cell surface receptors by anti O IgG binding to the O polysaccharide chain of LPS; IgM alone was found to enhance the bactericidal activity of serum (Traub,1981).

The prompt killing of M10B (K^- O^-) in fresh serum was unaffected by a range of added concentrations of anti M10B serum. Therefore the blocking effect of antibody is only manifested in the presence of the O antigen. The loss of the

O antigen rendered the organism very sensitive to rapid complement mediated serum killing. This effect was delayed upon chelation of the serum with MgEGTA to isolate alternative complement pathway activity. This may reflect the slower kinetics of activation of the alternative pathway (Root et al,1972) although the rapid lethal effect may involve other as yet unidentified serum bactericidal factors such as the those reported by Chedid (Chedid *et al*,1968). Another factor might be the heat labile, calcium dependent, complement requiring factor specific for the Ra mutant of *Salmonella* (I hara *et al*,1982). This mutant is equivalent to M10B inasmuch that both lack the O antigen whilst retaining the core polysaccharide and lipid A moeities of LPS (Poxton & Sutherland,1976). M10 was resistant to the bactericidal capacity of fresh serum but, like M10B was sensitive to serum chelated with MgEGTA. This effect on M10 was not due to a toxic effect of MgEGTA alone as the organism multiplied in heat inactivated serum chelated with MgEGTA. A synergistic effect between complement and EGTA cannot, however be completely ruled out.

In the absence of specific antisera, the encapsulated *K.aerogenes* strains NCTC 5055 and DL1 were not ingested to any appreciable extent by PMNs in whole human blood. As expected, addition of homologus antisera to the bacteria resulted in a dramatic increase in phagocytic ingestion. Anti M10 serum (lacking anti-capsular antibodies) opsonised NCTC 5055 as effectively as the homologous antiserum, but failed to opsonise DL1 unless the amount of capsule was

reduced by gentle homogenisation of the cells followed by resuspension in fresh 0.85% saline. Ehrenworth and Bayer (1956) studied the effect of the amount of capsule on phagocytosis of *Klebsiella* capsular type 2 mutants and found that this did not affect phagocytic ingestion to any great extent. Our findings showed that, whilst physically reducing the amount of DL1's capsule did not itself enhance phagocytosis, addition of antisera containing anti O antibodies rendered the organism susceptible to opsonisation and subsequent phagocytic engulfment.

More than 70 distinct *K.aerogenes* capsular types have been recognised (Richard,1982;Powell,1980) but they appear to share just 8 different O antigens (Mizuta *et al*,1983). Since we have shown that under certain conditions it is possible to opsonise encapsulated strains of *Klebsiella* with antibody to the O antigen, this opens the possibility that a vaccine conferring protection against a wide range of *Klebsiella* capsular types might be composed of the 8 O antigens together with K antigens from those strains in which the capsule masks the O antigen.

Several investigators (McCabe,1972; Ziegler *et al*,1978; Young *et al*,1975) have reported that antiserum raised against rough mutants of the Enterobacteriaceae contain antibodies to the core glycolipid of LPS and could passively transfer broad spectrum protection against parenteral challenge with smooth strains of the Enterobacteriaceae. Whilst this effect is thought by some to be mainly antitoxic

(Braude *et al*,1973) others e.g. Crowley *et al* (1982) showed that opsonisation of smooth *E.coli* with anti Re *Salmonella minnesota* serum could be observed. There was however, some doubt as to whether antibodies to surface components other than the core glycolipid were also involved since similar findings occurred when both *E.coli* and *Klebsiella* strains were tested in opsonisation studies with anti Re *S.minnesota* serum in which the core glycolipid antibodies had been absorbed.

Anti M10B serum presumably containing anti core glycolipid antibodies, not only opsonised M10 but also the encapsulated parent, though to a much lesser extent. However, in terms of the formulation of an effective anti-*Klebsiella* vaccine, the opsonisation due to the anti core glycolipid is not as significant as that conferred by the anti O and anti K sera.

