ANTIBIOTIC RESISTANCE AND OUTER MEMBRANE PROTEIN ANTIGENS OF *PSEUDOMONAS AERUGINOSA*

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Antibiotic resistance and outer membrane protein antigens of *Pseudomonas aeruginosa*.

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P.aeruginosa and other Gram-negative bacteria were isolated directly from the urine of patients with urinary tract infection. The outer membrane protein (OMP) profiles of the bacteria were investigated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The results indicated that bacteria grew under iron deprived conditions as revealed by the expression of several high molecular weight OMPs which could also be observed when the same isolates were grown under iron-depleted conditions in laboratory media. The antigenicity of outer membrane components was studied by immunoblotting using serum and urine samples from the patients, and hyperimmune rabbit serum. The results indicated that serum and urine contained antibodies against major outer membrane components of the bacteria, including iron regulated membrane proteins. Antibody titres in serum and urine determined by enzyme linked immunabsorbent assay using iron-depleted cultures confirmed the results obtained by immunoblotting. Iron-depleted cultures of *P.aeruginosa* were also found to be more virulent than iron plentiful cultures in a mouse intraperitoneal challenge study.

Antibiotic sensitivities of a number of strains of *P.aeruginosa* from epidemiologically diverse sources were determined by an agar diffusion method. OMP profiles of strains with a wide range of antibiotic sensitivity were investigated. No major differences could be determined between the OMP profiles of the strains investigated. Minor differences in OMP profile did not correlate with differing antibiotic sensitivities. Antisera raised against iron-depleted cultures of different O-serotypes reacted differently with strains of all antibiotic sensitivities but each antisera recognized the major proteins F, H and I of the *P.aeruginosa* outer membrane.

The OMP profile of a trained polymyxin-resistant variant of *P.aeruginosa* showed reductions in the level of proteins D,E,F and H1. This reduction in protein levels was reflected in immunoblots of the outer membrane. Protein H1 is induced in the wild type cell under conditions of magnesium-depletion. H1 induction has been correlated with polymyxin resistance, however, this protein was not induced in the resistant variant in the presence of polymyxin even under conditions of magnesium-depletion. Polymyxin resistance in *P.aeruginosa* is probably multi-factorial and thus not simply the result of protein H1 induction.

Key words: *Pseudomonas aeruginosa*, outer membrane proteins, iron, antibiotic sensitivity, immunoblotting.

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Origin and scope of the work.

Pseudomonas aeruginosa is a potentially pathogenic Gram-negative bacteria which has an inherent resistance to the effect of many antimicrobial agents (Brown, 1975). In addition to this intrinsic resistance an increasing number of clinical isolates of P.aeruginosa are found to be resistant even to those antipseudomonal antibiotics which inhibit normally sensitive strains. The high mortality rate of certain P.aeruginosa infections, even in the face of intensive antibiotic therapy (O'Grady, 1984), has generated an interest in the development of an effective Pseudomonas vaccine (Pennington, 1979), possibly based on somatic components of the cell.

The property of resistance in P.aeruginosa is thought to reside partly in the lack of permeability of the outer membrane to harmful agents and as such may be influenced by the composition of the membrane (Brown, 1975) and by its environment (Brown, 1977). Vaccines based on outer membrane components are currently available but their use is limited by the fact that they contain lipopolysaccharide which itself is toxic and which confers a serotype specificity on the host immune response (Pennington, 1979). A vaccine based on outer membrane protein components common to all strains might avoid the possibility of toxicity and confer protection against all serotypes (Homma, 1982). Consequently, this study was undertaken to investigate the immunogenic properties of proteins in the outer membrane of Pseudomonas aeruginosa.

In the study techniques are described for separating

bacterial outer membrane proteins and for investigating the immunogenicity of the separated proteins. The outer membrane protein profiles of drug-resistant and drug-sensitive clinical isolates of *P. aeruginosa*, and an adaptive variant trained to polymyxin resistance, have been investigated. The reaction of these proteins with antibodies has also been studied to determine differences in immunogenicity between drug-resistant and -sensitive strains.

The outer surface of Gram-negative bacteria is remarkably plastic, it alters with changing environment to adopt a composition characteristic of that environment (Brown & Williams, 1985a, 1985b). Gram-negative bacteria growing *in vivo* in human infections have been shown to grow under conditions of iron-restriction (Anwar *et al*, 1984; Brown *et al*, 1984) and this study confirms these findings. Accordingly, the study has been made with cells cultivated under conditions of iron depletion so as to mimic as closely as possible those growing *in vivo*.

1. INTRODUCTION

1.1 Composition and structure of the Gram-negative cell envelope.

The cell envelope of Gram-negative bacteria is a complex structure that confers shape and rigidity on the cell and which regulates the organisms interaction with its environment. The envelope serves both to mediate the uptake of nutrients and to protect the cytoplasm from the ingress of harmful agents. The conformation of the cell envelope is extremely variable and is characteristic of the particular environment in which the cell is growing (Ellwood & Tempest, 1972; Holme, 1972; Harder & Dijkhuizen, 1983; Brown, 1975,1977; Brown & Williams, 1985b). Since the environment constantly changes so in turn does the cell envelope. The ability to change in this way confers major survival advantages on the cell. The response to antimicrobial agents, whether acting upon the cell envelope or traversing it to a target within the cell may be altered by the composition of the cell envelope (Brown, 1977; Costerton & Clegg, 1975) and by the changes that are bought about in the envelope by the cell's environment (Brown, 1975; Brown & Williams, 1985a). The pathogenic nature of bacteria also depends to a large extent on the characteristics of the cell surface (Smith, 1977; Brown & Williams, 1985b). The surface of pathogenic bacteria mediates adherance to host cells, assists the pathogen to multiply in hostile environments and elaborates mechanisms capable of resisting the host's defence systems (Ogata,1983).

The cell envelope structure of Gram-negative bacteria may be divided structurally into three layers which support different functions; an outer membrane (OM), a peptidoglycan (PG) layer and an

inner cytoplasmic membrane (CM) (Costerton et al 1974; Beveridge, 1981). Outside this structure may be a more or less organised layer of polysaccharide slime.

Rigidity, shape and the ability to withstand osmotic pressure is conferred upon the structure by the PG layer. PG is a macromolecule consisting of a backbone of N-acetylmuramic acid and N-acetylglucosamine molecules. To each molecule of N-acetylmuramic acid is attached a peptide chain. Cross bridges of amino acids connect the tetrapeptides from one molecule of muramic acid to the next (Hammond et al, 1984). It has been proposed that the outer part of the PG molecule is more highly cross-liked than the inner and that the PG molecule is hydrated so that it forms a gel filling the space between the outer and cytoplasmic membranes (Hobot et al 1984). The periplasmic gel contains oligosaccharides involved in regulation of osmolarity (Lugtenberg & van Alphen, 1983) and proteins. There are three classes of proteins (Hammond et al 1984): those with a catabolic function which convert nutrients for which no specific transport system exists into a form which can be transported; binding proteins that have affinity for certain nutrients, and degradative enzymes that inactivate harmful substances such as antibiotics. Periplasmic enzymes, which include beta-lactamases, proteases, acetylases, and phosphatases, play an obvious and important role in antibiotic resistance in Gram-negative organisms (Murray & Moellering, 1978; Costerton et al, 1979; Bryan, 1982). The PG layer in Gram-negative cell walls may be from 0.8-30nm in thickness (Costerton et al 1974) and comprises 10-20% of the dry weight of the cell.

Located inside the PG layer is the cytoplasmic membrane which is

a bilayer composed of phospholipids and containing embedded proteins. These proteins carry out a number of transport and enzymatic functions including electron transport through the cytochrome system, transport of nutrients and metabolites, synthesis and translocation of PG and OM components, and the secretion of extracellular enzymes (Inouye, 1979). The cytoplasmic membrane is joined to the OM at the zones of adhesion, estimated at between 200 and 400 per cell. Adhesion zones are thought to be involved in the export of OM components (Smit & Nikaido, 1978; Bayer, 1979).

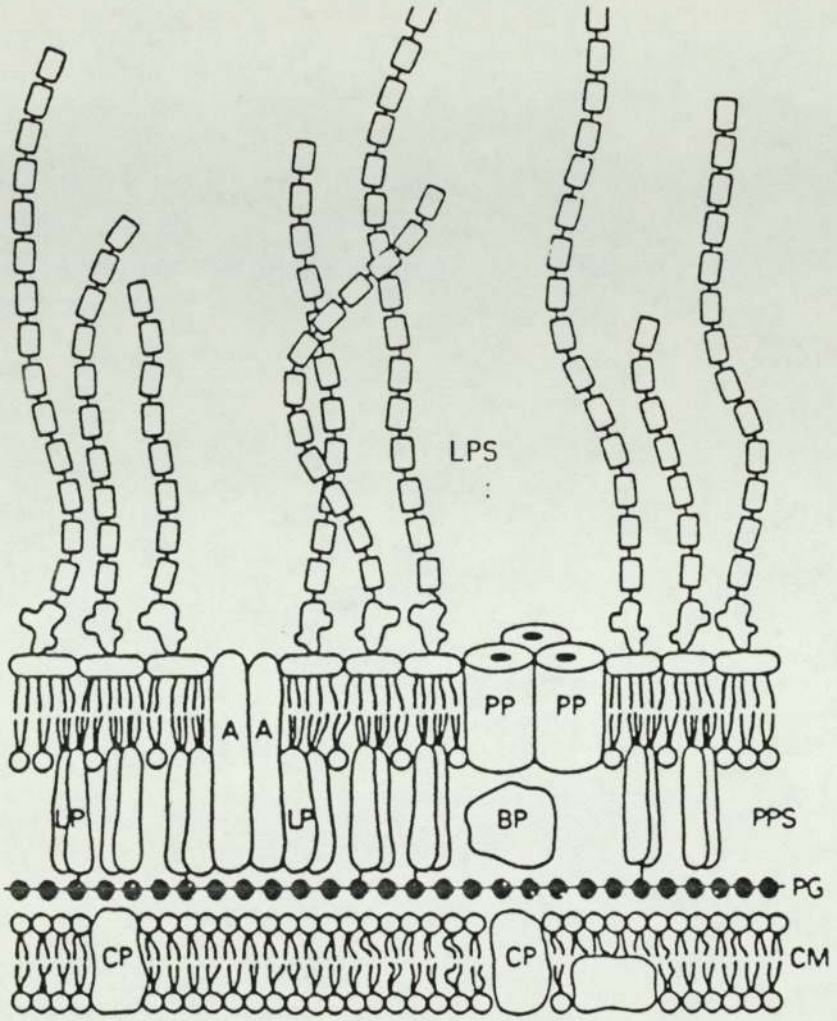
The OM lies outside the PG layer to which it is covalently attached via a lipoprotein (Braun, 1975; Lugtenberg & van Alphen, 1983)(figure 1.1).

1.1.1 Composition of the outer membrane

The outer membrane is a highly specialized bilayer made up of phospholipids, proteins and lipopolysaccharide (LPS)(Inouye, 1979; Nikaido & Nakae,1979; Lugtenberg & van Alphen, 1983) The OM constitutes a physical and functional barrier between the cell and its surroundings. The barrier function is necessarily selective and is accompanied by permeability properties which allow entry of nutrients from the environment. The OM components also function as receptors for bacteriophages and bacteriocins and facilitate cell-cell interactions during conjugation (Osburn & Wu, 1980).

Figure 1.1

Schematic diagram of the cell envelope of Gram-negative bacteria



from Lugtenberg & van Alphen (1983)

Key.

LPS - lipopolysaccharide

A - outer membrane protein, Omp A type

PP - porin protein

LP - lipoprotein

BP - binding protein

PPS - periplasm

PG - peptidoglycan

CP - transport protein

CM - cytoplasmic membrane

1.1.1.1 Phospholipids

The major phospholipids present in the outer and cytoplasmic membranes are phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol (Lugtenberg & van Alphen, 1983; Hammond *et al*, 1984). Phosphatidylethanolamine is usually the predominant species in the OM, possibly because it has the ability to form stable bilayers with LPS (Fried & Rothfield, 1978). The phospholipid composition of the cell wall is liable, however, to vary considerably with growth rate and nutrient limitation (Gilbert & Brown, 1978; Gunter *et al*; Minnikin *et al*, 1974)

1.1.1.2 Lipopolysaccharide (LPS)

LPS is a unique molecule characteristic of the outer surface of Gram-negative bacteria. It is amphipathic and consists of a hydrophobic moiety, lipid A, linked to a hydrophilic sugar chain (Westphal *et al* 1983; Wicken & Knox, 1980). The hydrophilic portion is made up of an oligosaccharide core and a polymer consisting of repeating sugar units and known as the O-antigen (figure 1.2)

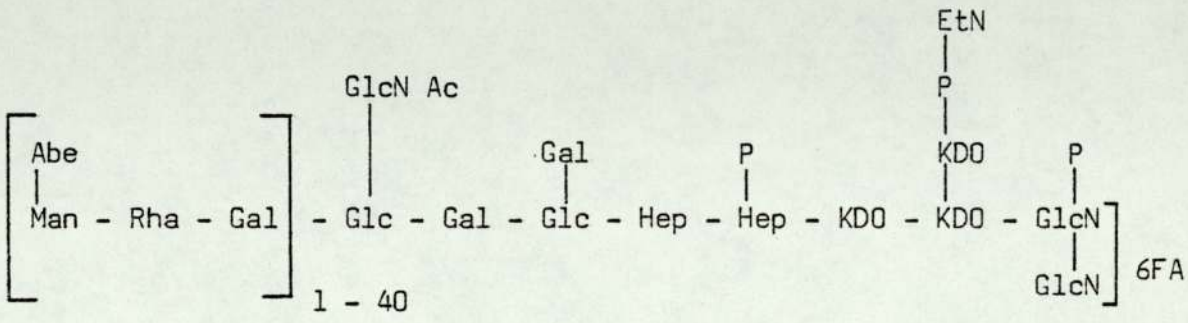
The number of repeating units in the O-antigen can vary even in the culture of one strain (Hitchcock & Brown, 1983; Jann *et al* 1975) and each may contain between 3 and 6 sugars. The outer surface of the bacterial cell is thus subject to considerable variation in its molecular make up. (Chester & Meadow, 1975;Goldman & Leive, 1980; Munford *et al* 1980; Pavla & Makela, 1980; Tsai & Frasch, 1982). The

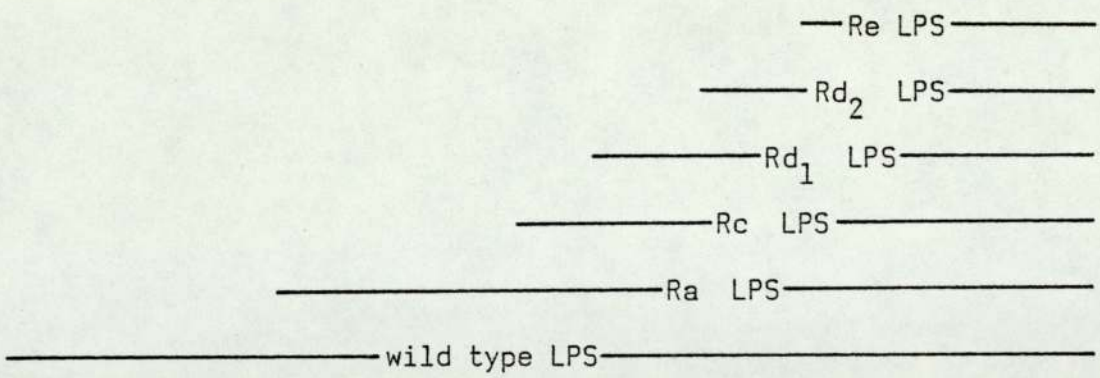
Figure 1.2

Structure of lipopolysaccharide from strains of Salmonella typhimurium (Nikaido & Nakae, 1979)

Key.

Abe	Abequose
Man	D- Mannose
Rha	Rhamnose
Glu	Glucose
Gal	Galactose
GlcN Ac	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 of different degrees of 'roughness'


EtN
P
GlcN Ac
Abe
Gal
P
KDO
P
Man - Rha - Gal - Glc - Gal - Glc - Hep - Hep - KDO - KDO - GlcN
GlcN
6FA
1 - 40


Re LPS
Rd2 LPS
Rd1 LPS
Rc LPS
Ra LPS
wild type LPS

structure of the subunit of the O-antigen is extremely variable and this property is used in immunological typing of substrains (O-serotyping (Bergan, 1975)). The O-specific side chains in Pseudomonas aeruginosa are rich in rhamnose and the amino sugars hexosamine, fucosamine and quivosamine (Wilkinson, 1983)

The morphological 'smooth' to 'rough' mutation co-incides with the disappearance of O-antigen leaving a 'rough' LPS consisting only of core oligosaccharide bound to lipid A (Westphal et al, 1983).

The core polysaccharide has an inner and an outer region. The inner core region contains heptose and the unique sugar 3-deoxy-D-manno-octulosonic acid (KDO) while the outer core contains glucose, galactose and in some cases N-acetyl glucosamine. KDO links the core polysaccharide to lipid A via an acid labile bond (Wicken & Knox, 1980; Wilkinson & Galbraith, 1975) The hydrophobic lipid A region can easily be separated from isolated LPS by mild acid hydrolysis. The core region is generally regarded as being of constant composition for smooth strains of a particular genus (Wilkinson & Galbraith, 1975).

Lipid A regions of LPS from different species of Gram-negative bacteria share a general structural similarity in which there are phosphatidyl glucosamine residues forming a disaccharide to which are attached fatty acids by ester and amide bonds (Westphal et al 1983). This structure is relatively invariable and is conserved in a wide range of Gram-negative bacteria (Matsby-Baltzer et al,1984). The absence from lipid A of 3-hydroxytetradecanoic acid and the presence of 2-hydroxydodecanoic acid, 3-hydroxydecanoic acid and 3-hydroxydodecanoic acid are characteristic of Pseudomonas aeruginosa (Wilkinson & Galbraith, 1975). The lipid A moiety of LPS is responsible for many of the endotoxic properties of LPS (Wicken & Knox, 1980).

Pseudomonas aeruginosa core LPS is highly phosphorylated (Wilkinson & Galbraith, 1975). Phosphate groups in the KDO-lipid A region of the LPS have a high affinity for divalent cations (Mg^{++} and Ca^{++}) and, by the formation of cross bridges, these are assumed to be important in the structural organisation of the OM. Chelation of divalent cations by ethylene diamine tetraacetic (EDTA) removes stabilizing cross-bridges which explains the characteristic, and unusual, sensitivity of Pseudomonas aeruginosa to EDTA (Wilkinson & Galbraith, 1975; Wilkinson, 1983).

1.1.1.3 Outer membrane proteins (OMP)

The outer membrane is poor in enzymic activity and identification of protein components has depended upon techniques of separation using gel electrophoresis (Hames & Richmond, 1980). A comparison of CM and OM preparations by sodium dodecyl

sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) reveals relatively few, but often heavy, bands of protein in the OM (Lugtenberg et al, 1975). The OM of Escherichia coli K-12 which has been well characterized (Lugtenberg & van Alphen, 1983) contains three major classes of proteins: the pore proteins (porins), OmpA protein and lipoprotein. These proteins facilitate the transport and diffusion of nutrients from the environment, stabilise the structure (Nogami & Mizushima, 1983) and anchor the OM to the PG layer (Nikaido & Nakae, 1979; Lugtenberg & van Alphen, 1983).

Porins are a group of transmembrane proteins that form hydrophilic pores which allow passive non-specific diffusion of solutes across the membrane (Nakae, 1976; Nikaido, 1979; Hancock, 1984; Nikaido & Vaara, 1985). There are approximately 1.5×10^5 molecules of porin protein per cell (Stephen et al 1977). Porin proteins share a number of unusual physical properties relevant to their structure and functional duties. They are characterized by their tight but not covalent association with the PG layer so that they remain attached to PG when cells are extracted with SDS at non-denaturing temperatures. (Osborn & Wu, 1980). Porin proteins are resistant to denaturing by SDS at temperatures below 85-100°C, resistant to proteolysis, have a high content of β-structure and exibit extremely strong self-association. In their biologically active form they exist as trimers associated with LPS (Osborn & Wu, 1980). It is not clear whether each porin molecule contains a water-filled channel or if the channel is formed between the three molecules of the trimer. Electron microscopic studies show that each trimer contains a triplet of holes (Dorset et al, 1983) and that the 3 channels so formed fused in the middle of the membrane to exit inside the membrane

as a single, central channel (Dorset et al,1984). If this molecular arrangement can be confirmed it would explain the tight association of three subunits that is neccessary to form a functional channel (Nikaido & Vaara, 1985).

The Escherichia coli K-12 OM contains two PG associated porins known as OmpC (M_r 36K) and OmpF (M_r 37K) after the genes which code for them. Other porin proteins can be induced by growth conditions (Hancock & Carey, 1980; Overbeeke & Lugtenberg, 1980; Nicas & Hancock, 1980) or coded for by a plasmid (Iyer, 1979).

Diffusion studies in the enteric bacteria indicate that oligosaccharides of M_r 600-700 are the largest molecules which can pass through the porin channel. This size limit corresponds to a pore diameter of about 1nm (Nikaido, 1979). The figure agrees well with pore diameters of 0.9-1.4nm calculated from conductivity through black lipid films (Nikaido, 1979; Schindler & Rosenbusch, 1978).

The diffusion rate of solutes through the pores is determined by the difference in concentration at the two sides of the membrane and by factors such as molecular size, charge and hydrophobicity (Nikaido, 1979). The relevance of these factors to antibiotic sensitivity is discussed in section 1.2.

The OmpA protein of Escherichia coli is also present in large numbers in the OM, approximately 10^5 copies per cell. The OmpA protein is heat modifiable, that is its molecular weight is higher (35K) after denaturing by heating with SDS than in the denatured form (28K) (Schnaitman, 1974). This implies that a conformational change has taken place in the molecule and not a degradation.The function of the OmpA protein is not completely understood, it is required for F-pilus-mediated conjugation and acts as a receptor for phages (Osborn

& Wu, 1980). The OmpA protein may be involved in maintaining the shape and structural integrity of the cell (Lugtenberg & van Alphen, 1983).

Lipoprotein is an unusual polypeptide that is lipid substituted at the N-terminus and is covalently bound at the C-terminus to PG. The lipoprotein was first reported by Braun & Rehn (1969). The bound form of the molecule is present in approximately 2.4×10^5 copies (Braun, 1975). However, twice as many copies have been found to exist in a free form. (Inouye et al 1972). The total of free and bound lipoprotein makes this the most abundant protein in the cell (Inouye, 1979). Lipoproteins have been found in all the Enterobacteriaceae studied and in Pseudomonas aeruginosa (Lugtenberg & van Alphen, 1983). The exact function of the lipoprotein is unknown but it is thought to play an important role in maintaining the integrity of the OM (Inouye, 1979). In mutants where both OmpA and lipoprotein are missing, cells are unable to grow in the rod form and the PG layer is no longer connected to the OM (Sonntag et al, 1978).

A number of minor proteins are also present in the OM. Some of these proteins function as receptors for phages and colicins (Osborn & Wu, 1980) or as specific transport proteins. An example is the M_r 60K OMP of Escherichia coli which facilitates translocation of the large molecule vitamin B12 across the OM. Some minor proteins may be induced in particular growth environments: the bacteriophage lambda protein of Escherichia coli is induced in the presence of maltose and becomes a major protein comparable in abundance with the pore proteins (Braun & Kreiger-Brauer, 1977).

When many Gram-negative bacteria are grown under conditions of iron restriction the synthesis of a number of high molecular weight

proteins is de-repressed (Neilands, 1974) and they may be present in quantities equal to those of the major OMPs (Griffiths et al, 1983). the regulation and function of these iron regulated membrane proteins (IRMPs) is discussed in section 1.4.

1.1.2 Molecular organization of the outer membrane

The Gram-negative OM is composed of LPS, protein and phospholipid. Freeze fracture studies of the OM show that it can be cleaved into two halves, suggesting a bilayer structure. Freeze-fracture of the OM of Escherichia coli (van Gool & Naninga, 1971) and Salmonella typhymurium (Smit et al 1975) show that the concave, or outer, fracture face is densely filled with particles while the convex, inner, face contains pits complementary to the particles. The major constituents of the particles were shown to be proteins (Smit et al, 1975). In Pseudomonas aeruginosa similar particles seen in the concave fracture appear to be protein-LPS complexes since EDTA treatment which releases protein-LPS complexes from the whole cell (Rogers et al, 1969) results in a disappearance of the majority of the particles (Gilleland et al, 1973; Gilleland, 1977).

Studies with ferritin labelled antibodies to the O-antigen of LPS (Muhlradt & Gelecki, 1975) show that LPS molecules are distributed only on the outer leaflet of the lipid bilayer of the OM. A substantial proportion of the LPS molecules in the OM form tight complexes with the OM proteins (van Alphen et al, 1978), the biological activity of which is often dependent on the presence of LPS (Lugtenberg & van Alphen, 1983). The formation of complexes between OMP and LPS has been shown to require divalent cations, notably

magnesium (Nakamura & Mizushima, 1975; van Alphen et al, 1978). The release of LPS from Escherichia coli (Leive, 1974) and LPS-protein complexes from Pseudomonas aeruginosa (Rogers et al ,1969) by treatment with the chelating agent EDTA emphasizes the role of divalent cations in maintaining the structural integrity of the OM. The cation content of the cell wall varies with growth conditions (Kenward et al, 1979). Alteration in the divalent cation content of simple salts media causes variation in a number of other cell wall components as well as cell wall cation content (Kenward et al, 1979). Divalent cation content in the media also alters the sensitivity of calls to EDTA and to polymyxin (Boggis et al, 1979)

The phospholipids of wild type strains of enteric bacteria are located largely on the inner surfaces of the OM where they are inaccesible to the activity of detergents and bile salts (Nikaido, 1979). Evidence provided by electron spin resonance studies confirm that phospholipids and LPS are segregated into separate domains (Nikaido et al 1977). It is possible that relegation of phospholipids to the inner face of the OM is an adaptation of enteric bacteria to prevent diffusion of bile salts in the highly specialized environment of the gut (Nikaido, 1979). Freeze-fracture electron microscopy of Pseudomonas aeruginosa revealed large featureless patches on the concave fracture face (Gilleland et al 1973) suggesting patches of symetrical phospholipid bilayer. OM proteins in the complex bilayer have sites exposed at the outer surface (Kamio & Nikaido, 1977; Swanson,1981; Lambert & Booth, 1982) which is consistent with their function as transmembrane pores and as receptors for phages, bacteriocins and nutrients requiring special pathways of uptake.

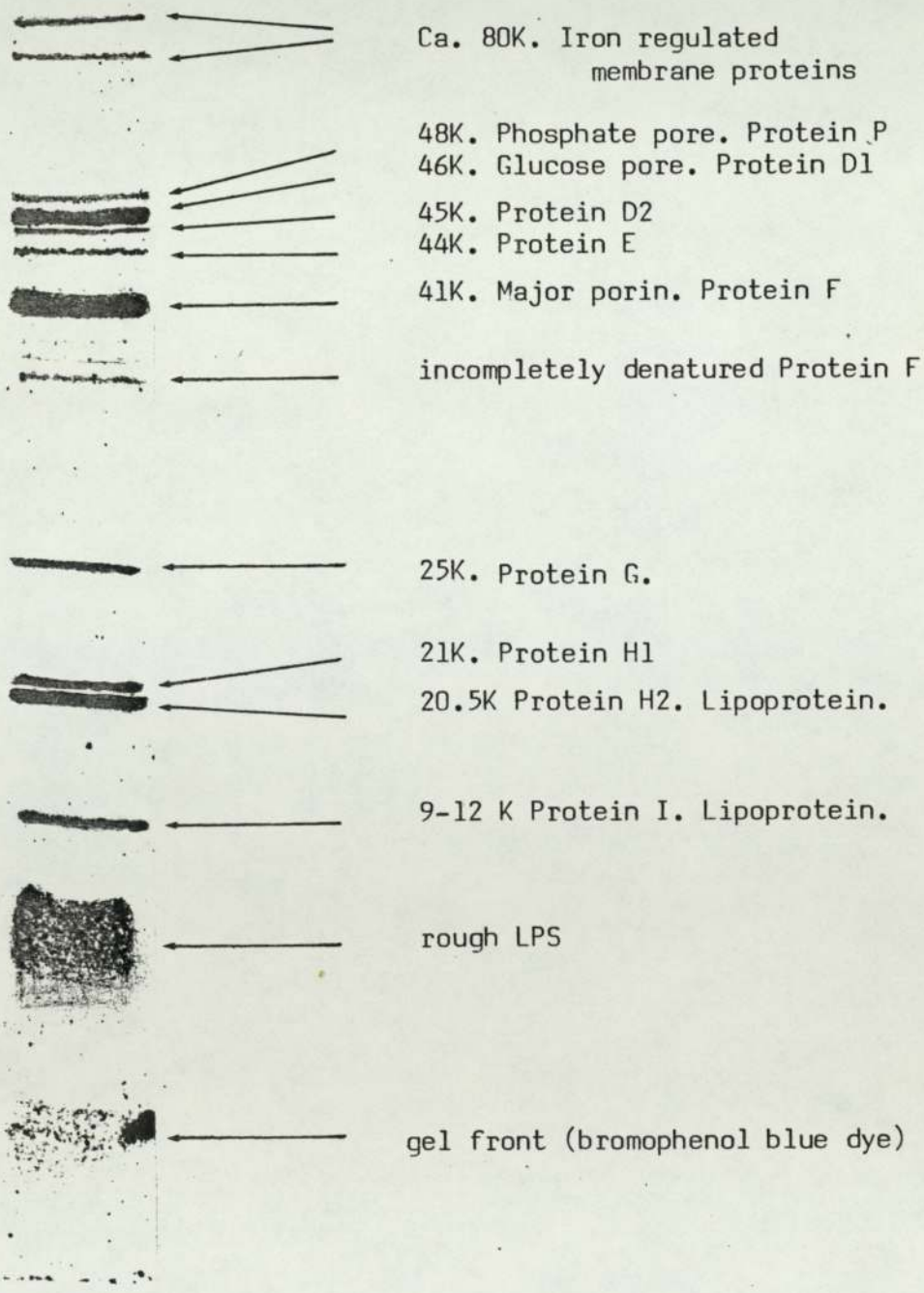
1.1.3 OMPs of *P. aeruginosa*

The presence of several major protein bands was noted when the protein composition of the OM of *P. aeruginosa* was first investigated in the early seventies (Stinnett & Eagon, 1973; Sadoff & Artenstein, 1974). At that time, however, work was concentrated on the enteric bacteria, possibly as a result of difficulties experienced in separating the outer and cytoplasmic membranes of *P. aeruginosa* . These difficulties were caused by the hypersensitivity of *P.aeruginosa* to EDTA (Matshushita *et al*, 1978), a reagent which is commonly used in the preparation of the OM of Enterobacteriaceae (Lugtenberg & van Alphen, 1983). In addition, the information already accumulated about the characteristics of *E.coli*, in particular knowledge of its genetics, may have made this a more attractive candidate for study.

More recent investigations of the OM of *P. aeruginosa* have enabled a number of proteins to be identified and characterized (Hancock and Nikaido, 1978 Matshushida *et al*, 1978; Mizuno and Kageyama, 1978). The OMPs of *P.aeruginosa* were initially given different systems of nomenclature but the system described Mizuno and Kageyama (1978, 1979a, 1979b) has been generally adopted in the literature (Anwar *et al*, 1983b). Hancock and Carey (1979) have described conditions for the separation of at least 8 major OM proteins. *P. aeruginosa*.

Figure 1.3

Pattern of OMP of P.aeruginosa produced by electrophoresis on 15% acrylamide gels after denaturing at 100°C in pure SDS and mercaptoethanol. The M_r and function of the proteins are shown, where known.



OMPs separated according to the method of Hancock & Carey (1979) are shown in figure 1.3, which also lists known functions of the major proteins. Of the 8 major proteins 5 are heat modifiable, three are non-covalently bound to the underlying peptidoglycan (peptidoglycan associated proteins) and three are in some degree induced when P.aeruginosa is grown with glucose as carbon source.

Proteins D1, D2, F, G and H1 are described as heat modifiable because their electrophoretic mobilities on SDS-PAGE are affected by the temperature used in the process of denaturation (Hancock & Carey, 1979). Proteins D1, D2, G and H1 gave similar results to those obtained with the OmpA protein of E.coli K12 (Schnaitman, 1974) in that heating of the proteins between 70 -100°C in SDS caused a decrease in the mobility of the proteins on SDS-PAGE, that is, an increase in their apparent M_r. Heat modifiability implies a re-arrangement within the protein molecule under denaturing conditions so that M_r appears to be increased, this is not a process of degradation which would reduce apparent M_r. When LPS was added to the heat modified forms of D1,D2, G and H1 the modification was reversed to the same extent as with similar treatment of heat modified OmpA protein. This led to the conclusion that these four polypeptides belonged to the same class of heat modifiable proteins as OmpA (Hancock & Carey, 1979).

The major porin protein of P.aeruginosa has been identified as protein F. (Hancock & Nikaido, 1978). Like the OmpF and OmpC proteins it has a M_r of approximately 35-37K. It is also heat modifiable (Mizuno & Kageyama, 1978, 1979a; Hancock & Carey, 1979) but does not belong to the same group of heat modifiable proteins as do D1, D2, G

and H. Protein F requires long periods of boiling in SDS to effect the change from the unmodified to the heat modified form (Hancock & Carey, 1979). It has been shown that this protein is rich in β sheet structure (Mizuno & Kageyama, 1979b) as are the matrix proteins of E.coli and S.typhimurium, which may explain the mechanism by which the heat modification occurs (Nakamura & Mizushima, 1976). As in the enteric bacteria, the porin protein of P. aeruginosa ,protein F, has been found to be non-covalenty bound to the underlying peptidoglycan (Mizuno & Kageyama ,1979b; Hancock et al, 1981; Yoshimura et al, 1983).

Protein I and protein H2 are also associated with the PG (Hancock et al, 1981a). The M_rs of proteins H1 and H2 are very similar, in earlier studies proteins H1 and H2 were not separated and were identified on SDS-PAGE as one band labelled H (Mizuno & Kageyama, 1979b). Protein H has been identified as a novel lipoprotein with a counterpart of similar amino acid composition and M_r in E.coli (Mizuno 1979). It is probable that the lipoprotein identified initially as protein H is in fact protein H2 as this, unlike protein H1, was PG-associated (Hancock et al 1981a). Protein H1 becomes a major OMP only when the organism is grown in Mg^{++} deficient media (Nicas & Hancock 1980, 1983a). A linear reciprocal relationship has been found between Mg^{++} levels in the cell envelope and the amount of protein H1 present (Nicas & Hancock, 1980)

Protein I in P. aeruginosa exists in both the free and bound form (Mizuno & Kageyama 1979a; Hancock et al, 1981). It is rich in α helical structure (Mizuno & Kageyama 1979b) and is analogous to the Brauns lipoprotein of E. coli (Mizuno & Kageyama, 1979a). Proteins D1 and D2 are of similar M_r and because of this have not always been identified as separate polypeptides (Mizuno & Kageyama 1979b, Hancock

Carey 1979). Both are strongly induced in cells grown in media containing glucose as the sole carbon source (Hancock & Carey 1979). Protein D1 is not observed in cells grown under nutritionally rich conditions, whereas D2 is present at low levels. The glucose inducible protein D1 functions as a pore (Hancock & Carey, 1980).

Protein E is also slightly induced after growth of the organism on glucose, although it is generally present in the OM irrespective of growth conditions. Proteins D1, D2, E, G and H1 are released as a complex with LPS when P. aeruginosa is treated with EDTA/Tris (Hedstrom et al, 1981). Proteins F and D have been shown to be exposed at the surface of the cell (Lambert & Booth, 1982).

A 48K protein induced in conditions of phospate depletion forms an anion selective channel when isolated and incorporated in artificial lipid bilayer membranes (Benz et al, 1983). This protein, named protein P, has been proposed to be a porin involved in a phosphate uptake system (Benz et al, 1984).

As in the enteric bacteria several high M_r OMP are induced when P.aeruginosa is grown in iron deficient conditions. These proteins have been reported as having M_rs between 70-80K (Mizuno & Kageyama,1978; Meyer et al, 1979; Ohkawa et al, 1980). The regulation and function of these proteins is discussed in section 1.4.

1.1.4 Flagella and pili of P. aeruginosa

Flagella and pili are protein structures anchored in the OM. Flagella are threadlike structures which propel the bacteria by

rotation. Each flagellum is made up of a number of repeating polypeptide units called flagellin (Kobayashi et al, 1959). The isolated and purified flagella of P. aeruginosa has a M_r of approximately 53K (Montie & Stover, 1983). Pili are shorter and straighter than flagella and are usually found at the poles of the cell. They consist of a single polypeptide unit of M_r approximately 18K (Frost & Paranchych, 1977; Paranchych et al, 1979). Pili serve as attachment sites for bacteriophages (Bradley & Pitt, 1974) and have been shown to bring the phage into contact with the cell surface by retracting into the cell (Bradley, 1978). Bacteria possessing pili adhere to each other and to host tissue. Both are implicated in virulence of P.aeruginosa (Cheng et al, 1981; Holder et al, 1982; Holder & Naglick, 1984).

1.1.5 Surface exopolysaccharide of P. aeruginosa

Most strains of P. aeruginosa secrete a high M_r polysaccharide slime distinct from the polysaccharides of LPS (Sensakovic & Bartell, 1974). Mucoid strains produce a slime which differs from the exopolysaccharides of other Gram-negative bacteria in that the heterosaccharide appears to lack a repeating unit (Jann & Jann, 1983). Exopolysaccharide of mucoid P.aeruginosa is a co-polymer of β mannronic acid and α -L-guluronic acid, and closely resembles the alginates produced by marine algae (Evans & Linker, 1973). Acid hydrolysis shows that the polysaccharide contains some homogenous sequences of either poly-mannuronic acid or poly-guluronic acid interspersed with sequences containing both polymers. Alginate synthesis is very expensive in energy terms (Jarman & Pace, 1984) which suggests that a clear survival advantage is gained by the

organism from alginate synthesis in those environments where the mucoid state predominates. The change to the mucoid state is under genetic control (Fyfe & Govan, 1984; Dazins & Chakrabarty, 1984) and may be coded by a phage (Martin, 1973; Miller & Rubero, 1984). Alginate production is unstable and mucoid organisms revert after culture *in vitro*. (Govan & Fyfe, 1978). Mucoid strains appear to be more stable under conditions of iron limitation (Boyce & Miller, 1982; Ombaka *et al*, 1983) and iron restriction may be a selective pressure *in vivo*.

1.2 Role of the outer membrane in antibiotic resistance

Bacteria resist the action of antibiotic agents in a variety of ways, which may be grouped into three basic processes: elaboration of enzymes that inactivate the antibiotic, modification of the target site to make it resistant to the antibiotic, or prevention of access of the antibiotic to the target site. (Gale et al, 1972; Richmond, 1975; Lambert, 1983;)

With the exception of membrane active agents (Lambert, 1978) and of the β lactam antibiotics, whose targets are the penicillin binding proteins PBPs) located in the CM (Spratt, 1983), the target site of the majority of antibiotics are intracellular. To reach the CM and cytoplasm antibiotic molecules must cross the OM and the periplasm, which contains inactivating enzymes (Nordstom & Sykes, 1974). The unique structure of the OM functions as a permeability barrier which prevents the penetration of many antimicrobial agents to the CM and cytoplasm while permitting the uptake and transport of nutrients.

1.2.1 Diffusion of hydrophobic antibiotics across the OM

Hydrophobic compounds are able to pass through lipid bilayer membranes by a process of diffusion. The uptake of a wide range of antimicrobial substances has been investigated with a series of mutants of S.typhimurium differing only in the amount of polysaccharide in the core and O-antigen region of LPS (Nikaido ,1976) (figure 1.2). Wild type strains of E.coli and S.typhimurium are naturally sensitive to a number of low molecular weight hydrophilic

compounds such as neomycin, cycloserine, ampicillin and cephalothin and sensitivity to these antibiotics is not much affected by alterations in LPS structure. The same strains are much more resistant to hydrophobic antibiotics such as actinomycin D, erythromycin, novobiocin and rifampicin. However, the sensitivity to these agents increases considerably in deep rough mutants (figure 1.2). In addition removal of LPS from wild type S.typhimurium or E.coli by treatment with EDTA (Leive, 1974) again renders them sensitive to hydrophobic antibiotics in this case novobiocin and rifampicin (Nikaido ,1979).

The conclusion reached by Nikaido (1979) was that there are two pathways by which agents can cross the OM: a hydrophilic pathway involving the water filled porin channels, and a hydrophobic pathway involving diffusion across the OM bilayer. The more hydrophobic the compound the more readily will it diffuse across the membrane. Among molecules of similar hydrophobicity smaller molecules penetrate more rapidly. The porin mediated pathway for small hydrophilic molecules is available in both rough and smooth strains. The hydrophobic pathway is less available in wild type smooth strains where O side chains of LPS prevent access of the hydrophobic molecules of the cell surface and where there are fewer hydrophobic phospholipid sites on the cell surface to interact with the hydrophobic molecules. Only in the deep rough strains can the hydrophobic molecules approach the OM, bind, and cross by diffusion. It has been proposed that loss of LPS from smooth strains results in re-orientation of the OM constituents so that phospholipids are present on the outer leaflet of the envelope (Smit et al, 1975). The significance of these changes in composition on the outer surface of rough strains has, however, been questioned (Shales & Chopra, 1982). These workers repeating the procedure of Smit et al (1975) found that differences in phospholipid levels in the outer

leaflet of smooth and deep rough strains of E. coli were small and not statistically significant. Possibly very small changes in phospholipid conformation in the bilayer are sufficient to account for enhanced passage of hydrophobic antibiotics across the membrane.

Some antibiotics do not fit in well with the scheme devised by Nikaido. Chloramphenicol and tetracycline (Sawai, 1983) are both hydrophobic but are active against wild type Gram-negative bacteria.

1.2.2 Diffusion of hydrophilic antibiotics across the OM

The majority of antibiotics used to treat Gram-negative infections are small hydrophilic molecules. Hydrophilic compounds pass across the OM by way of the water filled porin channels (Nikaido & Nakae, 1979; Nikaido, 1979). The pores only allow passage of hydrophilic molecules up to a certain size. The functional size of porins for β lactams have been established with intact cells by measuring the rate of hydrolysis of a chromagenic cephalosporin, nitrocefin, as it diffuses across the OM (Zimmermann & Rosselet, 1977; Nikaido et al, 1983). For other molecules the functional size of porins has been studied with reconstituted phospholipid vesicles containing porin protein in the bilayers (Nakae ,1976a; Nikaido & Rosenburg, 1983). Small hydrophilic molecules diffuse rapidly through the pores, diffusion reduces with increasing hydrophobicity and with negative charge. In enteric bacteria molecules above M_r 600-700 are excluded (Decad & Nikaido, 1976) although hydrophobic compounds below this size are also excluded, possibly because water in the pores is highly structured through hydrogen bonding to ionic groups within the channel (Lambert, 1983). Little is known about factors which may influence the functional state of the porins.

Alterations in antibiotic resistance in porin deficient mutants have been reported. Resistant mutants of E.coli K12 selected for resistance to carbenicillin were found to be deficient in OmpF and overproducing OmpC. The MIC of the basic β lactams carbenicillin, ticarcillin and sulbenicillin were increased up to 16-fold, but the MIC of the zwitterionic compounds cephaloridine and ampicillin were practically unchanged (Harder et al, 1981). Selection for cefoxitin resistance yielded mutants in ompB, a regulatory locus for proteins OmpF and OmpC, and mutants in ompF of E.coli K12. Both types of mutants had a decreased susceptibility for almost the whole spectrum of β lactam antibiotics including the third generation cephalosporins cefotaxime and moxalactam. Both types of mutant remained susceptible to imipenem (N-formimidoyl thienamycin). The mutants in which only the OmpF porin were absent remained susceptible to cefazolin and cephaloridine (Jaffe et al, 1982). Similar mutants were selected using ampicillin and a monobactam, aztreonam, but not with imipenem or mecillinam (Jaffe et al, 1983).

Mutants of E.coli , Proteus mirabilis and Enterobacter cloacae with reduced levels of 39-40K OMP had decreased susceptibility to the cephalosporins cefazolin, cephaloridine and cefoxitin, but not to the penicillins benzylpenicillin, ampicillin, carbenicillin and piperacillin (Sawai et al, 1982). All these antibiotics are hydrophilic and small enough to pass through the porin channels according to the criteria of Nikaido (1979). Sawai et al suggest that ampicillin might be capable of crossing the lipid bilayer of the OM by passive diffusion (Sawai et al, 1982, 1983). However, it is possible that even the reduced levels of porin protein in these mutants was sufficient to allow diffusion of antibiotic.

1.2.3. Resistance to aminoglycoside antibiotics.

The uptake of the polycationic aminoglycoside antibiotics is a complex process thought to involve three distinct processes. The initial rapid phase involves binding to LPS by displacement of divalent cations. The second and third proposed stages involve energy dependant active uptake across the CM (Bryan & Van Den Elzen, 1977b; Hancock, 1981; Hancock et al, 1981). Mechanisms of aminoglycoside resistance in Gram-negative bacteria may involve plasmid mediated inactivating enzymes (Bryan, 1982; Shannon & Phillips, 1982) or reduced binding and uptake of drug. Many strains of P.aeruginosa which do not produce drug-inactivating enzymes are resistant to aminoglycosides (Bryan & Van Den Elzen, 1977a; McNeill et al, 1984). Resistance in these cases is thought to be due to reduced binding and uptake . Changes in LPS repeating side chain sugar units, which possibly reduce binding of aminoglycoside molecules, have been correlated with resistance to aminoglycosides (Bryan et al, 1984; Day, 1980). Changes such as these may be selected for by repeated exposure to low levels of these agents in, for instance, cystic fibrosis lung infection (McNeill et al, 1984)

Aminoglycoside antibiotics including gentamicin, kanamycin and streptomycin pass rapidly through porin pores in reconstituted vesicles (Nakae & Nakae, 1982). The aminoglycoside antibiotics diffuse as rapidly as small sugars despite the fact that they have molecular weights close to or higher than the exclusion limits of the pores. The susceptibility of mutant strains producing only 3-4% of normal porin levels was not significantly different from that of wild type strains. It was concluded that the aminoglycosides diffuse very efficiently

through porin pores and that diffusion is not a rate limiting step in the action of aminoglycoside antibiotics (Nakae & Nakae, 1982). If this were also the case in intact cells *in vivo* resistance due to altered porin levels would be more likely to be encountered with β lactam antibiotics than with aminoglycosides (Lambert, 1983).

1.2.4 Plasmid mediated changes in OM permeability

The production of enzymes which inactivate antibiotics is a formidable obstacle in the chemotherapy of infectious diseases (Bryan, 1982; Lambert, 1983; Guiney, 1984). Most clinically significant antibiotic resistance is determined by genes located on transferable extrachromasomal DNA elements called plasmids or R-factors (Foster, 1983; Murray & Moellering, 1978). Resistance genes may also be carried on discrete genetic units called transposons (Murray & Moellering, 1978; Nugent *et al*, 1979; Schmidt *et al*, 1983).

A plasmid mediated tetracycline resistance reported in *E.coli* involved an inducible decrease in uptake of the antibiotic (Franklin, 1967). The report of a new protein in membranes of *E.coli* containing a plasmid mediating resistance to tetracyline (Levy & McMurray, 1974) led to the suggestion that plasmid-mediated resistance involves synthesis of a protein which alters permeability. Transferable resistance to chloramphenicol in *E.coli* (Nagai & Mitsuhashi, 1972) and *P.aeruginosa* (Mitsuhashi *et al*, 1975) has been reported in strains which were unable to inactivate this antibiotic and there have also been reports of plasmid-mediated sulphonamide resistance due to decreased permeability (Mitsuhashi, 1977). The suggestion that the RP1 plasmid specifying β lactamase production in *E.coli* also specifies a decrease in OM permeability has been proposed to explain high levels

of resistance to carbenicillin in E.coli (Curtis & Richmond, 1974; Yamamoto & Yokata, 1977).

In general, the evidence for plasmids specifying resistance due to alterations in envelope permeability is based on a lack of evidence which would explain the resistance as enzyme inactivation or alteration of target site. In those cases where low permeability has been measured by uptake of radio-labelled antibiotic (Mitsuhashi et al, 1975; Mitsuhashi, 1977) the reduction in uptake has been at a much lower level than the increase in resistance. Reduced uptake may, however, be a factor in resistance specified by a plasmid.

1.2.5 Role of the OM in resistance of P.aeruginosa

P.aeruginosa is intrinsically resistant to a wide range of antimicrobial agents and this resistance has been attributed to the low permeability of the OM (Brown, 1975; Bryan, 1982). The exclusion limit of P.aeruginosa pores has been reported to be much higher than those in enteric bacteria (Nikaido, 1979). Exclusion limits of M$_r$ 4000-6000 have been reported from determinations in reconstituted liposomes (Hancock & Nikaido, 1978; Hancock et al, 1979) and by measurements of electrical conductivity across lipid films containing porin molecules (Benz & Hancock, 1981). A number of factors may, however, affect the properties of pore-forming proteins in the extraction procedure. The functions of the pores may also be affected by the conditions under which the cells were originally grown. Thus determinations made in reconstituted liposomes and lipid films alone are not convincing and the results may well be artefacts. An assay comparing permeability to a series of labelled oligosaccharide molecules in intact cells found an exclusion limit of about M$_r$ 360 for

P.aeruginosa (Caulcott et al, 1984). The method used in this study was a measure of efflux (Decad & Nikaido, 1976) which assumes that molecules can diffuse freely in and out of cells through the pores. This may not be the case since the oligosaccharides may interact with macromolecules present in the periplasm and not be able to diffuse freely out. Further work, probably using a method based on that of Zimmerman & Rosselet (1977), is needed to clarify the question of pore size in P.aeruginosa. A large pore size may be advantageous to the growing bacteria in that it would permit entry of large nutrient molecules, however, large pores would also allow entry of molecules harmful to the cell. The resistance to low molecular weight hydrophilic antibiotics is problematic if the pores are large. To explain this apparent paradox it has been hypothesized that only a small proportion of the available porin molecules form open functioning channels (Angus et al, 1982; Nicas & Hancock, 1983b).

All strains of P.aeruginosa produce a chromasomally mediated inducible β-lactamase (Nordstrom & Sykes, 1974; Livermore, 1982). This enzyme is capable of hydrolysing the β-lactam antibiotics developed before the mid-1960s such as penicillin G and ampicillin. Carbenicillin and later antipseudomonal acylureidopencillins, cephalosporins, thienamycins and monobactams are stable to this lactamase. Plasmid-mediated β-lactamases in an increasing number of isolates are capable of hydrolysing these newer β-lactams and conferring resistance (Lowbury et al, 1969; Livermore, 1982; Dalhoff & Cullmen, 1984). However chromosomal or plasmid encoded β-lactamases account for only a minority of isolates of P.aeruginosa resistant to B-lactam antibiotics (Williams et al, 1984). Resistance in the majority of strains has been termed 'intrinsic' (Richmond, 1975) and

may involve modification of target PBPs (Rodriquez-Tebar et al, 1982) or an impermeability barrier (Brown, 1975).

Evidence for resistance due to a permeability barrier in P.aeruginosa comes from studies of a hypersensitive mutant and comparison with its normally resistant parent strain (Zimmermann, 1979,1980). The mutant, Z61, was derived by chemical mutagenesis from its parent, K799. Antibiotic sensitivity could not be explained by alteration of target sensitivity as binding of radio-labelled benzylpenicillin to penicillin binding proteins of both strains was essentially the same. Synthesis of inducible β-lactamase was deficient in the Z61 mutant but sensitivity to β lactam antibiotics was not due to enzymatic inactivation since the difference between resistant and sensitive strains was maintained against a number of antibiotics stable to hydrolysis by P.aeruginosa lactamases. The data was consistent with resistance in the wild-type parent being due to the permeability barrier of the outer layer of the cell envelope. An investigation of the permeability of 6 carbenicillin-resistant and -hypersusceptible strains, including the Z61 mutant, showed that resistant strains were also resistant to other β-lactams and to the unrelated compounds quinoline, tetracycline or chloramphenicol. Carbenicillin-hypersusceptible strains were very sensitive to these compounds (Livermore, 1984). Resistance in these resistant strains did not involve β-lactamase or target PBP alterations and a permeability-type resistance was again proposed. The level of porin protein was quantitatively the same in resistant and sensitive strains (Livermore, 1984).

Attempts to discover those features of the cell which would explain the high intrinsic resistance of P.aeruginosa have been made by comparison of the OM of the sensitive mutant and its normally

resistant parent (Angus et al, 1982; Kropinski et al, 1982). These studies confirmed the difference in permeability and showed that the major porin protein, protein F, was quantitatively and qualitatively the same in both organisms. (Angus et al, 1982). Analysis of LPS revealed minor differences in the fatty acids of lipid A and the neutral sugars of the core region. The high permeability of the mutant was tentatively explained by the minor differences in LPS influencing the functional state of porin channels (Kropinski et al, 1982). Minor changes in LPS composition have also been found in chemically .induced mutants of the PAO strain resistant to a number of β-lactam antibiotics (Godfrey et al, 1984). Porin levels in the mutant strains examined in this study were identical as analysed by SDS-PAGE. Some minor changes in OMP profile were observed but did not correlate with resistance. Again the observed changes in LPS may affect porin function or may themselves affect permeability by changes in the hydrophobic-hydrophilic nature of the outer layers of the cell (Godfrey et al, 1984; Hiruma et al, 1984)

Further evidence for the low permeability of the P.aeruginosa OM has come from measurements of the hydrolysis of the chromogenic cephalosporin nitrocefin (Angus et al, 1982). Comparison with cephalosporin permeability in E.coli has been used to show that diffusion through the P.aeruginosa OM is much lower than diffusion through the E.coli OM. This evidence is not wholly convincing since different cephalosporins (albeit of the same hydrophilicity as judged by partition in octanol/water) were used with each organism. Direct evidence of low permeability in P.aeruginosa strains PAO1 and K799 has been determined by measuring the rate of hydrolysis of a series of cephalosporins and phosphate esters by lactamases and alkaline phosphatase of the periplasm (Yoshimura & Nikaido, 1982). The

permeability of these compounds was about 100-fold lower in P.aeruginosa strains that in E.coli K12. The authors conclude that the low permeability to hydrophilic compounds afforded by the OM acts as a rate-limiting step in active transport processes. The rate-limiting aspect of resistance mediated by the OM permeability barrier is important because it controls the rate at which the drug is delivered to inactivating enzymes in the periplasm. When the OM barrier limits the rate at which the drug reaches the periplasm enzymic inactivation need only take place for that small amount of drug, that is, only a small fraction of the total drug in the medium. Lack of a permeability barrier may explain why some bacteria which produce enzymes capable of inactivating a particular drug nonetheless remain susceptible to that drug (Murray & Moellering, 1978).

An additional barrier situated deeper within the OM, possibly a protein barrier in the periplasm or the outer surface of the CM, has been hypothesized (Scudamore & Goldner, 1982). This suggestion is based upon a study of the action of a number of antibiotics on growth of P.aeruginosa treated with low levels of EDTA and comparison with untreated cells. The hypothesis offers a possible explanation of the finding that porin levels are the same in sensitive and resistant cells (Angus et al, 1981; Livermore, 1984) in that the porin molecules themselves need not form a part of this permeability barrier (Livermore, 1984).

1.2.6 Exopolysaccharide as a barrier to antibiotics

Many bacteria secrete large polymers which adhere to the OM to form an extracellular slime layer or capsule. Most strains of P.aeruginosa form a polysaccharide slime containing glycoprotein (Bartell & Krikszens,1980; Sensakovic & Bartell, 1974). Mucoid strains produce copious quantities of an alginic acid-like exopolysaccharide (Evans & Linker, 1973). The contribution of the capsule and extracellular slime to the permeability barrier is highly variable. The exopolysaccharide constitutes a largely anionic matrix (Brown, 1975) in which charged molecules may be partitioned, this may bind an antibiotic so that few molecules are able to reach their targets in the cell. Alternatively, partition in the matrix may serve to concentrate molecules at the cell surface and this has been proposed in a study with tetracycline (Govan & Fyfe, 1978; Govan et al, 1983). The positively charged aminoglycosides antibiotics and polymyxins are potentially subject to binding in the anionic matrix of P.aeruginosa polysaccharides. Diffusion of aminoglycosides has been shown to be slowed by diffusion in agar plates containing sodium alginate or P.aeruginosa exopolysaccharides (Slack & Nichols, 1981). This effect was not observed with neutral or negatively charged β-lactams.