The conditions used to grow the organism for the preparation of antisera and for serum and phagocytosis assays were not the same as those encountered *in vivo*. The nutritional environment will greatly affect the composition of the outer envelope of gram negative bacteria and therefore the response of the organism to serum (Taylor *et al*,1981) and phagocytosis (Finch & Brown,1978; Anwar *et al*,1983). Sensitivity of gram negative bacteria to serum has been shown to vary with a number of factors including growth rate and the availability of magnesium and carbon (Taylor *et al*,1981). The effect of these parameters on phagocytosis has not been extensively explored but to mimic *in vivo*

conditions, the phagocytosis and whole blood killing of virulent and avirulent strains of *K.aerogenes* were compared. No difference in the phagocytic ingestion of either strain whether log phase or iron depleted was observed. However, comparison of the killing kinetics in whole human blood showed that iron depleted cells did not rapidly multiply upon incubation as did nutrient agar or log phase CDM1+Fe grown cells. This is presumably because stationary phase iron depleted cells remained in the lag phase for the whole of the incubation period, as resuspension of iron depleted DL1 in fresh CDM1+Fe was known to result in a lag phase of at least six hours.

7. Development of a Polyvalent *Klebsiella* Vaccine

7.1. Immunogenicity of the Culture Filtrate Vaccines

The minimum lethal doses (MLD) for some virulent *K.aerogenes* strains are listed in Table 11. MLD determination for each strain used in the study enabled a concentration of one MLD to be used as the intraperitoneal challenge dose for both vaccinated and control mice. As variation in the MLD may arise as a result of subculturing or because of alterations in the response of the mice to the challenge inoculum, MLDs were checked before starting a series of mouse protection tests.

The mouse protection tests described in 2.2.5.1. were used to investigate the protective and cross protective properties of the culture filtrates. Culture filtrate vaccines obtained from the supernatants of *K.aerogenes* DL1(K1) and *K.aerogenes* K44 grown for 5 days in the Liu medium (Carney & Jones,1968) were tested alone or in combination against seventy seven capsular types. The results are shown in Table 12. This procedure was repeated and a combination of ten culture filtrate fractions from ten different virulent strains (*K.aerogenes* K1, K2, K3, K15, K20, K35, K44, K50 and K63) gave complete cross protection against 54 capsular types (Table 13). Table 13 also lists the strains against which a pooled culture filtrate vaccine (obtained from five strains grown for five days in CDM1-Fe)

Table 11. Minimum Lethal Doses of Some Virulent strains of *Klebsiella aerogenes*

Capsular Type	*Dilution	Organisms/ml
K1	1 in 200	1×10^7
K2	1 in 20	1×10^7
K3	4 in 10	8×10^8
K15	4 in 10	8×10^8
K20	1 in 20	1×10^8
K35	5 in 10	1×10^9
K36	1 in 10	2×10^8
K44	1 in 20	1×10^8
K50	8 in 10	1.6×10^9
K63	1 in 20	1×10^8

The dilution is that of a stock suspension consisting of 2×10^9 organisms/ml. MLDs were determined as described in section 2.2.5.1. The *Klebsiella* strains described above were clinical isolates collected by Drs. R.J.Jones and E.A.Roe, MRC Vaccines Research Laboratories, Medical School, University of Birmingham.

Table 12. Protection against Lethal i.p. Infection with *K.aerogenes* Conferred by Vaccination with Culture Filtrate Vaccines (1)

Culture Filtrate Vaccine (Single or Pooled)	K1	K44	K1,K44
Protection Conferred Against (Capsular Type)	1,7,10,13, 14,16,19,26, 29,30,31,33, 42,43,45,73, 77.	16,19,22,27, 30,31,33,42, 43,44,45,47, 51,54,77.	1,4,7,13, 14,15,16,18, 19,21,22,23, 26,27,30,31, 33,34,42,43, 44,45,50,54, 58,60,61,64, 66,72,76,77.
Total Number of Capsular Types Protected Against	17	15	32

Mice were vaccinated as described in 2.2.5.1. with culture filtrate vaccines prepared from K1 or K44 or a pooled K1 and K44 vaccine. Both strains were grown in the Liu medium. They were subsequently challenged with 1MLD of each of 77 *Klebsiella* capsular types. The capsular types and total number of strains which the vaccine(s) protected against were recorded. All control non-vaccinated mice died.