Binding of a number of antibiotics to purified P.aeruginosa alginate has been calculated from equilibrium dialysis data (Tannenbaum et al, 1984). Positively charged antibiotics such as streptomycin and tobramycin initially bound to the ionic matrix whereas the neutral species clindamycin and penicillin did not. However, when dialysis was repeated in the presence of physiological

concentrations of saline none of the antibiotics bound. This suggests that binding of these antibiotics to P.aeruginosa mucoid exopolysaccharide does not occur in vivo. The hydrophilic nature of the slime layer may also interfere with antibiotic penetration by repelling hydrophobic molecules, making penetration to deeper layers difficult (Bryan, 1982).

There appears to be a complete heterogeneity of response to antibiotics in mucoid strains (Sheehan et al, 1982; Irvin et al, 1981). Mucoid strains from the sputum of CF patients have been shown to be more resistant to tobramycin, flucloxacillin, carbenicillin (Govan & Fyfe, 1978). Irvin et al (1981) have shown that hypersusceptibility in a mucoid strain was related to the ability to synthesize alginate and that the sensitive strain possessed two additional OM proteins (25 and 32K) the larger of which was lost on reversion to normal carbenicillin sensitivity.

Bacteria present in infected body fluids such as urine or sputum are present in exopolysaccharide enclosed microcolonies adherent to epithelial cells and, in smaller numbers, free in the fluid (Costerton, 1979; Costerton et al, 1983). Growth of the adherent microcolonies whilst not restricting their viability appears to confer a considerable measure of protection from antibacterial agents. P.aeruginosa colonizing latex catheter material in a test system simulating part of the urinary tract survived exposure to 1000 µg/ml tobramycin while the MIC and minimum bactericidal concentration (MBC) of the free floating cells were 0.4µg/ml and 50µg/ml respectively (Nickel et al,1985). The mechanism for antibiotic resistance in exopolysaccharide enclosed microcolonies is not known. The microcolonies are unlikely to be large enough for altered rates of diffusion of antibiotics in alginate alone to account for increased

resistance (Slack & Nichols, 1982). Although drug inactivating enzymes are normally confined to the periplasm they may diffuse from the cell (Nordstrom & Sykes, 1974) and the local concentration of drug inactivating enzyme within the microcolony may be high. Alternatively, the physiological state of the surface growing organisms within the microcolony may be different from those free in the medium.

1.2.7 Resistance to polymyxin in *P.aeruginosa*.

The polymyxins are decapeptide antibiotics which were first isolated from the aerobic sporing bacilli *Bacillus polymyxa* in 1947 (Ainsworth *et al*, 1947). The basic structure of this group of antibiotics is a seven membered peptide ring with a linear tripeptide side chain ending in a fatty acid residue. Five polymyxins have been described A,B,C,D & E, of which A,C & D are too toxic for therapeutic use. (Horton & Pankey, 1984). Polymyxin B (Px) is largely inactive against Gram-positive organisms but has a bactericidal action against a number of Gram-negative bacilli, notably *P.aeruginosa* (Garrod *et al*, 1981; Horton & Pankey, 1984; Fekety, 1985).

The bactericidal action of Px results from interaction with and disruption of the cell membranes (Newton, 1956) with a consequent leakage of cytoplasmic contents and cell death (Storm *et al*, 1977). The exact mechanism of membrane disruption is not known but two models have been proposed. In the first (Hartman *et al*, 1978; Sixl & Galla, 1981) an interaction takes place in which the hydrophobic side chain of the Px molecule is inserted into the hydrophobic portion of the membrane while the charged head interacts with the negatively charged

membrane surface. It is proposed that this interaction disrupts membrane lipid packing and distorts the membrane with the result that it becomes permeable. In the second model (El Maskah & Tocanne, 1980) the whole Px molecule enters the lipid bilayer causing it to expand sideways and distort. Studies in which Px was immobilised by covalent attachment to agarose beads so that it could not cross the OM indicate that an interaction with the OM alone is sufficient to alter the structural integrity of the cell (Laporte et al, 1977). Evidence that disruption of the CM is a lethal mode of action of Px comes from observations that the OM of Px-resistant organisms is not susceptible to Px disruption while the separated CM, and the OM of sensitive organisms, remains susceptible (Gilleland & Farley, 1982; Vaara et al, 1979). Support for this mode of action comes from work with P.mirabilis, an organism usually resistant to the action of Px. Studies of P.mirabilis mutants (Sud & Feingold, 1970) and penicillin induced speroplasts (Teuber, 1969) suggest that resistance may be attributed to exclusion of the antibiotic from the CM by the OM.

To reach a site of action on the CM, Px must first interact with and possibly pass through the outer layers of the cell. Px has a high affinity for isolated anionic components of the Gram-negative cell envelope. Stoichiometric complexes with the lipid A region of LPS (Bader & Teuber, 1973) and with negatively charged phospholipids (Teuber, 1973) have both been characterised. Both have been proposed as the initial binding sites for Px in the whole cell, where it is proposed that Px competitively displaces Mg^{++} and Ca^{++} from negatively charged groups on membrane lipids and LPS (Brown, 1975; Storm et al, 1977; Teuber & Bader, 1976a). Divalent cations are effective antagonists of Px activity (Boggis et al, 1979; Kenward et al, 1979)

presumably by competing with Px for these negatively charged binding sites. The highly phosphorylated LPS core polysaccharide of P.aeruginosa (Wilkinson & Galbraith, 1975) may provide additional binding sites for which cations and antibiotic compete in this organism (Schindler & Osborn, 1979).

How the Px molecule, with its large hydrophilic headgroup passes through the OM is not known. Two mechanisms have been suggested. The first (Storm et al, 1977) is that after initial binding has taken place there is a local disruption and breakdown of the OM which allows the molecule to pass through. The other (Gilleland & Lyle, 1979) is that Px is initially bound by OM phospholipids or LPS but then gains access to the CM by way of a porin protein. Resistance to the action of Px may be mediated by changes in the OM which modify the initial binding of the Px molecule or prevent its passage through the membrane (Brown, 1975; Gilleland & Murray, 1976).

Evidence that binding to phospholipids is involved in the activity of Px comes from the observations that the phospholipid content of cell walls is reduced in some Px resistant variants of P.aeruginosa (Brown & Watkins, 1970; Brown & Wood, 1972) and from experiments in which the naturally Px resistant organism Acholeplasma laidlawii was converted to Px sensitivity by treatment with the isolated phospholipids of a susceptible strain of S.typhimurium (Teuber & Bader, 1976b).

Resistance by exclusion could be determined by loss of a porin protein. Working with Px resistant variant, isolate A, derived from the Px sensitive PA01 by stepwise adaptation to 6000 units/ml, Gilleland & Murray (1976) showed in a series of EM studies of freeze-etched cells that in the resistant isolate there was a large reduction in the number of particles normally found on the concave

face of the OM. Treatment of sensitive cells with EDTA produced the same effect and removed the particles in the form of vesicles which were shown to be protein-LPS complexes. Biochemical alterations in the resistant isolate were a reduction in LPS (Gilleland & Lyle, 1979), divalent cations and phospholipids of the cell wall and an increase in readily extractable lipids (Conrad & Gilleland, 1981). SDS-PAGE of OMPs showed a reduction in proteins 5, 9 and 10 in their system (Conrad & Gilleland, 1981; Gilleland & Beckham, 1982), which correspond to proteins D2, F and H of the Mizuno and Kageyama nomenclature (1978). The gel system used did not separate proteins H1 and H2 (Gilleland & Beckham, 1982). Proteins D and F are porin proteins. These findings are consistent with either of the two suggested models of Px resistance and Gilleland proposed that Px resistance followed a loss in permeability due to a decrease in porin levels (Gilleland & Beckham, 1982).

Resistance to EDTA and to Px by P.aeruginosa resulting from growth in media low in magnesium was first reported by Brown and Melling (1969a ,1969b). Sensitivity could be restored by adding back magnesium or other metal cations. Further studies indicated a correlation between the lytic action of these agents and the stability constants of the various cations for the agents (Boggis et al 1979; Kenward et al, 1979). The similarity of the effects of these ions on EDTA and Px sensitivity served to confirm the hypothesis proposed by Brown & Melling (1969a,b) that these agents both act at a common initial site. It was further proposed that specific antagonism by cations of both aminoglycosides and Px action in P.aeruginosa involves the same EDTA/Px sensitive LPS attachment site on the outer surface of the OM (Brown, 1975).

Nicas & Hancock (1980) using SDS-PAGE showed that the level

of protein H1 in the OM of P.aeruginosa was inversely proportional to cell envelope Mg^{++} concentration. When cells were subcultured from a medium low in Mg to one of higher concentration the time course of decrease in protein H1 levels correlated well with an increase in sensitivity to Px (Nicas & Hancock, 1983a). Nicas & Hancock (1980) were also able to show that two mutants, H181 and H185, which are both resistant to 75 μg/ml Px (Sigma Px, 8000 units/mg) both exhibit increased levels of protein H1. The mutants H181 and 185 are derived from PAO1 by chemical mutagenesis with diethyl sulphate. Px resistance was proposed to be due to increased levels of protein H1 which acts by replacing Mg^{++} cross-bridges at a site in the OM which would otherwise be susceptible to attack by Px. When Gilleland and his colleagues grew the strains used by Nicas & Hancock (1980) in the presence of 50 units/ml Px, however, they once again found reductions in proteins D2, F and H, and in phospholipid levels, and concluded that Px resistance in these mutants could not be due to increased H1 production (Gilleland & Conrad, 1982).

Recently there have been further reports of lipid alterations (Champlin et al, 1983; Gilleland et al, 1984) in the Px resistant isolate A compared with the sensitive parent strain and revertants. These studies again showed significant decreases in the phospholipid content of resistant cells and the appearance of a major new lipid lacking either phosphate or amino moieties (Champlin et al, 1983). The new lipid was constituted from essentially identical fatty acids, both qualitatively and quantitatively, to those esterified to membrane phospholipids in the sensitive parent. This suggests that the new lipid is formed by hydrolytic degradation of phospholipids. Lipid altertions resulting from exposure to low concentrations of Px are

proposed to account for adaptive resistance to Px (Gilleland et al, 1984). These changes reduce the availability of acidic binding sites for the antibiotic, thus allowing the cell to tolerate higher concentrations.

1.3 Infections due to Pseudomonas aeruginosa.

P.aeruginosa is a gram-negative aerobic rod which varies in length from 1-3 µm and in width from 0.5-1 µm. Many strains are piliated and are mobile by means of a single polar flagellum. The organism will grow readily on unenriched media; colonies on nutrient agar are typically rounded, flat and translucent with a dull, matt surface and butyrous consistency (Gilardi, 1979). A number of other colonial types may be observed on solid media - notably a mucoid type with a smooth shiny surface and a viscid, sticky consistency (Gilardi, 1979). Many strains produce a characteristic blue-green water-soluble pigment, pyocyanin, (Rhame, 1979) which is commonly used to identify the organism. Pyoverdin, a water-soluble, yellow-green fluorescent pigment is also formed by most strains of P.aeruginosa, particularly in iron-deficient media (Meyer & Abdullah, 1978). Because it has the ability to grow over a wide temperature range in almost any moist surroundings and is not fastidious in its nutrient requirements P.aeruginosa is widely distributed in the environment (Young, 1977; Rhame, 1979). The species rarely causes infection in the healthy host and may be isolated from the faeces of 10% of normal humans (Stoodley & Thoma, 1970). This intestinal carriage rate is increased in hospitalised patients (Shooter et al, 1966).

P.aeruginosa may be isolated from a wide variety of sources in hospitals (Baird & Shooter, 1976; Hurst, 1966; Rhame 1979; Richards, 1975; Young, 1977), where it may act as a reservoir for outbreaks of infection in susceptible patients (Levin et al, 1984; Noone et al, 1984). The organism has become an increasingly important

'opportunist' pathogen in hospital acquired infections (Bennett, 1974; Bodey et al, 1983; Bryan et al, 1983a). The incidence of Pseudomonas infection is highest in patients with severe burn injuries, cystic fibrosis, and conditions in which the immune system is compromised by disease or cytotoxic and immunosupressive drugs (Cates, 1983; Froland, 1981). The reasons for the increase in the incidence of infections with P.aeruginosa in the hospital setting are not fully understood. The fact that these infections most often occur in debilitated patients whose normal immune defence mechanisms are compromised (Peterson, 1979; Bryan et al, 1983a) indicate that a major factor is the nature of the patient population and the more complex therapeutic and surgical procedures which have been introduced into hospital practice.

Pseudomonas is notoriously resistant to the action of antibacterial agents (Brown, 1975). This resistance increases the capacity of the organism to cause disease as it is likely to take over as the primary cause of infection in sites from which other organisms have been eliminated by antibiotic treatment. Infections due to P.aeruginosa occurred infrequently until after the introduction of the sulphonamides and penicillin (McGowan et al, 1975; Cross et al, 1983). However, it is now reported as being the most common pathogen recovered from patients hospitalised for more than one week in studies in a cancer center (Bodey et al, 1983) and a respiratory intensive care unit. (Geddes, 1979).

Pseudomonas may give rise to a wide variety of diseases, from acute and rapidly fatal septicaemias to chronic suppurative infections, or to a less harmful colonisation of a number of different body sites (Geddes, 1979; Bodey et al, 1983).

1.3.1 Virulence of P.aeruginosa

Virulence of P.aeruginosa is probably multifactorial (Ogata, 1983) and involves proliferation at the site of infection and the elaboration of a number of extracellular and somatic metabolites (Cryz, 1984b). The specific contribution of P.aeruginosa enzymes and toxins to wound invasion and infection is poorly defined (Morrison & Ulerich, 1978). P.aeruginosa is unusual among Gram-negative bacteria in that it secretes a number of protein exoproducts (Neilsen et al, 1983). Enzymes produced by P.aeruginosa include collagenase, elastase,lecithinase, protease, esterase, lipase, nucleases (Liu, 1974) and at least two haemolysins (Wretland et al, 1973; Hommaet al 1984). Pathogenesis of disease has been ascribed to endotoxin (the LPS component of the cell wall) and to the glycoprotein of slime (Sensakovic & Bartell, 1974; Lynn & Lotz, 1984). Death from Gram-negative endotoxin is the result of a complex interaction involving direct dammage to host cells, activation of the complement and coagulation cascades, release of inflammatory mediators and deleterious effects upon granulocytes and macrophages (Young, 1979).

Other evidence indicates that a toxin, exotoxin A, is the most potent exoproduct of P.aeruginosa (Liu, 1974; Cryz et al, 1980; Ohman et al, 1980; Middlebrook & Dorland, 1984). Exotoxin A is a protein with a molecular weight of ~50,000 which inhibits protein synthesis by interfering with polypeptide translocation on messenger RNA, a mechanism of action identical to that of diphtheria toxin (Middlebrook & Dorland, 1984). Injection of exotoxin A in animals produces tissue necrosis, neutropaenia and death (Liu, 1974). Alkaline protease and elastase are extracellular proteolytic enzymes produced by P.aeruginosa which degrade gelatin, elastin, fibrin and collagen (Blackwood et al, 1983; Woods & Iglewski, 1983; Doring et al, 1984).

When injected into the skin of animals, or introduced in to the lung or eye these enzymes induce haemorrhage and necrosis (Homma, 1982) and severely damage tissues and blood vessels (Cash et al, 1983). There is evidence that elastase producing strains are more virulent in lung infection than elastase-deficient mutants (Blackwood et al, 1983). The production of proteolytic enzymes at the site of infection may break down mechanical barriers in the tissues and allow P.aeruginosa to become invasive. In eye infections due to P.aeruginosa proteolytic enzymes are considered to be the cause of rapid globe invasion. (Ohman et al, 1980; Howe & Iglewski, 1984).

1.3.2 P.aeruginosa in human infection.

Infection is the most frequent cause of morbidity and mortality in severly burned patients (Pruit, 1983,1984). Burn injury destroys the mechanical barrier of the skin (Mimms, 1977) and has a deleterious effects on both humoral and cellular immunity (Pruit, 1984). Physiological deficiencies have been noted in complement (Bjornsen, 1978), immunoglobulins (Munster et al, 1979; Schorr et al, 1984), leukocyte (Eurhenius & Brouse, 1973) and monocyte (Hansbrough et al, 1983) function following burn injury. McEuen et al (1976) have found in a murine burn model that the virulence of P.aeruginosa was related to the organism's resistance to phagocytosis and that phagocytosis was inhibited by a decrease in serum factors. These manifold immunological defects (Munster, 1984) combine to make infection the principal cause of mortality in burns patients (Pruit, 1984). Any of a spectrum of opportunist organisms may cause infection. P.aeruginosa is prominent in this group. The burn eschar produced by necrosis of the skin provides a rich growth environment for bacteria and permits access to the underlying tissues. The burn wound is

avascular and lack of blood supply limits both the delivery of elements of the host defence system and systemically administered antibiotics to the eschar (Order, 1965).

P.aeruginosa is seldom recovered from a burn injury during the first 24 hours following the injury but thereafter the rate of colonisation increases rapidly. In one study at 30 hours following injury 20% of burnt patients were found to have P.aeruginosa recoverable from their wounds. By 48 hours 48% and by 5 days over 60% of burn wounds harboured P.aeruginosa (Pruit, 1983). Motility .appears to be an important microbial virulence factor in terms of the penetration of the burn wound and invasion of underlying tissue. Non-motile mutants of a strain of P.aeruginosa have been shown to produce a significantly lower incidence of invasive wound infection in a mouse burn model than the motile parent (McManus, 1980).

The prophylactic use of topical sulphonamide antimicrobials such as mafenide and silver sulphadiazine has reduced mortality from burn wound sepsis but it remains a significant problem (Pruit, 1984).

P.aeruginosa is also an extremely troublesome pathogen in the damaged airways of patients with cystic fibrosis (CF)(Doggett, 1969) where, since the introduction of effective antistaphylococcal antibiotics P.aeruginosa infections of the lungs have largely replaced those due to Staph.aureus. Reported rates of colonisation of the respiratory tract of CF patients vary from 18-80% (Hoiby & Olling, 1977) with mucoid strains predominating (Hoiby, 1978, 1982). The reasons for colonisation by P.aeruginosa (Johanson et al, 1972) and the change to the mucoid form are far from clear (Costerton et al, 1979). However, the colonization and change to the mucoid form are strongly associated with CF and chronic lung conditions in which their

is bronchiectasis (Rivera & Nicotra, 1982). Mucoid P.aeruginosa colonizing the CF lung has been shown to be growing under iron-restricted conditions (Brown et al, 1984), mucoid strains appear to be more stable under iron-limited conditions (Boyce & Miller, 1982; Ombaka et al, 1983) thus iron deprivation in the lung may be a selective pressure which maintains the mucoid form.

On the mucosal surface of the lung bacterial adherence is a major determinant of pathogenicity. Organisms unable to attach are removed by secretions and fail to colonize (Mimms, 1977). Both mucoid and non-mucoid strains have demonstrated adherence to epithelial cells of the upper respiratory tract (Ramphal et al, 1984; Ramphal & Pier, 1985; Woods et al, 1980). Once P.aeruginosa has colonized the CF lung it is almost impossible to eradicate (Hoiby et al, 1982) possibly because of poor penetration of antibiotics in the CF lung (Pennington & Richards, 1973; Hoiby et al, 1981). Frequent therapy with high doses of antibiotics improves the clinical picture in CF but does not materially alter the microbiological flora (Szaff et al, 1983).

Although mucoid strains of P.aeruginosa resist phagocytosis (Schwarzman & Boring, 1971; Baltimore & Mitchell, 1980) there does not seem to be a major specific defect in the immune defences of the CF lung (Shiotz, 1982). Indeed, crossed immune electrophoresis studies (Hoiby & Axelsen, 1973) reveal a large number of reactions between patient's antibodies and bacterial antigens (Hoiby & Schiotz, 1982) including extracellular toxins and enzymes (Doring et al, 1981, 1983). Inflammatory reactions induced by the formation of immune complexes may be the cause of tissue damage in the CF lung (Hoiby & Schiotz, 1982). Infective lung disease remains a major cause of morbidity and mortality in CF.

P.aeruginosa is one of the more important pathogens causing respiratory infections and septicaemias in myelosuppressed or immunosuppressed individuals (Froland, 1981; Hughes, 1983). Prominent amongst these individuals are cancer patients following chemotherapy or radiotherapy and those undergoing transplant surgery. This population continues to increase in size because of more aggresive treatment of cancer and wider application of organ transplantation. Granulocytopaenia is the most important factor predisposing the cancer patient to infection (Topper & Armstrong, 1974). Those patients with a neutrophil count > 1,000/mm^3 rarely develop septicaemia but when the level of circulating neutrophils is below 100/mm^3 the risk of infection is considerable; over 50% of neutropaenic patients develop febrile infection requiring antimicrobial therapy (Klastersky, 1983b), the mortality rate in those who do not respond quickly to therapy is high (Klastersky, 1983a). Initial antimicrobial therapy is generally broad spectrum as the causative organism is often unknown until the reports of blood cultures are available. This may cause even greater problems by further encouraging the emergence of opportunist and resistant organisms.

1.3.3 Infections of the lower urinary tract

Urinary tract infection (UTI) is a major cause of human morbidity and mortality. The prevalence of bacteriuria in adult women is 5-6% and is higher among hospital in-patients than in ambulant patients at home (Maskell,1982). 20% of cases of end stage renal failure presenting for haemodialysis or transplantation are due to chronic pyelonephritis (Brynger *et al*,1980). Predisposing factors for UTI include female sex, anatomical or physiological conditions causing

urinary stasis (O'Grady et al, 1968; Sobel & Kaye, 1984), and instrumentation or catheterisation. This last factor is important in hospital practice where many patients are catheterised because of neurological disease or in the course of major surgery. The hospital surveillance programme of the Atlanta Centre for Disease Control has reported that 10% of UTIs in hospitalised patients were due to P.aeruginosa (Bennett,1974). The figure in the United Kingdom is 3-6% (Shanson, 1982).

Bacteria may invade the urinary tract via ascending or, more rarely, heamatogenous routes. The large majority of infections result from the ascending route when bacteria colonising the periurethral area (O'Grady et al, 1970), or present on instruments or catheters, gain access to the urethra. If pathogenic bacteria become established in the urethra, symptomatic or asymptomatic infection of the bladder may result. A few organisms may finally ascend the ureters and reach the kidneys to cause pylonephritis (Sobel & Kaye, 1984).

Urinary tract infections are more common in women that in men, probably due to the shorter length of the female urethra (Maskell, 1982; Shanson, 1982) and possibly the state of perineal hygeine. Urinary tract infections are common in the elderly, the greater prevalence of bacteriuria in women continues in those over 65 but is not as pronounced as in young adults (Bendall, 1984; Freedman, 1983). Four percent of men and 20% of women over the age of 70 have been estimated to have bacteriuria (Freedman,1983. Kaye,1980).

The normal aerobic flora of the periurethral area in female subjects includes lactobacilli, staphylococci, corynebacteria and streptococci (Marrie et al,1978). The majority of urinary tract pathogens are coliform organisms from the patients bowel flora (O'Grady et al, 1970; Nitzan et al, 1983) but may also be introduced

from the hospital environment. Little is known about the factors that lead the colonizing organisms of the periurethral area to cause UTI. Attachment of bacteria to uroepithelial cells is believed to be a crucial step in the establishment of UTI (Smith, 1977. Svannborg-Eden & Hanson, 1978; Arbuthnott & Smyth, 1979; Jones & Isaccson; 1983. Reid et al, 1984). The adhesive ability of E.coli strains to uroepithelial cells correlates strongly with the presence of pili on the bacterial surface (Svannborg-Eden & Hanson, 1978). Woods et al, (1980) have described pili-dependent adherence of P.aeruginosa to . buccal epithelial cells. The observation that non-piliated strains of P.aeruginosa adhere well to rat bladder epithelium suggests that pili independent mechanisms also exist (Vardi et al, 1983). Strains of bacteria which show enhanced adhesion to uroepithelial cells may be more likely to cause UTI than others (Brooks et al, 1980). A surface layer of mucin synthesized by the bladder epithelium (Parsons, et al, 1980) may be a non-specific bladder antiadherence mechanism. Removal of surface mucin by acid washing increased adherence of a number of bacterial species (Sobel & Vardi, 1982; Vardi et al, 1983). Indwelling catheters may increase the risk of UTI by erosion through the protective mucin layer.

Mucoid strains of P.aeruginosa may be isolated from UTIs (Marrie et al, 1979). Electron microscopy studies suggest that mucoid exopolysaccharide is involved in the adherence of mucoid P.aeruginosa in UTI (Costerton et al, 1983). Non-specific adherence mediated by mucoid exopolysaccharide has been confirmed in radio-labelled binding studies in trachea of mice (Ramphal & Pier, 1985). A preponderance of Gram-negative bacteria isolated in UTI have been found to have a high surface hydrophobicity as measured by hydrophobic interaction chromatography and salt aggregation tests (Ljungh & Wadstrom, 1984).

Non-specific adherence to the mucosal surface may be due to the pronounced hydrophobic properies or surface charge of these bacteria.

The most common organism isolated in UTI is E.coli. Proteus sp., Klebsiella sp. and P.aeruginosa are also frequently isolated and are more common in the elderly patient. Acute infections of the urinary tract are commonly caused by a single bacterial species. More than one kind of organism may be isolated in significant numbers from chronic infections, particularly where there are structural or functional abnormalities of the urinary tract (Garrod et al,1981). P.aeruginosa and Klebsiella are common in chronic infections (Gould,1968). Infections due to P.aeruginosa often follow instrumentation and may be associated with chronic bladder dysfunction (Slade, 1977). Infection with Klebsiella sp. are endemic in certain hospital environments (Orskov, 1972) where they are transmitted via improperly sterilized instruments or the hands of hospital staff (Gardner and Smith, 1969). Proteus infections are also a frequent cause of hospital-acquired UTI following instrumentation (Stamm et al, 1977). The frequency with which these Gram-negative organisms are isolated and their distribution in different patient populations varies considerably (Nitzan et al, 1983).

The outcome of bacterial invasion of the urinary tract depends on the size of the origional inoculum, the virulence of the invading organism and the state of the defence mechanisms of the host. In the absence of structural or functional abnormalities of the urinary tract infections may clear spontaneously by a dilution effect (1.4.4). Where an abnormality leads to residual urine in the bladder following micturation the chances of infection persisting are much greater (O'Grady et al, 1968). Acute infections due to sensitive organisms in ambulatory patients may be treated with oral antibiotics, which are

excreted in high concentrations in the urine (Rapoport et al, 1981; Shanson, 1982). Even with antibiotic therapy frequent micturation is necessary to reduce residual volume and the persistence of infection (O'Grady et al, 1973).

1.3.4 Treatment of P.aeruginosa infections.

Until the mid-1960's septicaemia due to infection with P.aeruginosa was associated with a very high level of mortality. This was due to the relative lack of antipseudomonal activity of all the available antibiotics with the exception of the polymyxins (Gillett, 1982). This state of affairs was improved with the introduction of two drugs with good activity against P.aeruginosa : gentamicin and carbenicillin (Selwyn, 1982). These antibiotics have been followed by other aminoglycosides and semi-synthetic penicillins with equal or better activity (Garrod et al, 1981) and by the newer antipseudomonal cephalosporins (Cunha & Ristuccia, 1982). Although the aminoglycosides represented a significant step forward their usefulness as single agents in the treatment of Pseudomonas infections is limited by their potential for auditory and nephro toxicity in high doses, which prevents their administration to patients with renal impairment in quantities that would provide optimal bactericidal activity. Resistance to aminoglycosides has become a problem (Lowbury & Jones, 1975; Bridges et al, 1979; Gillett, 1982) nevertheless they remain useful first line drugs in sensitive infections. The antipseudomonal penicillins are more effective than aminoglycosides in neutropaenic patients, but again emergence of resistance limits their usefulness.

There are reports of synergy between combinations of carbenicillin, ticarcillin, mezlocillin, azlocillin or piperacillin with gentamicin, tobramycin or amikacin against many strains of P.aeruginosa (Anderson et al, 1978; Bruckner et al, 1983; Hughes et al, 1983; Walsh & Schimpff, 1983). Studies in vitro (Takahashi &

Kanno, 1984) and in experimental animal infections (Andriole, 1974) have demonstrated the efficacy of these combinations. Synergistic combinations between antipseudomonal β-lactams and aminoglycosides offer the added advantage that they enable the effective dose of the potentially toxic aminoglycoside to be reduced (Angehern, 1983). Although this form of therapy has not been subjected to definitive controlled studies (Krogstad & Moellering, 1980) it has become accepted practice to use a combination of these drugs in serious infections (Hughes et al, 1983; Klastersky, 1983a). The introduction of the newer penicillins and cephalosporins offers the possibility of other, more effective combinations (Van Laetham et al, 1983; Zuravleff et al, 1983).

Although new agents continue to be synthesised at a rate which has so far kept pace with the emergence of resistant strains the efficacy of currently available antipseudomonal therapy has been questioned (Pennington, 1979; Bryan et al, 1983; Klastersky, 1983a) as has the value of synergistic antibiotic combinations (Heinman & Lofton, 1978). The introduction of new antibiotics may indeed create selective pressures for the emergence of novel drug inactivating enzymes (Simon et al, 1980). Furthermore, antibiotics, even when they are effective, may prevent the further accumulation of toxic bacterial exoproducts but cannot ameliorate their effects. It is apparent that antibiotic therapy alone is not a satisfactory treatment for Pseudomonas infections, accordingly there is considerable interest in the development of antipseudomonal vaccines. (Introduction 1.5)

The polymyxins and their sulphomethyl derivatives are now used infrequently for systemic therapy since they have proved to be

ineffective in the treatment of severe P.aeruginosa infection (Lowbury & Jones, 1975; Fekety, 1985). Systemic use of the drugs is further limited by neurotoxicity and a dose-related nephrotoxicity (Garrod et al, 1981, Fekety, 1985). The polymyxins are excreted via the kidneys and high concentrations are achieved in urine. Good results have been obtained in the treatment of UTIs or septicaemias arising from the urinary tract (Brumfitt et al, 1966, Goodwin, 1970) where relatively low and well tolerated doses of the drug may be used. In a model simulating conditions of bacterial growth in the urinary bladder polymyxins were effective whereas the sulphonomethyl derivatives had little effect (Greenwood & O'Grady, 1977).

Polymyxins are now used systemically almost exclusively for life-threatening Gram-negative infections caused by organisms resistant to other drugs (Horton & Pankey,1984, Fakety, 1985). Synergistic combination of the polymyxins with tetracycline (Lieberson, 1969), sulfamethoxole and trimethoprim (Nord et al, 1974; Rosenblatt & Stewart, 1974) and rifampin (Ostenson et al, 1977) have been reported in the treatment of infections due to multi-resistant Gram-negative bacteria. Although there are no well controlled studies in the efficacy of these drug combinations their chief value is probably that they allow the removal of aminoglycosides from environments where multi-resistant organisms require aminoglycosides pressure to thrive (O'Callaghan et al, 1978).

Aerosolised polymyxins have been used to prevent P.aeruginosa infection in respiratory intensive care units (Klick et al, 1975). The use of polymyxin B aerosols decreases the incidence of colonization by susceptible organisms. The emergence of resistant organisms, however, limits the usefulness of this therapy (Horton & Pankey, 1984).

Polymyxin B is commonly included in topical preparations for

treatment of otic and ocular infections and the prevention and treatment of skin infections (Horton & Pankey, 1984; Fekjety, 1985)

1.4 Host defences against *P.aeruginosa* infections.

Bacteria that succeed in overcoming the mechanical barriers imposed by the skin and mucous membranes and defence mechanisms such as the muco-ciliary lining of the respiratory tract are able to invade the tissues and fluids of the body and then become exposed to the specific and non-specific defences of the body's immune system. (Mims, 1977). Non-specific defences are mediated by fever, inflammation and restriction of iron availability, and by antimicrobial factors such as complement and lysozyme. Specific immune defences rely on the co-ordinated response of antibodies, phagocytic cells, lymphocytes and complement. These defences interfere with bacterial growth, promote the uptake and killing of bacteria by phagocytes and inhibit the toxic properties of bacterial components and exoproducts.

1.4.1 Humoral immunity.

Antigenic foreign material which gains entrance to the body induces a specific immune response whereby plasma cells derived from lymphocytes are stimulated to produce globulin antibodies which react with soluble antigens to cause precipitation and with insoluble antigens to cause agglutination. Globulin antibodies will act in concert with the complement system to lyse foreign cells or will act as opsonins so that foreign particulate matter is more easily taken up by phagocytes. There is ample evidence that production of antbodies to *Pseudomonas* cell wall components and toxins increases in infection and correlates with survival. Cross *et al* (1980) found that a high level

of antibody to exotoxin A correlated with a successful outcome of infection with Pseudomonas. Patients in this study who survived had six times the level of antibody as those who died from bacteraemia. In another study Pollack et al (1983) showed that antibody to LPS and antibody to exotoxin A each confered protection independently and additively. Antibodies against LPS have been associated with a good prognosis in cases of Pseudomonas bacteraemia (Young & Armstrong, 1972).

1.4.1.1 Complement

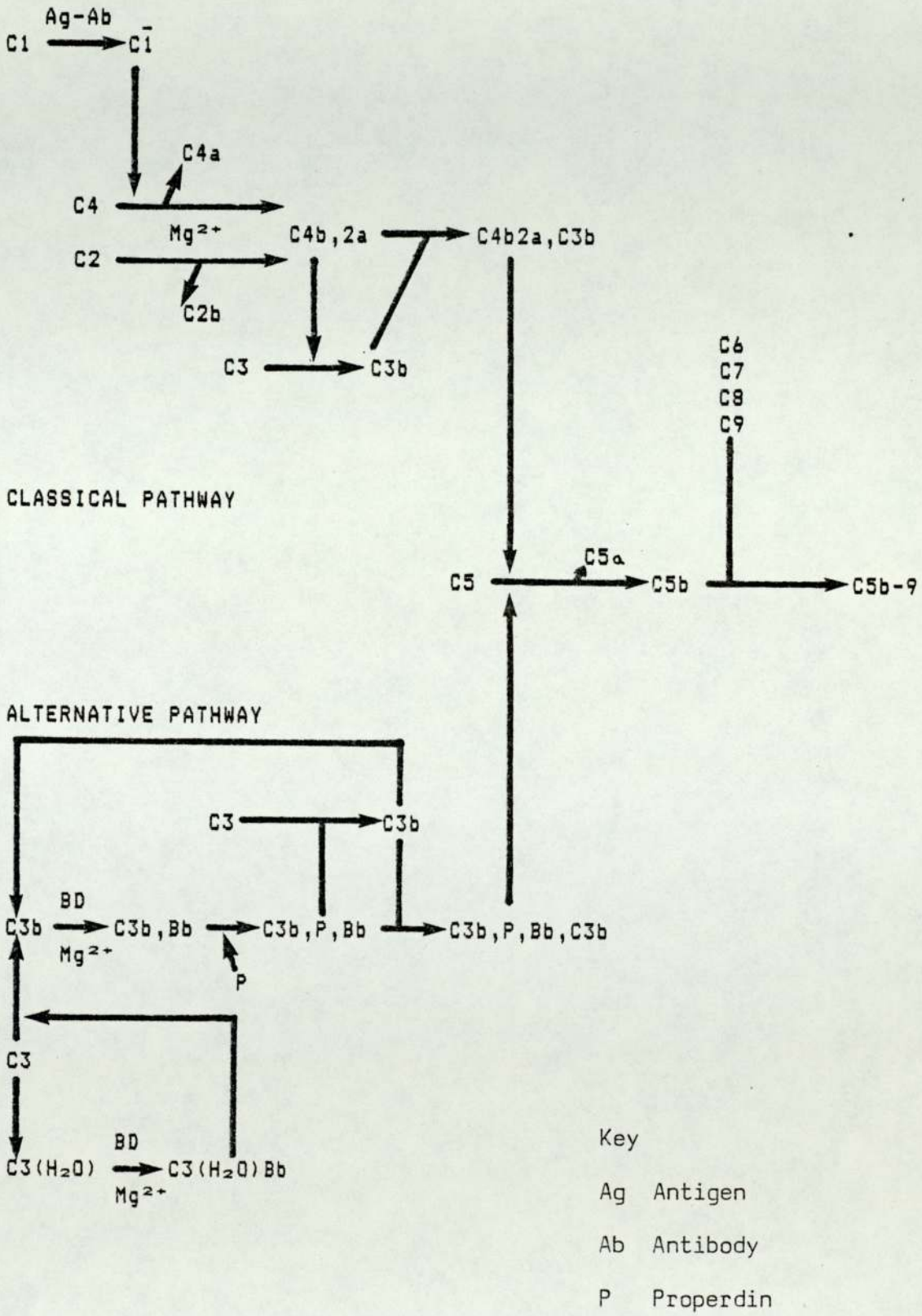
Complement is a complex, multi-component system in serum comprising at least 12 different proteins and capable of causing lysis and death of Gram-negative bacteria (Peterson, 1979; Taylor, 1983). In addition to lysis of invaders some components of the system cause a local inflammation which focusses the body's defensive mechanisms on to the site of infection, some are chemotaxins, and some act as opsonins which enhance the ingestion of invading bacteria by phagocytes.

The activity of complement depends upon the operation of nine major plasma protein components, C1 to C9 (Inoye et al, 1968). As each component of the sequence is activated it aquires the ability to activate several molecules of the next component in the sequence; this produces a cascade effect with amplification. The terminal components of the cascade, The 'membrane attack complex', C5b-C9 forms a hollow protein cylinder which becomes inserted into the OM of the bacterial cell resulting in leakage, access of lysozyme, and eventual lysis (Bhakdi & Tranum-Jensen, 1983; Koski et al, 1983). The 'classical' pathway of the complement sequence is initiated when the first

component, C1, reacts with bacteria which have been opsonized by IgM or IgG antibodies (Lachman, 1979). C1 attaches through its C1q subunit to a binding site on the Fc portion of the immunoglobulin molecule. More than one Fc must bind before activation of C1 can take place. This explains the greater efficiency of the pentamer IgM over the monomer IgG in activating complement. The activated C1 catalyses, in the presence of Mg^{++}, the assembly of C4 and C2. C42 cleaves C3 into two fragments C3a and C3b. The C3b fraction binds to receptors on the bacterial cell membrane and then combines with C42. This complex splits C5 into C5a and C5b. The larger fragment, C5b, attaches to the bacterial cell membrane and binds C6 and C7. This complex in turn binds C8 and C9 to form the membrane attack complex (Taylor, 1983).(figure 1.4)

Figure 1.4

Schematic representation of the Classical and Alternative pathways of complement (Taylor, 1983)



Key

Ag Antigen

Ab Antibody

P Properdin

The 'alternative pathway' (Gotze & Muller-Eberhard, 1974) depends upon activation of C3 without the formation of C142 and is thus independent of specific antibody. Factor P (properdin) in serum stabilizes C3b, again in the presence of Mg^{++}, which binds another C3b to form a complex which cleaves C5. In addition to Mg^{++} lysozyme and Ca^{++} are components of the extracellular fluids required for complement mediated killing. Lysozyme is a single polyeptide chain of low molecular weight (14K) which cleaves the glycosidic link between N-acetylmuramic acid and N-acetylglucosamine of cell wall PG (Taylor, 1983). The end result of the alternative pathway is the same C5b-C9 membrane attack complex as the classic pathway. Activation of complement via the classic pathway is immediate but bactericidal action may take several hours when mediated solely by the alternative pathway. Secretory IgA at mucosal surfaces is able to activate complement by the alternative , but not by the classical, pathway (Van Furth, 1981). Membrane receptors for complement components have been reported on a number of cell types which modulate the immune response (Weigle, 1983). The C567 complex and the split products C3a and C5a are chemotactic, with C5a playing a major chemotactic role. C3a and C5a are also anaphylotoxins which, in low concentrations, cause degranulation of mast cells with release of histamine and consequent enhancement of the inflammatory response.

Pseudomonal elastase has a destructive effect on immunoglobulins (Doring et al, 1981) and complement. Of the protein components of the complex only C4 and C7 are not degraded by elastase (Schultz & Miller, 1974). Elastase thus depresses the inflammatory response, inhibits chemotaxis of neutrophils and lowers their phagocytic activity

(Kharazami et al, 1983).

Some Gram-negative bacteria are rapidly killed by human serum (Offredo-Hammer et al, 1983) while others are completely resistant even in the presence of opsonizing antibody (Taylor,1983; Hughes et al, 1982). Serum-resistant cells may be sensitized to the bactericidal effect of serum by agents which disrupt the integrity of the OM such as EDTA, Tris (Anwar et al, 1983c; Reynolds & Pruul, 1971) or polymyxin (Pruul & Reynolds, 1972)

Most strains of P.aeruginosa are resistant to the bactericidal effects of serum when examined in short term assays (Meshulam et al, 1982; Taylor, 1983). Growth phase and conditions of culture considerably affect sensitivity to complement lysis (De Matteo et al, 1981). Nutrient limitation and growth rate modified the serum sensitivity of chemostat grown E.coli isolated from a UTI (Taylor, 1978). Iron depleted and oxygen depleted P.cepacia have been shown to be more resistant to serum killing than log phase or carbon depleted cells (Anwar et al, 1983a)

1.4.1.2 Phagocytosis

The encounter between bacteria and phagocytic cells in body fluids may be functionally divided into phases of attraction, recognition, attachment, ingestion and killing (Stossel, 1974a,b,c). The majority of isolates of P.aeruginosa are serum resistant and are not killed by serum even when it contains high titres of antibody. However, bacteria that have interacted with fresh serum or specific antibody are efficiently taken up and killed by granulocytes with great avidity (Young & Armstrong, 1972). This facilitated ingestion and subsequent killing is caused by opsonins, the best characterised

of which are IgG and fragment C3b of the complement sequence (Peterson, 1979). The very specific nature of opsonic attachment is due to the presence of specific receptors for IgG and C3b on the phagocytic cell membrane (Stossel, 1974a; Fearon, 1983). A correlation has been demonstrated between the relative degree of hydrophobicity between the surface of phagocytes and bacteria (Van Oss, 1978). The more hydrophobic the bacterial surface relative to the phagocyte the more easily is the bacteria engulfed. One action of opsonins may be to increase surface hydrophobicity to render the bacteria more susceptible to ingestion.

Following ingestion lysosomal granules within the phagocyte fuse with the phagosome. Bacteria are killed by the formation of a group of highly reactive oxidising agents (Babior, 1978) and by the action of granular contents which include lysozyme and lactoferrin (Stossel, 1974b,c).

Granulocytopaenia is one of the most important factors predisposing susceptible individuals to Pseudomonas infections (Young & Armstrong, 1972). In addition to quantitative deficiencies patients with serious underlying disease may have granulocytes with functional abnormalities (Peterson, 1979). Granulocyte transfusion has been advocated as a therapy for Gram-negative septicaemia in neutropaenic patients (Herzig et al, 1977; EORTC, 1983). Although this is logical, the risks associated with the treatment, ethical considerations, and its cost, may outweigh the benefits (Klastersky, 1983a).

The evasion of phagocytosis is one of the principal ways in which bacteria escape host defences (Smith, 1977). Somatic and extracellular products of P.aeruginosa interfere at all stages of the phagocytic process (Peterson, 1979). Mucoid strains of P.aeruginosa resist phagocytosis even in the presence of specific antisera (Baltimore &

Mitchell, 1980). Slime glycoprotein from P.aeruginosa is toxic to granulocytes (Sensakovic & Bartell, 1974). Leukocidins of P.aeruginosa kill phagocytic cells (Scharmann, 1976; Homma, 1982).

Nocardicin A, a B-lactam antibiotic with limited anti-pseudomonal activity in vitro, enhances the intracellular killing of P.aeruginosa by phagocytes. Doses of nocardicin A which produce serum levels of drug below the MIC controlled an otherwise fatal P.aeruginosa infection in mice (Banks & O'Grady, 1983). This novel effect is interesting in that it advances the possibility of antimicrobial drugs with no measurable in vitro MIC, which act in concert with host defence mechanisms.

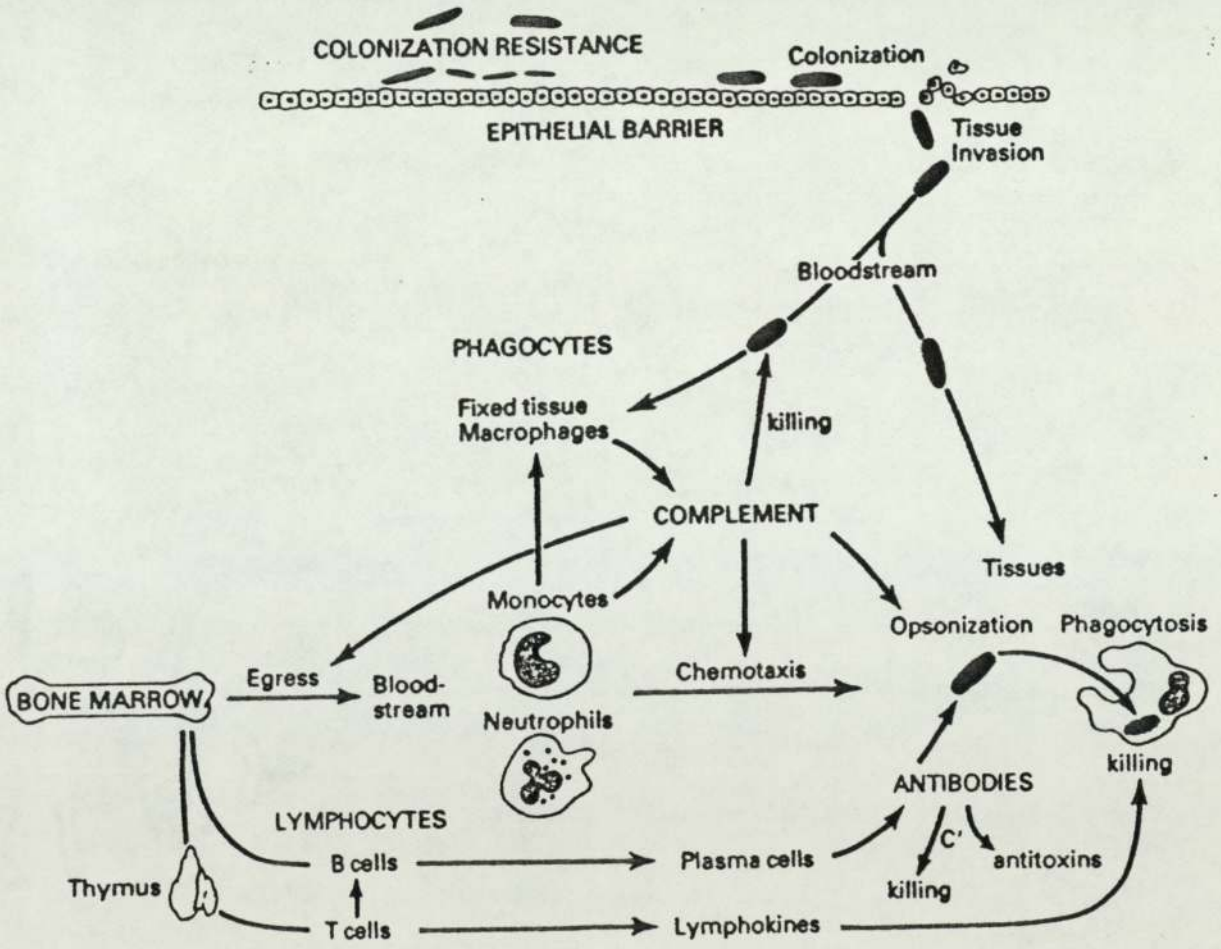
1.4.2 Cell-mediated immunity.

Host resistance to P.aeruginosa depends mainly on humoral immune phagocytic action (Homma, 1982) and cell-mediated immunity has not been thought to be important in P.aeruginosa infections. However, since T-lymphocyte cooperation is essential for many B-lymphocyte responses T-cells may be involved in the overall response. A recent study has shown that there is an antigen-specific proliferation of T-cells in response to challenge with P.aeruginosa (Porwoll et al, 1983), this response may be important in patients with burns and CF, who have high antibody titres which do not appear to improve the prognosis. (Aduan & Reynolds, 1979).

Host defences against P.aeruginosa are summarized in figure 1.5.

Figure 1.5

Host defences against P.aeruginosa



from Peterson,1979.

1.4.3 The role of iron in infection and host defence

Iron is a ubiquitous element which readily accepts or donates electrons; complexed with various proteins it serves as a catalyst in biological processes requiring oxidation or reduction and is an essential constituent of all living organisms (Neilands, 1981). At neutral pH in aerobic conditions iron is extremely insoluble and exists in the form of a ferric hydroxide polymer. The avidity of Fe^{3+} to polymerize is so great that the equilibrium concentration of free ferric iron cannot exceed about 10^{-18}M. This is too low to allow involvement of free Fe^{3+} in biochemical reactions (Aisen, 1977). Most forms of life have had to develop iron uptake systems to solubilize iron and deliver it in a usable form to their cells. With the possible exception of some Lactobacillus (Archibald, 1983), iron is an essential growth factor for all bacteria. Pathogenic bacteria equipped with an iron uptake system which sequesters iron more successfully than that of the host will have an advantage. Host mechanisms designed to deprive bacteria of iron while retaining their own access to the element will convey an obvious advantage to the host. This type of defence strategy has been termed nutritional immunity (Weinberg, 1978).

The body contains iron far in excess of the minimal requirements of bacterial growth, however, this iron is located intracellularly as ferritin, haemosiderin, haemoglobin and myoglobin and is relatively unavailable to the bacteria. The concentration of free iron in body fluids is only about 10^{-18}M Fe^{3+} (Weinberg, 1978; Bullen, 1981). The small amount of iron in body fluids is strongly bound to carrier and

transport proteins synthesised by the host. These proteins have association constants of about 10^{36} for iron. (Bullen, 1981; Griffiths, 1983). There are two types of iron-binding proteins in the human host, transferrin in blood and lymph, and lactoferrin in external sectetions and polymorphonuclear leukocytes (PMNLs) (Bullen & Armstrong, 1979). A closely related compound, ovotransferrin, occurs in avian egg white (Mazurier et al, 1983).

1.4.3.1 Host iron carrier proteins

Transferrin is a glycoprotein with a single polypeptide chain bearing two iron binding sites capable of reversibly binding one ferric iron each with the simultaneous incorporation of two bicarbonate ions, it is not a homogenous entity, at least 22 different genetic variants have been described with molecular weights between 77K and 80K (Werner et al, 1983). The molecular weight of the dominant allele in human serum is 79,570 (MacGillivray et al, 1983). As well as depriving invading bacteria of iron and thus preventing growth there is evidence that there is synergy between transferrin, lactoferrin, specific antibody and complement in the bactericidal activity of serum (Fletcher, 1971; Rivier et al, 1983).

Lactoferrin, which differs from transferrin in that it has a higher affinity for iron and will bind it at a lower pH (Aisen & Liebraman, 1972) is one of the components of the specific granules of PMNLs. Lactoferrin is not itself directly bactericidal but may maintain a low-iron environment within the acidic (pH 4) phagolysosome which is neccessary for the bactericidal function of other components. Evidence for this hypothesis comes from experiments in which the lactoferrin in PMLs was saturated with ferritin, resulting in the

survival and eventual rapid growth of phagocytosed P.aeruginosa (Bullen & Armstrong, 1979). The presence of lactoferrin is also neccessary for the generation of the oxidising hydoxyl radical within the PML (Ambruso & Johnson, 1981).

A rapid decrease in serum iron concentrations is observed during infection, inflammation and fever (Cartwright et al, 1946). Inflammation triggers synthesis of transferrin and ceruloplasmin (an iron transport protein) and induces hypoferraemia (Beumier et al, 1984). Leukocyte lactoferrin, released as a response to inflammation, is responsible for removing iron from the serum (Weinberg, 1978; Reiter, 1983). Release of lactoferrin, which can also be stimulated by bacterial endotoxin (Kampschmidt & Upchurch, 1962) binds iron in serum and the iron saturated lactoferrin is removed from the circulation by the macrophages of the reticuloendothelial system (Van Snick et al, 1974).

An iron-rich environment in the host, produced experimentally or by trauma or by medication with injected iron can saturate the normally partially saturated transferrins and exacerbate infections (Bullen, 1981; Griffiths, 1983; Van Asbeck & Verhoef, 1983; Finkelstein et al, 1983). Excess iron not only compromises the host by increasing the availability of iron for bacterial growth but also impairs the function of monocytes and granulocytes (Van Asbeck et al, 1984a,b) and macrophages and lymphocytes (Keown & Descamps-Latscha, 1983). However, iron-deficiency, as in anaemia, is also assocciated with an increased incidence of infection because of its adverse effects on the immune system (Van Asbeck & Verhoef, 1983).

1.4.3.2 Bacterial iron uptake

Neilands (1984) has differentiated iron uptake systems in microorganisms into 'high' and 'low' affinity categories. The low affinity uptake system is assumed to be widely distributed since deletion of the high affinity uptake system in many microorganisms is not lethal unless another ligand is introduced which binds iron in a form unavailable to the cell. In high affinity systems bacteria inhabiting environments low in free iron secrete metabolites known as siderophores which chelate iron and transport it in a usable form into the bacterial cell. Siderophores are small (M_r 500-1000) iron specific ligands whose biosynthesis is regulated by iron (Neilands, 1981). A number of siderophores have been described (Neilands, 1974,1984) the majority of which are phenolic or hydroxamic acid compounds. The phenolic compounds, or catechols, are derivatives of 2,3 dihydroxybenzoic acid and are all produced by bacteria. Hydroxamates are derivatives of hydroxamic acid and are produced by fungi, actinomycetes and some, like aerobactin, by bacteria (Neilands, 1981). Almost every aerobic or facultatively anaerobic bacteria examined has synthesized a siderophore from one of these two classes (Neilands, 1981). P.mirabilis does not synthesize phenolate or hydroxamate siderophores. α-hydroxyisovaleric acid which is capable of solubilising iron and supplying it to the bacteria has been proposed as the siderophore in this species (Evanylo et al, 1984). The best characterised siderophores are the phenolic enterochelin and hydroxamate aerobactin of Escherichia, Salmonella and Klebsiella sp. (figure 1.6). Enterochelin, a cyclic trimer of dihydroxy benzoyl serine, is the most effective iron chelating compound known, and has an association constant of 10^{52} for iron (Hartman & Braun, 1981).

1.4.3.2.1 Mechanism of bacterial iron uptake

All the genes involved in siderophore synthesis and iron transport in E.coli are regulated by one gene, designated fur (ferric uptake regulation) which is negatively controlled by iron concentration in the medium (Hantke, 1982). At a crucially low iron concentration siderophore is synthesized and secreted into the medium (Hartman & Braun, 1981). A ferric-siderophore complex is formed (enterochelin is able efficiently to remove iron from transferrins) and transported into the bacterial cell by way of a membrane receptor (Neilands, 1982). Inside the cell the ferric-enterochelin complex is hydrolysed to release the iron. The reduced iron is incorporated into the intracellular pool making it available to promote bacterial growth (Griffiths, 1983). Ferric-aerobactin is recycled and is thus more efficient in use than enterochelin (Braun et al, 1984). Excess iron may be stored and released for cellular metabolism during iron deprivation (Klebba, 1982).

Bacteria which rely on the iron associated with the iron-binding proteins for growth must not only synthesise efficient siderophores but must also produce receptors on the cell surface to take up the iron-siderophore complex (Neilands, 1982). The molecular weights of ferric-siderophores are within the exclusion limits of the water filled OM pores of enteric bacteria (M_r 600-700) but do not diffuse through them. The diffusion rate of the larger compounds may be too slow to support growth but the smaller compounds also will not diffuse even when present in high concentration (Braun, 1985). Receptors on the cell surface are probably a means of concentrating the scarce iron.

The best characterised high affinity uptake systems are in enteric bacteria (Braun et al, 1983). E.coli produces several new high

molecular weight proteins and a new 25K protein when grown under conditions of iron deprivation (Klebba, 1982), these are called iron-regulated membrane proteins (IRMPs). It is known that the 81K (FepA) protein is the receptor for ferric-enterochelin (Braun et al, 1983). The 78K protein (Fhu) is the receptor for a ferrichrome-type hydroxamate siderophores (Braun et al, 1983) as well as T1, T5 and ϕ 80 phages and colicin M (Osborn & Wu, 1980). Ferrichrome is a fungal hydroxamate siderophore which can be utilised by E.coli The OMP of M.W. 80.5K (FecA) is produced in iron restricted media conaining citrate and is thought to be part of a citrate transport system (Braun, 1985). FhuE and Iut are OM receptors for Fe^{3+}-coprogen and Fe^{3+}-aerobactin respectively (Braun et al, 1983; Hantke, 1983). E.coli harbouring colV plasmids induce a 74K protein involved in aerobactin uptake (Williams, 1979; Grewal et al, 1982). Two other high molecular weight protein, Fiu and Cir, are strongly expressed under iron-deprived conditions but have no known siderophore receptor function (Klebba, 1982; Braun et al, 1983). Apart from colV another plasmid mediated iron sequestering system has been described in the fish pathogen Vibrio anquillarum (Crosa et al, 1980; Crosa, 1984).