Table 13. Protection against Lethal i.p. Infection with *K.aerogenes* Conferred by Vaccination with Culture Filtrate Vaccines (2)

Mice were vaccinated as described in 2.2.5.1. with pooled culture filtrate vaccines prepared from *Klebsiella* strains grown in either the Liu medium or in CDM1-Fe. They were challenged with 1MLD of each of 77 *Klebsiella* capsular types. The capsular type and the total number of strains protected against were recorded.

All control non-vaccinated mice died.

Culture Filtrate Vaccine (Pooled)	K1,K2,K3,K15, K20,K35,K36, K44,K50,K63.	K1,K2,K20 K35,K44.
Protection Conferred Against (Capsular Type)	1,2,3,4,5, 7,9,10,11, 12,14,15,18, 19,20,22,24, 25,27,28,29, 30,32,33,34, 35,36,38,40, 41,42,44,45, 46,47,48,50, 51,53,54,55, 56,59,60,61, 62,63,64,65, 66,67,75,77.	1,2,4,5,7, 8,9,10,12, 18,19,20,22 23,25,27,28, 30,31,32,33 34,35,37,38, 40,41,44,45, 48,50,51,53, 54,55,56,59, 61,62,64,65, 66,67,69,71, 72,73,76,77.
Failed to Protect Against	70,74.	6,15,16,17, 36,60,63,74, 70
Total Number of Capsular Types Protected Against	54	50

gave protection. Protection against fifty of the fifty nine capsular types tested was achieved using this combination.

7.2. Chemical Composition of the Culture Filtrate Vaccines

To compare the protection against lethal challenge conferred by culture filtrate vaccines prepared from some virulent strains of *K.aerogenes* grown in the medium designed by Liu and described by Carney and Jones (1968), five of these strains were cultured for five days at 37°C in 400mls of CDM1-Fe. The yields of culture filtrate vaccines obtained following extraction and freeze drying are given in table 14.

The culture filtrate vaccines were initially examined by gel filtration in phosphate buffered saline (pH 7.2) on a column of Sepharose 4B (50cm x 2.5cm diameter). Effluent from the column was monitored by refractive index measurements and u.v. light absorption. The refractive index monitoring showed a broad and slightly irregular spread, indicating that the samples were probably polydispersed in molecular weight. Samples applied to the column were noticeably viscous so the ionic strength of the buffer was increased by the addition of sodium chloride in an attempt to overcome the electroviscous effect of charged macromolecules. The broad distribution persisted confirming the polydispersity of the samples. The low u.v. absorption observed suggested that proteins and nucleic acids were present in low

Table 14. Culture Filtrate Vaccines Yields Prepared from *Klebsiella aerogenes* grown in CDM1-Fe

Capsular Type	[1]A_{470}	[2]pH	Yield of Vaccine (mgs)
1	3.92	5.4	130.2
[3]1(+Fe)	4.64	5.1	104.2
2	3.04	6.0	77.2
20	2.72	7.15	135.0
35	2.48	6.6	141.7
44	2.96	6.8	125.0

The initial pH of each culture was pH7.4; 1 and 2 were recorded after 5 days growth; 3 denotes the culture to which 2×10^{-5}M Fe^{2+} had been added.

quantities and the samples consisted mainly of polysaccharide. Hydrolysis and paper chromatography of several culture filtrate vaccines revealed a selection of sugars confirming the presence of polysaccharides (Fig.61). Positive identification of the monosaccharide composition of the culture filtrate polysaccharides was obtained by ion exchange chromatography of the borate-sugar complexes (carried out by the Chemistry Department, University of Birmingham, Edgbaston, Birmingham). The results are given in table 15 . The monosaccharide components of the polysaccharides present in the culture filtrate vaccines derived from *Klebsiella aerogenes* capsular types K1, K20 and K44 are consistent with those reported to be present in the respective capsular polysaccharides (Erbing *et al* 1976; Sutherland,1977; Dutton & Folkman,1980).

Analysis of the gross chemical composition of several culture filtrate vaccines prepared from cells grown in both CDM1-Fe and in the Liu medium revealed that they consist mainly of polysaccharide, LPS and protein (Tables 16, 17 & 18). Nucleic acids have a strong absorption band in the uv with a maximum at 260nm and a minimum at 230nm (in contrast to proteins which absorb maximally at 280nm). An ultraviolet scan between 350nm and 225nm of 1mg/ml solutions in distilled water failed to reveal any absorption maxima. Therefore the culture filtrate vaccines do not contain significant amounts of nucleic acid.

The analyses shown in tables 16,17 and 18 do not account for

Figure 61. Paper Chromatogram showing the Monosaccharide Components Derived from the Acid Hydrolysis of some *Klebsiella* Polysaccharides

Key:

Glu	Glucose
Man	Mannose
Rha	Rhamnose
Gal	Galactose
Fuc	Fucose

Glu
K1
K2
K20
K44
K2
K20
Glu
Glu
Glu
Gal
Glu
Man
Glu
Man
Glu
Gal
Glu
Man
Fuc
Rha

100% by weight of the freeze dried culture filtrate vaccines, a result which to some degree reflects the insensitivity of the assay methods employed. The phenol-sulphuric acid assay used to estimate the total carbohydrate content of each vaccine is based on glucose equivalents and therefore does not account for the presence of substituents such as pyruvate, acetal or ketal groups (Sutherland,1977; Powell,1979) or for the differences in reactivity of the various monosaccharides components. LPS can be estimated by assaying for the presence of KDO in the samples. KDO has been calculated to consist of approximately 1.5% by weight of the LPS of a smooth strain of *K.aerogenes* (Sutherland & Poxton,1976). KDO determination therefore enabled the LPS content of the vaccines to estimated.

The major components of the culture filtrate vaccines, whether prepared from cells cultured in CDM1-Fe or in the Liu medium, were present in similar proportions. Therefore, the use of either medium does not appear to affect the gross chemical composition of the culture filtrate vaccines to any great extent.

Table 15. Principle Monosaccharide Components of some *Klebsiella aerogenes* Capsular Polysaccharides

Strain	Sugar	Mole Proportions
K1 (a)	Fucose, Glucose	0.89 : 1.12
K1 (b)	Fucose, Glucose	1.05 : 1.12
K20	Mannose, Galactose, Glucose	0.72 : 0.67 :0.81
K44	Rhamnose, Glucose	1.33 : 1.68
KX	Mannose, Galactose	0.54 : 2.31
KY	Fucose, Galactose	1.24 : 1.76

Where K1 (a) and K1 (b) were two different strains with the same capsular type, KX was non typable and KY gave muliple cross reactions. Positive identification of the monosaccharides was obtained by ion exchange chromatography of the borate-sugar complexes (carried out by the Chemistry Department, University of Birmingham).

Table 16. Carbohydrate Content of Culture Filtrate Vaccines Prepared from *Klebsiella* Strains Grown in Two Different Media

Capsular type	Mean Carbohydrate Content (%)	
	CDM1-Fe	Liu
K1	40.4±0.2	42.6±1.5
K1(+Fe)	28.8±1.5	-
K2	71.4±3.6	57.5±2.7
K3	-	57.5±2.7
K20	37.9±2.7	61.2±2.5
K35	30.9±1.3	50.7±2.8
K44	60.5±1.6	64.4±7.5

Where K1(+Fe) denotes growth in CDM1 supplemented with 2×10^{-5}M Fe^{2+}

Table 17. Protein Content of Culture Filtrate Vaccines Prepared from *Klebsiella* Strains Grown in Two Different Media

Capsular Type	Mean Protein Content(%) CDM1-Fe	Liu Medium
K1	9.9±1.2	4.7±1.2
K1 (+Fe)	4.0±0.4	-
K2	4.1±1.1	4.3±0.9
K3	-	3.5±0.41
K20	7.9±0.0	1.5±0.3
K35	2.7±0.7	7.0±0.36
K44	5.5±0.5	2.2±0.3

Where K1(+Fe) denotes growth in CDM1 supplemented with 2×10^{-5}M Fe^{2+}

Table 18. KDO Content of Culture Filtrate Vaccines Prepared from *Klebsiella* Strains Grown in Two Different Media