Elevated temperature, as a host defence in fever, considerably reduces the efficiency of iron uptake systems. Production of siderophore in Salmonella is reduced at 40°C compared with 36°C (Garibaldi, 1972). Enterochelin production has also been reported to be reduced in S.typhimurium at 42°C and the ability to transport Fe^{3+}-enterochelin reduced in both S.typhimurium and E.coli when compared with cells grown at 37°C (Worsham & Konisky, 1984).

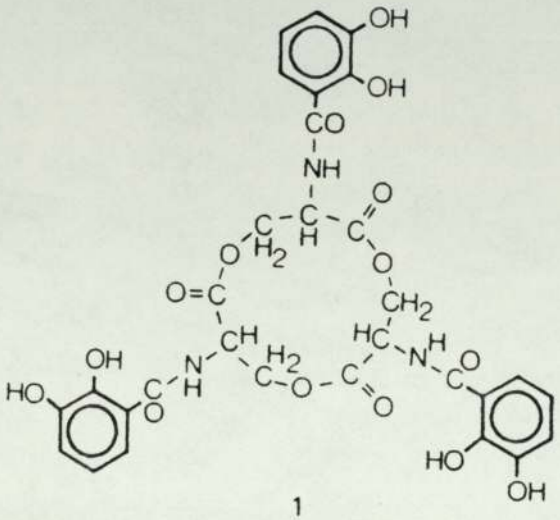
Enterochelin complexed with indium or scandium has been shown to have a significant therapeutic effect on experimental K.pneumoniae and E.coli infections in mice (Playa & Rogers, 1983; Rogers et al,

1984). These complexes are thought to act as specific anti-metabolites which interfere with transport of Fe^{3+}-enterochelin into the cell.

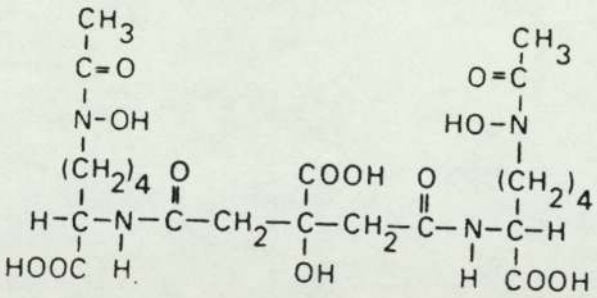
The possibility that iron can be removed directly from host transferrin by receptors on the bacterial cell without the participation of a siderophore has been suggested in Neisseria meningitidis (Archibald & DeVoe, 1979).

Figure 1.6

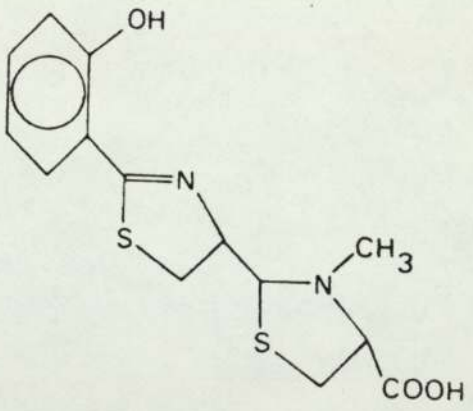
Structures of bacterial siderophores (Griffiths, 1983)



1



2



3

Key.

1. Enterochelin
2. Aerobactin
3. Pyochelin

1.4.3.2.2 Iron uptake in *P.aeruginosa*

Iron limitation in *P.aeruginosa* also induces production of a number of high molecular weight OM proteins (Meyer *et al*, 1979; Ohkawa *et al*, 1980) which are assumed to be involved in the uptake of iron because of their appearance in iron deprived conditions and their similarity in M_r (70,000-90,000) to the iron-binding proteins of the enteric bacteria. It cannot definately be stated that they are iron-binding proteins because the genes coding for these proteins have not been mapped as they have for *E.coli*. A low M_r (14K) iron-binding protein has also been reported in *P.aeruginosa* (Sokol & Woods, 1983).

P.aeruginosa produces a siderophore, pyochelin (Liu & Shokrani, 1978), which is synthesised when the organism is grown in medium containing $< 5 \times 10^{-6}$ added iron (Cox & Graham, 1979). When added to iron deficient cultures of *P.aeruginosa* pyochelin promotes its growth and also reverses growth inhibition caused by the iron chelator EDDA (Cox & Graham, 1979). *P.aeruginosa* can also utilise enterochelin as a siderophore, although it does not synthesise it, and ferric citrate (Cox & Graham, 1979). As with enterochelin synthesis of pyochelin and the mechanism of iron uptake appear to be controlled by iron concentration in the medium. Separate mechanisms may, however, control pyochelin synthesis and the formation of the transport mechanism (Cox, 1980). Pyochelin has been shown to increase the lethality of a virulent (but not an avirulent) strain of *P.aeruginosa* *in vivo* (Cox, 1982). It is postulated that pyochelin stimulation of growth *in vivo* allows the bacteria to express their pathogenic potential. Pyochelin may be an important virulence factor in Pseudomonas infections (Ankenbauer *et al*, 1985).

Pyoverdin, the yellow-green fluorescent pigment produced by P.aeruginosa when it is grown in iron-limiting conditions, has been shown to have siderophore activity (Cox & Adams, 1985). Pyoverdin is produced by other fluorescent Pseudomonads, notably P.fluorescens (Garibaldi, 1971; Meyer & Abdallah, 1978; Meyer & Hornsberger, 1978). Pyoverdin is reported to have a hydroxamate structure (Cox & Adams, 1985) very similar to that of pseudobactin, a siderophore from a plant rhizosphere Pseudomonad (Tientze et al, 1981). P.aeruginosa pyoverdin has been shown to bind Fe^{3+} and to stimulate growth of P.aeruginosa in human plasma and serum (Cox & Adams, 1985; Ankenbauer et al, 1985). It is not known why P.aeruginosa should produce both pyochelin and pyoverdin siderophores, it is possible that there is sequential derepression of siderophores under various degrees of iron deprivation. Cox & Adams (1985) report that they have isolated pyoverdin-negative strains from human infections but never pyochelin-negative strains. Pyochelin production may thus correlate better with virulence than does pyoverdin.

1.4.3.3 Iron and Virulence

A number of potentially pathogenic bacterial strains are unable to cause infection if the high affinity uptake systems are not functioning (Crosa, 1984). Jackson & Burrows (1956) first observed that wild type Yersinia pestis could utilize exogenous heamin but that mutants incapable of accumulating the compound were avirulent. Wake et al (1975) later showed a correlation between siderophore production and virulence in Y.pestis. The ability of S.typhimurium to grow in human serum and cause lethal infection in mice was shown to be dependent on the production of enterochelin (Hoseith & Stocker, 1981).

Virulence related to siderophore production in P.aeruginosa has been described above (Cox, 1982). Brown et al (1984) have demonstrated that mucoid P.aeruginosa directly isolated from the lungs of cystic fibrosis patients expressed IRMPs. Similar observations have been made in experimentally induced E.coli infections in the peritoneal cavities of guinea pigs (Griffiths et al, 1983) and Vibrio cholerae infection in the intestinal tract of infant rabbits (Sciortino & Finkelstein, 1983). It is noteworthy that pathogenic strains of E.coli produce considerably more of these proteins than does the non-pathogenic laboratory strain K12 under the same conditions (Griffiths & Humphries, 1978).

Other determinants of bacterial virulence are under the control of iron. Synthesis of P.aeruginosa toxin A is induced in conditions where iron is limiting and suppressed by addition of iron. (Bjorn et al, 1978, 1979; Sokol et al, 1982). Addition of iron to the medium also reduces the yield of other exoproducts of P.aeruginosa implicated in virulence such as protease and elastase (Bjorn et al, 1978; Ombaka et al, 1983). P.aeruginosa grown in a medium treated by iron exchange in a Chelex 100 column were more virulent in a chronic pulmonary infection model in rats (Sokol & Woods, 1984). These workers attributed the differences to the action of iron. Chelex 100 treatment of media lowers the content of constituents other than iron (Kadurugamuwa, 1985) so that micronutrients need to be added back to the treated media (Brown et al, 1984; Anwar et al, 1984; Kadurugamuwa, 1985) and this may have influenced the results.

1.4.4 Host defenses against UTI

Normal human urine is a suitable growth medium for many non-fastidious aerobic bacteria (Asscher et al, 1968). A number of non-specific mechanisms of host defence such as an inhibiting indigenous flora (Hentges et al, 1985) or secretion of lysozyme or lactoferrin are not usually present in the urinary tract (Asscher et al, 1968). Factors in urine which may inhibit bacterial growth are very high or low osmolarity, high concentration of organic acids and a low pH (Asscher et al, 1968). Urine is normally sterile and flushing of the lower tract every few hours during micturition prevents bacteria gaining access and becoming established (Van Furth, 1981). The major role of dilution and displacement of infected urine as a defence mechanism in UTI has been demonstrated by in vitro models (O'Grady et al, 1968; Greenwood & O'Grady, 1978). The flushing effect of micturition alone is probably not sufficient completely to clear infecting bacteria from the bladder since a film of urine containing bacteria remains adhering to the mucosa (Costerton et al, 1983). The finding that bacteria introduced into the bladder even in large numbers tend to clear spontaneously lead to the postulation of antibacterial mechanisms at the bladder mucosa whose function was to prevent adherence and inhibit bacterial multiplication and thus allow clearance by flushing (Hand et al, 1971).

A non-specific antibacterial role for surface mucin in the bladder has been proposed (Parsons et al, 1980). Antibodies present at the mucosal surface influence bacterial attachment which is a pre-requisite for many bacterial infections (Tomasi & Grey, 1972; Jones & Isaacson, 1983). In urine from patients with UTI antibodies of

the IgA class predominate but IgG antibodies are also commonly found (Sohl-Akelund et al, 1979). Antibodies of both types have been shown to be effective in blocking E.coli attachment in vitro (Svannborg-Eden and Svennerholm, 1978).

The presence of secretory IgA (sIGA) at mucosal surfaces and the fluids which bathe them is an important feature of the specific immune host defence (McNabb and Tomasi, 1981; Van Furth, 1981). A majority of the antibody that appears in external secretions is produced locally by plasma cells in the submucosal tissues (McNabb & Tomasi, 1981) although a direct transport of IgA from serum may occur (Sheldrake et al, 1984). sIgA differs from the major IgA species of serum in that it is a dimer composed of two IgA molecules covalently linked by a glycoprotein called the J-chain (Tomasi & Grey, 1972). The secretory component of secretory IgA stabilises the dimer and renders it more resistant to denaturation with agents such as organic acids and urea, and to proteolytic attack. This increased stability in the conditions likely to occur in external secretions may give a selective advantage to sIgA compared with other immunoglobulins. sIgA has no bactericidal activity except when complement and lysozyme are present (Van Furth, 1981). The mechanism of the antiadhesive effect of antibody is not well understood. It is possible that specific antibody may agglutinate bacteria or interact with receptors on bacteria or the epithelial cell. sIgA also inhibits antigen uptake by mucosa and neutralizes toxic exoproducts of bacteria (Van Furth, 1981; McNabb & Tomasi, 1981). Binding of sIgA has been shown to reduce the hydrophobicity of bacteria (Magnusson, 1979). Reduction in hydrophobicity may result in a reduction in adhesiveness of the bacteria.

1.5 Cell envelope structures of *P.aeruginosa* as antigens

The cell envelope of *P.aeruginosa* contains a number of macromolecules which may interact with host cells to produce an immune response (Pennington, 1979).

1.5.1 Lipopolysaccharide

LPS antigens confer O-serotype specificity (Chester *et al*, 1973) and there is evidence to indicate a shared core region among strains of *P.aeruginosa* (Chester *et al*, 1973; Wilkinson and Galbraith, 1975). Most systems for the serological classification of *P.aeruginosa* are based on the ability of antisera raised against heat-killed whole cell antigens to agglutinate bacteria. (Habs, 1957; Verder, 1961; Homma, 1976; Lanyi, 1966-67). The Fisher system (Fisher *et al*,, 1969), in contrast, uses a mouse protection assay to identify seven immunotypes which together cover more than 90% of clinical strains isolated in the United States. The antigenic determinants governing reactions in at least two of these typing schemes have been shown to be LPS (Fisher *et al*, 1969; Habs, 1957). This is to be expected in view of the heat-stable nature of the antigens used to elicit the response. Serotyping has been standardized in the International Antigen Typing Scheme (IATS; Bergan, 1975). The IATS is based on the 12 serotypes of Habs (1957) with some additions (Pitt & Erdman, 1977). This system defines 17 serotypes some of which may be subdivided by minor antigens to define a total of 21 identifiable groups. 90 - 95% of clinical strains encountered in British hospitals can be typed using this scheme (Pitt, 1980). Certain O-serotypes predominate, the two most frequent groups, O:6 and O:11, account for over 40% of all strains

typed (Pitt, 1980). Serotypes O:10 and O:16 account for a further 10% each and polyagglutinating (PA) strains , which are agglutinated by more than one serum, 5% (Pitt, 1980; Meadow et al, 1984). The IATS was used to O-serotype clinical isolates of P.aeruginosa used in this study.

The ability of LPS vaccines and antisera raised against LPS to protect has been evaluated in a number of animal models. Passive transfer of gamma globulin raised against heptavalent LPS vaccine provided high levels of protection against an intraperitoneal (i.p.) challenge (Fisher, 1977). Protection was serotype specific and virtually no protection was conferred by immune plasma given to moribund mice. These findings were confirmed in another study (Pier et al, 1981) which showed that antisera given as little as 3 hours post challenge was ineffective. Later studies by Pier (1982) and Cryz (1984a) have shown LPS to be highly immunogenic. However, in these studies low doses effectively gave protection only against organisms of an homologous O-serotype. Passive immunotherapy with LPS antisera gave high levels of protection in both mouse (Cryz et al, 1983a) burn wound models and a mouse model where mice had been rendered leukopenic by injection of cyclophosphamide (Cryz et al, 1983b). Antibodies to LPS in this study (Cryz et al, 1983a) gave far greater protection than did antibodies to exotoxin A or elastase. In another study (Tegtmeier & Anderson, 1983) passive immune therapy with LPS antisera provided minimal protection while significant protection was observed when passive transfer of antibody was combined with active immunisation. Passively transferred LPS antisera did not protect immune compromised dogs (Horvath et al, 1976) against lethal challenge with P.aeruginosa whereas active serotype-specific immunisation significantly improved survival (Horvath & Andersen, 1976).

The serotype specificity of protection conferred by LPS antigens means that to be generally effective against live bacteria a vaccine must contain a mixture of a wide variety of O-antigens. There are serious problems associated with the use of mixed LPS vaccines because of adverse side effects (Homma, 1982). The inoculating dose of the vaccine may be restricted by these side effects and make it difficult to achieve adequate antibody titres. In addition it is not desirable to use an endotoxin containing preparation in already debilitated patients. These disadvantages have prompted a search for less toxic immunising agents against P.aeruginosa and specifically for 'common antigens' from which a monovalent vaccine might be derived.

1.5.2 Polysaccharide

The outer layer of the cell envelope contains high M_r polysaccharides (PS) associated with cell wall LPS. A high M_r PS fraction separated from culture supernatants of P.aeruginosa evoked a protective immune response in mice i.p. (Pier et al, 1978) and burn wound (Pollack et al, 1984) infections. The purified PS antigen (Pier et al, 1983a) confers the same seological specificity as O-antigens of LPS in animals (Pier et al, 1981) and man (Pier & Thomas, 1983). PS differs from O-antigen in molecular weight and lacks the core-type sugars KDO, heptose and glucosamine (Pier et al, 1983a). PS is considerably less immunogenic than LPS (Pier, 1982) but because it is not toxic (Pier et al, 1978. Pier et al, 1981) could form the basis of a protective vaccine.

Acid treatment of P.aeruginosa LPS generates a low M_r polysaccharide fraction which by itself does not generate antibodies or resistance to infection in mice. When the low M_r polysaccharide was conjugated to bovine serum albumin, however, the conjugate vaccine

stimulated production of IgG reactive against LPS in an ELISA and protected mice challenged i.p. with P.aeruginosa (Tsay & Collins, 1984). Serum from immunised mice protected burned and normal mice challenged with an otherwise lethal inocula of P.aeruginosa. Mice immunised with the conjugate vaccine showed no evidence of toxicity or inflammation at the site of infection (Tsay & Collins, 1984).

The mucoid exopolysaccharide produced by some strains of P.aeruginosa , especially those isolated from sputa of CF patients is thought not to be immunogenic (Hoiby & Axelsen, 1973). An antibody response has, however, been demonstrated in rabbits immunised with whole mucoid organisms or purified mucoid exopolysaccharide (Pier et al, 1983b) and in patients with CF lung infection. Exopolysaccharide induces antibodies to an apparently common serological epitope in most strains although some strains also produced a type specific determinant. If these findings can be confirmed and are not due to contamination with LPS they may be useful in a vaccine to prevent colonisation of the respiratory tract with P.aeruginosa (Johanson et al, 1972).

1.5.3 OMPs

Several studies have indicated that OMPs are highly conserved in different strains of P.aeruginosa (Sadoff & Artenstein, 1974; Mizuno and Kageyama, 1978, 1979a,b; Mutharia et al, 1982). Furthermore, OMPs F, H2 and I have been shown to be antigenically related in a study of 20 strains (Mutharia et al, 1982). A monoclonal antibody specific for Protein H2 confirmed that a single antigenic site on this protein was present in the OMs of 25 P.aeruginosa strains (Hancock et al, 1982). Antibodies which react with P.aeruginosa OMPs have been demonstrated in experimental animal infections (Hedstrom et al, 1984) and in CF

patients colonised with P.aeruginosa (Fernandes et al, 1981; Brown et al, 1984). Proteins F and H2 have been shown to be exposed on the cell surface (Lambert & Booth, 1982) and may thus be good condidates for inclusion in a protective vaccine. Protein F separated from P.aeruginosa strain PAO1 OM by column chromatography or extraction from gels after SDS-PAGE succesfully protected mice from an i.p. challenge with PAO1 and also with two other strains of different O-serotype (Gilleland et al, 1984b). The protective ability was lost when the vaccine preparation was treated with papain, indicating that protection was due to the protein component and not to contaminating LPS. After immunisation with protein F mice showed an increase in antibody titre determined by ELISA. Administration of rabbit antisera raised to the protein F preparation protected mice against challenge with three times the LD_{50} of a heterologous serotype strain (Gilleland et al, 1984b). The three challenge strains of P.aeruginosa used in this study were not isolated directly from infection and their virulence or ability to cause clinical infection was not stated. In another study (Sawada et al, 1984) monoclonal antibodies to OMPs afforded poor protection in mice challenged with a virulent strain of P.aeruginosa i.p. or at a burn site.

Homma (1982) has described a protein normally complexed with LPS in the cell wall which he has isolated from toluene-lysed cells and named original endotoxin protein (OEP). Separated OEP is composed of protein (77%) and small amounts of polysaccharides, lipid, hexosamine and KDO. This material appears to be a common antigenic component of most serotype strains of P.aeruginosa and to have protective properties against infection due to all serotypes. Mice vaccinated with OEP from a single Pseudomonas serotype were protected from i.p. injections of heterologous serotypes. Rabbit antisera raised against

OEP also conferred passive protection to mice from infection with different P.aeruginosa serotypes (Abe et al, 1977). Passive protection of mice by OEP antisera has been confirmed in immunosuppresed animals (Homma, 1982). A vaccine consisting of OEP with toxoids of elastase and protease has been described (Okada et al, 1980). This vaccine gave significant protection against the same strain in a burn wound model and against a serotype 5 strain but not against two serotype 16 strains. Serotype 16 strains appear not to contain antigens cross-reacting with OEP (Okada et al, 1980).

1.5.4 Flagella and pili

Highly purified P.aeruginosa flagella preparations have been isolated and characterised (Montie et al, 1982; Montie & Stover, 1983). The efficacy of flagella antigens as protective vaccines has been tested in a burn wound model (Holder et al, 1982). Mice immunised with either of two flagella antigens were protected against challenge with live P.aeruginosa in the burn area. The protection afforded by immunisation was independent of O-seroytype of the challenge strain but was specific to H (flagella) serotype. Anti-flagellar antibody appears to immobilise the bacteria at the site of challenge and thereby reduces systemic infection.

Pili have been shown to mediate attachment of P.aeruginosa to epithelial cells of the respiratory tract (Woods et al, 1980). Attachment could be blocked by homologous anti-pilus antisera but not by antisera raised against pili from another strain. This indicates the existence of more than one pilus immunotype. There are problems associated with using Flagella and pili as protective antigens. There are a variety of serotypes, not yet characterized for pili (Woods et al, 1980). Furthermore flagella and pili are easily sheared and may

not be essential to the organism so that an antibody effect would not be bactericidal. Vaccines against flagella and pili may, however be useful in blocking mobility or attachment of bacteria if it can be established that these are determinants of virulence.

1.6 Current P.aeruginosa vaccines and immune sera

A number of P.aeruginosa vaccines have been described. The majority of these are based on cell wall products. The recognition of toxic P.aeruginosa exoproducts has also lead to the development of toxoid vaccines.

1.6.1 Whole cells

Two clinical trials have evaluated the efficacy of killed whole cell vaccines against P.aeruginosa infections. Feller & Pierson (1968) used a monovalent heat-killed vaccine prepared from a strain isolated in a burns unit. Burn wound patients were treated with a combination of active and passive immunisation with human antisera raised against the vaccine strain. Mortality in this study was reduced from 44% in a historical control group to 20% in the immunised group. Although immunotherapy improved survival part of the protection noted may have been due to improved patient management and antimicrobial therapy in the 8 year period of the study. Another trial of whole cell vaccine (Sachs, 1970) employed a multicomponent vaccine consisting of six formalin-heat-killed strains of P.aeruginosa, two strains of Staphylococcus pyogenes and staphylococcal toxoid to vaccinate 39 burn patients. A trend towards increased survival was noted in the vaccinated group compared with historical controls but the small number of vaccinated patients and the design of the trial did not allow firm conclusions to be drawn.

1.6.2 Heptavalent LPS vaccine (Pseudogen, Parke,Davis and Company)

Heptavalent LPS vaccine is a phenol extract of P.aeruginosa

described by Hannessian et al, (1971). The vaccine is based on the 7 protective antigens of the immunotyping system of Fisher et al, (1969). Chemical analysis of the vaccine shows it to be comprised primarily of cell wall LPS. The LPS nature of the vaccine was confirmed by treatment with protease, lysozyme or nuclease which does not adversely affect its protective capacity in animals. Human volunteers hyperimmunised with Pseudogen exhibit increased titres of antibody, predominantly of the IgM class. This is potentially important since IgG antibodies have been shown to offer better protection against experimental P.aeruginosa infection (Bjornsen & Michael, 1970). In addition IgG antibodies have a better half-life in serum (23 days) than IgM (5.1 days) and better penetration into mucous secretion (Pennington, 1979).

The vaccine has been used in several different patient populations at high risk of P.aeruginosa infection. Immunization in burn wound patients has been extensively evaluated over a period of 6 years (Alexander & Fisher, 1974) using various immunisation schedules and routes of administration. Overall mortality did not change during the 6 year period but mortality due to P.aeruginosa septicemia was considerably reduced. Unfortunately no control group was included in this study.

In the largest study of Pseudogen immunisation in cancer patients (Young et al, 1973) vaccination did not significantly decrease the incidence of P.aeruginosa infections but did offer limited protection against mortality due to P.aeruginosa infection. Mortality in bacteraemic cases was associated with pronounced neutropenia (< $1000/mm^3$) and a low titre of opsonic antibodies. In a smaller trial involving children with acute leukemia (Hagbin et al, 1973) vaccination was found to give no significant protection against

mortality due to P.aeruginosa infection. The rise in antibody titre in patients vaccinated in this study was low, predominantly IgM in nature and short-lived. In another study with leukemic patients (Pennington et al, 1975) antibody titres were higher but the limited number of patients treated did not allow the efficacy of the vaccine to be evaluated.

No clinical value of the vaccine could be demonstrated in CF patients (Pennington et al, 1975; Pennington, 1979). This is not perhaps suprising as the antibody titre in CF serum is already greatly elevated (Hoiby & Axelson, 1973. Hancock et al, 1984). The reasons for the persistence of infecting organism in the presence of high titres of circulating antibody are not clear. High titres of antipseudomonal antibodies in CF are associated with severe pulmonary disease and it is thought that alveolar damage is due to the formation of immune complexes (Hoiby & Schiotz, 1982).

In all studies of Pseudogen the antibody response was found to be primarily IgM and short-lived. Use of Pseudogen in human subjects has consistently caused local pain and systemic reactions such as fever, headaches and malaise. Burns patients tolerated the vaccine reasonably well and the reactions were considered acceptable (Alexander & Fisher, 1974) but reactions severe enough to limit the immunisation schedule occured in a substantial proportion of cancer patients.

1.6.3 Polyvalent Extract Vaccine (PEV-01, Wellcome Research Laboratories)

PEV-01 is a polyvalent vaccine containing cell wall extracts from 16 serotypes of P.aeruginosa (Miler et al, 1977). Each of the 16 vaccine components was prepared by extracting viable cells with EDTA-glycine for 2 minutes at 37°C. The aim of this extraction was to

eliminate toxic cell wall components thought to be responsible for the high incidence of side effects associated with the use of Pseudogen. The presence of cell wall LPS in the vaccine has not been directly determined but the method of extraction suggests that LPS is a component ((Wilkinson, 1983). Immunisation of mice with PEV-01 has been shown to give good protection against i.p. challenge (Miler et al, 1977). Guinea pigs vaccinated with PEV-01 (Pennington & Miler, 1979) survived an intratracheal challenge with P.aeruginosa better than unvaccinated controls in an experimental pneumonia. model. Histological examination of lung tissue also showed less tissue damage in vaccinated animals following infection. Experimental animal pneumonia does not mimic CF but these positive reports prompted prophylactic vaccination with PEV-01 in CF. A small number of children were involved in the trial, no significant differences were observed in the numbers colonised with P.aeruginosa or in the disease progress of vaccinated children and unvaccinated controls (Langford & Hiller, 1984)

A number of clinical trials of PEV-01 in burn patients have been undertaken. In two of the trials no difference in overall mortality was found between vaccinated and control groups (Jones et al, 1979). However, a large majority of deaths in these burn patients were not from bacterial sepsis and none were due to P.aeruginosa The results of a trial in India, where the risk of P.aeruginosa sepsis is high, were more encouraging. In unvaccinated adults the overall mortality was 40.6% with 6 deaths due to P.aeruginosa whereas in the vaccinated group mortality was 6.6% and no fatalities were due to P.aeruginosa (Jones et al, 1980). Children with burn wounds were included in this trial. None of those passively immunised with immunoglobulin from plasma of volunteers vaccinated with PEV-01 died while mortality in a

group of unimmunised controls was 21%. The Indian study cannot be compared directly with other vaccine trials (Alexander & Fisher, 1974) as the burn wound area was substantially smaller. Also, no topical burn wound therapy was used in the Indian trial. The use of topical therapy is a routine procedure in Western burn units. (Pruit, 1984).

PEV-01 is reported to be well tolerated (Jones et al, 1979,1980) but has not been compared directly with Pseudogen for incidence of adverse reactions.

A polyvalent vaccine consisting of extracts of the 24 serotypes of the Lanyi scheme (1966-67) has been evaluated in mouse protection studies (Merle et al, 1984). The vaccine was prepared using the same glycine EDTA extraction as PEV-1 (Miler et al, 1977). However, the cells were broken by homogenization during extraction and the authors claim a high protein content (25%). Mice given a single injection of polyvalent vaccine were protected against i.p. challenge with heterologous strains Response to the vaccine was shown to be largely IgG by ELISA. Passive immunization with the IgG fraction of immune serum protected against 10 of 12 challenge strains in normal mice and 3 of 6 challenge strains in leukopenic mice.

1.6.4 OEP

Enzootic pneumonia is an economically important pseudomonal disease of mink. A multi component vaccine consisting of toxoids of elastase and protease with the protein cell wall antigen OEP has been shown in a number of trials to prevent enzootic P.aeruginosa pneumonia (Homma, 1980). The multicomponent vaccine plus exotoxin toxoid has also been reported to reduce the incidence of mortality due to P.aeruginosa in patients with severe burns and to prevent P.aeruginosa

superinfection in patients with severe respiratory disease (Homma, 1982). The number of human clinical cases vaccinated with OEP multi component vaccine is too small to allow judgements to be made about its effectiveness.

1.6.5 Immunoglobulin prerarations

A number of investigators have shown that protection against experimental P.aeruginosa infection in mice can be conferred by passive immunisation with rabbit (Jones 1970) and human (Davis, 1975; Fisher, 1977) immune serum. Passive therapy in human P.aeruginosa infections, as in the study by Alexander & Fisher (1974), may be benificial in immune compromised individuals where there is an increased risk of P.aeruginosa sepsis and an inadequate response to active immunisation. Treatment of human infection with immune globulin has been frustrated by the need to use intramuscular preparations which limits the dose (Collins & Dorsey, 1984). Immune globulin for intravenous use has been prepared by reduction and alkylation of human IgG. Intravenous immune globulin protected mice when administered 3 hours after challenge with P.aeruginosa in a burn wound model (Collins & Dorsey, 1984) and also significantly enhanced the activity of tobramycin and carbenicillin (Collins & Dorsey, 1984). Some human plasmas have been found to contain abnormally high levels of IgG to P.aeruginosa LPS (Collins & Roby, 1984). Immune globulin prepared from this plasma was several fold more effective than normal human immune globulin in protecting burned mice from P.aeruginosa challenge (Collins & Roby, 1984). An S-sulphonated human gamma globulin preparation given passively protects mice against i.p. P.aeruginosa infection (Kamimura, 1984). The degree of protection conferred against

different strains by the sulphonated preparation in this study correlated well with agglutinin titres against formalised cells. The administration of immune antipseudomonal globulin may be useful as adjunctive treatment but has yet to be proved in human trials.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Preparation of glassware.

All glassware was rinsed in tap water, fully immersed in Extran 5% v/v (BDH Chemicals. Atherstne, Warwickshire.) and allowed to stand overnight at room temperature. It was then rinsed once in distilled water, once in 1% v/v HCl, six times in distilled water and twice in double distilled water before drying at 60°C. Glassware was sterilised by dry heat at 160°C for three hours.

2.1.2 Bacteria.

The laboratory organisms used in this study were Pseudomonas aeruginosa PAO1 (ATCC 15692), P.aeruginosa NCTC 6750 and P.aeruginosa ATCC 10662. Clinical isolates of P.aeruginosa were kindly collected from Selly Oak Hospital and Birmingham Accident Hospital by Dr.Malcolm Noy and Mrs. Catherine Webb (Microbiology Department, Selly Oak Hospital, Birmingham). Clinical isolates were also generously donated by Dr.W.Nichols (Bacteriology Department, John Radcliffe Hospital, Oxford), Dr Ty Pitt (Division of Hospital Infection, Public Health Laboratory Service, Colindale Avenue, London), Dr David Livermore (Department of Medical Microbiology, The London Hospital Medical College, London) and Dr.George Miller (Schering Corp., Bloomfield, N.J., USA.). Clinical isolates of P.aeruginosa are listed in table 2.1. Clinical strains were identified as P.aeruginosa by Gram stain, positive oxidase reaction, growth and pigment production on

Pseudomonas Isolation Agar (PIA)(Difco, West Molesey, Surrey) and growth on an agar isolation medium containing 15µg/ml of the antimicrobial agent 9-chloro-9-(4-diethylaminophenyl) 10-phenyl acridan (C-390) (Davis, 1983; Araj, 1984). C-390 was a generous gift of Norwich-Eaton Pharmaceuticals, New Jersey, USA.

Strains of P.aeruginosa were serotyped by slide agglutination (Pitt, 1980; Collins & Lynne, 1984) using Colindale typing sera (PHLS, Colindale Avenue, London)

All strains were maintained on nutrient agar slopes at 4°C and were subcultured at approximately monthly intervals.

2.1.3. Clinical material

Serum and infected urine from which P.aeruginosa, Escherichia coli, K.pneumoniae, Proteus mirabilis, and Proteus morganii were isolated was supplied by Dr. Stanley Silverman (Clinical Teaching Unit, General Hospital, Birmingham.). Serum and infected burn wound tissue from which P.aeruginosa was isolated was supplied by Dr. Kaya Alpar (Burns Unit, Birmingham Accident Hospital).

Table 2.1 *P.aeruginosa* strains: source, serotype and colonial morphology

Strain	Source	Colony type	Sero-type
Selly Oak Hospital			
CI4	urine	smooth	O:11
CI5	urine	smooth	PA
CI6	urine	smooth	PA
CI7		smooth	O:1
CI8		smooth	O:3
CI9		smooth	O:4
CI10		smooth	O:7
CI11		smooth	O:8
CI12		smooth	O:9
CI13		smooth	O:13
CI14		smooth	O:14
CI16		smooth	O:10
Birmingham Accident Hospital			
119X	burn	smooth	O:11
PANT	burn	smooth	O:11
2168	burn	smooth	O:11
PARH	burn	smooth	O:11
PAEW	burn	smooth	O:4
4496	urine	mucoid	NT
4696		mucoid	NT
4697		mucoid	NT
4786	blood	mucoid	O:6
4940	urine	mucoid	NT
Colindale			
0072		smooth	O:5d
0014		smooth	O:12
9841		smooth	O:14
9766		smooth	O:15
17TS		smooth	O:17
John Radcliffe Hospital			
WN8	urine	smooth	O:6
WN10	wound	smooth	O:12
WN12	trach	smooth	PA
WN14	urine	smooth	NT
WN15	urine	smooth	PA
WN32	sputum	smooth	O:10
WN34	trach	smooth	O:10
WN50	ulcer	smooth	O:6
General Hospital			
PAGS	urine	smooth	O:6
PACG	urine	smooth	O:11
PAGW	urine	smooth	O:6
The London Hospital			
Ps50	mutant	smooth	
799WT		smooth	O:2a/b
799/Z61	mutant	smooth	O:2a/b
Schering			
7506		smooth	NT
7511		smooth	NT
Laboratory Strains			
PAO1		smooth	PA
6750		smooth	PA
10662		smooth	O:2b

PA polyagglutinating

NT non-typeable

trach tracheotomy site

2.1.4 Chemically defined media (CDM).

Chemically defined growth media are described in table 2.2. Strains of Pseudomonas were grown in CDM 10 or CDM 12. CDM 10 is a defined medium designed by Noy (1982) for P.aeruginosa 6750 . CDM 10 was formulated so that the concentration of each essential nutrient was sufficient to allow exponential growth to a theoretical optical density of 10 (2.2.1.). CDM 10 was made isosmotic with serum (Ca. 300 m.osmols) by increasing the concentration of NaCl in the medium from 0.5mM to 5mM. P.aeruginosa has no requirement for sodium or chloride (Boggis, 1971).

In order to avoid the early onset of nutrient limitation in those studies comparing growth in iron sufficient and iron depleted media (Williams et al, 1984), CDM 10 was reformulated as CDM 12 by increasing the concentration of all ingredients except iron by a factor of x 1.2 and the concentration of iron by a factor of x 2 to a final concentration of 0.12mM.

Klebsiella strains were grown in the CDM 1 medium designed by Williams (Williams et al, 1984) to enable exponential growth of K.pneumoniae DL1 to an OD of 10 before the onset of stationary phase.

Chemicals used in the preparation of CDM were Analar grade or equivalent. The constituents of the media were sterilised separately by autoclaving for 15 minutes at 121°C.

Table 2.2 Chemically defined media.

Nutrient	Molar Concentration		
	CDM 10	CDM'12'	CDM 1
NaCl	0.5mM [d]	0.6mM	0.045mM
KCl	0.62mM	0.74mM	1.5mM
$K_2HPO_4.3H_2O$	3.2mM	3.8mM	
$(NH_4)_2SO_4$	40mM	48mM	2mM
NH_4Cl			25mM
$MgSO_4.7H_2O$	0.4mM	0.48mM	0.4mM
Glucose	40mM	48mM	35mM
MOPS [a]	50mM	60mM	
Na_2HPO_4/NaH_2PO_4 [b]			66.6mM
$FeSO_4.7H_2O$ [c]	0.062mM	0.12mM	0.02mM

a MOPS (Morpholinopropane sulphonic acid), buffered to pH 7.8 with NaOH

b Sodium phosphate buffer, pH 7.4. For Fe- CDM 1 the phosphate buffer was passed twice through a column of Chelex-100 ion exchange resin to remove iron.

c $FeSO_4$ solution was acidified with conc. H_2SO_4 to prevent precipitation when autoclaving.

d The molar concentration of NaCl in CDM 10 was increased from the origional 0.5 to 5mM to make the medium isosmotic with serum. CDM'12' was isosmotic with serum as formulated.

Iron depleted CDM (Fe- CDM) contained no added iron salts.

Magnesium limited CDM (Mg- CDM) contained 0.02mM added magnesium.

2.1.5. Complex media

Nutrient agar was obtained from Oxoid (London, S.E.1.). Pseudomonas Isolation Agar was obtained from Difco (West Molesey, Surrey) Both were made up according to the manufacturers instructions and sterilised by autoclaving at 121°C for 15 minutes. A single. batch of Drug Sensitivity Test (DST) agar of defined cation content was obtained from Oxoid and was made up according to the manufacturers instructions. DST used for antibiotic disc sensitivity testing (2.2.5.2.) was supplemented with calcium and magnesium to a final concentration of Ca^{++} 50mg/l and Mg^{++} 25mg/l (Reller et al, 1974). Supplementation was carried out by addition of sterile solutions of $CaCl_2$ and $MgCl_2$ to liquid DST after it had been sterilised by autoclaving but before it had set.

Unbound iron was removed from tryptone soy broth (TSB) (Lab M, London Analytical and Bacteriology Media Ltd., Salford, Lancs.) by passing it 5 times through a column of Chelex 100 ion exchange resin (Bio-Rad, Watford, Herts.) This treatment decreased its iron content by approximately 90% (Fe- TSB)(Kaduragamuwa, 1985). The Chelex 100 column was prepared by washing with 1M HCl (2 vols), distilled water (5 vols), 1M NaOH (2 vols.), distilled water (5 vols) and 0.7M sodium phosphate buffer pH 7.4 until the pH of the eluant was constant. Five-fold treatment with Chelex 100 removed all metal cation (Kadurugamuwa, 1985) and treated broth would not support bacterial growth. After treatment essential metal ions were replaced by addition of $MgSO_4$, 0.4mM and a micronutrient solution of essential metal ions

to a final concentration of $CaCl_2$, $5x10^{-7}M$; HBO_3,$5x10^{-7}M$; $CoCl_2$, $5x10^{-8}M$; $CuSO_4$, $1x10^{-8}M$; $ZnSO_4$,$1x10^{-8}M$; $MnSO_4$,$1x10^{-7}M$ and $(NH_4)_6$ Mo_7O_{24},5 x $10^{-9}M$. For iron-sufficient TSB (Fe+ TSB) $FeSO_4$ was added to a final concentration of 0.02mM.

2.1.6. Blood and Serum

2.1.6.1. Hyperimmune serum.

Antisera was raised in rabbits to whole cells grown to early stationary phase in (Fe- CDM). Cells were harvested by centrifugation at 5000 x g for 10 minutes at 4°C, washed in 0.85% saline and resuspended to a concentration of 10^9/ml. The cells were killed by exposure to formalin at a final concentration of 1% for one hour, washed twice with saline and again resuspended to a concentration of 10^9/ml. Killed suspension (1 ml) was injected intradermally into two young Half Lop rabbits (Ranch Rabbits, Crawley Down, Sussex) each week for 6 weeks. After 6 weeks the rabbits were bled by cardiac puncture. Blood was allowed to clot for 2 hours at 37°C then separated by centrifugation at 2000 x g for 5 minutes at room temperature. Supernatant serum from each pair of rabbits was pooled(Harboe & Ingeld, 1973) and stored at -20oC.

Mouse antisera was raised in adult Thailers Original mice (Bantin and King, Hull, Yorkshire) by intraperitoneal injection of 0.1ml of formalin killed bacterial suspension on alternate days for two weeks. After two weeks the mice were bled by cardiac puncture and serum separated as above.

2.1.6.2. Non immune human blood and serum.

Blood was taken by venipuncture from volunteers (5 female, 2 male; ages 19-47) with no history of infection due to P.aeruginosa. For antibacterial assays a portion of the blood was transferred to sterile heparin coated tubes and was used immediately. The remaining blood was allowed to clot at 37°C for 2 hours. The clotted blood was centrifuged at 2000 x g for 5 minutes at room temperature and the supernatant serum either used immediately or stored at -20°C.

Serum iron concentration and total iron binding capacity (TIBC) of the serum sample in which bacteria were to be grown for analysis of OMP profile (3.2) were measured by the Clinical Chemistry Department, General Hospital, Birmingham using the ferrozine method (Leggett & Crookes, 1972), and were 11μM and 47μM respectively. For iron sufficient serum 100μM iron was added as $FeSO_4$ to saturate TIBC and to make 6.4 x 10^{-3}M free iron available.

Serum heated to 56°C for 30 minutes to destroy complement was known as heat inactivated serum (HIS).

2.1.6.3. Patient's serum.

Patient's serum was separated from blood obtained by venipuncture during the course of clinical investigation. A small portion of the blood was allowed to clot at 37°C for 2 hours, then centrifuged at 2000 x g for 5 minutes and the supernatant serum collected and stored at -20°C.

2.1.7 Urine

Urine was collected from volunteers with no history of UTI and kept at 4°C. After 24 hours the collected urine was pooled and used immediately as a growth medium. For iron sufficient urine iron was added as $FeSO_4$ to a final concentration of 0.12mM.

2.1.8. Chemicals.

Chemicals and reagents used in the study and not specified in the text were supplied by :-

Acrylamide - Sigma Chemical Company, Poole, Dorset.

Amido black - BDH Chemicals Ltd., Poole, Dorset.

Ammonium Hydroxide solution - BDH Chemicals Ltd, Poole, Dorset.

Ammonium persulphate - BDH Chemicals Ltd., Poole, Dorset.

Antibody/Horseradish peroxidase conjugates

Rabbit anti-human IgG heavy chain - Dakopatts, Mercia Brocades, Weybridge, Surrey.

Goat anti-human IgA, IgG, IgM, heavy and light chain

Rabbit anti-mouse IgG, IgM.

Miles-Yeda; Rehovot, Israel.

Bovine serum albumin - Sigma Chemical Company, Poole, Dorset.

Bromophenol Blue - BDH Chemicals Ltd., Poole, Dorset.

Coomassie brilliant blue R-250 - Sigma Chemical Company, Poole, Dorset.

Citric acid - BDH Chemicals Ltd., Poole, Dorset.

4-chloro-1-naphthol - Sigma Chemical Company, Poole, Dorset.

Dimethylsulphoxide - Sigma Chemical Company, Poole, Dorset.

Formaldehyde 37% solution (formalin) - BDH Chemicals Ltd., Poole , Dorset.

Gentamicin was a generous gift of Roussel Laboratories Ltd. It was supplied as a sterile aqueous solution containing 2mg/ml gentamicin and was stored at 4°C.

Glycine (Biochemical grade) - BDH Chemicals Ltd., Poole , Dorset.

Lipopolysaccharide, E.coli O:111 - Sigma Chemical Company, Poole, Dorset. 2- mercaptoethanol - Sigma Chemical Company, Poole, Dorset.

N,N'- methylene bisacrylamide - Sigma Chemical Company, Poole, Dorset.

Morpholinopropanesulphonic acid (MOPS) - BDH Chemicals Ltd., Poole, Dorset.

N- lauroyl sarcosine (sodium salt) -Sigma Chemical Company, Poole, Dorset. Organic solvents - BDH Chemicals Ltd., Poole, Dorset.

Periodic acid - Fisons Laboratory reagents, Loughborough, Leics.

Polymyxin B was a generous gift of Wellcome Research Laboratories, Beckenham, Kent. Polymyxin was supplied as sterile vials of 500,000 units each and as non-sterile powder. Stock solutions of 100,000u/ml and 10,000u/ml were made up in sterile distilled water and stored at 4°C, solutions made from non-sterile powder were sterilised by filtration. Unused stock solutions were discarded after 14 days.

Phenol - Fisons Laboratory Reagents, Loughborough, Leics.

Polyoxyethylene sorbitan monolaurate (Tween 20) - Sigma Chemical Company, Poole, Dorset.

RPMI 1640 medium - Imperial Laboratories, Salisbury, Wilts.

Silver nitrate - Hopkin & Williams Ltd., Chadwell Heath, Essex.

Sodium dodecyl sulphate: specially purified for biochemistry - BDH Chemicals Ltd., Poole, Dorset.

3,3',5,5'- tetramethylbenzidine (TMB) - Miles-Yeda, Rehovot, Israel.

N,N,N',N'- tetramethylene diamine (TEMED) - BDH Chemicals Ltd, Poole, Dorset.

N,N,N',N'- tetramethyl-p-phenylene-diamine HCl - BDH Chemicals Ltd., Poole, Dorset.

Tris(hydroxymethyl)aminomethane (tris) - Sigma Chemical Company, Poole, Dorset.

2.1.9. Equipment.

Equipment and apparatus used in the study and not specified in the text was supplied by:-

Automatic pipettes - Gilson Pipetman, P-200 and P-1000: Anachem, Luton, Beds.

Blender - Kenwood Model A 524 blender, Fisons Scientific Apparatus, Loughborough, Leics.

Blood collecting tubes - Lithium heparin coated tubes & polypropylene stoppered tubes, Sterilin Ltd., Teddington, Middx.

Centrifuges

Beckman J2-21 high speed centrifuge,

Beckman-RIIC Ltd., High Wycombe, Bucks.

MSE Superspeed 50 ultra centrifuge

MSE bench centrifuge,

Measuring and Scientific Equipment, Crawley, Surrey.

Freeze Dryer - Edwards Modylo freeze dryer: Edwards High Vacuum Ltd, Crawley, Surrey.

Gel electrophoresis apparatus - made in house by Aston Services.

Gel dryer - Bio-Rad Model 224 gel slab dryer, Bio-Rad Laboratories Ltd., Watford, Herts.

Immunoblotting tank - Trans Blot Cell, Bio-Rad Laboratories Ltd., Watford, Herts.

Incubators

Mickle reciprocating water bath,

Cam Lab Ltd, Cambridge.

Gallenkamp orbital shaking incubator, Gallenkamp, London.

Membrane Filters - Gelman Acrodisc, Gelman Sciences, Brackmills, Northampton.

Microscopes - Wild model B 20 binocular phase contrast microscope, Micro Instruments Ltd., Oxford.

pH meter - PTI-15, Fisons Scientific Apparatus, Loughborough, Leics.

Power pack (electrophoresis) - Bio-Rad Model 500/200.

(blotting) - Bio-Rad Model 250/2.5, Bio-Rad Laboratories Ltd., Watford, Herts.

Rotary Evaporator - Buchi Rotavapor-R, Fisons Scientific Apparatus, Loughborough, Leics.

Sonicator - Dawe Soniprobe, Dawe Instruments Ltd., Acton, London.

Spectrophotometers

Unicam SP600, Pye-Unicam instruments, Cambridge.

Cecil CE292 Digital, Cecil Instruments, Cambridge.

Plastic cuvettes for spectophotometry, Brand, Gallenkamp, Loughborough, Leics.

Scanning UV Spectrophotometer - Unicam 8000, Pye-Unicam Instruments, Cambridge.

Quartz cuvettes for UV spectrophotometry,

Hellma, Westcliff-on-sea, Essex.

Syringes - Gillette Surgical, Isleworth, Middx.

Whirlimixer - Fisons Scientific Apparatus, Loughborough, Leics.

2.2 Experimental methods

2.2.1 Measurement of bacterial concentration

The concentration of bacteria in suspension was determined by measurement of the optical density (OD) of the suspension. At low cell concentrations the OD is directly proportional to cell concentration (Kenward, 1975). This relationship is expressed by the Beer-Lambert law

$$OD \propto \log(Io/I)$$

where Io = incident light

I = emergent light

At higher cell concentrations this relationship does not hold due to secondary scattering of light. It has been shown that the relationship obeys the Beer-Lambert law up to an OD of 0.3 (Kenward, 1975), above this absorbance the OD increases less than proportionally with increasing cell concentration. If the cell suspension is diluted with fresh media to an absorbance less than 0.3 proportionality is restored (Kenward, 1975). Measurements of OD were made at a wavelength of 470nm to minimise absorbtion by metabolic products of the bacteria such as pyocyanin. Normal saline was used as a blank. An optical density of 1 at 470nm indicates a concentration of $\sim 10 \times 10^8$ cells/ml (Anwar, 1981).

2.2.2 Growth measurements.

Growth of bacteria in CDM was measured by following the change in optical density of the medium with time. Inocula for growth experiments were prepared from a culture grown overnight in complete CDM. The overnight culture was harvested by centrifugation at 5000 x g for 10 minutes at room temperature and resuspended to an OD of 1.0 in CDM lacking glucose and 0.1ml of this suspension added to 20ml of pre-warmed medium in a 100ml conical flask. The flasks were agitated at a rate of 120 strokes/minute in a shaking waterbath at 37°C. Samples for measurement of OD were removed aseptically at appropriate intervals and were diluted where necessary. Undiluted samples were returned to the flask to prevent undue reduction in volume, diluted samples were discarded.

2.2.3 Magnesium depletion studies.

To determine the magnesium requirements of PAO1 the process described in 2.2.2. was repeated except that the inoculum, resuspended in media lacking glucose and magnesium, was added to a series of flasks containing graded concentrations of magnesium in CDM and the increase in OD of each flask measured with time. The magnesium requirement of PAO1 adapted to polymyxin resistance (2.2.8.1.) was determined in the same way in CDM containing 6000u/ml polymyxin

2.2.4 Osmolarity of CDM and serum.

Osmolarity was measured by a depression of freezing point method (Wallwork & Grant, 1977) using a Knauer osmolarity meter (Knauer Wissenschaftliche Gerate. Biotech Instruments, Caxton Hill, Herts.). Calibration curves (figure 2.1) were constructed by measuring the depression of freezing point produced by Knauer standard solutions of known osmolarity and the osmolarity of CDM and serum calculated from the curves.

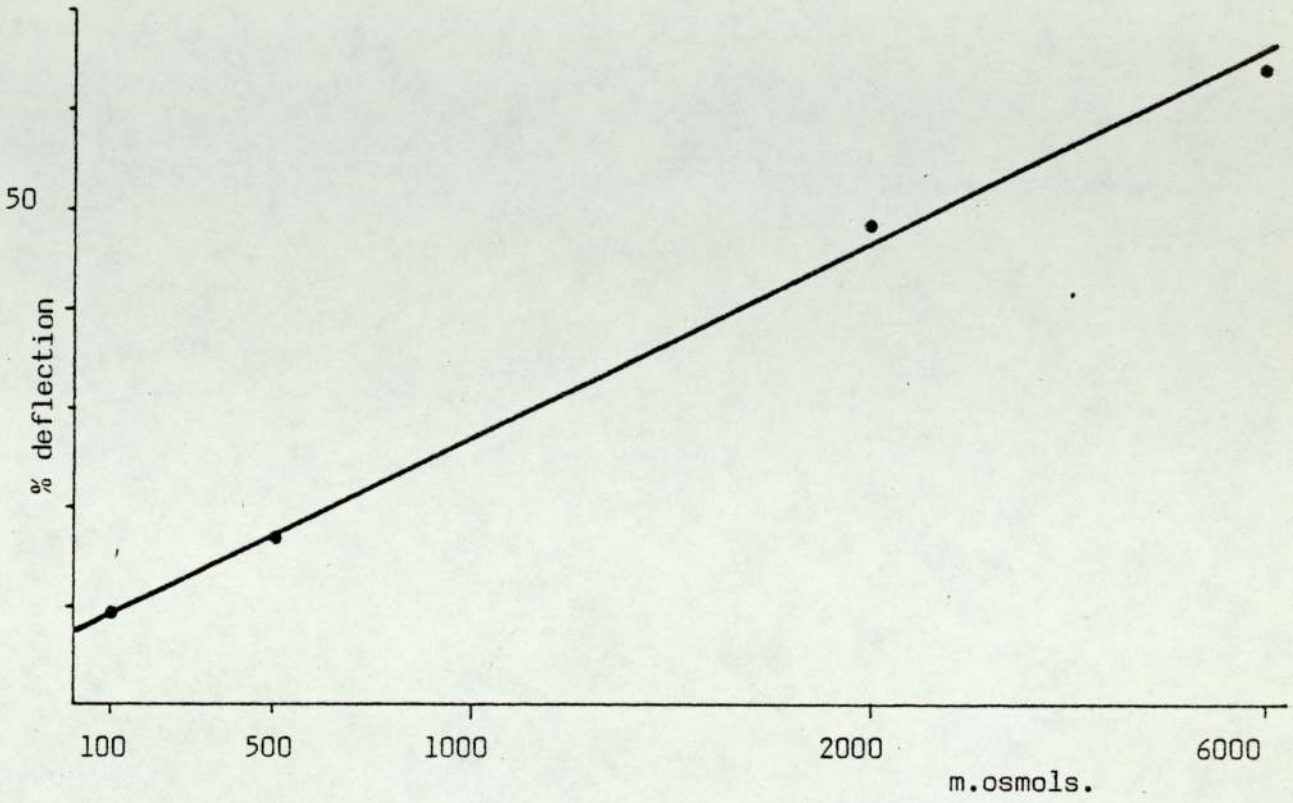


Figure 2.1

Calibration curve for the estimation of osmolarity by depression of freezing point.

2.2.5 Antibiotic assays

2.2.5.1 Minimum inhibitory concentration (MIC).

MICs were determined by a tube dilution method (Washington, 1974). Tubes containing a series of dilutions of the antibiotic in 5ml CDM were inoculated with a fixed inoculum of 10^5 bacteria. The lowest concentration of antibiotic which resulted in inhibition of bacterial growth (as judged by absence of visible turbidity) after 24 hours incubation at 37°C was considered to be the MIC. All determinations were carried out in duplicate, drug free and sterility controls were included in each determination.

2.2.5.2 Antibiotic Disc sensitivity.

Antibiotic sensitivities were determined by an agar diffusion method (Brown & Blowers, 1978) using Neo-sensitab antibiotic impregnated discs (A/S Rosco; Taastrup, Denmark.). Sensitivity testing was carried out according to the manufacturers instructions (Casals & Pedersen, 1975) using an inoculum size sufficient to produce semi-confluent growth on the surface of freshly prepared test plates of DST agar supplemented with Ca^{++} and Mg^{++} (2.1.5.). Test plates were filled to a uniform depth of 4mm (Barry & Fay, 1973) and discs were placed in the same relative position on the plate for each assay

(Cunningham & Flournoy, 1983). Plates were kept for one hour at 4°C before incubation to ensure uniform diffusion of antibiotic from the disc in the absence of bacterial growth (Brown & Blowers, 1978), then incubated at 37°C for 24 hours. The diameter of the zone of inhibition round each tablet was measured with calipers after 24 hours.

2.2.6 Determination of metal cation concentration by atomic absorbtion spectophotometry.

Metal cation concentrations in urine and media were determined by atomic absorption using a Perkin-Elmer type 360 SG flameless atomic absorbtion spectrophotometer (AAS) fitted with deuterium background corrector and HGA-74 graphite furnace (Perkin-Elmer, Beaconsfield, Bucks.). A series of standard solutions of iron, manganese, calcium and magnesium in triple glass distilled water was made using stock solutions (BDH Chemicals Ltd., Poole, Dorset) and a calibration curve constructed for each metal cation. Calibration curves were constructed the same day as the analysis of samples was to be carried out. Determinations were carried out at least in duplicate. Peak heights were averaged and cation concentrations calculated from the calibration curves. The calibration curve for iron is shown in figure 2.2. and instrumental conditions for AAS in table 2.3.