	CDM1-Fe		Liu Medium	
	KDO(%)	LPS(%)	KDO(%)	LPS (%)
Capsular Type				
K1	0.4	26.7	0.21	14.0
K1(+Fe)	0.5	33.0	-	-
K2	0.31	20.7	0.4	26.7
K3	-	-	0.2	13.3
K20	0.23	15.3	0.31	20.7
K35	0.34	22.7	-	-
K44	0.42	28.0	0.44	29.0

Where K1(+Fe) denotes growth in CDM1 supplemented with 2×10^{-5}M Fe^{2+}. The LPS content of the culture filtrate vaccines was calculated on the basis that KDO consists of approximately 1.5% by weight of the LPS of a smooth strain of *Klebsiella* (Poxton & Sutherland, 1976).

7.3. Discussion

Pooled culture filtrate vaccines prepared from several virulent strains of *K.aerogenes* cultured in the Liu medium, protected mice against lethal intraperitoneal challenge by autologous, homologous and heterologous strains. A combination of vaccines from ten *Klebsiella* strains gave good cross protection against fifty four capsular types and partial protection against further twenty types.This partial protection was later discovered to be dose related, complete protection being attained by increasing the dose (personal communication from Dr. E Roe).

The preliminary results presented in this study are encouraging inasmuch that wide cross protection can be achieved using culture filtrate vaccines prepared from relatively few *Klebsiella* capsular types. Bearing in mind the increasing clinical problems associated with drug resistant *Klebsiella* strains (S.Young,1982; Ullman,1983), the development of a broad spectrum anti *Klebsiella* vaccine would provide a possible alternative or complementary form of treatment. As culture filtrate vaccines do not need to be prepared from every *Klebsiella* capsular type, such a vaccine would also be a commercially viable proposition.

Analysis of the gross chemical composition of several culture filtrate vaccines revealed that the major constituents were capsular polysaccharide, LPS and protein.

These vaccines are therefore complex antigenic mixtures, more than one component probably contributing to the overall protection. Further development of this anti *Klebsiella* vaccine for human use, will require extensive toxicological testing as it contains LPS which although a potent immunogen, is extremely toxic at high doses (Braude, Douglas & David, 1973). Capsular polysaccharides are the major culture filtrate vaccine components and antibodies against them will act as opsonins rendering the organism much more susceptible to phagocytosis (Jones, 1981). Many *Klebsiella* capsular polysaccharides cross react with pneumococcal and *E.coli* capsular polysaccharides (Heidelberger & Nimmich,1976; Lee & Fraser, 1981). Treatment with an anti *Klebsiella* vaccine may therefore confer some protection against infection with some strains of *Streptococcus pneumoniae* and *E.coli*.

Culture filtrate vaccines prepared from *Klebsiella* strains grown in CDM1-Fe also protected mice against lethal challenge with a wide variety of *Klebsiella* capsular types. As this CDM has been quantitatively defined for the growth of *K.aerogenes*, its use ought to enable vaccines to be prepared under nutritional conditions which are optimal for the production of protective antigens whilst reducing the levels of any potentially toxic components.

8. CONCLUSIONS

8. Conclusions

The work described in this thesis stresses the importance of bacterial surface components in enabling a micro-organism to evade host defence mechanisms and antibiotics.

The presence of both K and O antigens of *Klebsiella aerogenes* was found necessary to protect the organism from either complement-mediated serum killing or phagocytosis in the absence of specific antisera. Optimal phagocytic ingestion of *K.aerogenes* NCTC 5055 could be achieved in the presence of either anti-K or anti-O sera or to a much smaller extent in antisera raised against a rough unencapsulated mutant (M10B) derived from NCTC 5055. Anti-O sera failed to opsonize a clinical *Klebsiella* isolate (DL1) possessing immunologically cross reacting LPS, but did so when the amount of capsule was physically reduced. The serum sensitivity of the encapsulated strains was unaffected by the addition of specific antisera. Fresh serum was bacteriostatic for an unencapsulated smooth mutant (M10) derived from NCTC 5055. This bacteriostatic effect was reduced by heat-inactivation of the serum or by the addition of anti-O serum. M10 was rendered sensitive to the bactericidal action of serum in the presence of antisera raised against M10B or after chelation with MgEGTA to isolate alternative complement pathway activity. The rough unencapsulated mutant (M10B) was rapidly killed by fresh

serum, an effect which could be delayed by chelation with MgEGTA. The serum sensitivity of M10B was unaffected by the presence of anti-M10B sera. Thus the O antigen, unlike the K antigen, of these *Klebsiella* strains is not antiphagocytic but it does confer some protection against the rapid bactericidal activity of serum complement. However, possession of these surface antigens did not appear to impede the access of a range of hydrophilic and hydrophobic antibiotics to their target sites. Both the parent strain NCTC 5055 and the isogenic mutants, M10 and M10B showed comparable antibiotic sensitivities.