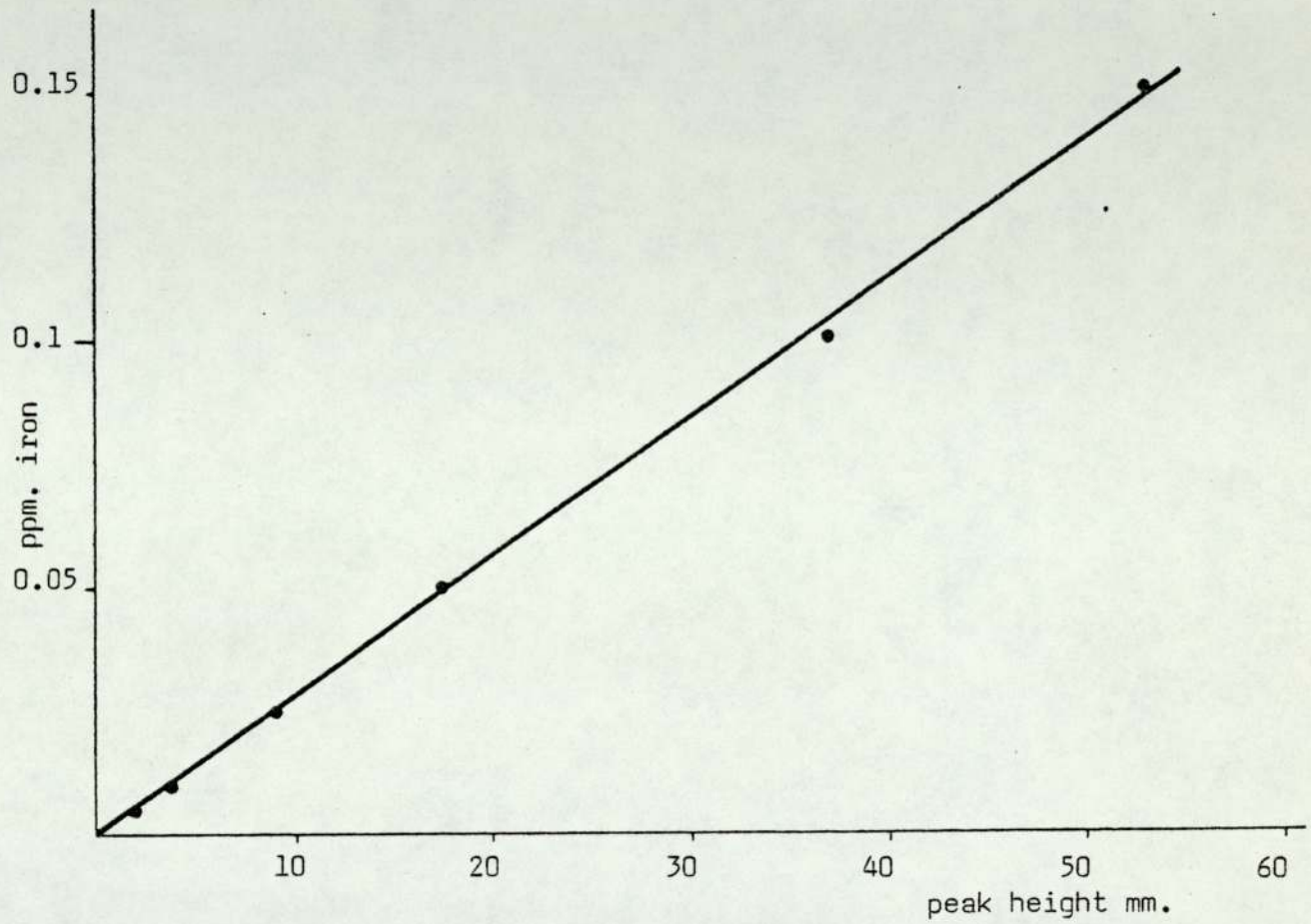


Figure 2.2

Calibration curve for the estimation of iron by AAS.

Table 2.3

Instrumental conditions for the estimation of metal cation concentrations by atomic absorbtion spectophotometry.

	Calcium	Iron	Magnesium	Manganese
Wavelength, nm	422.7	248.3	285.2	279.5
Spectral slit width, nm	0.7	0.2	0.7	0.2
Source	Ca hollow cathode lamp	Fe hollow cathode lamp	Mg hollow cathode lamp	Mn hollow cathode lamp
Charring temperature, ^{o}C	1100	1100	1200	1100
Atomising temperature, ^{o}C	2700	2700	2700	2700

2.2.7. Biological Assays

2.2.7.1. Serum resistance of bacteria.

Strains of *P.aeruginosa* chosen to represent the common O serotype strains (Pitt, 1980) were subjected to a preliminary screen for serum resistance by mixing 0.1ml of a suspension of 10^5 organisms/ml with 0.9ml of fresh serum in sterile stoppered polypropylene tubes and incubating at 37°C. Serum resistant strains were defined as those which showed growth at 24 hours as judged by visible turbidity. Where there was no visible growth, 0.1ml of the mixture was plated out onto nutrient agar and incubated for a further 24 hours at 37°C. Those strains which showed no growth or only isolated colonies after 24 hours were defined as serum sensitive.

2.2.7.2. Antibacterial activity of blood and serum.

Bactericidal activity was measured using the method of Jones *et al*, (1979) as modified by Williams *et al*, (1983). 0.9ml of fresh heparinized blood (or serum in serum killing experiments) and 0.1ml of bacterial suspension were placed in a sterile stoppered polypropylene tube and the tube shaken to ensure complete mixing. A 0.1ml sample was immediately withdrawn (time 0). The sample was mixed with 0.9ml of sterile distilled water which osmotically lysed blood cells, destroying their bactericidal activity, but did not impair the viability of the bacteria (Jones *et al*, 1979). Five tenfold dilutions

of the sample were made in sterile distilled water and the number of bacteria in the diluted sample counted by the method of Miles and Misra (1938). The remaining mixture was incubated in a shaking water bath at 37°C and further 0.1ml samples withdrawn and counted at 30 minute intervals for 90 minutes. The bacterial counts at each 30 minute interval were expressed as a percentage of the original count and plotted against time.

2.2.7.3 Mouse intraperitoneal infection model

2.2.7.3.1. Virulence of P.aeruginosa

The virulence of a number of clinical isolates of P.aeruginosa grown in iron sufficient and iron depleted conditions was determined in a peritoneal infection model in mice (Cleeland & Grunberg, 1980).

20g Porton Laboratory mice (PHLS, Porton Down, Wiltshire) were challenged by intraperitoneal injection of organisms of O serotypes O:6, O:10, and O:11, and a polyagglutinating (PA) strain. Strains used in the virulence study had previously been screened to determine their sensitivity to serum (2.2.7.1)

Inocula were prepared by growing challenge strains to logarithmic phase in either Fe+ or Fe- CDM 12. Cells were harvested by centrifugation at 5000 x g for 10 minutes at room temperature and resuspended in 0.85% saline to graded concentrations between 10^6 and 10^8/ml. 0.5ml of each concentration was injected immediately after resuspension into the peritoneal cavity of each of six mice. The mice were returned to their cages and allowed food and water ad lib. The animals were observed at 24 hour intervals for 4 days and the number of mice alive in each group of six noted. As a control to ensure that any death occurring was due to infection and not toxicity induced by the large number of bacteria injected (endotoxic shock) each graded concentration of Fe- inocula was divided into two portions one of which was injected immediately and the other killed by heating to 80°C for 30 minutes. 0.5ml of each concentration of the heat killed cells was used to inoculate another group of six mice as above.

Data were analysed and the 50% lethal dose (LD_{50}) calculated by the method of Litchfield and Wilcoxon (1949).

2.2.7.3.2. Antibody response in mouse intraperitoneal infection model.

To determine the nature of the antibody response in mice infected via the intraperitoneal route the viscera were removed from one group of four mice which were dead at 24 hours after a challenge with 8 x 10^7 P.aeruginosa WN50. A group of mice which had survived a challenge with 8 x 10^6 of the same organism were sacrificed after 4 days and their viscera also removed. The viscera from each group were homogenised in a blender. The homogenate was freeze dried, resuspended in 100ml of tris buffered saline (0.85% saline/10mM tris. pH 7.4)(TBS) containing 0.3% Tween and reacted with separated OMPs of P.aeruginosa WN50 which had been electrophoretically transferred to nitrocellulose paper (2.2.9.5.). Anti-mouse IgG or IgM were used as second antibody. A suspension of freeze dried viscera from two Porton Laboratory mice which had not been exposed to pseudomonas infection were use as a control. Hyperimmune serum raised in mice (2.1.6.1.) was used as a positive control.

Animal work was carried out at the Vaccine Research and Production Laboratory, Porton Down by Nigel Bailey and Shaun Baker.

2.2.8. Preparative techniques.

2.2.8.1 Training of P.aeruginosa to polymyxin and to gentamicin resistance.

P.aeruginosa was trained to Px resistance in CDM by serial subculture in increasing concentrations of Px (Brown & Tomlinson, 1979). A series of tubes containing increasing Px concentrations were inoculated with bacteria and incubated at 37°C. The ratio of inoculum size to volume of CDM was standardised using a fixed inoculum of 10^7 cells and a fixed volume of 5ml of CDM 10 containing Px. Cells from the tube containing the highest concentration of Px permitting growth after one week were used as the inoculum for the subsequent series. All steps were carried out in duplicate, drug free and sterility controls were included at each step. A sample of cells growing at the highest concentration at each step were diluted in CDM and an inoculum of 10^2 cells subcultured into 10ml of CDM 10 containing the appropriate concentration of Px and designated as stores. Stores were kept at room temperature and subcultured monthly into fresh CDM containing Px.

The training procedure described above was repeated using gentamicin instead of polymixin.

The OMP and LPS profile of resistant organisms produced in this way was analysed by gel electrophoresis and immunoblotting (2.2.9.2 and 2.2.9.5).

2.2.8 2. Isolation of bacteria directly from infected urine.

Urine was collected from hospitalised patients with UTI every day for 5 days during hospitalisation and stored at 4°C. Refrigeration at 4°C results in essentially static bacterial counts in infected urine (Traub et al, 1983). Microbiological analysis of the infected urine was carried out according to the criteria of Cowan & Steel.(Cowan, 1974). The urine from each patient was coarse filtered to remove cell debris. Material retained by the filter was washed with 0.85% saline and the filtered washings added to the bulk of the filtered urine. The filtered urine from each patient was pooled and bacteria harvested by centrifugation at 3000 x g for 10 minutes.

To determine whether storage in urine at 4°C affected OMP profiles a strain of K.pneumoniae, isolated from urine was cultivated for 24 and 72 hours in urine from a healthy volunteer. Half the bacteria in each culture were stored at 4°C, the other half were harvested and OMs prepared immediately. After 5 days the cultures stored at 4°C were also harvested and OMs prepared. SDS-PAGE analysis of the OMPs (figure 2.3) showed no differences between the fractions harvested immediately and those stored at 4°C.

Supernatant urine for chemical and immunological determinations was stored at -20°C or was concentrated by freeze drying and resuspending in a smaller volume of distilled water or buffer. Concentrated urine was stored at -20°C.

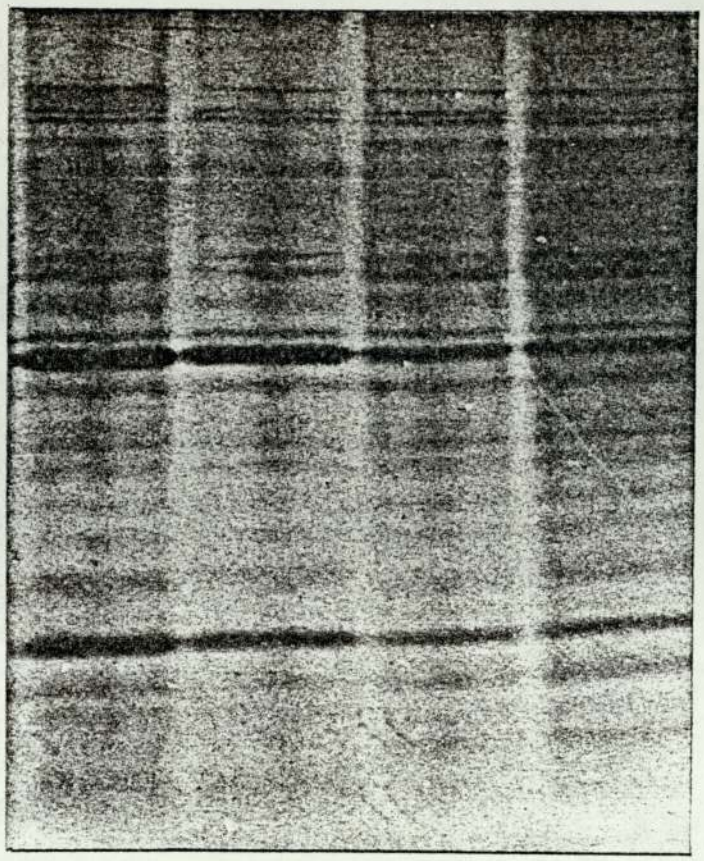


Figure 2.3

OMP profiles of K.pneumoniae grown in vitro in urine for 24 and 72 hours. OMs were prepared from a portion of the cells harvested at 24 and 72 hours (lanes 1 and 3) and from the remainder of the cells after storage for 5 days at $4^{o}C$ (lanes 2 and 4).

2.2.8.3. Preparation of outer membranes.

Outer membranes were prepared by the Sarkosyl method of Filip et al (1973). Cells grown in an orbital shaking incubator at 37°C were harvested by centrifugation at 5,000 x g for 10 minutes at 4°C. The cells were washed twice with 0.85% saline at 4°C by centrifugation and the resultant pellet resuspended in 20ml of distilled water. The cells were broken by sonication in an ice bath for 10 x 1 minute periods separated by 30 second intervals to allow cooling. Sarkosyl (N-lauroyl sarcosine) was added to the broken cell suspension to a final concentration of 2% and the mixture incubated at room temperature for 30 minutes. Sarkosyl solubilizes the CM of P.aeruginosa but leaves the OM intact (Lambert & Booth, 1982). The suspension was then spun at 3,000 x g for 10 minutes to remove unbroken cells and the supernatant centrifuged at 38,000 x g for 40 minutes at 4°C. The supernatant containing solubilised CM was removed and the pellet, consisting of OMs, resuspended in 1ml of distilled water. OM preparations not used immediately were stored at -20°C.

2.2.8.4. Isolation of flagella.

Flagellar preparations were isolated from a number of strains of P.aeruginosa using a modification of the method of Montie and Stover (1983). Two litres of culture grown for 48 hours in Fe-CDM 10 in a slowly rotating orbital incubator at 37°C, were harvested by centrifugation at 5000 x g for 10 minutes at 4°C. The cells were resuspended in 100ml of phosphate buffered saline (8.1mM Na_2HPO_4, 1.5mM KH_2PO_4, 140mM NaCl, 2.7mM KCl. pH 7.4) (PBS) then agitated vigorously in a blender to shear the flagella. Flagella were isolated by a process of differential centrifugation. The suspension from the blender was centrifuged first at 5000 x g for 15 minutes at 4°C to remove whole cells then the supernatant centrifuged at 38,000 x g for a further 4 hours at 4°C to separate flagella. The alternation of low and high speed centrifugation was repeated until a water clear pellet was obtained. The purity of the flagella was determined by gel electrophoresis which resulted in a single band of M_r about 55K. Optimal shearing time as determined by relative purity on polyacrylamide gels was 10 seconds.

Strains from which flagella were separated were H serotyped by Dr.Ty Pitt, PHLS, Colindale Avenue, London (Pitt, 1981).

2.2.8.5. Extraction and purification of lipopolysaccharide.

LPS was extracted by the phenol-water method of Westphal & Luderitz (1952). Two litres of culture grown to early stationary phase in an orbital shaking incubator at 37°C were harvested by centrifugation, the cells were washed twice with distilled water, resuspended in 175ml of distilled water and the suspension warmed to 65°C. An equal volume of 90% w/v aqueous phenol at the same temperature was added and the mixture stirred vigorously in a large beaker until glutinous strands of DNA had dispersed. The temperature was maintained at 65°C in a water bath throughout this procedure. The mixture was cooled rapidly by immersing the beaker in crushed ice then centrifuged at 3,000 x g for 30 minutes to permit phase separation. The upper aqueous phase containing LPS was removed with a pipette, care being taken not to disturb proteinaceous material at the interface and dialysed against tap water for 24 hours to remove phenol. Magnesium chloride was added to the non-diffusible material to a final concentration of 50mM and the resultant solution spun at 100,000 x g for 4 hours.

RNA, DNA, and protein were removed from the crude LPS pellet by the method of Hasin et al (1975). Crude LPS was suspended in 0.01M Tris/acetate buffer, pH 7.5 containing 5mM magnesium sulphate. Ribonuclease (Bovine pancreas type 1-AS. Sigma Chemical Company, Poole, Dorset) and deoxyribonuclase (Bovine pancreas type III. Sigma Chemical Company, Poole, Dorset) were added in quantities of 10 µg/mg crude LPS. The mixture was incubated at room temperature for 24 hours in the presence of 1mM sodium azide. Protease (Strep.griseus type XIV.

Sigma Chemical Company, Poole, Dorset) was then added and incubation continued for a further 24 hours at room temperature. The solution was again centrifuged at 100,000 x g for 4 hours and the clear viscous pellet of pure LPS resuspended in 1ml of distilled water. The absence of nucleic acids and protein in the purified product was confirmed by absence of UV absorbtion at 260 and 280nm. Purified LPS was stored at -20°C.

2.2.9. Analytical Techniques.

2.2.9.1. Fatty acid analysis.

Fatty acids were extracted from bacteria by the alkali hydrolysis method of Moss (1978) and were analysed by gas liquid chromatography (GLC). Cells grown to stationary phase in a shaking incubator at 37°C were harvested by centrifugation at 5000 x g for 10 minutes, washed twice in 0.85% saline and resuspended in 2.5ml 5%NaOH/50% aqueous methanol. Fatty acids were saponified by heating the mixture to 100°C for 30 minutes in screw top culture tubes with teflon-lined caps (Sterilin, Teddington, Middx.). The saponified mixture was allowed to cool and adjusted to pH2 with concentrated HCl. The fatty acids were derivatised to methyl esters by addition of 2.5ml boron trifluoride methanol complex (14% boron trifluoride, BDH Chemicals Ltd., Poole, Dorset) and heating to 80°C for 5 minutes. Fatty acid methyl esters were extracted from the mixture with 5ml chloroform/petroleum ether 60-80 (1:4). The solvent layer containing the fatty acid methyl esters was separated in a separating funnel and evaporated to dryness under vacuum in a rotary evaporator. The residue was redissolved in a small quantity of petroleum ether and the fatty acids analysed by GLC.

GLC conditions

Column - 10ft x 2mm I.dia. x 1/4 in. O.dia glass packed with 3% SP-2100 DOH on 100/120 Supelcoport (Supelco

Chromatography supplies, Supelchem, Sawbridgeworth, Herts)

Column temperature - 150-225°C at 2°C/min increases

Gas pressures - Hydrogen 14.5 psi.

Air 6.5 psi.

Sample size - 2 microlitre

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

2.2.9.2. Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE)

Whole cell or OMP preparations were separated by gel electrophoresis using the method of Lugtenberg et al (1975) as modified by Anwar et al (1983b). Polyacrylamide gels were prepared according to the schedules in table 2.4. Solutions were mixed in a 250ml beaker and stirred with a magnetic stirrer. Polymerisation was started by the addition of NNN'N'-tetramethylethylene diamine (TEMED) and the solution poured between glass plates separated by 1mm plastic spacers where it formed a gel. Samples were denatured before loading onto the gel by heating at 100°C for 10 minutes with an equal quantity of sample buffer (table 2.4).

Both electrode buffers contained 0.025M Tris, 0.19M glycine and 0.1% pure SDS, pH 8.3.

Samples were routinely analysed on 15% gels which allow complete separation of the major OMPs of P.aeruginosa and the Enterobacteriaceae used in this study. 10% gels, which allow greater separation of high molecular weight proteins at the expense of reduced separation at lower molecular weights, were used in some cases to

improve separation.

Electrophoresis was carried out using a constant current of 40mA and stopped when the tracking dye (bromophenol blue) had moved 10-12cm from the top of the running gel, usually after 4-5 hours. Gels were stained for protein overnight in a solution of 0.1% coomassie brilliant blue in 50% methanol-10% acetic acid, then destained in 5% methanol-10% acetic acid.

Molecular weights of proteins separated by SDS-PAGE were estimated by comparison with protein standards of known molecular weight. The protein standards used were phosphorylase A (97.4K), bovine albumin (66K), egg albumin (45K), pepsin (34.7K), trypsinogen (24K) and lysozyme (14.3K)

Destained gels were photographed using diffuse light from below, or were scanned with an LKB 2202 Ultrocsan Laser Densitometer run by an Apple II Europlus computer (Gelscan software: LKB Instruments Ltd., Croydon, Surrey). Completed gels were dried for storage.

2.2.9.3. Characterisation of LPS.

Purified lipopolysaccharides were separated by electrophoresis on polyacrylamide gels as described by Jann et al (1975). LPS preparations were subjected to SDS-PAGE using a 15% separating gel and buffer systems similar to that used for separation of OMPs (table 2.4) but with the addition of 4M urea in both stacking and running gels (Tsai & Frasch, 1982). Electrophoresis was carried out at a constant current of 20mA for 6 hours. The gel was stained by the silver stain method of Tsai & Frasch (1982).

Table 2.4 Gels and denaturing buffer for sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE)

Ingredient	Running gel		Stacking gel	Denaturing Buffer
	10%	15%	5%	
Stock 1	12.5ml	18.75ml		
Stock 2			5ml	
10% SDS soln.	1.5ml	1.5ml	0.3ml	5ml
1.5M tris [a] pH 8.8	18.75ml	18.75ml		
0.5M tris pH 6.8			7.5ml	2.5ml
Dist. H_2O	26ml	20ml	16ml	5ml
TEMED [b]	0.14ml	0.14ml	0.08ml	
10% APS [c]	0.2ml	0.2ml	0.1ml	
Glycerol				2.5ml
2-mercapto-ethanol				0.25ml
5% bromphenol blue				0.2ml

Stock 1 - 44G acrylamide + 0.8G N,N'- methylenebisacrylamide (bis) in 100ml distilled water.

Stock 2 - 30G acrylamide + 0.8G bis in 100ml distilled water

a - Tris (hydroxymethyl) aminomethane

b - N,N,N',N'- tetramethylene diamine

c - Ammonium persulphate, prepared daily.

2.2.9.4. Silver stain of LPS.

LPS was fixed in the polyacrylamide gel by immersion overnight in a 40% IMS-5% acetic acid solution. The fixing solution was replaced by fresh fixing solution containing 0.7% periodic acid and oxidation allowed to procede for one hour. The gel was washed in copious quantities of double distilled water to remove unreacted periodic acid then drained and placed in 150ml of staining reagent in which it was agitated for 45 minutes. Staining reagent was prepared by adding 5ml of 20% silver nitrate solution to a mixture of 2ml conc. ammonium hydroxide and 28ml of 1N NaOH. A transient brown precipitate formed which disappeared with mixing, the volume of staining reagent was made up to 150ml with double distilled water. After staining the gel was again washed with copious quantities of double distilled water. The stain was developed with a solution containing 50mg of citric acid and 0.5ml 37% formaldehyde/litre double distilled water. LPS in the gel is stained brown, the colour developing in 2-5 minutes. Development was terminated when the stain had reached the desired intensity, or when the clear background began to discolour, by replacing the developing solution with 40% IMS-5% acetic acid solution. Gels were photographed and dried as before.

2.2.9.5. Immunoblotting.

OMPs separated on polyacrylamide gels were transferred to nitrocellulose (NC) paper (Trans Blot Membrane: Bio-Rad Laboratories Ltd; Watford, Herts.) and antigenic sites visualised by a modification

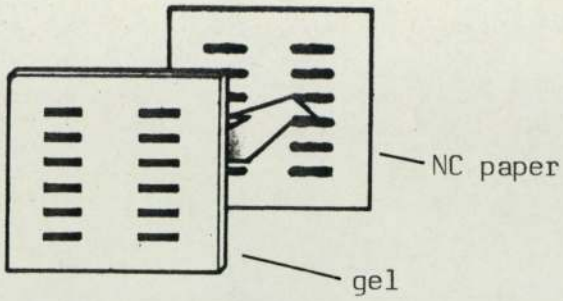
of the method of Towbin et al (1979). Following electrophoresis (2.2.9.2.) the gel and NC paper were sandwiched first between sheets of chromatography paper (Whatman Ltd., Maidstone, Kent) then between Scotch-brite pads (Bio-Rad Laboratories Ltd., Watford, Herts.) and finally between perforated plastic support grids. The sandwich was placed in a trans-blot cell containing blotting buffer with the NC sheet facing the cathode. Blotting buffer contained 25mM Tris, 193mM glycine and 20% v/v methanol, pH 8.3. The sandwich was subjected to a voltage of 50V overnight then increased to 80V for 2 hours to ensure complete transfer of proteins. Under these experimental conditions LPS O-antigen, which has a lower affinity for NC paper than does protein, passes through the paper (Sturm et al, 1984). The temperature of the cell was maintained at 4°C by an ice-bath.The NC sheet was removed from the transblot apparatus and soaked in 0.9% saline/10mM tris.HCl, pH 7.4 containing 0.3% Tween 20 (TBS/Tween)(Batteiger et al, 1982; Tsang et al, 1983) for one hour to saturate non-specific protein-binding sites. The paper was then incubated with patient's serum diluted 1 in 50 in TBS/Tween for 4 hours at 37°C or with patients urine concentrated by freeze drying and resuspending in one tenth its original volume with TBS/Tween. The paper was then washed thoroughly with TBS and incubated for a further 2 hours at 37°C with horseradish peroxidase goat anti-human IgG, IgM or IgA conjugate 1 in 1000 in TBS/Tween. After incubation the NC paper was again washed thoroughly and antigenic sites visualised with a 25µg/ml solution of 4-chloro 1-napthol in TBS containing 0.01% H_2O_2.

To show complete qualitative transfer of protein bands from acrylamide gel to the NC paper blotted protein bands on duplicate NC papers were directly visualised by staining with 1% amido black.

The process of immunoblotting is illustrated in figure 2.4.

Figure 2.4

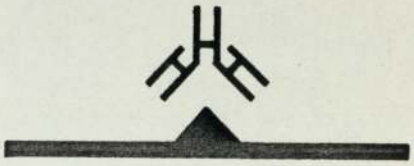
Procedure for visualization of antigens after electrophoretic transfer from polyacrylamide gels to nitrocellulose (NC) sheets. (western blotting; immunoblotting)



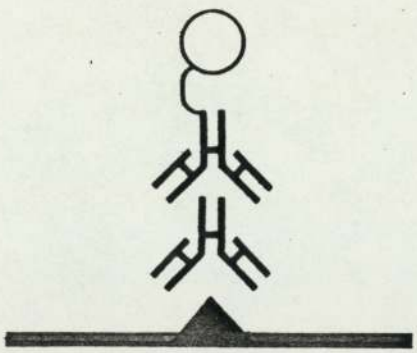
1. Electrophoretic transfer of antigens

2. Antigen on NC Paper

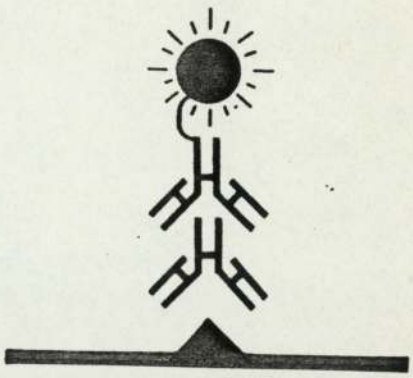
3. Blanking of non-specific binding sites



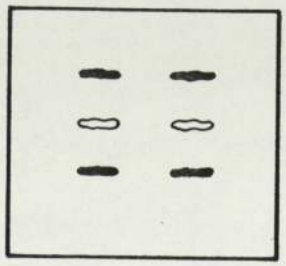
4. Reaction with antibody



5. Reaction with 2nd antibody tagged with HRP



6. Reaction with chromagen



7. Antigenic sites visualised

2.2.9.6. Enzyme linked immunabsorbent assay (ELISA).

The ELISA was a modification of the method of Engvall et al (1971). Immulon ELISA plates were coated with either whole cells (5 x 10^7/ml.) or outer membrane preparation (0.035mg/ml) suspended in PBS by incubating for 5 minutes with shaking at room temperature then overnight at 4°c. Plates were washed four times with phosphate buffered saline, pH 7.4, containing 0.05% Tween. Non-specific protein binding sites were blanked with a solution of 1% (w/v) bovine serum albumin in RPMI 1640 containing 10% (v/v) foetal calf serum by incubating for 30 minutes with shaking at room temperature then 2 hours at 37°C. The plates were washed as above and 50µl samples of serum, urine or concentrated urine serially diluted in gelatin phosphate buffer (0.07M sodium phosphate buffer, pH 6.5, containing 0.2% gelatin) added to each well. The plates were incubated for a further 2 hours at room temperature with shaking. One row of wells on each plate was incubated with plain gelatin phosphate buffer as a blank. The plates were again washed as above then incubated for 2 hours with 100µl anti-antibody/horseradish peroxidase conjugates diluted in the blanking solution. Conjugates used in this determination were rabbit anti-human IgG (1:200), goat anti-human IgA (1:1000) and goat anti-human IgM (1:1000). Plates were washed again and 100µl of substrate added to each well. The substrate solution was prepared by dissolving 10mg of 3,3',5,5'-tetramethylbenzidine (TMB) in 1ml of dimethylsulphoxide to 100ml of 0.1M sodium acetate/citrate buffer, pH6. Immediately before use 8µl of H_2O_2 was added and the plates shaken at room temperature until a blue colour developed. The

reaction was stopped by adding 50µl of 2N H_2SO_4 to each well and a yellow colour developed. The absorbance of each well was measured at 450nm. using a Dynatech plate reader coupled to a BBC model B computer (Porton software: Dynatech Ltd., Billingshurst, Sussex). The ELISA titre was taken as the dilution at a net absorbance of 0.1.

Pooled serum and urine from volunteers with no history of Pseudomonas infection were used as controls. Each determination was carried out at least in duplicate.

ELISA assays were carried out at the Vaccine Research and Production Laboratory, Porton Down. I am grateful to Drs. Howard Tranter and Narendra Modi for showing me how to use the technique and for helpful discussions.

2.2.9.7.Estimation of transferrin and lactoferrin.

Levels of lactoferrin and transferrin in serum and in urine were made by radialimmunodiffusion using NOR-Partigen plates (Behring Institut, Behringwerke, Marburg, W.Germany). The plates were used in accordance with the manufacturers instructions. Precipitation rings formed by serum and urine were measured with calipers and values take from calibration curves constructed using standard preparations (lactoferrin) or from tables supplied with the standard preparation (transferrin).

3-8 RESULTS AND DISCUSSIONS

3 Effect of iron in laboratory media and body fluids on the outer membrane protein profile of P.aeruginosa.

3.1 Effect of transferrin on growth rate and OMP profile.

The effect of iron restriction induced by the iron chelator ovotransferrin (Otr) was determined by growing P.aeruginose PAO1 in CDM to which Otr had been added. Ovotransferrin (Conalbumin, Sigma Chemical Co., Poole, Dorset) as supplied is iron free but must be dialysed against buffer containing 0.15M sodium chloride and 0.024M sodium bicarbonate at 4°C for 24 hours to remove citrate (Griffiths & Humphries, 1978). The dialysed Otr was freeze dried, resuspended in double distilled water and added to Fe- and Fe+ CDM at a final concentration of 0.2mg/ml immediately before use. Growth curves of PAO1 cultured in Fe+ and Fe- CDM 12 in the presence and absence of Otr are shown in figure 3.1. Growth in both Fe+ and Fe- medium was slowed by Otr, that of the Fe- medium considerably. The lag phase in Fe+ medium was prolonged by Otr and the growth rate somewhat slowed (doubling time 72 minutes as against 54 minutes), however the point at which exponential growth ceased was in both cases very similar (Fe+ CDM, 3.26; Fe+ Otr+ CDM, 3.09) as was the OD in the stationary phase (5.6 and 5.2 respectively). The growth rate of the organism in Fe+ Otr+ medium was very similar to that in simple Fe- medium (doubling times 72 and 78 minutes respectively). Growth in Fe- Otr+ medium was very much slower (doubling time 132 minutes) than in simple Fe- medium but again OD in the stationary phase was very similar (1.36 and 1.46 respectively). Copious amounts of bright green pigment were expressed

at stationary phase in both media containing Otr.

Figure 3.2 shows the OMP profile of PAO1 grown under the conditions described above and separated by SDS-PAGE using a 10% acrylamide gel. Three proteins (88, 85 and 64K) which were not present in the OM of cells grown in Fe+ CDM (lane 1) were induced in the OM of cells grown in Fe- CDM (lane 2) and in both Fe- and Fe+ media with added Otr (lanes 3 and 4). There were no apparent differences between the proteins induced in media with no added iron (iron-depleted media) and media in which iron was present but was withheld from the growing bacteria by a chelator (iron-restricted media). Apart from the induction of a number of high M_r proteins there were no differences in OMP profile between iron-sufficient and iron-depleted or -restricted preparations.

Figure 3.1

Growth of P.aeruginosa PAO1 in Fe- and Fe+ CDM 12 in the presence and absence of ovotransferrin (Otr)

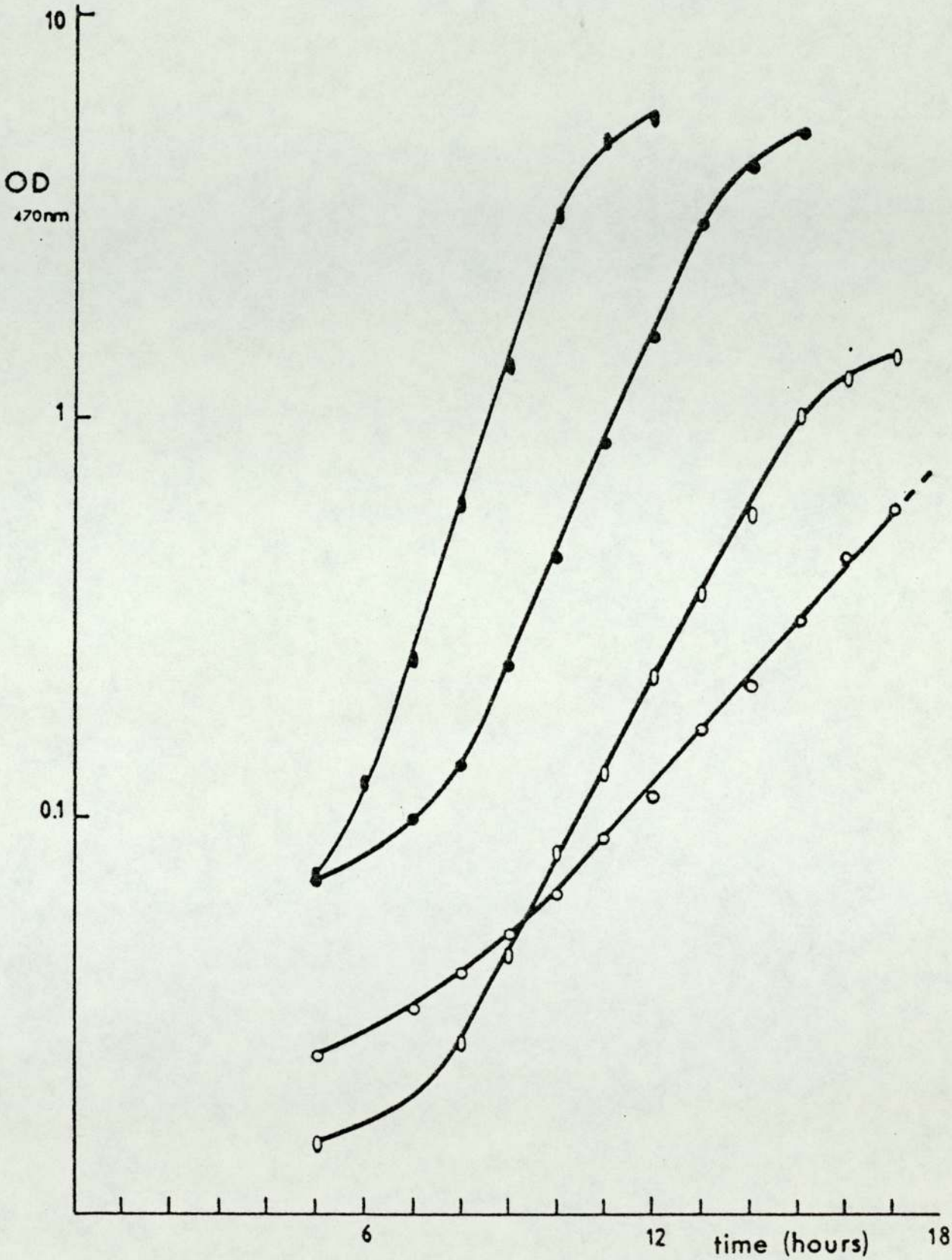
●Fe+ CDM

○Fe- CDM

●Fe+ CDM with added Otr

○Fe- CDM with added Otr

The OD_{470} of the Fe- CDM culture with added Otr at 24 hours was 1.36.


10
OD
470nm
1
0.1
6
12
time (hours)
18

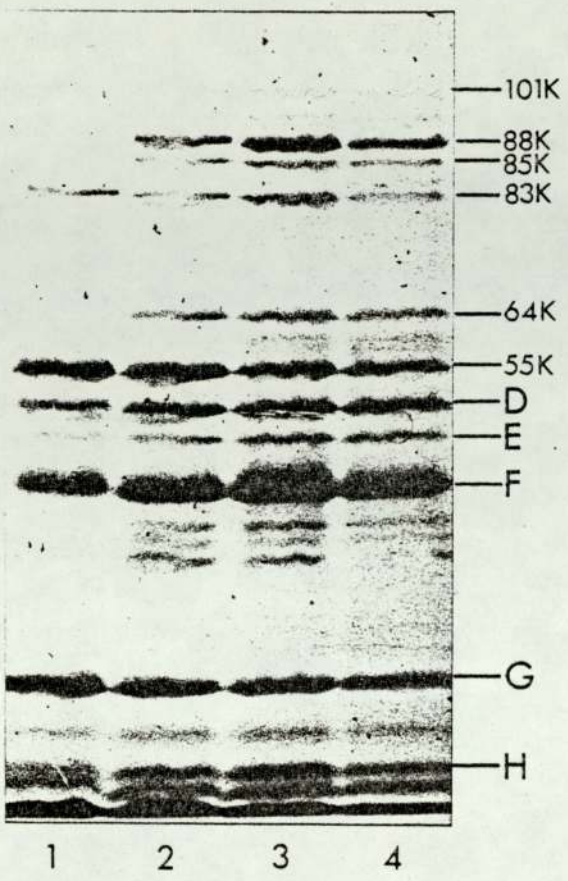


Figure 3.2

The OMP profile of P.aeruginosa PAO1 grown in Fe+ CDM (lane 1), Fe- CDM (lane 2), Fe+ CDM with added Otr (lane 3) and Fe- CDM with added Otr (lane 4) on a 10% polyacrylamide gel.

The letters in the right hand column show proteins labelled according to the scheme of Mizuno & Kageyama (1978).

Those protein bands which are not described in the scheme of Mizuno & Kageyama are expressed in molecular weights in thousands.

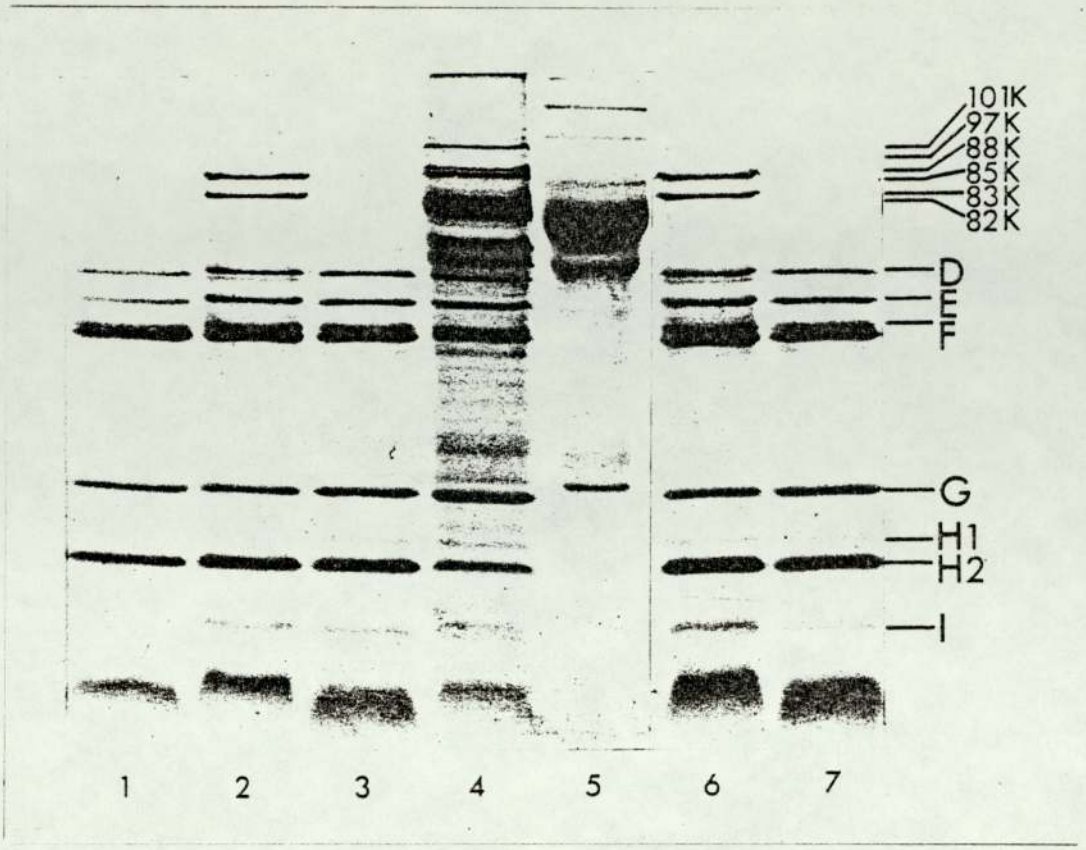


Figure 3.3

The OMP profile of P.aeruginosa WN50 grown in Fe+ CDM (lane 1), Fe- CDM (lane 2), Fe+ serum (lane 3), serum (lane 4), Fe- TSB (lane 6) and Fe+ TSB (lane 7). Proteins present in the serum alone are shown in lane 5.

15% acrylamide gel.

3.2 Effect on OMP profile of growth in serum.

The OMP profiles of P.aeruginosa strain WN50 grown in laboratory medium and serum are shown in figure 3.3. WN50, a strain isolated from an infected ulcer, was used as it had been shown to be serum resistant (2.2.7.1) and, in fact, to grow avidly in serum. WN50 was serotyped by slide agglutination and found to be serotype O:6. The OMP profile of WN 50 grown in Fe+ and Fe- CDM 12 is shown in lanes 1 and 2 respectively. Two major high molecular weight proteins - 83 and 88K- and four minor high molecular proteins - 82, 85, 93 and 101K- which were present in the OM of the iron depleted cells were wholly or partly repressed in iron sufficient conditions. The increase in the relative intensity of these protein bands in iron depleted conditions is shown graphically in the densitometer scan of the OMP profiles (figure 3.4,a and b). Very similar alterations in OMP profile were produced when WN50 was grown in TSB under iron sufficient (figure 3.3,lane 6) and iron depleted conditions (figure 3.3,lane 7).

Lane 3 shows the OMPs of the same organism grown in serum to which iron had been added (2.1.6.2). All the IRMPs were repressed. Protein D2 was also repressed. Figure 3.4 indicates the close similarity in OMP profiles between the organism grown in iron sufficient CDM (3.4,a) and serum to which iron had been added in excess (Fe+ serum)(3.4,c). When WN50 was grown in serum without added iron, however, major changes occured in the OMP profile (3.3,lane 4) and densitometer scan (3.4,d). In addition to the previously recognised IRMPs at 101, 88, 85 and 83K there were major protein bands between 60 and 80K. The new bands do not appear to be caused by

contamination with serum proteins as they do not co-migrate with proteins in a sample of the same serum in which the cells were grown (fig. 3.3,lane 5). Neither do they appear to be complement proteins as an identical OMP profile was obtained when WN50 was grown in HIS (not shown).

Figure 3.5 shows that these protein bands did not react with antibodies in hyperimmune rabbit serum raised against a serotype O:6 organism (PAGS) when electrophoretically transferred to NC paper (3.5,iii), although an amido black stain of a duplicate blot showed complete qualitative transfer of proteins (3.5,ii). The immunoblot of the OMPs of WN50 grown in serum (lane 4) was essentially similar to that grown in Fe- CDM (lane 2). The new protein bands do, however, co-migrate with human transferrin. Figures 3.4,d and e shows the close alignment between the densitometer scan of WN 50 grown in human serum and a scan of human transferrin after SDS-PAGE.

Figure 3.4

Densitometer scans of the OMP profiles of P.aeruginosa WN50 grown in a) Fe+ CDM, b) Fe- CDM, c) Fe+ serum, d) serum. 3.4e) is the densitometer scan of human transferrin in the same gel system. Gels were stained with Coomassie blue and scanned at 580nm.

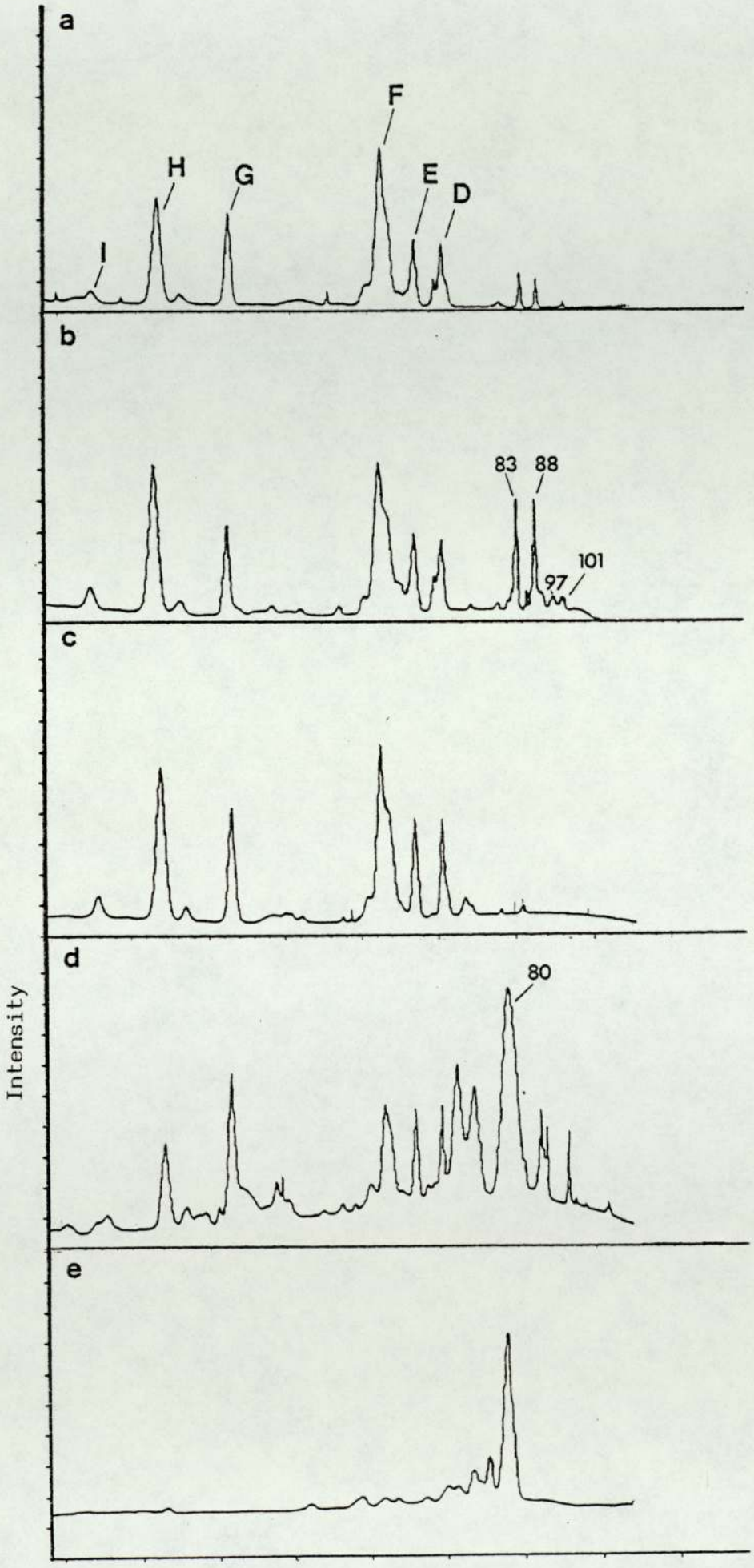

a
F
H
G
E
D
I
b
83
88
101
97
c
d
80
e
Intensity
Distance from top of gel (cms)

Figure 3.5,i

The OMP profiles of P.aeruginosa WN50 grown in Fe+ CDM (lane 1)., Fe- CDM (lane 2), Fe+ serum (lane 3) and serum (lane 4). 15% polyacrylamide gel.

Figure 3.5,ii

OMPs shown in figure 3.5 i electrophoretically transferred to NC paper and stained with 1% amido black.

Figure 3.5,iii

Immunoblot of OMPs shown in figure 3.5 i electrophoretically transferred to NC paper and reacted with hyperimmune rabbit serum.

Figure 3.5,i

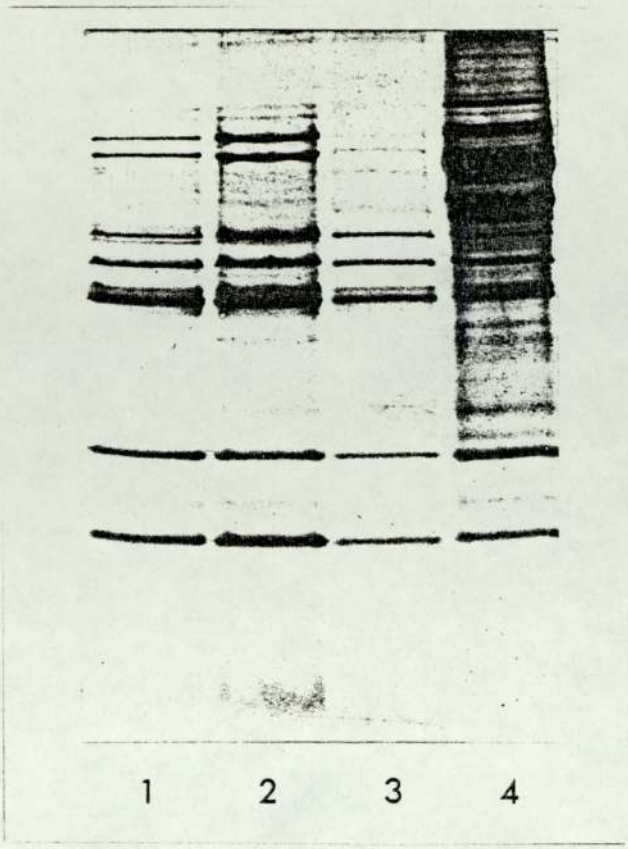


Figure 3.5,ii

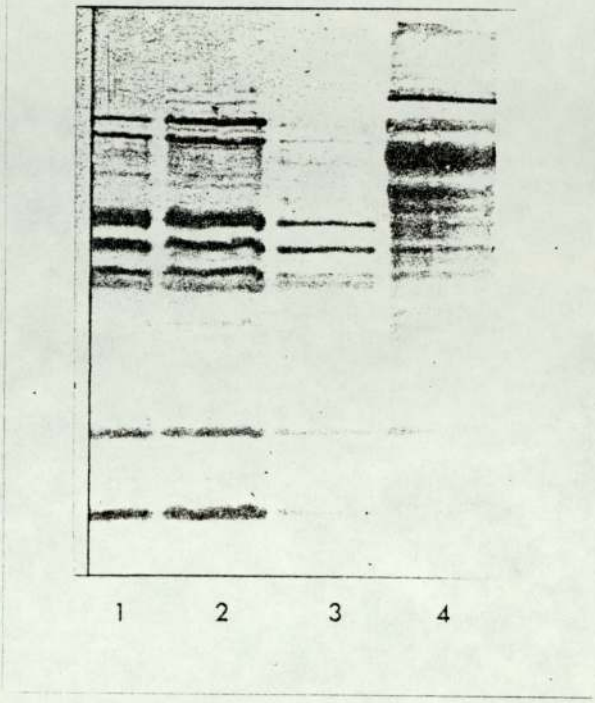
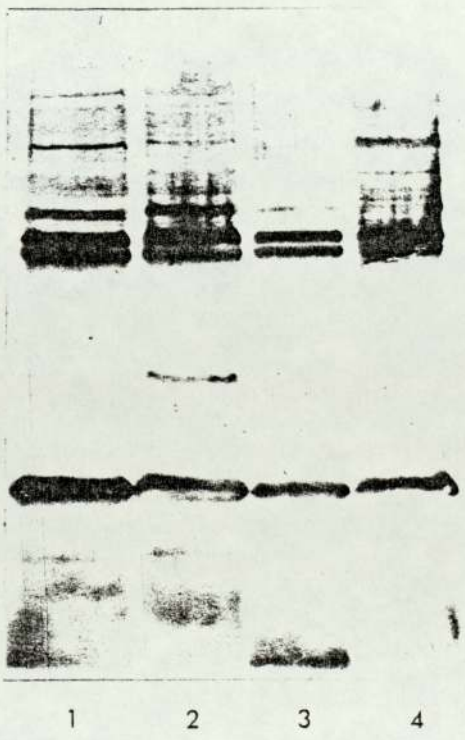


Figure 3.5,iii



3.3 Effect on OMP profile of growth in urine.

P.aeruginosa PAGS is a strain isolated from a human UTI. Figure 3.6 shows the effect on the OMP profile of strain PAGS of growth in iron depleted (lane 1) and iron sufficient (lane 2) CDM. In PAGS proteins D1 and D2 are pronounced and easily separated on a 15% acrylamide gel (figure 6.6). On a 10% gel (figure 3.6) they were well separated and protein E which runs as a single band on 15% gels was also separated into two bands. The OMP profile of PAGS grown in urine and urine to which 0.12mM iron had been added (Fe+ urine) is shown in lanes 3 and 4 respectively. The profile of cells grown in Fe+ urine was similar to that of those grown in Fe+ CDM except for the presence of a protein band at 64K. Protein D2 was repressed in both OMs grown in iron sufficient conditions (lanes 2 and 4). Four high M_r proteins were induced in the OM of cells growing in urine without added iron (lane 3), two of these, at 83 and 88K, were also present in the OM from Fe- CDM grown cells but the others, 97 and 101K, were not. The expression of proteins G and H was partly repressed in urine (lanes 3 and 4).

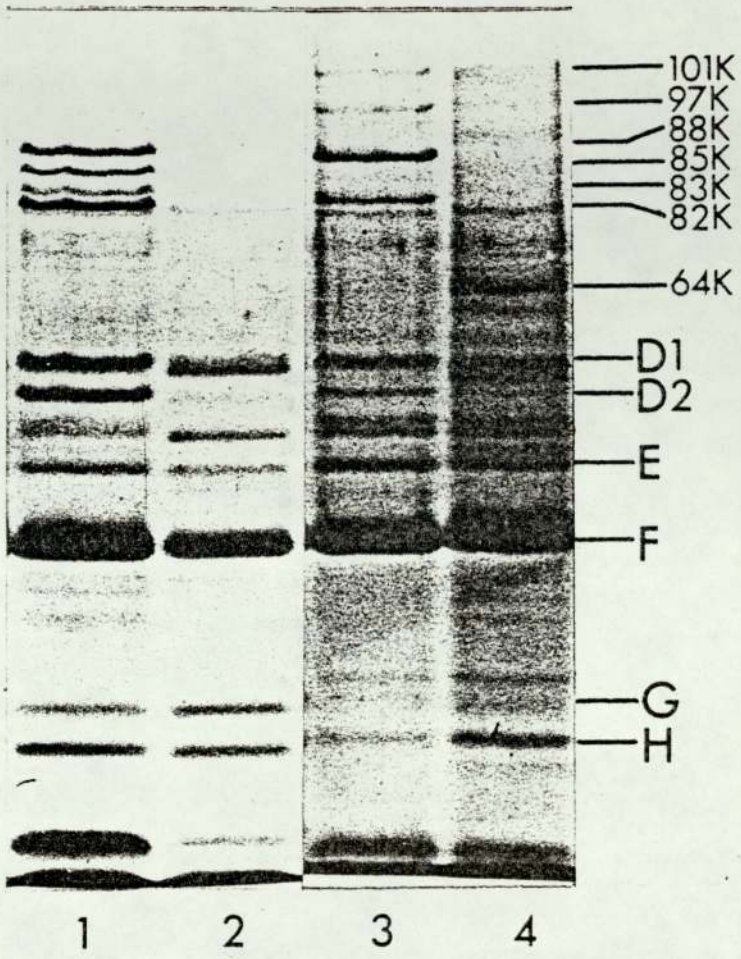


Figure 3.6

The OMP profile of P.aeruginosa PAGS grown in Fe- CDM (lane 1), Fe+ CDM (lane 2), urine (lane 3) and Fe+ urine (lane 4). 10% acrylamide gel.

3.4 Discussion

Iron is an essential nutrient for bacterial proliferation. Growth of many Gram negative bacteria in iron deficient environments results in the induction of a number of high M_r OMPs (Neilands, 1982). Bacteria obtained from human CF lung infection (Brown *et al*, 1984) and from experimental animal models (Griffiths *et al*, 1983; Sciortino & Finkelstein, 1983) expressed these high M_r proteins *in vivo*. In *E.coli* some of the induced OMPs are known to function as receptors for high affinity (Braun *et al*, 1983) and low affinity (Hartman & Braun, 1981) iron uptake systems. The kinetics of iron uptake have not been so extensively studied in other organisms but it is assumed that the induced high M_r proteins in *P.aeruginosa* (Meyer *et al*, 1979; Ohkawa *et al*, 1980) also function as receptors for iron salts or iron-siderophore complexes.

P.aeruginosa is known to secrete a phenolic siderophore, pyochelin, (Cox & Graham, 1979) and a hydroxamate, pyoverdin, (Cox & Adams, 1985) which have been shown to promote bacterial growth in iron-deficient conditions (Cox & Graham, 1979; Ankenbauer *et al*, 1985) and to act as a substrate for iron transport (Cox, 1980; Cox & Adams, 1985). Sokol and Woods (1983) have reported the induction of a low M_r OMP (14K) in *P.aeruginosa* grown under conditions of iron depletion which they claim specifically binds [Fe^{59}]-pyochelin. A 14K protein is also claimed to be induced in strains of *P.cepacia*, *P.mutivorans*, *P.fluorescens*, *P.putida* and *P.maltophila* grown in low iron conditions and to bind [Fe^{59}]-pyochelin in all these strains and in *P.stutzeri* (Sokol, 1984).

Induction of high M_r proteins in *P.aeruginosa* has been observed in laboratory media (Ohkawa *et al*, 1980) but little is known about the

effect on protein profile of added iron in body fluids. Figures 3.2 to 3.6 indicate the changes in OMP profile of P.aeruginosa bought about by growth under iron-sufficient and iron-restricted conditions in laboratory media, human serum and human urine. They show that a number of high M_r proteins which are present in the OM of cells grown in media in which iron is depleted or restricted are repressed by the addition of iron. Growth in CDM in the presence of transferrin alters the growth kinetics of PAO1 but produces the same OMP profile as growth in media without added iron. Because transferrin did not have any significant effect on the OMP profile and because there are practical difficulties associated with its use and it is expensive it was not used routinely to restrict iron availability in media. Instead, cells were grown in media without iron (iron-depleted media).

The effect of iron in body fluids on OMs is not simply one of induction or repression of high M_r proteins since the pattern of proteins induced by lack of iron in laboratory media is not the same as those induced when the same organism grows in serum or urine. The picture is a complex one and the factors which influence the induction or repression of OMPs are not well understood. Further work is needed to characterize the induced high molecular weight proteins.

The protein bands which appear at about 80K in the OM of WN50 cells grown in human serum may be cell wall proteins strongly induced by the conditions of growth, but they are not recognised as a bacterial protein by hyperimmune serum raised against a strain of the same serotype. The 80K protein may be one of the components of complement even though the OMP profile of cells grown in HIS is the same as that grown in unheated serum. Complement proteins whose enzymic activity have been destroyed by heating may still have the ability to bind to the bacterial surface. The avirulent laboratory

strain P.aeruginosa PAO1 is serum-resistant and will grow in normal human serum (Lambert & Booth, 1981). The isolated OM of serum grown PAO1 contained a number of high molecular weight proteins not present in the OM of cells grown in nutrient broth. Two of these proteins (73.5K and 77K) were repressed by iron (Lambert & Booth, 1981). PAO1 grown in serum did not, however, express heavy protein bands at about 80K as did WN50 (figure 3.3, lane 4). It is possible that the virulent strain WN50 is able to remove iron directly from serum transferrin. Such a mechanism has been proposed for N.meningitidis (Archibald & DeVoe, 1980; Mickelson & Sparling, 1981). A possible explanation for the appearance of the circa. 80K protein is that it is serum transferrin which has bound to the cell surface strongly enough to withstand treatment with sarkosyl during the preparation of the OM.

An iron regulated protein at 14K reported by Sokol and Woods (1983) is not apparent in the OM of any of the strains grown under iron depleted conditions. A number of experimental points in the Sokol & Woods protocol raise doubts as to whether the [Fe^{59}]-pyochelin receptor is a 14K protein. In their protocol OMs prepared from PAO1 grown in simple salts medium without added iron were incubated with [Fe^{59}]-pyochelin for five minutes at 4 and 25°C. After treatment membrane filtered and washed OM preparations were analysed by SDS-PAGE. Bound [Fe^{59}]-pyochelin was visualised by autoradiography. OMPs were also transferred to NC sheets and reacted with [Fe^{59}]-pyochelin and again visualised by autoradiography. In both cases radio-labelled pyochelin was associated with a protein band of molecular weight 14K. This low molecular weight protein was expressed in higher concentrations in OMs grown in media without added iron than media to which $FeCl_3$ had been added. Concentrations of protein in the OM were based on densitometer scans. This is dubious since the scanner

measures only the density of coomassie blue colour. It would be necessary to show that binding of coomassie blue dye to all OMPs of P.aeruginosa was quantitative and linear before making conclusions about relative concentrations. The apparent binding of [Fe^{59}]-pyochelin may also be an artefact since denaturing in the presence of SDS is likely to split [Fe^{59}]-pyochelin from the protein. The observed position of the radio-labelled enterochelin on gels and blots may result from [Fe^{59}]-pyochelin binding to a small protein fragment, or non-specific binding to cell debris running at the gel front.

4.1 Outer membrane protein antigens of P.aeruginosa

The IATS defines 17 different O serotype strains of P.aeruginosa (Bergan, 1975) of which the four most common types isolated in British hospitals are O:6, O:10, O:11 and O:16 (Pitt, 1980). Figure 4.1,i shows the OMP profiles of strains representative of each of these serotypes grown in iron sufficient (lanes 1-4) and iron depleted (lanes 5-8) CDM 12. The OMP profiles of all four strains are basically similar and conform to the format described by Mizuno and Kageyama (1978). A number of proteins of M_r between 82 and 101K which were expressed in iron depleted conditions were repressed by the presence of iron in the medium in three of the serotype strains shown but not in the O:10 strain (lane 4). This strain (CI 16) continued to express proteins in this M_r range when grown in iron rich complex media such as nutrient broth (not shown). Expression of these high M_r proteins appears to be constitutive in this strain.

Figure 4.1,ii is the immunoblot obtained when the proteins separated in 4.1,i were transferred to NC paper and reacted with hyperimmune rabbit serum raised against an O:11 strain (PARH). Proteins F, H and I and rough LPS react strongly with antibody and are visualised as intense grey/black bands in all four strains grown under both iron sufficient and iron depleted conditions. The dark shadows below the intense protein F band were probably due to incompletely denatured protein F present in concentrations too low to be visualised in the coomassie blue stained gel. Three high M_r proteins, 83,85 and 88K, give rise to bands in the serotype O:11 strain (lane 7) and to a lesser extent in other strains grown under iron depletion (lanes 5,6 and 8). There were also faint reactions in the 80K region in lanes

1-4.

Figure 4.2,i shows flagellar preparations isolated from H serotype H:4 (lane 1) and H:3 (lane 2) strains and the immunoblot obtained by transferring these proteins to NC paper and reacting with hyperimmune rabbit serum (figure 4.2,ii). The hyperimmune serum used had been raised against the PARH strain (as in figure 4.1,ii) which was of H:1,2 serotype. Both flagellar preparations appeared as single bands of 55K on the SDS-PAGE gel (figure 4.2,i) and give rise to intense bands at 55K on the immunoblot (figure 4.4,ii). The H:4 preparation (figure 4.2,i, lane 1) also gave rise to an intense band at 18K on the immunoblot (figure 4.2,ii, lane 1). This low M_r band may be a flagellin subunit present as a result of the flagella breaking down in the gel or it may be pili. In both cases rough LPS, not visible in the coomassie blue stained gel but visualised on the blot, showed that there was some contamination of the flagellar preparation with LPS. Both flagellar preparations were highly immunogenic.

Figure 4.1,i

OMP profiles of serotype O:6, O:10, O:11 and O:16 strains of P.aeruginosa grown in Fe+ CDM (lanes 1-4) and Fe- CDM (lanes 5-8).

15% acrylamide gel.

Figure 4.1,ii

Immunoblot of OMPs shown in figure 4.1 i electrophoretically transferred to NC paper and reacted with hyperimmune rabbit serum.

Figure 4.1,i

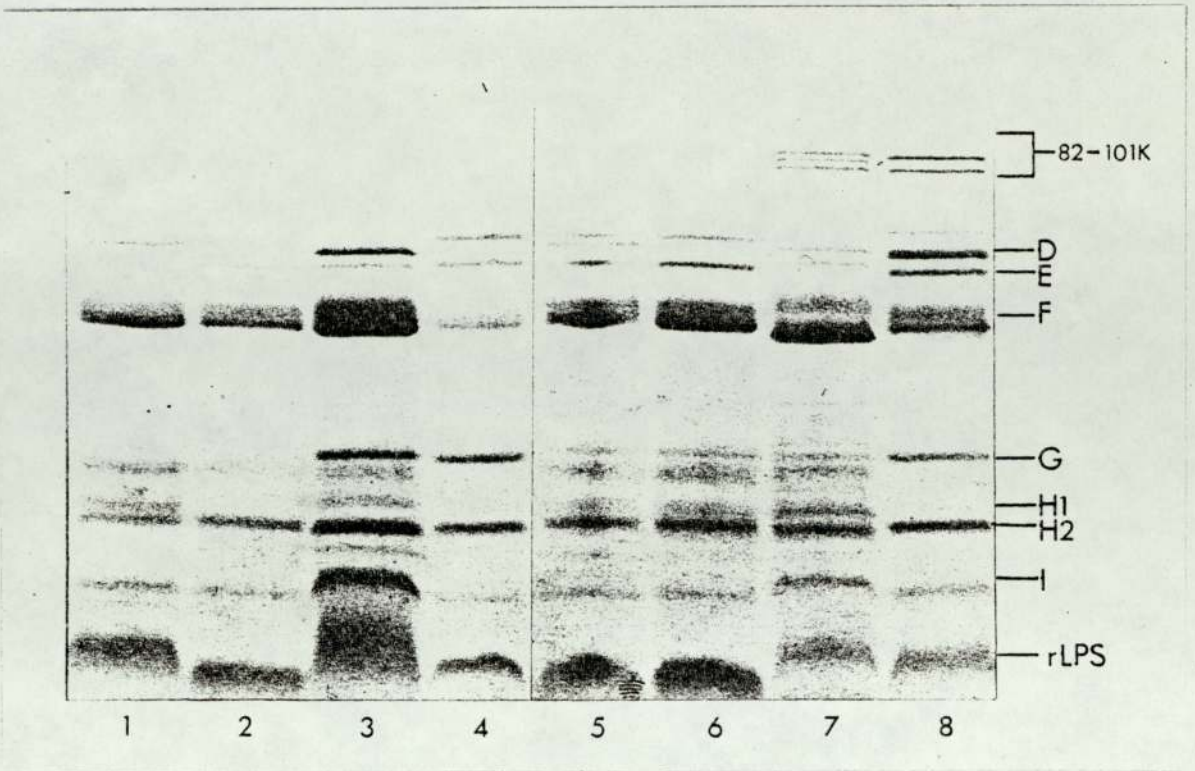
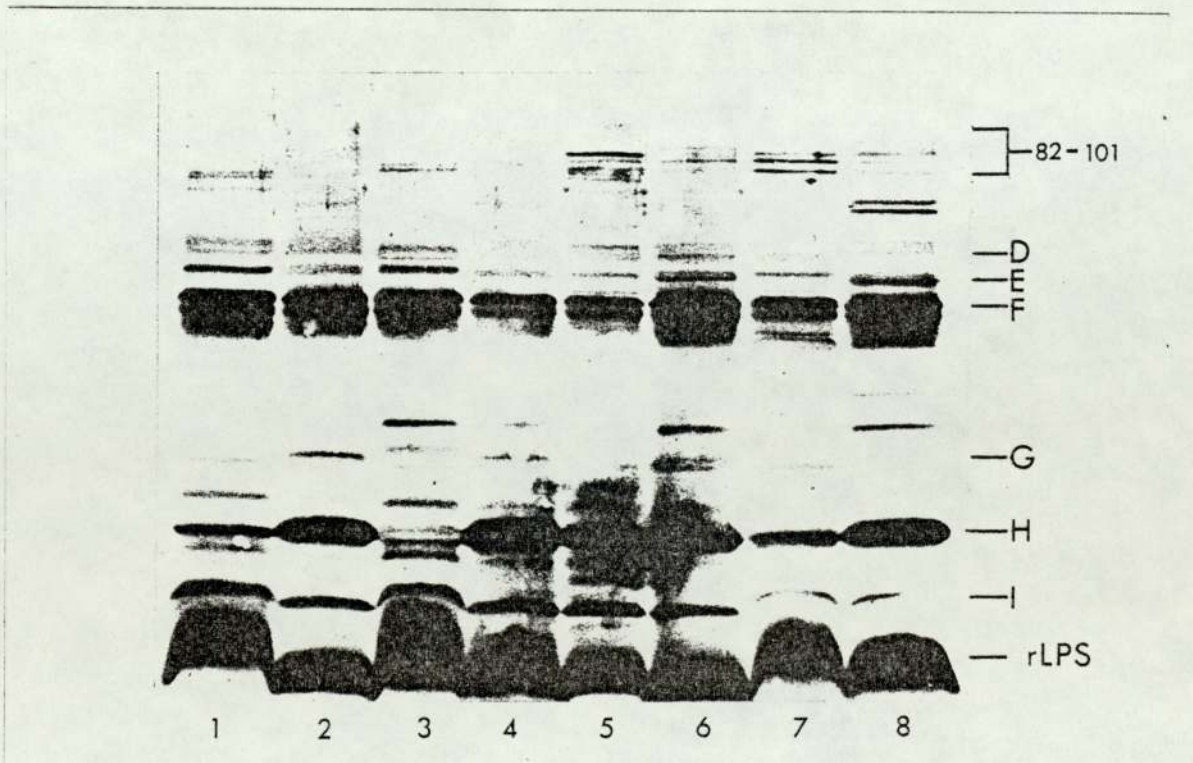


Figure 4.1,ii



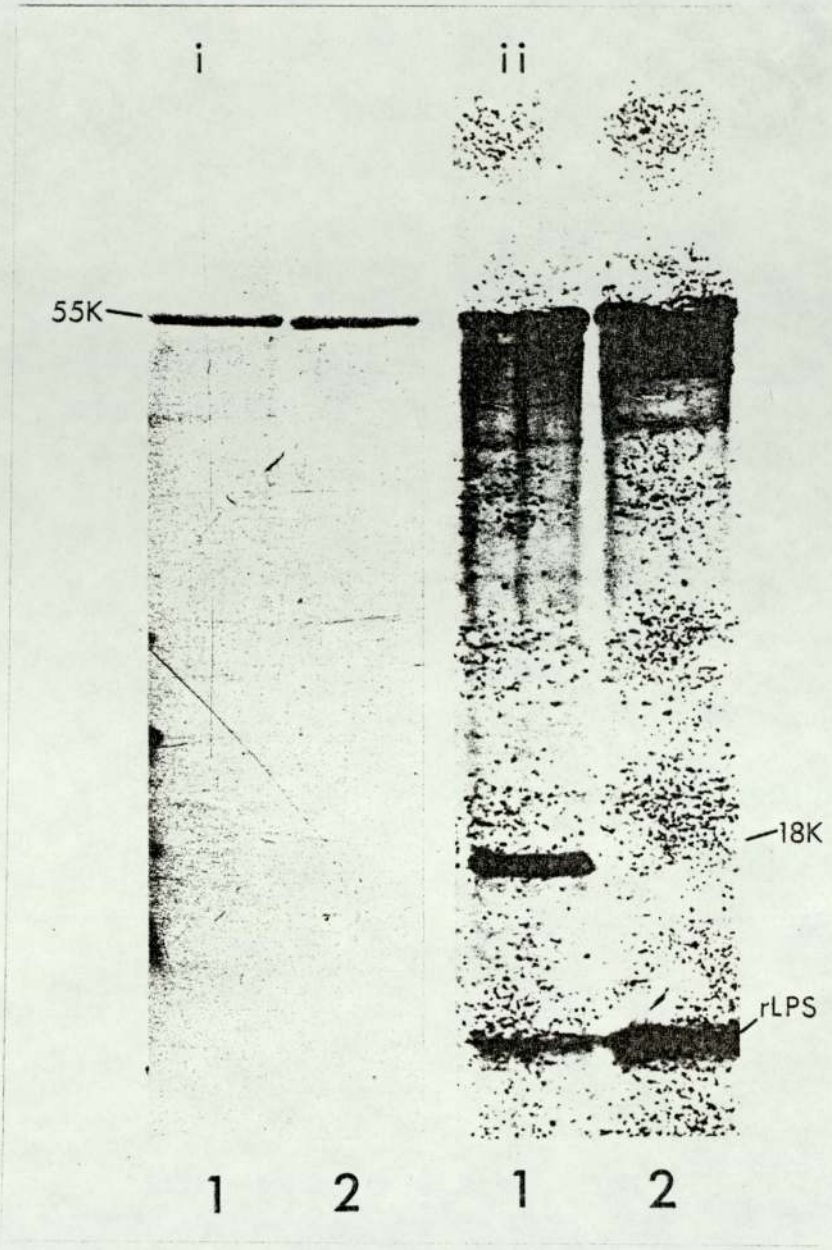


Figure 4.2,i

Flagellar preparations from H serotype H:4 (lane 1) and H:3 (lane 2) strains of P.aeruginosa analysed by SDS-PAGE, 15% acrylamide gel.

Figure 4.2,ii

Immunoblot of the flagellar preparations shown in figure 4.2,i electrophoretically transferred to NC paper and reacted with hyperimmune rabbit serum.

4.2 Reaction of human serum antibodies with OM antigens of P.aeruginosa and other Gram negative bacteria.

Serum from patients with infections due to P.aeruginosa and from volunteers with no history of Pseudomonas infection was used to investigate the antibody response to OM antigens of a number of Gram negative bacteria. Figure 4.3,i shows the OMP profile of strains of K.pneumoniae (lane 1), P.mirabilis (lane 2), E.coli (lane 3) and P.aeruginosa PAO6 (lane 4) all isolated from human UTI and grown under iron restricted conditions in Fe- TSB. Proteins D,E,F,G,H and I are present in the OM of P.aeruginosa (lane 4). The predominant protein in the OM of the three Enterobacteriaceae (lanes 1-3) are the porin proteins with a M_r about 40K. In each strain a number of high M_r (Ca. 80K) proteins have been induced.

Figures 4.3,ii to 4.3,v show the immunoblots obtained when the proteins separated in figure 4.3,i were transferred to NC paper and probed with control serum from a volunteer with no history of P.aeruginosa infection (4.3,ii) and also with sera from patients with urinary tract (4.3,iii), burn wound (4.3,iv) and CF lung infection (4.3,v) due to P.aeruginosa. In figure 4.3,ii there is a reaction of moderate intensity with the porin proteins and IRMPs of the Enterobacteriaceae (lanes 1-3) but a barely perceptible reaction with P.aeruginosa antigens (lane 4). Only an antigen with a low M_r, which may be rough LPS, was visualised as a clear band. This reaction was typical of all control sera investigated.

Figure 4.3,iv shows the immunoblot obtained when the OMPs were transferred to NC paper and probed with serum from a patient with

a burn wound infection due to P.aeruginosa The blot shows that the patient had antibodies to major OMP antigens of all three Enterobacteriaceae. A low molecular weight protein antigen of approximately M_r 15K is highly immunogenic with this patients serum in all four strains of Enterobacteriaceae (lanes 1-3) but is absent from the immunoblot of P.aeruginosa (lane 4). Proteins D and E and the 83K M_r IRMP of P.aeruginosa were the only protein antigens to be readily detected by immunoblotting. In addition a protein with M_r of approximately 60K, which may be flagella, was also recognised.

Figure 4.3,iii shows a strong reaction between antibodies in the serum of a patient with UTI and proteins D,E and F in the OM of P.aeruginosa (lane 4) and with protein antigens, especially porins, in the OM of the Enterobacteriaceae (lanes 1-3). Proteins G,H and I and the IRMPs at 83 and 88K reacted much less strongly and gave rise only to faint grey bands in the immunoblot.

The immunoblot obtained when the serum from a patient with P.aeruginosa CF lung infection (4.3,v) was used to probe the OM antigens separated in 4.3,i is more intense than that seen from patients with burn wound infection and UTI (4.3,iii and iv) and differs from them in that antigens of low M_r are strongly visualised. Most noiceably rough LPS of P.aeruginosa gives rise to an intense band which is not seen in figures b,c and d. The blot of the P.aeruginosa OM with CF serum is very similar to that seen with hyperimmune rabbit serum (figure 4.1,ii).

The degree of immunological cross-reactivity between different species of Gram-negative bacteria was investigated using sera from two patients with UTI, one due to P.aeruginosa and the other due to E.coli. Figure 4.4,i and ii show the immunoblots obtained when the OMs described in figure 4.3,i were transferred to NC paper and probed

with patients sera. Figure 4.4,i shows that serum from a patient with E.coli infection reacted with OM antigens of K.pneumoniae, P.mirabilis and E.coli (lanes 1-3) but only faintly reacted with OM antigens of P.aeruginosa (lane 4). Serum from a patient with an infection due to P.aeruginosa, however reacted with with antigens from all four strains used (figure 4.4,ii).

Figure 4.3,i

OMP profiles of strains of K.pneumoniae (lane 1), P.mirabilis (lane 2), E.coli (lane 3) and P.aeruginosa (lane 4) isolated from UTI and cultivated in TSB. 15% acrylamide gels. Arrows in the left margin indicate molecular weight markers.

Figure 4.3,ii

Immunoblot of OMPs separated in 4.3,i electrophoretically transferred to NC paper and reacted with serum from a volunteer with no history of P.aeruginosa infection.

Figure 4.3,iii

Immunoblot of OMPs separated in 4.3,i electrophoretically transferred to NC paper and reacted with serum from a patient with UTI due to P.aeruginosa.

Figure 4.3,i

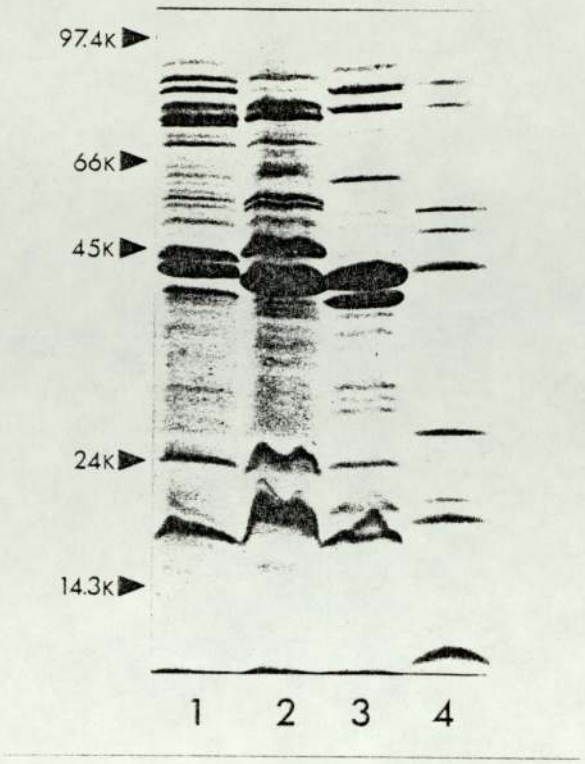


Figure 4.3,ii

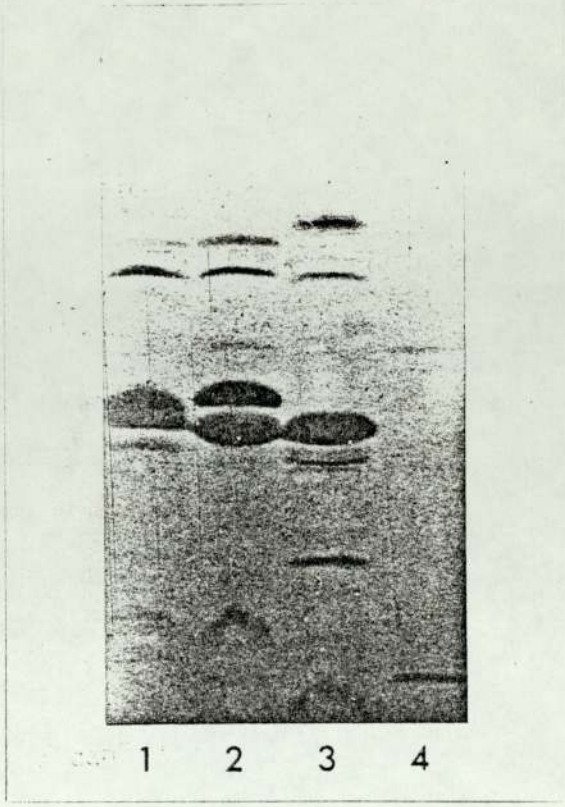


Figure 4.3,iii

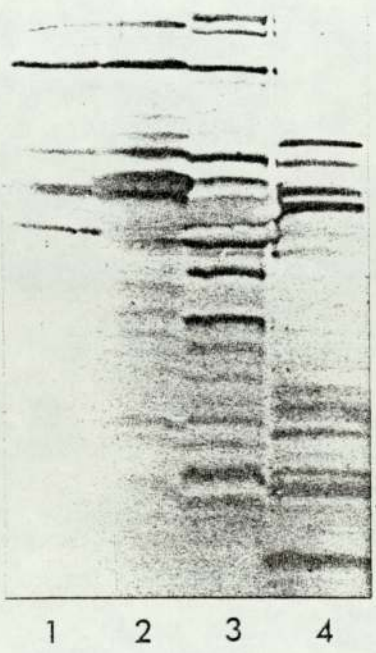


Figure 4.3,iv

Immunoblot of OMPs separated in 4.3,i electrophoretically transferred to NC paper and reacted with serum from a patient with a burn wound infection due to P.aeruginosa.

Figure 4.3,v

Immunoblot of OMPs separated in 4.3,i electrophoretically transferred to NC paper and reacted with serum from a cystic fibrosis patient with lung infection due to P.aeruginosa.

Figure 4.3,iv

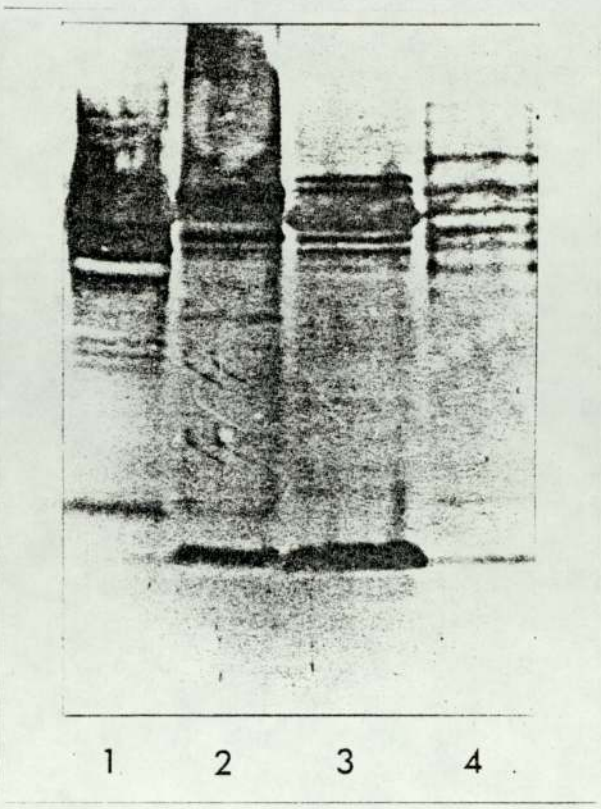
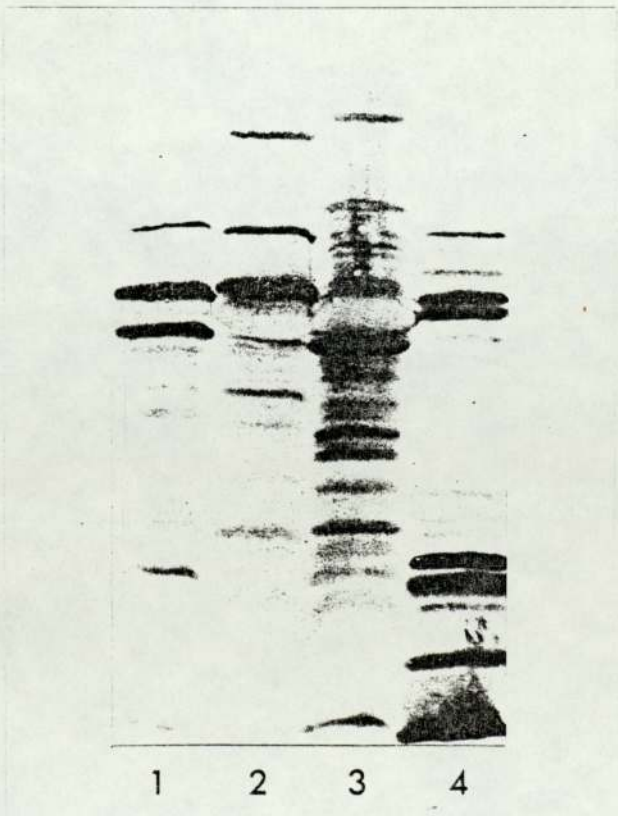


Figure 4.3,v



4.3 Discussion

Antibodies in hyperimmune rabbit sera reacted with proteins E, F, H and I in representatives of all 4 serotype strains after electrophotetic transfer to NC paper (figure 4.1). Proteins D and G reacted faintly or not at all. These antigen-antibody reactions were the same in cells cultured in iron-sufficient and iron-depleted conditions. Antibodies in serum also reacted with 3 to 5 protein bands of M_r 82-101K in serotype strains grown under iron-depleted conditions. Faintly reactive bands in the 80K region in cells grown in iron-sufficient conditions indicate that even in iron-sufficient conditions there are a few copies of the high M_r proteins normally associated with iron depletion present in the OM.

Pili of *P.aeruginosa* have been characterised biochemically (Paranchych *et al*, 1979; Watts *et al*, 1982) and shown to have a molecular weight of 18K (Frost & Paranchych, 1979). Flagella and an antigen of about 18K thought to be pili were highly immunogenic (figure 4.2). This immunological response to flagella and pili is not surprising given their location on the cell surface. Pili, like flagella, are not always apparent in OM preparations separated by SDS-PAGE or the blots derived from them. They are easily sheared and are lost during the process of separation of the OM.

Serum antibodies from patients with infections due to *P.aeruginosa* were investigated by reaction with OMs of a number of pathogens (figure 4.3). The three species of Enterobacteriaceae and

one strain of P.aeruginosa used were chosen as being representative of Gram-negative pathogens which are able to cause infection in the urinary tract, burn wounds and CF lung. OMP antigens, especially the porin proteins, of the three species of Enterobacteriaceae reacted with serum from 3 patients with P.aeruginosa infections. Serum from patients with UTI and CF lung infection reacted with the major protein antigens of P.aeruginosa . The CF serum had a notably high antibody titre and the immunoblot with this serum is similar to that obtained with hyperimmune rabbit serum. This result is in agreement with experimental findings that ELISA titres of antibody were at the same high level in CF patients chronically colonized with P.aeruginosa as in hyperimmunized rabbits (Hancock et al, 1984).

The reaction of serum from a burn wound patient with OMPs of P.aeruginosa is rather different from that seen with UTI or CF serum in that only a few higher M_r proteins are recognised as antigens. Unfortunately this patient died two days after P.aeruginosa was isolated from the burn wound and the result may reflect the initial antibody response in acute infection. Further studies of this type need to be carried out to determine the nature of the acute antibody response and to determine how it changes as the infection becomes chronic, or as the patient recovers.

The reaction of a control serum from a volunteer with no history of P.aeruginosa infection (figure 4.3,ii) is typical of all control sera studied (five women, two men; ages 19-47). None of the serum from the volunteers contained antibodies which reacted strongly with P.aeruginosa OMP antigens. All 7 volunteers were hospital or research laboratory personel who might be expected to experience a higher than usual environmental exposure to P.aeruginosa . A study of 4000 blood donors who were screened for serum IgG antibody to

P.aeruginosa LPS antigens showed that less than 5% had levels greater than pooled reference serum (Collins & Roby, 1984). The high antigen titres persisted in these subjects for the 40 weeks of the study. The investigators speculate that the individuals with high antibody titres had trivial epidermal infections or asymptomatic colonisation of gastro-intestinal or genito-urinary tracts (Collins & Roby, 1984). It seems probable from these studies that only those individuals who are colonized or have had an infection with P.aeruginosa carry antibodies to specific P.aeruginosa OM components.

The control experiments (figure 4.4) showed that the patient sera examined contained antibodies which reacted with members of the Enterobacteriaceae and indicates a cross-reactivity between the three species of Enterobacteriaceae used. This is to be expected as members of the Enterobacteriaceae are part of the normal gut flora and it is probable that all normal individuals have a low titer of antibodies against them. This antibody titer may be increased when an individual contracts an infection with one of these organisms. The experiments taken as a whole show a very weak cross-reactivity between antibodies to Enterobacteriaceae and P.aeruginosa antigens and indicate the specificity of the response to P.aeruginosa antigens. The exact nature of the cross-antigenicity between strains remains to be investigated with a more specific system, possibly one using monoclonal antibodies against these antigens.

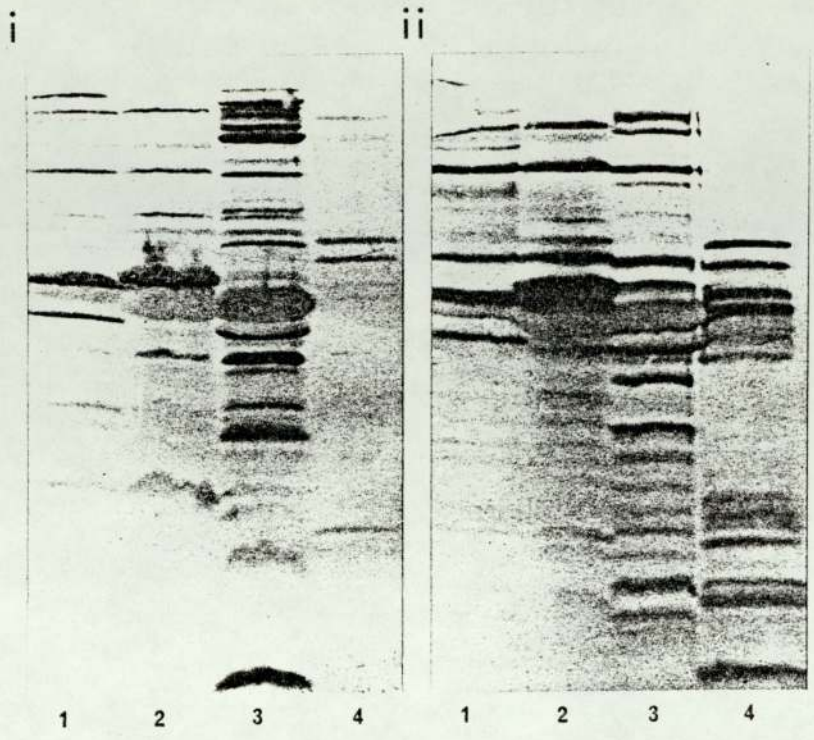


Figure 4.4

Immunoblots of the OMPs shown in figure 4.3,i electrophoretically transferred to NC paper and reacted with serum from i) a patient with UTI due to E.coli and ii) a patient with UTI due to P.aeruginosa.

5. Effect of iron on virulence of P.aeruginosa in a mouse intraperitoneal infection model

5.1 Effect of iron depletion on virulence of P.aeruginosa

The virulence of a number of strains of P.aeruginosa was investigated in mice infected by intraperitoneal injection (Cleeland & Grunberg, 1980). Strains were chosen to cover a range of common serotypes and included both serum-resistant and serum-sensitive types (2.2.7.1). The LD_{50}s of strain WN50 (serotype 0:6) and a serotype 0:11 (2168) and PA strain (CI6) cultured in iron-sufficient and iron-depleted media are shown in table 5.1. In both WN50 and CI6 strains a smaller inoculum of Fe- cultured cells was required to kill 50% of the mice in each group, in the case of strain WN50 the difference was more than seven-fold. The LD_{50} for strain 2168 mice injected with Fe- cultured cells was 4.8×10^7. Data for mice in the strain 2168 Fe+ group was non-linear and did not allow LD_{50} to be estimated. However, at 48 hours there were 27 mice surviving in the Fe- group and 33 in the Fe+ group, indicating that the trend towards greater virulence of Fe- cells observed in strains WN50 and CI6 was also the case in strain 2168. Strains WN50, CI6 and 2168 were all serum resistant. When the assay was repeated with a serum sensitive strain, WN34 (O:10), cultured in both Fe- and Fe+ CDM none of the mice died at inoculum sizes up to 8×10^7. Likewise no mice died in the control groups in which mice were injected with heat-killed Fe- cells in inoculum sizes up to 8×10^7

Table 5.1 The effect of iron-sufficient and iron-depleted culture of inocula on virulence of P.aeruginosa in a mouse intraperitoneal infection model.

strain WN50	Fe-	Fe+
Inoculum size range	$4 \times 10^6 - 8 \times 10^7$	$4 \times 10^6 - 8 \times 10^7$
LD_{50} (48 hours)	2.19×10^7	15.9×10^7
correlation coefficient	0.98	0.99
95% confidence limits	$1.85 - 2.6 \times 10^7$	$15.2 - 16.7 \times 10^7$
strain CI6		
Inoculum size range	$4 \times 10^6 - 8 \times 10^7$	$4 \times 10^6 - 8 \times 10^7$
LD_{50} (48 hours)	3.36×10^7	4.64×10^7
correlation coefficient	0.97	0.99
95% confidence limits	$2.21 - 5.09 \times 10^7$	$3.48 - 6.17 \times 10^7$
strain 2168		
Inoculum size range	$4 \times 10^6 - 8 \times 10^7$	$4 \times 10^6 - 8 \times 10^7$
LD_{50} (48 hours)	4.8×10^7	
correlation coefficient	0.99	
95% confidence limits	$2.5 - 9.25 \times 10^7$	
strain WN34		
Inoculum size range	$4 \times 10^6 - 8 \times 10^7$	$4 \times 10^6 - 8 \times 10^7$
LD_{50} (48 hours)	$> 8 \times 10^7$	$> 8 \times 10^7$

5.2 Investigation of the antibody response to intraperitoneal infection.

At the completion of the virulence study the antibody response in groups of mice which had either died or survived the intraperitoneal challenge with strain WN50 was investigated by immunoblotting. The OMs of strain WN50 which had been transferred to NC paper were probed with suspensions of the viscera of mice from groups which had (a.) died within 24 hours of a challenge with 8 x 10^7 Fe- WN50 or (b.) survived more than 4 days after a challenge with 8 x 10^4 Fe- WN50. Both anti-mouse IgG and anti-mouse IgM were used as second antibody. A suspension of viscera removed from mice which had not been challenged with P.aeruginosa was used as control. In all three cases there was no observable antibody reaction with OMP antigens of WN50 although the experiment was repeated on a number of occasions. A positive control using hyperimmune mouse serum raised against strain PAEW gave a reaction with all major OMPs of WN50 (not shown) which verified that the assay procedure was capable of detecting antibodies in mouse serum. The negative result thus appears to show that there was no antibody response to intraperitoneal infection over the 5 days of this experimental infection.

5.3 Discussion

In order to cause infection bacteria must be able to enter host tissue by penetrating skin or mucous membrane or colonizing a

site of trauma. Bacteria cause disease by multiplying at the site of infection and by the elaboration of toxic materials. To survive in the body the bacteria must also interfere with the host defence mechanisms (Van Furth, 1981; Ogata, 1983). The biochemical characteristics of bacteria which enable these disease processes to occur are referred to as virulence determinants (Smith, 1977). Rational design of vaccines against bacterial pathogens depends upon an understanding of the mechanisms involved in the pathogenesis of a particular disease (Hambleton & Melling, 1983). Understanding of the disease process may lead to the development of vaccines designed to neutralize one or more of the virulence determinants and so interfere with the pathogenesis of disease. An animal model of infection which mimics the natural disease is a valuable tool with which to assess experimentally the effect of vaccination. Unfortunately it has proved to be difficult to reproduce human Pseudomonas disease in animals. There appears to be an intimate relationship between these infections and the defence systems of the host. (for references see Introduction 1.3). A number of animal models have been proposed which compromise the host defences (for references see Introduction 1.5). This virulence study was undertaken to investigate the effect of iron limitation of inocula and to evaluate the antibody response in a mouse infection model.

The results of the virulence study indicate that a smaller number of cells grown in iron-depleted conditions were needed to produce a fatal infection in mice than where cells grown in iron-sufficient conditions. The control study in which equal numbers of heat-killed cells were injected by the same route shows that death in the live cell groups was due to infection and not to endotoxic shock caused by LPS associated with the large number of cells injected (Pruit et al, 1983). Since bacteria need iron to proliferate and

thereby cause an invasive infection (Bullen, 1981) the ability of bacteria to store iron or to acquire it fron the environment can be considered to enhance their virulence (Weinberg, 1978,1984). Administration of iron (2mg ferric ammonium citrate) with the inoculum in a mouse intraperitoneal infection (Joo, 1980) has been reported to increase the virulence of K.pneumoniae and all 7 Fisher immunotypes of P.aeruginosa many hundred-fold compared to an inoculum without added iron. Culture of P.aeruginosa in iron depleted conditions has been shown to induce a number of high M_r proteins in the OM. If these IRMPs constitute part of an iron uptake system they can be considered virulence factors. Iron deprived inocula with IRMPs already induced in the OM should be able to acquire iron immediately whereas rapid growth of iron-sufficient inocula could not occur until IRMPs had been induced.

The bactericidal activity of serum is an important component of host defence against Gram-negative bacteria (Taylor, 1983). It is tempting to correlate the avirulent nature of the WN34 strain in this model with its serum sensitivity. However, the susceptibility of P.aeruginosa strains to serum appears to be quite heterogeneous (Sheehan et al, 1982) and the nature of serum sensitivity not well understood (Taylor, 1983). The evidence that serum-sensitive strains can be trained to serum-resistance by passage in increasing concentrations of human serum (Schiller et al, 1984) makes even a clear definition of serum-resistance or -sensitivity complicated. Changes in LPS O-chain substitution and hence serotype concomitant with training to serum-resistance (Goldman et al, 1984) emphasise the plastic nature of the cell surface. Only 2 serum sensitive smooth strains were isolated in this study so it was not possible to correlate avirulence with serum-sensitivity in any statistically

significant way.

An investigation of antibody response was undertaken to determine whether experimental infection in a mouse intraperitoneal model would elicit an immunological response to OMPs. The 'classic' mouse intraperitoneal model was chosen because of the ease of obtaining large numbers of mice and their relative economy of use. The inoculation procedure is straightforward and reproducible infections can be achieved without the need to lower the host's resistance by artificial stress (Berk, 1977). Mice with systemic Gram-negative infections in this model die in 24-72 hours so that results are obtained reasonably quickly. The disadvantage of this model is that the process of inoculating large numbers of organisms directly into the peritoneum does not correspond to any naturally acquired infection.

Repeated i.p. injections of a suspension of formalin-killed cells will produce an antibody response in mice. Hyperimmune mouse serum was prepared by this method (2.1.6.1). A single injection of live cells did not produce an antibody response which could be measured by the immunoblotting technique under the conditions used in this study. It is possible that too short a time was allowed for formation of antibodies to be assessed. Repeated injections of live cells would probably have evoked an antibody response but results would not then have been comparable with those from the dead mice group. The lack of a measurable antibody response means that this model would not be suitable to monitor the protective effect of immunisation with separated OM components since the antibody response to individual components could not be evaluated. Hedstrom -et al- (1984) have recently reported the antibody response to an P.aeruginosa infection model in which mice were injected subcutaneously with 10^7 - 10^8

organisms. This challenge caused the formation of an acute abcess which usually resolved within 3-14 days. An ELISA of serum taken from the mice at 3 days showed an antibody response to isolated OM of the infecting strain. Serum taken at 7 and 14 days showed an increasing titer of antibodies. Immunoblotting with a ^{125}I labelled second antibody showed after autoradiography that 14 day serum contained antibodies to OMPs F, H2, I and a 16K protein believed to be pili. The inoculum in this study was grown in complex medium and nutrient limitations were not specified. This experiment, like the intraperitoneal infection model, had the disadvantage that it did not mimic the natural acquisition of infection (except perhaps infection from trauma) and it could not easily be used to study the protective effect of immunisation since the infection was local and not lethal. Nevertheless, since a measurable antibody response was produced it may be useful as a means of monitoring the effect of vaccines composed of OMPs.

6. Interaction of antibodies in serum and urine with bacteria isolated directly from human UTI.

6.1 *Klebsiella* and *Proteus* species in human UTI

The OMP composition and antigenicity of OM components was investigated using organisms isolated from the urine of three patients with UTI and studied without subculture. The first patient (female, age 69) had an asymptomatic UTI following catheterisation made necessary by a stroke. Microbiological analysis of the urine (Cowan, 1974) yielded *K.pneumoniae*, *Streptococcus faecalis* and *Staphylococcus aureus*. The second patient (female, age 86) was catheterised following surgical closure of an entero-vesical fistula. Microbiological analysis of the urine yielded *K.pneumoniae*, *P.Mirabilis* and *Proteus morganii*. One daily sample of urine from this patient was found to contain a pure culture of *P.mirabilis* and this was harvested separately. After five days the patient received one dose of ceftriaxone (Rocephin, Roche) and urine subsequently collected was sterile. The Gram-negative bacteria isolated from the two patients were cultivated *in vitro* in CDM and also TSB under iron-sufficient and iron-depleted conditions.

Figure 6.1

SDS-PAGE of OMPs of K.pneumoniae isolated directly from urine (lane 1) and the same isolate grown in Fe- TSB (lane 2) and Fe+ TSB (lane 3).

Figure 6.2

Immunoblot of the OMPs shown in 6.1 electrophoretically transferred to NC paper and reacted with the patient's own serum.

Figure 6.1

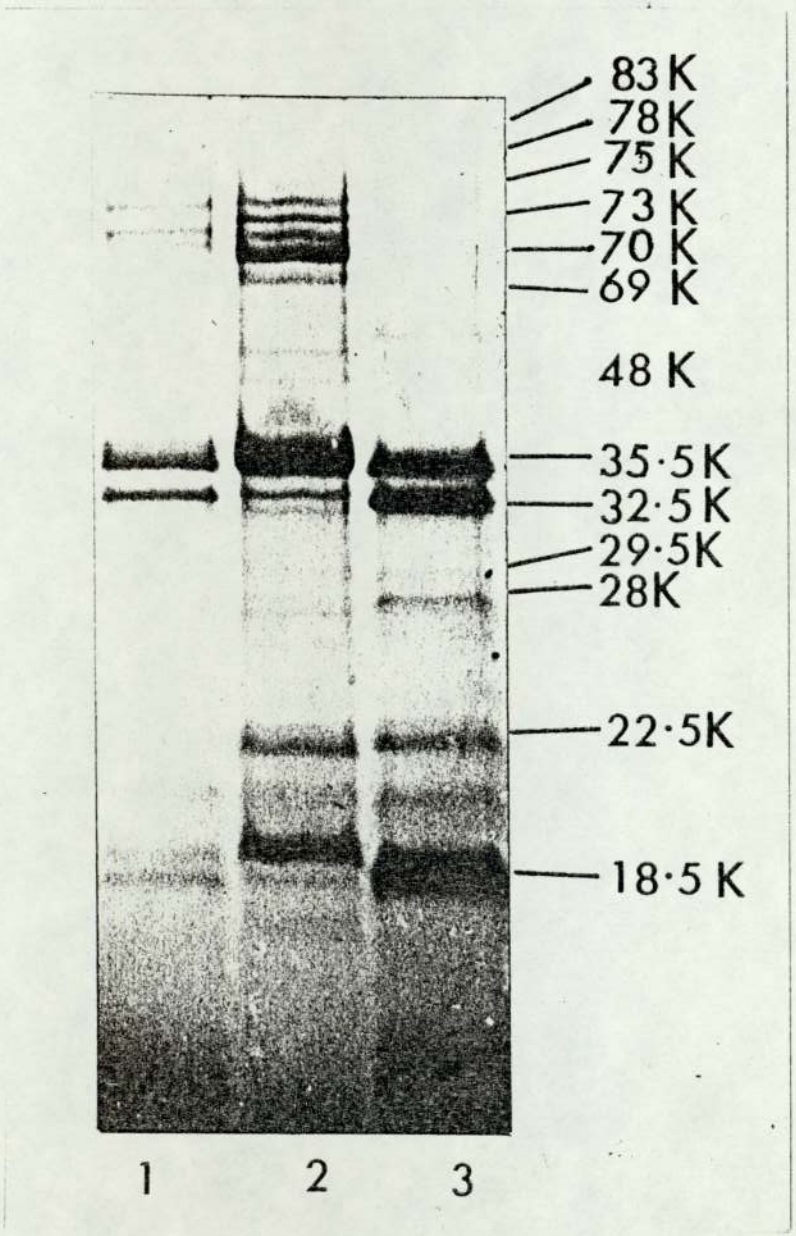


Figure 6.2

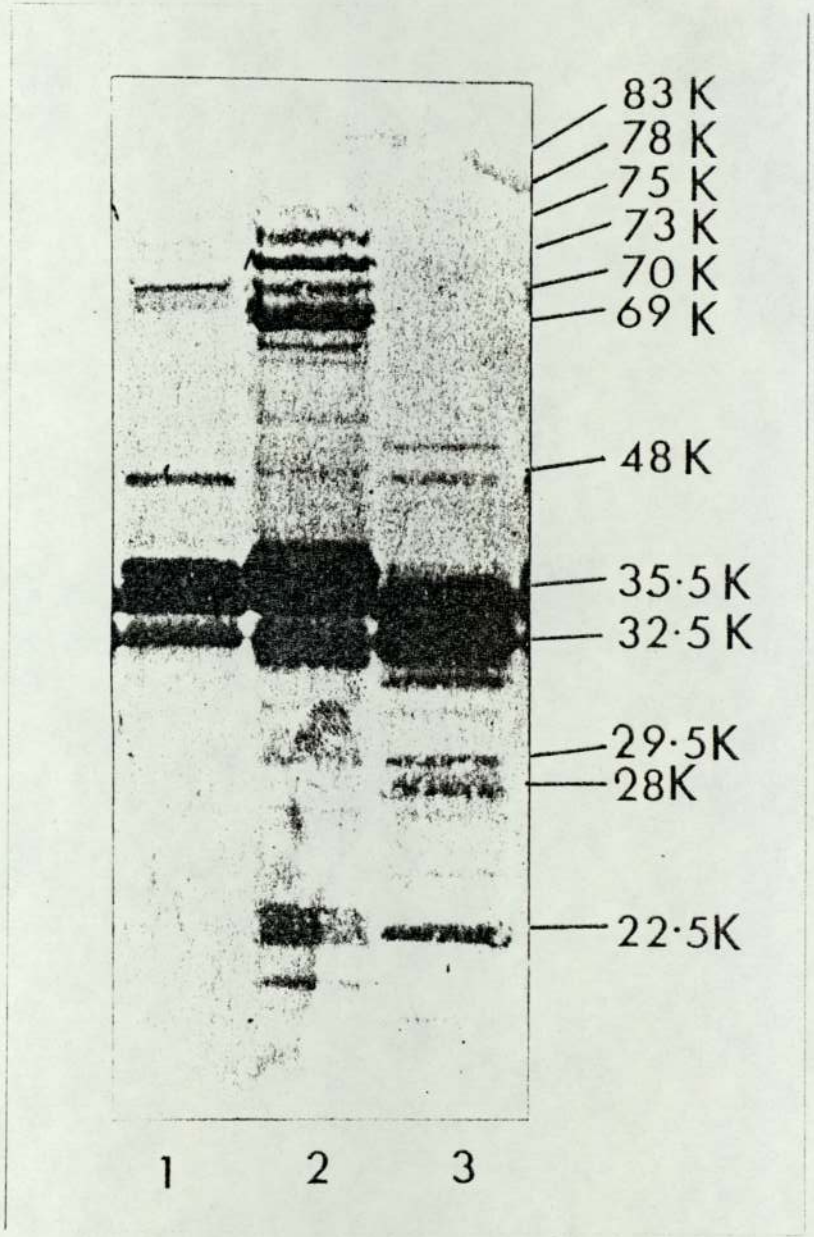


Figure 6.3

SDS-PAGE of OMPs of Gram-negative bacteria isolated directly from urine and the same isolates grown in laboratory media. Lanes 1 and 2, P.mirabilis grown in Fe+ TSB and Fe- TSB respectively; Lane 3, P.mirabilis isolated directly from urine. Lanes 4 and 5, P.morganii grown in Fe+ TSB and Fe- TSB respectively. Lane 6, mixed culture isolated directly from urine. Lanes 7,8,9 and 10, K.pneumoniae grown in Fe- TSB, Fe+ TSB, Fe- CDM and Fe+ CDM respectively.
15% acrylamide gel.

Figure 6.4

Immunoblot of the OMPs shown in figure 6.3 electrophoretically transferred to NC paper and reacted with the patient's serum.

Figure 6.3

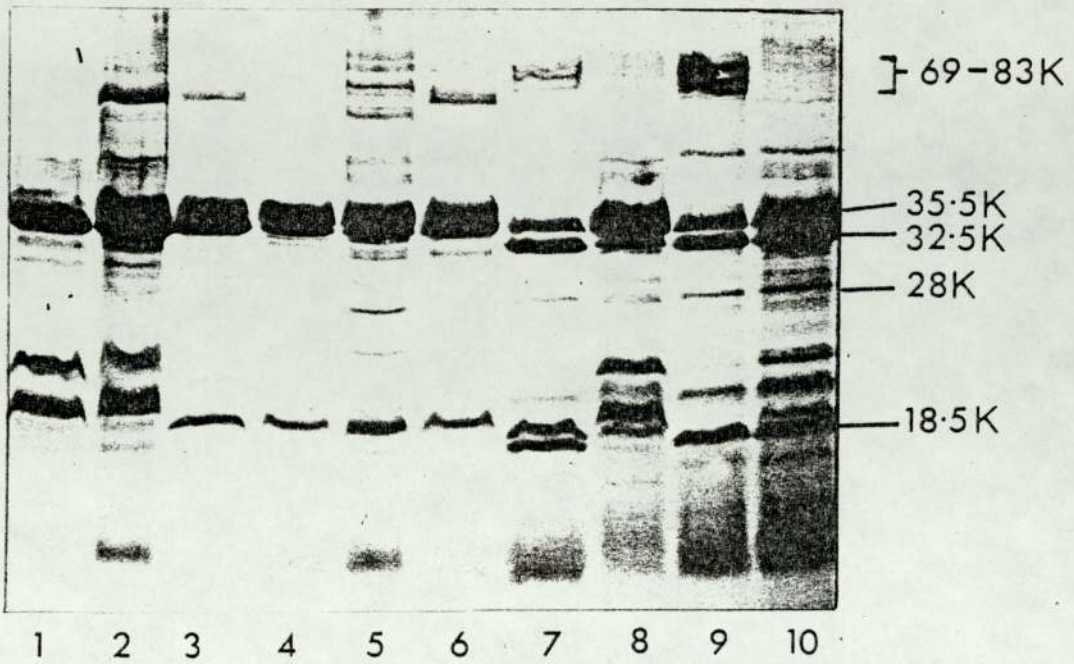


Figure 6.4

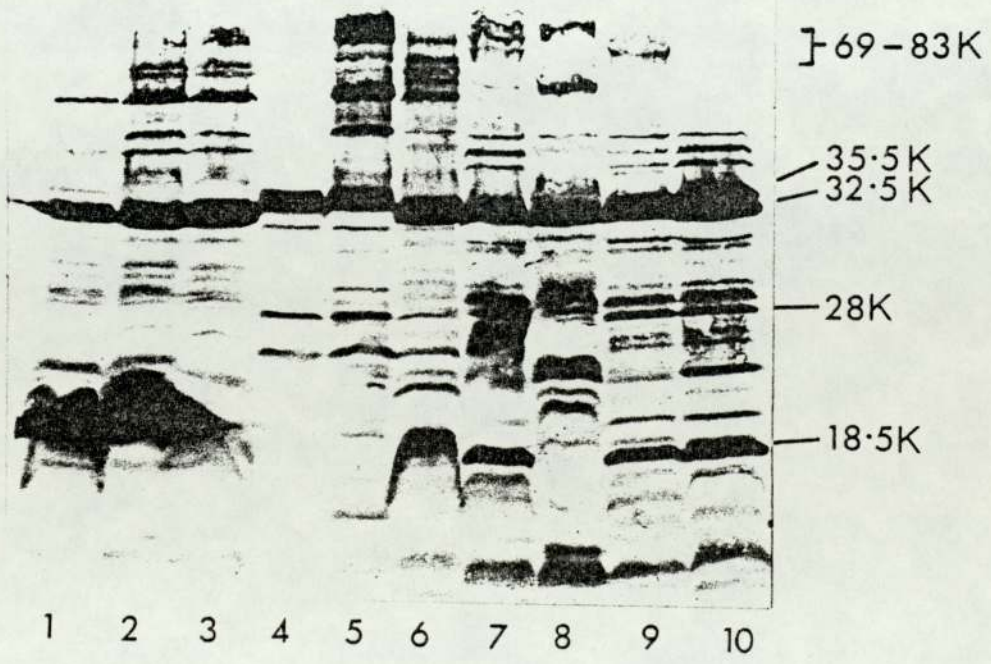


Figure 6.1 shows the OMP profiles of K.pneumoniae isolated directly and without subculture from the urine of patient 1 (lane 1) and the same isolate grown in vitro in Fe- TSB (lane 2) and Fe+ TSB (lane 3). Three high molecular weight proteins were present in the OM of the cells grown in vivo (73,75 and 83K), these correspond to proteins induced in the iron-depleted medium (lane 2) and were repressed when the organism was grown in iron-sufficient medium (lane 3). The number of high molecular weight bands observed in lane 2 was greater than those seen in lane 1 and it is possible that other factors such as pH, osmolarity or growth on epithelial surface affected the expression of these high M_r proteins. The osmolarity of pooled urine from this patient was 350 m.osmols which is within the normal range (Asscher et al, 1968). Growth of this organism in iron depleted media at a number of different pH in the range pH 5-7 always produced at least six IRMPs (P. Williams, personal communication), indicating that factors other than pH are involved. It is not known whether these proteins were involved in specific iron uptake mechanisms. The observation that they are induced when the same organism is grown in iron-depleted medium (lane 2) and repressed in an iron-sufficient medium (lane 3) indicated that organisms growing in urine were iron-restricted. The concentration of iron in the pooled urine of patient 1 was 1.8×10^{-6}M and of patient 2, 3.3×10^{-6}M.