An important attribute of invasive gram-negative pathogens is the ability to multiply in host tissues, an ability which is greatly influenced by the availability of iron (Griffiths, 1983). Restriction of the available body iron can therefore be considered as an important component of the host defences. To study the physiological response of *Klebsiella aerogenes* to iron depletion, a simple salts medium (CDM2-Fe) was designed in which all components except iron were present in a gross excess. The outer membrane protein profile of iron depleted *Klebsiella aerogenes* DL1 revealed at least six new proteins in the M_r 69,000; 70,000; 73,000, 75,000; 78,000 and 83,000. These proteins were also apparent in the OM of DL1 grown in serum. Following growth in CDM2+Fe, in iron saturated serum and in citrate-supplemented CDM2, a protein of M_r 18,500 was present in the OM of stationary phase DL1. This protein was repressed in CDM2-Fe and in serum. Enterochelin but not

aerobactin was detectable in the spent culture supernatants of iron deficient *K.aerogenes* DL1. A study of the effect of growth phase on the appearance of the high affinity iron sequestering mechanisms showed that within two generations in CDM2 containing <17 x 10^{-7}M Fe^{3+}, iron replete DL1 manufactured an OM complete with the IRMP and enterochelin was detected in the supernatant. Enterochelin biosynthesis paralleled the growth cycle and a maximum of 27 μg/ml was detected in the late stationary phase. Therefore, enterochelin and the IRMP were produced several generations before any change in the growth rate was apparent. Inoculation of iron depleted cells into CDM2+Fe resulted in dilution rather than active excretion of the IRMP from the OM, 1.5 generations being required for the initial relative level to decrease by one half and four generations to return to that observed after growth to stationary phase in CDM2+Fe. A low level of IRMP was always just detectable by Coomassie Blue staining of the SDS polyacrylamide gels of the OM of iron replete DL1 grown in CDM2+Fe. The appearance of the IRMP of DL1 was unaffected by the prior growth of the organism in a gross excess of iron (up to 100 μM) which suggested that whether or not DL1 was capable of storing iron, it rapidly responded to the extracellular iron concentration.

These results reflect the marked changes which occur in the bacterial envelope in response to the lack of a particular nutrient. By understanding the effects of phenotypic variation on bacterial cell envelope components, a rational

basis for the development of new immunotherapeutic measures to combat infection can be made. Preliminary experiments showed that culture filtrate vaccines prepared from *Klebsiella* strains grown in CDM1-Fe or in the Liu medium, protected mice against lethal intraperitoneal challenge with homologous and heterologous *Klebsiella* strains. A combination of vaccines from ten *Klebsiella* strains gave good cross protection against seventy four capsular types. Chemical analysis of the vaccines revealed that they consisted mainly of capsular polysaccharide, LPS and to a lesser extent, protein.

The cross protection achieved may be explained, at least in part, by our understanding of the role of the O and K antigens in determining the resisatnce of *Klebsiella aerogenes* to serum killing and phagocytosis. *Klebsiella* species share just eight O antigens (Mizuto *et al*, 1983). The culture filtrate vaccines may therefore consist of those eight O antigens together with K antigens from those strains in which the capsule masks the O antigen. However, the vaccines were prepared from culture supernatants of iron depleted cells and contain proteins as well as LPS and capsular polysaccharide. The vaccines may therefore contain IRMPs, antibodies against which could interfere with the acquisition of iron by *Klebsiella in vivo*. Anti-IRMP antibodies may be particularly effective, since the ability of *Klebsiella* to multiply rapidly in serum appears be an important virulence determinant.

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