Figure 6.3 shows the OMP profiles of P.mirabilis from the urine of patient 2 cultured in Fe+ TSB (lane 1) and Fe- TSB (lane 2); P.morganii in Fe+ TSB (lane 4) and Fe- TSB (lane 5); and K.pneumoniae in Fe- TSB (lane 7), Fe+ TSB (lane 8), Fe- CDM (lane 9) and Fe+ CDM (lane 10). The pure culture directly isolated from the urine is in

lane 3 and the mixed culture in lane 6. The OMP profile of the pure culture is very similar to that of the iron-depleted P.mirabilis in lane 2. Thus, in this patient, the organisms isolated directly from the urine had an OMP profile which could be closely mimicked when the same isolates were grown in laboratory media under iron-depleted conditions. The mixed culture in lane 6 appears to be a mixture of Proteusand Klebsiella protein profiles.

6.1.1 Interaction of antibodies in patient's serum with Klebsiella and Proteus OM antigens.

Figure 6.2 shows the immuno-blot of the OM proteins seen in figure 6.1 reacted with the patient's own serum. The porins (32.5K and 35.5K) of the organism under all three growth conditions were highly immunogenic. IRMPs were also seen to be immunogenic but were fainter on the blot of the isolate from the patient's urine (lane 1) than when the cells were grown in Fe- TSB (lane 2). In addition an antigen with a molecular weight of 48K seems to be highly immunogenic although it was not evident on the coomassie blue stained polyacrylamide gel (figure 6.1). Those proteins with a molecular weight below 32.5K were not recognised by the patient's serum in the sample directly isolated from the urine (figure 6.2,lane 1).

Figure 6.4 shows the antigenic profile after blotting the proteins separated in figure 6.3 onto NC paper and probing with the patient's own serum. Again the porin proteins and IRMPs were immunogenic. The antigenic profile of the organism isolated directly from the urine (lane 3) was very similar to that of P.mirabilis grown in Fe- TSB (lane 2). The patient's serum contains antibodies which react with antigens from all three organisms present in the urine.

To determine the cross-reactivity of antibodies present in the patient's serum a control experiment was carried out using OM antigens of Enterobacteriaceae and P.aeruginosa. Figure 6.5a shows the OMP profiles of K.pneumoniae (lane 1), P.mirabilis E.coliand P.aeruginosa isolated from human UTI and grown in Fe- TSB. Figures 6.5,b and c show immunoblots obtained when the antigens described in 6.5a were transferred to NC paper and probed with sera from each of the two patients. The patient's sera contained antibodies which reacted with antigens in the OM of K.pneumoniae , P.mirabilis and E.coli (lanes 1-3) but demonstrated a very weak response to antigens on the OM of P.aeruginosa (lane 4).

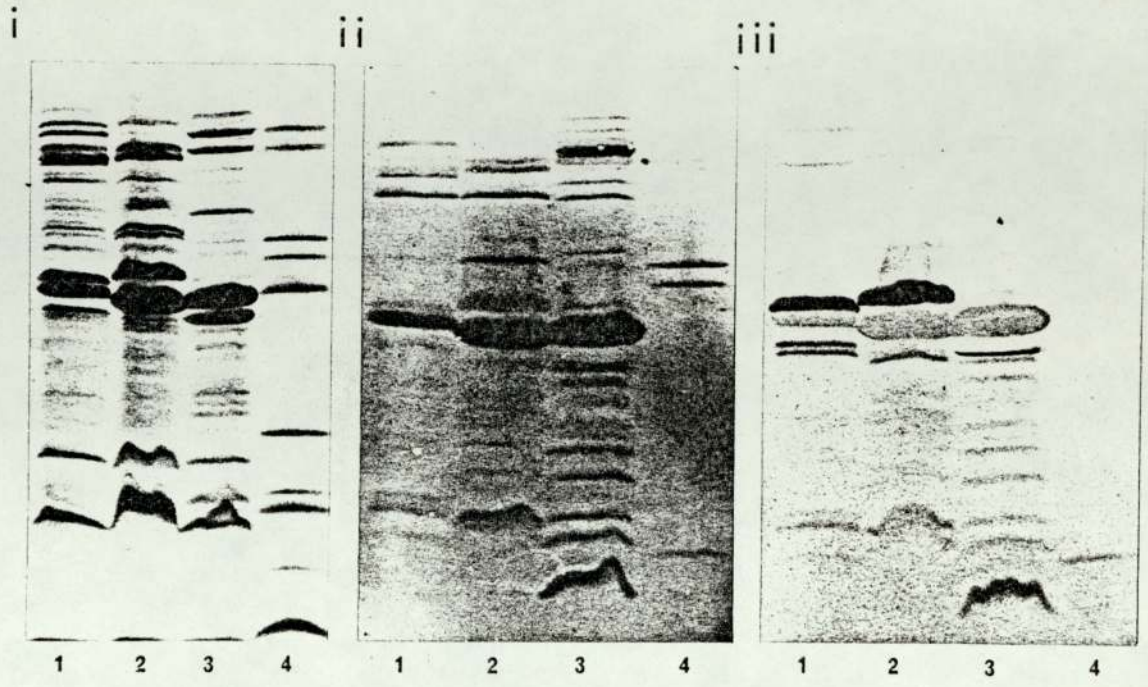


Figure 6.5,i

SDS-PAGE of OMPs of K.pneumoniae (lane 1), P.mirabilis (lane 2), E.coli (lane 3) and P.aeruginosa (lane 4) isolated from UTIs. All strains were grown in Fe- TSB. 15% acrylamide gel.

Figure 6.5,ii and iii

Immunoblots of the OMPs shown in figure 6.5,i reacted with patient's serum.

6.2 *P.aeruginosa* in human UTI

P.aeruginosa PAGS was isolated as a pure culture from urine collected from two hospitalized female patient (ages 62 and 75) with UTI following catheterisation after surgery (patient 3) and following a stroke (patient 4). The urine sample from patient 4 did not contain sufficient bacteria to allow membranes to be isolated without subculture. The outer membrane protein profile of bacteria isolated directly from the urine of patient 3 is shown in figure 6.6, lane 3. The protein profiles of the same isolate grown in iron sufficient and in iron-depleted CDM 12 are shown in lanes 1 and 2 respectively. Growth in iron-depleted media induced the production of two major high molecular weight proteins (83 and 88K) and four minor high molecular weight proteins (82,85,93 and 101K). These proteins were absent in the OM of bacteria grown in iron sufficient conditions. A corresponding cluster of high molecular weight proteins was present in the OM of bacteria isolated directly from the urine (lane 3) indicating that bacteria were growing in the urine under conditions of iron-restriction.

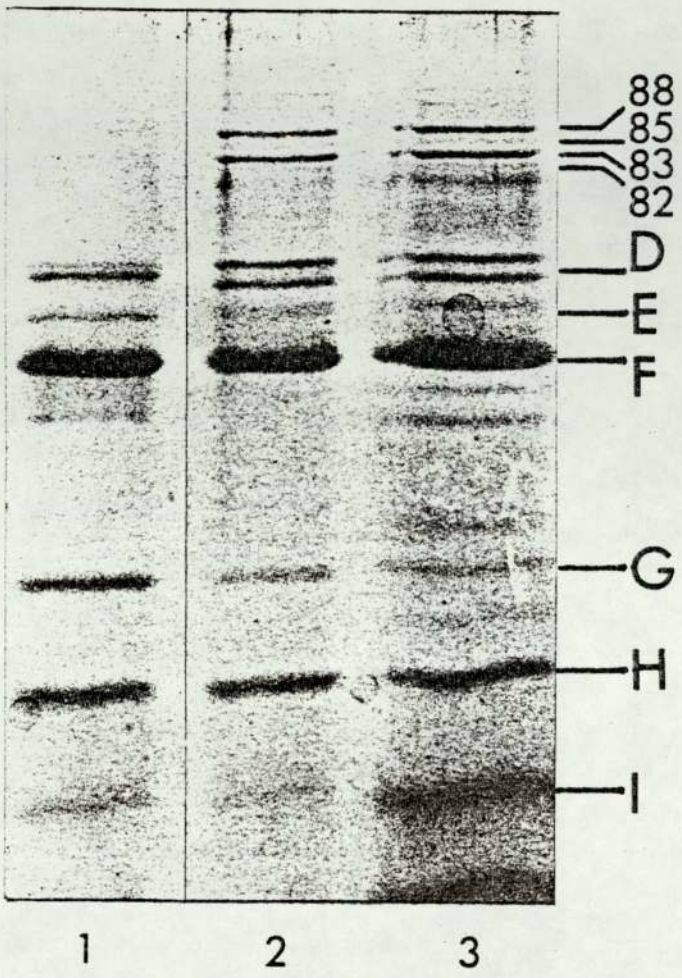


Figure 6.6

OMP profiles of P.aeruginosa isolated directly from the urine of a patient with UTI (lane 3) and the same isolate grown in Fe+ CDM (lane 1) and Fe- CDM (lane 2).

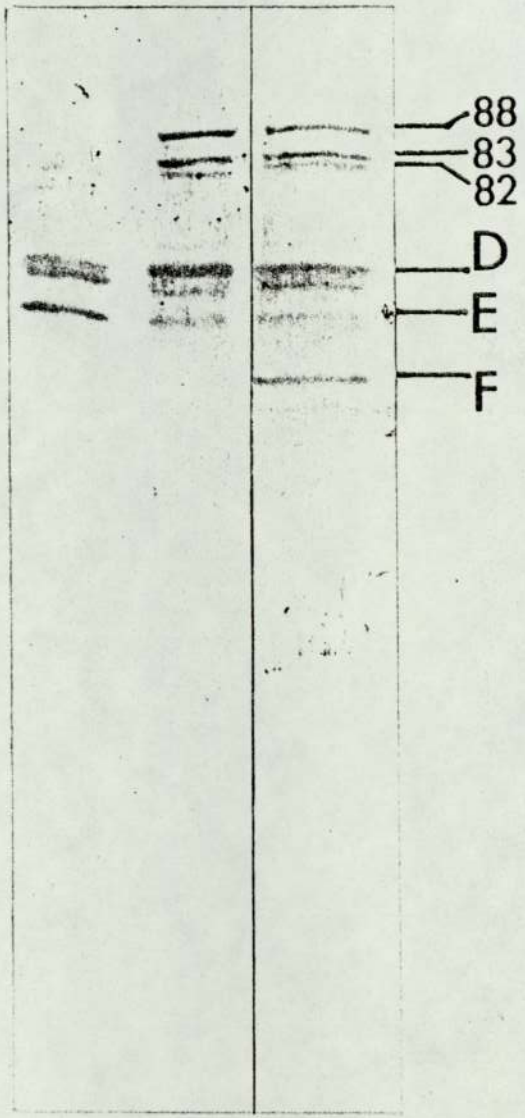
15% acrylamide gel.

Figure 6.7,i and ii

Immunoblot of the OMPs shown in Figure 6.6 electrophoretically transferred to NC paper and reacted with i) patient's serum, and ii) patient's urine, using IgG as second antibody.

Figure 6.7,i

Figure 6.7,ii



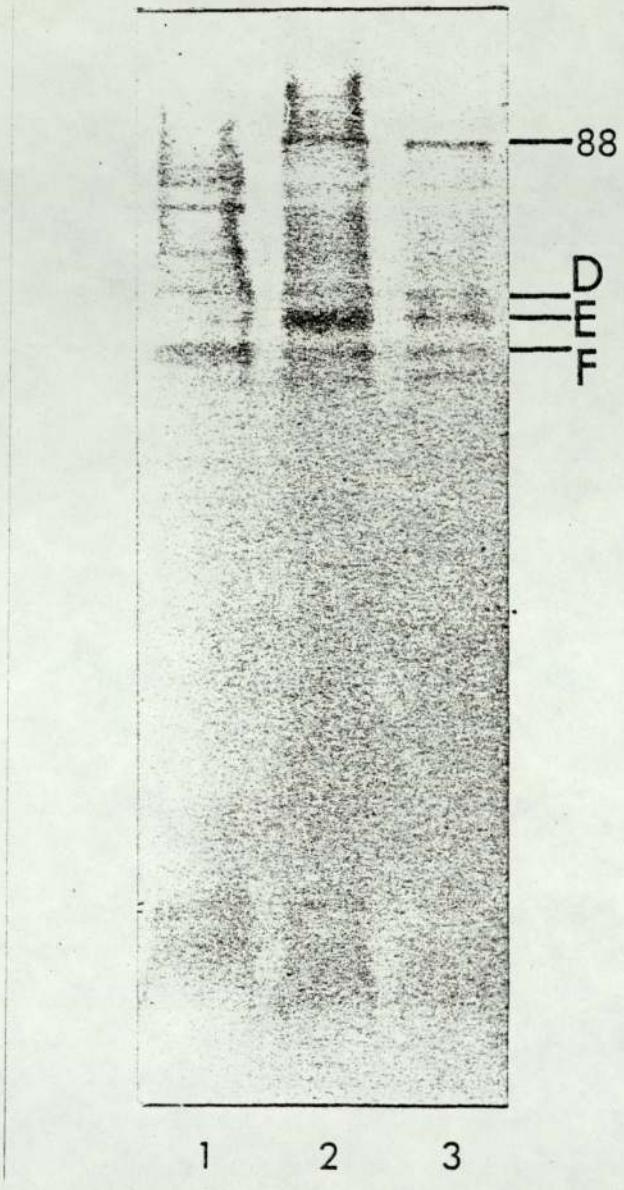


Figure 6.8

Immunoblot of the OMPs shown in figure 6.6 electrophoretically transferred to NC paper and reacted with patient's serum, using IgA as second antibody.

6.2.1 Interaction of antibodies in patient's serum and urine with P.aeruginosa OM antigens.

Figure 6.7 shows the immunoblot obtained by electrophoretically transferring OMPs to nitrocellulose paper and probing with the patients own serum (figure 6.7a.) and urine which had been concentrated five-fold by freeze-drying (figure 6.7b.). Goat anti-human IgG tagged with HRP was used as the second antibody. The patient's serum contained IgG antibodies which reacted with proteins D,E,F and H. In addition two proteins with molecular weights slightly below that of protein F were apparent in the immunoblot of bacteria directly isolated from the urine (lane 3). These may be incompletely denatured protein F fragments (Mizuno & Kageyama, 1978; Anwar et al, 1983b) whose origin can be seen more clearly in lanes 1 and 2. Proteins in these positions were seen faintly in the coomassie blue stained gel (figure 6.6,lane 3). Three of the high molecular weight proteins (82,83 and 88K) were visualised faintly in the serum blot but are much more clearly evident in the urine blot (6.7b, lanes 2 and 3) where they were as strongly reactive as proteins D,E and F. In both blots faint reactions were seen with antigens in the Ca. 80K region of cells grown in iron sufficient CDM (lane 1).

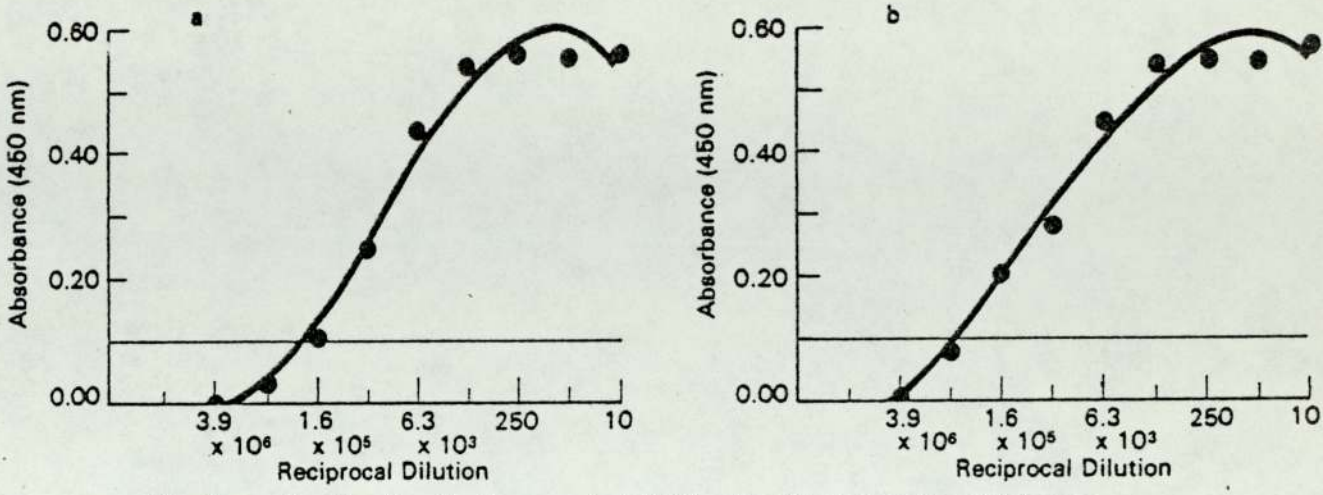
The immunoblotting procedure was repeated with anti-human IgA-HRP and anti-human IgM-HRP conjugates as second antibody. Figure 6.8 shows the immunoblot obtained after probing OMPs transferred to NC paper with patient's serum and goat anti-human IgA as second antibody. The patient's serum contained IgA antibodies which reacted with OMPs D,E

and F and with the IRMP of 88K molecular weight, these reactions were, however, fainter than those seen with IgG serum antibodies. Immunoblots probed with patient's urine gave no reaction with goat anti-human IgA conjugate. Likewise there was no reaction when blots were probed with serum and urine and anti-human IgM conjugate was used as second antibody.

6.2.2 Enzyme linked immunosorbent assay.

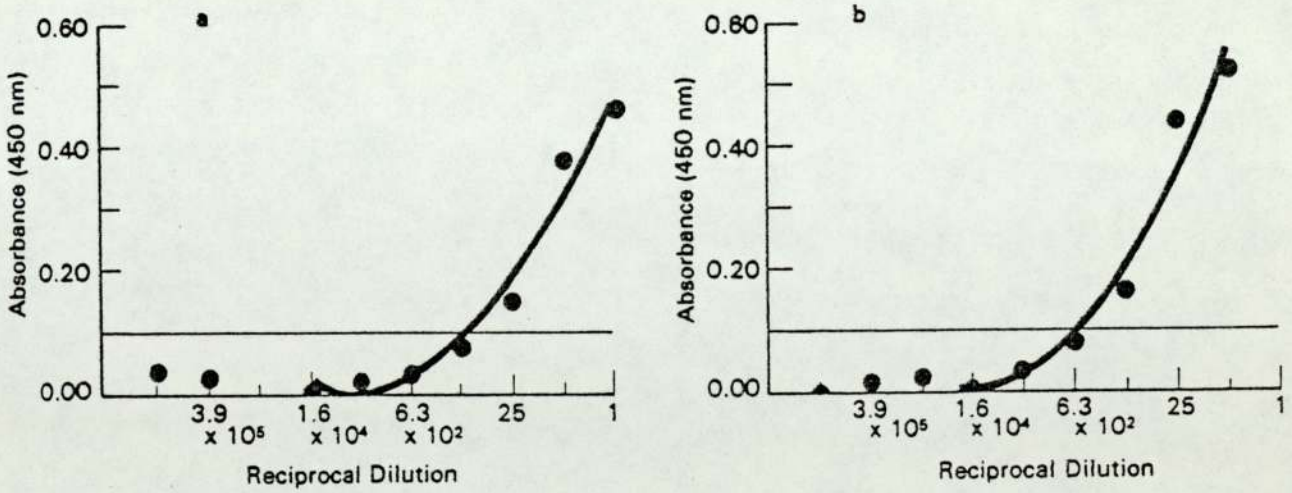
Antibodies in patient's serum and urine were quantified by enzyme linked immunosorbent assay (ELISA). Figure 6.9 shows the binding of serum IgG to a) whole cell antigens and b) OM antigens of P.aeruginosa. Similar titration curves were observed in the assay for IgA and IgM in serum (data not shown). Comparison of the titre of IgG, IgA and IgM at a specific endpoint (0.1 asbsorbance units above background) indicated that the plates coated with OMs were twice as sensitive as those coated with whole cells (table 6.1). The reason for this may be that the antigenic determinants on the OM preparation are more accessible to antibody or that the concentration of these determinants is greater in the OM. In the patient's serum titres of IgA and IgM were approximately 10-fold and 100-fold lower respectively than those of IgG (table 6.1). All three immunoglobulin subclasses studied gave some reaction with control serum (table 6.1), in all cases this was significantly below the levels detected in patient serum but indicated some degree of non-specific binding of serum antibodies.

Figure 6.9



Titration of 1g G antibodies in serum by ELISA using (a) whole cells and (b) outer membranes of P. aeruginosa as antigens. Serum samples were initially diluted 10-fold followed by 5 fold serial dilution.

Figure 6.10



Titration of 1g G antibodies in (a) urine and (b) concentrated urine by ELISA using outer membranes of P. aeruginosa as antigens. The first urine sample was undiluted, each subsequent dilution was 5 fold.

Table 6.1

	Patient's Urine		Concentrated Patient's Urine		Control Urine		Patient's Serum		Control Serum	
	OM	WC	OM	WC	OM	WC	OM	WC	OM	WC
IgG	490	90	4.7×10^3	10^3	3.4	1	7×10^5	2.7×10^5	9.8×10^3	850
IgA	4.4	0	11.6	11.2	0	35	5.5×10^4	2.6×10^4	576	50
IgM	0	0	2.9	0	0	0	4.3×10^3	2.2×10^3	719	180

ELISA titer expressed as a reciprocal of the dilution at a net absorbance of 0.1.

Titre of antibodies in patient's serum and urine against antigens of iron depleted whole cells and isolated outer membranes of infecting P.aeruginosa.

Figure 6.10 illustrates the binding of IgG in a) urine and b) 5-fold concentrated urine to the OM antigens of P.aeruginosa. Titres of IgG in urine and concentrated urine (table 6.1) indicate that concentration of urine by freeze drying resulted in an increase in sensitivity greater than the concentration factor. The same increase in sensitivity with concentrated urine was also observed when whole cells were used as antigens although as in the case of serum the use of OMs as antigens (cf. whole cells) increased the sensitivity of detection in urine. This disproportionate increase in sensitivity may have occured because the reaction with unconcentrated patient urine is at the lower end of the detection limits of the system. Even after concentration the ELISA titres for IgA and IgM were significantly lower than those of IgG (table 6.1).

Urine was freeze-dried to reduce its bulk. Comparison of antibody titers using ELISA on urine and urine concentrated by freeze-drying indicated that the process of freeze-drying had no adverse effect on antibody titre (table 6.1). Freeze drying was thus an acceptable method of reducing the bulk of urine to manageable proportions and also increased the sensitivity of immunological procedures.

All three subclasses of immunoglobulin were present at very low levels in control urine. A similar pattern of results to those given in table 6.1 were obtained when the assay was repeated on separate occasions. The results indicate that the antibody response at this chronic stage in the infection was predominantly IgG in both serum and urine. There was a smaller IgA response in serum but none in urine. IgM levels in both serum and urine were below the level of detection. Although the levels of IgM in serum were higher than in urine, these were significantly lower than corresponding levels of IgG. These

results reflect the findings obtained by immunoblotting described above.

6.2.3 Cross-reaction of antibodies in patient's serum and urine with antigens of P.aeruginosa serotypes.

The IATS defines 17 O-antigen serotypes of P.aeruginosa (Bergan, 1975). Some of these O groups may be subdivided to give a larger number of serotypes. The two groups most frequently isolated in the United Kingdom are seroptypes O:6 and O: 11 (Pitt, 1980). The organism isolated in this study was identified as serotype O:6 by slide agglutination. Figure 6.11 shows the OMP profiles of a representative of each of the 17 serotypes of P.aeruginosa and two polyagglutinating strains, all grown under iron-depleted conditions. Athough there are minor differences between the strains shown in the figure they all conformed basically to the scheme described by Mitzuno and Kageyama (1978). Protein F of the O:11 strain was not completely denatured by the conditions used so that it appeared in both F and F* positions. Figure 6.12 shows the immunoblot obtained by probing the OMPs described in figure 6.11 with (6.12,a) serum and (6.12,b) urine after transfer to NC paper. The reaction of IgG antibody with the porin, protein F, is observed consistently in all serotypes and the PA strains used in the study. Proteins D and E were also observed in all of the strains represented but were noticeably less reactive in some strains than in others (cf. O:10). Proteins G,H and I reacted only faintly or not at all with antibodies in both serum and urine. There were two noteworthy differences between the two immunoblots. Firstly the presence of antibodies to rough LPS in the urine but not in serum and secondly the reaction of antibodies in urine with between 3 and 6

discrete proteins with molecular weights in the range 82-101K. Serum antibodies to these proteins were indistinct in most serotype strains investigated.

In strains O:1, O:2, O:3, O:4, O:5, O:7, O:12, O:13 and O:16 another major antigen with a molecular weight of 55K was visualised. A protein of this M_r was faintly visible in the coomassie blue stained gel of OMPs (figure 6.11). To investigate the possibility that this antigen might be flagella, the flagellar preparation from strain PAGS was electrophoretically transferred from a polyacrylamide gel (figure 6.13, lane 1) onto nitrocellulose paper and probed with patients serum and urine. The resulting immunoblots (figure 6.13, lanes 2 and 3) indicated that patient's serum and urine both contained antibodies which reacted with flagella. Other workers have also found the flagella of P.aeruginosa to be highly immunogenic (Mutharia et al, 1982; Anwar et al, 1984).

OMs prepared from the strain of P.aeruginosa isolated from patient 4 were cultivated in Fe+ and Fe- CDM 12. OMs were prepared, separated by SDS-PAGE and electrophoretically transferred to NC paper. The NC paper was probed with five-fold concentrated urine from the same patient. The immunoblot produced (figure 6.14) demonstrated the presence of antibodies to OMPs, including IRMPs, in the patient's urine. This immunoblot confirms the findings from patient 3 (figure 6.7).

6.2.4 Transferrin and lactoferrin levels in serum and urine.

The concentration of transferrin in the serum of patient 3 was 1.65mg/ml, 56% of the normal reference value (Table of calibration values for NOR-Partigen transferrin; 1983, Behring institute, Behringwerke, Marburg, W.Germany). The concentration of lactoferrin in patients serum was too low to be measured. The concentration of transferrin and lactoferrin in urine was also too low to be measured directly but the concentration of lactoferrin was 3.6×10^{-5}µg/ml in urine which had been concentrated twenty-fold by freeze drying, that is 5×10^{-6}µg/ml in undiluted urine. A small precipitin ring formed on the transferrin diffusion plate with urine concentrated twenty-fold but the size of the ring was below that ascribed any value in this system. The concentration of iron in the urine was 0.8×10^{-6}M.

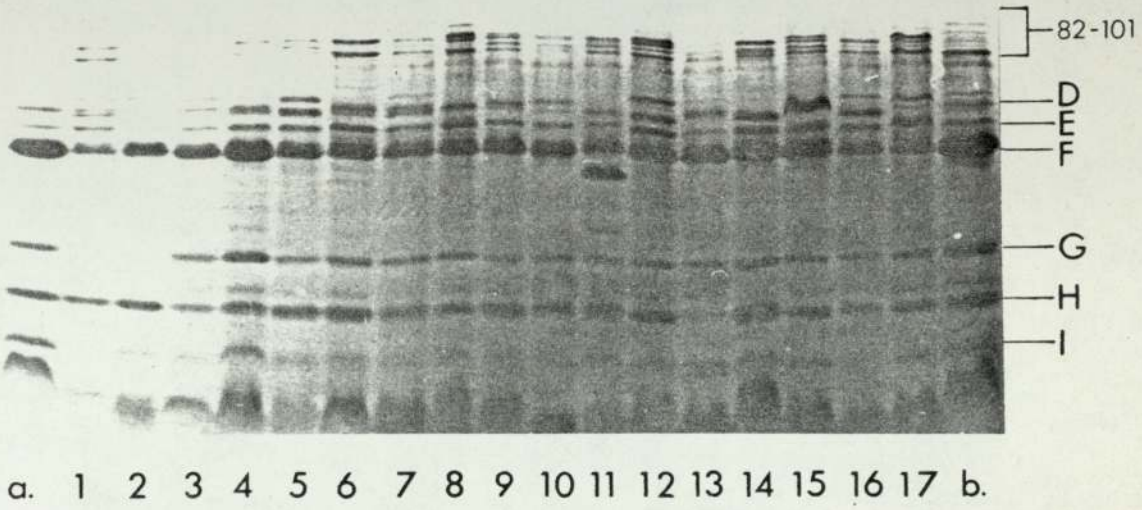


Figure 6.11

The OMP profiles of a representative strain of each of the 17 O-serotypes of P.aeruginosa (lanes 1-17), and two polyagglutinating strains (lanes a and b.), all grown in Fe- CDM.

15% acrylamide gel.

Figure 6.12

Immunoblot of the OMPs shown in figure 6.11 electrophoretically transferred to NC paper and reacted with i) patient's serum and ii) patient's urine.

Figure 6.12,i

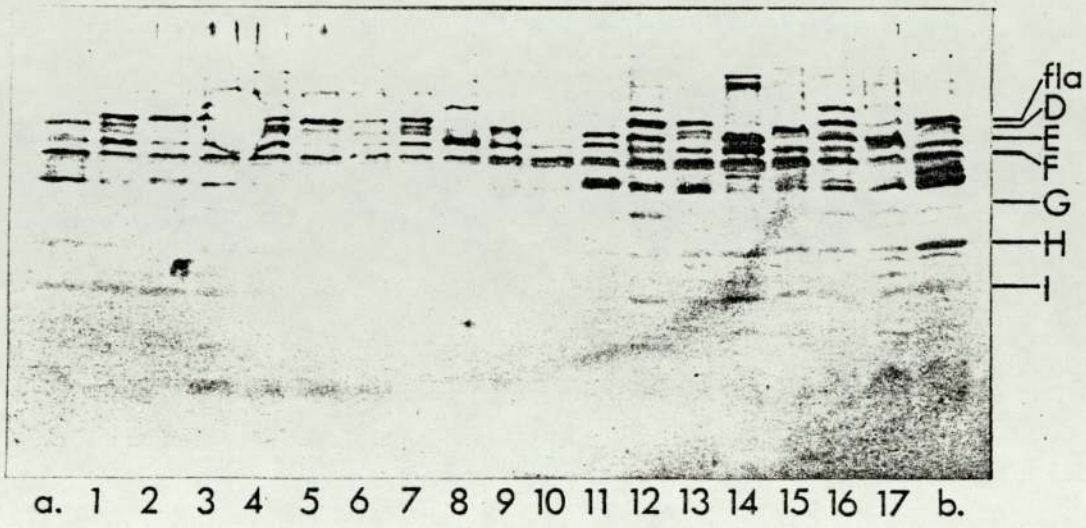
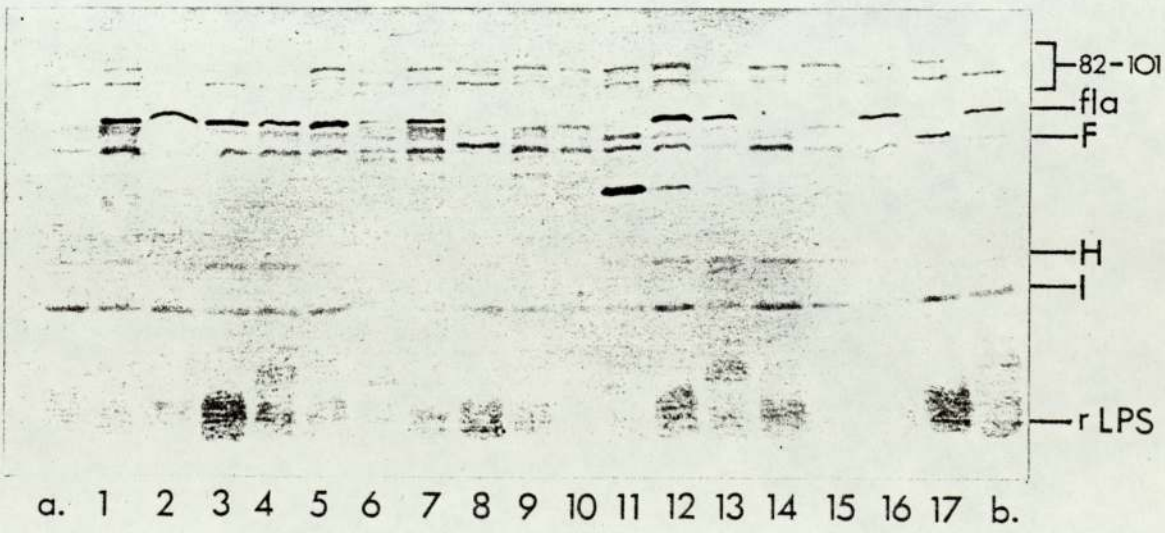


Figure 6.12,ii



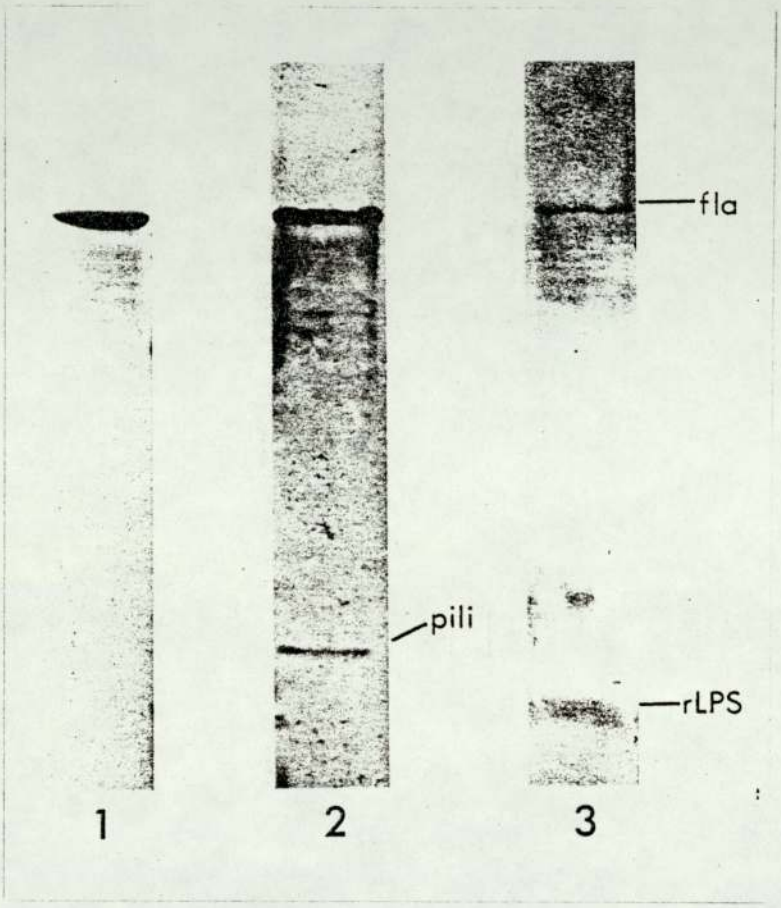


Figure 6.13

SDS-PAGE of the flagellar preparation from P.aeruginosa PAGS (lane 1) and the immunoblots obtained when the flagellar preparation was transferred to NC paper and reacted with patient's serum (lane 2) and urine (lane 3).

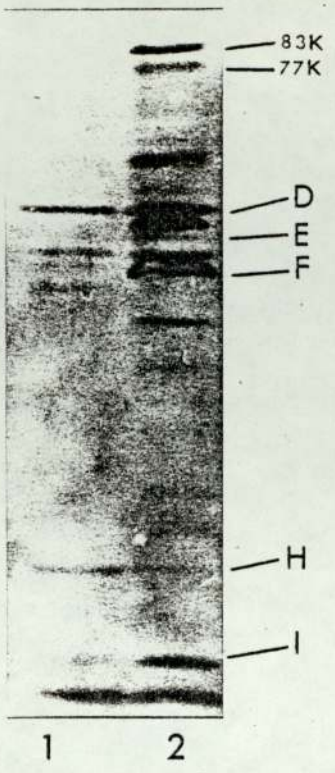


Figure 6.14

Immunoblot of OMPs from a strain of P.aeruginosa isolated from infected urine and grown in Fe+ (lane 1) and Fe- (lane 2) CDM electrophoretically transferred to NC paper and reacted with the patient's urine. IgG was used as second antibody.

6.3 Discussion

These data indicate that P.aeruginosa, P.mirabilis and K.pneumoniae were growing in urine under conditions of iron deprivation as judged by expression of IRMPs. Similar OMP profiles could be obtained when the same isolate was grown under iron-depletion in laboratory media. The finding that Gram-negative bacteria in UTI are growing in conditions of iron deprivation have recently been confirmed (Lam et al, 1984). In the three patients studied the iron concentration in the urine was found to be less than 10^{-5}M. These concentrations of iron in the urine are low (Washington & Boggs, 1975). The low concentration of iron in the urine may be a physiological response to the infection (Beumier et al, 1984) or to the underlying illness (Fitzsimons & Levine, 1983).

The OMP profile of P.aeruginosa and P.mirabilis growing in urine could be mimicked by growing the same organism in iron-depleted medium in vitro, but that of K.pneumoniae could not. Growth of K.pneumoniae in vivo in a rabbit peritoneal infection model also produced an organism whose OMP profile showed three high M_r proteins (Kadurugamuwa, 1985). Further work is necessary to mimic more closely the growth environment in vivo. The limitation of nutrients, including iron, has been shown to have profound effects on envelope composition (Brown & Williams 1985 a, 1985b), including PBPs (Turnowsky et al, 1983) of bacteria, with consequent changes in sensitivity to antibiotics (Brown, 1977) and host defence mechanisms (Anwar et al, 1983; Finch & Brown, 1978) as well as virulence factor production (Ombaka et al, 1983). If it can be shown that the majority of bacteria causing UTI are growing in iron restricted conditions these findings

would support the use of iron restricted growth conditions for the *in vitro* study of UTI.

The radial immunodiffusion method was not sufficiently sensitive precisely to quantify the amount of transferrin and lactoferrin in urine. It did show that these concentrations were low, a more sensitive method of quantifying these iron binding proteins such as an ELISA (Hetherington *et al*, 1983) could with advantage be used in future studies in urine.

The initial antibody response to infection in the urinary tract is a local secretion of IgA and an IgM response in serum (Dick & Kirkwood, 1984). In the chronic phase of infection these responses are both replaced by IgG. IgA is not usually detectable in serum in an acute infection but may become so in the chronic phase (Dick & Kirkwood, 1984).

Immunoblotting using the patient's own serum showed in all cases IgG antibodies in serum from patients with UTI which reacted with OMPs of the infecting organism, including IRMPs. Control experiments (figures 4.3b, 4.4 and 6.5) have shown that there is cross reactivity between OMP antigens of the Enterocacteriaceae but only a weak cross-reactivity between these antigens and those of *P.aeruginosa*. These controls indicate, especially in the case of the *P.aeruginosa*, infection that the antibody response was specific.

ELISA was used to confirm the experimental observations made by immunoblotting *P.aeruginosa* OMPs and to quantify the relative amounts of antibodies of each subclass present in patient's serum and urine. Borowski *et al* (1984) have recently reported an ELISA to determine antibody titres in serum to whole cells of *P.aeruginosa*. However, the cells used were cultured in complex laboratory media and may not represent cells as they occur in the human host during

infection. Immunoblotting and ELISA findings taken together indicate that the antibody response in both serum and urine to P.aeruginosa UTI was predominantly IgG. There was a small IgA response in serum and a much smaller IgM response. In urine the levels of IgA and IgM were both very low and were undetectable by immunoblot. The low level of IgA in the urine of this patient probably reflects the chronic nature of the infection but may also be due in part to the action of IgA proteases secreted by the bacteria (Plaut, 1983). Strains of P.aeruginosa which secrete IgA proteases have been identified in UTI (Milazzo & Delisle, 1984).

Figure 3.22 shows that there is an immunological cross reaction between OMP antigens of the 17 serotypes of the IATS and indicates that there are antibodies in both serum and urine which react with OMP antigens of all 17 serotypes strains, and with the protein antigen of molecular weight 56K identified as flagella. The homcgeneity of the P.aeruginosaOMP antigens is further investigated in Chapter 7.

ELISA results show that urine from volunteers with no history of P.aeruginosa infection contain no specific antibody to P.aeruginosa whole cells or OMs. The presence in the urine of bacteria coated with antibody has been recognised in UTI for some time (Giamarellou, 1984). Other studies of bacteriuria in elderly patients also report that patient's sera contained antibody to the bacteria responsible for the UTI (Reid et al, 1984). IgG and sIgA antibodies in urine against the O antigen of infecting strain of E.coli have been reported (Sohl-Akerlandet al, 1979) but antibodies to specific protein outer membrane components have not previously been demonstrated in urine. It is not known whether these antibodies were derived from serum, produced locally in the bladder or excreted from the kidneys (Sobel & Kaye, 1984). None of the patients included in the study had clinical

upper urinary tract infection but the possibility of kidney involvement cannot be ruled out. The demonstration of antibodies in the urine of a second patient indicates that these results were not idiosyncratic.

Impaired immunological status has been suggested as a possible contributory reason for the high incidence of UTI in elderly hospitalised patients (Freedman, 1983). This study apears to show that the immune systems of the three patients studied effectively recognised the OMP antigens of the infecting organisms.

These results show that it is possible to measure immunoglobulin levels in urine and that the sensitivity of this determination may be increased by freeze drying the urine to concentrate it. Using estimations of this type which combine immunoblotting and ELISA techniques it may be possible to chart the time course of immunoglobulin response in both urine and serum during an infection.

7 Immunogenicity of OMPs of P.aeruginosa strains of different serotype, colony type and antibiotic sensitivity.

7.1 Antibiotic sensitivity.

The antibiotic sensitivities of a number of different strains of P.aeruginosa were determined to construct a table of relative sensitivity (table 7.2). This index would be used when comparisons were made of the immunological properties of OMPs of sensitive and resistant strains.

The antibiotic disc sensitivity of P.aeruginosa strains used in the study is shown in table 7.1. The table shows the mean diameter of the zone of inhibition round each disc averaged from at least 3 determinations. The mean diameter indicates the relative susceptibility of the strains used in the study. Each strain has been designated resistant or sensitive by comparison with a reference control strain, ATCC 10662 (Brown & Blowers,1978). For each antibiotic tested a strain was defined as having the same susceptibility as the control strain (C) if its zone diameter was within $\pm$ 1SD of the mean diameter of the control strain. Outside this range those strains whose zone diameter differed from the control by more than +1 SD were designated sensitive (S), those which differed by more than -1 SD were designated resistant (R). Zone diameters beyond the range $\pm$ 3 SD from the mean were very sensitive (SS) or very resistant (RR). Where there was growth of cells up to the tablet (i.e. no zone at all) the strain was designated very resistant (RR) (table 7.2).

Table 7.1 Inhibition of growth of P.aeruginosa strains by Neosensitab antibiotic discs. Average diameter of zones of inhibition in mm. ± standard deviation.

Strain	Ampicillin	Azlocillin	Carbenicillin	Pipracillin	Amikacin	Gentamicin	Kanamycin	Tobramycin	Polymyxin	Sulphonamide	Tetracycline
10662	-	24.7±1.15	26.3±0.58	32.3±1.15	24.7±1.15	26.3±0.58	12.3±0.58	29.3±0.58	24.7±0.58	13.3±0.58	13.7±0.58
CI4	-	26.0	24.3±0.58	32.3±0.58	29.3±0.58	31.0	23.3±0.58	32.0	23.8±0.29	24.7±0.58	17.0
CI5	-	26.7±1.53	28.0±2	33.7±0.58	27.7±0.58	27.7±0.58	16.0	29.7±1.53	27.3±0.58	-	14.0±1
CI6	-	23.7±1.53	23.8±0.29	30.0	29.0	29.7±0.58	21.0	30.7±0.58	24.3±0.58	-	11.3±0.58
CI7	-	24.0±1	23.0	29.7±0.58	27.3±0.58	27.7±0.58	23.3±0.58	29.7±0.58	24.3±0.58	-	-
CI8	-	22.7±1.15	22.7±0.58	29.7±0.58	22.7±0.58	23.3±0.58	13.7±0.58	26.3±1.15	23.3±0.58	20.7±0.58	14.3±1.15
CI9	-	25.0	25.3±0.58	30.7±0.58	26.3±0.58	26.7±0.58	20.0±1	27.7±0.58	24.0	22.7±0.58	16.7±0.58
CI10	-	25.0±1	23.7±0.58	29.7±0.58	26.3±0.58	26.0	14.0±1	29.7±1.53	23.7±1.15	18.7±2.08	14.3±0.58
CI11	-	29.0±1	27.3±0.58	34.3±0.58	33.0	33.7±0.58	20.7±0.58	36.0±1	27.0	18.3±0.58	23.3±0.58
CI12	-	25.3±1.15	23.7±1.15	30.0	25.7±0.58	26.0±1	15.7±0.58	29.3±0.58	24.7±0.58	16.7±1.15	15.3±0.58
CI13	-	29.3±1.53	31.7±0.58	39.7±0.58	37.0±1	38.0	27.0	40.0±1.79	33.0	30.0±1	20.0±1
CI14	-	28.3±0.58	28.3±0.58	35.3±1.53	31.7±0.58	31.0	17.3±0.58	30.7±0.58	25.7±1.15	18.3±0.58	18.7±1.15
CI16	15.7±1.15	27.0±1	25.3±0.58	33.7±0.58	33.3±0.58	34.0±1	22.0	33.7±1.15	27.3±0.58	19.7±0.58	21.3±1.15
119X	-	26.7±1.5	26.7±0.58	34.0±1	26.0±1	15.0	11.0	12.7±0.58	25.3±0.58	-	15.3±0.58
PANT	-	26.6±0.58	23.7±0.58	31.3±0.58	26.7±0.58	13.8±0.29	11.0	12.0	24.0±1	-	13.3±0.58
2168	-	25.3±1.5	26.3±0.58	31.0±1	25.0	25.0	18.0±1	28.7±0.58	23.7±1.5	21.7±0.58	16.0
PARH	-	28.3±0.58	25.0	33.0±1	22.0	22.3±0.58	14.7±0.58	26.7±0.58	25.3±0.58	22.7±1,53	16.0
4496	12.7±0.58	33.0±1	45.0±1	39.7±1.15	31.0	31.7±0.58	24.7±0.58	36.0	28.0	34.7±1.53	20.3±0.58
4696	-	26.0	24.3±0.58	31.7±0.58	28.0	27.7±0.58	18.0	31.3±1.15	27.7±1.53	19.3±1.15	19.0±1
4697	11.0	35.0±1	44.0	40.0	24.7±0.58	27.3±0.58	17.7±0.58	29.7±0.58	30.0	-	20.3±0.58
4786	-	-	-	24.3±0.58	24.0±1	25.7±0.58	16.0	28.3±0.58	26.0	11.0	-
4940	-	26.0	26.0±1	33.0	35.7±0.58	39.0	27.0-1	41.7±0.58	34.0	12.7±0.58	-

Table 7.1 (continued)

Strain	Ampicillin	Azlocillin	Carbenicillin	Pipracillin	Amikacin	Gentamicin	Kanamycin	Tobramycin	Polymyxin	Sulphomamide	Tetracycline
0072	-	35.0±1	28.7±1.15	45.0±1	38.0	38.3±0.58	28.3±1.53	43.0±1	32.7±0.58	21.3±0.58	23.0±1
0014	-	-	-	12.3±0.58	26.0	-	-	-	27.0	-	16.0
9841	-	24.7±0.58	27.3±0.58	32.0	29.3±0.58	29.3±0.58	19.3±0.58	32.0±1	24.7±0.58	16.3±0.58	16.0
9766	-	14.0±1	23.0±2	24.0±2.6	27.7±0.58	28.0±1	19.3±0.58	31.0	26.6±0.58	15.7±1.15	16.3±0.58
17TS	-	25.6±0.58	24.3±0.58	33.0	30.7±0.58	29.7±1.53	17.0±1	34.3±0.58	26.0±1	23.7±1.53	15.3±0.76
WN8	-	24.0±1	24.7±1.15	31.3±0.58	16.7±0.58	18.7±0.58	11.0	21.3±1.15	25.3±0.58	-	17.3±0.58
WN10	-	-	-	11.3±0.58	21.7±0.58	-	-	-	-	-	-
WN12	-	11.3±0.58	23.3±1.53	27.7±0.58	28.7±0.58	27.7±1.53	13.3±0.58	30.7±0.58	23.0±1	24.0	18.0
WN14	-	26.7±0.58	24.3±1.15	31.7±1.15	16.7±0.58	18.3±0.58	13.0	22.3±1.15	25.3±1.53	-	15.7±0.58
WN15	-	14.7±0.58	16.3±1.15	25.3±0.58	25.0±1	16.3±0.58	12.7±0.58	16.0±1	25.3±0.58	-	-
WN32	-	15.0±1	15.3±0.58	23.0	25.0	12.3±0.58	12.3±0.58	14.0±1	25.7±0.58	-	14.3±0.58
WN34	-	12.7±0.58	14.3±0.58	22.3±0.58	24.0	12.0	12.0	13.7±0.58	25.7±0.58	-	±
WN50	-	13.0±1	15.3±0.58	22.0±1	28.0	19.3±0.58	16.0	19.3±0.58	25.3±0.58	-	22.0
PAGS	-	24.7±2.5	25.0±1	33.0	24.7±0.58	26.3±0.58	15.3±0.58	29.0±1	24.3±0.58	18.0	18.3±0.58
PACG	-	24.7±1.15	22.0±1	30.0	25.7±0.58	26.3±0.58	15.3±0.58	28.3±0.58	23.7±0.58	22.7±0.58	19.0
Ps50	-	-	24.3±1.15	18.0		31.7±1.53	25.7±0.58	35.7±0.58	26.5±0.5	30.0±1	15.0±1
799	-	27.7±0.58	26.3±0.58	32.0	30.0	29.7±0.58	18.3±1.53	32.3±0.58	14.0	24.3±0.58	23.0±1
761	19.7±0.82	38.2±0.75	36.8±2.64	42.7±1.5	33.0	38.7±0.58	27.0	41.0±1	29.3±0.58	27.3±0.58	26.3±1.15
7511	-	25.3±0.58	26.6±0.58	32.0±1	24.0	24.3±0.58	13.3±0.58	27.7±0.58	24.0±1	19.0	13.0
PA01	-	26.0±1	25.3±1.53	33.7±0.58	29.7±0.58	28.3±0.58	18.5±1.32	31.3±1.15	25.3±0.58	19.0±1	16.7±0.58
6750	-	27.3±1.15	26.6±0.29	33.0	28.7±1.15	28.0	21.3±0.58	30.7±0.58	22.0±1	20.3±1.15	13.7±0.58

Table 7.2 Antibiotic sensitivity of P.aeruginosa strains.

Strain	Ampi.	Azlo.	Carb.	Pipr.	Amik.	Gent.	Kana.	Tobr.	Poly.	Sulp.	Tetr.
CI4	RR	S	RR	C	SS	SS	SS	SS	R	SS	SS
CI5	RR	S	S	S	S	S	SS	C	SS	RR	C
CI6	RR	C	RR	R	SS	SS	SS	S	C	RR	RR
CI7	RR	C	RR	R	S	S	SS	C	C	RR	RR
CI8	RR	R	RR	R	R	RR	S	RR	R	SS	C
CI9	RR	C	R	R	S	C	SS	R	R	SS	SS
CI10	RR	C	RR	R	S	C	S	C	R	SS	C
CI11	RR	SS	S	S	SS	SS	SS	SS	SS	SS	SS
CI12	RR	C	RR	R	C	C	SS	C	C	SS	S
CI13	RR	SS	SS	SS	SS	SS	SS	SS	SS	SS	SS
CI14	RR	SS	SS	S	SS	SS	SS	S	S	SS	SS
CI16	SS	S	R	S	SS	SS	SS	SS	SS	SS	SS
119X	RR	S	C	S	S	RR	R	RR	C	RR	S
PANT	RR	S	RR	C	S	RR	R	RR	R	RR	C
2168	RR	C	C	R	C	R	SS	C	R	SS	SS
PARH	RR	SS	R	C	R	RR	SS	RR	C	SS	SS
4496	SS	SS	SS	SS	SS	SS	SS	SS	SS	SS	SS
4696	RR	S	R	C	S	S	SS	SS	SS	SS	SS
4697	SS	SS	SS	SS	C	S	SS	C	SS	RR	SS
4786	RR	RR	RR	RR	C	C	SS	R	SS	RR	RR
4940	RR	S	C	C	SS	SS	SS	SS	SS	C	RR
0072	RR	SS	SS	SS	SS	SS	SS	SS	SS	SS	SS
0014	RR	RR	RR	RR	S	RR	RR	RR	SS	RR	SS
9841	RR	C	S	C	SS	SS	SS	SS	C	SS	SS
9766	RR	RR	RR	RR	S	S	SS	S	SS	SS	SS
17TS	RR	C	RR	C	SS	SS	SS	SS	S	SS	S
WN8	RR	C	R	C	RR	RR	R	RR	C	RR	SS
WN10	RR	RR	RR	RR	RR	RR	RR	RR	RR	RR	RR
WN12	RR	RR	RR	RR	SS	S	S	S	R	SS	SS
WN14	RR	S	RR	C	RR	RR	S	RR	C	RR	SS
WN15	RR	RR	RR	RR	C	RR	C	RR	C	RR	RR
WN32	RR	RR	RR	RR	C	RR	C	RR	S	RR	S
WN34	RR	RR	RR	RR	C	RR	C	RR	S	RR	RR
WN50	RR	RR	RR	RR	S	RR	SS	RR	C	RR	SS
PAGS	RR	C	R	C	C	C	SS	C	C	SS	SS
PACG	RR	C	RR	R	C	C	SS	R	R	SS	SS
Ps50	RR	RR	RR	RR		SS	SS	SS	SS	SS	S
799	RR	S	C	C	SS	SS	SS	SS	C	SS	SS
Z61	SS	SS	SS	SS	SS	SS	SS	SS	SS	SS	SS
7511	RR	C	C	C	C	RR	S	R	R	SS	R

7.2 Immunogenicity of OMPs

The immunogenicity of OMP antigens from strains of differing serotype, colony type and antibiotic activity was investigated by transferring separated OMs to NC paper and probing with hyperimmune rabbit serum. The rabbit sera used had been raised against serotype O:6, O:11 and PA strains. To allow a direct comparison of a large number of strains small (5µl) OM samples were loaded onto a gel using a narrow comb made for this purpose. This technique enabled up to 20 samples to be run together.

The immunoblots obtained when the 17 O-serotypes and two PA strains in figure 6.11 were blotted against O:6, O;11 and PA serum are shown in figure 7.1. Serum raised against all three serotypes reacted with proteins F and H from all 17 serotype strains and the two PA strains. It was not possible to distinguish H1 from H2 with this technique. Serotype O:6 antisera (figure 7.1,i) also reacted faintly with proteins D and E from all serotype strains except 9, 10 and 16. In addition O:6 antisera reacted with protein I from all 19 strains and with protein G from strains 1,3,4,5,7,11,12,13 and the two PA strains.

Figure 7.1

Immunoblots of the OMPs shown in figure 6.11 electrophoretically transferred to NC paper and reacted with antisera raised against i) an O:6 strain, PAGS ii) an O:11 strain, PARH, and iii) a PA strain, PAO1. The strains shown in figure 6.11 were isolates which represent all 17 IATS O-serotypes (lanes 1-17). These were; O:1, CI7; O:2, 10662; O:3, CI8; O:4, CI9; O:5, 0072; O:6, PAGW; O:7, CI10; O:8, CI11; O:9, CI12; O:10, CI16; O:11, PANT; O:12, 0014; O:13, CI13; O:14, 9841; O:15, 9766; O:16, CI14 and O:17, 17TS. The two PA strains were CI5 (lane a.) and CI6 (lane b.)

Figure 7.1,i

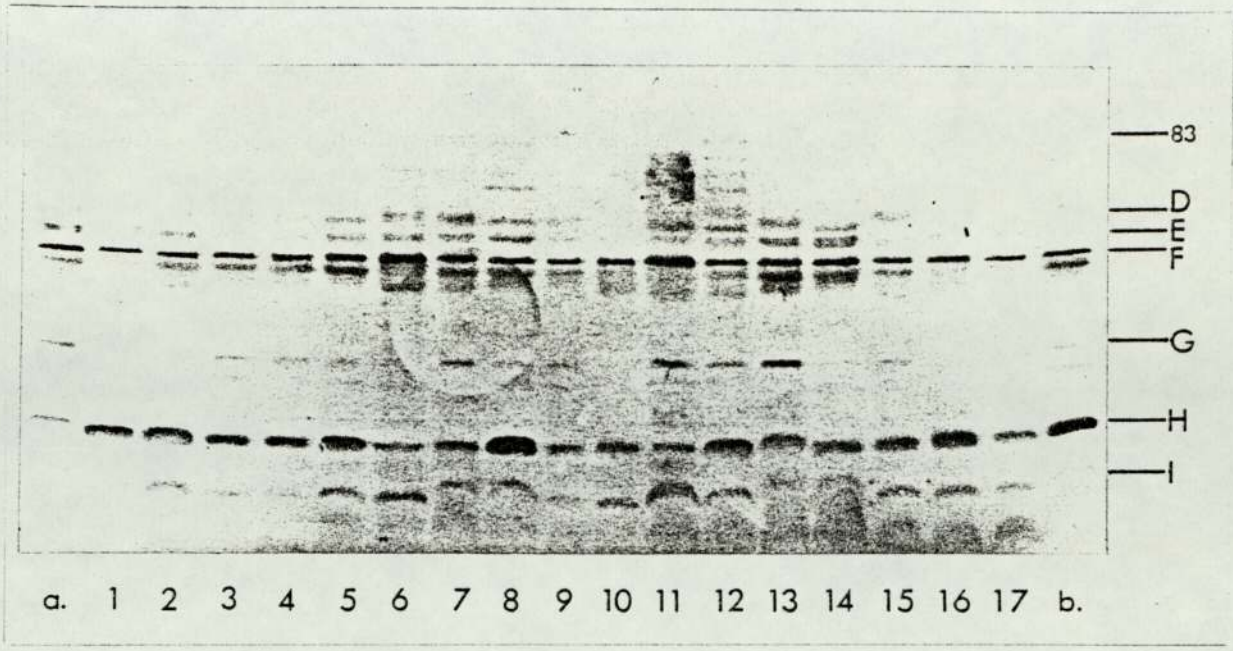


Figure 7.1,ii

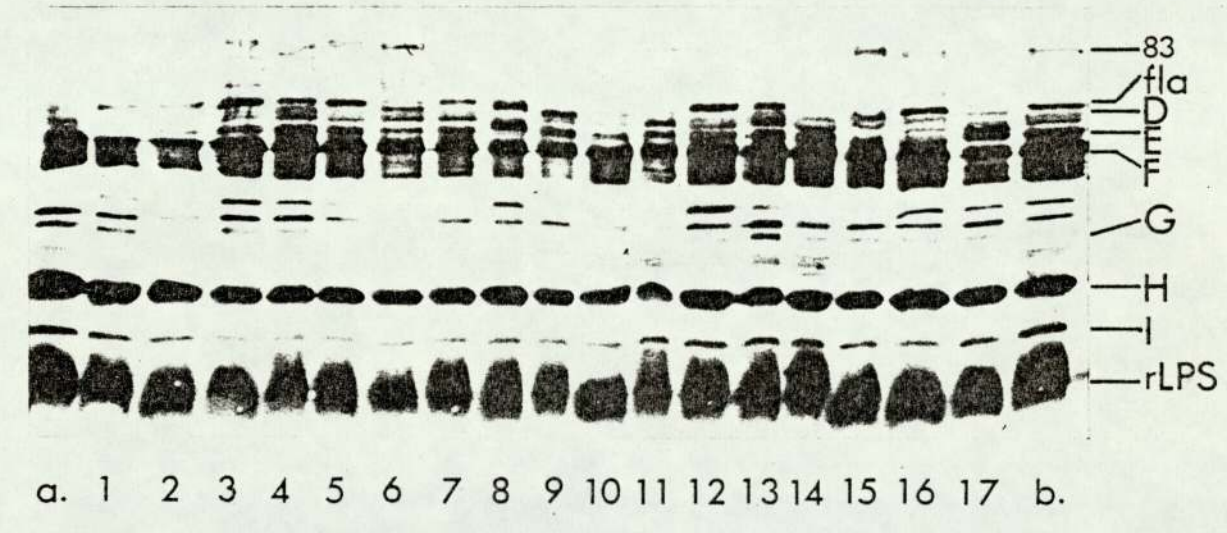
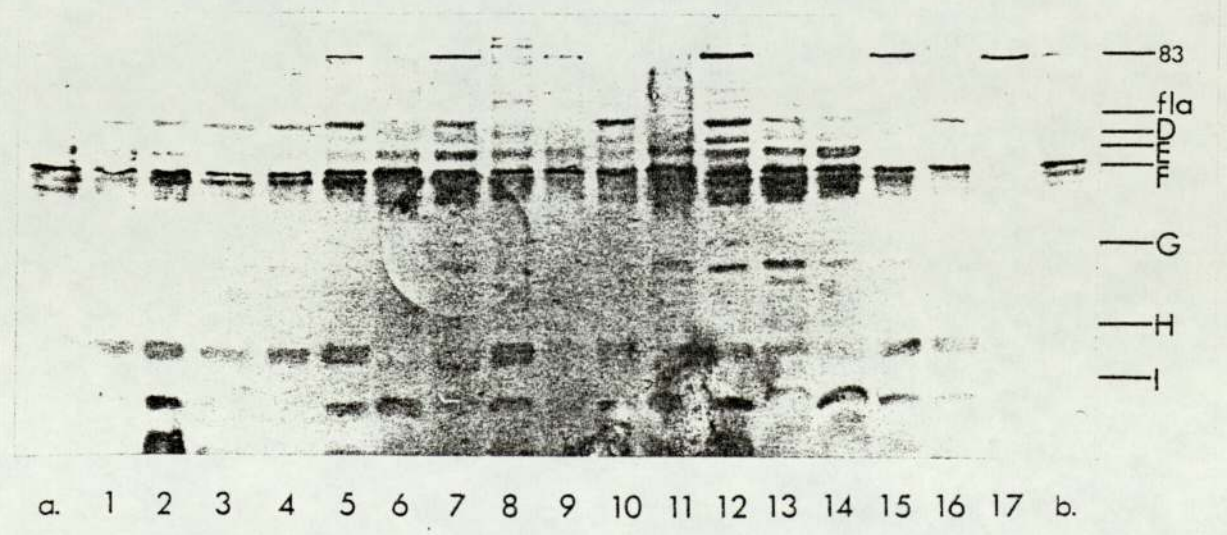


Figure 7.1,iii



The reaction of serotype O:11 antisera (figure 7.1,ii) with OMPs of all serotype strains was considerably stronger than O:6 and PA antisera (figures 7.1,i and iii). Proteins F,H and I were all visualised strongly by O:11 antisera, protein F as a wide intense band between F and F* positions. Serum antibodies also reacted with proteins D and E in all strains, and flagella in all serotype strains except 6,9,10,11,14 and 15. Anchoring of flagellar in the OM may vary from strain to strain so that some may be sheared more easily during preparation of the OM. Non-flagellated strains of P.aeruginosa have been reported (Gilardi, 1979; Pugashetti, 1984). No protein bands were visible at this position on serotype strains 6,8,9,11 and 14 in the coomassie blue stained gel (figure 6.11). There was a strong reaction between rough LPS and the O:11 antisera which was less visible in 7.2,i and iii. It is possible that rough LPS of this O:11 strain may be exposed at the surface in such a way that it is readily accessible to antibody. A reaction with rough LPS may be related to the apparently high antibody titre of this O:11 antisera.

O:11 antisera reacted moderately with an antigen of M_r 83K in the majority of serotype strains. The same antigen reacted strongly with antibodies in the PA antisera in serotype strains 1,5,7,9,12,15,17 and the two PA strains (figure 7.1,iii). OMP antigens with M_r below about 40K reacted faintly with this antisera.

Figure 7.2,i

SDS-PAGE of clinical isolates of P.aeruginosa comparing mucoid, non-typeable and polyagglutinating strains with serotype O:6 and O:11 strains. The strains used were; 4496, non-typeable, mucoid (lane 1); 4696, non-typeable, mucoid (lane 2); 4786, serotype O:6, mucoid (lane 3); PAGW, serotype O:6, non-mucoid (lane 4); PACG, serotype O:11, non-mucoid (lane 5); 7511, non-typeable, non-mucoid (lane 6) and 6750, polyagglutinating, non-mucoid (lane 7). 15% acrylamide gel.

Figure 7.2,ii, iii and iv

Immunoblots of the OMPs shown in figure 7.2,i electrophoretically transferred to NC paper and reacted with antisera raised against ii) an O:6 strain, PAGS iii) an O:11 strain, PARH, and iv) a PA strain, PAO1.

Figure 7.2,i

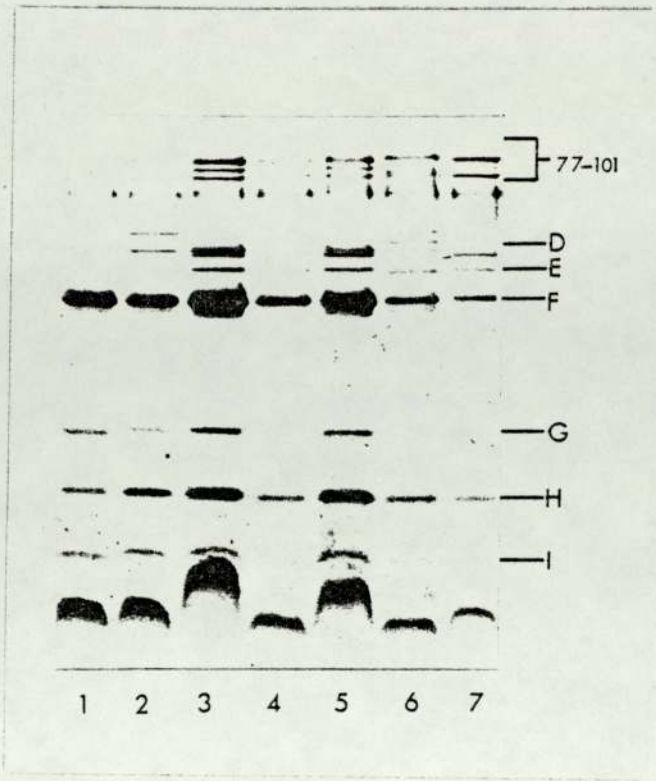


Figure 7.2,ii

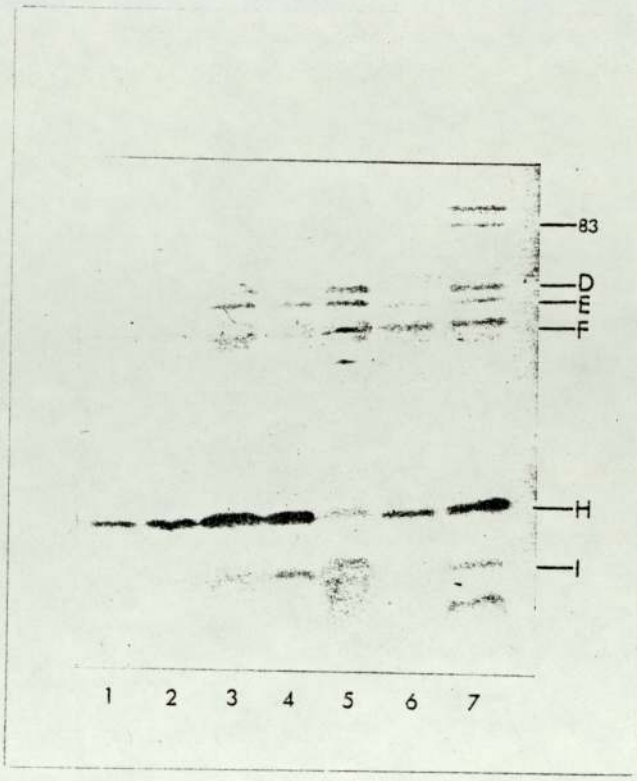


Figure 7.2,iii

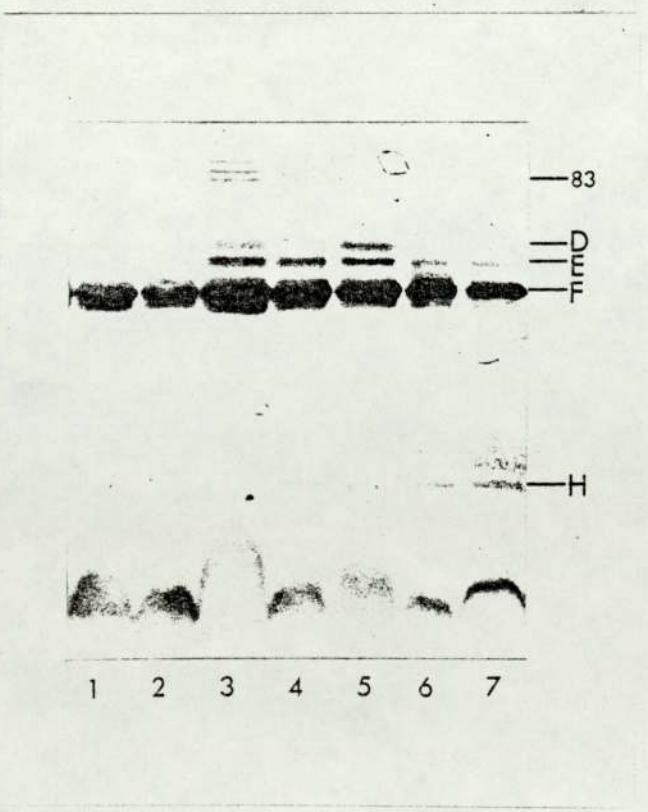
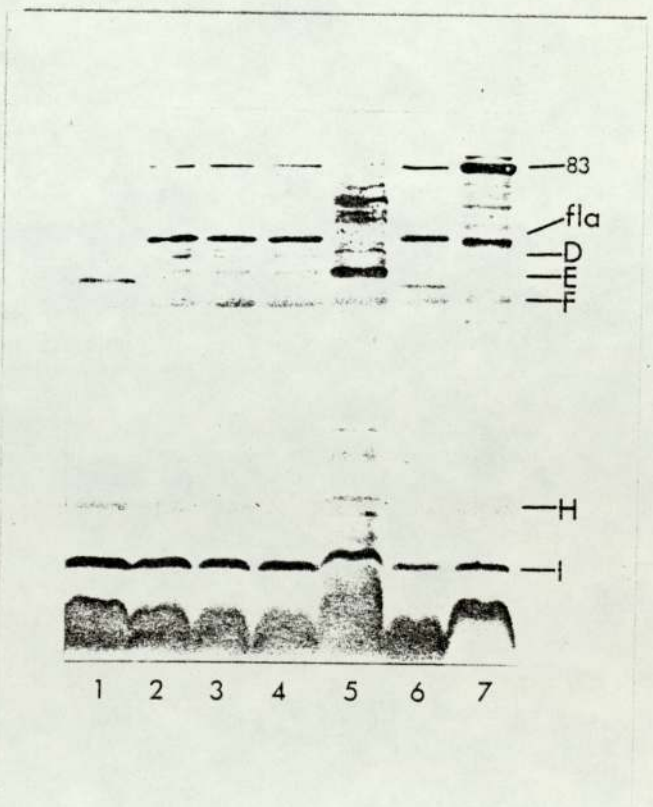


Figure 7.2,iv



The OMP profiles of mucoid (figure 7.2,i, Lanes 1,2 and 3), non-typeable (lane 6) and PA strains (lane 7) were essentially the same as strains representative of serotype O:6 and O:11 (lanes 4 and 5). All strains were grown in Fe- CDM. As in figure 6.11 all strains conform basically to the format described by Mizuno & Kageyama (1978). Very similar results to those in figure 7.1 were obtained when the OMs separated in figure 7.2,i were transferred to NC paper and reacted with antisera to the O:6 (figure 7.2,ii), O:11 (figure 7.2,iii), and PA (figure 7.2,iv) strains. Proteins F and H reacted with antibodies in all three antisera. The O:6 antisera reacted also with proteins D, E and I in all strains. The PA antisera reacts strongly with flagella and a protein at 83K in all strains except 4496 (lane 1) and PACG (lane 5). Protein E of strain PACG, however, does react strongly with this antisera. None of the antisera reacted with protein G in any of these seven strains.

Figure 7.3,i

SDS-PAGE of strains of P.aeruginosa of differing antibiotic sensitivity. The strains used were 799 wild-type (lane 1), 799/Z61 mutant (lane 2), Ps50 (lane 3), WN8 (lane 4), WN10 (lane 5), WN12 (lane 6), WN14 (lane 7), WN15 (lane 8), WN32 (lane 9), WN34 (lane 10), WN50 (lane 11) and 10662, reference strain (lane 12). All strains were grown in Fe- CDM. 15% acrylamide gel.

The antibiotic sensitivities of these strains are shown in Tables 7.1 and 7.2.

Figure 7.3,ii

Immunoblot of the OMPs shown in figure 7.3,i electrophoretically transferred to NC paper and reacted with antisera raised against an O:6 serotype strain.

Figure 7.3,i

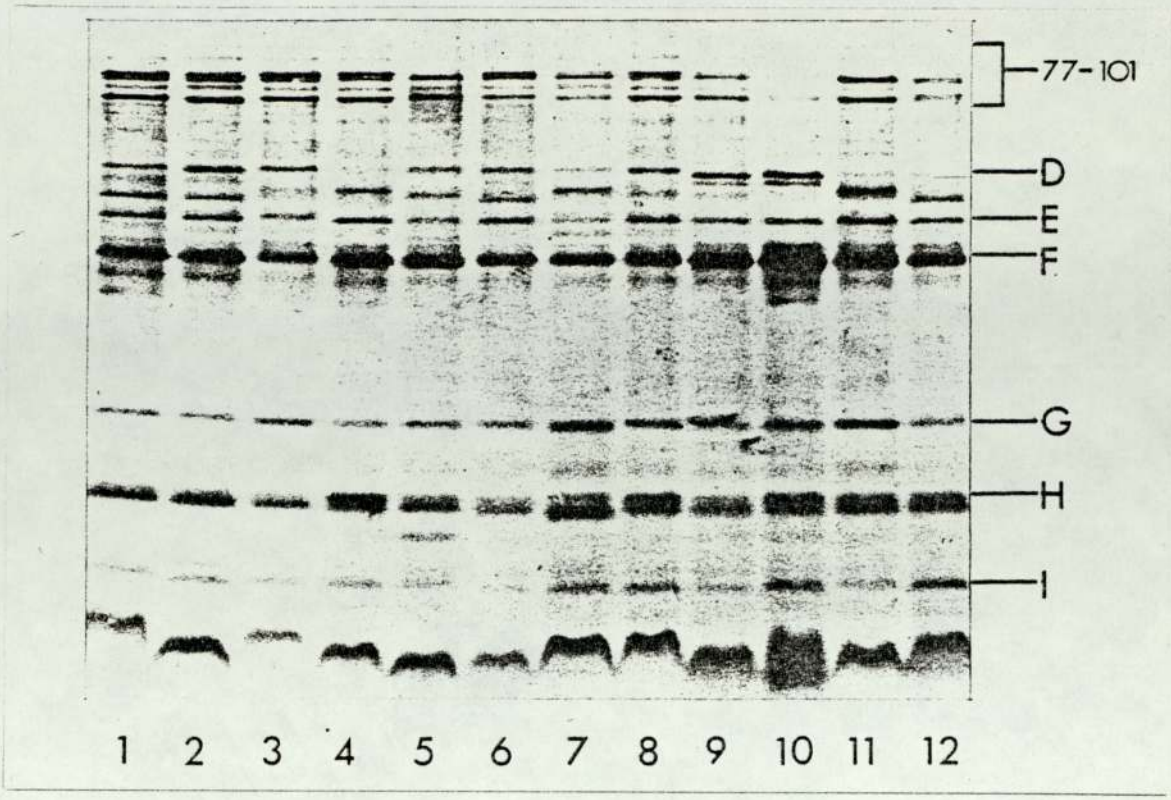


Figure 7.3,ii

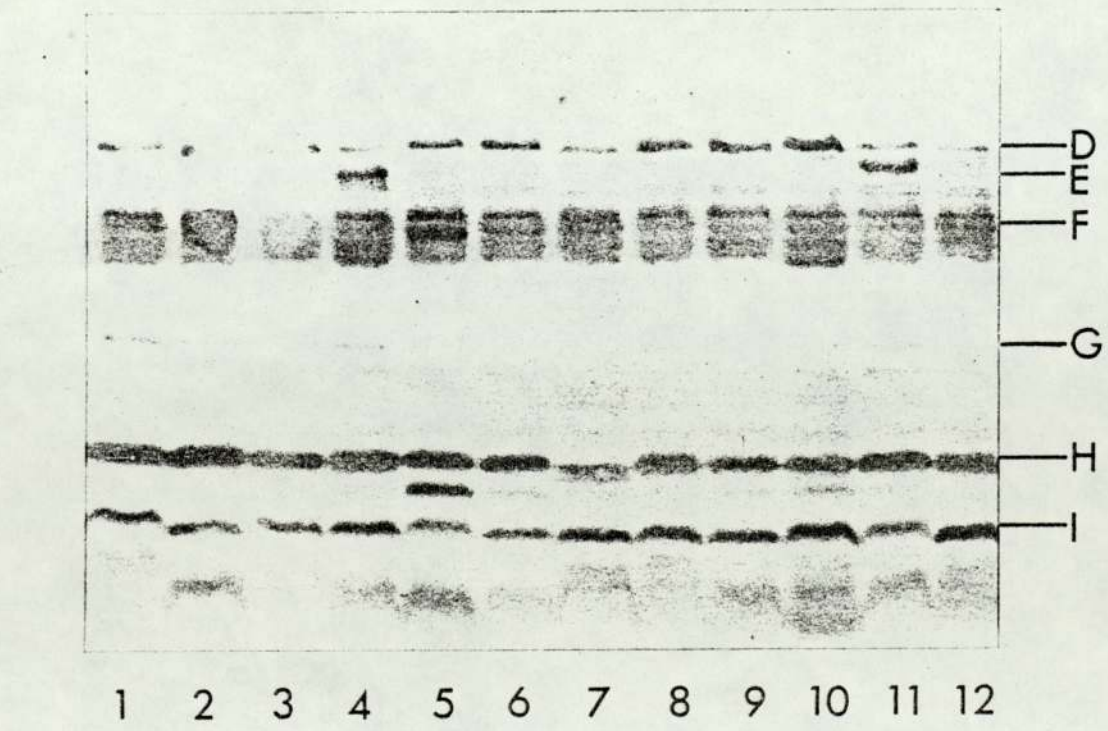


Figure 7.3,iii

Immunoblot of the OMPs shown in figure 7.3,i electrophoretically transferred to NC paper and reacted with antisera raised against an O:11 serotype strain.

Figure 7.3,iv

Immunoblot of the OMPs shown in figure 7.3,i electrophoretically transferred to NC paper and reacted with antisera raised against a PA strain.

Figure 7.3,iii

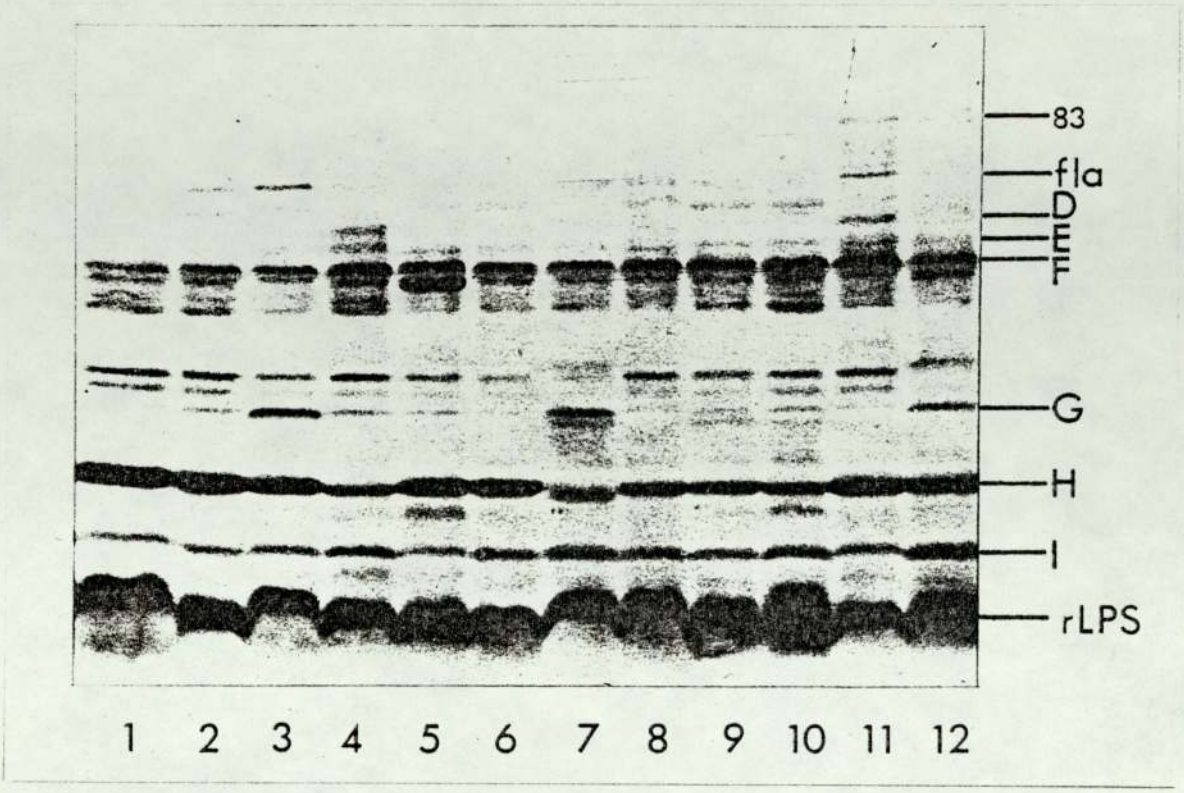
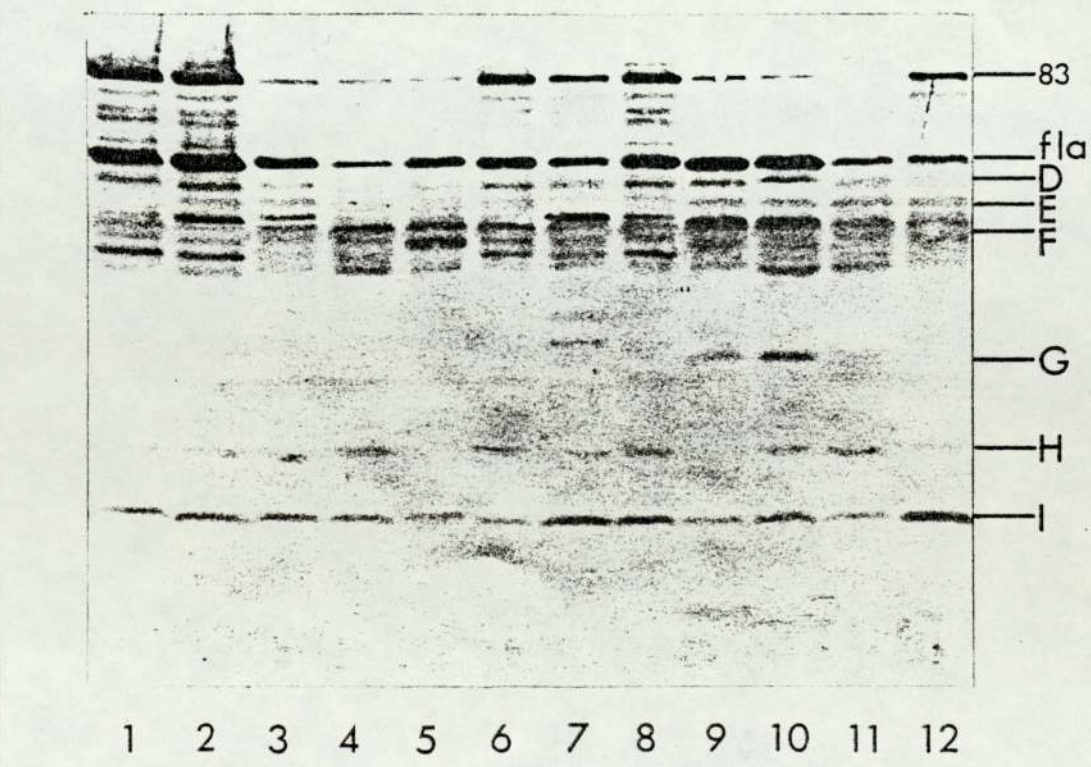


Figure 7.3,iv



The 26 strains used to represent the 17 O-serotypes, PA and non-typeable strains cover a spectrum of antibiotic resistance from very sensitive strains such as CI11, CI13 and CI16 through strains selectively resistant to certain classes of antibiotics such as 4786 and 9766 to the very resistant 0014 (table 7.2). Very similar patterns of OMP profile and immunogenicity were observed when OMs of P.aeruginosa strains of extreme variation in antibiotic sensitivity were separated by SDS-PAGE and immunblotted against rabbit sera (figure 7.3). The OM profiles of 12 such strains (figure 7.3,i) all conformed, as before, to the scheme of Mizuno and Kageyama (1978). The multi-resistant WN strains (lanes 3-10) were in this respect very similar to the strains of different serotype (figure 6.11) and colonial morphology (figure 7.2,i). There is no substantial difference in OMP profile between these multi-resistant strains and the constitutive B-lactamase producer Ps50SAS^{con1} (Lane 11) or the hypersensitive Z61 strain (lane 2). The Zimmerman mutant Z61 and its more resistant wild type parent 799 also cannot be distinquished from their OMP profiles (Fig 7.3,i lanes 1 and 2). When these sensitive and resistant strains of P.aeruginosa were transferred to NC paper and reacted with immune sera (figure. 7.3,ii, iii and iv) an essentially similar pattern of results to those seen in figures 7.1 and 7.2 was produced. As before, immune serum derived from the three serotype strains reacted with proteins F and H. The reaction with proteins F and H occured in all 12 strains studied regardless of the nature of their antibiotic sensitivity. O:6 antisera (figure. 7.3,ii) also reacted with protein D and protein I of all 12 strains but not with protein G. O:11 antisera again reacted strongly with rough LPS. Flagella and the IRMP at 83K react strongly with PA antisera (figure

7.4iv) to form dark bands. As with the OMP profiles it is not possible to characterize sensitive and resistant strains from their immunoblots.

7.3 Discussion

The sensitivity of P.aeruginosa to antibiotics, especially to the aminoglycosides (Garrod & Waterworth, 1969; Gilbert et al, 1971; Zimelis & Jackson, 1973) and polymyxin (Newton, 1953; Brown & Melling, 1969b; Boggis et al, 1979) is greatly influenced by the concentration of Mg^{++} and Ca^{++} in the medium (D'Amato et al, 1975). Increased concentrations of these metal cations increase the apparent resistance of the organism probably by an increased competition for binding sites on the cell surface (Brown, 1975). This phenomena is a peculiar property of P.aeruginosa (Garrod & Waterworth, 1969; Gilbert et al, 1971) and is not the result of antibiotic inactivation. Different media vary markedly in their divalent cation content (Garrod & Waterworth, 1969; Gilbert et al, 1971; Reller et al, 1974). A high degree of reproducibility of results can be maintained with Mg^{++} concentrations in the range 20-35mg/l and Ca^{++} concentrations between 50 and 100mg/l (Reller et al, 1974) and the Mg^{++} and Ca^{++} concentrations in the medium used in this study have been adjusted to be within these values (2.1.5). Strain 10662 is known to be susceptible to variations in ionic concentration in the media (Reller et al, 1974; Brown & Blowers, 1978) and to give reproducible zones of inhibition at the specified Mg^{++} and Ca^{++} concentrations (Reller et al, 1974).

Sensitivity testing was carried out to construct an index of sensitive and resistant strains which would be used when comparisons were made of OMP constitution and immunogenicity. In view of the large number of strains involved and the large number of antibiotics to be used it was decided to employ a disc sensitivity technique in which sensitivity was related to the size of a zone of inhibition. Table 7.2 represents the comparative sensitivity or resistance of P.aeruginosa strains to a number of antibiotics defined by reference to a control strain, P.aeruginosa ATCC 10662. The designation sensitive or resistant is comparative and not meant to imply values which might be related to clinical antibiotic treatment (Bauer et al 1966). The designation sensitive or resistant does, however, correlate well with the documented sensitivity of strains Ps50SAS^{cont} (Livermore, 1983) and Z61 (Zimmerman, 1979,1980) and the clinically demonstrated gentamicin resistance of strains 119X, PANT and 2168 from Birmingham Accident Hospital, and multiple drug resistance of the WN strains from John Radcliffe Hospital.

It was not the purpose of this study to investigate mechanisms of antibiotic resistance, nevertheless, some general observations may be derived from the table of antibiotic sensitivities (table 7.2). The non-typeable mucoid strains 4496,4696,4697 and 4940 as a group are more sensitive to the action of the antibiotics tested than are the majority of typable strains. The sensitivity of these strains is emphasised by the inhibition of 4496 and 4697 by ampicillin, as were CI16 and the Z61 mutant. Ampicillin is an antibiotic to which P.aeruginosa is normally resistant. NT strains have been reported to lack O-antigen (Hancock et al, 1983; Ojeniyi et al, 1985). In mucoid NT strains the O-antigen may be inaccessible to

antibody due to occlusion by slime. Absence of O-antigen alone is not sufficient to account for their sensitivity as demonstrated by the sensitivity of the CI11 (serotype O:7), CI13 (O:13), CI14 (O:16), CI16 (O:10) and Z61 (O:2) strains. LPS in the Z61 mutant (Zimmerman, 1979,1980) has been shown to be only subtly different to its more resistant 799 parent (Kropinski et al, 1982). The mucoid character of the cells also does not correlate well with antibiotic sensitivity since the mucoid 4786 (O:6) is sensitive only to kanamycin and Px. Heterogeneity of antibiotic sensitivity in mucoid strains is well documented (Sheehan et al, 1982).

Strain Ps50SAS^{con1} is a constitutive lactamase producer (Livermore, 1983) which is resistant to all the B lactam antibiotics tested and especially ampicillin and azlocillin. Ps50SAS^{con1} is not unduly sensitive to other antibiotics. Lactamase production can also be presumed to account for the resistance of strains 9766, WN12 and 4786 which to some extent share the same pattern as Ps50SAS^{con1}. A large number of the strains tested were resistant to carbenicillin (Lowbury et al, 1969).

Strains which are resistant to gentamicin, kanamycin and tobramycin but sensitive to other antibiotics such as 119X, PANT and WN8 probably secrete enzymes which inactivate aminoglycosides (Shannon & Phillips, 1982). Sensitivity to the enzyme-resistant amikacin in 119X and PANT tends to confirm this (Shannon & Phillips, 1982). A combination of impermeability and enzyme inactivation possibly accounts for the multiple antibiotic resistance of strains such as CI8, WN10, WN15, WN34, WN50 and 0014.

In this comparison of 35 strains of P.aeruginosa of different serotype, colony type and antibiotic sensitivity major proteins D,E,F,G,H and I could all be identified in the OM. Under the

denaturing and electrophoretic conditions described (Anwar et al, 1983b) each class of protein had a characteristic electrophoretic mobility so that closely similar OMP patterns were produced on SDS-PAGE. Strain PANT (serotype O:11) was unusual in that protein F was incompletely denatured under these conditions and appeared on the gel in both F and F* positions (figure 6.11, line 11).

The relative levels of individual proteins varied somewhat. For example, the level of protein H in the serotype O:12,O:13 and O:14 strains varies considerably while the level in these three strains is consistent (figure. 6.11, lanes 12,13 and 14). Similarly, protein G is almost completely repressed in the serotype O:2 strain compared with the O:3 strain while again the level of protein F is much the same (figure 6.11, lanes 2 and 3). Protein D2 was missing from a number of strains. It is, for example, readily visible in the serotype O:1 strain but absent from the O:2 and O:3 strains (figure 6.11, lanes 1,2 and 3). A number of IRMPs of M_r between 77 and 101 were present in the OM of all strains. Although two of these proteins (83 and 88K) were present in a majority of strains there was considerable diversity in the number and the concentration of high M_r protein bands between individual strains. Similar small variations in the pattern of OMPs can be seen in the comparisons of OMP profiles of strains of different colony type and antibiotic sensitivity.

A number of strains of serotype O:6 and O:11 have been compared. Among other serotypes sometimes only one representative has been studied. It is not possible to determine from this study whether small variations in OMP profile are serotype related. The results show, however, that patterns of OMP profile were substantially similar in P.aeruginosa strains of all degrees of antibiotic sensitivity. These results confirm and expand on those of others (Sadoff and Artenstein,

1974. Mizuno and Kageyama,1978; Mutharia et al, 1982). Previous studies did not specify degrees of antibiotic sensitivity and were not carried out with cells grown under iron-depleted conditions. Similar heterogeneous patterns of OMP profile are not seen in other Gram-negative organisms. A number of different OMP patterns have been observed in Haemophilus influenzae (Barenkamp et al,1981), Neisseria gonorrhoeae (Buchanan & Hildebrandt, 1981), Neisseria meningitidis (Tsai et al, 1981), E.coli (Overbeeke & Lugtenberg, 1980), Bacteroides fragilis (Kaspar & Seiler, 1975) and Vibrio cholerae (Kabir,1980).

Antibodies in rabbit immune sera reacted with proteins F,H and I of P.aeruginosa strains transferred to NC paper. Each of the sera used showed a different reaction with the OMPs. The high resolving power of SDS-PAGE combined with the sensitivity and specificity of the western blotting technique (Towbin & Gordon, 1984) may be used to separate and analyse OM components and investigate their antigenic properties. The distinctive feature of this combination of methods is the ability to retain the original pattern of the separated components on a solid phase which enables identification of the reacting antigens (Towbin & Gordon, 1984). The technique has the additional advantages that proteins are concentrated in a thin layer on the surface of the NC paper and are freed from denaturing SDS used in preparation of the sample (Hjerten, 1983). Proteins transferred to NC paper may be stained directly with amido black or indian ink to determine the degree of qualitative transfer of protein from the gel. Alternatively the gel may be stained in coomassie blue following the transfer procedure to identify those proteins not transferred. OMP antigens from N.gonorrhoeae (Batteiger et al, 1982; Swanson & Barrera, 1983), B.fragilis (Cousland & Poxton, 1983), B.nodosus (O'Donnell et al, 1983) and P.mirabilis (Driver & Lambert, 1984) as well as P.aeruginosa

(Mutharia et al, 1982) have been characterised using this combination of methods.

In this study antibodies in rabbit immune serum reacted with proteins F, H and I in the separated OMs of strains of P.aeruginosa representative of 17 O-serotypes, Non-typeable and PA strains and strains of a wide spectrum of antibiotic sensitivity. In addition protein E was immunogenic in many strains. Flagella was also strongly immunogenic in many strains and reacted especially strongly with the PA antiserum. A number of high M_r IRMPs reacted with antisera, most consistently a protein at 83K. Each of the three antisera used showed a different pattern of reaction with the separated OMs but the pattern of reaction for each serum was consistent (figures 7.2, 3 and 4).

Lambert & Booth (1982) have investigated exposure of proteins at the surface of P.aeruginosa with [^{125}I]-lactoperoxidase, a compound which reacts with tyrosine and histidine residues. [^{125}I]-lactoperoxidase itself is too large to penetrate the OM and is therefore assumed to label only those proteins on the cell surface. Proteins F and D were labelled with this technique. Proteins F and D both form pores in the OM and might be expected to be exposed on the surface. Proteins E, H1, H2 and I were not labelled. These proteins were either not exposed at the surface or did not expose tyrosine or histidine residues at the surface. Two proteins (M_r 72.5K and 38K) were labelled but did not appear in the coomassie blue stained gel of separated OMPs. The 38K protein may be incompletely denatured protein F, the 72.5K protein may be an IRMP. The function of the IRMPs has not been investigated in P.aeruginosa. If one of these proteins were to be shown to be a receptor for an iron siderophore complex it might have considerable potential as a protective antigen. A receptor protein

could be expected to be exposed on the cell surface, interaction of antibody with a receptor protein might prejudice the cells ability to acquire iron neccessary for growth.

8 Outer membrane antigens of a polymyxin resistant variant of *P.aeruginosa*

8.1 General description.

P.aeruginosa PAO1 is normally sensitive to polymyxin (MIC 10 units/ml). PAO1 was trained in CDM 10 to resistance to 60,000u/ml Px in 12 incremental steps. The cells used in subsequent procedures were those trained in 8 steps to a resistance of 6000 units/ml Px as this level of resistance had been used previously (Brown & Watkins, 1970; Brown & Wood, 1972) and by other workers (Gilleland & Beckham, 1982; Gilleland & Farley, 1982). Resistant organisms produced in this way were designated RPAO1. Growth of RPAO1 in CDM containing Px was slow and was characterised by a long lag phase. Growth on agar containing 100 units/ml Px was also slow and resulted in very small translucent colonies which did not produce pigment. The resistant cells were not stable mutants and would revert to full Px sensitivity if washed and subcultured into medium without Px.

8.2 Fatty acids of polymyxin-resistant *P.aeruginosa*.

GLC analysis of cellular fatty acids (figure 8.1, table 8.1) shows that fatty acids in both parent and resistant derivative are qualitatively and quantitively very similar. Both organisms contain relatively large amounts of 16:1, 16:0 & 18:1 acids, and 12:0 & 12:0,2OH acids. This is characteristic and may be used to identify *P.aeruginosa* and to distinquish it from other Pseudomonas species (Moss, 1978; Oyaizu & Komogata, 1983).

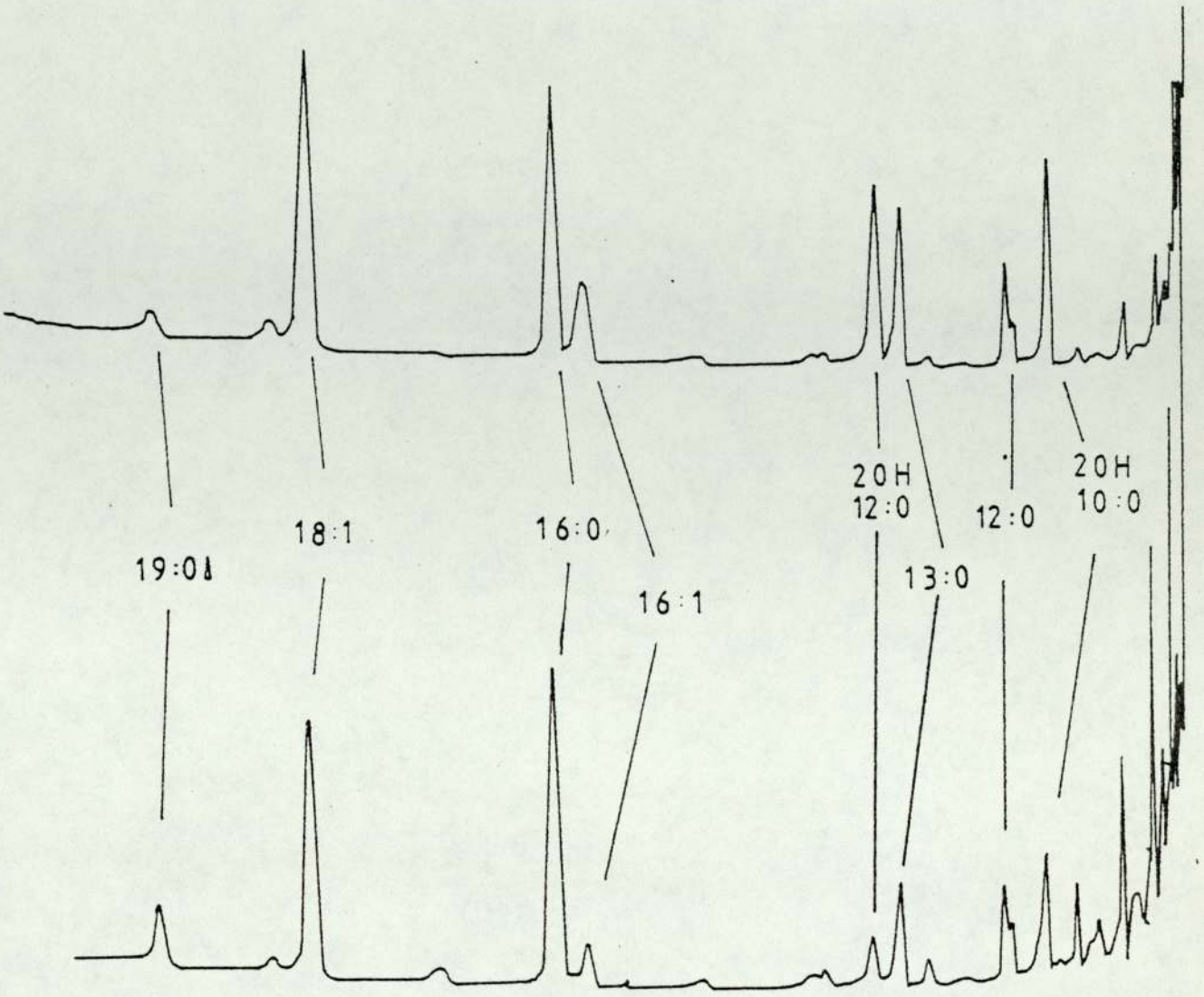
The presence of 19:0 cyclopropane is also characteristic and may be used to distinquish the fluorescent species *P.aeruginosa*, *P.putida* and *P.fluorescens* from other pseudomonads (Moss, 1978). The

Figure 8.1

Chromatograms from GLC analysis of fatty acids of P.aeruginosa PAO1 and a polymyxin-resistant variant, RPAO1

Table 8.1

GLC analysis of cellular fatty acids of P.aeruginosa PAO1 and a polymyxin-resistant variant, RPAO1



Fatty Acid	% total	
	PA01	RPA01
C10:0 20H	9.7	10.5
C12:0	5.3	5.2
C12:0 20H	3.6	13.5
C13:0	9.1	7.8
C16:0	32.6	24.3
C16:1	3.4	6.9
C18:1	30.3	30.1
C19:0cyc.	6.0	1.7
	100	100

concentration of 19:0 cyclopropane fatty acid in the resistant organism was less than that in its sensitive parent.

8.3 Antibiotic sensitivity of polymyxin-resistant P.aeruginosa.

Antibiotic sensitivity of the Px-resistant organisms was compared with that of their wild type parents using Neo-sensitabs (2.2.5.2). Unbound Px was removed from cells prior to testing by washing three times in 0.85% saline. The results in table 8.2 show that the Px-resistant cells have greatly increased sensitivity to all the antibiotics tested. RPA01 cells were exquisitely sensitive to the recognised anti-pseudomonal antibiotics and also two antibiotics, ampicillin and erythromycin, to which they are normally resistant. The results imply that the RPA01 cell's permeability barrier had been disrupted. This conclusion also explains an observation that cells trained to a Px-resistance greater than 80 units/ml Px would not grow on PIA. Cells washed in growth media (CDM + 6000 units/ml Px) were included as a control to test the possibility that washing in saline alone had damaged the permeability barrier. Control cells had closely similar antibiotic sensitivity to RPA01 washed in saline.

Table 8.2

Antibiotic sensitivity of P.aeruginosa PAO1 compared with a variant trained to polymyxin resistance (RPAO1), using Neosensitab antibiotic discs. Average diameter of zones of inhibition in mm. ± standard deviation.

	PAO1	RPAO1	RPAO1 (control)
Ampicillin	-	36.8 ± 3.3	37
Azlocillin	26.0 ± 1	40.5 ± 5.3	50
Carbenicillin	25.3 ± 1.53	42.0 ± 4.3	52
Piperacillin	33.7 ± 0.58	47.0 ± 2.6	
Amikacin	29.7 ± 0.58	35.8 ± 1.7	38
Gentamicin	28.3 ± 0.58	40.3 ± 1.7	40
Kanamycin	18.5 ± 1.32	42.7 ± 1.5	36
Tobramycin	31.3 ± 1.15	42.8 ± 2.2	42
Erythromycin	13.0 ± 1	34.0 ± 0.58	36
Sulphonamide	19.0 ± 1	32.8 ± 0.95	
Tetracycline	16.7 ± 0.58	31.3 ± 1.3	30

8.4 Killing of polymyxin-resistant P.aeruginosa by human serum and whole blood.

Viable counts of wild type PAO1 and Px-resistant RPAO1 subcultured into serum (figure 8.2) show that RPAO1 were very sensitive to serum. All Fe- cultured cells were killed within 40 minutes and Fe+ cells within 20. Although the mechanism of serum resistance in P.aeruginosa is not well understood these results, like those of antibiotic sensitivity, indicate that the protection afforded by the permeability barrier of the OM was affected by training to Px-resistance. It is noteworthy that wild type PAO1 cells multiplied in HIS (a positive control) while RPAO1 cells were killed. RPAO1 was possibly killed by lysozyme and cationic proteins in serum to which PAO1 is normally resistant. This is another indication that the permeability barrier of the cells had been disrupted. Wild type cells in serum were slowly reduced in number over a 60 minute period, Fe-grown cells were somewhat more resistant to serum than Fe+ (figure 8.2). This difference in kinetics of killing of Fe- and Fe+ grown cells was small but was consistent when the assay was repeated on separate occassions. Wild type cells were killed more efficiently by blood (figure 8.3) than by serum. As before RPAO1 cells were killed much more rapidly, all Fe- cells were killed within 30 minutes and Fe+ cells within 20 minutes. Again the more rapid killing of Fe+ cultured cells was reproducible when the assay was repeated on separate occasions.

Figure 8.2

Kinetics of killing of P.aeruginosa PAO1 and a polymyxin-resistant variant, RPAO1, by fresh human serum and heat inactivated serum (HIS).

Serum

- ○ PAO1 cultured in Fe- CDM
- ● PAO1 cultured in Fe+ CDM
- □ RPAO1 cultured in Fe- CDM
- ■ RPAO1 cultured in Fe+ CDM

HIS (controls)

- ● PAO1 cultured in Fe- CDM
- ▽ RPAO1 cultured in Fe- CDM

Percentage survival indicates viable counts relative to time zero (100%).

Figure 8.2

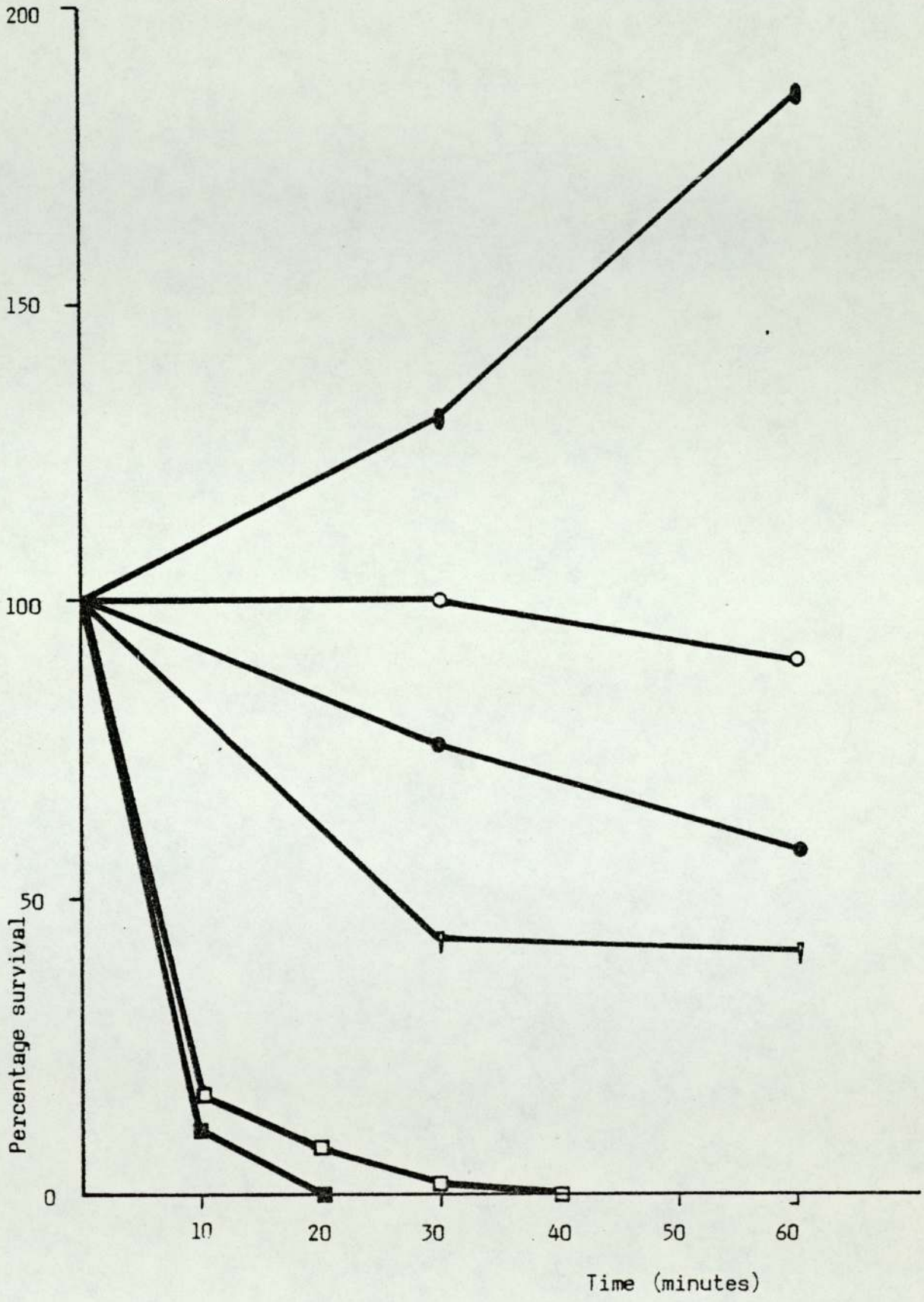

200
150
100
50
0
Percentage survival
10
20
30
40
50
60
Time (minutes)

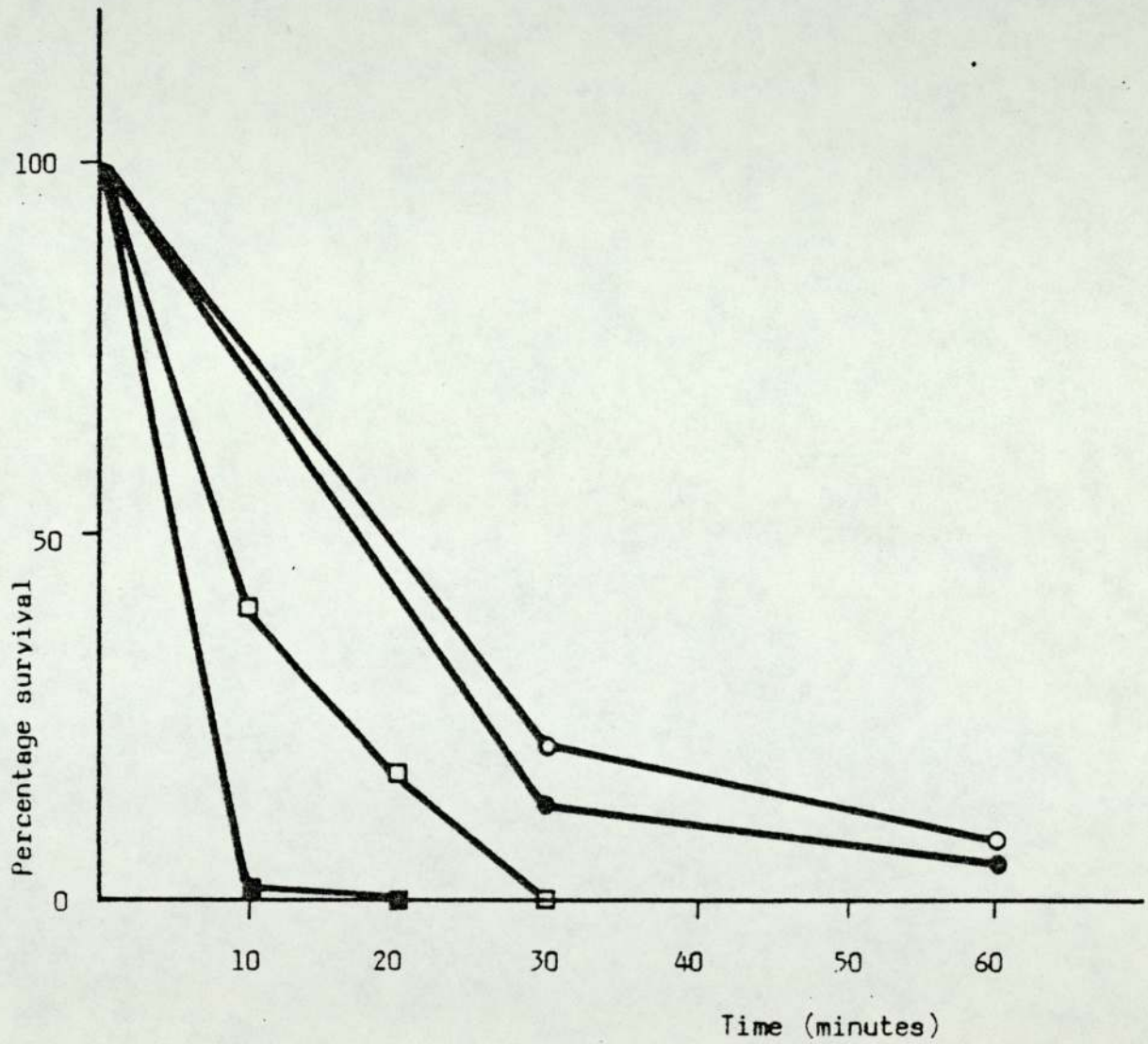
Figure 8.3

Kinetics of killing of P.aeruginosa PAO1 and a polymyxin-resistant variant, RPAO1, by fresh human whole blood.

- ○ PAO1 cultured in Fe- CDM
- ● PAO1 cultured in Fe+ CDM
- □ RPAO1 cultured in Fe- CDM
- ■ RPAO1 cultured in Fe+ CDM

Percentage survival indicates viable counts relative to time zero (100%).

Figure 8.3



8.5 Outer membrane proteins of polymyxin-resistant P.aeruginosa.

Outer membranes were prepared by the Sarkosyl method (2.2.8.3), cells grown in the presence of Px (and under conditions of magnesium depletion) were extremely resistant to physical disruption and required two sessions of 10 x 1 minute periods of sonication to break. Figure 8.4 shows the changes produced in the OM protein profile of PAO1 as it becomes exposed to increasingly higher concentrations of Px during the training procedure. Lane 1 shows the OM protein profile of the untreated parent PAO1. Levels of prteins H1 and H2 remain unchanged at 36 units/ml Px (lane 2). However, at 80 units/ml Px protein H1 - the protein whose induction has been proposed to explain polymyxin resistance - has dissappeared. At this step the level of all major proteins in the OM except H2 & I was considerably reduced compared with the wild type (Lane 1). At a resistance step of 160 units/ml (lane 4) the protein F band is much reduced compared with lane 1, again H1 is absent. H2 remains a major membrane protein. Thereafter the OM protein profile of the organism does not change materially with succeeding training steps up to 6000 units/ml. Figure 8.5 shows the OMP profile of PAO1 (lane 1) and RPAO1 grown to early stationary phase in CDM 10 (lane 2), and Mg- CDM 10 (lane 3) both with 6000 units/ml Px. The OMP profiles of RPAO1 grown in complete CDM and under Mg-limitation were the same, in both cases the level of protein F was reduced and other major OMPs with the exception of H2 and I much reduced. These changes are consistent with the OM protein profile of a trained resistant variant reported by Gilleland & Beckham (1982) in which the loss of proteins with M_r of 24,000, 36,500, and 47,000 (in their gel system) was observed.

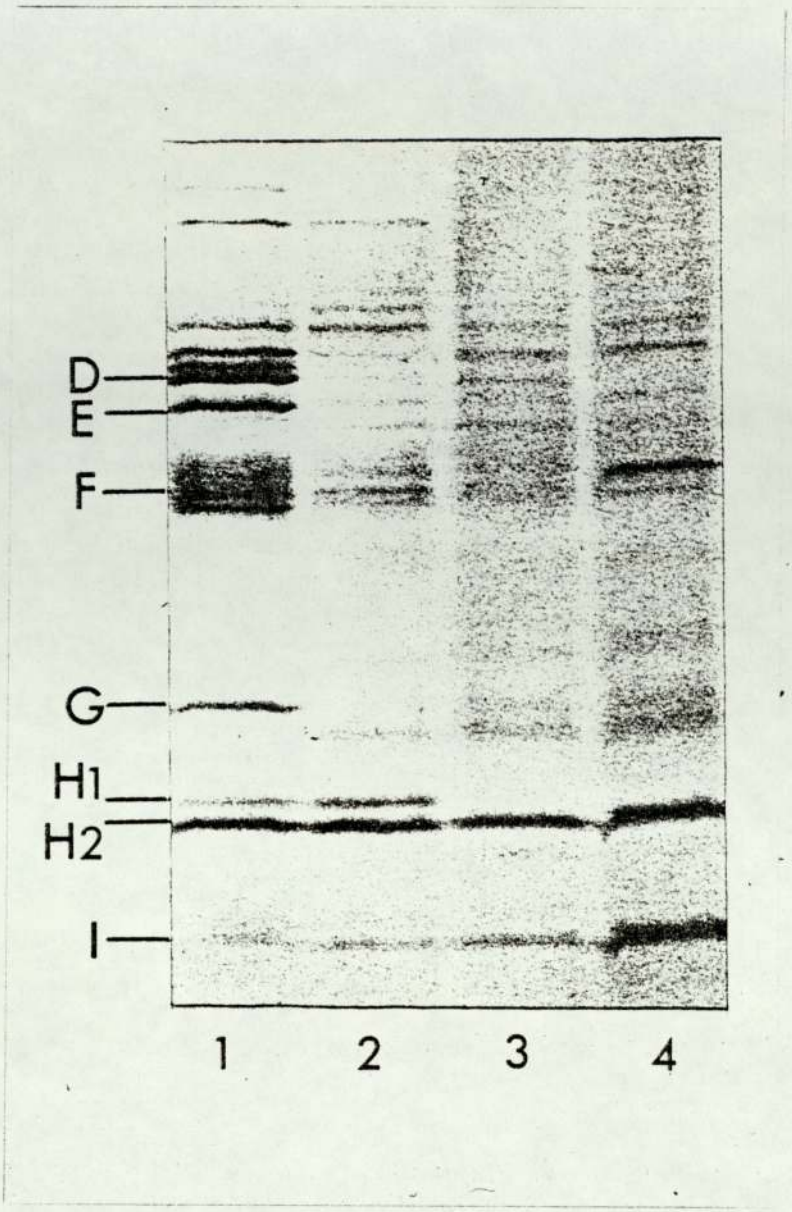


Figure 8.4

OMP profiles of P.aeruginosa PAO1 and a resistant variant, RPAO1, at 3 stages in the stepwise adaptation to polymyxin resistance. Wild type PAO1 grown in CDM is in lane 1, RPAO1 trained to 36, 80 and 160 units/ml Px and grown in CDM + Px at these concentrations are in lanes 2-4 respectively.

Gels are 15% acrylamide to allow separation of OMPs H1 and H2.

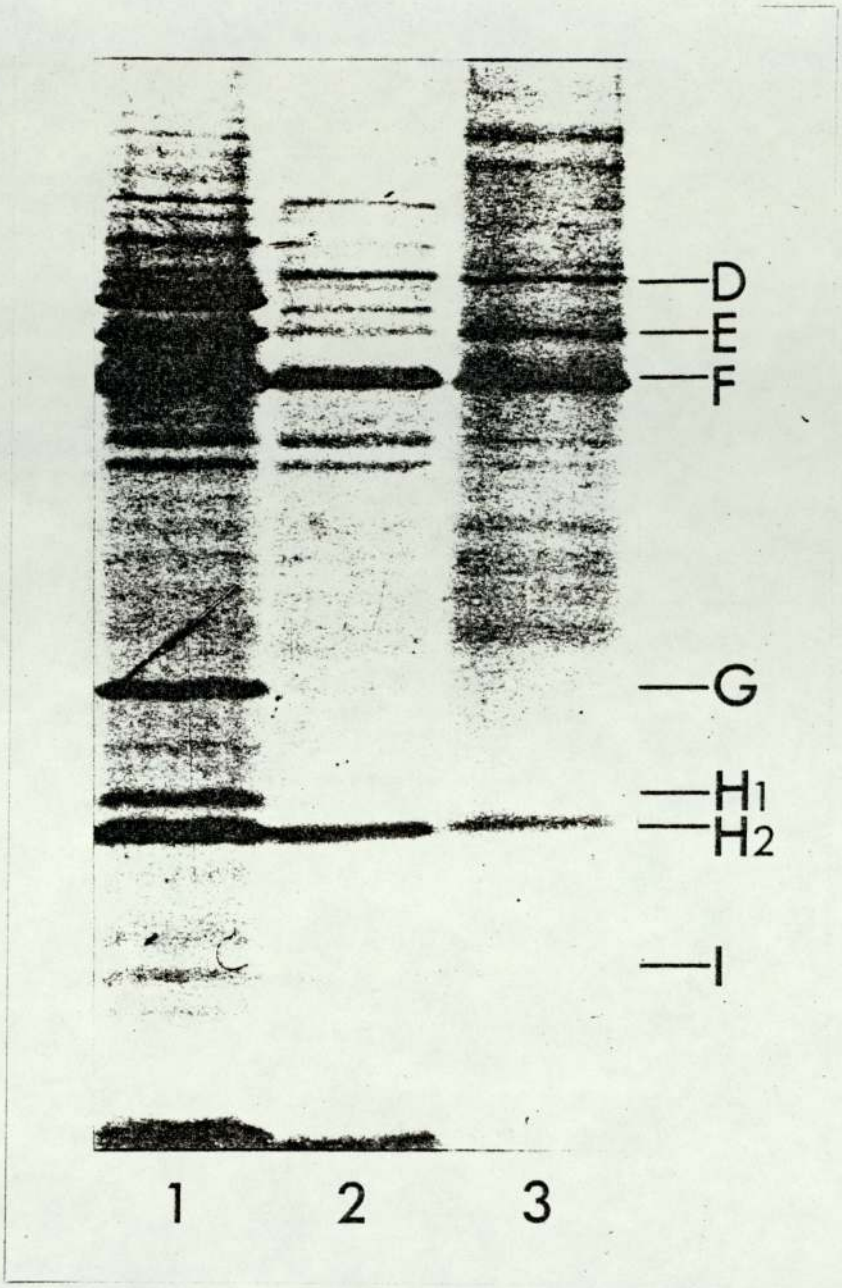


Figure 8.5

OMP profiles of P.aeruginosa PAO1 grown in CDM (lane 1) and a resistant variant, RPAO1, trained to polymyxin resistance of 6000 units/ml and grown in CDM + 6000 units/ml (lane 2) and Mg- CDM + 6000 units/ml Px. (lane 3)

Similar changes were seen in the OM protein profile of a resistant variant of PAO1 trained to a gentamicin resistance of 50µg/ml (figure 8.6). Figure 8.6, lane 1 shows wild type PAO1 grown in complete CDM, lane 2 shows the gentamicin resistant variant. Like the Px resistant variant in figure 1 proteins F & H1 have disappeared. Unlike the Px resistant variant, however, proteins D,E,G & I remain largely unchanged.

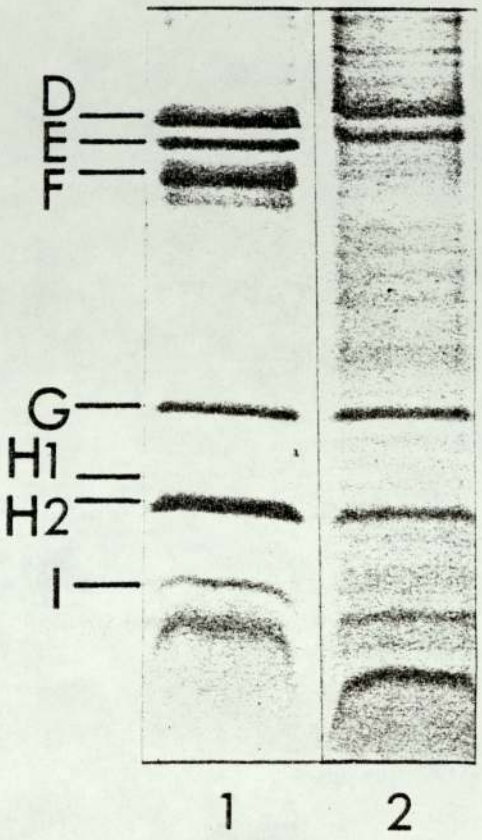


Figure 8.6

OMP profiles of P.aeruginosa PAO1 grown in CDM (lane 1) and a variant of PAO1 trained to a gentamicin resistance of 50μg/ml and grown in CDM + 50μg/ml gentamicin (lane 2).

8.6 OM antigens of polymyxin-resistant P.aeruginosa

OMP antigens visualised in immunoblots of OM preparations transferred to NC paper and probed with antisera raised to PAO1 reflect the OMP profiles shown in figure 8.5. In figure 8.7 lanes 1 and 2 show the OM profile of major protein antigens of P.aeruginosa grown in Fe+ and Fe- CDM 10 respectively. A number of high M_r bands in lane 2 show the position of IRMPs induced in the OM of Fe- grown cells. Lanes 3 and 4 show OM preparations of RPAO1 also grown in Fe+ and Fe- CDM. Bands corresponding to proteins D,E and F in lanes 1 and 2 are absent. The complete disappearance of protein F, which in the coomassie blue stained gel (figure 8.3, lanes 2 and 3) is only reduced, implies that the configuration of this protein may have changed during the training procedure in such a way that it is no longer antigenic. IRMPs were not induced in the OM of RPAO1 grown under conditions of iron derivation. There are no corresponding bands in the immunoblot of these cells (figure 8.7, lane 4).

Whole cells blotted onto NC paper and probed with antisera raised to PAO1 show the ladder-like pattern of LPS. There is very little differentiation in figure 8.8 between whole cells of PAO1 (lanes 1 and 2) and RPAO1 (lanes 3 and 4) or between Fe+ grown cells (lanes 1 and 3) and Fe- cells (lanes 2 and 4). LPS isolated from PAO1 and RPAO1 grown under the same conditions is shown in figure 8.9 and exhibits the characteristic ladder pattern.

8.7 Effect of magnesium depletion at different Px concentrations.

Induction of OMP H1 is a property of Mg depletion in P.aeruginosa and has been correlated with Px and gentamicin resistance. The disappearance of protein H1 from the OM of P6x-resistant cells (figure 8.5, lane 2) even under conditions of Mg depletion (lane 3) is thus of interest. The growth conditions under which it might be induced to re-appear were investigated by subculturing RPAO1 into a series of increasing concentrations of Px. A standard inoculum (2 x 10^6 .cells) of late logarithmic phase RPAO1 cells growing in CDM containing 6000 units/ml Px was subcultured into flasks containing 10,20,40,80,160,1000,2000, and 6000 units/ml of Px in Mg- CDM. The same number of RPAO1 cells, washed twice with 0.85% saline to remove unbound Px, were subcultured into Mg- CDM without Px. Bacteria were cultivated at 37°C for 24 hours using an orbital shaking incubator and harvested by centrifugation at 5,000 x g for 10 minutes and washed once with saline.

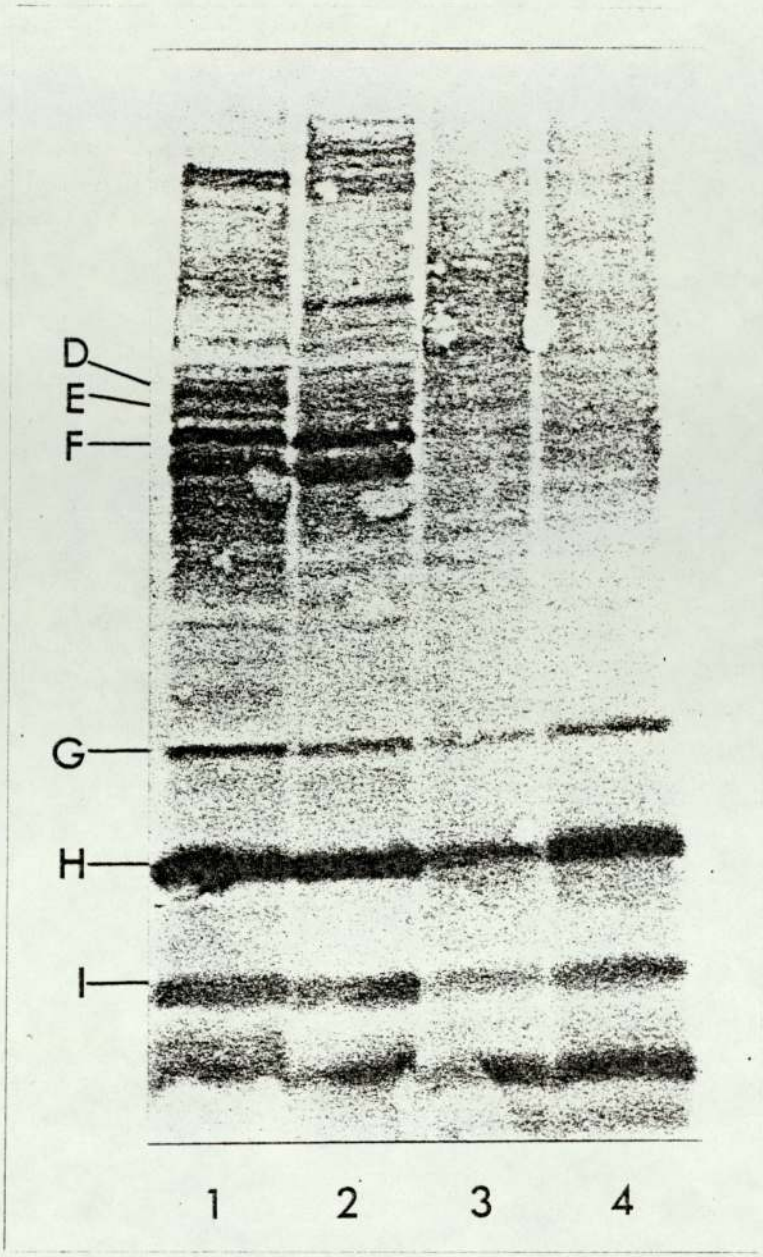


Figure 8.7

Immunoblot of OMPs of P.aeruginosa PAO1 grown in Fe+ (lane 1) and Fe- (lane 2) CDM and a Px resistant variant, RPAO1, grown in Fe+ (lane 3) and Fe- (lane 4) CDM + 6000 units/ml Px electrophoretically transferred to NC paper and reacted with rabbit antisera raised to PAO1.

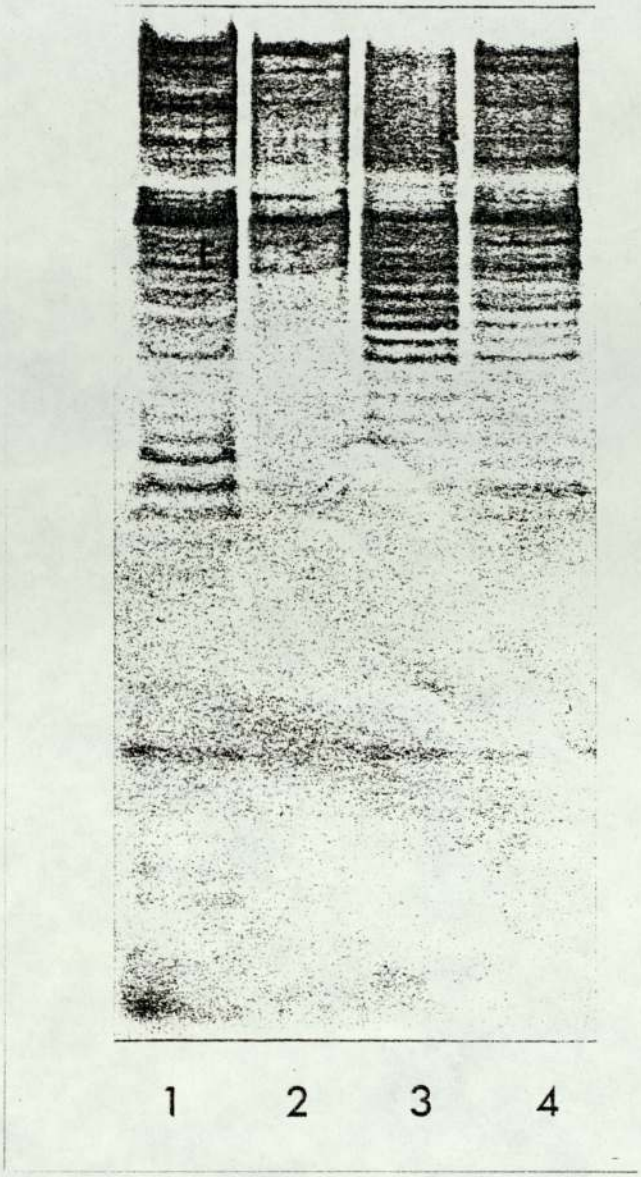


Figure 8.8

Immunoblot of whole cells of P.aeruginosa PAO1 grown in Fe+ (lane 1) and Fe- (lane 2) CDM and a Px resistant variant, RPAO1, grown in Fe+ (lane 3) and Fe- (lane 4) CDM + 6000 units/ml Px electrophoretically transferred to NC paper and reacted with rabbit antisera raised to PAO1

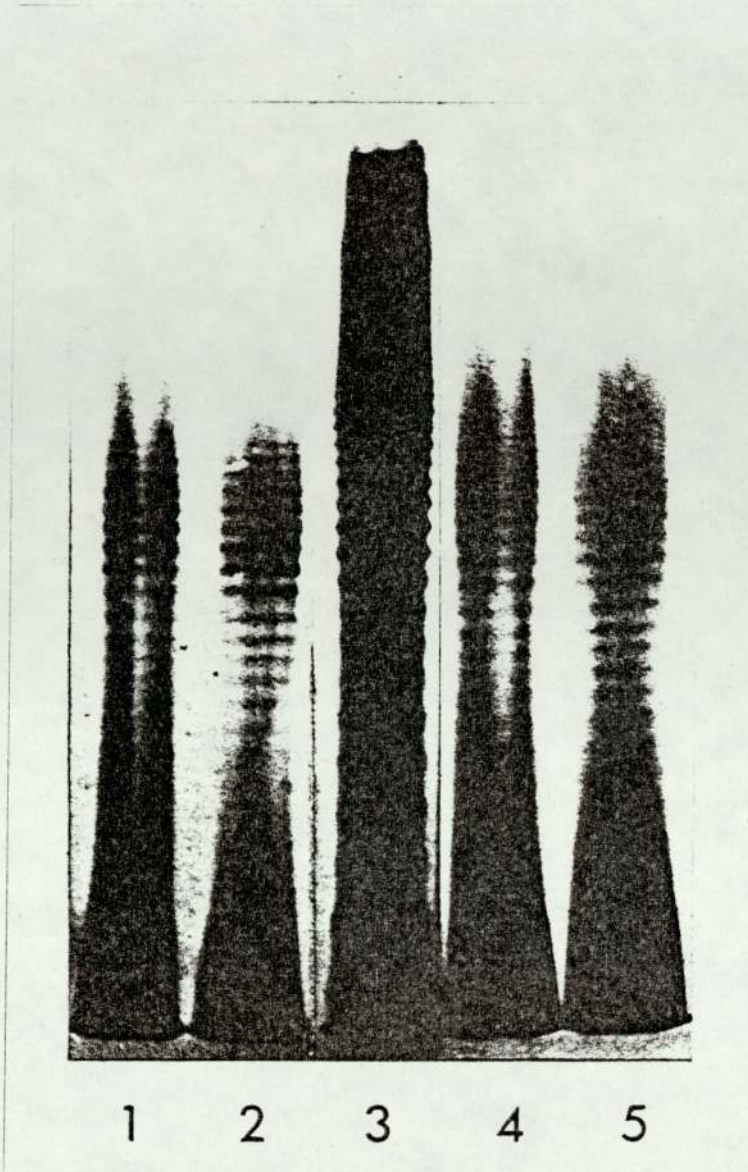


Figure 8.9

LPS of P.aeruginosa PAO1 grown in Fe+ (lane 1) and Fe- (lane 2) CDM and a Px resistant variant, RPAO1, grown in Fe+ (lane 4) and Fe- (lane 5) CDM + 6000units/ml Px. E.coli O:111 LPS (control) is in lane 3.

15% acrylamide gel containing 4M urea.

Figure 8.10 shows the effect of Mg depletion on the OM protein profile of the parent PAO1 and a variant resistant to 6000 units/ml of Px as it was grown in increasing concentrations of the antibiotic. Lane 1 shows PAO1 grown under conditions of Mg depletion. Protein H1 has been induced. Lane 2 shows RPAO1 grown in Mg- CDM in the absence of Px. The level of protein H1 was induced and the OMP profile is similar to that of the wild type grown in complete CDM. It requires at least 2 serial subcultures of RPAO1 into CDM without polymyxin before the pattern of its OM proteins reverts exactly to that of the parent strain (data not shown). Lanes 3-8 show RPAO1 grown in various concentrations of Px from 10 units/ml - 1000 units/ml in Mg- CDM. Protein H1 was not induced as would be expected under these conditions of Mg depletion. The level of protein G was also significantly reduced. Lanes 9 and 10 show RPAO1 grown in 2000 and 6000 units/ml of Px in Mg- CDM. The levels of proteins D and F were considerably reduced compared with the wild type. Again the level of protein G was reduced as is seen in Lanes 3-8. Protein H1 was eliminated completely. The MIC of Px of the resistant cells grown in the presence of different concentrations of the antibiotic remained at 6000u/ml. Thus the Px resistance of RPAO1 could be maintained at a high level by the presence of even small amounts of Px in the medium. RPAO1 grown in the absence of Px (lane 2) reverted to the normal Px sensitivity of PAO1, 10u/ml.

To rule out the possibility that divalent cations in the Px solution itself were depressing H1 levels the concentrations of Ca^{++}, Mg^{++}, and Mn^{++} in the Px solution were measured by AAS (2.2.6).

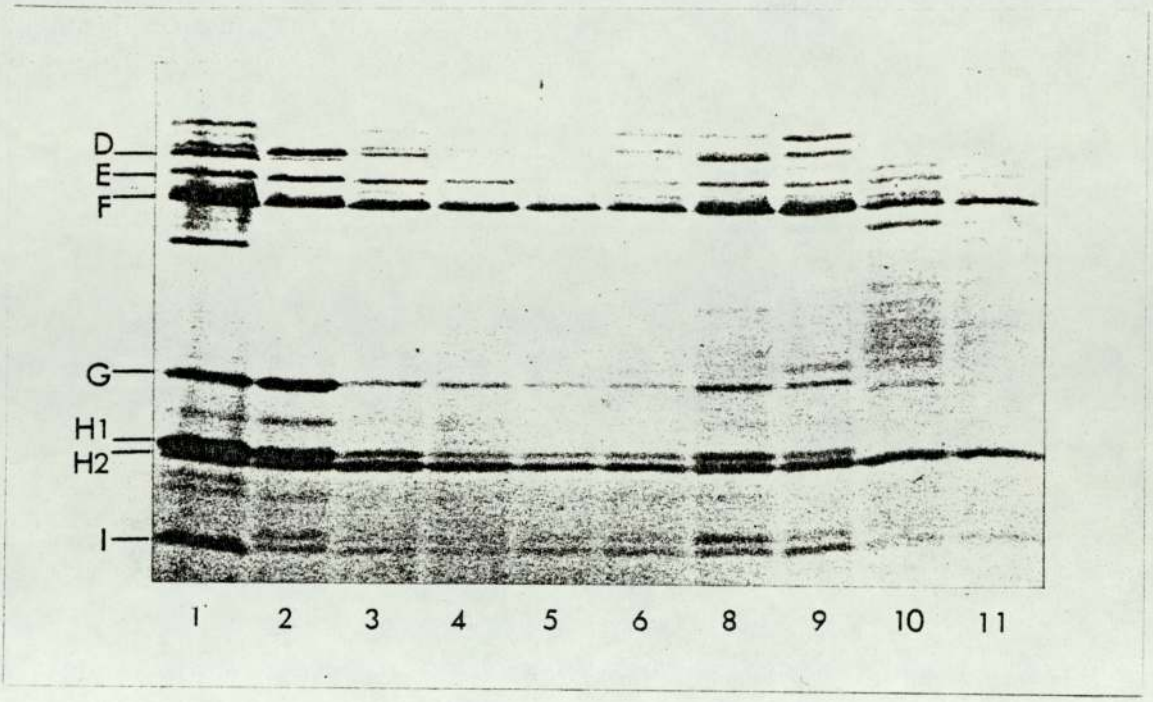


Figure 8.10

OMP profiles of P.aeruginosa PAO1 grown in Mg- CDM (lane1), and a resistant variant, RPAO1, trained to resistance in 6000 units/ml Px and grown in Mg- CDM containing increasing concentrations of Px (lanes 3-11). Concentrations of Px were 10, 20, 40, 80, 160, 1000, 2000, and 6000 units/ml respectively. Lane 2 shows RPAO1 cells subcultured in CDM without Px.

Figure 8.11

Effect of magnesium concentration on the growth of

P.aeruginosa PAO1

Added magnesium concentration ($M \times 10^{-5}$)

× 0.5

▼ 1.0

■ 2.0

□ 4.0

● 8.0

○ 16.0

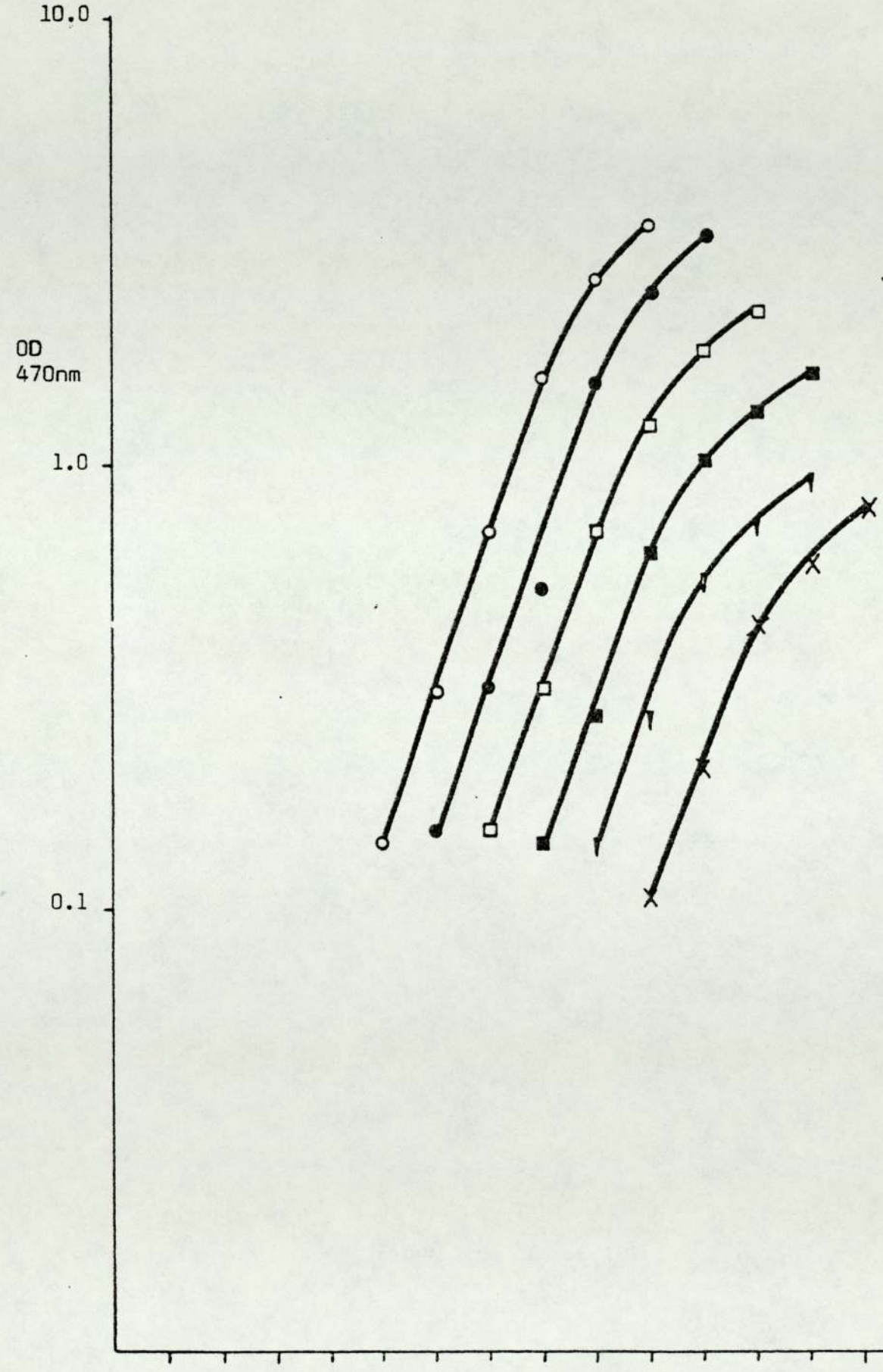

10.0
OD
470nm
1.0
0.1
Time (60 minute intervals)

Figure 8.12

Relationship between maximum exponential growth of

P.aeruginosa PAO1 and the added magnesium concentration

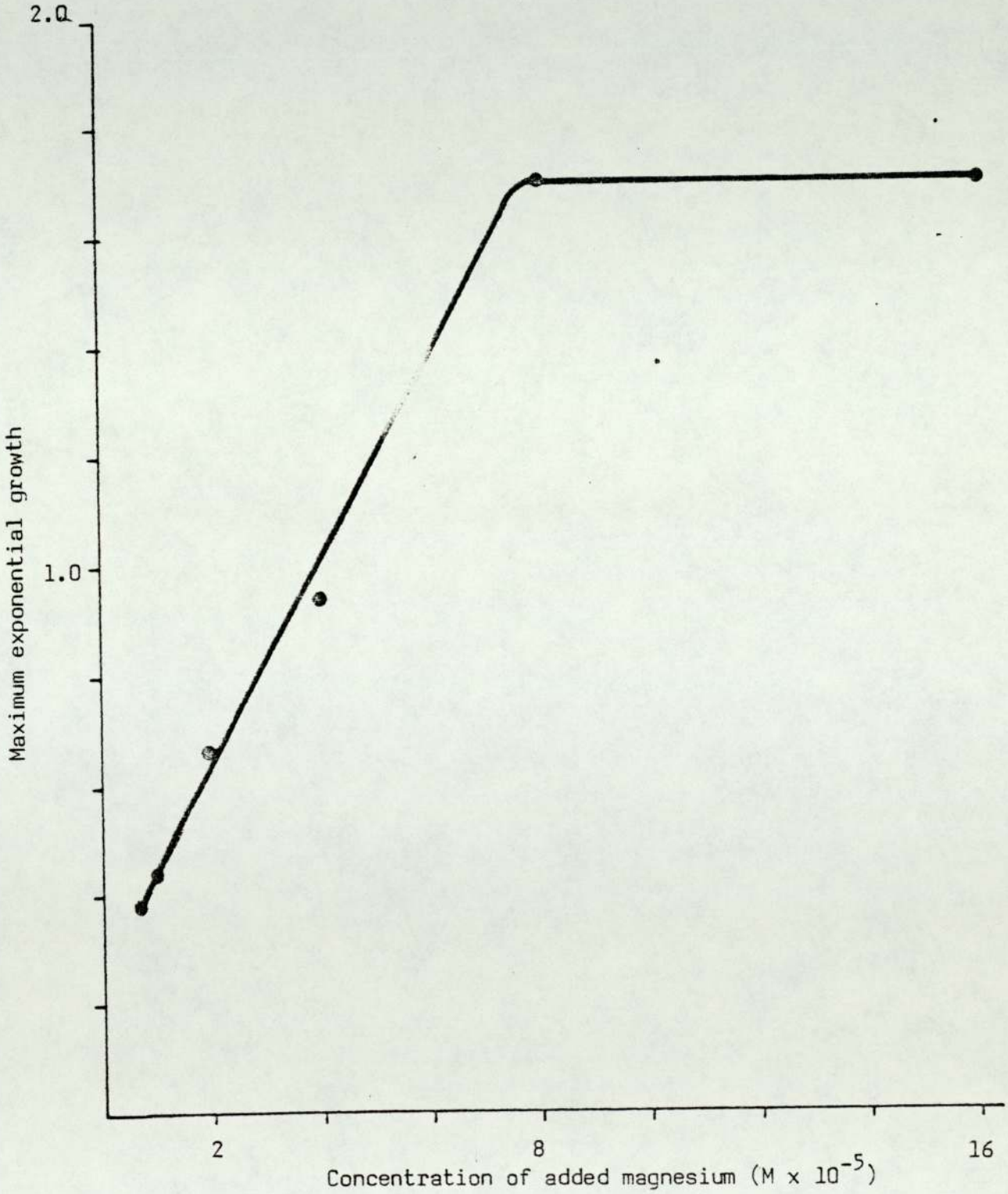


Figure 8.13

Effect of magnesium concentration on the growth of a resistant variant of P.aeruginosa PAO1 (RPAO1)

Added magnesium concentration ($M \times 10^{-5}$)

× 0.5

▼ 1.0

■ 2.0

□ 4.0

● 8.0

○ 16.0

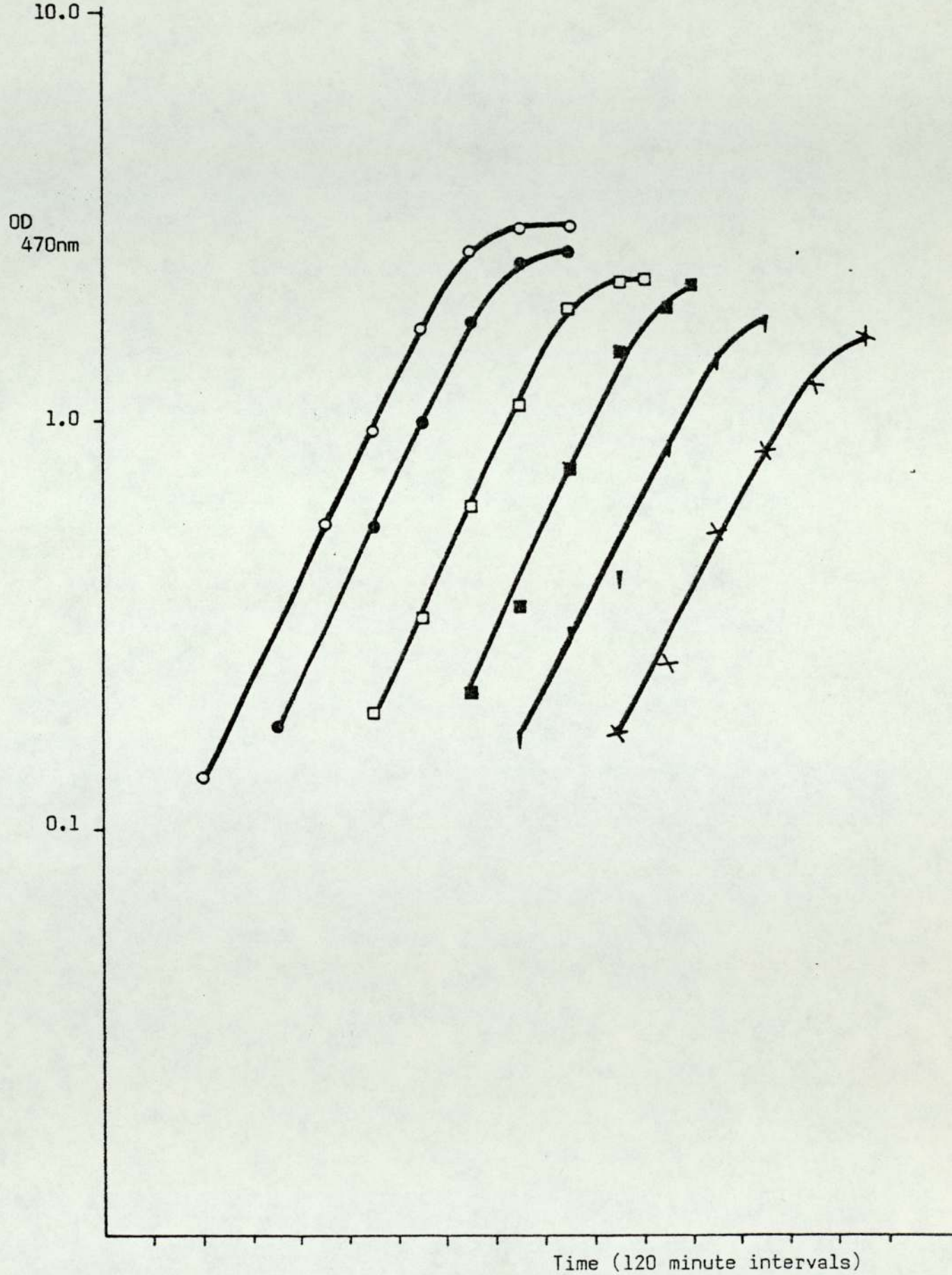
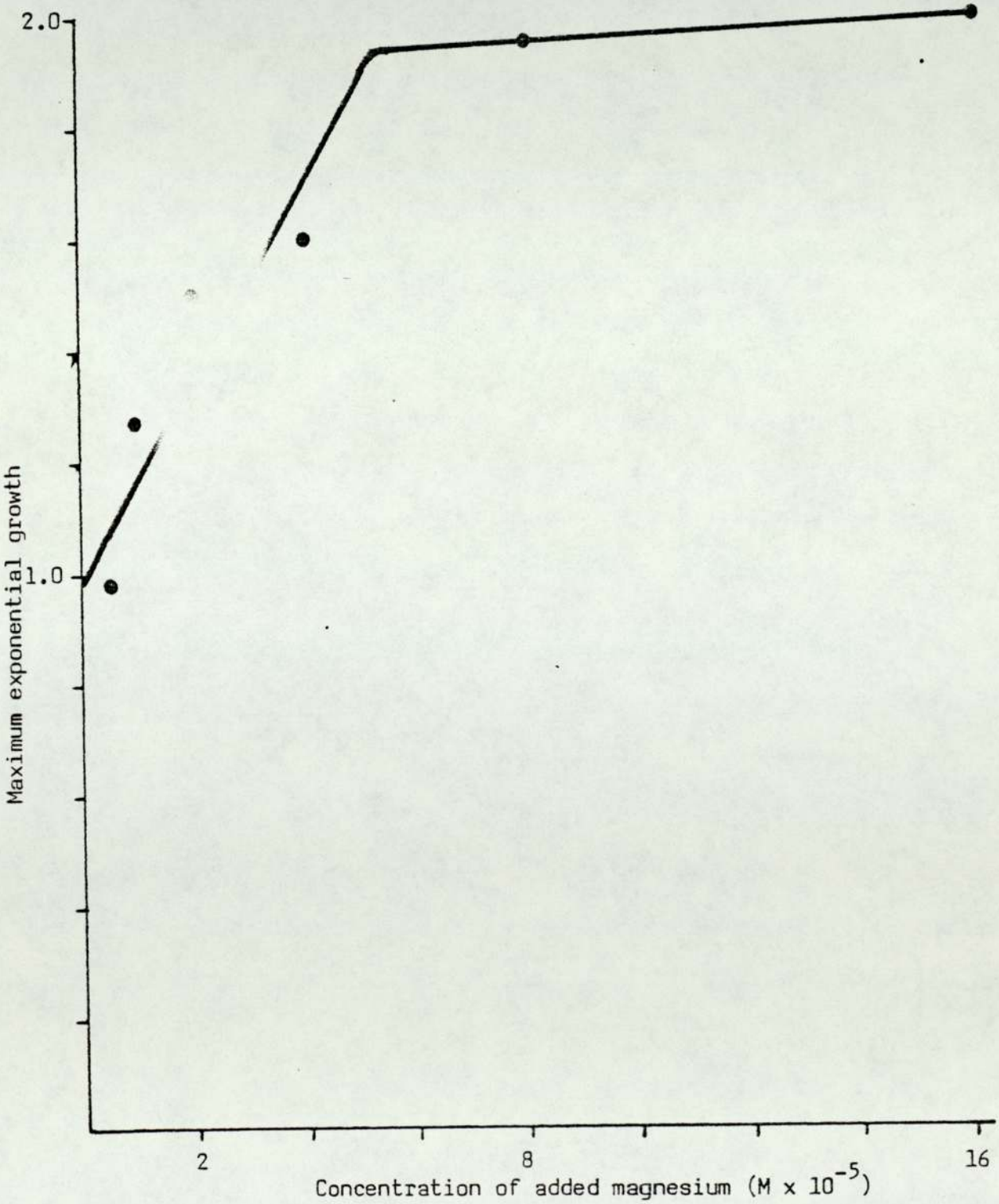

10.0
OD
470nm
1.0
0.1
Time (120 minute intervals)

Figure 8.14

Relationship between maximum exponential growth of RPA01 and the added magnesium concentration



In all cases only trace amounts, less than 1ppm, could be detected in the Px solution.

The magnesium requirements of PAO1 in CDM and RPAO1 in CDM + 6000 units/ml Px were determined by the method of Klemperer et al (1979). Growth of PAO1 (figure 8.11) and RPAO1 (figure 8.13) were measured turbidimetrically at graded magneium concentrations (2.2.3). The point at which exponential growth ceased was plotted against added Mg concentration. In both cases a linear relationship was observed, to an absorbance of OD 1.7 (figure 8.12) and 1.95 (figure 8.14), after which addition of more magnesium did not enable exponential growth to continue. The Mg requirement of the RPAO1 cells was lower than PAO1 but in both cases the concentration of magneium in Mg- CDM (table 2.2) was growth limiting

8.8 Discussion

Growth of P.aeruginosa PAO1 in increasing concentrations of Px produced a Px-resistant organism with a characteristic outer membrane protein profile deficient in proteins D,F,G & H1. This reduction in the number of OMPs is reflected in immunoblots of the OM.

LPS extracted from Px-resistant cells appears to be qualitatively very similar to that from the sensitive wild type. Immunoblots of whole cells reveal the characteristic ladder pattern of LPS. The pattern is very similar for PAO1 cells and for RPAO1 which infers that that LPS is not changed immunologically by adaptational resistance to Px.

Fatty acids of both wild type and resistant strains are qualitatively and quantitatively similar (Champlin et al, 1983). Fatty acid composition is a useful biochemical fingerprint (Moss, 1978; Oyaizu et al, 1983) which serves to confirm that RPAO1 is derived from PAO1 despite their different OMP profiles. The decrease in C19:0 cyclopropane fatty acid content noted in the resistant organism is interesting. A number of resistant strains of Gram-negative organisms, including a Px-resistant K.pneumoniae, show decreases in cyclopropane acids when compared with sensitive strains (Dunnick & O'Leary, 1970). These changes are inherent and not due to growth stage (Dunnick & O'Leary, 1970). Cyclopropane ring structures in a membrane lower its melting point compared with the same membrane containing saturated straight chain acids. Alterations in cyclopropane concentration may alter the nature and integrity of the membrane and affect the ability of an antibiotic to interact with it. Alterations in membrane fluidity induced by changes in temperature cause striking differences in susceptibility of bacteria to Px (Hodate & Bito, 1982).

Antibiotic sensitivity and serum killing experiments indicate that the OM permeability barrier is deficient in the Px-resistant cells. This is interesting because of recent findings (Vaara & Vaara, 1983a,1983b) that a derivative of Px, polymyxin B nonapeptide, modified by enzymatic removal of the terminal diaminobutyric acid-fatty acid of the side chain, loses its bactericidal activity but retains its ability to disrupt the OM. Bacteria treated with this agent are not killed but show greatly increased sensitivity to hydrophobic (and to a lesser extent hydrophilic) antibiotics and to killing with serum. It is possible that adaptational resistance to Px has a similar effect. Changes in the bacterial outer membrane during

the period of training (Champlin *et al* 1983; Gilleland *et al*, 1984a) could be such that Px molecules bind to the altered surface by replacing divalent cations but are then immobilised in the cell wall and do not kill the cell. Comparison of the growth kinetics of PAO1 in CDM (figures 8.11 & 8.12) and RPAO1 in CDM + Px (figures 8.13 & 8.14) show that the exponential phase of growth ceases at a lower OD in PAO1 when a limiting quantity of Mg was added to the medium. It is possible that Px has replaced Mg at binding sites in the OM thus reducing the magnesium requirements in RPAO1.

In vitro activity of Px against *P.aeruginosa* is high and there are no reports of plasmid mediated resistance to the antibiotic yet treatment of *P.aeruginosa* infections with Px has yielded disappointing results (Lowbury & Jones, 1975) and it is now rarely used. This is partly because of its toxicity to mammalian cells. The findings of Vaara & Vaara (1983a,b) are interesting in that they hold out the possibility of treating bacterial infection with a non-toxic, non-bactericidal agent which sensitises the bacterial invader to the body's defence mechanisms and to other antibiotics. It is possible that adaptational resistance to Px of the type demonstrated here *in vitro* may also occur *in vivo* and thus explain the disappointing results seen with polymixin therapy. This effect of adaptational resistance to polymyxin has been noted with *E.coli* in batch and dilution cultures (Greenwood, 1975; Greenwood & O'Grady, 1977).

PAO1 cells trained by serial subculture to resistance to Px and to gentamicin lose protein H1 from their outer membranes as analysed by SDS-PAGE. Overproduction of protein H1 is a property of *P.aeruginosa* when grown under Mg- depleted conditions. The ability of the organism to overproduce H1 was lost when resistant cells were grown under magnesium depletion in the presence of Px. Amounts of Px

as low as 10units/ml are capable of repressing Hi overproduction and cells grown at this concentration of Px retain their resistance to high concentrations of the antibiotic. These observations make it extremely unlikely that the overproduction of protein Hi seen in some cells (Nicas & Hancock, 1980) is in itself an explanation of Px resistance.

Mg++ is thought to play a crucial role in cross-linking of LPS which is known to be highly phosphorylated in P.aeruginosa (Wilkinson, 1983). The binding sites of Mg ions can also be occupied by other cations such as Ca or Mn when the growth environment lacks Mg (Boggis et al, 1979; Kenward et al, 1979; Nicas & Hancock, 1983a). It has been suggested that polymyxin acts initially by binding the acidic groups in the lipid A part of the LPS molecules of the OM and thus displaces the stabilising divalent metal cation cross-bridges between LPS molecules and between LPS and OM proteins (Brown, 1975; Schindler & Osborn, 1979; Vaara & Vaara, 1983a,b). Analysis of the envelope composition of Mg-depleted cells and of polymyxin trained cells has revealed changes in ultrastructure (Gilleland & Murray, 1976; Gilleland, 1977) and qualitative or quantitative alterations in cell envelope lipids (Brown & Watkins, 1970; Brown & Woods, 1972; Conrad & Gilleland, 1981; Gilleland & Conrad, 1982; Champlin et al, 1983; Gilleland et al, 1984), LPS (Gilleland & Lyle, 1979) and divalent cations (Brown & Watkins, 1970; Brown & Woods, 1972; Kenward et al, 1979) as well as proteins (Nicas & Hancock, 1980; Gilleland & Conrad, 1982; Gilleland & Beckham, 1982).

The phospholipid composition of sensitive and resistant cells has been studied with results closely similar to those reported by Gilleland et al (1984) (data not shown). Preliminary studies also show an increase in the amount of extracellular polysaccharide produced by

RPA01 (J.W.Costerton, personal communication). Resistance to Px is probably multifactorial and involves alterations in a number of components in the cell envelope which in turn modify the interaction between Px and the OM. It is unlikely that an isolated change in protein concentration in the membrane will be a sufficient explanation of Px resistance. The OMP profiles of a number of clinical isolates of P.aruginosa resistant to a spectrum of antibiotics (Chapter 7) show no evidence of elevated H1 levels. It is probably only co-incidental that protein H1 levels are induced in some chemically induced polymyxin resistant mutants (Nicas & Hancock, 1980).

9 CONCLUSIONS

P.aeruginosa infections occur with increasing frequency in hospitalized patients with compromised immune defence mechanisms. These patient populations, especially the immunosuppressed may be expected to increase in number as more aggressive chemotherapy and surgery are used in cancer and transplant therapy (Bodey et al, 1983; Bryan et al, 1983a; Klastersky, 1983a).

Antibiotic development is continuing to provide agents with activity against P.aeruginosa, nevertheless antibiotic resistance and emergence of new resistant strains remains a problem in clinical practice (O'Grady, 1984).

Optimal host defence against P.aeruginosa occurs in the presence of adequate numbers of phagocytic cells, complement and type-specific P.aeruginosa antibodies and P.aeruginosa infection rarely occurs in immune competent individuals (Peterson, 1979). Thus there is considerable interest in immunization or immune therapy in high risk groups (Pennington, 1979).

In P.aeruginosa infection the bacteria multiplies at the site of infection and elaborates metabolites which have been shown to be toxic. Some of these metabolites are somatic, some are extracellular. Immune comlex disease may be a problem in patients exposed to P.aeruginosa exoproducts over a long period of time Hoiby & Shiotz, 1982). It has not been established if the way to prevent P.aeruginosa infection in the various diseases caused by the organism is to kill bacteria or neutralize toxic metabolites or both.

This study has obtained direct biochemical evidence that bacteria grown under iron restricted conditins in UTI. Similar OMP

profiles can be obtained by cultivation of the same isolates under conditions of iron-depletion in laboratory media. Results obtained by other workers have confirmed observations made in this study (Lam et al, 1984). The need to acquire iron before proliferation can occur in vivo makes possession of IRMPs a virulence factor. The high Mr OMPs induced in vivo or in vitro under iron depleted conditions were recognised by antibodies present in patients serum or urine. Antibodies against IRMPs may play an imprtant role in host defence (Griffiths et al, 1983,1985). The IRMP antigens are therefore potential candidates for vaccine development. Proteins F, H & I of P.aeruginosa strains grown under conditions which mimic those in the body were also immunogenic and reacted with antibodies in immune rabbit serum, these are also potential candidates for vaccines. However, antibodies raised in hyperimmunized animals may give a different picture to that which can be obtained with material directly from infection because immunization with killed cells does not involve the same physiological stress as those which occur in infection. The immune response to infection should be studied in animal models using conditions which mimic as far as possible the actual infection in vivo (Hambleton & Melling, 1983). Protective studies using antibodies raised against separated and purified OMPs will provide useful information about the ability of these purified components to protect against bacterial infection due to clinically important resistant strains.

Studies on the chemical changes in Px-resistant organisms show that antibiotic resistance is complex and multifactorial. The pattern of OMPs in clinical isolotes as revealed by SDS-PAGE and by immunoblotting was not significantly different in antibiotic-sensitive

and -resistant strains. Antibiotic resistance did not correlate with serotype, colony type or serum resistance. The reasons for the high intrinsic resistance of P.aeruginosa are not known (Brown, 1975). It is possible that subtle changes in the molecular conformation of surface components is responsible for resistance. These changes were not detected by SDS-PAGE analysis and immunoblotting, techniques which give information about the presence of OM components but no information about the arrangement of the molecules at the surface of the bacteria. Studies of surface exposure or surface accessibility as described by Swanson (1981) could be useful in providing new information about the surface exposure of antigens. Monoclonal antibodies against these surface components could provide more useful information at the molecular level.

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